Journal of Cellular Biochemistry 45:196–206 (1991)

Progesterone Augments Proliferation Induced by Epidermal Growth Factor in a Feline Mammary Adenocarcinoma Cell Line

Jaime F. Modiano, Yasuo Kokai, David B. Weiner, Mark J. Pykett, Peter C. Nowell, and C. Richard Lyttle

Department of Pathology and Laboratory Medicine (J.F.M., Y.K., D.B.W., M.J.P., P.C.N.) and Department of Obstetrics and Gynecology (C.R.L.), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Abstract Steroid hormones and peptide growth factors promote growth and development of normal mammary tissues and some types of breast cancer. Ovarian steroids may influence mammary growth directly or indirectly. The epidermal growth factor (EGF) family of proteins may also regulate mammary growth. These two pathways may function independently of each other or they may act in concert, with steroids inducing transcription of genes that encode growth factors or growth factor receptors. We used a feline mammary adenocarcinoma cell line (K12) to address whether there was an interrelation between progesterone (PGN) and EGF-associated growth pathways. K12 cells responded to EGF by a dose-dependent increase in proliferation. PGN or promegestone (R5020, a synthetic progestagen) alone did not stimulate K12 growth, but when EGF and PGN, or EGF and R5020 were combined, they were synergistic. This synergistic response was abrogated by the PGN receptor antagonist RU486 or by antibodies that blocked binding of EGF to its receptor. K12 cells expressed characteristic double-affinity EGF receptors, as well as p185 (a functionally and structurally related protein, product of the *neu* gene) on their surface. PGN receptors were also found on intact cells and in cleared cytosols. Stimulation of K12 cells by PGN or by R5020 induced a two- to threefold increase in the number of high-affinity surface EGF receptors after 24 h. Stimulation of these cells by PGN also affected the relative levels of phosphorylation of the EGF receptor and p185 within minutes, but not of other cellular phosphoproteins. Our results show that PGN enhances the EGF-induced growth of K12 cells and suggest that this effect may be mediated at least partly via an increase in the number or function of high-affinity EGF receptors.

Key words: steroids, tyrosine kinases/phosphorylation, RU486, p185 *neu*, phosphatases

There is a growing body of evidence that suggests that mammary gland growth and development is regulated by the combined action of steroid hormones and polypeptide growth factors such as epidermal growth factor (EGF). Female mice made EGF-deficient by pregestational sialoadenectomy have reduced mammary gland growth during pregnancy [1], and this effect is fully reversible by the administration of EGF. Furthermore, steroid-induced mammary development is accompanied by specific increases in phosphorylation events [2], some of which can be attributed to the activation of the EGF receptor kinase.

Aberrant regulation of the pathways controlled by either EGF or by ovarian steroids may contribute to the development of mammary tumors. Human breast tumors frequently bear receptors for estrogen (E_2, [3–5]) and/or progesterone (PGN, [4,5]), and some are exquisitely sensitive to hormonal therapy [6]. Likewise, some breast tumors have been shown to express elevated levels of EGF receptors [5,7].

In fact, these two apparently independent pathways may act in concert to induce some types of mammary tumors, since both E_2 [8] and PGN [9] specifically upregulate transcription of the EGF receptor gene in hormone-responsive epithelial cells and cell lines. EGF stimulates

Abbreviations used: EGF, epidermal growth factor; PGN, progesterone; R5020, promegestone; E_2, estrogen; TPA, 12-O-tetra-decanoyl phorbol 13 acetate.

Yasuo Kokai's present address is 2044 Hiranuma, Yoshikawa-machi, Kitakatsu-shika-gun, Saitama, 342 Japan.

Received May 18, 1990; accepted September 28, 1990.

Address reprint requests to Jaime F. Modiano, Dept. of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6082.

proliferation of MCF-7 cells (a human E_2 receptor positive mammary carcinoma cell line [10]), and the antiestrogen tamoxifen inhibits this effect [10]. Conversely, the inhibitory effect of antiestrogens on another steroid receptor positive human mammary cell line (T47D) can be overcome by EGF [11], suggesting that the response of these cells to EGF is regulated by the function of steroid hormone receptors. These cell lines can also be stimulated by ovarian steroids to produce and secrete EGF [12] or transforming growth factor α [13], both of which bind to the EGF receptor. Some or all of these factors may help to establish autocrine growth loops that give the cells a growth advantage in the mammary gland environment and that may eventually lead transformed cells to a hormone-independent phenotype.

Another gene that may be important in mammary cell growth is c-*neu* (c-*erb*-B2). This gene encodes a protein of 185 kDa that is closely related to the EGF receptor both structurally and functionally [14]. The role of the *neu* oncogene in the genesis and progression of mammary tumors is unclear, but in women with breast cancer, amplification of this gene may inversely correlate with prognosis and outcome of the disease [15,16].

The cat is a good model for the study of hormone-induced mammary growth and tumorigenesis [17]. Mammary tumors in both humans and felines share a number of common features. Specifically, both occur spontaneously, primarily in middle-aged females [18]; they exhibit similar histological patterns [18]; and in both species, the majority are derived from secretory tissues [19]. These tumors appear to arise in a hormonally dependent fashion in both species [19]; however, in contrast to humans, feline mammary tumors commonly have high-affinity receptors for PGN [19], but not for E_2 [19,20]. Progestagen therapy has been correlated with the development of mammary fibroadenoma complex [21] and anecdotally with mammary adenocarcinomas in both male and female cats [22]. The *neu* gene product, p185, is also expressed in normal and transformed feline mammary tissues (J. Modiano, M. Goldschmidt, and H. Maguire, unpublished data), although whether its expression is subject to hormonal control is unknown.

We have analyzed the possibility that PGN- and EGF-associated growth pathways (including those that may use p185) work cooperatively to promote growth of a feline mammary tumor cell line.

MATERIALS AND METHODS

Cells

The K12-72.1 cell line was a gift of William Hardy, Jr. This cell line was obtained by serial passage of a primary tumor explant from a female 14 year old pet cat [23]. An epithelial cell line was established after 15 passages in culture, and it was cloned at passage 72 [23]. Karyotypic examination of K12-72.1 cells on the 77th passage showed them to be near-diploid with a modal chromosome number of 37 (2n in cats is 38) and a small percentage of hypotetraploid cells [23]. The EGF-response pathways in these cells seem to be genetically intact (see Results), making them a useful model system to study the interplay between ovarian steroids and EGF-mediated growth regulation.

K12 cells from the original stock were maintained in continuous culture for 5–20 passages in complete medium [RPMI-1640, GIBCO, Grand Island, NY, supplemented with 2% [v:v] heat-inactivated fetal bovine serum (GIBCO), 2 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM HEPES, pH 7.3]. Confluent cells were trypsinized off the tissue culture flasks, gently washed in Hank's balanced salt solution (HBSS, Ca^{++}, Mg^{++} free, GIBCO), and seeded at a density of 1×10^5 cells/ml in 75 cm^2 flasks (Corning, Corning, NY).

Radiolabeled Compounds and Chemical Reagents

^{3}H-R5020 (promegestone), ^{3}H-thymidine, and ^{32}P-orthophosphate were obtained from Dupont-NEN (Wilmington, DE). Unlabeled R5020 was obtained from Dupont-NEN and solubilized in EtOH to a concentration of 75 μg/ml. The concentration of R5020 was adjusted immediately before use to 7.5 μg/ml in 10 mM Tris, pH 7.5, 1.5 mM Na_2EDTA, 10% glycerol, and 4 mg% gelatin (buffer A). RU38486 (RU486) was a gift from Roussel Uclaf (Romainville, France), and it was solubilized in EtOH to a concentration of 1 mM and further diluted in buffer A as necessary.

Antibodies

Rabbit anti-EGF antisera and the anti-EGF receptor monoclonal antibody 29.1. were purchased from ICN immunobiologicals (Lisle, IL).

The antibodies were mixed and used as a cocktail (α-EGF) at a 1:500 dilution from the stock provided by the manufacturer.

^{125}I was obtained from Dupont-NEN, and highly purified murine salivary gland EGF was iodinated using the Enzymobeads® (Bio-Rad, Rockville, NY) method as described by the manufacturer. Alternatively, ^{125}I-EGF was purchased from Dupont-NEN. All chemicals and biologicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Proliferation Assays

K12 cells were allowed to grow to near-confluency and were then trypsinized, washed three or more times in HBSS, and seeded in 96-well microtiter plates at a density of 1×10^5 cells/ml in serum-free HL1 medium (Ventrex, Portland, ME) in a final volume of 100 μl/well (10,000 cells). The cells were allowed to adhere for 16–20 h, and at this time they were stimulated in triplicate with EGF, PGN, R5020, RU486, α-EGF antibody cocktail, or combinations thereof added in 100 μl of serum-free HL1 medium. The stimulated cells were then incubated further for 6–104 h, and ^{3}H-thymidine (0.5 μCi/well) was added for the last 6–8 h of culture. At the end of the culture period, the plates were freeze/thawed to ensure 100% of cell lysis, and the DNA was then adsorbed onto fiberglass filters with a cell harvester (model M12V, BRI, Rockville, MD). Thymidine incorporation into trichloro-acetic acid precipitable DNA was determined by liquid scintillation counting in an LS 380 counter (Beckman Instruments, Sommerset, NJ).

Cell Counts

Monolayers were stimulated as above and cultured for 70–74 h, at which time the cultures were trypsinized and mechanically disrupted to form single-cell suspensions. The concentration of cells/ml was determined by manual counting on a hemacytometer (≥ 3 fields), and viability was assessed by exclusion of trypan blue dye.

Receptor Binding Assays

EGF and PGN receptors in K12 cells were measured using radioligand binding assays. For measurement of EGF receptors, 1×10^5 K12 cells were incubated in duplicate in 24-well plates (Corning) in 1 ml of complete medium with and without PGN (10–100 nM) or R5020 (100 nM). Eighteen to 24 h later, the medium was aspirated and the cells were washed three times with binding medium (RPMI-1640, 1% bovine serum albumin). ^{125}I-EGF ($7–10 \times 10^5$ μCi/mmole) was then added to the cells over a range of concentrations from 1 pM to 5 nM in 100 μl of binding medium. This was followed by a 1 h incubation at 25°C, after which the cells were washed three times with binding medium and then lysed in 1 N NaOH for 1 h at 4°C.

Cell-associated radioactivity was counted in a γ-counter (1270 Rackgamma counter, LKB Piscataway, NJ). Non-specific binding was determined by adding a 100-fold molar excess of unlabeled EGF to parallel cultures, and final cell numbers were corrected to the average number of adherent cells recovered from two control wells for each condition.

The measurement of cellular PGN receptors was carried out as described above for EGF receptors, except the cells were always unstimulated. ^{3}H-R5020 (0.1–2 μCi/ml) was used as an agonist over a concentration range of 1 to 20 nM. Cell associated radioactivity was determined by liquid scintillation spectrometry. Non-specific binding was determined as above using unlabeled R5020 (Dupont-NEN) as a competitor.

Cytosolic PGN receptors were measured using a ^{3}H-progestin receptor assay kit (Dupont-NEN) as described by the manufacturer. Briefly, cytosols were extracted from $> 1 \times 10^7$ K12 cells by homogenization in a monothioglycerol-containing buffer followed by centrifugation at 100,000*g* for 1 h at 4°C. Protein concentration in the cytosols was determined by the method of Waddell [24]. An aliquot of 100 μl of "adjusted cytosol" (2–3 mg protein/ml cytosol) was then incubated with ^{3}H-R5020 overnight followed by charcoal adsorption and centrifugation. Non-specific binding was determined as above. Cytosol-associated radioactivity in the supernatants was counted by liquid scintillation.

Receptors for E_2 were measured by radioimmunoassay using an anti human E_2 receptor antibody as described [25]. All the data were analyzed using the method of Scatchard as described by Berson and Yalow [26] and Rodbard et al. [27].

Phosphorylation Assays

To study the relative levels of protein phosphorylation in K12 cells, 2×10^6 cells were

incubated in complete medium for 16–24 h. The cells were then washed three times and finally incubated in phosphate-free RPMI medium supplemented with 2% dialyzed fetal bovine serum and 250 μCi/ml of ^{32}P-orthophosphate with or without Na-orthovanadate (400 μM) for 4–6 h to saturate their ATP stores with reactive ^{32}P. The cells were then exposed to PGN (100 nM) for 1 to 15 min and lysed in RIPA buffer (1% NP-40, 1% deoxycholate, 0.1% lauryl sulfate, 0.15 M NaCl, 0.01 M NaH_2PO_4, pH 7.4, 200 U/ml aprotinin, 2 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 400 μM Na_3VO_4, and 10 mM Iodoacetamide) overnight. EGF receptors or p185 were immunoprecipitated using the antibodies AHER (an anti-human EGF receptor antisera provided by Dr. Stuart Decker [28]) or DBW2 (an antisera against a synthetic p185 peptide [29]), respectively, and visualized by SDS-PAGE [30] and autoradiography on XAR5 X-ray film (Eastman Kodak, Rochester, NY).

RESULTS

Proliferative Response of K12 Cells to Stimulation by Epidermal Growth Factor or Progestagens

Epidermal growth factor (EGF) induced an increase in the proliferation of K12 cells as measured by DNA synthesis (Fig. 1) or by an absolute increase in cell numbers (Table I) that peaked 50 to 80 h after stimulation. Neither progesterone (PGN) nor promegestone (R5020) stimulated proliferation of K12 cells (Fig. 2) at any dose used (up to ~5 mM). However, R5020, which binds to the PGN receptor irreversibly, induced a consistent, dose-dependent increase of 75–130% in the response of K12 cells to EGF when used at concentrations of 50–500 nM (Figs. 1, 2; Table I). PGN also enhanced the response of K12 cells to EGF. The magnitude of this effect was more variable, ranging from a 10% to a 100% increase over the basal proliferation of

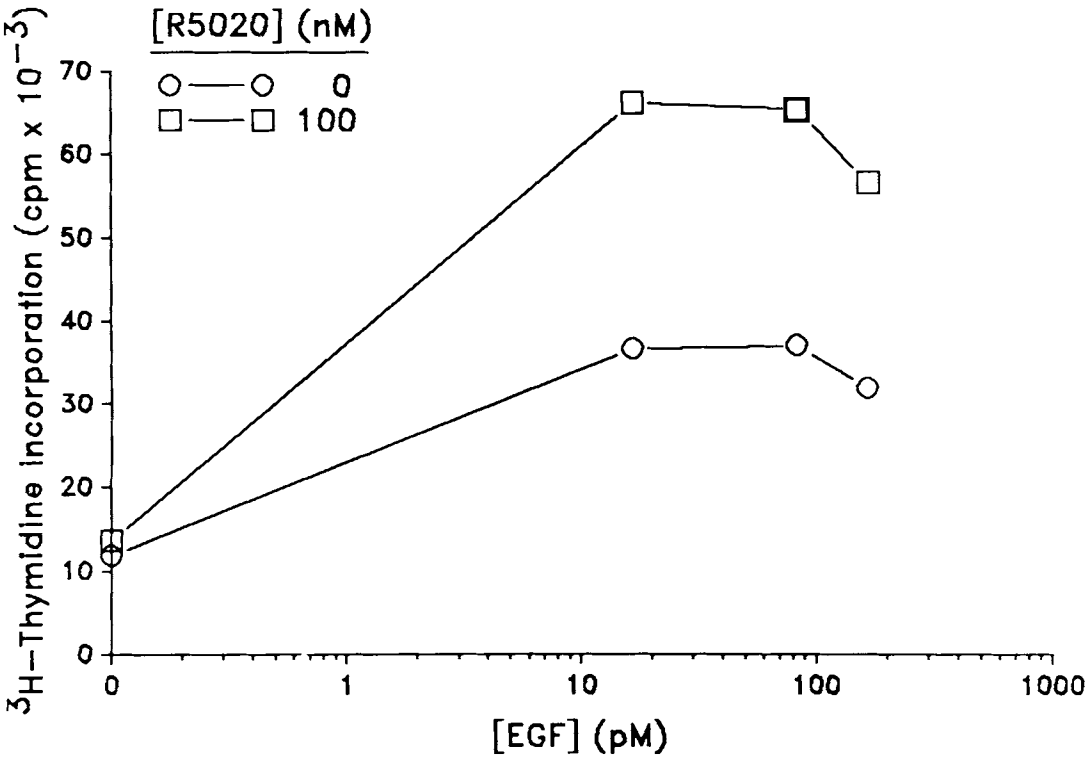

Fig. 1. Subconfluent K12 cells were trypsinized and plated in 96-well microtiter plates at a density of 1 × 10^5 cells/ml in 100 μl of serum-free HL1 media (10,000 cells/well). The cells were incubated for 18 h to allow them to adhere to the plates, after which time they were stimulated in triplicate and incubated for an additional 52 h. ^{3}H-thymidine (0.5 μCi/well) was added to the cultures for the last 6–8 h of incubation. The cells were then freeze-thawed, and DNA was harvested onto fiberglass filters and precipitated using 10% trichloro-acetic acid. Open circles represent cells stimulated by EGF alone, and open squares represent cells stimulated by EGF + R5020 (100 nM). Results show the mean of triplicate samples from one of eight experiments performed; the SEM within each experiment were routinely ≤12%.

TABLE I. Effect of EGF and PGN on K12 Cell Growth

Stimulus[a]	DNA synthesis[b] (^{3}H-Tdr incorporation in cpm)	Viable cells/10^5 plated[c] ($\times 10^{-5}$)
Media	20,083 ± 582 (n = 5)	11.7 ± 1.0 (n = 3)
EGF	35,074 ± 3,293 (n = 5)	17.8 ± 2.1 (n = 3)
EGF + R5020	60,667 ± 7,692 (n = 5)	30.6 ± 1.7 (n = 2)
EGF + RU486	35,287 ± 8,386 (n = 3)	16.9 ± 2.5 (n = 2)
EGF + R5020 + RU486	31,609 ± 5,745 (n = 3)	15.7 ± 2.9 (n = 2)
EGF + R5020 + αEGF	22,653 ± 2,113 (n = 3)	9.6 (n = 1)

[a]K12 cells were plated at a density of 1×10^5/ml in HL1 serum-free media and stimuli added 18 h later as described in Materials and Methods. The concentration of the stimuli used was as follows: EGF, 5 pM; R5020, 100 nM; RU486, 1 or 2 μM; and α-EGF antibody cocktail was used at a final dilution of 1:500 from the manufacturer stock.

[b]DNA synthesis was measured 52 h following the addition of stimuli by incorporation of ^{3}H-thymidine into TCA-precipitable material during an 8 h pulse. The results are shown as the mean cpm of triplicate samples ± SEM of three to five independent experiments (n).

[c]The number of viable cells/10^5 cells plated at the initiation of culture was determined after 70 h. The cell monolayers were trypsinized and mechanically disrupted to achieve a single-cell suspension; the cells were then counted manually on a hemacytometer. A minimum of three fields/condition were counted to determine the concentration of cells/unit volume and the absolute cell numbers. Viability was assessed by exclusion of trypan blue dye and was routinely ≥ 90%. The results are expressed as the mean number of live cells in each condition divided by 10^5 ± SD in one to three independent experiments.

K12 cells stimulated by EGF alone (not shown), but was consistently statistically significant ($P < 0.05$ as determined by Student's t-test).

The PGN receptor antagonist RU486 abrogated the increase in proliferation induced by R5020 in EGF-stimulated K12 cells (Table I), but had no effect on the response of EGF itself. An anti-EGF/anti-EGF receptor antibody cocktail inhibited both the proliferative response of K12 cells to EGF or to the combination of EGF and R5020 (Table I), although they did not affect the basal proliferation of K12 cells (not shown).

Expression of Receptors for EGF in K12 Cells

Expression of EGF receptors in K12 cells was assessed by radioligand binding and immunoprecipitation studies. High-affinity binding of EGF to K12 cells was saturable at 350–500 pM EGF at 25°C (Fig. 3). Scatchard analysis revealed that unstimulated K12 cells expressed 1.5–2.5 $\times 10^5$ EGF receptors/cell (Figs. 3, 4), of which approximately 5% (~10,000) were high-affinity receptors (K_d 35 pM, Table II).

To determine whether the EGF binding activity in these cells reflected a receptor akin to that which has been characterized in human and other cells, we immunoprecipitated EGF receptors from metabolically labeled K12 cells. Figure 5 shows that the AHER antisera precipitated a protein of 175 kDa and a protein of 150 kDa from unstimulated cells and from cells stimulated by EGF or PGN. In other species, the EGF receptor undergoes autophosphorylation after stimulation by EGF [28]. In K12 cells, stimulation by EGF also induced a five- to eightfold increase in the relative levels of phosphorylation of the 175 kDa protein (Fig. 5, lane B). The 150 kDa protein probably reflects an immature species of the EGF receptor [28] consistent with those observed in other species.

Expression of Receptors for Ovarian Steroids in K12 Cells

To determine whether the effects of PGN and R5020 were mediated by a specific receptor, we used radiolabeled R5020 to measure binding to intact K12 cells or to cleared K12 cytosols. We determined in three experiments that K12 cells expressed an average of 22,300 PGN receptors/cell with a K_d of 2.82 nM, and a binding capacity of 280 fmoles of R5020/mg of cytosol protein.

Similarly, we used an anti-human E_2 receptor antibody to measure E_2 receptor expression in K12 cells. This anti-E_2 receptor antibody is cross-reactive among humans and rodents [25]; thus, it should be likely to recognize the feline E_2 receptor. By this method, K12 cells appeared to express few to no E_2 receptors.

Effects of PGN on Expression of EGF Receptors, and Phosphorylation of EGF Receptors and p185

We examined whether the synergistic effect of PGN on EGF-induced proliferation of K12 cells might be associated with an increased number of surface EGF receptors in these cells. K12 cells treated with PGN (20–200 nM) or R5020 (100

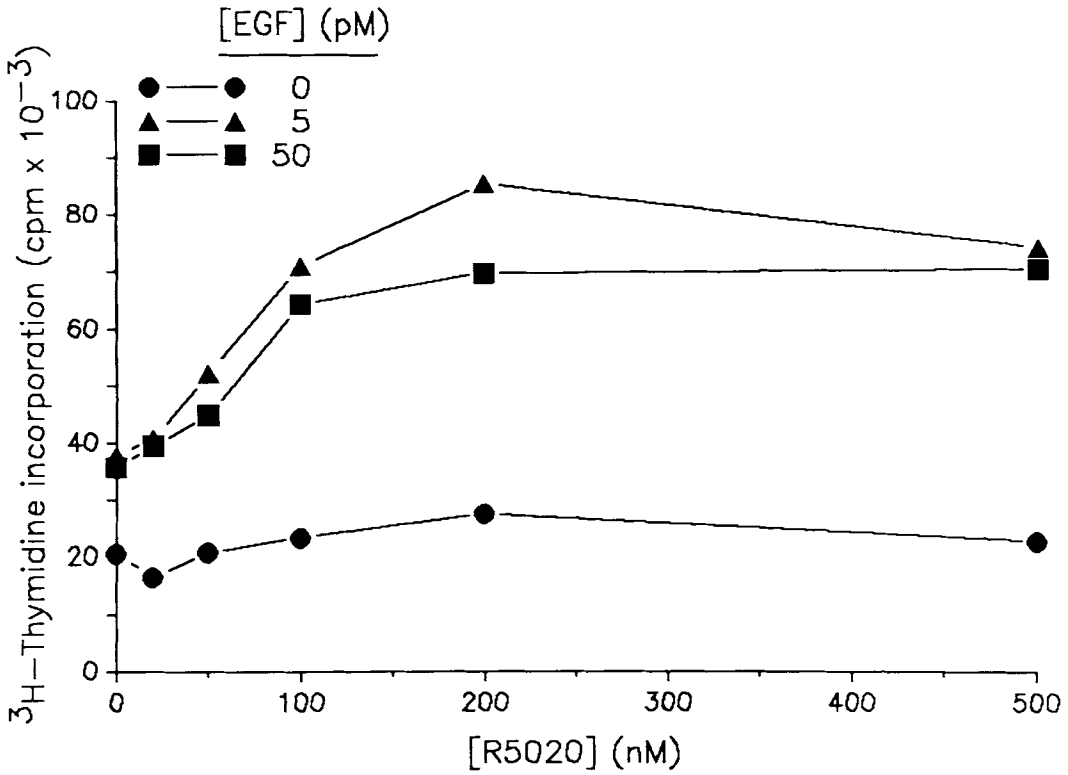

Fig. 2. Cell cultures were established, stimulated, and harvested as in Figure 1. Circles represent cultures stimulated by R5020 alone; triangles represent cultures stimulated by R5020 + EGF used at 5 pM; squares represent cultures stimulated by R5020 + EGF used at 50 pM. Results show the mean of triplicate samples from one representative experiment of three done; the S.E.M. within each experiment were routinely $\leq 12\%$.

nM) for 18–24 h expressed two to three times as many high-affinity EGF receptors on their surface as unstimulated cells (Figs. 3, 4; Table II), but exhibited no significant changes in the numbers of low-affinity receptors. This effect was inhibited specifically by a tenfold molar excess of the PGN receptor antagonist RU486 (Fig. 4).

The increase in surface EGF receptors observed in K12 cells stimulated by PGN was also abolished by the protein synthesis inhibitor cycloheximide used at 5 μg/ml (3,000 receptors/cell, K_d = 40 pM) and by TPA, a phorbol ester that induces a rapid modulation of EGF receptors off the cell surface, used at 20 nM (300 receptors/cell, K_d = 20 pM).

The augmentation of EGF-mediated growth in K12 cells by PGN could also reflect a post-transcriptional change in the EGF receptor (or in other proteins) that modifies its function. Thus, we also determined whether PGN affected the relative levels of phosphorylation of the EGF receptor and the closely related protein product of c-*neu,* p185. In our initial experiments, we observed that PGN induced a dramatic (50–90%) dephosphorylation of p185 (Fig. 6) and of the EGF receptor (data not shown). This dephosphorylation could be mediated by 1) inactivation of the EGF kinase; 2) activation of one or more specific phosphatases; 3) increased receptor modulation and lysosomal destruction; or 4) a combination of all of the above. Therefore, to investigate these possibilities, we used sodium-orthovanadate, an inhibitor of phosphatase activity, and stimulated K12 cells with EGF or PGN for 10 min. As also shown in Figure 5, there was up to an 800% (lane B) and a 300% (lane C) increase over the endogenous phosphorylation of the EGF receptor protein (and p185, data not shown) in cells stimulated by EGF or PGN, respectively, as compared to the unstimulated controls. PGN also stimulated a small increase in the phosphorylation of an unidentified protein of 110–120 kDa (Fig. 7), but, importantly, the effect of PGN was not a generalized one affecting every cellular phosphoprotein (Fig. 7). Taken together, these observations suggest that 1) the dephosphorylation we observed initially was at least partly medi-

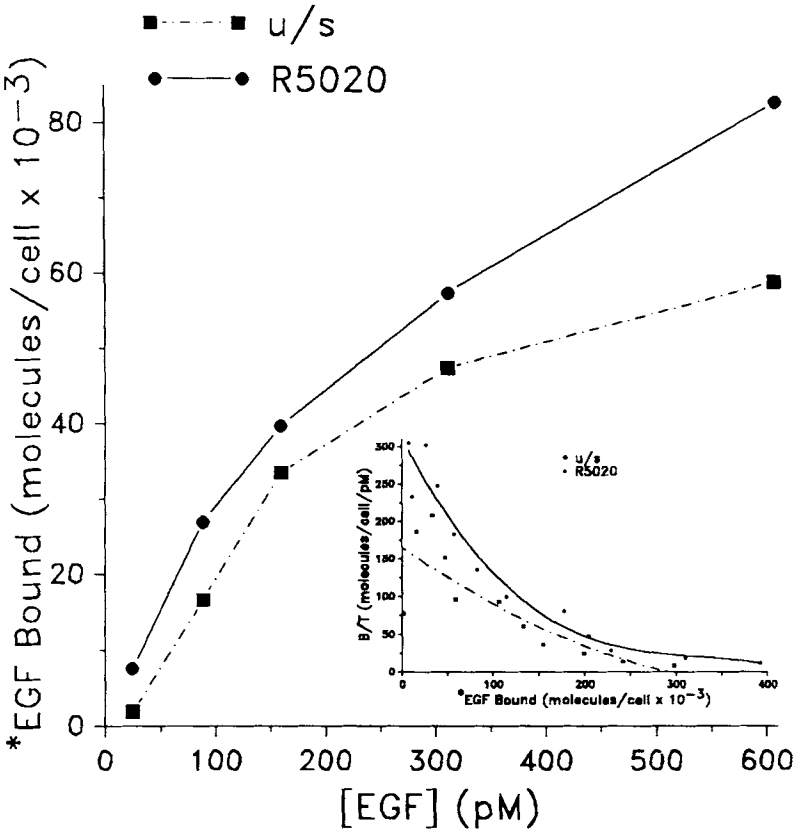

Fig. 3. Subconfluent K12 cells were trypsinized and plated in duplicate in 24-well plates at a density of 1×10^5 cells/ml in 1 ml of complete media (100,000 cells/well). After 18 h, the media was changed to fresh serum-free media (squares, dashed-dotted line) or fresh serum-free media with 100 nM R5020 (circles, solid line) for an additional 24 h. The cells were then washed twice in binding media (RPMI/1% BSA). Serial dilutions of *I-EGF (1–620 pM) were then added to the wells and the cells incubated for 1 h at 25°C. At this time, the cells were washed two more times in binding media and lysed for 30 min in NaOH. Cell-associated radioactivity was determined by γ-counting. Data show the mean of duplicate samples of increasing molecules of *EGF bound (specific binding) as a function of increasing concentrations of *EGF. Inset shows the Scatchard plots for the same data points. These data show one of three experiments done; the S.E.M. within each experiment were ≤ 10% of the mean values.

ated by a phosphatase(s) and 2) PGN-stimulation induced the activation of one or several kinases that phosphorylated the EGF receptor and p185. The basal phosphorylation state of the EGF receptor and p185 are a steady state mediated by both kinases and phosphatases. Thus, it is also possible that the PGN-induced increase in phosphorylation could have been mediated by the specific inactivation of an additional distinct phosphatase(s) that was insensitive to vanadate, and that contributed to the maintenance of this steady state. Finally, to determine if receptor degradation played a role in these changes, we immunoprecipitated EGF receptors or p185 from K12 cells labeled with ^{35}S-methionine. The results from these experiments indicate that PGN had no apparent effect on the relative quantity of intact EGF receptors or p185 present in the cells after 15 min (data not shown).

DISCUSSION

Ovarian steroids and epidermal growth factor (EGF) stimulate growth of mammary cells in vivo [1] and in vitro [14,31,32], but the signaling mechanisms used by these hormones to induce proliferation and the interrelation between them are poorly understood. We used the K12 feline mammary tumor cell line to investigate whether there was an interrelation between the growth regulatory mechanisms of EGF- or PGN-induced proliferation. Our results indicate that EGF stimulates growth of K12 cells (Fig. 1) and that these neoplastic cells bear receptors for EGF that appear not to be amplified or physically altered (Table II; Figs. 3–5). Furthermore,

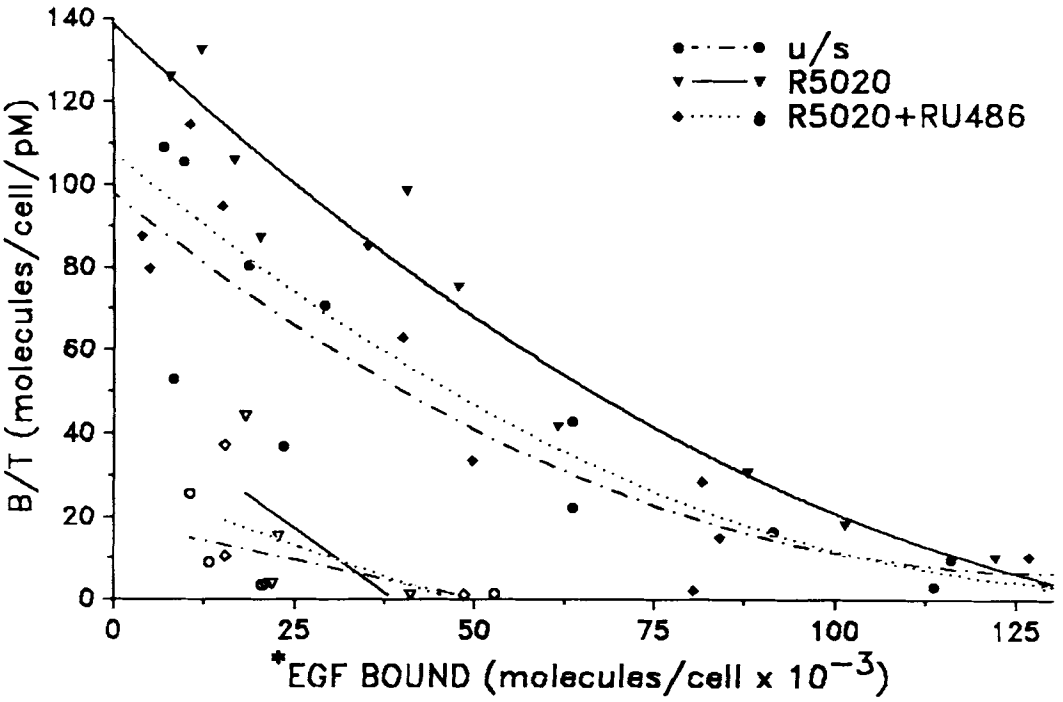

Fig. 4. Cell cultures were established, stimulated, and quantified as in Figure 3. Data points reflect the means of duplicate samples where the SEM were ≤10% of the mean values. Closed symbols represent specific binding of *EGF; open symbols represent non-specific binding in the presence of a 100-fold molar excess of unlabeled EGF. Best-fit regression (second order) was fitted using Sigmaplot 3.0. Circles and dashed-dotted lines show data points from cells that received fresh serum-free media only; inverted triangles and solid lines show data points from cells that received 100 nM R5020; and diamonds and dotted lines show data points from cells that received 100 nM R5020 + 1 μM RU486. These results are representative of two independent experiments.

TABLE II. EGF Receptor Levels in K12 Cells*

Stimulus	High-affinity receptors[a] (K_d = 1–5 × 10^{-11} M)	Low-affinity receptors[a] (K_d = 1–4 × 10^{-9} M)
A.		
Media	9,506 ± 2,282	235,000 ± 13,400
PGN	19,264 ± 7,415	232,000 ± 11,300
B.		
Media	10,474 ± 886	195,000 ± 77,800
R5020	32,950 ± 4,173	215,000 ± 106,000

*Subconfluent K12 cells were trypsinized and plated in duplicate in 24-well plates at a density of 1 × 10^5 cells/ml in 1 ml of complete medium (100,000 cells/well). After 18 h, the media was changed to fresh serum-free media or fresh serum-free media with (A) 10, 100, or 200 nM PGN or (B) 100 nM R5020 for an additional 24 h. The cells were then washed twice in binding media (RPMI/1% BSA). Serial dilutions of *I-EGF (1–5,000 pM) were then added to the wells, and the cells incubated for 1 h at 25°C. At this time, the cells were washed two more times in binding media and lysed for 30 min in NaOH. Cell-associated radioactivity was determined by γ-counting, and the data were analyzed by the method of Scatchard.

[a]The data represent the means ± SEM of (A) five different experiments or (B) the means ± SEM of three different experiments.

our data show that despite having functional receptors for PGN, these cells do not proliferate in response to progestagens (Fig. 2). However, PGN and R5020 synergized with EGF to significantly enhance the proliferation of K12 cells (Fig. 2), and this response was specifically inhibited by the PGN receptor antagonist RU486 or by antibodies that prevented binding of EGF to its receptor (Table I).

We next investigated the mechanisms that may regulate this synergistic response. Our data indicate that the number of high-affinity EGF

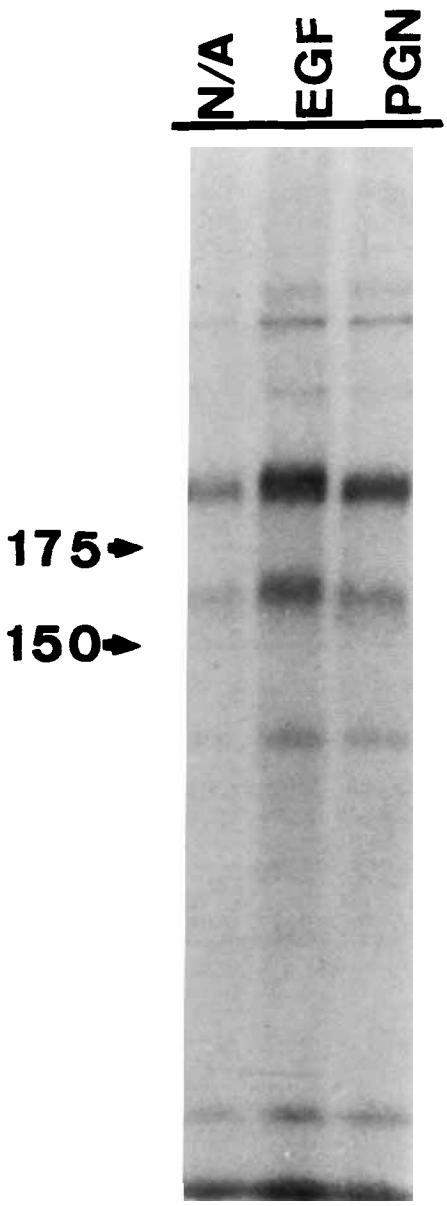

Fig. 5. Subconfluent K12 cells were trypsinized and plated in 6-well plates at a density of 1×10^6 cells/ml in 2 ml of complete media (2×10^6 cells/well). The cells were incubated for 18 h to allow them to adhere to the plates and were then washed in phosphate-free RPMI for 30 min. The cells were then incubated in 1 ml phosphate-free RPMI with 2% dialyzed fetal bovine serum, sodium-orthovanadate (a phosphatase inhibitor), and 250 μCi ^{32}P-orthophosphate/ml. Stimuli were added for the last 10 min of culture. The cells were then lyzed in RIPA buffer and EGF receptors immunoprecipitated from the lysates. The immunoprecipitates were separated by SDS-PAGE followed by autoradiography. N/A: unstimulated cells; EGF was 85 pM and PGN was 100 nM. Shown is one representative experiment of three performed; exposure of the autoradiograph was 14 h.

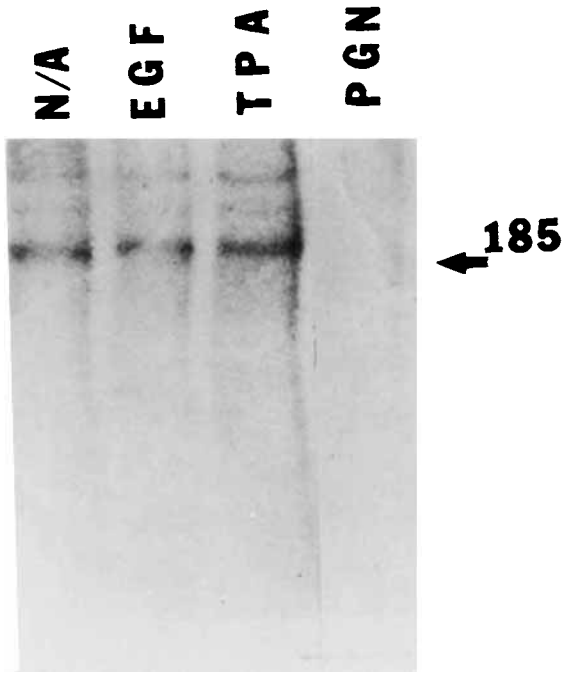

Fig. 6. K12 cells were treated as for Figure 5, except that no sodium-orthovanadate was used in the cultures. p185 was immunoprecipitated from the cell lysates, and the immunoprecipitates were separated by SDS-PAGE followed by autoradiography. N/A: unstimulated cells; EGF was 85 pM; TPA was 20 nM; and PGN was 100 nM. Shown is one representative experiment of four performed; exposure of the autoradiograph was 20 h.

receptors is indeed two- to three-fold greater in K12 cells stimulated by PGN or R5020 as compared to unstimulated cells after 18–24 h (Fig. 3; Table II). The increased expression of surface EGF receptors induced by R5020 was inhibited by RU486, a PGN-receptor antagonist (Fig. 4). Thus, this effect is likely to be mediated directly through the PGN receptor. Interestingly, the total number of EGF receptors (high + low affinity) was not significantly different in unstimulated cells vs. cells stimulated by PGN (Table II). The increment in high-affinity EGF receptors was also at least partly dependent on new protein synthesis since it could be blocked by cycloheximide. This suggests that PGN (through its receptor) regulates the expression of the EGF receptor gene in these cells.

The fact that EGF at concentrations as low as 5 pM (100-fold below the concentration required to saturate the high-affinity EGF receptors) could induce growth of these cells suggested that the occupancy of a small percentage of these receptors was sufficient for signaling. Therefore, the increased response to EGF induced by PGN may not be completely attribut-

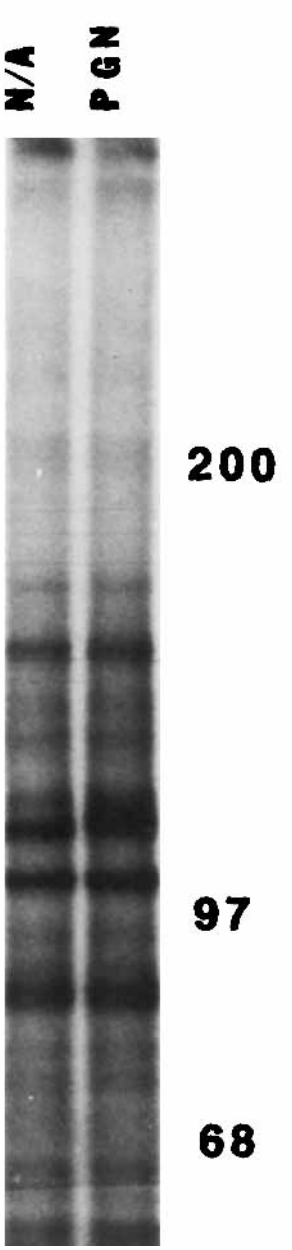

Fig. 7. Cells were treated as for Figure 5. The supernatants from the immunoprecipitates were separated by SDS-PAGE followed by autoradiography. N/A: unstimulated cells; PGN was added at 100 nM. One representative experiment of three performed is shown; the exposure of the autoradiograph was 2 h.

able to an increase in the number of surface EGF receptors. It is possible then that this effect could also be partially mediated by a functional alteration of the EGF receptor or other related proteins. To determine if this occurred in K12 cells, we asked whether PGN could modify the EGF receptor (and p185) post-translationally, specifically in its phosphorylation status, since phosphorylation of the EGF receptor by its endogenous kinase [33] and by other proteins [33] may be an important mode of regulating the ability of this receptor to deliver transmembrane signals. Also, a marked increase in endogenous tyrosine kinase activity has been shown to exist in the oncogenic forms of both the EGF receptor [34] and p185 [35], and this activity is critical to their transforming potential. Finally, following cognate binding, steroid hormone receptors may mediate effects that are not directly related to DNA binding or transcriptional regulation [36,37,38]. However, there have been no enzymes that are known to be directly modified by these steroid hormone-receptor complexes to date.

Our initial data show that PGN induced an alteration in the relative level of phosphorylation of the EGF receptor and p185 (Figs. 5, 6). These changes in phosphorylation may be regulated directly or indirectly by PGN via the activation of one or several phosphatases and one or several kinases that affect the EGF receptor within minutes.

It is interesting to speculate that these changes in EGF receptor phosphorylation status could contribute to convert a percentage of EGF receptors from the low- to the high-affinity state or otherwise modify their signaling function such as by affecting the EGF receptor kinase itself. The observations that stimulation of K12 cells by PGN had similar effects on p185 as on the EGF receptor (Figs. 5, 6), and that both of these proteins are substrates for the EGF receptor kinase [39,40], are consistent with this hypothesis.

These various possibilities are not mutually exclusive, and further studies will be necessary to determine their relative importance on the proliferative response of K12 cells.

ACKNOWLEDGMENTS

The authors wish to thank Valerie Brown and Takuro Wada for technical assistance in iodination of EGF, William Hardy, Jr., and Stuart Decker for providing the K12 cell line and AHER antibody, respectively, and Stuart C. Helfand and Mark I. Greene for helpful discussions. This work was supported in part by NIH grants CA 15822 (P.C.N.) and HD 20025 (C.R.L.). D.B.W. was supported by a grant from the Council for Tobacco Research.

REFERENCES

1. Okamoto S, Oka T: Am J Physiol 249 (Regulatory Integrative Comp. Physiol. 18):R285–R289, 1985.
2. Sheffield LG, Ayslworth CF, Welsch CW: Proc Soc Exp Biol Med 185:283–290, 1987.
3. Knight WA, Livingston RB, Gregory EJ, McGuire WL: Cancer Res 37:4669–4671, 1977.
4. McGuire WL: Cancer Res 38:4289–4291, 1978.
5. Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS: Cancer Res 44:3448–3453, 1984.
6. Fisher B, Redmond C, Brown A, et al.: J Clin Oncol 1:227–241, 1983.
7. Skoog L, Macias A, Azavedo E, Lombardero J, Klinterberg C: Br J Cancer 54:271–276, 1986.
8. Mukku VR, Stancel GM: J Biol Chem 260:9820–9824, 1985.
9. Murphy LJ, Sutherland RL, Stead B, Murphy LC, Lazarus L: Cancer Res 46:728–734, 1986.
10. Vignon F, Bouton M-M, Rochefort R: Biochem Biophys Res Commun 146:1502–1508, 1987.
11. Koga M, Sutherland RL: Biochem Biophys Res Commun 146:739–745, 1987.
12. Murphy LC, Murphy LJ, Dunik D, Bell GJ, Shiu RPC: Cancer Res 48:4555–4560, 1988.
13. Dickson RB, Huff KK, Spencer EM, Lippman ME: Endocrinology 118:138–142, 1986.
14. Bargman CI, Hung M-C, Wienberg RA: Nature 319:226–230, 1986.
15. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Science 235:177–182, 1987.
16. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Science 244:707–712, 1989.
17. Misdorp W, Weijer K: Am J Pathol 98:573–576, 1980.
18. Weijer K, Hart AAM: J Natl Cancer Inst 70:709–716, 1983.
19. Johnston SD, Hayden DW, Kiang DT, Handschin B, Johnson KH: Am J Vet Res 45:379–382, 1984.
20. Hamilton JM, Else RW, Forshaw P: Vet Rec 99:477–479, 1976.
21. Hayden DW, Johnston SD, Kiang DT, Johnson KH, Barnes DM: Am J Vet Res 42:1699–1703, 1981.
22. Hernandez FJ, Fernandez BB, Chertack M, Gage PA: Feline Pract 5:45–48, 1975.
23. Kahn MW: Doctoral dissertation. Cornell University School of Medical Sciences, New York, 1978.
24. Waddell WJ: J Lab Clin Med 48:311–314, 1956.
25. Greene GL, Jensen EV: J Steroid Biochem 16:353–359, 1982.
26. Berson SA, Yalow RS: J Clin Invest 38:1996–2016, 1959.
27. Rodbard D, Munson PJ, Takur AK: Cancer [Suppl] 46:2907–2918, 1980.
28. Decker S: Arch Biochem Biophys 228:621–626, 1984.
29. Cohen JC, Weiner DB, More KF, Kokai Y, Williams WB, Maguire HC Jr, LiVolsi VA, Greene MI: Oncogene 4:81–88, 1989.
30. Laemmli UK: Nature 227:680–685, 1970.
31. Taketani Y, Oka T: FEBS Lett 152:256–260, 1983.
32. Taketani Y, Oka T: Proc Natl Acad Sci USA 80:2647–2650, 1983.
33. Schlessinger J, Screiber AB, Levi A, Lax I, Liberman T, Yarden Y: CRC Crit Rev Biochem 14:93–111, 1983.
34. Chen WS, Cheri SL, Poenie M, Tsien RY, Gill GN, Rosenfeld MG: Nature 328:820–823, 1987.
35. Weiner DB, Kokai Y, Wada T, Cohen JA, Williams WV, Greene MI: Oncogene 4:1175–1184, 1989.
36. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB: Cell 48:417–428, 1987.
37. Pash JM, Bailey JM: Faseb J 2:2613–2618, 1988.
38. Adler S, Waterman ML, He X, Rosenfeld MG: Cell 52:685–695, 1988.
39. Kadowaki T, Kasuga M, Tobe K, Takaku F: Biochem Biophys Res Commun 144:699–704, 1987.
40. Kokai Y, Dobashi K, Weiner DB, Myers JN, Nowell PC, Greene MI: Proc Natl Acad Sci USA 85:5389–5393, 1988.