

Identification of GIYWHHY as a Novel Peptide Substrate for Human p60^{c-src} Protein Tyrosine Kinase

Qiang Lou, Margaret E. Leftwich and Kit S. Lam*

Arizona Cancer Center and Department of Medicine, Microbiology and Immunology, University of Arizona College of Medicine, Tucson, AR 85724, U.S.A.

Abstract—We have recently determined that -Ile-Tyr- were the two critical residues as a peptide substrate for p60^{c-src} protein tyrosine kinase (Lou, Q. et al., *Lett. Peptide Sci.*, **1995**, 2, 289). Here, we report on the design and synthesis of a secondary 'one-bead, one-compound' combinatorial peptide library based on this dipeptide motif (XIYXXXX, where X = all 19 eukaryotic amino acids except for cysteine). This secondary library was screened for its ability to be phosphorylated by p60^{c-src} PTK using [γ -³²P]ATP as a tracer. Five of the strongest [³²P]-labeled peptide-beads were identified and microsequenced: GIYWHHY, KIYDDYE, EIYEENG, EIYEEYE, and YIYEEED. A solid-phase phosphorylation assay was used to evaluate the structure–activity relationship of GIYWHHY. It was determined that Ile², Tyr³, His⁵, and His⁶ were crucial for its activity as a substrate. Copyright © 1996 Elsevier Science Ltd

Introduction

Protein tyrosine kinases (PTKs) are key enzymes in the signaling pathways of many cellular functions.^{1,2} The p60^{c-src} protein is the protein product of the first discovered proto-oncogene and the first PTK described.³ It belongs to the non-receptor class of PTK and is localized at the inner surface of the plasma membrane via its N-terminal myristate.^{4,5} Human p60^{c-src} has been implicated in the development of leukemia, breast, and colon cancer.^{6–8} However, the exact physiological role of p60^{c-src} or its physiological target proteins are not known, and the mechanism by which the p60^{c-src} activity is regulated is not completely understood.

To understand the physiological role and mechanism of action of p60^{c-src}, it is important to study its substrate specificity. In the absence of clear physiological target substrates, a number of exogenous substrates have been used to study this enzyme function and its substrate specificity. These substrates include casein,⁹ enolase,¹⁰ and synthetic peptides such as autophosphorylation sites of p60^{c-src} itself^{11,12} and cdc(26–20) peptide.^{13,14} However, most of these substrates are phosphorylated only at very high substrate concentrations. In an attempt to define the primary sequence requirements for tyrosine phosphorylation, much effort has been devoted to finding peptide substrates with low K_m values.^{15,16}

Recently, we¹⁷ and Songyang et al.¹⁸ reported the use of combinatorial peptide library methods to identify

efficient and specific peptide substrates for p60^{c-src} PTK. We employed the 'one-bead, one-peptide' combinatorial peptide library method^{19–21} and solid-phase phosphorylation approach to screen a completely random heptapeptide library XXXXXXX (where X = 19 eukaryotic amino acids except Cys, and only 500,000 peptide-beads were screened) and identified YIYGSFK as a relatively potent and specific substrate for p60^{c-src} PTK.¹⁷ The library was prepared by a split synthesis method^{19,22} such that each peptide-bead displayed only one peptide entity.¹⁹ The peptide-bead library was then incubated with [γ -³²P]ATP and p60^{c-src} PTK. After thorough washing, the bead library was then immobilized on a glass plate by agarose, and the [³²P]-labeled peptide-beads were localized by autoradiography and isolated for microsequencing.^{17,21}

Songyang et al.¹⁸ used a completely different screening approach. A biased 15-mer peptide library, MAXXX-XYXXXXAKKK (where X = 15 eukaryotic amino acids except Cys, Trp, Tyr, Ser, and Thr) was synthesized and cleaved off the resin. After phosphorylation in the solution-phase, the phosphopeptides were isolated by ferric chelation column chromatography. Concurrent sequencing of all the eluted peptides was then performed to determine the optimal substrate motif for p60^{c-src} PTK or other protein kinases. Despite the two completely different approaches, our peptide YIYGSFK does share considerable sequence homology with the EEIYGEFF motif determined by Songyang et al.:¹⁸ _ _ IYG _ F _ . Despite their similarity, the net charge of these two peptides are completely different.

We have recently used a rapid solid-phase phosphorylation assay to study the structure–activity relationship (SAR) of over 70 YIYGSFK analogues and deter-

Key words: combinatorial libraries, peptide substrate, p60^{c-src} protein tyrosine kinase.

mined that a hydrophobic L-residue at position 2 and a L-tyrosine at position 3 are critical for activity.²³ Combinatorial peptide library methods, besides facilitating the discovery of initial leads, can also be used to optimize initial leads using a sequential library approach.^{24,25} In this paper, we report on the screening of a secondary peptide library (XIYXXXX), in which the second and third positions are fixed with Ile and Tyr, respectively, and the rest of the residues were randomized with 19 eukaryotic amino acids, except cysteine.

Results

The reduction of background phosphorylation by HCl treatment

The background phosphorylation of the peptide-bead library can drastically be reduced by the treatment of the [³²P]-labeled library with 1 N HCl at 100 °C for 15 min. This is illustrated in Figure 1. Figure 1A shows the representative autoradiogram of a library screen on which no acid treatment was performed. After acid treatment, the background phosphorylation was minimal (Fig. 1B).

Screening of secondary peptide library XIYXXXX

Approximately 1 million peptide-bead were screened. Five strongly labeled-beads were picked and sequenced: GIYWHHY, KIYDDYE, EIIYEENG, EIIYEEYE, and YIIYEEED. These five peptides together with the initial lead YIYGSKF were resynthesized and their phosphorylation by p60^{c-src} on solid-phase confirmed. In addition, the Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate their relative level of phosphorylation on solid-phase as described elsewhere.²³ The result is shown in Table 1. GIYWHHY was almost two-fold more potent than that of the parent compound YIYGSKF. However, the rest of the four acidic peptides were significantly weaker.

Phosphorylation studies of GIYWHHY analogues on solid-phase

The structure–activity relationship of GIYWHHY analogues (Table 2) was examined by quantitative solid-phase phosphorylation studies. Eleven analogues of GIYWHHY were examined. ‘Alanine-walk’ of GIYWHHY indicated that Ile² and Tyr³ were crucial for p60^{c-src} activity. In addition, His⁵ and His⁶ were also extremely important, and replacement of either of those two histidines by alanine resulted in a great loss in activity. Tyr⁷ was not essential, as it could readily be replaced by alanine or totally deleted. This also strongly suggests that Tyr³, but not Tyr⁷, is the phosphorylation site. Although Gly¹ could be replaced by alanine with only a loss of half of the activity, further decrease in activity (down to 20%) was observed when that residue was deleted. Further

deletion of both Gly¹ and Ile² rendered the peptide totally inactive.

Discussion

Over the last 5 years, the combinatorial library field has grown enormously. The library concept was first applied to peptide^{19,26–28} and nucleic acids,^{29–35} and more recently it has been rapidly expanded to non-peptide or small organic molecules.^{36–40} This latter development is primarily driven by the realization that the combinatorial library methods can greatly facilitate the drug discovery process. New solid-phase chemistries that can be applied to the library format are currently under intense development, both in academia and pharmaceutical industries. In addition to facilitating drug development, combinatorial library methods also serve as powerful tools for basic research.

There are several general approaches for screening peptide libraries: (1) Biological libraries with the filamentous phage,^{27,41} plasmid⁴² or polysomes,⁴³ (2) synthetic peptide libraries using iterative approach^{26,28,44} or positional scanning,⁴⁵ (3) the ‘one-bead, one-compound’ approach or the ‘Selectide Process’,^{19,20} (4) synthetic peptide libraries using affinity chromatography selection approach,^{18,46} (5) spatially addressable parallel solid-phase library methods such as the multi-pin technology,⁴⁷ spots-membrane,⁴⁸ or light-directed parallel synthesis on chips.⁴⁹ The biological library method, in general, can only be applied to peptides with 20 eukaryotic amino acids, whereas, the latter three methods can be applied to unnatural amino acids and even non-peptide small organic molecules.⁵⁰

We first described the ‘one-bead one-compound’ combinatorial library approach 4 years ago.¹⁹ Since then, we have successfully applied this method in the identification of ligands for various targets: monoclonal antibodies,^{19,51,52} streptavidin,^{19,53} avidin,⁵³ lymphoma specific surface idiotype,⁵⁴ MHC-class I molecules,⁵⁵ cAMP-dependent protein kinase,²¹ p60^{c-src} PTK,¹⁷ glycoprotein IIb/IIIa integrin,⁵⁶ and small organic indigo carmine.⁵⁷ Others have also used this method in many other systems.^{58–64} In this paper, we describe the use of the ‘one-bead one-compound’ combinatorial library method in the identification of potent and specific peptide substrates for p60^{c-src}. The structure of the peptide library used in this study was XIYXXXX. The design of this secondary library was based on the SAR study²³ of the YIYGSKF peptide identified from our primary screen with a totally random heptapeptide library.¹⁷ After screening a limited secondary library (approximately one million peptide-beads) with p60^{c-src}, we identified and sequenced five strongly positive beads (Table 1). Interestingly, of these five peptides, four were highly acidic. This is similar to the highly acidic motif (EEIYGEFF) identified by Songyang et al.¹⁸ who used a ferric chelation column chromatographic selection approach. The remaining peptide GIYWHHY, on the other hand, does not contain any

acidic residues. In solid-phase phosphorylation assay, GIYWHHY is a much more potent substrate than the other four acidic peptides. This peptide would have been missed in Songyang's affinity chromatographic selection approach as their eluted peptides were

sequenced concurrently and only the residues with highest occurrence will be reflected in their final motif.

With our solid-phase phosphorylation on-bead screening approach, treatment of the peptide-bead

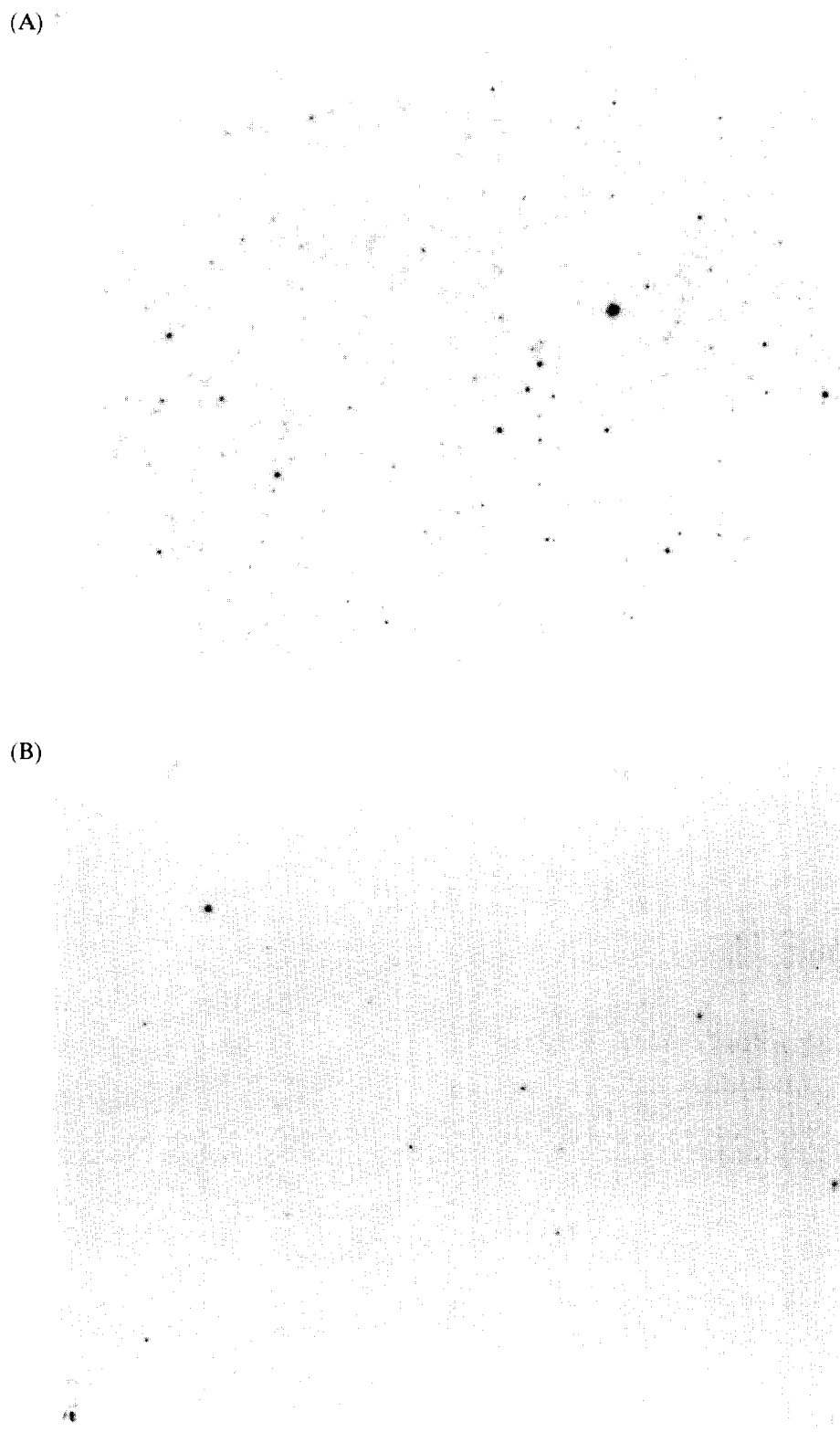


Figure 1. Autoradiogram showing the screening of a secondary peptide library (XIYXXXX) of which the 1 N HCl treatment step is (A) omitted, or (B) included. The high background of (A) is due to non-covalent interaction of [γ - 32 P]ATP with highly basic peptides. See text for explanation.

Table 1. Peptide substrate identified from a limited secondary peptide library (XIYXXXX) and quantitation of peptide phosphorylation with PhosphorImager

| Peptide | Relative phosphorylation (% of YIYGSKF) |
|---------|--|
| YIYGSKF | 100 |
| GIYWHHY | 198 |
| KIYDDYE | 12 |
| EIYEENG | 10 |
| EIYEEYE | 20 |
| YIYEEED | 8 |

library prior to autoradiography is crucial in eliminating the background label. The background label is primarily due to the noncovalent interaction of [$\gamma^{32}\text{P}$]ATP with some highly basic peptides (Lam, K. S., unpublished data). Acid treatment hydrolyzes the ATP but leaving the tyrosyl, seryl, and threonyl phosphate intact, thereby eliminating the undesirable noncovalent [^{32}P]-labeling. For those who desire to study tyrosine phosphorylation only, the peptide-beads may be treated with alkali condition prior to autoradiography since the seryl or threonyl phosphate is base-labile. The p60^{c-src} PTK preparation used in our present study is highly purified and there are no contaminating serine or threonine kinases, therefore alkaline treatment is not necessary.

Solid-phase phosphorylation assay is quantitative and convenient. It is invaluable in rapidly surveying the SAR of numerous analogues at the same time.²³ However, it does not necessarily reflect the true phosphorylation rate when both the substrate and kinase are in solution. Therefore any peptide of potential interest should be resynthesized and its kinetic parameters determined in solution-phase. Preliminary solution-phase kinetic studies in our laboratory indicate that GIYWHHY has a K_m of approximately 20 μM and substituting Tyr³ with 2-naphthylalanine generates a potent and very specific pseudosubstrate-based inhibitor for p60^{c-src} PTK, with an IC_{50} of approximately 10 μM .

Table 2. Solid-phase phosphorylation of GIYWHHY analogues by p60^{c-src} PTK

| Peptide | Relative phosphorylation (% of GIYWHHY) |
|---------|--|
| GIYWHHY | 100 |
| AIYWHHY | 49 |
| GAYWHHY | 16 |
| GIAWHHY | 5 |
| GIYAHHY | 67 |
| GIYWAHY | 14 |
| GIYWHAY | 15 |
| GIYWHHA | 88 |
| IYWHHY | 20 |
| YWHHY | 7 |
| WHHY | 6 |
| GIYWHH | 89 |

Experimental

Materials

Human p60^{c-src} was purchased from UBI (Lake Placid, New York). [$\gamma^{32}\text{P}$]ATP was obtained from ICN Biomedicals Inc. (Irvine, California). BSA (fraction V), agarose, (MES), magnesium chloride, phenol, anisole, and ethanedithiol were purchased from Sigma (St. Louis, Missouri). Fmoc-protected amino acids, Rink resin, TFA, piperidine, BOP, HOBt, and DIEA were purchased from Advanced ChemTech (Louisville, Kentucky). TentaGel S resin was obtained from Rapp Polymere (Tubingen, Germany). DMF and methanol were purchased from Baxter (McGaw Park, Illinois). Glogos II autoradiogram marker was purchased from Stratagene (La Jolla, California). X-ray film (Kodak X-OMAT LS) was also obtained from Sigma. Thin-layer chromatography (TLC) plates [Cellulose MN 300 polyethyleneimine (PEI) impregnated] were purchased from Bodwen (Aston, Pennsylvania).

Synthesis of the secondary peptide library (XIYXXXX)

The 'split synthesis' method^{19,22} was used in the synthesis of the secondary peptide library as previously described.^{19,20} TentaGel S and Fmoc chemistry were used.^{65,66} TentaGel S already has a polyoxyethylene linker and, therefore, no further linker is needed in the synthesis of peptide libraries. The resins were first divided into 19 equal aliquots, and a four-fold excess of each Fmoc-protected amino acid was added to each aliquot (all 19 eukaryotic amino acids except for cysteine were used). The coupling reaction was started by the addition of 4 molar excess of HOBt, BOP, and DIEA. Coupling was continued for 1 h. The ninhydrin test was used to evaluate the completion of the coupling reaction. The resins were then mixed, thoroughly washed, and the N- α Fmoc group was removed with 20% piperidine in DMF (v/v), washed, and ready for another coupling cycle. After three additional cycles of split synthesis coupling with 19 amino acids, the resins were pooled, deprotected, and coupled with Fmoc-Ile alone without any resin splitting. Similarly, Fmoc-Tyr alone were added at the sixth coupling cycle. For the seventh and last cycle of coupling, the resins were again splitted into 19 aliquots and coupled with all the 19 amino acids as described above. After all the couplings were completed, the N- α Fmoc group was removed with 20% piperidine in DMF (v/v) and side chain protected groups were then removed with a mixture of TFA:phenol:anisole:ethanedithiol (94:2:2:2; v/w/v/v). The resin beads were then washed thoroughly with DMF and stored in 0.01% HCl.

Screening of secondary peptide library (XIYXXXX)

About one million peptide-beads from the secondary peptide library (XIYXXXX) were washed six times with the MES buffer (pH 6.8) containing 30 mM MES, 10 mM MgCl_2 , and 0.4 mg/mL BSA. Phosphorylation of the peptide library was conducted in 2 mL MES

buffer containing 60 units p60^{c-src}, and 0.1 μ M [γ -³²P]ATP as previously described.¹⁷ After incubation at room temperature for 2 h with gentle shaking, the beads were washed three times with 1 M HCl, then treated with 1 M HCl at 100 °C for 15 min to hydrolyze the ATP and to eliminate any non-covalent labelling. Following 1 M HCl treatment, the beads were washed three times with PBS-Tween (0.68 M NaCl, 10 M KCl, 40 mM Na₂HPO₄, 7 mM KH₂PO₄, and 0.05% Tween 20, pH 1). The thoroughly washed library beads were then suspended in 1% agarose solution at 70–80 °C and carefully poured onto glass plates (16 × 18 cm) and air-dried overnight at room temperature. Glogos™ II autoradiogram markers were taped on each corner of dried agarose plate prior to exposure in order to be able to later align the immobilized beads with the autoradiogram. The immobilized beads on the plate were then exposed to an X-ray film (Kodak X-OMAT LS) for 20–30 h at room temperature and the film developed. The agarose gel with embedded beads corresponding the dark spots on the X-ray film was excised with a razor blade, swollen in double-distilled water and then carefully transferred to a tube with 5 mL water. The agarose gel was dissolved in water at 80–85 °C and the beads treated with 1 M HCl and washed with PBS-Tween in order further to decrease noncovalent labelling. The washed beads were diluted with additional agarose, dried on glass plates, and autoradiography performed as described above. Because at this time, individual beads were relatively far away from each other, a single [³²P]-labeled peptide-bead can be precisely localized and readily removed with a micropipettor. The positive beads were washed with water, transferred to a glass-fiber filter, and inserted into a ABI protein sequencer (Model 477A, Applied Biosystems, Foster City, CA) for peptide structure determination as described.^{19,53}

Quantitation of peptide phosphorylation on solid-phase

The positive-beads were synthesized on TentaGel S resin according to the method described above. A quantitative solid-phase phosphorylation assay of various peptide analogues was performed as described previously.²³ About 100 peptide-beads from each sample were washed extensively (6 ×) with MES buffer (pH 6.8). The phosphorylation reaction was performed in 20 μ L MES buffer containing 1.5 units human p60^{c-src}, 0.1 μ M [γ -³²P] ATP (specific activity 25 Ci/mmol) and about 100 peptide-beads. After 2 h of incubation at room temperature with gentle shaking, the beads were then washed with 1 M HCl 2 × and treated with 1 M HCl at 100 °C for 15 min. After treatment, the peptide-beads were washed 3 × with PBS-Tween. About 20 thoroughly washed [³²P]-labeled beads were randomly picked up and suspended in 0.5 mL 1% agarose solution (w/v) at 80–85 °C and carefully poured onto a section of a glass plate that had been sectioned into 2 × 4 cm sections with a wax pencil, and air-dried overnight. The immobilized beads were then exposed to the storage phosphor screen for 12 h

at room temperature. The exposed screen was then read by the 425S PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the relative phosphorylation of the peptide-beads was quantitated.

Acknowledgments

This work is supported by NIH grants CA17094, CA57723, and CA23074. Kit S. Lam is a Scholar of the Leukemia Society of America.

References

1. Ullrich, A.; Schlessinger, J. *Cell* **1990**, *61*, 203.
2. Pawson, T. *Trends Genet.* **1991**, *7*, 343.
3. Taylor, S. J.; Shalloway, D. *Curr. Opin. Genet. Dev.* **1993**, *3*, 26.
4. Resh, M. D. *Cell* **1994**, *76*, 411.
5. Rudd, C. E.; Janssen, O.; Prasad, K. V. S.; Raab, M.; Silva, A.; Telfer, J. C.; Yamanoto, M. *Biochim. Biophys. Acta* **1993**, *1155*, 239.
6. Lynch, S. A.; Brugge, J. S.; Fromowitz, F.; Glantz, L.; Wang, P.; Caruso, R.; Viola, M. V. *Leukemia* **1993**, *7*, 1416.
7. Luttrell, D. K.; Lee, A.; Lansing, T. J.; Crosby, R. M.; Jung, K. D.; Willard, D.; Luther, M.; Rodriguez, M.; Berman, J.; Gilmer, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 83.
8. Talamonti, M. S.; Roh, M. S.; Curley, S. A.; Gallick, G. E. *J. Clin. Invest.* **1993**, *91*, 53.
9. Richert, N. D.; Blithe, D. L.; Pastan, I. *J. Biol. Chem.* **1982**, *257*, 7143.
10. Cooper, J. A.; Esch, F. S.; Taylor, S. S.; Hunter, T. *J. Biol. Chem.* **1984**, *259*, 7835.
11. Patschinsky, T.; Hunter, T.; Esch, F. S.; Cooper, J.; Sefton, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 973.
12. Hunter, T. *J. Biol. Chem.* **1982**, *257*, 4843.
13. Cheng, H.; Litwin, C. M. E.; Hwang, D. M.; Wang, J. H. *J. Biol. Chem.* **1991**, *266*, 17919.
14. Cheng, H.; Nishio, H.; Hatase, O.; Ralph, S.; Wang, J. H. *J. Biol. Chem.* **1992**, *267*, 9248.
15. Geahlen, R. L.; Harrison, M. L. In *Peptides and Protein Phosphorylation*; Kemp, B. E., Ed.; CRC: Boca Raton, FL, 1990.
16. Nair, S. A.; Kim, M. H.; Warren, S. D.; Choi, S.; Songyang, Z.; Cantley, L. C.; Hangauer, D. G. *J. Med. Chem.* **1995**, *38*, 4276.
17. Lam, K. S.; Wu, J. Z.; Lou, Q. *Int. J. Pept. Protein Res.* **1995**, *45*, 587.
18. Songyang, Z.; Carraway, K. L.; 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.; Cantley, L. C. *Nature* **1995**, *373*, 536.
19. Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82.

20. Lam, K. S.; Lebl, M. *Methods: A Companion to Methods in Enzymology* **1994**, 6, 372.
21. Wu, J. Z.; Ma, Q. N.; Lam, K. S. *Biochemistry* **1994**, 33, 14825.
22. Furka, A.; Sebestyen, F.; Asgedom, M.; Dobo, G. *Int. J. Pep. Protein Res.* **1991**, 37, 487.
23. Lou, Q.; Wu, J. Z.; Salmon, S. E.; Lam, K. S. *Lett. Pept. Sci.* **1995**, 2, 289.
24. Lebl, M.; Krchnak, V.; Sepetov, N. F.; Seligmann, B.; Strop, P.; Felder, S.; Lam, K. S. *Biopolymers* **1995**, 37, 177.
25. Lam, K. S.; Lake, D.; Salmon, S. E.; Smith, J.; Chen, M. L.; Wade, S.; Abdul-Latif, F.; Leblova, Z.; Ferguson, R.D.; Krchnak, V.; Sepetov, N.F.; Lebl, M. *Immunomethods*, in press.
26. Geysen, H. M.; Rodda, S. J.; Mason, T. J. *Mol. Immunol.* **1986**, 23, 709.
27. Scott, J. K.; Smith, G. P. *Science* **1990**, 249, 386.
28. Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, 354, 84.
29. Oliphant, A. R. Struhl, K. *Meth. Enzymol.* **1987**, 155, 568.
30. Horwitz, M. S. Z.; Loeb, L. A. *J. Biol. Chem.* **1988**, 263, 14724.
31. Robertson, D. L.; Joyce, G. F. *Nature* **1990**, 344, 467.
32. Green, R.; Ellington, A. D.; Szostak, J. W. *Nature* **1990**, 347, 406.
33. Tuerk, C.; Gold, L. *Science* **1990**, 249, 505.
34. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, 346, 818.
35. Tsai, D. E.; Harper, D. S.; Keene, J. D. *Nucleic Acids Res.* **1991**, 19, 4931.
36. Bunin, B. A.; Ellman, J. A. *J. Am. Chem. Soc.* **1992**, 114, 10997.
37. Nikolaiev, V.; Stierandova, A.; Krchnak, V.; Seligman, B.; Lam, K. S.; Salmon, S. E.; Lebl, M. *Pept. Res.* **1993**, 6, 161.
38. Cho, C. Y.; Moran, E. J.; Cheng, S.R.; Stephens, J. C.; Fodor, S. P. A.; Adams, C. L.; Sundaram, A. Jacobs, J. W.; Schultz, P.G. *Science* **1993**, 261, 1303.
39. Chen, C.; Randall, A.; Lisa, A.; Miller, B. R.; Jones, D.A.; Kurth, M. J. *J. Am. Chem Soc.* **1994**, 116, 2660.
40. Stankova, M. Issakova, O.; Sepetov, N. F.; Krchnak, V.; Lam, K. S.; Lebl, M. *Drug Dev. Res.* **1994**, 33, 146.
41. Cwirla, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, 87, 6578.
42. Cull, M. G.; Miller, J. F.; Schatz, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, 89, 1865.
43. Kawasaki, G. PCT International patent application WO 91105058, 1991.
44. Geysen, M. H.; Rodda, H., M.; Mason, T.; Tribbick, G.; Schoofs, P. J. *Immunol. Meth.* **1987**, 102, 259.
45. Pinilla, C.; Appel, J. R.; Blanc, P.; Houghten, R. A. *Biotechniques* **1992**, 13, 901.
46. Songyang, Z.; Blechner, S.; Hoagland, N.; Hoekstra, M. F.; Piwnica-Worms, H.; Cantley, L. C. *Curr. Biol.* **1994**, 4, 973.
47. Geysen, H. M.; Melven, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, 81, 3998.
48. Frank, R.; Doring, R. *Tetrahedron Lett.* **1988**, 44, 6031.
49. Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Strter, L.; Lu, A. T.; Solas, D. *Science* **1991**, 251, 767.
50. Gorden, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gallop, M. A. *J. Med. Chem.* **1994**, 37, 1385.
51. Lam, K. S.; Lebl, M.; Krchnak, V.; Wade, S.; Abdul-Latif, F. R. Cuzzocrea, C.; Wertman, K. *Gene* **1993**, 137, 13.
52. Lam, K. S.; Hruby, V. J.; Lebl, M.; Knapp, R. J.; Kazmierski, W. M.; Hersh, E. M.; Salmon, S. E. *Bioorg. Med. Chem. Lett.* **1993**, 3, 419.
53. Lam, K. S.; Lebl, M. *Immunomethods* **1992**, 1, 11.
54. Lam, K. S.; Lou, Q.; Zhao, Z. G.; Smith, J.; Chen, M. L.; Pleshko, E.; Salmon, S. E. *Biomed. Pept., Proteins Nucleic Acids* **1995**, 1, 205.
55. Smith, M. H.; Lam, K. S.; Hersh, E. M.; Lebl, M.; Grimes, W. J. *Mol. Immunol.* **1994**, 3, 419.
56. Salmon, S. E.; Lam, K. S.; Lebl, M.; Kandola, A.; Khattri, P. S.; Wade, S.; Patek, M.; Kocis, P.; Krchnak, V., Thorpe, D.; and Felder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, 90, 11708.
57. Lam, K. S.; Zhao, Z. G.; Wade, S.; Krchnak, V.; Lebl, M. *Drug Dev. Res.* **1994**, 33, 157.
58. Meldal, M.; Svendsen, I.; Bressam, K.; Auzanneau, F. I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, 91, 3314.
59. Boyce, R.; Li, G.; Nestler, H. P.; Suenaga, T.; Still, W. C. *J. Am. Chem. Soc.* **1994**, 116, 7955.
60. Brummel, C. L.; Lee, I. N. W.; Zhou, Y.; Benkovic, S. J.; Winogard, N. *Science* **1994**, 264, 399.
61. Yoon, S. S.; Still, W. C. *Tetrahedron* **1995**, 51, 567.
62. Yu, H.; Chen, J. K.; Feng, S.; Dalgarno, D. C.; Braues, A. W.; Schreiber, S. L. *Cell* **1994**, 76, 933.
63. Kassarian, A.; Schellenberger, V.; Turck, C W. *Pept. Res.* **1993**, 6, 129.
64. Jayawickreme, C. K.; Graminski, G. F.; Quillan, J. M.; Lerner, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, 91, 1614.
65. Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical: Rockford, Illinois, 1984.
66. Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis*; IRL: Oxford, 1989.

(Received in U.S.A. 12 November 1995; accepted 19 December 1995)