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Inhibition of Protein Serine/Threonine Phosphatases by Fumonisin B_1, a Mycotoxin

Hirokazu Fukuda,* Hiroshi Shima,* Ronald F. Vesonder,† Harukuni Tokuda,‡ Hoyoku Nishino,‡ Seishi Katoh,§ Shinri Tamura,¶ Takashi Sugimura,* and Minako Nagao*,[1]

**Carcinogenesis Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku Tokyo 104, Japan; †Mycotoxin Research, National Center for Agricultural Utilization Research, USDA, Peoria, Illinois 61604; ‡Department of Biochemistry, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku Kyoto 602, Japan; §Sagami Chemical Research Center, 4-4-1 Nishi-Ohnuma, Sagamihara, Kanagawa 229, Japan; and ¶Department of Biochemistry, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-77, Japan*

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Fumonisin B_1 (FB_1), a mycotoxin produced by the fungus *Fusarium moniliforme,* which is a common contaminant of corn, is suspected to be a cause of human esophageal cancer. FB_1 is hepatotoxic and hepatocarcinogenic in rats, and although the mechanisms involved have not been clarified, the latter is associated with a weak initiating activity. The effects of FB_1 on the activity of protein serine/threonine phosphatases (PPs) (PP1, PP2A, PP2B, PP2C and PP5/T/K/H) were investigated in the present study. Inhibition of dephosphorylation was noted for all five PPs with IC_{50} values of 80 μM−3000 μM. Among the five PPs examined, PP5 was most sensitive with an IC_{50} of 80 μM. This concentration is comparable to that estimated to be reached in the rat body by feeding FB_1 to obtain hepatic tumors. Inhibition of PP5 could thus play important roles in the toxicity and carcinogenic action of FB_1.

Fumonisin B_1 (FB_1) is a mycotoxin which can be isolated from corn contaminated with *Fusarium moniliforme* (1, 2, 3) and from culture of this fungus (4, 5). FB_1 is hepatotoxic and hepatocarcinogenic in rats (6, 7) and a causative agent for equine leukoencephalitis (8) and porcine pulmonary edema syndrome (9). Further, it is suspected to be associated with the high prevalence of human esophageal cancer in southern Africa and China (10–12). FB_1 is, however, not mutagenic in the Salmonella assay (13) and proved negative in a DNA repair assay using primary rat hepatocytes (14). These data coincide well with the established characteristics of FB_1 regarding rat hepatocarcinogenesis and mouse skin carcinogenesis; weak initiating activity but strong promotion (15, Tokuda *et al.*, unpublished results). FB_1 is similar to sphingosine in structure and inhibits the activity of sphingosine *N*-acyltransferase that results in accumulation of sphingoid bases in Swiss 3T3 fibroblasts and rat hepatocytes (16, 17). Although the possibility that the inhibition of sphingosine biosynthesis plays some role in the FB_1 cancer-promotion cannot be excluded, little is known about the mechanism of FB_1 carcinogenesis. It should be noted, however, that there are some similarities between FB_1 and okadaic acid (OA), an inhibitor of protein phosphatases (PPs), in biological activities. Both are tumor promoters in two-stage carcinogenesis experiments in mouse skin initiated with a single application of 7,12-dimethylbenzanthracene (18); they are not mutagenic in Salmonella; they are negative for induction of Epstein-Barr virus early antigen (EBV-EA) and activation of protein kinase C, differing from the typical tumor promoter, 13-*O*-tetradecanoyl phorbol acetate (18, Tokuda et al., unpublished results). These findings motivated us to analyze the effects of FB_1 on PP activities, including PP1, PP2A, PP2B, PP2C and PP5/T/K/H.[2]

[1] To whom correspondence should be addressed. Fax: 81-3-5565-1753.

[2] We isolated a novel protein serine/threonine phosphatase from rat brain cDNA library and named it PPH. However, before publication of our paper on PPH (19), details of the same enzyme had been published and the name PP5/T/K has been used in literature (24–26). Therefore, in this paper PP5 was used to refer to the enzyme identical to that described in our previous paper (19).

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MATERIALS AND METHODS

Enzymes. PP2A and PP2B were purchased from Seikagaku Corp. (Tokyo). PP2Cα and PP1γ2, an isotype of the PP1 catalytic subunit, were bacterially expressed and purified (20,21). PP5 was expressed in *Escherichia coli* as a GST-fusion protein containing the region from Met^{107} to Met^{499} (C-terminal) of rat PP5, lacking 106 amino acids of the N-terminal part, and purified (Fukuda et al., unpublished results). FB_1 was purified as described (3).

Phosphorylase a phosphatase assays. The ^{32}P-labeled phosphorylase *a*, a substrate for PPs, was prepared essentially according to MacKintosh (22) using phosphorylase kinase (Life Technologies, Inc., Buckinghamshire), glycogen phosphorylase *b* (Life Technologies, Inc.), and [γ-^{32}P] ATP (Amersham, Gaithersburg). The standard dephosphorylation reaction assay was performed as follows: Ten microliters of protein phosphatase in TEMB buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 1 mg/ml BSA) were mixed with 10 μl of ^{32}P-labeled phosphorylase a in buffer B (50 mM Tris-HCl pH 7.5, 0.1% 2-mercaptoethanol) (final 1 mg/ml) and 10 μl of FB_1 in TEMB buffer (final concentrations were as indicated in Fig. 1). The final concentrations of PP1, PP2A, PP2B, PP2C and PP5 were 0.029 mU/ml, 0.021 mU/ml, 0.015 mU/ml, 0.059 mU/ml, and 0.022 mU/ml, respectively. The reaction of PP2C was performed in the presence of 10 mM $MgCl_2$. To obtain full enzyme activity, PP2B was activated by preincubation in TEMB buffer containing 50 mM $MgCl_2$, 5 mM $MnCl_2$, and 0.3 mM Na_3VO_4 at room temperature for 30 min and then added to the reaction mixture. After incubation of the mixture at 30°C for 20 min, the reaction was stopped by adding 100 μl of ice cold 25% trichloroacetic acid. Samples were incubated on ice for 10 min, centrifuged at 15,000 × g for 5 min and then the radioactivity of the supernatant was measured in a liquid scintillation counter (Beckman LS 1801, Palo Alto).

Histone H1 phosphatase assays. The ^{32}P-labeled histone H1 was prepared by phosphorylation with cdc2 kinase as follows: 250 μg of histone H1 (Life Technologies, Inc.) was incubated with 50 ng of cdc2 kinase (Seikagaku Corp.) and 4 μl of [γ-^{32}P] ATP (Amersham) at 30°C for 4 h in 100 μl of the buffer (10 mM 2-sodium glycerophosphate pH 7.0, 0.4 mM EDTA, 0.2 mM EGTA, 0.2 mM ATP, 2.5 mM $Mg(OAc)_2$). The reaction was stopped by adding 10 μl of the stop solution (100 mM EDTA, 500 mM NaF, 10 mM sodium pyrophosphate) and incubating at 30°C for 10 min. After the mixture was centrifuged at 15,000 × g for 1h, the supernatant was applied to a Sephadex G-50 NICK column (Pharmacia Biotech Inc., Uppsala) equilibrated with buffer B and the histone H1 fractions was obtained. The dephosphorylation reaction assay was performed essentially according to the method for the phosphorylase a phosphatase assays with the following modifications: The final concentrations of the substrate and PP5 were 0.35 mg/ml and 0.061 mU/ml, respectively. Just before adding 100 μl of ice cold 25% trichloroacetate acid to stop the reaction, 2 μl of 10 mg/ml BSA was added as a carrier protein.

RESULTS AND DISCUSSION

PPs have been classified into four major groups: PP1, PP2A, PP2B, and PP2C (23). The effects of FB_1 on the phosphorylase a phosphatase activity of PP1, PP2A, PP2B, PP2C and PP5, which is a novel type PP isolated by ourselves (19) and others (24–26), were examined. The enzyme preparations used were as follows; the heterodimer of A and C subunits of PP2A purified from human red blood cells, the calcineurin (PP2B) catalytic subunit purified from bovine brain, the bacterially expressed PP2Cα isozyme and PP1γ2, an isotype of PP1 catalytic subunit (20, 21), and a GST-PP5 fusion protein expressed in *Escherichia coli* (Fukuda *et al.*, unpublished results).

FB_1 inhibited all five PPs tested (Fig. 1). Among these, PP5 was most sensitive with an IC_{50} of 80 μM (Fig. 1B). FB_1 inhibited PP2Cα, PP2A, PP1γ2 and PP2B with IC_{50} values of 300 μM, 400 μM, 500 μM and 3000 μM, respectively (Fig. 1). These results suggest the possibility that PP5 is a biochemical target for the cancer initiating and promoting effects of FB_1. The IC_{50} of 80 μM is much high compared with the IC_{50} values for other PP-inhibitors such as okadaic acid, calyculin A and microcystin LR acting on PP1 and PP2A. It is interesting that PP2C, which is not inhibited by OA, microcystin LR or trifluoperazine (27), was inhibited by FB_1 as much as PP1 or PP2A. Because the enzyme-specificity of FB_1 was relatively low, it remained a possibility that FB_1 inhibited PPs by the specific binding to phosphorylase a. Thus we investigated the effect of FB_1 on dephosphorylation of another substrate, histone H1 labeled by cdc2 kinase. FB_1 inhibited the dephosphorylation of histone H1 catalyzed by PP5 with IC_{50} value of 150 μM (Fig. 2A). This result excludes the possibility that FB_1 inhibits PPs only when phosphorylase a is used as a substrate.

Okadaic acid has so far not been reported to have tumor initiating activity, in contrast to the weak action found for FB_1: In a short-term carcinogenesis study FB_1 induced enzyme-altered foci in the

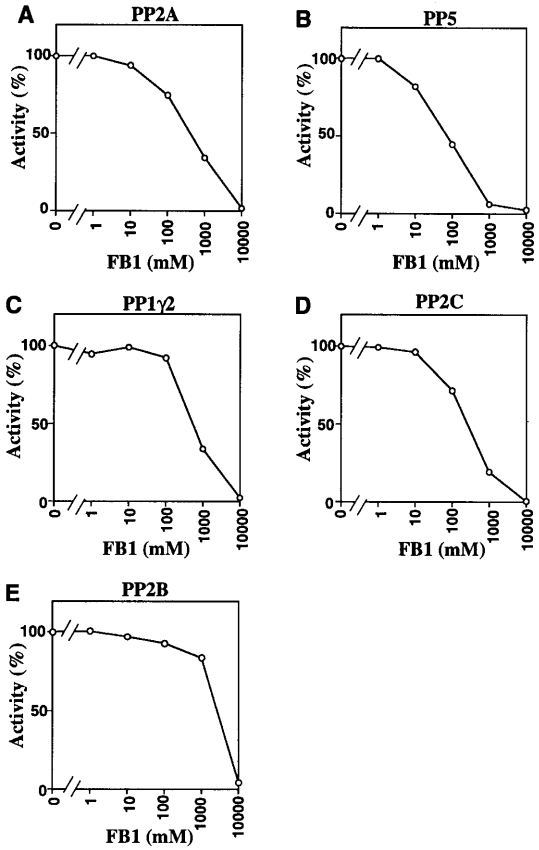

FIG. 1. Inhibition by fumonisin B_1 of PPs. PP2A (A), PP5 (B), PP1γ2 (C), PP2C (D), and PP2B (E). The concentrations of PP2A, PP5, PP1γ2, PP2C, and PP2B were 0.021, 0.022, 0.029, 0.059, and 0.015 mU/ml, respectively. Activity was measured *in vitro* using phosphorylase *a* as a substrate, as described in the text. Each point represents the mean of three separate experiments.

BDIX rat liver after 26 days-feeding at a dietary level of 0.1% (14), and in a long-term carcinogenesis study FB_1 induced hepatocellular carcinomas in BDIX rats at 18–26 months when given at a dietary level of 0.005% (6). If it is assumed that all FB_1 given to rats in feeding studies (1–10 mg) (28–30) become distributed uniformly throughout the body, the achieved concentrations could be between 10 and 100 μM (17). Further, other biological effects of FB_1 have been reported; it

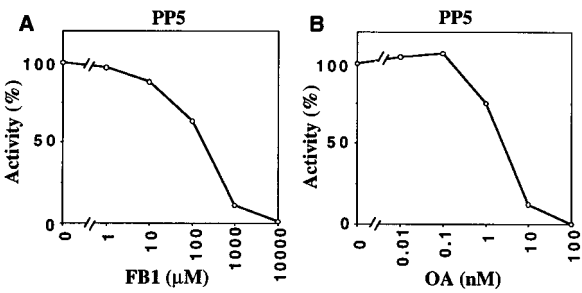

FIG. 2. Inhibition by fumonisin B_1 (A) and okadaic acid (B) of PP5 using ^{32}P-labeled histone H1 as a substrate. The concentration of PP5 was 0.061 mU/ml. Activity was measured *in vitro* as described in the text. Each point represents the mean of the two separate experiments.

inhibits epidermal growth factor (EGF)-induced DNA synthesis *in vitro* in rat hepatocytes up to 90% at concentrations of 150 to 300 μM (31) and it stimulates DNA synthesis in Swiss 3T3 fibroblasts at concentrations of 10 to 50 μM (16). These concentrations are in line with the lC50 value for PP5 on the whole, suggesting that PP5 may be a target for FB_1, leading to various biological responses.

OA has various effects on mammalian cells, causing flattening of NIH 3T3 cell transformed by *raf* and *ret*-II (32), diphtheria toxin-resistant mutations (33), sister chromatid exchanges (34), aneuploidy (35), gene amplification (36) and loss of transfected genes (19). These data suggest that inhibition of protein phosphatases by OA induces genomic instability. The PP2A catalytic subunit is much more sensitive to OA (lC50 of 0.04 nM) than PP1 (lC50 of 50 nM) or PP2B (lC50 of 5000 nM). We recently showed that OA resistant CHO-mutants (CHO-OAR) have a mutation in the PP2A catalytic subunit (19, 37, 38), but also expressed a high level of the MDR protein that pumps out OA. Thus, so far we could not clarify yet which PP is involved in maintaining genome stability. There remains a possibility that other PP(s) are involved in the instability and enhanced tumor development caused by OA. The phosphorylase a phosphatase activity (0.4 mU in control) of rat PPT (PP5) was found to be fully suppressed in the presence of 11 μM OA (24). Our phosphatase samples of PP5 and the heterodimer of A and C subunits of PP2A showed responses that coincided well with those reported earlier, with lC50 of 5 nM (0.022mU/ml) and 0.05 nM (0.021 mM/ml), respectively (Fig. 3). OA also inhibited the histone H1 phosphatase activity of PP5 with lC50 of 3 nM (Fig. 2B). PP5 is also known to be inhibited by OA in an enzyme-concentration dependent manner, as observed with PP2A, and the cellular amount of the PP5 molecule was estimated to be 5- to 10-fold less than that of PP2A (24). Thus the possibility that PP5 is a target of OA for induction of genomic instability cannot be excluded completely. PP4, another novel type PP, is sensitive to OA with lC50 of 0.2 nM (0.076 mU/ml) (39) and is also a possible target of OA. Further, it is known yet whether PP5 exists as monomer, homomultimer or heteromultimer, and sensitivity to OA of the most common form of PP5 in cells is not clear. This is also the case for PP5 inhibition by FB_1. One of the possible mechanisms of FB_1 tumor initiating and promoting activity is that it induces genetic alterations through inhibition of PP5. We are now examining effects of FB_1 in this direction.

The function of PP5 is unknown, but it has the tetratricopeptide repeat (TPR) motifs often detected in nuclear cell-division gene products, and proteins regulating RNA synthesis, as well as receptor-associated, and stress-inducible proteins (26, 40). The TRP motifs are suspected to provide a suitable interface for protein-protein interaction (40). FB_1 represses the expression of protein kinase C, stimulates a simple promoter containing a cyclic AMP response element (41), exerts a mitoinhibitory effect on rat hepatocytes (31) and is mitogenic in mouse fibroblasts Swiss 3T3 (16). It is thus conceivable that it acts on PP5 to alter the function of this phosphatase in signal transduction pathways, and that this disruption cause predisposing to cancer.

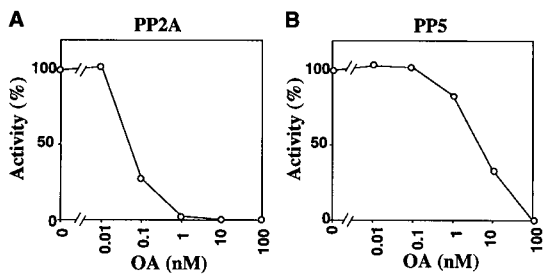

FIG. 3. Inhibition by okadaic acid of PPs. PP2A (A) and PP5 (B). The concentrations of these enzymes were 0.021 (PP2A) and 0.022 mU/ml (PP5), respectively. Activity was measured *in vitro* using phosphorylase *a* as a substrate as described in the text. Each point represents the mean of three separate experiments.

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REFERENCES

1. Voss, K. A., Norred, W. P., Plattner, P. D., and Bacon, C. W. (1989) *Food Chem. Toxicol.* **27,** 89–96.
2. Sydenham, E. W., Gelderblom, W. C. A., Theil, P. G., and Marasas, W. F. O. (1990) *J. Agric. Food Chem.* **38,** 285–290.
3. Vesonder, R., Peterson, R., Plattner, R., and Weisleder, D. (1990) *Mycotoxin Res.* **8,** 85–87.
4. Ross, F. F., Nelson, P. E., Richard, J. L., Osweiler, G. D., Dice, L. G., Plattner, R. D., and Wilson, T. M. (1990) *Appl. Environ. Microbiol.* **56,** 3225–3226.
5. Bezuidehaut, S. C., Gelderblom, W. C. A., Gorst-Allman, C. P., Horak, R. M., Marasas, W. F. O., Spiteller, G., and Vleggaar, R. (1988) *Onderstepoort J. Vet. Res.* **55,** 197–203.
6. Gelderblom, W. C. A., Kriek, N. P. J., Marasas, W. F. O., and Thiel, P. G. (1991) *Carcinogenesis* **12,** 1247–1251.
7. Gerderblom, W. C. A., Jaskiewiz, K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vleggaar, R., and Kriek, N. P. J. (1988) *Carcinogenesis.* **9,** 1405–1409.
8. Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C. A., Coetzer, J. A. W., Thiel, P. G., and Van der Lugt, J. J. (1988) *Onderstepoor J. Vet. Res.* **55,** 197–203.
9. Harrison, L. R., Colvin, B., Greene, J. T., Newman, L. E., and Cole, J. R. (1990) *J. Vet. Diagn. Invest.* **2,** 217–221.
10. Lin, M., Lu, S., Ji, C., Wang, M., Cheng, S., and Tian, G. (1980) *in* Genetics and Environmental Factors in Experimental and Human Cancer (Gelboin H. V., Ed.), pp. 139–148, Japan Scientific Societies Press, Tokyo.
11. Yang, C. S. (1980) *Cancer Res.* **40,** 2633–2644.
12. Marasas, W. F. O., (1982) *in* Cancer of the Oesophagus (Pfeiffer C. J., Ed.), Vol. 1, pp. 29–40, CRC Press, Boca Raton, FL.
13. Gelderblom, W. C. A., and Snyman, S. D. (1991) *Mycotoxin Res.* **7,** 46–52.
14. Gelderblom, W. C. A., Semple, E., Marasas, W. F. O., and Farber, E. (1992) *Carcinogenesis.* **13,** 433–437.
15. Gelderblom, W. C. A., Jaskiewicz, K., Marasas, W. F. O., Thiel, P. G., Horak, R. M., Vleggaar, R., and Kriek, N. J. P. (1988) *Appl. Environ. Microbiol.* **54,** 1806–1811.
16. Schroeder, J. J., Crane, H. M., Xia, J. X., Loitta, D. C., and Merrill, A. H. (1994) *J. Biol. Chem.* **269,** 3475–3481.
17. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. Jr. (1991) *J. Biol. Chem.*, **266,** 14486–14490.
18. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Yamada, K., and Sugimura, T. (1988) *Proc. Natl. Acad. Sci. USA.* **85,** 1768–1771.
19. Nagao, M., Shima, H., Nakayasu, M., and Sugimura, T. (1995) *Mutat. Res.* **333,** 173–179.
20. Fukunaga, K., Kobayashi, T., Tamura, S., and Miyamoto, E. (1993) *J. Biol. Chem.* **268,** 133–137.
21. Zang, Z., Bai, Z., Shima, H., Zhao, S., Nagao, M., and Lee, E. Y. C. (1993) *Arch. Biochem. Biophys.* **303,** 402–406.
22. MacKintosh, C. (1993) *in* Protein Phosphorylation: A Practical Approach (Hardie, D. G., Ed.), Oxford Univ. Press, Oxford.
23. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508.
24. Becker, W., Kentrup, H., Klumpp, S., Schultz, J. E., and Joost, H. G. (1994) *J. Biol. Chem.* **269,** 22586–22592.
25. Chen, M. X., MaPartlin, A. E., Brown, L., Chen, Y. H., Barker, H. M., and Cohen, P. T. W. (1994) *EMBO J.* **13,** 4278–4290.
26. Chinkers, M. (1994) *Proc. Natl. Acad. Sci. USA.* **91,** 11075–11079.
27. Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256,** 283–290.
28. Voss, K. A., Plattner, R. D., Bacon, C. W., and Norred, W. P. (1990) *Mycopathologia.* **112,** 81–92.
29. Jaskiewicz, K., van Rensburg, S. J., Marasas, W. F., and Gelderblom, W. C. (1987) *J. Natl. Cancer Inst.* **78,** 321–325.
30. Marasas, W. F. O., Kriek, N. P. J., Fincham, J. E., and van Rensburg, S. J. (1984) *Int. J. Cancer.* **34,** 383–387.
31. Gelderblom, W. C. A., Snyman, S. D., van der Westhuizen, L., and Marasas, W. F. O. (1995) *Carcinogenesis.* **16,** 625–631.
32. Sakai, R., Ikeda, I., Kitani, H., Fujiki, H., Takaku, F., Rapp, U., Sugimura, T., and Nagao, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9946–9950.
33. Aonuma, S., Ushijima, T., Nakayasu, M., Shima, H., Sugimura, T., and Nagao, M. (1991) *Mutat. Res.* **250,** 375–381.
34. Tohda, H., Nagao, M., Sugimura, T., and Oikawa, A. (1993) *Mutat. Res.* **289,** 275–280.

35. Afshari, C. A., Kodama, S., Bivins, H. M., Willard, T. B., Fujiki, H., and Barrett, J. C. (1993) *Cancer Res.* **53,** 1777–1782.
36. Wang, S.-J., Scavetta, R., Lenz, H.-J., Danenberg, K., Danenberg, P. V., and Schöthal, A. H. (1995) *Carcinogenesis.* **53,** 637–641.
37. Sikorski, R. S., Michaud, W. A., and Hieter, P. (1993) *Mol. Cell. Biol.* **13,** 1212–1221.
38. Shima, H., Tohda, H., Aonuma, S., Nakayasu, M., DePaoli-Roach, A. A., Sugimura, T., and Nagao, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9267–9271.
39. Brewis, N. D., Street, A. J., Prescott, A. R., and Cohen, P. T. W. (1993) *EMBO J.* **12,** 987–996.
40. Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., and House, A. K. (1993) *J. Biol. Chem.* **268,** 13287–13192.
41. Huang, C., Dickman, M., Henderson, G., and Jones, C. (1995) *Cancer Res.* **55,** 1655–1659.