Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF

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An elevation in total MAP kinase activity and expression has been observed in breast cancer tissue. However, the mechanisms underlying these changes in kinase activity and regulation by growth factors are not well characterized. In these studies, the effect of the potent mammary mitogen, epidermal growth factor (EGF), on the activation of the mitogen-activated protein kinases, ERK1 and ERK2 (extracellular regulated protein kinases 1 and 2, respectively), was compared in primary cultures of normal mouse mammary epithelial cells and in a hormone-responsive mouse mammary tumor. In normal epithelium, EGF stimulated an early rise in ERK activity at 4 min followed by a rapid decline, whereas a sustained (1 h) elevation of ERK activity was observed in the tumor cells. The time course of ERK activity in both cell types coincided with the phosphorylation state of the EGF receptor, suggesting that altered regulation of EGF receptor phosphorylation or EGF receptor turnover produces an enhanced ERK response to EGF in tumor cells. The MEK inhibitor, PD 098059 inhibited EGF-stimulated proliferation and ERK activity in a parallel, dose-dependent manner showing that ERK activation is at least permissive for the proliferative response to EGF. In addition, tumor cells showed a 4-fold elevation in basal (or ligand-independent) activity over normal cells without an increase in total enzyme level, and a preferential activation of ERK1 by EGF. These EGF-dependent and -independent changes in ERK regulation in the hormone-responsive mammary tumor underscore how multiple alterations in the regulation of this pathway may play a role in mammary tumorigenesis.

Introduction

Epidermal growth factor (EGF) is a potent mitogen for normal and tumor mammary epithelial cells. Overexpression of EGF and its receptor (EGFR or ErbB-1) has been observed in breast cancer, raising the possibility of potential autocrine growth regulation as observed in some breast cancer cell lines (1). Elevations in EGFR levels can also play a role in mammary tumor progression from hormone dependence to hormone independence, as shown in mouse mammary tumors (2) and in human breast cancer cells when the EGFR is overexpressed (3). In addition, an elevated level of EGFR is associated with a poor prognosis in breast cancer (1). The EGFR receptor is a transmembrane tyrosine kinase that is activated by tyrosine autophosphorylation after ligand-induced dimerization (4). The ligand-bound receptor is capable of phosphorylating multiple signal transduction molecules, leading to the activation of multiple signal transduction pathways including protein kinase cascades (5). One of these kinase cascades is the ras–raf pathway leading to the activation of the extracellular regulated protein kinases (or ERKs), one of the mitogen-activated protein (MAP) kinase cascades. This pathway has been the subject of intense interest because of its role in the regulation of proliferation, differentiation and cell–matrix interactions. ERK1 and ERK2 are dually phosphorylated on threonine and tyrosine by the upstream MAP kinase, MEK. ERKs then phosphorylate and activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases leading to positive or negative regulation of signaling cascades (5).

Disruption of the regulation of the ERK pathway can predispose cells to undergo tumorigenic conversion as illustrated by the ras oncogene (6) which lies upstream of ERK, and transfection studies showing that constitutively active mutants of MEK can lead to in vitro transformation, increased sensitivity to, or independence from growth factors and tumor formation in vivo (7,8). Recent studies have shown an increase in the level and kinase activity of ERKs in human renal cell carcinoma (9) and breast cancer (10), indicating that deregulation resulting in overstimulation of this pathway may play a role in tumorigenesis.

These latter studies in primary human tumors did not address the regulation of the activation of the ERK cascade by exogenous factors. It was not possible to discern if an apparent constitutive elevation in basal activity was due to an inherent alteration in the regulation of the pathway or if the pathway was more sensitive to stimulation by an exogenous ligand. In our studies, we have sought to directly compare the effect of EGF on the activation of the ERKs in primary cell cultures of normal and tumor mammary epithelium to better recognize alterations that may occur during tumorigenesis. Primary culture using a biomatrix-based culture system was chosen to more closely approximate the in vivo state and as an alternative to immortalized cell lines adapted for growth on a plastic substrate. These experiments show that the mitogenic effect of EGF in normal and tumor mammary epithelium is dependent, at least in part, on ERK activation. Furthermore, mammary tumor epithelium may exhibit an elevation in basal ERK activity and sustained ERK activation by EGF, the latter sustained activation reflecting a difference in the regulation of EGF receptor activity. This altered regulation of the MAP kinase pathway may be an example of an alteration in regulation by growth factors that may occur in some breast cancers impacting both tumor growth and progression.

Materials and methods

Reagents
Cell culture: Ham’s F-12, Medium 199 and Dulbecco’s Modified Eagle’s medium (DMEM) were from Gibco BRL (Grand Island, NY); collagenase

Abbreviations: EGF, epidermal growth factor; ERK, extracellular regulated protein kinase; MAP kinase, mitogen-activated protein kinase.
sequence (amino acids 95–98 of MBP) was from Santa Cruz Biotechnology; kinase substrate peptide containing the MAP kinase consensus phosphorylation (PY20) was from Transduction Laboratories (Lexington, KY). Biochemicals: MEK1 inhibitor PD 098059 was from Calbiochem (San Diego, CA); MAP kinase substrate peptide containing the MAP kinase consensus phosphorylation sequence (amino acids 95–98 of MBP) was from Santa Cruz Biotechnology; γ-32P[ATP was from DuPont-NEN (Wilmington, DE). EGF was from Collaborative Research (Waltham, MA) and Protein A agarose was from Sigma (St Louis, MO).

Cell culture and tissues

Mammary tumors were raised from intrafat pad transplants of an undifferentiated mammary carcinoma derived after transfection of primary mammary epithelial cells from virgin Balb/c mice with the BAG (12) retroviral vector containing a mouse Gαz cDNA insert. This tumor arose originally from transfected cells transplanted into cleared fat pads and is stored as a frozen stock. Tumors are raised in virgin hosts in vivo by transplantation into the fat pad. In this tumor, the retrovirial insert appears to have undergone rearrangement and a full length retroviral transcript is not detectable by northern analysis (unpublished data). In vivo, this tumor contains undifferentiated, hormone-responsive, non-tumorigenic hosts, while in vitro, progesterone and prolactin but not estrogen stimulate the proliferation of this tumor (unpublished observations). This tumor is, thus, classified as hormone responsive, not hormone dependent. Normal tissues were from mature virgin Balb/cAnNcrlBR mice obtained from Charles River. Animals were maintained and killed according to NIH guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Normal and tumor tissues were dissociated with collagenase (0.05%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (11,13). These cells have been shown in extensive studies to be composed primarily of ductal epithelium with a minor population of myoepithelial cells located basally to the ductal epithelium (14,15). For growth experiments, cell organelles were mixed with neutralized, isosmotic rat collagen, and 0.5 ml containing ~2×10⁵ cells pipetted into individual wells of 12-well culture plates containing a preformed bottom layer of collagen gel (0.25 ml) as described (11). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham’s F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 µg/ml insulin, 100 µM soybean trypsin inhibitor, 1 µg/ml α-tocopherol succinate and other additives as indicated in legends to figures. Cells were grown at 37°C in a 2% CO₂/98% air atmosphere. Medium was changed every other day. When PD 098059 (in dimethyl sulfoxide) was added, it was mixed well by vortexing in fresh medium at the time of medium changes. For determination of cell number, collagen gels containing cells were transferred to 12×75 mm glass tubes, acidified with 1/10 vol 25% acetic acid and incubated at 37°C until the collagen had dissolved. Cells were pelleted, extracted with 70% ethanol overnight and the cell pellets dried for fluorometric DNA assay using dianminobenzoic acid (16). Cell cultures for kinase assays were established as monolayers on collagen-coated culture dishes to facilitate rapid termination. Dishes were coated with collagen by covering the surface of the dish with a thin film of denatured rat tail collagen and air drying overnight. Cell organoids were seeded onto collagen I-coated 10 cm culture dishes in basal F12/DMEM (above) supplemented with 2% porcine serum (for tumor) or 5% BSA (for normal MEC). After 4–6 days, when the cells had grown to near confluence, the cells were rinsed three times with basal serum-free medium and incubated in this medium for 24–36 h prior to the initiation of experimental treatments. Control experiments showed that serum pre-exposure had no effect on the time course of ERK activation for tumor or normal cells.

MAP kinase assay

MAP kinase assays were performed using an immune complex kinase filter assay as described previously with minor modifications (17). Briefly, 0.3 µg of sample protein and 10 µl Protein A-Sepharose beads, washed previously in Triton lysis buffer and conjugated with 3 µg anti-MAP kinase rabbit polyclonal antibodies (ERK1 and ERK2), were mixed in a total volume of 20 µl assay buffer [50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol] and resuspended in 40 µl of kinase reaction buffer containing 20 µM ATP, 0.25 mg/ml myelin basic protein peptide and 10 µCi (γ-32P)ATP. The samples were incubated for 30 min at 4°C and the reaction was terminated by the addition of 20 µl of 200 mM EDTA, pH 7.0. Samples were centrifuged (13 000 g, 2 min, 4°C) and 50 µl of the supernatant was spotted on p81 cation-exchange filter paper (Whatman). The filters were washed three times (8 min each) in 200 ml of 180 mM phosphoric acid, once in 200 ml of 96% ethanol and then air-dried. Radioactivity was quantitated by liquid scintillation counting (Packard).

Western blotting and immunoprecipitation of EGFR

Samples containing 20 µg of total protein were electrophoresed on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBS buffer containing 5% (w/v) dry milk and 0.1% Tween and incubated with anti-phospho-ERK antibody (diluted at 1:3000), ERK1 and ERK2 antibodies (diluted at 1:10 000), or anti-EGF receptor (EGFR) antibody (diluted at 1:2000) as indicated. For immunoprecipitation of the phosphorylated EGFR, 200 µg of total protein was immunoprecipitated overnight with anti-phosphorytrosine antibody (PY20). The immunoprecipitates were resolved on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with EGFR antibody (diluted at 1:1000). For all immunoblotting, a horseradish peroxidase-conjugated secondary antibody was utilized to allow detection of the appropriate bands using enhanced chemiluminescence (Amersham). Immunoprecipitation with antisera to the EGFR followed by immunoblotting with PY20 yielded results similar to the above protocol (data not shown), showing that immunoprecipitation with PY20 precipitated phosphorylated EGFR not just receptor-associated tyrosine phosphorylaited phosphoproteins complexed to non-phosphorylated EGFR.

Results

Effect of EGF on proliferation

The concentration dependence and time course for EGF-stimulated growth of normal and mammary tumor cells in serum-free, basal medium containing insulin was examined (Figure 1). Compared with normal mammary epithelial cells in collagen gel culture, the tumor cells showed similar sensitivity to EGF. The relative cell numbers for normal and tumor cells (5.9- versus 3.7-fold, respectively). The time course of growth for tumors exhibited a rapid phase of growth beginning at 4 days and reaching a plateau at 10 days. This is a typical response pattern in collagen gel culture. Actual increase in cell number is not observed immediately after culture since the cell organoids first have to attach to, and cells begin to migrate into, the collagen gel matrix. In contrast to normal mammary epithelium (18), the proliferative response to EGF is not dependent upon insulin, since EGF was growth promoting in medium lacking insulin supplementation (not shown).

Effect of EGF on MAP kinase activity

Normal and tumor mammary epithelial cells were cultured on collagen-coated culture plates and exposed to EGF in serum-free medium for different times. Cells cultured in the monolayer system also proliferate in response to EGF over a long time course similar to cells cultured within gels (19). The monolayer system was originally chosen to facilitate rapid termination of cultures. At termination, cells were lysed and total ERK1 and
ERK2 activities determined after immunoprecipitation with combined ERK1 and ERK2 antisera. Figure 2A shows that in normal cells, ERK activity in immunoprecipitates rose to a peak at 4 min and declined rapidly within 10 min to a level ~2-fold higher than the basal activity. In comparison, tumor cells (Figure 2B) showed a 4-fold higher basal activity and a sustained increase in kinase activity in response to EGF. Although the fold activation by EGF was less than for normal cells (due to the elevated basal activity), the final level of activity was ~2-fold higher. At times >60 min, there was a gradual decline in ERK activation in tumor cells. The difference in the time course for kinase activation (maximum in minutes to 1 h) versus proliferation (maximum after days) reflects the difference in the way the assays are performed. Kinase assays on monolayer cultures are designed to detect an EGF response in a ‘synchronized’ population i.e. re-exposed to EGF after a period in its absence. Under these conditions, a response to EGF is measurable and illustrative of the effect of EGF. During long term continuous exposure to EGF in proliferation assays, we assume a similar activation is occurring (also accompanied by a prolonged elevated basal level) in dividing cells over time as cells enter the proliferating cell pool (represented by cells at the periphery of colonies or monolayers).

Immunoblot analysis of cell lysates was performed using antisera against phosphorylated and total ERK1 and ERK2 to examine the relative activation of ERK1 and ERK2. Shown in Figure 3 is a comparison of normal (A) and tumor cells (B) cultured in basal medium or stimulated by EGF. For both cell types, ERK1 and ERK2 were activated with no apparent change in total ERKs. Examination of the immunoblotting data suggests that in tumor cells, EGF activates ERK1 (p44) to a greater extent than ERK2 (p42). Densitometry of the ERK bands and comparison of the ratio (ERK1:ERK2) of the fold increases caused by EGF at 4 min showed that this ratio was greater in tumor cells compared to normal cells.
higher in tumor cell cultures (mean = 5.2, range 2.8–11.8, n = 8) compared with normal cell cultures (mean = 1.35, range 1–2.6, n = 6). These results suggest that ERK1 is more important for the EGF response in tumor cells, whereas ERKs 1 and 2 were activated to a similar extent in normal cells.

Recent work has indicated that the total level of MAP kinases is higher in breast cancer compared with normal breast (10). We compared the total level of ERKs in normal and tumor cells by electrophoresing, side-by-side, equal amounts of protein from lysates of these cells cultured in basal medium, and performed immunoblot analyses with antisera to ERK1 and ERK2. ERK bands were subjected to scanning densitometry, summed and normalized to the lowest value. By this analysis, total ERK levels for normal and tumor cells, 1.06 ± 0.19 versus 1.14 ± 0.27 (mean ± SD arbitrary units, n = 6), respectively, were found not to be significantly different (Student’s t-test, P > 0.05). However, the ratio of cytosolic protein to cell number was an average of 73% higher (mean of two experiments) in tumor cells than normal cells, indicating that the basal level of kinases, if normalized to cell number, is higher in tumor cells. Thus, the difference in basal activity between normal and these tumor cells most probably results from differences in the regulation of their state of activation (4-fold) and a smaller contribution from an increased level of kinase per cell.

Dependence of EGF-stimulated cell proliferation on ERK activation

The MEK (ERK kinase) inhibitor, PD 098059, has been shown to specifically and reversibly block ERK activation and inhibit proliferation (20,21). We have shown that EGF, a potent growth stimulant, activates the ERK pathway. To determine if ERK activation by EGF is necessary for the proliferative response to this growth factor, normal and tumor mammary epithelial cells were cultured in the presence of EGF and different concentrations of PD 098059. The effect of PD 098059 on EGF-induced proliferation and ERK activation was then assessed. This compound inhibited the proliferation of tumor and normal cells cultured in the presence of EGF in a concentration-dependent manner (Figure 4A and B). The growth of cells exposed to dimethyl sulfoxide vehicle alone was not affected (not shown). This inhibitory effect is consistent with the interpretation that the ERK pathway is a major signaling pathway involved in EGF mitogenesis in mammary epithelial cells but does not establish that this is the only pathway or that this pathway may not act in synergism with other pathways. That other cellular functions could be affected by PD 098059 was shown by the effect of the inhibitor on the morphology of the colony outgrowths for normal and tumor cells. The typical stellate morphology was reduced and/or maintenance of matrix interactions, phenomena that may be related.

To confirm that PD 098059 was inhibiting ERK activity,
MAP kinase and mammary growth

**Fig. 6.** Reversibility of PD 098059 growth inhibition in tumor cells. PD was added to EGF-stimulated cultures for 6 days (EGF + PD 6 days). At this time, parallel cultures in PD were switched to EGF-only medium and cultured for an additional 2, 4 or 6 days before termination. Control cultures containing only EGF were terminated at 6 days (EGF 6 day) and 12 days (EGF 12 days). Similar results were obtained in a second experiment.

**Fig. 7.** Effect of PD 098059 on MAP kinase activation in (A) normal and (B) tumor mammary epithelial cells. Cells were preincubated in inhibitor for 1 h before EGF was added. After 4 min in EGF, cultures were terminated and western blotting for phosphorylated and total ERKs was performed as described in Figure 3. Representative of three experiments.

**Fig. 8.** Phosphorylation of the EGF receptor in response to EGF and PD 098059. (A) Normal and (B) tumor mammary epithelial cell cultures were stimulated by EGF for 4–60 min. PD 098059 was also included in parallel for cells exposed to EGF for 4 min (peak response). Cell lysates were immunoprecipitated with anti-phosphotyrosine antisera then the immunoprecipitates were subjected to western blot analysis with EGF receptor antisera. The 170 kDa band is the phosphorylated EGF receptor. A431 cell lysates were run as a positive control. Representative of three experiments. B, basal untreated culture.

**Discussion**

These studies were initiated to examine the hypothesis that the regulation of the activity of the MAP kinases, ERK1 and ERK2 could be altered during mammary tumorigenesis. This hypothesis was tested by comparing the effect of EGF, a known activator of this pathway, on proliferation and activation of ERKs in normal and tumor mammary epithelial cells. Previous work examining the effect of growth factor stimulation of confluent and quiescent cultures of fibroblasts has shown that after an early peak in ERK activation, there is a slower elevation in activity over several hours to a level above basal. This later rise or sustained increase in activity is associated with the stimulation of DNA synthesis (22–25).

The time course of ERK activation in our primary epithelial cell cultures differs from that observed in fibroblasts by lacking this biphasic quality. In normal mammary epithelium, EGF stimulates peak ERK activity at 4–5 min post-exposure, with a decline to a steady level ~2-fold above basal by 30 min; this is presumably sufficient and necessary to maintain mitogenesis as revealed using the upstream MEK inhibitor, PD 098059. Thus, during proliferation occurring over a period of 7–10 days, the continuous presence of EGF would stimulate ERK activity in the proliferating pool of cells shown to be localized at the periphery of the colonies or tips of growing projections (26).

In the tumor cells, EGF stimulation caused ERK activity to rise to a plateau at 4–5 min before declining slowly but suggesting that the extended time course of activation in tumor cells is due to continued stimulation of the ERK pathway initiated at the EGF receptor. Figure 8 also shows that PD 098059 pretreatment of cells did not block EGF receptor autophosphorylation (or EGF-induced c-jun kinase (JNK1 and JNK2) activation (unpublished observation)), in agreement with the reported specificity of this agent. Western immunoblotting of cell lysates with antisera to the EGF receptor showed that there was no apparent change in total receptor level during a 1 h time course in normal and tumor cells (data not shown).

cells were cultured in the presence and absence of this compound and ERK activation was assayed by electrophoresis and immunoblotting of cell lysates with antisera to phosphorylated ERK1 and ERK2. Figure 7 shows that ERK phosphorylation was inhibited in normal and tumor cells in a concentration dependent manner with complete inhibition of EGF-stimulated ERK phosphorylation at 50–100 µM.

**EGF receptor phosphorylation in normal and tumor cells**

As shown in Figure 1, maximum EGF-stimulated ERK activation was prolonged in the tumor and relatively transient in normal cells. EGF binding to its receptor results in receptor dimerization and transphosphorylation of tyrosines by the receptor kinases (4). We monitored tyrosine phosphorylation of the receptor to compare the time course of receptor activation to ERK activation. Figure 8 shows that the time course of EGF-induced receptor autophosphorylation differed between normal and tumor cells. Receptor phosphorylation was sustained in tumor but not normal cells where it declined significantly by 30 min. Thus, we find a correlation between ligand-induced EGF receptor activation and ERK activation, sug-
remaining elevated ~3-fold above the basal level. A preferential activation of ERK1 by EGF was also observed. Both enzymes appear to be present in approximately equal levels as observed by immunoblotting, suggesting that MEK is preferentially phosphorylating ERK1 or that the dephosphorylation of these proteins is differentially regulated in the tumor cells. Both ERKs phosphorylate the same sequence in peptide substrates but may differ in their nuclear translocation or association with cell surface receptors (27).

In fact, nuclear translocation may be associated with sustained ERK activation observed in fibroblasts or PC12 cells that is associated with mitogenesis or differentiation, respectively (28,29). We can hypothesize that sustained activation and nuclear localization of ERK1 in tumor cells may play a role in tumorigenesis.

In spite of the enhanced stimulation of ERK activity by EGF, we observed no dramatic enhancement of EGF mitogenesis in vitro. This could reflect a growth limitation of the in vitro system in which proliferation plateaus at 10–14 days, or suggest that EGF also affects non-proliferative signaling pathways affecting tumor cell invasion, differentiation or cell–cell interactions. EGF may also affect proliferation indirectly in vivo by modulating responses to other growth-stimulatory factors such as hormones. Estrogenic control of proliferation has been linked to EGF (30–32) and recently, an estrogen non-responsive breast cancer cell line was found to possess an elevated basal MAP kinase activity (33). Below, we speculate on possible roles that EGF may play in regulating matrix interactions, especially the regulation of matrix-responsive signaling pathways that may play a role in tumor progression.

For both cell types, we observed a correlation between tyrosine phosphorylation of the EGF receptor and ERK activation. Thus, the difference in the time course of EGF-induced ERK activation between normal and tumor cells was due to a sustained activation of the EGF receptor, not a direct modulation of ERK activity downstream of MEK that is independent of EGF receptor activation. This response is reminiscent of a mutant EGFR found in human cancers, which exhibits a low level of constitutive activity combined with a decrease in down regulation resulting in an amplification of signaling and possibly tumorigenesis (34).

The prolonged EGFR activation could be explained by attenuated down regulation or an alteration in the regulation of its phosphorylation state by other kinases or phosphatases. For example, EGFR kinase activity is inhibited by phosphatidylinositol-3-phosphatases regulated by protein kinase C acting through the MAP kinase pathway (35,36), arachidonic acid metabolites can augment the mitogenic effect of EGF by enhancing the phosphorylation of the EGF receptor (37), and phosphatases (PTP1C) have been found associated with the EGFR that can dephosphorylate the receptor upon co-stimulation of cells by ligands to G protein-coupled receptors (38). Also, treatment of primary cultures of mammary epithelium with the tyrosine phosphatase inhibitor, pervanadate, could potentiate cell proliferation stimulated by a suboptimal concentration of EGF that itself is not mitogenic (39).

Another key difference between normal and tumor cells was an elevation in the level of basal kinase activity. We could not reproducibly detect any difference in basal EGFR phosphorylation between normal and tumor cells, although, in breast cancer cells overexpressing ErbB-2, an elevation in ligand-independent basal activity was observed (6). This tumor does not proliferate in the absence of EGF or other mitogens, rendering autocrine stimulation an unlikely explanation for the elevation in ERK activity in basal medium.

It is possible that altered cell matrix interactions seen as changes in tumor morphology in vivo can affect basal ERK activity. Many studies have shown that membrane receptors for matrix molecules (integrins) can signal via the ERK pathway, illustrating how proliferation control can be linked to matrix interactions (40–43). Cell matrix interactions can be EGF dependent as well. We have also observed an effect of PD 098059 on the morphology of the outgrowths in collagen gels. While not directly showing an effect on matrix binding, we speculate that this result indicates that the ERK pathway is required for proper matrix interaction and possibly also cell migration. The synthesis and deposition of matrix molecules (Type IV collagen, laminin, fibronectin) is regulated by substrate (44) and growth factors such as EGF (45). EGF in this way then can affect (directly or in cooperation with other pathways) proliferation and morphogenesis by ERK-directed events that establish matrix interactions such as adhesion complexes and then act as positive or negative co-regulators of matrix-dependent signaling. As an example, overexpression of the EGFR has been associated with a defect in integrin function in human cutaneous squamous carcinoma cells (46).

Another possible explanation for an elevation in basal activity is a change in the regulation of the expression or activity of ERK specific threonin/tyrosine phosphatases that inactivate ERK (24,47,48). Recently, it has been shown in Rat-1 cells that the expression of the ERK phosphatase MKP-1 is controlled by growth factors acting via ERK- and calcium-dependent pathways. Interestingly, treatment with sodium orthovanadate, a phosphatase inhibitor, elevated basal ERK activity in the absence of growth factor (24). Thus, an attenuation in the expression or activation of phosphatases might be manifest as a rise in basal ERK activity.

In conclusion, we find that a hormone-responsive mammary tumor exhibits multiple alterations in the regulation of the activity of the MAP kinases, ERK1 and ERK2 with a smaller change in enzyme level. These alterations are related in part to a change in the regulation of the EGFR but also involve pathways that are independent of EGF. These kinds of alterations in a pathway associated with proliferation and estrogen receptor regulation may play a role in tumor progression and the evolution of hormone independence in breast cancer.

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