Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 440-447

REGULATION OF PROTEIN PHOSPHOTYROSINE CONTENT BY CHANGES IN TYROSINE KINASE AND PROTEIN PHOSPHOTYROSINE PHOSPHATASE ACTIVITIES DURING INDUCED GRANULOCYTIC AND MONOCYTIC DIFFERENTIATION OF HL-60 LEUKEMIA CELLS

David A. Frank and Alan C. Sartorelli

Department of Pharmacology and Developmental Therapeutics Program,
Comprehensive Cancer Center,
Yale University School of Medicine,
New Haven, CT 06510

Received September 10, 1986

Summary: About 1.5% of phosphorylated amino acid residues of HL-60 promyelocytic leukemia cells are phosphotyrosine. Induction of granulocytic differentiation by exposure to dimethylsulfoxide decreased tyrosine phosphorylation to 0.2%. A maximum 3-fold increase in tyrosine kinase activity and a 7-fold increase in protein phosphotyrosine phosphatase activity accompanied this change. Monocytic differentiation induced by 12-O-tetradecanoylphorbol-13-acetate, caused a decrease in phosphotyrosine levels to 0.1%; tyrosine kinase activity maximally increased 2-fold, and protein phosphotyrosine phosphatase activity increased 11-fold in these differentiated cells. Thus, although total tyrosine kinase activity markedly increased during differentiation, this was counteracted by an even greater elevation in protein phosphotyrosine phosphatase activity. The findings support the concept that tyrosine phosphorylation is important in the regulation of growth and differentiation of leukemia cells.

Tyrosine phosphorylation has been shown to be intimately involved in cellular responses to growth factors (1), embryogenesis (2), and tumorigenesis induced by several oncogenic viruses (3). Thus, phosphotyrosine residues may be central intracellular mediators of signals for proliferation and differentiation. The HL-60 promyelocytic leukemia represents an excellent model system for studying differentiation (4), since these cells, which grow predominantly as promyelocytes in suspension culture, can be induced to mature along the granulocytic (5) or monocytic (6) pathways. The differentiation process is terminal, involving the programmed shut down of cellular replicative capacity. Since tyrosine phosphorylation appears to be inextricably linked to cellular growth, we have measured the effects of the granulocytic inducer of differentiation DMSO and the monocytic inducer TPA on protein phosphotyrosine content of HL-60 cells, and on tyrosine kinase and protein phosphotyrosine phosphatase enzyme activities

Abbreviations: DMSO, dimethylsulfoxide; TPA, 12-O-tetradecanoylphorbol-13-acetate; GAT, poly(glutamate:alanine:tyrosine, 6:3:1); Hepes, (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); NBT, nitroblue tetrazolium; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's medium; TCA, trichloroacetic acid; EGF, epidermal growth factor; PNPPase, p-nitrophenylphosphatase.

0006-291X/86 $1.50

involved in regulating the intracellular quantity of protein phosphotyrosine residues during the maturation process.

Materials and Methods

GAT, Hepes, and TPA were obtained from Sigma Chemical Co., St. Louis, MO. [γ-^{32}P]ATP and [^{32}P]orthophosphate were purchased from Amersham Corp., Arlington Heights, IL.

Culture conditions: HL-60 promyelocytic leukemia cells were a gift from Dr. R. C. Gallo, National Cancer Institute. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56°C for 30 min) FBS at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells were seeded at a level of 2 x 10^5 cells/ml, and were allowed to attain a maximum density of 1.5 x 10^6 cells/ml before being passed into fresh medium. All studies employed cells that were between passages 32 and 60. Under these conditions, HL-60 cells doubled in 32 hr and reached a plateau at a concentration of 2.5 x 10^6 cells/ml. A431 cells were obtained from the American Type Culture Collection (Rockville, MD); they were seeded at 0.5 to 1 x 10^6 cells/100 cm^2 and were grown in DMEM supplemented with 10% (v/v) FBS in a humidified 90% air/10% CO_2 incubator at 37°C. Cells were routinely removed from the flask by exposure to 0.05% trypsin and subcultured before reaching confluence.

Induction of differentiation: HL-60 leukemia cells were treated with either DMSO (1.2%, v/v) or TPA (10^{-8} M). A stock solution of 10^{-3} M TPA was prepared in either ethanol or DMSO and stored at -20°C. The final vehicle concentration in TPA treated cultures was 0.001% (v/v); neither ethanol nor DMSO affected cell growth or differentiation at this concentration. Granulocytic differentiation was measured by the ability of cells to reduce nitroblue tetrazolium (7). Monocytic differentiation was determined by the percentage of cells containing non-specific esterase activity (8).

Leukocyte separation: Human blood was fractionated using LSM (Litton Bionetics, Kensington, MD). Monocytes were purified from the mononuclear layer by adhesion to plastic (9); neutrophils were separated from red blood cells by a combination of dextran sedimentation (10) and hypotonic lysis (11).

Cell fractionation: HL-60 cells, human neutrophils, or monocytes (4 to 5 x 10^7) were collected, washed twice in phosphate-buffered saline (8 g/l NaCl; 0.2 g/l KCl; 1.8 g/l $Na_2HPO_4.7H_2O$; 0.2 g/l KH_2PO_4), resuspended at a level of 10^7 cells/ml in buffer A (5 mM Hepes (pH 7.4), 1 mM $MgCl_2$ and 1 mM EDTA) and disrupted with a Branson sonicator (Danbury, CT) using two 10-second bursts at a setting of 20. Nuclei and unbroken cells were removed by low speed centrifugation (1000 x g for 10 min) and the supernatant was centrifuged at 30,000 x g for 30 min. The resulting supernatant was designated the soluble fraction. The pellet was resuspended in 0.3 ml of buffer B (25 mM Hepes (pH 7.4), 5 mM 2-mercaptoethanol and 0.1% (v/v) Nonidet P-40), shaken vigorously and centrifuged for 5 minutes at 12,000 x g in a microcentrifuge. The resulting supernatant was designated the particulate fraction. Protein was determined by the method of Bradford (12) using bovine serum albumin as a control.

Phosphoaminoacid analysis: Quantitation of phosphoaminoacids was performed according to the method of Cooper et al. (13).

Tyrosine kinase assay: Tyrosine phosphorylation of artificial substrates was measured by a modification of the method of Braun et al. (14). Briefly, 10 to 20 µg of protein was added to the tyrosine kinase buffer containing 20 mM Hepes (pH 7.4), 12 mM $MnCl_2$, 10 µM $ZnCl_2$, and 0.5% (v/v) Nonidet P-40 with or without GAT (1 mg/ml) as substrate. After 3 min at 22°C, the reaction was initiated by the addition of 16.7 µM [γ-^{32}P]ATP (3 Ci/mmol). The reaction was terminated by the addition of 7 µl of unlabeled ATP (10 mM). Fifty µl of the mixture was then applied to a 1 cm x 1 cm square of Whatman 3 MM filter paper, which was washed and counted as described by Corbin and Reimann (15), except that the TCA washes contained 10 mM sodium pyrophosphate. Net phosphorylation represented the difference between tubes with and without GAT.

Protein phosphotyrosine phosphatase assay: Protein phosphotyrosine phosphatase was measured using a modification of the method of Shriner and Brautigan (16). The substrate [Tyr(^{32}P)]GAT was prepared by incubating 20 to 30 µg of A431 cell particulate fraction protein with 1 to 2 mg of GAT, 10^{-7}M EGF (Collaborative Research, Lexington, MA) and 25 µM Na_3VO_4 in a total volume of 1 ml of tyrosine kinase buffer. The reaction

was allowed to proceed overnight at 22°C and was terminated by the addition of 111 µl of 100% TCA. A precipitate was allowed to form at 4°C for 1 hour and the pellet was collected by centrifugation in a microcentrifuge at 12,000 x g for 5 min. The pellet was washed three times in 10% TCA at room temperature and was solubilized in 100 µl of 1 M NaOH. Two ml of protein phosphotyrosine phosphatase buffer (50 mM Hepes (pH 7.0) and 25 mM 2-mercaptoethanol) were added and the solution was diafiltrated repeatedly (10,000 MW cutoff; Amicon Corp., Danvers, MA) at 5,000 x g for 30 min until the ^{32}P in the filtrate was $<1\%$ of that of the retentate. The retentate was then collected and designated the protein phosphotyrosine phosphatase substrate.

Protein phosphotyrosine phosphatase activity was measured by the release of [^{32}P]orthophosphate from [Tyr(^{32}P)]GAT in a 50 µl reaction mixture containing substrate and 10 to 20 µg of protein in protein phosphotyrosine phosphatase buffer containing 0.5 mg/ml of bovine serum albumin at 37°C. The reaction was initiated by the addition of the substrate and was allowed to proceed for one minute, at which time the reaction was terminated by the addition of 50 µl of 20% TCA. The tube was mixed well, chilled at 4°C for 5 min, and centrifuged at 12,000 x g for 5 min. Fifty µl of the supernatant was mixed with 5 ml of Hydrofluor (National Diagnostics, Sommerville, NJ) and radioactivity therein determined with a scintillation spectrometer. Specific activity was represented as the difference in the amount of radioactivity released in the presence and absence of protein.

Results

Changes in protein phosphotyrosine content occurred as a result of chemically induced granulocytic and monocytic differentiation. When HL-60 leukemia cells were treated with 1.2% DMSO over a course of 7 days they attained the characteristics of mature granulocytes, as measured by their capacity to reduce NBT to insoluble formazan granules (Fig. 1). Over this time period, phosphotyrosine residues decreased from 1.5 $\pm$ 0.2% of total phosphorylated amino acids to 0.2 $\pm$ 0.1%, with phosphoserine and phosphothreonine making up the remainder in a ratio of approximately 9:1 (Table 1). Monocytic differentiation was induced in these cells by treatment with 10^{-8}M TPA for 2 to 3 days and was measured by the acquisition of non-specific esterase activity (Fig. 1). After three days of exposure to TPA, 60% of the cells displayed this differentiation marker and the phosphotyrosine content of the mature population decreased from the control level of 1.5 $\pm$ 0.2% in untreated cells to 0.1 $\pm$ 0.1%.

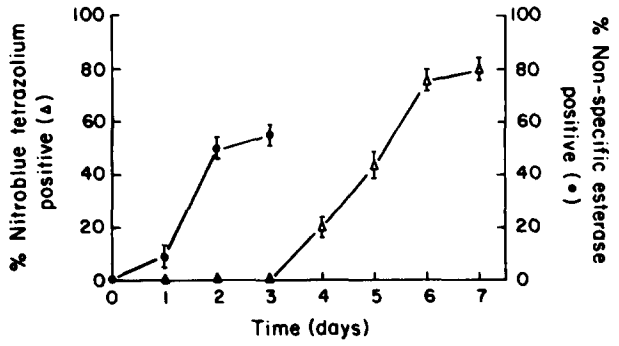

Figure 1. Differentiation of HL-60 leukemia cells treated with DMSO or TPA. Cells were exposed to 1.2% (v/v) DMSO (△) or 10^{-8} M TPA (●) and the ability of cells to reduce nitroblue tetrazolium or the presence of non-specific esterase activity was used to assess granulocytic and monocytic maturation, respectively. Each point represents the mean $\pm$ standard deviation of 3 separate experiments.

Table 1

Phosphoaminoacid Content of HL-60 Leukemia Cells

Treatment	P-TYR	P-SER	P-THR
None	1.5 ± 0.2	88.2 ± 0.9	10.3 ± 0.4
DMSO	0.2 ± 0.1	89.4 ± 1.1	10.4 ± 0.4
TPA	0.1 ± 0.1	89.4 ± 1.1	10.5 ± 0.5

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser) and phosphothreonine (P-Thr). HL-60 cells were treated with DMSO (1.2%, v/v) for 7 days or TPA (10^{-8} M) for 3 days, and the phosphoaminoacid distribution was determined as described in the Methods section. Values represent the means ± standard deviation of 3 separate determinations.

Changes in tyrosine kinase activity occurred with the attainment of the differentiated state. Greater than 90% of cellular tyrosine kinase activity was found in the particulate fraction of untreated and all drug-treated HL-60 cells. Tyrosine kinase activity increased approximately 3-fold during granulocytic differentiation (Fig. 2), and doubled as a result of monocytic differentiation (Fig. 3). Given the seemingly paradoxical increase in tyrosine kinase activity in the presence of a fall in phosphotyrosine content during cellular maturation, the level of protein phosphotyrosine phosphatase activity was determined in HL-60 cells treated with DMSO or TPA. Marked increases in protein

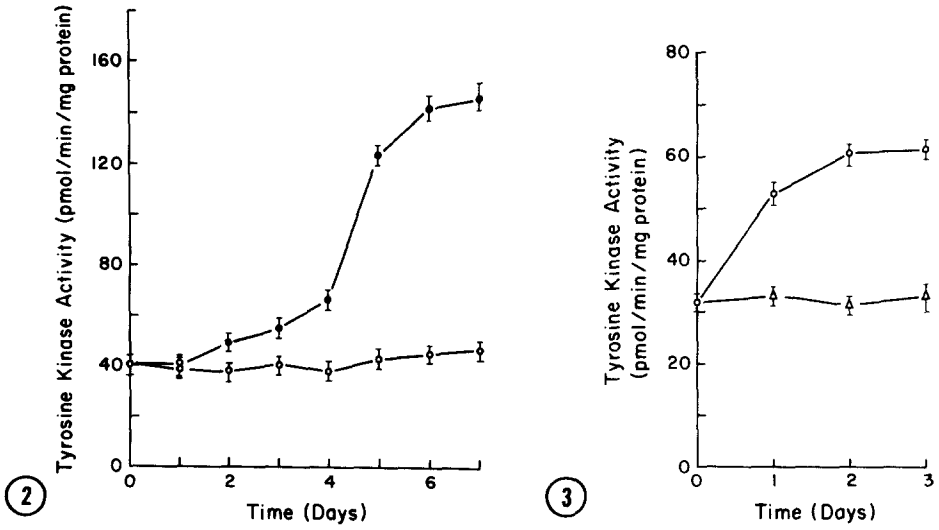

Figure 2. Tyrosine kinase activity of HL-60 leukemia cells treated with DMSO. Cells were cultured in the presence of 1.2% DMSO (v/v, ●) or were untreated (O), and tyrosine kinase activity was measured at various times. Each point represents the mean ± standard deviation of 3 separate experiments, each done in duplicate.

Figure 3. Tyrosine kinase activity of HL-60 leuekmia cells treated with TPA. Cells were cultured in the presence (O) or absence (△) of 10^{-8}M TPA, and tyrosine kinase activity was measured at various times. Each point represents the mean ± standard deviation of 3 separate experiments, each done in duplicate.

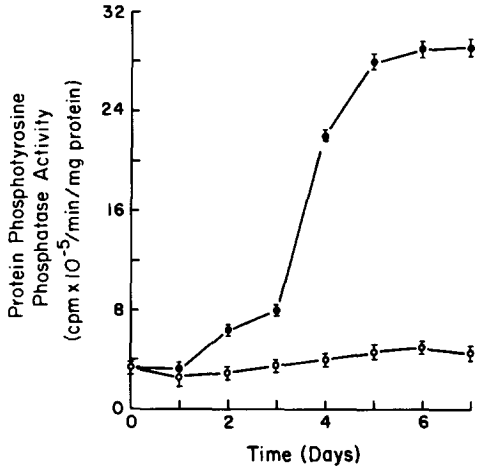

Figure 4. Phosphotyrosine phosphatase activity of HL-60 cells treated with DMSO. Cells were cultured in the presence (●) or absence (○) of 1.2% DMSO, and phosphotyrosine phosphatase activity was measured at various times. The specific activity of the [Tyr(^{32}P)]GAT was 5.2 x 10^6 dpm/mg. Each point represents the mean ± standard deviation of 3 separate experiments, each done in duplicate.

phosphotyrosine phosphatase activity also occurred with differentiation. Using release of [^{32}P]orthophosphate from [Tyr(^{32}P)]GAT as a measure of activity, >90% of this enzymatic activity was isolated in the particulate fraction. Granulocytic maturation was accompanied by a 7-fold increase in protein phosphotyrosine phosphatase activity (Fig. 4), and monocytic differentiation by an 11-fold increase in this enzymatic activity (Fig. 5). These changes preceded functional differentiation as measured by NBT positivity by approximately one day in both granulocytic and monocytic maturation.

To assess how these activities compared to those found in normal peripheral blood granulocytes and monocytes, human blood was separated into appropriate fractions. Mature granulocytes contained approximately 20% the tyrosine kinase activity of DMSO-

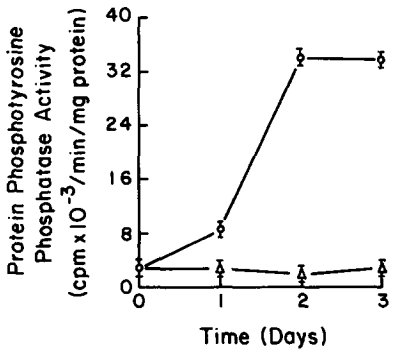

Figure 5. Phosphotyrosine phosphatase activity of HL-60 cells treated with TPA. Cells were cultured in the presence (○) or absence (△) of 10^{-8}M TPA, and phosphotyrosine phosphatase activity was measured at various times. The specific activity of the [Tyr(^{32}P)]GAT was 8.6 x 10^3 dmp/mg. Each point represents the mean ± standard deviation of 3 separate experiments, each done in duplicate.

Table 2

Tyrosine Kinase and Protein Phosphotyrosine Phosphatae Activities of Peripheral Blood Granulocytes and Monocytes and of HL-60 Cells

Cell type	Tyrosine kinae activity (pmol/min/mg)	Phosphotyrosine phosphatase activity (cpm x 10^3/min/mg)
Peripheral blood granulocytes	23.8	12.4
Peripheral blood monocytes	71.0	12.1
HL-60, untreated	39.4	3.6
HL-60, granulocytic	114.6	26.4
HL-60, monocytic	72.8	32.8

Mature peripheral blood granulocytes and monocytes were isolated and purified as described in the text, and tyrosine kinase and phosphotyrosine phosphatase activities were measured. These were compared to those of HL-60 cells induced to mature along the granulocytic [1.2% DMSO (v/v) for 6 days] or monocytic (10^{-8} TPA for 3 days) pathways. Values represent the mean of 3 separate experiments each done in duplicate; the range was less than 5% of the mean.

treated HL-60 cells and about 50% of the phosphotyrosine phosphatase activity; peripheral blood monocytes contained nearly equal levels of tyrosine kinase, but only 40% of the phosphotyrosine phosphatase activity of TPA-treated HL-60 cells (Table 2).

Discussion

The finding that many of the acutely transforming retroviruses are able to cause tumors by virtue of their tyrosine kinase activity (17) provided the first evidence that this unique protein modification has profound effects on cellular function. The importance of this activity was highlighted further by the discovery that a number of growth factor receptors appear to transmit the signal of ligand binding by activating tyrosine kinase activities (1). Thus, a biochemical basis for the link between growth factors and transformation was established. Terminal differentiation may be considered to represent a reversal of the transformation process, in that cells with an unlimited life span acquire a finite capacity to proliferate. For this reason, it appeared to be worthwhile to explore the control of tyrosine phosphorylation in a system undergoing terminal differentiation.

As early as 1978, work by Graf et al. (18) suggested that the presence of an active oncogene product, now known to be a tyrosine kinase, could block erythroid differentiation. Using a temperature sensitive mutant, avian erythroblastosis virus infected erythroblasts were transformed at the permissive temperature; at the non-permissive temperature, a differentiated phenotype, measured by hemoglobin synthesis, was induced (18). The present study sought to determine how phosphotyrosine levels and the activities of the enzymes which regulate these levels would be modulated with the differentiation process. HL-60 leukemia cells were used for this purpose, since these cells are bipotent in

their differentiation capacity and, thereby, serve as an excellent model for maturation specific changes. The initial observation of an order of magnitude decrease in protein phosphotyrosine residues with both granulocytic and monocytic differentiation appeared to be consistent with the concept that tyrosine phosphorylation was intimately related to cellular proliferation. Since terminal differentiation includes as part of the maturation process the programmed shut down of proliferative capacity, a decrease in total phosphotyrosine levels did not appear unreasonable. The finding that tyrosine kinase activity increased significantly during the progression of cells along both pathways of differentiation (also, recently reported by Earp et al. (19)), however, seemed paradoxical given the decrease in cellular phosphotyrosine content of mature cells. The finding that protein phosphotyrosine phosphatase activity rose to a greater extent than tyrosine kinase activity with the attainment of the mature state appeared to explain the decrease in cellular phosphotyrosine levels in differentiated cells (20). Evidence with temperature sensitive mutants (21) has suggested that the phosphate group linked to tyrosine turns over rapidly, as would be expected for a critical modulator of function, and that protein phosphotyrosine phosphatase must be active in these cells. Since critical substrates for tyrosine kinases have not yet been identified in the present studies, it is difficult to conclude that a given change in these enzyme activities is critical for the commitment to enter a differentiation pathway. Nonetheless, the major increase in protein phosphotyrosine phosphatase activity suggests that this activity is the critical factor in modulating the level of protein phosphotyrosine residues in differentiating HL-60 cells. Given this model, protein phosphotyrosine phosphatase enzymes would also fulfill the criteria for a product of the recently described recessive oncogenes (22). The attainment of homozygosity at such a locus is generally associated with the loss of genetic material and the loss of an active gene product (23). Thus, the loss of protein phosphotyrosine phosphatase activity in a cell with normal tyrosine kinase activity could lead to an unbalanced accumulation of protein phosphotyrosine residues on critical substrates, with resultant transformation by such a recessive mechanism. Although a disparity exists in the enzyme levels between mature peripheral blood cells and differentiated HL-60 cells, one consistent finding was that phosphotyrosine phosphatase activity was always much higher in mature cells than in undifferentiated HL-60 cells, suggesting that this change is important for maturation. The differences in tyrosine kinase activity are less easily explained, and may reflect changes in subcellular compartmentalization or changes in intracellular substrate concentrations. The results of the present study support the concept that protein phosphotyrosine phosphatase activity is an important element in the regulation of both proliferation and differentiation.

Acknowledgements

This research was supported in part by U.S. Public Health Service Grant CA-02817 from the National Cancer Institute.

References

1. Heldin, C.-H., and Westermark, B. (1984) Cell 37, 9-20.
2. Dasgupta, J. D., and Gaibers, D. L. (1983) J. Biol. Chem. 258, 6174-6178.

3. Bishop, J. M. (1985) Cell 42, 23-38.
4. Collins, S. S., Gallo, R. C., and Gallagher, R. E. (1977) Nature 270, 347-349.
5. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2458-2462.
6. Rovera, G., Santoli, D., and Damski, C. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2779-2783.
7. Collins, S. J., Bodner, A., Ting, R., and Gallo, R. C. (1980) Intl. J. Cancer 25, 213-218.
8. Yam, L. T., Li, C. Y., and Crosby, W. H. (1971) Am. J. Clin. Path. 55, 283-290.
9. Gamba-Vitalo, C., Gallicchio, V. S., Watts, T. D., and Chen, M. G. (1983) Exptl. Hematol. 11, 382-388.
10. Skoog, W. A., and Beck, W. S. (1956) Blood 11, 436-454.
11. Fallon, H. J., Frei, III, E., Davidson, J. D., Trier, J. S., and Burk, D. (1962) J. Lab. Clin. Med. 62, 779-791.
12. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
13. Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) Meth. Enzymol. 99, 387-405.
14. Braun, S., Raymond, W. E., and Racker, E. (1984) J. Biol. Chem. 259, 2051-2054.
15. Corbin, F. D., and Reimann, E. H. (1974) Meth. Enzymol. 38, 287-290.
16. Shriner, C. L., and Brautigan, D. L. (1984) J. Biol. Chem. 259, 11383-11390.
17. Hunter, T., and Sefton, B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1311-1315.
18. Graf, T., Ade, N., and Beug, H. (1978) Nature 275, 496-501.
19. Earp, H. S., Austin, K. S., Gillespie, G. Y., Buessow, S. C., Davies, A. A., and Parker, P. J. (1985) J. Biol. Chem. 260, 4351-4356.
20. Frank, D. A., and Sartorelli, A. C. (1985) J. Cell Biol. 101, 238a.
21. Martin, G. S. (1970) Nature 227, 1021-1023.
22. Cavenee, W. K., Hansen, M. F., Nordenskjold, M., Kock, E., Maumenee, I., Squire, J. A., Phillips,. R. A., and Gallie, B. L. (1985) Science 228, 501-503.
23. Fearon, E. R., Feinberg, A. P., Hamilton, S. H., and Vogelstein, B. (1985) Nature 318, 377-380.