Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 377-384

REDUCTION OF EPIDERMAL GROWTH FACTOR RECEPTOR AFFINITY BY HETEROLOGOUS LIGANDS: EVIDENCE FOR A MECHANISM INVOLVING THE BREAKDOWN OF PHOSPHOINOSITIDES AND THE ACTIVATION OF PROTEIN KINASE C

Kenneth D. Brown*, Jonathan Blay*, Robin F. Irvine*,
John P. Heslop† and Michael J. Berridge†

*A.F.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

†A.F.R.C. Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, U.K.

Received July 23, 1984

The tetradecapeptide bombesin converts epidermal growth factor (EGF) receptors on Swiss 3T3 cells from a high affinity state (K_D = 9.8 x 10^{-11}M) to a lower affinity state (K_D = 1.8 x 10^{-9}M). This conversion occurs when the cells are incubated with bombesin at 37°C but not when incubated at 4°C. Previously, a number of other (chemically unrelated) cell growth-promoting peptides and polypeptides have been shown to induce a similar indirect, temperature-dependent reduction of EGF receptor affinity. We have now demonstrated that hormones and growth factors which cross-regulate EGF receptor affinity in Swiss 3T3 cells have a common ability to stimulate the breakdown of phosphoinositides in these cells. We propose that the reduction of EGF receptor affinity is a consequence of the activation of protein kinase C by the diacylglycerol generated by this breakdown. In support of this proposal we have found that exogenously added diacylglycerol reduces the affinity of the Swiss 3T3 cell EGF receptor.

Various agents including platelet-derived growth factor (PDGF) and related polypeptides (1-7) and vasopressin (8), and the tumour-promoting agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (9-11) inhibit the binding of ^{125}I-labelled epidermal growth factor to its receptors on Swiss 3T3 fibroblasts. In all cases the inhibition is due to a reduction in EGF receptor affinity with no change in the number of cellular EGF receptors (2-10). The mechanism by which this reduction in receptor affinity occurs is unknown, but the mechanism is indirect and secondary to the binding of heterologous ligand to its own cellular receptor (2-11). PDGF, vasopressin or TPA are all able to individually synergise with insulin to promote DNA synthesis and proliferation of Swiss 3T3 cells (12, 13) and recently, the peptide bombesin has been shown to have a similar ability (14). We now report that bombesin also reduces the affinity of the EGF receptor of Swiss 3T3 cells.

Abbreviations

EGF, epidermal growth factor; IP_3, inositol 1,4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; IP_1, inositol 1-phosphate; PDGF, platelet-derived growth factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; protein kinase C, Ca^{++}-activated, phospholipid dependent protein kinase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

0006-291X/84 $1.50

Studies on calcium-mobilizing agonists, including vasopressin, have established that their primary action is to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generating diacylglycerol which activates protein kinase C and inositol 1,4,5-trisphosphate (IP_3) which releases calcium from intracellular stores (for reviews see 15-17). Recently it has been shown that TPA, and related tumour promotors, bind to and directly activate (18-21) protein kinase C. In this report we demonstrate that PDGF, vasopressin, and bombesin, at mitogenic concentrations, all stimulate phosphatidylinositol metabolism in Swiss 3T3 cells with the consequent generation of IP_3 and diacylglycerol. We therefore propose that the reduction in 3T3 cell EGF receptor affinity brought about by these diverse agents may be due to activation of protein kinase C.

MATERIALS & METHODS

Materials. Culture medium, antibiotics, trypsin and newborn calf serum were from Flow Laboratories. $Na^{125}I$ and myo[^{3}H]inositol were from Amersham International. EGF was purified from male mouse submaxillary glands (22) and ^{125}I-EGF was prepared as described (6). Bombesin was purchased from Sigma and 1-oleoyl-2-acetylglycerol was a generous gift from Prof. Y. Nishizuka (Kobe, Japan).

Cell cultures. Stock cultures of Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn-calf serum as described (6). For experimental use cells were seeded into 3-cm dishes (Nunc) and grown for at least 6 days.

^{125}I-EGF binding assay. Confluent cultures of cells were washed with 1-2 ml of binding medium which consisted of DME containing 0.1% crystalline bovine serum albumin, 0.1 μM-KI and 50 mM-Bes [N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid] adjusted to pH 7.0. The cells were incubated at 4°C or 37°C with 1 ml of binding medium containing ^{125}I-EGF at the required concentration. The test dishes also received the concentration of test agent indicated in the Figures. After incubation, unbound radioactivity was removed by washing the cells four times with cold (4°C) phosphate-buffered saline (pH 7.4) containing 0.1% albumin and 0.1 μM-KI. The washed cells were solubilized (60 min at 37°C) into 1 ml of 0.5 M-NaOH, and cell-associated radioactivity was determined in a gamma counter. Non-specific ^{125}I-EGF binding measured as cell-associated radioactivity in the presence of unlabelled EGF (2 μg/ml) was less than 5% of the total. Cell numbers were obtained from replicate dishes of cells using a Coulter counter.

Extraction and determination of inositol phosphates. Cells were prelabelled for 60 hours with myo-[2-^{3}H]-inositol (20 μCi/dish) added directly to the growth medium. The labelled medium was removed, the cells were rinsed twice and incubated for 1 h in phosphate-buffered saline (pH = 7.4) to remove as much as possible of the free [^{3}H]inositol. After a further rinse, the cells were incubated for 5 min at room temperature with or without the test peptide. The medium was removed by aspiration and incubations were ended by adding 1 ml of cold 15% trichloracetic acid. The water-soluble inositol phosphates were extracted and separated by anion-exchange chromatography as described (23).

RESULTS

Influence of temperature and bombesin concentration on bombesin-mediated inhibition of ^{125}I-EGF binding to Swiss 3T3 cells. The dose-response curves

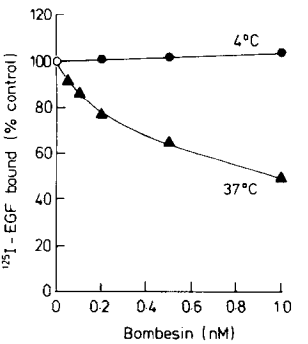

Fig. 1. Dose-response and temperature-dependence of the inhibition of ^{125}I-EGF binding to Swiss 3T3 cells by bombesin. Quiescent cultures of cells were rinsed with binding medium and incubated for 60 min at 37°C (▲) or 175 min at 4°C (●) with 1 ml of binding medium containing ^{125}I-EGF (0.6 ng/ml; 82,000 c.p.m./ng) and bombesin at the indicated concentration. The cells were washed and cell-associated radioactivity was determined as described in the Methods section. Each point represents the mean value (n = 3 or 4) for ^{125}I-EGF binding expressed as a percentage of the mean control value for ^{125}I-EGF binding measured at the same temperature.

for the bombesin-mediated inhibition of ^{125}I-EGF binding to Swiss 3T3 cells at 37°C and 4°C are shown in Fig. 1. Bombesin potently inhibited ^{125}I-EGF binding at 37°C. The maximal inhibition varied between experiments from 35%-65% and was achieved at a peptide concentration 0.5 - 2 nM, with a half-maximal response at a bombesin concentration of ∿ 0.25 nM. In contrast to its effect at 37°C, bombesin was unable to inhibit the binding of ^{125}I-EGF to Swiss 3T3 cells at 4°C (Fig. 1). Since unlabelled EGF is an equally effective competitive inhibitor of ^{125}I-EGF binding at both 37°C and 4°C (6), this result suggests that the bombesin-mediated inhibition of ^{125}I-EGF binding is indirect. Further evidence for an indirect inhibitory mechanism is found in the observations (not shown) that bombesin does not down-regulate EGF receptors and does not inhibit ^{125}I-EGF binding to several other cell lines including A431 cells.

Effect of ^{125}I-EGF concentration on the bombesin-mediated inhibition of ^{125}I-EGF binding. The ability of bombesin to inhibit ^{125}I-EGF binding to Swiss 3T3 cells was measured at different ^{125}I-EGF concentrations. Analysis of the data (Fig. 2) shows that bombesin does not alter the maximal ^{125}I-EGF binding capacity (B_{max}) of 3T3 cells but causes a shift in receptor affinity. The curvilinear Scatchard plots (B/F v. B) shown in Fig. 2 suggest that the cells contain more than one class of receptor site. The curves were analysed using a two-site model and the B_{max} and dissociation constants ($K_{D(1)}$ and $K_{D(2)}$) were estimated with a general optimising routine E04 JBF (NAG MK10 Subroutine Library, Numerical Algorithms Group, Banbury Road, Oxford). The data for both control and bombesin-treated cells can be well-fitted by a model with

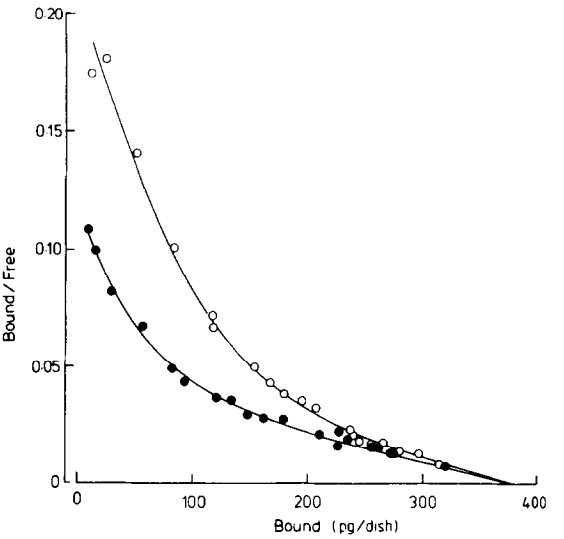

Fig. 2. Scatchard plot of ^{125}I-EGF binding to Swiss 3T3 cells in the presence and absence of bombesin. Quiescent cultures of cells were rinsed with binding medium and incubated (60 min at 37°C) with 1 ml of binding medium containing concentrations of ^{125}I-EGF in the range 0.1 - 40.0 ng/ml (59,000 c.p.m./ng) with (●) or without (○) bombesin (2 nM). Immediately before washing the cells, 200 µl of the binding medium was removed to plastic tubes for measurement of free ^{125}I-EGF. The cells were washed and cell-associated radioactivity was determined as described in the Methods section. Each point represents the value obtained from a single dish of cells.

two classes of receptors ($K_{D(1)}$ = 9.8 x 10^{-11}M; $K_{D(2)}$ = 1.8 x 10^{-9}M) and a constant total binding (B_{max} = 378 pg/dish). The effect of bombesin is to change the proportion of receptors in each affinity class from a $K_{D(1)}$:$K_{D(2)}$ ratio of 28%:72% to 14%:86%. Thus, bombesin converts higher-affinity receptors to lower-affinity receptors without altering total receptor number. No statistically better fit could be achieved by a less restricted model in which the K_D and B_{max} values were allowed to vary independently in the control and treated cells.

Taken together, these results indicate that bombesin inhibits ^{125}I-EGF binding to Swiss 3T3 cells by reducing receptor affinity through an indirect, temperature-sensitive mechanism. Thus the ^{125}I-EGF-inhibiting properties of bombesin are very similar to those that have previously been reported for TPA (9, 10), PDGF and related growth factors (2-7), and vasopressin (8). The mechanism by which these chemically diverse agents bring about a reduction in EGF receptor affinity remains unknown. The effect does not appear to be mediated via the cytoskeleton since neither colchicine nor cytochalasin B affect the modulation of EGF receptor affinity (8). Furthermore, the indirect inhibition of ^{125}I-EGF binding mediated by these agents does not require protein synthesis or degradation (8).

Table 1. The effect of cell growth-promoting peptides on inositol phosphate accumulation in Swiss 3T3 cells

Experiment	Treatment		IP	IP_2	IP_3
			(c.p.m./10^6 cells)		
I	Control		5229 ± 244	1421 ± 51	493 ± 60
	PDGF	33.3 µg/ml	4900 ± 99	1436 ± 104	691 ± 64*
		11.0 µg/ml	5904 ± 214*	1912 ± 49*	835 ± 119*
	Control		7672 ± 2020	1393 ± 220	377 ± 25
	bombesin	5 nM	11252 ± 1700	2670 ± 205*	622 ± 73*
		50 nM	16822 ± 1970*	2950 ± 128*	620 ± 21*
	vasopressin	100 nM	10232 ± 918	2130 ± 153*	582 ± 24*
		1 µM	10987 ± 1433	2205 ± 228*	575 ± 23*

* P versus control incubations < 0.01

Cells that had been prelabelled with [^{3}H]inositol (20 µCi/dish for 60 hours) were stimulated for 5 minutes with the indicated concentration of peptide. Values shown are the mean (± S.E.) for 4 or 6 determinations on separate dishes of cells.

Bombesin, vasopressin and PDGF stimulate the production of inositol phosphates by Swiss 3T3 cells. PDGF has been shown to stimulate phosphatidylinositol breakdown in Swiss 3T3 cells (24, 25). Vasopressin is known to stimulate the metabolism of phosphoinositides in hepatocytes (26, 27). We have therefore tested the effect of PDGF, vasopressin and bombesin on the breakdown of phosphoinositides in Swiss 3T3 cells prelabelled with myo[^{3}H]-inositol. The results in Table 1 demonstrate that bombesin and vasopressin, like PDGF, stimulate the generation of inositol phosphate (IP), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) in treated cells. In each case, these effects are generated at concentrations of ligands similar to those which are mitogenic (2-4, 8, 14) and which cause reductions in the affinity of the EGF receptor (2-4, 8, Fig. 1). This stimulation of the breakdown of cellular phosphoinositides will also generate diacylglycerol (see 15-17). An increase in the level of diacylglycerol has already been demonstrated in Swiss 3T3 cells following stimulation with PDGF (25). Taken together with the effect of TPA on EGF receptor affinity (9-11), these results suggest that the cross-reduction of EGF receptor affinity is due to activation of protein kinase C.

Synthetic diacylglycerol produces a reduction of EGF receptor affinity in Swiss 3T3 cells. In order to further investigate this possibility we have examined the effect of synthetic diacylglycerol (1-oleoyl-2-acetylglycerol) on ^{125}I-EGF binding to Swiss 3T3 cells. Diacylglycerol produced a dose-dependent inhibition of ^{125}I-EGF binding to Swiss 3T3 cells at 37°C. As

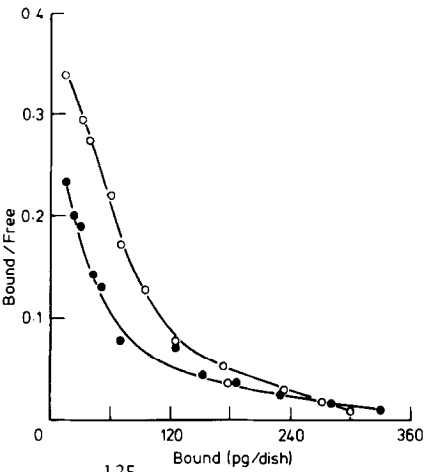

Fig. 3. Scatchard plot of ^{125}I-EGF binding to Swiss 3T3 cells in the presence and absence of synthetic diacylglycerol. Quiescent cultures of cells were rinsed with binding medium and incubated (60 min at 37°C) with 1 ml of binding medium containing concentrations of ^{125}I-EGF in the range 0.05 to 34 ng/ml (59,000 c.p.m./ng) with (●) or without (○) diacylglycerol (20 µg/ml). Bound and free ^{125}I-EGF were determined as described in the legend to Fig. 2. Each point represents the value obtained from a single dish of cells.

with bombesin, the maximal inhibition varied between experiments (25%-60%) and was achieved at a diacylglycerol concentration of ∿ 20 µg/ml (results not shown). Scatchard analysis of the diacylglycerol-mediated inhibition of ^{125}I-EGF binding indicates that the reduction in binding is due to a reduction in the proportion of higher affinity receptors with no change in the maximal binding capacity (Fig. 3). It is unlikely that this inhibition of ^{125}I-EGF binding is due to diacylglycerol acting as a non-specific membrane fusigen or perturber (16) since mono-olein, at the same concentration, did not reduce the affinity of the EGF receptor and because diacylglycerol failed to inhibit ^{125}I-EGF binding to isolated plasma membranes (results not shown).

DISCUSSION

The recent demonstration (18) that TPA activates a Ca^{++}- and phospholipid-dependent protein kinase suggested that this kinase may also be involved in mediating the TPA-induced switch of EGF receptor affinity in 3T3 cells (9-11). Recent work (for review see 16) has demonstrated that protein kinase C is activated by diacylglycerol produced from mebrane phosphoinositides on activation of certain receptors by their agonists. Our results demonstrate that the various growth-promoting peptides which have been shown to reduce EGF receptor affinity on 3T3 cells have a common ability to stimulate cellular phosphoinositide breakdown. We suggest, therefore, that the effect of these agents on EGF receptor affinity is due to the production of diacylglycerol and the activation of protein kinase C. This conclusion is

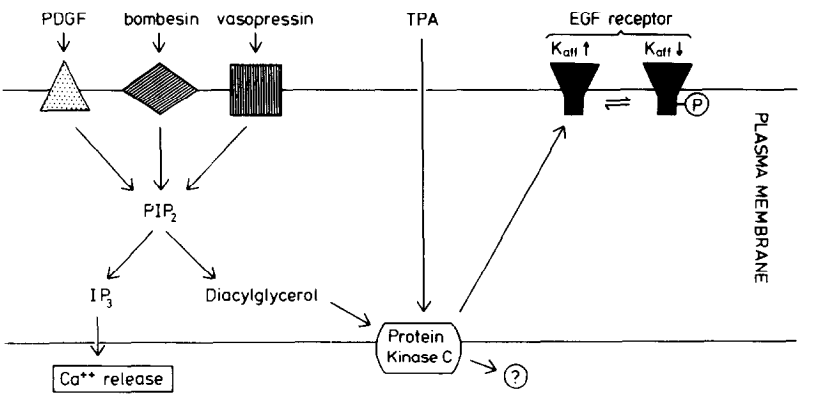

Fig. 4. Proposed mechanism for the cross-regulation of EGF receptor affinity by diverse growth-promoting factors. Peptides bind to their own receptors (indicated by the shaded symbols) stimulating PIP_2 breakdown and the generation of diacylglycerol which, like TPA, activates protein kinase C. The activated kinase phosphorylates higher-affinity EGF receptors causing a reduction in affinity for ^{125}I-EGF. Other substrates for protein kinase C are indicated by (?)

supported by our finding that synthetic diacylglycerol also causes switching of higher affinity EGF receptors to a lower affinity. Recently it has been shown (28) that purified protein kinase C is able to phosphorylate the EGF receptor on A431 cell membranes. Taken together these results indicate that cross-regulation of EGF receptor affinity by various heterologous ligands occurs via protein kinase C-mediated phosphorylation of EGF receptors following protein kinase C activation by those ligands as summarized in Fig. 4.

The cross-regulation of receptor affinity by protein kinase C-activating ligands is not restricted to Swiss 3T3 cells. TPA inhibits ^{125}I-EGF binding to a wide variety of cell types in culture (10,11). In addition, it has recently been reported that pancreatic secretagogues cause a reduction in ^{125}I-EGF binding to pancreatic acini cells (29, 30). Since these secretagogues are known to induce phosphoinositide breakdown by these cells (31) it seems probable that protein kinase C activation also causes a decrease in the EGF receptor affinity in pancreatic acini.

The significance of cross-regulation of EGF receptor affinity is not clear. Cross-regulating agents reduce EGF binding to Swiss 3T3 cells over the functional range of EGF concentrations (0.1 - 10 ng/ml). However in spite of the induced decrease in EGF binding, agents which activate protein kinase C either directly (TPA) or indirectly (e.g. vasopressin) synergistically increase the mitogenic response of Swiss 3T3 cells to EGF (9, 12). This result suggests that the residual binding to lower affinity receptors is sufficient to generate the necessary biochemical signals for EGF action. Furthermore, this synergistic interaction between peptides which stimulate phosphoinositide breakdown and EGF suggests that EGF activates additional biochemical pathways.

ACKNOWLEDGMENTS

We are most grateful to John Paterson for the computer analysis of binding data and to Professor Y. Nishizuka for the gift of 1-oleoyl-2-acetylglycerol. We thank Diane Blakeley for her help with cell culture and Janet Hood and Janet Tickner for typing the manuscript.

REFERENCES

1. Bowen-Pope, D.F., and Ross, R. (1983) Biochem. Biophys. Res. Comm. 114, 1036-1041.
2. Bowen-Pope, D.F., DiCorleto, P.E., and Ross, R. (1983) J. Cell Biol. 96, 679-683.
3. Brown, K.D., Blakeley, D.M., and MacDonald, M. (1983) Biosci. Rep. 3, 659-666.
4. Collins, M.K.L., Sinnett-Smith, J.W., and Rozengurt, E. (1983) J. Biol. Chem. 258, 11689-11693.
5. Rozengurt, E., Collins, M., Brown, K.D., and Pettican, P. (1982) J. Biol. Chem. 257, 3680-3686.
6. Brown, K.D., and Blakeley, D.M. (1983) Biochem. J. 212, 465-472.
7. Brown, K.D., and Blakeley, D.M. (1984) Biochem. J. 219, 609-617.
8. Rozengurt, E., Brown, K.D., and Pettican, P. (1981) J. Biol. Chem. 256, 716-722.
9. Brown, K.D., Dicker, P., and Rozengurt, E. (1979) Biochem. Biophys. Res. Comm. 86, 1037-1043.
10. Shoyab, M., De Larco, J.E., and Todaro, G.J. (1979) Nature 279, 387-391.
11. Lee, L.-S., and Weinstein, I.B. (1979) Proc. Natl Acad. Sci. U.S.A. 76, 5168-5172.
12. Dicker, P., and Rozengurt, E. (1981) J. Cell Physiol. 109, 99-109.
13. Rozengurt, E., Legg, A., and Pettican, P. (1979) Proc. Natl Acad. Sci. U.S.A. 76, 1284-1287.
14. Rozengurt, E., and Sinnett-Smith, J. (1983) Proc. Natl Acad. Sci. U.S.A. 80, 2936-2940.
15. Michell, B. (1983) Trends Biochem. Sci. 8, 263-265.
16. Nishizuka, Y. (1984) Nature 308, 693-698.
17. Berridge, M.J. (1984) Biochem. J. 220, 345-360.
18. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
19. Niedel, J.E., Kuhn, L.J., and Vandenbark, G.R. (1983) Proc. Natl Acad. Sci. U.S.A. 80, 36-40.
20. Ashendel, C.L., Staller, J.M., and Boutwell, R.K. (1983) Biochem. Biophys. Res. Comm. 111, 340-345.
21. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.
22. Savage, C.R., and Cohen, S. (1972) J. Biol. Chem. 247, 7609-7611.
23. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Biochem. J. 212, 473-483.
24. Berridge, M.J., Heslop, J.P., Irvine, R.F., and Brown, K.D. (1984) Biochem. J. (in press).
25. Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D., and Ross, R. (1981) J. Biol. Chem. 256, 12329-12335.
26. Rhodes, D., Prpic, V., Exton, J.H., and Blackmore, P.F. (1983) J. Biol. Chem. 258, 2770-2773.
27. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., and Kirk, C.J. (1983) Biochem. J. 212, 733-747.
28. Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A., and Hunter, T. (1984) J. Biol. Chem. 259, 2553-2558.
29. Logsdon, C.D., and Williams, J.A. (1983) FEBS Lett. 164, 335-339.
30. Korc, M., Matrisian, L.M., and Magun, B.E. (1983) Life Sci. 33, 561-568.
31. Rubin, R.P., Godfrey, P.P., Chapman, D.A., and Putney, J.W. Jr. (1984) Biochem. J. 219, 655-659.