BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 69–74 (1998)
ARTICLE NO. RC988912

Epitope Mapping of SHP-1 Monoclonal Antibodies Using Peptide Phage Display

Kishore K. Murthy,[1,2] S-H. Shen, and D. Banville
Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, National Research Council Canada, 6100, Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada

Received June 1, 1998

We have characterized the binding epitopes of four monoclonal antibodies for SHP-1, an SH2 domain containing protein tyrosine phosphatase, using two phage displayed random peptide libraries. Three of the antibodies are directed against the phosphatase domain of the molecule and the fourth is toward the NH2-terminal part of the second SH2 domain. The first two antibodies recognize the sequence N A N Y, amino acid 305 to amino acid 308, numbered in the non haematopoietic form of human SHP-1 sequence. The third antibody binds the sequence P Y W P (amino acids 365 to 368) located toward the middle of the phosphatase domain of the enzyme. The fourth antibody is directed against the first two amino acids, W Y (amino acids 112 and 113), of the second SH2 domain. The specificities of these antibodies are demonstrated by ELISA and western blot using different protein constructs expressed in bacteria. All the antibodies can detect wild type SHP-1, expressed in 293 cells, by western blot analysis, both under denaturing conditions as well as following renaturation. The data presented here show that the antibodies characterized in this study are raised against linear epitopes and suggest that these epitopes are accessible from the outside in the native SHP-1 molecule. © 1998 Academic Press

Transient phosphorylation of proteins on tyrosine residues is an important post translational modification which plays a central role in maintaining normal cellular function. The phosphorylated state of proteins is kept under a tight control by two classes of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (1).

SHP-1 (also known as PTP1C, HCP and SHP)(2-5) is a cytosolic protein tyrosine phosphatase expressed in both hematopoietic and in many nonhematopoietic and some malignant epithelial cells (3,4,6). It is a 68kDa protein with two src Homology-2 (SH2) domains at the amino terminus and a single phosphatase domain at its carboxy terminus. SHP-1 has been implicated in different functions including association with several cytokine and growth factor receptors (7-9), regulating antibody production by inhibiting B cell signalling (10) and a role in hematopoiesis (11,12). The crystal structure of this interesting molecule is yet to be established.

We are using monoclonal antibodies to SHP-1 to gain insight into its structure-function relationship and its role in cell signalling. The use of antibodies in such studies has been documented extensively for a variety of different proteins (13-17). In our laboratory four monoclonal antibodies are currently available and have been characterized in this study.

Several methods are used to characterize antibody epitopes (18). In our study we have used the technique of peptide phage display (19) to map the epitopes recognized by our antibodies. In this approach, a peptide library is constructed at the amino terminus of one of the coat proteins of a filamentous phage and used to determine the peptide involved in the interaction which would represent the epitope against which the antibody is raised. Epitopes of many monoclonal antibodies (18,20-22) as well as the peptides binding to structural domains of several proteins (23-26) have been characterized using this method.

In this paper we present the characterization of four monoclonal antibodies to SHP-1 by peptide phage display. The unique binding peptide sequences have been deduced from the DNA sequence of the binding phages. The specificities of these antibodies are demonstrated by ELISA and western blot analysis using full length SHP-1 as well as deletion constructs of the enzyme. The ability of the antibodies to bind to renatured SHP-1 expressed in 293 cells is also demonstrated giving insight into the structure of the SHP-1 molecule.

[1] To whom correspondence is to be addressed. Fax: (514) 496-5143. E-mail: kishore.murthy@nrc.ca.
[2] Present address: Biophage Inc., 6100, Royalmount Avenue, Montreal H4P 2R2, Canada.

0006-291X/98 $25.00
Copyright © 1998 by Academic Press
All rights of reproduction in any form reserved.

MATERIALS AND METHODS

Antibodies

Four different monoclonal antibodies, 6D41, 3E2, 3E7 and 4E6, generated against the full length SHP-1 molecule are characterized in this study. Antibodies were collected from the supernatants of hybridoma cell cultures and the immunoglobulins purified by protein A affinity chromatography (27).

Library Construction

Peptide phage display libraries were prepared essentially by the method of Christian *et. al.* (28). Two decapeptide libraries, one random (PDL-10R), for which the sequence $(NNK)_{10}$ (where K=G/T) was used as the degenerate oligonucleotide sequence, and the second (PDL-10Y) was biased, containing a tyrosine (TAT) at the third position of the insert sequence were constructed in fUSE-5 vector (kindly provided by Dr. George Smith, University of Missouri, Columbia, MO).

Affinity Panning

The panning method used was essentially as outlined by Sparks and co workers (25). Antibodies (10μg/ml) were immobilized in individual wells of a microtitre plate and panned against both PDL-10R and PDL-10Y libraries. After 3 rounds of panning, specifically bound phages were chosen for DNA sequencing. Phage DNA was sequenced using αS^{35}-dATP and T7 sequencing kit from Pharmacia Biotechnologies (Baie d'Urfe, Quebec, Canada).

Phage ELISA

Individual microtitre wells were coated with the monoclonal antibodies (1μg/ml) followed by blocking of the uncoated surfaces with 1% BSA. Phages eluted from round three of panning were used in this study. Specifically bound phages were detected using polyclonal anti-M13 antibody followed by goat anti rabbit IgG antibody cross-linked to horse radish peroxidase (HRP). Bound HRP was quantitated using tetra methyl benzidine (TMB) and hydrogen peroxide.

Preparation of SHP-1 Constructs

Several deletion constructs of SHP-1 were used in this study. The preparation of these constructs is outlined in an earlier publication (29). The portion of SHP-1 present in the various constructs is given below: SHP-1 - amino acids 1-597; SHP-1 ΔNSH2 (amino acids 107-597); SHP-1 ΔCSH2 (amino acids 1-109, 210-597) and SHP-1 ΔNSH2 ΔCSH2 (amino acids 209-597). The proteins were expressed in E.coli and purified as outlined in the above mentioned publication (29).

For expression in mammalian cells, a construct for expressing wild type SHP-1 was prepared in a vector containing the cytomegalovirus promoter as outlined earlier (30). Plasmid DNA was transfected into 293 cells by the calcium phosphate method and clones stably expressing wild type SHP-1 were selected as described earlier (31).

Western Blot

The various protein constructs of SHP-1 were resolved on a denaturing 10% polyacrylamide gel and transferred to a nitrocellulose membrane according to standard protocols. Individual antibodies were used at a concentration of 1μg/ml and the bound antibody was detected using goat anti mouse IgG antibody-HRP conjugate followed by the ECL system from Amersham.

In the case of SHP-1 expressed in 293 cells, the cellular proteins were solubilized in lysis buffer as outlined earlier (30), centrifuged at 14,000 rpm for 10 min in a microfuge at 4°C and the supernatant collected. An aliquot was taken in SDS sample buffer, the proteins resolved by SDS-PAGE and used for western blot analysis both under standard western blot conditions (as outlined earlier) as well as following renaturation of the proteins on the blot. For renaturation studies, the blot was renatured essentially as outlined by Horiuchi et. al. (32). In brief, the nitrocellulose membrane was incubated in renaturation buffer [50mM HEPES/KOH pH 7.2, 5mM magnesium acetate, 100mM potassium acetate, 3mM dithiothreitol, 30 mg/ml BSA, 0.1% Triton X-100 and 0.3% Tween-20] for 2 days at 4°C on a rocking platform with one change of buffer in-between. The blot was washed 2 times with TBS-T and incubated with the first and second antibodies as outlined above. The bound antibody was detected using the ECL system from Amersham.

RESULTS

Two decapeptide libraries were constructed in the M13 filamentous phage derived vector, fUSE-5. Transformation of these libraries into E.coli by electroporation gave 6×10^8 independent transformants. Sequencing of the library pool did not show any bias in the libraries. The diversity of the libraries were checked by sequencing several ($\approx$50) clones from each library. All the clones had complete inserts and each of these inserts had a different sequence confirming the diversity of these libraries.

Purified antibodies were immobilized in microtitre plates and panned against phages from the two libraries. With every round of panning there was an enrichment of sequences that bound to these antibodies. The specificity of this interaction was monitored by determining the number of phages that bound to BSA coated wells, which would represent the non-specific binding component of this interaction. The progress of specific binding was also followed by ELISA using anti-phage antibody. By round 3 considerable enrichment was observed and hence individual clones were chosen for sequencing.

Several binding clones were sequenced for each of these proteins. Figure 1a lists the amino acid sequence of peptides that are expressed on phages binding to monoclonal antibody 6D41. All the sequences have been aligned and yield a consensus motif **N A X Y**. At position +2, amino acid alanine was the most frequent with serine and glycine being present occasionally. All numbers presented in this paper are with reference to the primary structure of the non hematopoietic form of SHP-1. The amino acid at position +3 was variable. Identical results were obtained using either the random peptide library (PDL-10R) or the decapeptide library biased for a tyrosine at the third position (PDL-10Y). A sequence motif **N A N Y** is found from amino acid 305 to amino acid 308 of SHP-1. This region is in the phosphatase domain of SHP-1 (Figure 1a) suggesting that antibody 6D41 is raised against this portion of the molecule. In all the phages isolated from both libraries, a hydrophobic amino acid is present at position −1. Similarly an isoleucine is present at this

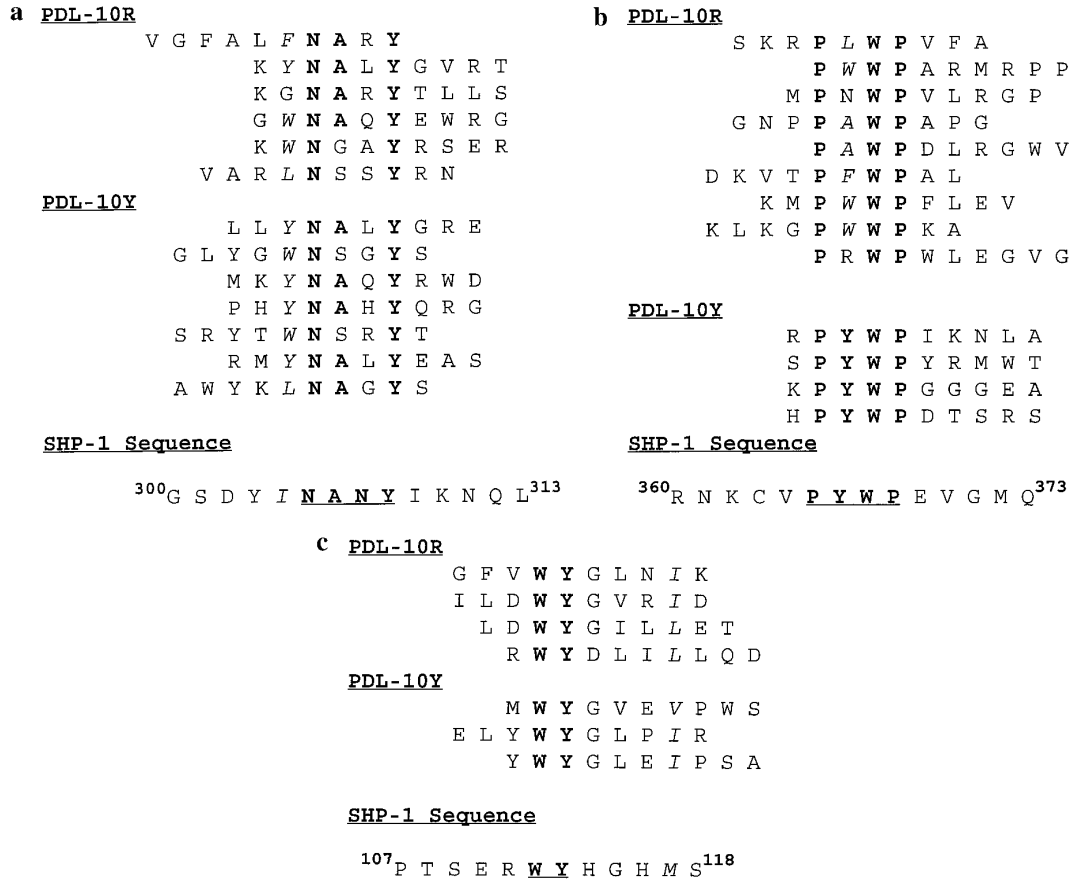

FIG. 1. Alignment of the deduced amino acid sequences of peptides displayed on the phages that bind to monoclonal antibodies 6D41 (a), 3E7 (b) and 4E6 (c). An exact match to a contact residue in SHP-1 molecule is denoted by bold letters. A conservative substitution for a contact residue in the SHP-1 sequence is italicised. The sequence of SHP-1 around this reactive region is also shown in the figure (the numbers refer to the position of the amino acids in the non hematopoietic form of SHP-1 molecule).

position in SHP-1, suggesting the importance of a hydrophobic amino acid at this location in antibody recognition. Similar results were obtained with antibody 3E2 (data not shown).

Peptide sequences that bind to the third monoclonal antibody, 3E7, are shown in Figure 1b. A consensus sequence **P X W P** was obtained using the random decapeptide library with the amino acid at position +2 being hydrophobic in a majority of the clones. Using the PDL-10Y library however, the consensus sequence obtained was **P Y W P**. This sequence is identical to that found from amino acid 365 to amino acid 368 in the phosphatase domain of SHP-1 (Figure 1b). This data suggests that antibody 3E7 is raised against the middle portion of the phosphatase domain of SHP-1.

Figure 1c shows the peptide sequences of clones binding to monoclonal antibody 4E6. Only two amino acids **W Y** were consistently found in all the clones sequenced. A third amino acid glycine was present at position +3 in most of the clones binding to this antibody. Identical consensus sequences were obtained using both the PDL-10R and PDL-10Y libraries. These conserved amino acids, **W Y**, represent amino acids 112 and 113 in the SHP-1 sequence and are the first two amino acids of the C-SH2 domain of this molecule (Figure 1c). A hydrophobic amino acid is present at amino acid +6 of all the clones. Similarly, a hydrophobic amino acid, methionine, is present at this position in SHP1. These data show that antibody 4E6 is directed against the first part of the C-SH2 domain of the molecule.

To demonstrate the specificity of binding, ELISA experiments were carried out. Microtitre plates were coated with each of the four antibodies being studied. Background binding was determined by coating wells with BSA instead of the antibodies. Phages isolated after 3 rounds of affinity panning on each of these antibodies were used in the experiments. Phages isolated by the panning process bound very efficiently to the respective antibodies with negligible background binding to BSA alone (data not shown).

The specificity of each of these antibodies was further

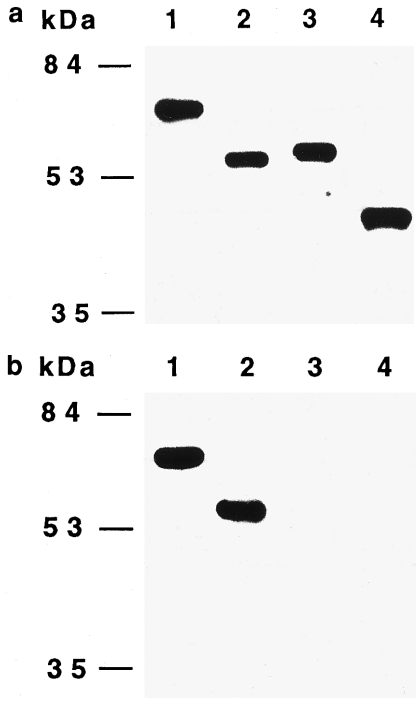

FIG. 2. Recognition of the various fragments of SHP-1 by the different antibodies characterised in this study. Different protein fragments were run on a denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the various antibodies as outlined in the materials and methods section. The proteins loaded in the various lanes are: lane 1, SHP-1; lane 2, SHP-1ΔNSH2; lane 3, SHP-1ΔCSH2 and lane 4, SHP-1ΔNSH2 ΔCSH2. The different antibodies used are (a) 6D41, and (b) 4E6.

established by western blot analysis using truncated SHP-1 proteins. The various constructs used here were: (1) SHP-1, full length (SHP-1), (2) SHP-1 minus N-SH2 (SHP-1ΔNSH2), (3) SHP-1 minus C-SH2 (SHP-1ΔCSH2) and (4) SHP-1 minus both SH2 domains (SHP-1ΔNSH2ΔCSH2). The results obtained with antibodies 6D41 and 3E2 are shown in Fig.2a. Using these antibodies which are directed against the same motif of the SHP-1 molecule (located in the phosphatase domain), all four protein constructs could be detected. Identical results were observed with antibody 3E7 which is raised against the middle portion of the phosphatase domain of the molecule (data not shown).

Antibody 4E6 on the other hand recognises only two constructs, the full length molecule and the one containing the C-SH2 domain of SHP-1 (Fig.2b). This agrees well with the results from affinity panning which showed that this antibody is directed against the first two amino acids of the C-SH2 domain. The antibody does not bind to either of the other two protein constructs which lack this domain. These results clearly establish the specificity of these antibodies towards the different portions of the SHP-1 molecule.

To evaluate if the epitopes identified by these monoclonal antibodies were exposed to the outside, western blot analysis was done using SHP-1 expressed in mammalian cells both directly after SDS-PAGE (protein in denatured state) as well as after renaturing the proteins on the blot. SHP-1 was expressed in 293 cells, the solubilized proteins subjected to SDS-PAGE and the proteins transfered to a nitrocellulose membrane. One blot was probed with the antibodies directly and the second was probed after incubating the blot in renaturation buffer for 2 days (proteins renatured on the blot). The results are shown in Fig.3. Only three of the four antibodies characterized here were used for the renaturation study. Since two of these antibodies, 6D41 and 3E2, recognize the same epitope (**N A N Y**), only antibody 6D41 was used for all further work. Wild type SHP-1 could be detected by all three antibodies under standard western blot conditions (proteins in a denatured state) (data not shown) as well as after renaturation of the proteins on the blot (Fig 3). In addition to SHP-1, antibody 6D41 crossreacts weakly with a 55kDa protein present in the 293 cell lysate (Fig3. lane 1). This band is observed under both denaturing and renaturing conditions. The other two antibodies, 3E7 and 4E6, are very specific and recognize only SHP-1 (Fig. 3, lanes 2 and 3).

DISCUSSION

Peptide phage libraries are now being widely used for characterizing binding epitopes of antibodies. Using this approach has the advantage of providing several sequences with different binding affinities which will help understand the nature of the interaction. Data obtained from these studies also help in studying the importance of different amino acids in the interaction.

Two peptide libraries (PDL-10R and PDL-10Y) were constructed for this study. Both the libraries had a titre of 6×10^8 independent clones. This titre is well within

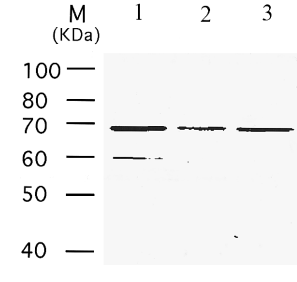

FIG. 3. Recognition of SHP-1 expressed in 293 cells by the different antibodies following renaturation. SHP-1 was expressed in 293 cells, the proteins solubilized in lysis buffer and resolved by SDS-PAGE. The proteins were transfered to nitrocellulose membrane and probed with various antibodies after renaturation of the proteins on the blot as outlined in materials and methods. The antibodies used are: lane 1, 6D41; lane 2, 3E7 and lane 3, 4E6.

the range reported for peptide libraries by several groups (23, 25, 28). The complexity of the libraries was confirmed by sequencing a number of random clones from both preparations.

Three of the four antibodies characterized are directed against the phosphatase domain and one against the first part of the C-SH2 domain of SHP-1. The specificity of these interactions is clearly demonstrated by both ELISA and by Western Blot analysis. In ELISA these antibodies showed negligible binding to BSA. As determined by Western Blot using deletion constructs of SHP-1, these antibodies recognized only fragments of the protein containing the motifs identified by peptide phage display.

To understand the location of the epitopes recognized by these monoclonal antibodies with respect to the secondary structure of SHP-1, western blot analysis was done after renaturation of the proteins. SHP-1 was expressed in 293 cells and used in these studies. The protocol used for protein renaturation on the nitrocellulose membrane was identical to that used earlier by Horiuchi and co-workers (32) for the identification of proteins interacting with GTPase Rab5. In the above study, cellular proteins from HeLa cells separated by SDS-PAGE were renatured on the blot and interacting proteins identified by protein overlay with Rab5 (32). Under these renaturation conditions, all the antibodies recognized SHP-1 suggesting that the three epitopes identified by these antibodies are on the outer surface of the molecule.

Since antibodies are generated against accessible regions of a protein molecule, mapping the binding epitopes gives us an insight into the secondary structure of the protein. Even though the crystal structure of SHP-1 has yet to be worked out, the data from this study could be compared to the crystal structure of other related proteins—the phosphatase domain of PTP1B (33) and the SH2 domains of SHPTP2 (34, 35). Antibodies 6D41 and 3E2 are directed against a sequence **N A N Y** (amino acids 305-308), which is located at the beginning of the phosphatase domain. Based on the crystal structure of PTP1B this corresponds to the region located in the first part of the β strand (β2) just after loop 2 (l2). The amino acids **N** and **A** are conserved between these proteins. In the PTP1B structure this region would be buried with only the last tyrosine being exposed to the solvent. The antibody mapping data presented here would therefore suggest that the secondary structure of the phosphatase domains of SHP-1 and PTP1B are slightly different in this region.

Antibody 3E7 binds the sequence **P Y W P** (amino acids 365-368) present in the middle portion of the phosphatase domain of SHP-1. Amino acids **Y W P** are conserved even in PTP1B and amino acids **Y W** are conserved among many PTPs. In the crystal structure of PTP1B these amino acids are located in loop 8 (l8) between beta sheets β6 and β7, which is located on the outer surface of the molecule. The data with antibody 3E7 suggests that the secondary structure of SHP-1 is similar to that of PTP1B in this region.

Antibody 4E6 recognizes two amino acids **W Y** (amino acids 112, 113) located at the beginning of the C-SH2 domain of SHP-1. This corresponds to the region **W F** (amino acids 112, 113) of SHP-2 which is located in the first part of a β strand, βA. This region appears in a groove between the two SH2 domains in the SHP-2 crystal structure and is exposed to the solvent. The monoclonal antibody data suggests that the structure of the SH2 domains of SHP-1 and SHP-2 are similar in this region. However these comparisons can be verified only when the crystal structure of SHP-1 is solved.

In conclusion, we have characterized the binding epitopes of 4 monoclonal antibodies of SHP-1. Three of these antibodies are directed against the phosphatase domain and the fourth against the first two amino acids of the C-SH2 domain. The data also suggest that these antibodies are raised against linear epitopes that are located on the outer surface of the native SHP-1 molecule and could shed some light on the secondary structure of this protein.

ACKNOWLEDGMENTS

We thank Dr.Marko J.Pregel for the purified SHP-1 proteins, Dr.George Smith, University of Missouri, Columbia, for the fUSE-5 vector and Ms.Jue Wang and Ms.Ming Shen for help with sequencing the phages. This work was supported by the National Research Council of Canada.

REFERENCES

1. Hunter, T. (1995) *Cell* **80,** 225–236.
2. Shen, S-H., Bastien, L., Posner, B., and Chretien, P. (1991) *Nature* **352,** 736–739.
3. Plutzky, J., Neel, B. G., and Rosenberg, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1123–1127.
4. Yi, T., Cleveland, J. L., and Ihle, J. (1992) *Mol. Cell Biol.* **12,** 836–846.
5. Matthews, R. J., Brown, D. B., Flores, E., and Thomas, M. L. (1992) *Mol. Cell Biol.* **12,** 2396–2405.
6. Banville, D., Stocco, R., and Shen, S-H. (1995) *Genomics* **27,** 165–173.
7. Yi, T., and Ihle, J. N. (1993) *Mol. Cell. Biol.* **13,** 3350–3358.
8. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) *Cell* **80,** 729–738.
9. Yi, T., Mui, A. L-F., Krystal, G., and Ihle, J. N. (1993) *Mol. Cell. Biol.* **13,** 7577–7586.
10. DeFranco, A. L., and Law, D. A. (1995) *Science* **268,** 263–264.
11. Shultz, L. D., Schweitzer, P. A., Rajan, T. V., Yi, T., Ihle, J. N., Matthews, R. J., Thomas, M. L., and Beier, D. R. (1993) *Cell* **73,** 1445–1454.
12. Tsui, H. W., Siminowitch, K. A., DeSouza, L., and Tsui, F. W. I. (1993) *Nature Genetics* **4,** 124–128.
13. Kanakura, Y., Cannistra, S. A., Brown, C. B., Nakamura, M.,

Seelig, G. F., Prosise, W. W., Hawkins, J. C., Kaushanky, K., and Griffin, J. D. (1991) *Blood* **77,** 1033–1043.

14. Restle, T., Pawlita, M., Sczakiel, G., Muller, B., and Goody, R. S. (1992) *J. Biol. Chem.* **267,** 14654–14661.
15. Tanaka, S., Matsuda, M., Nagata, S., Kurata, T., Nagashima, K., Shizawa, Y., and Fukui, Y. (1993) *Jpn. J. Cancer Res.* **84,** 279–289.
16. Oldfield, S., Jones, B. L., Tanton, D., and Proud, C. G. (1994) *Eur. J. Biochem.* **221,** 399–410.
17. Moreau, J. L., Bossus, M., de Groote, D., Francois, C., Jaques, Y., Tartar, A., and Theze, J. (1995) *Mol. Immunol.* **32,** 1047–1056.
18. Bottger, V., Stasiak, P. C., Harrison, D. L., Mellerick, D. M., and Lane, E. B. (1995) *Eur. J. Biochem.* **231,** 475–485.
19. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–390.
20. Burritt, J. B., Quinn, M. T., Jutila, M. A., Bond, C. W., and Jesaitis, A. J. (1995) *J. Biol. Chem.* **270,** 16974–16980.
21. Grihalde, N. D., Chen, Y-C. J., Golden, A., Gubbins, E., and Mandecki, W. (1995) *Gene* **166,** 187–195.
22. Stephen, C. W., Helminen, P., and Lane, D. P. (1995) *J. Mol. Biol.* **248,** 58–78.
23. Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G. H., French, S., Howk, R., Ricca, G. A., and Jaye, M. (1994) *J. Biol. Chem.* **269,** 24034–24039.
24. Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S., and Zoller, M. J. (1994) *EMBO J.* **13,** 5598–5604.
25. Sparks, A. B., Quilliam, L. A., Thorn, J. m., Der, C. J., and Kay, B. K. (1994) *J. Biol. Chem.* **269,** 23853–23856.
26. Gui, J., Moyana, T., and Xiang, J. (1996) *Biochem. Biophys. Res. Commun.* **218,** 414–419.
27. Harlow, E., and Lane, D. P. (1988) Antibodies: A Laboratory Manual, pp. 283–318. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
28. Christian, R. B., Zuckermann, R. N., Kerr, J. M., Wang, L., and Malcolm, B. A. (1992) *J. Mol. Biol.* **227,** 711–718.
29. Pregel, M. J., Shen, S-H., and Storer, A. C. (1996) *Protein Eng.* **8,** 1309–1316.
30. Su, L., Zhao, Z., Bouchard, P., Banville, D., Fischer, E. H., Krebs, E. G., and Shen, S-H. (1996) *J. Biol. Chem.* **271,** 10385–10390.
31. Bouchard, P., Zhao, Z., Banville, D., Dumas, F., Fischer, E. H., and Shen, S-H. (1994) *J. Biol. Chem.* **269,** 19585–19589.
32. Horiuchi, H., Lippé, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997) *Cell* **90,** 1149–1159.
33. Barford, D., Flint, A. J., and Tonks, N. K. (1994) *Nature* **263,** 1397–1404.
34. Lee, C-H., Kominos, D., Jaques, S., Margolis, B., Schlessinger, J., Schoelson, S. E., and Kuriyan, J. (1994) *Structure* **2,** 423–438.
35. Eck, M. J., Pluskey, S., Trub, T., Harrison, S. C., and Schoelson, S. E. (1996) *Nature* **379,** 277–280.