Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 31, Issue of August 4, pp. 18195–18197, 1995
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Both p16 and p21 Families of Cyclin-dependent Kinase (CDK) Inhibitors Block the Phosphorylation of Cyclin-dependent Kinases by the CDK-activating Kinase*

(Received for publication, April 5, 1995, and in revised form, May 5, 1995)

Olga Aprelikova‡§, Yue Xiong§¶||, and Edison T. Liu‡§||**

From the Departments of ‡Medicine and ¶Biochemistry and Biophysics, the ||Curriculum in Genetics and Molecular Biology, and the §Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7295

Phosphorylation of cyclin-dependent kinases (CDKs) by the CDK-activating kinase is required for the activation of CDK enzymes. Members of two families of CDK inhibitors, p16/p18 and p21/p27, become physically associated with and inhibit the activity of CDKs in response to a variety of growth-modulating signals. Here, we show that the representative members of both families of CDK inhibitors, $p21^{waf1,cip1}$, $p27^{kip1}$, and p18, can prevent the phosphorylation of their CDK partners, CDK2 and CDK6, by CDK-activating kinase. No direct interaction between CDK-activating kinase and the CDK inhibitors could be detected, suggesting that binding of these CDK inhibitors to CDK subunits renders CDK inaccessible to the CDK-activating kinase phosphorylation. These findings suggest that a general mechanism of CDK inhibitor function is to block the phosphorylation of CDK enzymes by CDK-activating kinase.

The primary control of the eukaryotic cell cycle is provided by a family of serine/threonine protein kinases known as cyclin-dependent kinases (CDKs).[1] The enzymatic activity of a CDK is regulated at three different levels: cyclin binding and activation, subunit phosphorylation, and inhibition by a group of heterologous small proteins. Phosphorylation of Thr-161 (or Thr-160 in CDK2) is required for $p34^{cdc2}$/$p33^{cdk2}$ activation (1, 2). The enzyme that causes the phosphorylation of $p34^{cdc2}$, $p33^{cdk2}$, and perhaps other members of CDK family on Thr-161, termed CDK-activating kinase (CAK) (3–7). In mammalian cells, the catalytic subunit of CAK, CDK7, is associated with and activated by a regulatory subunit, cyclin H (8, 9). CDK activity is also regulated by a number of small proteins that physically associate with cyclins, CDKs, or their complexes. There exist at least two distinct families of CDK inhibitors in mammalian cells: the p21/p27 family and the p16/p18 family. p21 and p27 proteins share considerable sequence homology and have been shown to be a potent inhibitors of almost all cyclin-CDK enzymes (8–18), In contrast, the p16 family of CDK inhibitors specifically interacts with the D-type cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). The p16 family currently includes four isolated genes that share sequence similarity and were evolved from a common ancestor, $p16^{INK4A/MTS1}$ (19), $p14/p15^{MTS2/INK4B}$ (20, 21), *p18* (21), and *p20*.[2] The prototype member of this family, *p16*, is homozygously deleted at a high frequency in a wide variety of human tumor-derived cell lines (23, 24) and is also mutated or deleted in several specific types of primary tumors (25–29).

Direct physical interaction between CDK inhibitors and CDK enzymes is necessary for CDK inhibition, but the precise mechanism remains to be elucidated. We asked whether CDK inhibitors would affect CDK phosphorylation by CAK. Here, we show that CAK phosphorylation of CDK2 is prevented by the CDK inhibitors, p21 and p27, and that the inhibition of CAK activity by p21 or p27 does not appear to involve a direct interaction between CAK and p21 or p27. In addition, we find that the CDK6-specific inhibitor p18 belonging to the p16 family of CDK inhibitors also blocks CAK phosphorylation of CDK6.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture Synchronization—Primary human foreskin fibroblasts (NHF) cultivation, serum starvation, and serum stimulation were performed as described in Ref. 30.

Antibodies to Human CDK7—Polyclonal antibodies were raised in rabbits against C-terminal 131 amino acids (see Ref. 31). Corresponding fragment was PCR-amplified and subcloned into a pQE31 vector (Qiagen, Chatsworth, CA) as a *Bam*HI-*Kpn*I fragment. Antigenic polypeptide was expressed in bacteria as a hexahistidine-tagged recombinant protein and purified according to Qiagen instruction. Antisera was affinity-purified using bacterially expressed antigenic polypeptide bound to Ni^{2+}-NTA-agarose (Qiagen). 1 ml of the antibodies was loaded into the column, the column was extensively washed with phosphate-buffered saline, and antibodies were eluted with 1 ml of ActiSep media (Sterogene, Arcadia, CA) and desalted according to manufacturer's instructions.

Construction of Mutagenic Recombinant CDK6—To replace threonine 177 in GST-CDK6 for alanine, the fragment containing two unique sites (*Sph*I and *Dra*III) was PCR-amplified using forward primer with *Sph*I site: 5′-CGAGGAGGGCATGCCGCTCT-3′ and reverse mutagenic oligonucleotide with *Dra*III restriction site: 5′-GTACCACAGCGTGACGACCACTGAGGCTAGAG-3′. The PCR fragment was digested with *Sph*I and *Dra*III and inserted into *Sph*I/*Dra*III-cut GST-CDK6. The presence of mutation was confirmed by sequencing reaction.

Purification of Recombinant Proteins from Bacteria—GST-cyclins A and D were purified according to Solomon *et al.* (1). GST-CDK2 and GST-CDK6 were purified according to Poon *et al.* (5). Purification of bacterially expressed CDK inhibitors will be described elsewhere.[3]

Kinase Assay—Cell lysates (100 μg of total protein) prepared in Nonidet P-40 lysis buffer (10) were used for immunoprecipitation with anti-CDK7 antibodies. CAK activation of GST-CDK2 and direct phosphorylation of GST-CDK2 were performed as in Ref. 5. To test an effect

* This work was supported by Grant P50-CA-58223 from the National Institutes of Health, NCI (SPORE in breast cancer, to E. T. L., Y. X., and O. A.), and start-up funds from the University of North Carolina at Chapel Hill (to Y. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Leukemia Society Scholar. To whom correspondence should be addressed: CB 7295, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-1352; Fax: 919-966-3015; E-mail: medetl@med.unc.edu.

[1] The abbreviations used are: CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; PCR, polymerase chain reaction; GST, glutathione *S*-transferase.

[2] K. L. Guan, C. W. Jenkins, Y. Li, M. Zariwala, S. Noh, X. Wu, and Y. Xiong, submitted for publication.

[3] D. R. Mayrose, M. A. Nichols, Y. Xiong, and H. Ke, submitted for publication.

This is an Open Access article under the CC BY license.

of CDK inhibitors, 1 μg of GST-CDK2 and 1 μg of GST-cyclin A were mixed with p21 or p27 in amounts indicated on the top of the pictures in a total volume of 20 μl in kinase buffer for 30 min on ice. Then 5 μCi of [γ-^{32}P]ATP was added, and the reaction mixture was transferred to the tube with immunoprecipitated CAK for 10 min at 23 °C, followed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Direct phosphorylation of GST-CDK6 by CAK was performed by incubation of immunoprecipitated CAK (100 μg of total protein) with 0.5 μg of GST-CDK6, 0.5 μg of GST-cyclin D1, and 10 μCi of [γ-^{32}P]ATP for 40–60 min at 30 °C in the kinase buffer. To assay for the activation of CDK6 by CAK, the first step was performed with 1 mM cold ATP as above. Then GST fusion protein with the 137 C-terminal amino acids of Rb (21) was added together with 10 μCi of [γ-^{32}P]ATP for another 20 min at 30 °C.

Direct Effect of p21 and p27 on CDK2 and CAK Activity—To analyze direct effect of CDK inhibitors on CDK2 or CAK (Fig. 1), 100 μg of cell lysates were incubated with indicated amounts of p21 or p27 for 1 h at 4 °C. Then CAK or CDK2 were immunoprecipitated, washed two times with lysis buffer and two times with kinase buffer, and activities were measured using GST-CDK2 as a substrate for the anti-CDK7 immunoprecipitates or histone H1 as a substrate for the anti-CDK2 immunoprecipitates.

RESULTS AND DISCUSSION

Sequence similarity between CAK catalytic subunit CDK7 and CDKs initially prompted us to ask whether CDK7 could also be regulated by the p21 CDK inhibitor. To address this question, we raised polyclonal antibodies against the bacterially expressed C-terminal portion of human CDK7. This antibody was found not to cross-react with different members of CDK family. The anti-CAK immunoprecipitates possess kinase activity toward GST-CDK2.As reported previously (5, 8, 9), CDK2 alone without its associated cyclin partner can be readily phosphorylated by CAK, but the cyclin subunit is necessary for the activation of the CDK2 histone H1 kinase activity.Under our conditions the amount of immunoprecipitated CAK was a limiting factor, therefore phosphorylation of histone H1 by GST-CDK2·GST-cyclin A complex always correlated with phosphorylation of GST-CDK2 by CAK (data not shown).

To test the potential effect of p21 on CAK activity, we first assessed whether p21 interacts with the catalytic subunit of CAK, CDK7, as is the case for other CDKs. Normal human fibroblasts (NHF) synchronized at different phase of the cell cycle by serum starvation and re-stimulation were metabolically labeled with [^{35}S]methionine and immunoprecipitated with antibody specific to CDK7. This experiment did not reveal the presence of a 21-kDa protein in anti-CDK7 immunoprecipitates. Furthermore, when anti-p21 immunoprecipitates were probed with antibodies against CDK7 we found no detectable CDK7 protein in asynchronously growing fibroblasts. Similarly, when anti-CDK7 immunoprecipates were probed with antibodies against p21, we found no p21 protein in fibroblasts synchronized by serum starvation and restimulation. The same results were also obtained in human lymphocytes stimulated to proliferate by PHA and IL-2 treatment (data not shown).

Since it remained possible that the failure to detect the presumptive p21-CAK association may have resulted from low levels of CDK7 expression or from antibody disruption, we tested the potential direct interaction of p21 and CAK by assaying the activity of CAK in presence of high quantities of bacterially expressed p21. In these experiments we also used a related CDK inhibitor p27. When added to NHF cell lysates, purified recombinant p21 and p27 effectively inhibited the histone H1 kinase activity of immunoprecipitated CDK2 (Fig. 1*A*). In contrast, no inhibition of CAK kinase activity (as assessed by CDK2 phosphorylation) by p21 or p27 was detected (Fig. 1*B*). Thus, from these data, we believe that neither p21 nor p27 physically interact with CAK.

Although p21 and p27 do not appear to interact with CAK directly, it is still possible that the binding of CDK inhibitors with a CDK may render the threonine at position 160 (in CDK2, or 161 in CDC2) inaccessible to the CAK enzyme, thereby preventing the phosphorylation and activation of the CDK. To test this possibility, we assembled the CDK2-cyclin A complex *in vitro* using bacterially expressed and purified GST-CDK2 and GST-cyclin A and used this complex to determine possible effect of p21 and p27 on CDK2 phosphorylation by CAK. Intriguingly, the addition of the p21 or p27 proteins to the CDK2-cyclin A complex dramatically decreased CDK2 phosphorylation by CAK (Fig. 2, *A* and *B*). To further address the specificity of p21 and p27 inhibition of CDK2 phosphorylation by CAK, and to exclude the possibility that this inhibition may be caused by a trace amount of *Escherichia coli* proteins copurifying with the p21 or p27 proteins, we tested the effect of another newly identified CDK inhibitor, p18, on CDK2 phosphorylation by CAK. p18 is a homolog of p16 that strongly inhibits the activity of CDK6 and weakly inhibits that of CDK4, but exhibits no detectable interaction with four other CDKs including CDK2 (21). When we applied a recombinant p18 protein that was prepared in a manner similar to that for p21 and p27, no inhibition of CDK2 phosphorylation by CAK was detected (Fig. 2*C*). The same result was obtained when we added bovine serum albumin instead of CDK inhibitors in the reaction (Fig. 2*D*). These results indicate that inhibition of CDK2 phosphorylation by CAK is dependent on p21/p27 and not on p18.

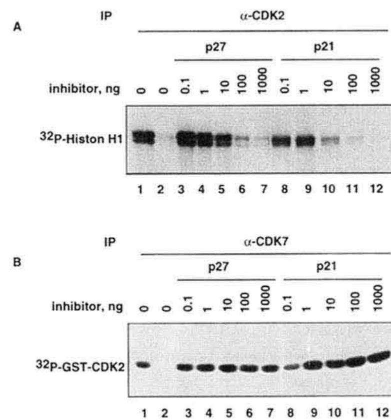

FIG. 1. **p21 and p27 do not directly inhibit CAK activity.** Extracts of NHF cells were incubated with indicated quantities of p21 or p27 and immunoprecipitated with anti-CDK2 (*A*) or anti-CDK7 (*B*) antibodies. The kinase activity of immunoprecipitated CDK2 was measured by histone H1 phosphorylation (*A*), and the kinase activity of CAK was measured by GST-CDK2 phosphorylation (*B*). *Lanes 1* of both *A* and *B* show the immunoprecipitated CDK2 or CAK activities from the lysates in the absence of CDK inhibitors. In *lanes 2* of both *A* and *B*, anti-CDK2 or anti-CDK7 antibodies were blocked with correspondent antigenic peptides before immunoprecipitation. Of note is the fact that none of the CDK inhibitors block the immunoprecipitation of CDK2.

The differences between two families of CDK inhibitors raise an important question of whether the p16 family of CDK inhibitors can also block the CAK phosphorylation on CDK. In order to analyze the effect of the p16/p18 family of inhibitors on CDK phosphorylation by CAK, we first determined whether the bacterially expressed GST-CDK6 protein could be phosphorylated and activated by CAK/CDK7. Unlike CDK2 activation that has been well studied, no data about phosphorylation and activation of CDK6 is available. Fig. 3 (*top panel*) shows that in the absence of immunoprecipitated CAK, no phosphorylation of CDK6 takes place. Some phosphorylation of GST-CDK6 can be detected when the recombinant CDK6 is coincubated with CAK in the absence of cyclin. However, the addition of GST-cyclin D1 enhances CAK phosphorylation of CDK6 and renders the CDK6 enzymatically active in the phosphorylation of the Rb

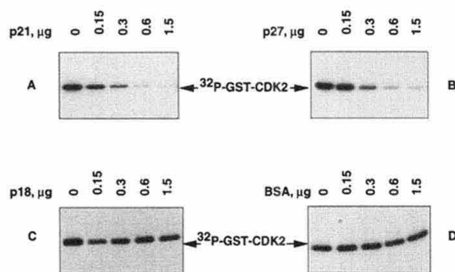

FIG. 2. **p21 and p27 proteins inhibit CDK2 phosphorylation by CAK.** Complexes of GST-CDK2, GST-cyclin A, and different amounts of p21 (*A*) or p27 (*B*) were used as a substrates for CAK immunoprecipitated from NHF cells. p18 (*C*) or BSA (*D*) were mixed with GST-CDK2·GST-cyclin A instead of p21/p27, showing no effect on CDK2 phosphorylation.

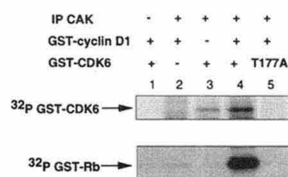

FIG. 3. **Immunoprecipitated CAK phosphorylates GST-CDK6 and activates its Rb kinase activity.** *Top panel*, immunopreciptated CAK was incubated with [γ-^{32}P]ATP and GST-CDK6, in the absence (*lane 3*) or presence (*lane 4*) of GST-cyclin D1 and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. *Bottom panel*, immunoprecipitated CAK was incubated in the presence of cold ATP and GST-CDK6, with or without cyclin D1. The reaction was then assayed for kinase activity using a purified GST-Rb protein as substrate. In *lanes 5* of both panels, a GST-CDK6 protein mutated at threonine 177 (threonine → alanine, GST-CDK6 T177A) was used.

protein (Fig. 3, *bottom panel*). To prove that CDK6 phosphorylation by CAK occurs at the unique site at threonine at position 177 that is homologous to Thr-160 in CDK2 and Thr-161 in CDC2, we used site-directed mutagenesis to replace Thr-177 by Ala. When GST-CDK6 T177A was treated with immunoprecipitated CAK, no phosphorylation of the mutant CDK6 protein (Fig. 3, *top panel*) or Rb kinase activity (Fig. 3, *bottom panel*) was detected. These data show that CDK6 is activated through phosphorylation by CAK, and that this phosphorylation is enhanced by cyclin D1.

We then tested the CDK6-specific inhibitor p18, a homolog of p16 (21), for its effect on CDK6 phosphorylation by CAK. In our experiment, the p18 protein and GST-cyclin D1 were first mixed, then added to GST-CDK6. After 30 min of incubation, the resultant complex was mixed with immunoprecipitated CAK and assessed for CAK-dependent phosphorylation. Fig. 4 (*top panel*) shows that p18 efficiently blocked the CDK6 phosphorylation by CAK. When recombinant Rb protein was added to the CDK6/p18/cyclin D mixture after CAK activation, no Rb phosphorylation was found (Fig. 4, *bottom panel*). These data show that, like CDK2, CDK6 activation by CAK is also regulated by p18 CDK inhibitor. Thus, we demonstrate that both families of CDK inhibitors block CDK phosphorylation and activation by CAK and that this block is most likely through the same mechanism: by rendering the CDKs inaccessible to CAK.

During the preparation of this manuscript, Kato *et al.* (7) reported that in mouse macrophages addition of cyclic AMP (cAMP) resulted in growth arrest in mid-G_1 phase, where CDK4 exerts its effect on Rb phosphorylation. The study of events that take place after cAMP treatment revealed that induction of the p27 CDK inhibitor blocked the CDK4 activation. Similar to our results, the authors did not find direct interaction of p27 with CAK; thus the inhibition of CDK activation by p27 bound to CDK4-cyclin D1 complex was inferred. Taken together, these data suggest that, in addition to the block of CDK phosphorylation of downstream substrates such as Rb or histone H1, another general mechanism by which CDK inhibitors regulate the cell cycle is by preventing CDK phosphorylation and activation by CAK.

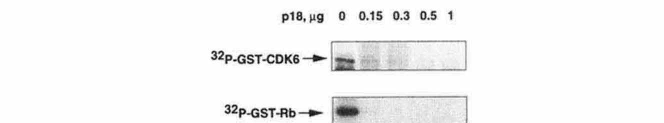

FIG. 4. **p18 inhibits GST-CDK6 phosphorylation by CAK.** In the *top panel*, increasing amounts of p18 were pre-mixed with GST-cyclin D1 and then added to GST-CDK6. This complex was then incubated with immunoprecipitated CAK showing inhibition of CDK6 phosphorylation by p18. In the *bottom panel*, Rb phosphorylation in presence of p18 was tested.

Acknowledgments—We are grateful to Rolf Craven for critical reading of the manuscript and to Aalok Kuthiala and Edward Baptist for help with experiments.

REFERENCES

1. Solomon, M. J., Lee, T., and Kirschner, M. W. (1992) *Mol. Biol. Cell* **3,** 13–27
2. Desai, D., Gu, Y., and Morgan, D. (1992) *Mol. Biol. Cell* **3,** 571–582
3. Fesquet, D., Labbe, J. C., Derancourt, J., Capony, J. P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J. C. (1993) *EMBO J.* **12,** 3111–3121
4. Solomon, M. J., Harper, J. W., and Shuttleworth, J. (1993) *EMBO J.* **12,** 3133–3142
5. Poon, R. Y., Yamashita, K., Adamczewski, J. P., Hunt, T., and Shuttleworth, J. (1993) *EMBO J.* **12,** 3123–3132
6. Shuttleworth, J., Godfrey, R., and Colman, A. (1990) *EMBO J.* **9,** 3233–3240
7. Kato, J., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. (1994) *Cell* **79,** 487–496
8. Fisher, R. P., and Morgan, D. O. (1994) *Cell* **78,** 713–724
9. Mäkelä, T. P., Tassan, J.-P., Nigg, E. A., Frutiger, S., Hughes, G. J., and Weinberg, R. A. (1994) *Nature* **371,** 254–257
10. Gu, Y., Turck, C. W., and Morgan, D. O. (1993) *Nature* **366,** 707–710
11. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75,** 805–816
12. Xiong, Y., Hannon, G., Zhang, H., Casso, D., Kobayashi, R., and Beach, D.(1993) *Nature* **366,** 701–704
13. Zhang, H., Hannon, G. J., and Beach, D. (1994) *Genes & Dev.* **8,** 1750–1758
14. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Lin, D. M., Mercer, W. E., Kinzler, K. W. V., and Vogelstein, B. (1993) *Cell* **75,** 817–825
15. Polyak, K., Kato, J., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. (1994) *Genes & Dev.* **8,** 9–22
16. Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J., Tempst, P., and Massague, J. (1994) *Cell* **78,** 59–66
17. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. (1994) *Cell* **76,** 1013–1023
18. Toyoshima, H., and Hunter, T. (1994) *Cell* **78,** 67–74
19. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366,** 704–707
20. Hannon, G. J., and Beach, D. (1994) *Nature* **371,** 257–261
21. Guan, K.-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994) *Genes & Dev.* **8,** 2939–2952
22. Deleted in proof
23. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavitgian, S. V., Stockert, E., Day, R. S., Johnson, B. E., and Skolnick, M. H. (1994) *Science* **264,** 436–440
24. Nobori, T., Mlura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. (1994) *Nature* **368,** 753–756
25. Mori, T., Miura, K., Aoki, T., Nishihira, T., Shozo, N., and Nakamura, Y. (1994) *Cancer Res.* **54,** 3396–3397
26. Spruck, C. H., III, Gonzalez-Zulueta, M., Shibata, A., Simoneau, A. R., Lin, M.-F., Gonzales, F., Tsai, Y. C., and Jones, P. A. (1994) *Nature* **370,** 183–184
27. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., Clark, W. H., Tucker, M. A., and Dracopoli, N. C. (1994) *Nature Genet.* **8,** 15–21
28. Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., McClure, M., Aitken, J. F., Anderson, D. E., Bergman, W., Frants, R., Goldgar, D. E., Green, A., MacLennan, R., Martin, N. G., Meyer, L. J., Youl, P., Zone, J. J., Skolnick, M. H., and Cannon-Albright, L. A. (1994) *Nature Genet.* **8,** 22–26
29. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. (1994) *Nature Genet.* **8,** 27–32
30. Li, Y., Jenkins, C. W., Nichols, M. A., and Xiong, Y. (1994) *Oncogene* **9,** 2261–2268
31. Levedakou, E. N., He, M., Baptist, E. W., Craven, R. J., Cance, W. G., Welcsh, P. L., Simmons, A., Naylor, S. L., Leach, R. J., Lewis, T. B., Bowcock, A., and Liu, E. T. (1994) *Oncogene* **9,** 1977–1988