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David H. Crean · Charles Liebow
Ming-Ting Lee · Angela R. Kamer
Andrew V. Schally · Thomas S. Mang

Alterations in receptor-mediated kinases and phosphatases during carcinogenesis

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Abstract Increased phosphorylation in cancers can stimulate growth and up-regulate certain receptors. To test whether the functional response of phosphatase receptors is up-regulated during carcinogenesis, we examined the effects of ligands on net phosphorylation in isolated membranes derived from hamster cheek-pouch tissues undergoing malignant transformation. The buccal mucosa of groups of Syrian golden hamsters was exposed thrice weekly to 0.5% dimethylbenzanthracene (DMBA) in acetone for 2–12 weeks to produce premalignant and malignant tissues. Homogenates of these tissues were then incubated with [32 P]ATP in the presence of epidermal growth factor (EGF), agonist of somatostatin analogue RC-160, luteinizing-hormone-releasing hormone (LH-RH) [D-Trp 6]LH-RH, or combinations of EGF, RC-160, and [D-Trp 6]LH-RH. Changes compared to controls in phosphorylation in response to ligands provided estimates of kinase or phosphatase activity. Phosphorylation increased continuously, from the first application

of DMBA in a linear fashion, and independently of EGF stimulation. RC-160 and [D-Trp 6]LH-RH reduced phosphorylation in vitro. This response occurred in premalignant (weeks 6–10 after DMBA application) as well as malignant tissues (week 12 after DMBA application), but was not significant in normal tissues. The results show a continuous augmentation in phosphatase activity prior to the appearance of cancers, but with a delay in expression following the primary event of increased kinase activity. Significantly less phosphorylation of substrates was induced by both RC-160 and [D-Trp 6]LH-RH after in vitro activation by EGF than in the absence of EGF. This suggests that EGF activates latent systems of hormonal receptors. Collectively, these results support the hypothesis that the enhancement of the hormonally stimulated phosphatase in cancers occurs secondarily to the increased kinase activity.

Key words Somatostatin analogue RC-160 · Luteinizing-hormone-releasing hormone [D-Trp 6]LH-RH Receptor kinases · Receptor phosphatases

Abbreviations EGF epidermal growth factor · RC-160 somatostatin analogue · [D-Trp 6]LH-RH luteinizing-hormone-releasing hormone · DMBA dimethylbenzanthracene

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D.H. Crean¹ (✉) · C. Liebow · A.R. Kamer · T.S. Mang
Great Lakes Biomedical Laser Institute, Department of Oral and Maxillofacial Surgery, School of Dental Medicine and Buffalo General Hospital, State University of New York at Buffalo, Buffalo, NY 14214, USA
Fax: (716) 829 3019

M.-T. Lee
National Taiwan University, School of Medicine, Taiwan

A.V. Schally
Endocrine and Polypeptide Research Center, V.A. Medical Center and Tulane University, School of Medicine, New Orleans, LA 70112, USA

Present address:

¹ PDT Inc., Santa Barbara, CA 93117, USA

Introduction

The relationship between increased phosphorylation and the development of cancer has been extensively examined over the past several decades. Any error increasing the phosphorylated signal stimulates the growth of cells, and could lead to cellular transformation. The additional phosphorylation could be caused by an augmentation of any step in the cascade of growth factor signal transduction, including ligands [e.g., excess epidermal growth factor (EGF), transforming

growth factor (TGF α), receptors (e.g., EGF receptor over-expression, constitutively active *erb-B2* product), or substrates for the tyrosine kinases (e.g., *ras* product).

To maintain the extent of the phosphorylated signal in balance, a system must exist to dephosphorylate and inactivate that signal (Liebow and Kamer 1992). The tyrosine phosphatases function as a counterbalance to the tyrosine kinases in controlling cell growth (Hunter 1989). Accordingly, specific phosphatases must exist that remove the phosphates placed on tyrosine residues by tyrosine kinases, which then turn off the signal. Evidence exists suggesting that the tyrosine phosphatases are involved in growth regulation (Klarlund 1985; Liebow et al. 1986, 1989).

The activities of the tyrosine phosphatases may be regulated by ligand binding. Among the ligands that are capable of stimulating tyrosine phosphatase and inhibiting the growth of cancers are analogues of somatostatin and luteinizing-hormone-releasing hormone (LH-RH) (Hierowski et al. 1985; Lee et al. 1991; Liebow et al. 1986, 1989, 1990; Schally 1988; Schally et al. 1990, 1993).

It has been assumed that both the expression of the oncogenes with tyrosine kinase activity (McCarley and Parsons 1987) and the loss of expression of anti-oncogenes with tyrosine phosphatase activity (Liebow and Kamer 1992) could promote cancer development. In fast-growing cancers, one would predict that, on the average, tyrosine kinase activity would be increased and tyrosine phosphatase activity would be decreased. Indeed, various investigators have found that an increase in the tyrosine kinase activity was linked with cancer presence. However, contrary to the predicted decline in tyrosine phosphatase, they have not consistently found that a statistical reduction in tyrosine phosphatase activity was associated with cancers. However, isolated examples of such decreases have been noted (McCarley and Parsons 1987).

Liebow and Kamer (1992) have suggested an explanation for the paradoxical increase in tyrosine phosphatase seen in some cancers. Anti-(growth factor/tyrosine phosphatase receptors) could themselves be activated and up-regulated by phosphorylation (Liebow et al. 1991). This receptor regulation would function as a negative-feedback system limiting excess growth (Liebow and Kamer 1992). As a result, it was predicted that the number of phosphatase-stimulating receptors would increase during the process of carcinogenesis.

In this study, we investigated the ligand-induced alterations in phosphorylation during malignant transformation. This was done to test the hypothesis put forth by Liebow and Kamer (1992). According to this hypothesis, we would expect the kinase activity of isolated tissues to increase first during the process of transformation. This would be followed by increased phosphatase activation. The model utilized was the

squamous cell carcinoma produced in the hamster buccal cheek pouch by dimethylbenzanthracene (Eveson 1981).

Materials and methods

Drugs

9,10-Dimethyl-1,2-benzanthracene [DMBA] was obtained from Sigma Chemical Company and dissolved in acetone to produce a 0.5% solution. Crude adenosine-5'-[32 P]triphosphate was obtained from ICN Biomedicals Inc., Irvine, Calif., with a specific activity of 7000 Ci/mmol. Epidermal growth factor (mouse, natural) was obtained from Collaborative Research Incorporated (Bedford, Mass.). Somatostatin analogue RC-160 and [D-Trp 6]LH-RH were synthesized in the laboratory of one of us [A.V.S.]. All hormones and analogues were used at 0.1 μ M.

Animal model

The experimental epidermoid carcinoma of hamster buccal cheek pouch is a widely studied oral mucous membrane tumor model. The squamous cell carcinomas were induced by thrice-weekly topical applications, by brush, of a 0.5% solution of DMBA in acetone for 12 weeks. A predictable sequence of histological changes transpires in the affected pouch mucosa after DMBA application: hyperkeratosis and chronic inflammation occur after 4–6 weeks, hyperkeratosis and dysplastic leukoplakia follow at 6–8 weeks, carcinoma in situ appears after 8–10 weeks, papillary and frankly invasive carcinoma appear after 10–12 weeks, and finally extensive tumors with invasion and surface necrosis appear after 12–14 weeks (Eveson 1981). In the present study, viral-antibody-free male Syrian golden hamster retired breeders weighing 200 g were obtained from Charles River Laboratories (Kingston, Ontario). The hamsters were housed five to a cage, fed pelleted laboratory diet food (Teklad Premier), and given tap water ad libitum.

Experimental protocol

The animals were anesthetized with an injection of 100 mg/kg Ketaset (ketamine hydrochloride; Avenco Inc.) intramuscularly into the hind leg. The buccal mucosa of groups of hamsters [five per group] was everted and a 3 to 7-cm 2 area exposed thrice weekly to a solution of 0.5% DMBA in acetone using a no. 4 sable brush for 2–12 weeks. Control group animals received no applications of the carcinogen or treatment with acetone. These animals were therefore completely normal compared to the experimental animals which had undergone the total treatment of carcinogen dissolved in acetone. Following the specified times, the animals were sacrificed by barbiturate overdose and the DMBA-initiated mucosal fields excised and used for kinase assays.

Enzymatic assay

Assay procedures were performed on isolated mucosal tissue membranes as described previously to determine net membrane phosphorylation (Lee et al. 1991). The excised tissues were minced in 0.02 M phosphate-buffered saline (pH 7.2, containing 0.5% NP-40 and 1 mM phenylmethylsulfonyl fluoride) using 3 ml/g tissue, and the homogenate was maintained at 4 °C overnight. The crude tissue proteins were then precipitated with 70% ammonium sulfate. After dialysis against 0.02 M HEPES buffer (pH 7.2), crude proteins were

stored at -70°C for future study. Protein concentration was determined using the BioRad protein assay kit (BioRad Laboratories, Richmond, Calif.). The reaction mixtures contained 100 μg hamster buccal mucosal proteins, 20 mM HEPES buffer (pH 7.4), and 1 mM MnCl_2 with various combinations of 0.1 μM EGF, somatostatin analogue RC-160, and/or $[\text{D-Trp}^6]\text{LH-RH}$ in a final volume of 0.06 ml. The reaction mixtures containing EGF, RC-160, and $[\text{D-Trp}^6]\text{LH-RH}$ were pre-incubated at room temperature for 2 h and then chilled at 4°C for 10 min. The reaction was initiated by the addition of $[\text{P}^{32}]\text{ATP}$ (6×10^6 – 12×10^6 cpm) and unlabeled ATP. The reaction was stopped by pipetting 0.05-ml aliquots of the reaction mixtures onto 2.3-cm diameter Whatman 3 MM paper and the filter-papers were immediately dropped into cold 10% trichloroacetic acid containing 0.01 M pyrophosphate. The filter-papers were then washed sequentially with 10% trichloroacetic acid, 50% and 100% ethyl alcohol (10 ml each), and diethyl ether, and dried. Radioactivity was measured in a Beckman scintillation counter.

Net phosphorylation was used to estimate kinase and phosphatase activities. EGF promotes phosphorylation on tyrosine residues of specific membrane proteins (Hunter 1987). RC-160 and $[\text{D-Trp}^6]\text{LH-RH}$ activate tyrosine phosphatase, which dephosphorylates these same residues (Lee et al. 1991).

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean. Tests of linear regression and correlation coefficients (r) of phosphorylation or receptor activity versus the time after DMBA application were analyzed by multiple regression using SPSS (SPSS Inc., Chicago, Ill.). Group means of the biochemical measurements were analyzed for significance by non-parametric paired (Wilcoxon signed-rank) and unpaired tests (Kruskal-Wallis) and Student t -tests with INSTAT (GraphPad, San Diego, Calif.).

Results

Phosphorylation was compared in normal and malignant tissue.

EGF-stimulated phosphorylation

To determine whether there is constitutive and stimulated phosphorylation in the DMBA-initiated hamster buccal cheek pouch oral mucosa, ^{32}P incorporation in the presence and absence of EGF was determined in membranes derived from normal and cancerous cells (Fig. 1). Protein extracts of oral mucosa ($n = 5$ per assay time per group) from normal tissues (week 0 of DMBA application) and invasive disease (after 12 weeks of DMBA application) were incubated with $[\text{P}^{32}]\text{ATP}$ in the presence or absence of 0.1 μM EGF.

It can be seen that kinase expression was different in normal tissues and cancerous tissues. The results demonstrated that constitutively expressed kinase activities of squamous cell carcinomas were significantly greater than kinase in normal cheek pouch ($P < 0.0001$). The EGF-stimulated total kinase activity was also significantly increased in carcinomas as compared to normal tissues ($P < 0.0001$). Stimulation of kinase activity by EGF resulted in significant increases over control

Fig. 1 Incorporation of labeled ^{32}P from ATP into proteins ($10^{-5} \times$ phosphorylation, cpm) of oral mucosal tissues ($n = 5$ for each assay time) from normal (week 0; solid bar) and invasive squamous cell carcinoma (week 12; solid bar) in the absence or presence of 0.1 μM epidermal growth factor (EGF), somatostatin analogue RC-160 (bar with lines rising to the right), or LH-RH agonist $[\text{D-Trp}^6]\text{LH-RH}$ (bar with lines rising to the left). Note the changes in phosphorylation in response to various ligands as the tissues undergo malignant transformation.

groups in which EGF was absent in both normal and cancerous tissues by 70% and 66% respectively ($P < 0.0001$). However, the level of EGF-stimulated phosphorylation in normal tissues was not statistically different from that in cancerous tissues. Thus, the results demonstrated an increase in constitutive and total kinase, but no apparent change in the EGF-stimulated increment.

Somatostatin and LH-RH-analogue-induced reduction in phosphorylation

Analogues of LH-RH and somatostatin are known to stimulate tyrosine phosphatase activity in some cancers (Hierowski et al. 1985; Lee et al. 1991; Liebow et al. 1986, 1989, 1990; Schally 1988; Schally et al. 1990, 1993). The ability of these hormones to reduce net phosphorylation was studied (Fig. 1). In normal tissue samples, the addition of either somatostatin analogue RC-160 or $[\text{D-Trp}^6]\text{LH-RH}$ did not significantly alter protein phosphorylation. However, in cancerous tissues, somatostatin analogue RC-160 significantly decreased the level of phosphorylation by 33% ($P < 0.0005$). $[\text{D-Trp}^6]\text{LH-RH}$ also significantly decreased phosphorylation ($P < 0.01$), but by only 12%. These results suggest that there is an elevation in phosphatase activity, which may be due to increased expression of somatostatin and LH-RH

receptor/phosphatase transduction in cancerous tissues that is not observed in normal tissues.

LH-RH receptors are activated *in vitro* by EGF (Liebow et al. 1991). To examine whether the tissue possessed latent receptors for somatostatin and LH-RH, we incubated the tissues with [32 P]ATP in the presence of EGF. Somatostatin analogue RC-160 significantly decreased phosphorylation in normal tissues by 8% ($P = 0.001$), but agonist [D -Trp 6]LH-RH failed to alter phosphorylation in normal tissues. In cancerous tissues induced by 12 weeks of DMBA application, RC-160 significantly decreased phosphorylation by 46% in the presence of EGF ($P < 0.0001$). [D -Trp 6]LH-RH also decreased phosphorylation in these samples by 39% ($P < 0.0001$). These results suggest that, in normal tissues in the presence of EGF, dephosphorylation occurs in response to somatostatin analogues, but not LH-RH analogues. In cancerous tissues, EGF caused increased response to both somatostatin and LH-RH analogues.

Determination of time course of changes during carcinogenesis

Phosphorylation in the presence and absence of EGF was determined in buccal cheek mucosal samples during various stages of carcinogenesis. Estimates of functional EGF receptors were derived from these measurements (assuming that EGF receptor is proportional to EGF-stimulated phosphorylation minus constitutive phosphorylation).

Figure 2 shows the observed EGF-stimulated and constitutive levels of kinase activities and the computed kinase stimulation in response to EGF for animals sacrificed after various periods of DMBA application. Constitutive kinase activity increased steadily from the initial application of DMBA ($r = 0.98$). EGF addition significantly enhanced levels of kinase activities of sample preparations from week 0 to week 12 after DMBA application compared to control tissues without EGF ($P < 0.0001$). However, slope comparisons of stimulated and constitutive kinase activities showed no significant difference, and there was no significant difference in the slope between the kinase stimulated by EGF and the constitutive kinase over the 12-week period of DMBA treatment. This suggests that EGF receptor remained relatively unchanged during transformation.

The appearance of phosphatase receptor activity during the process of malignant transformation

In membranes derived from normal tissues there was little or no change in phosphorylation after stimulation by somatostatin analogue RC-160 or [D -Trp 6]LH-RH, but in membranes derived from cancers a decrease was

Fig. 2 Phosphorylation ($10^{-5} \times {}^{32}\text{P}$, cpm) in the presence and absence of EGF (0.1 μM) in mucosal sample preparations ($n = 5$ for each assay time) as a function of the time after dimethylbenzanthracene (DMBA) application (weeks). Functional EGF receptor levels (■) were estimated by subtracting constitutive tyrosine kinase activity (○) from total tyrosine kinase activity (●). Note the significant increase in both constitutive and EGF-stimulated total tyrosine kinase activity

Fig. 3 Phosphorylation ($10^{-5} \times {}^{32}\text{P}$, cpm) in the presence or absence of 0.1 μM somatostatin analogue RC-160 or agonist [D -Trp 6]LH-RH of mucosal sample preparations ($n = 5$ for each assay time) as a function of the time after DMBA application (weeks). Tissue samples were examined for incorporation of ^{32}P from ATP in the absence (control; ○) or presence of somatostatin analogue RC-160 (□) or agonist [D -Trp 6]LH-RH (△). Note the decrease in phosphorylation induced by the hormonal analogues prior to the development of invasive squamous cell cancer.

found (Fig. 1). We examined the time course of induction of these responses. Results in Fig. 3 demonstrate that RC-160 did not decrease the levels of phosphorylation significantly from week 0 to week 6 after DMBA

application. However, after 6 weeks of DMBA application, RC-160 decreased net phosphorylation significantly ($P < 0.0001$). Examination of the first 6-week period shows that a y -axis intercept was significantly less for the RC-160-treated samples than for the control samples without RC-160 ($P = 0.02$). This suggests that some somatostatin-sensitive phosphatase receptors exist in the membranes of normal tissues.

Examination of the ability of [D-Trp⁶]LH-RH to decrease phosphorylation showed results similar to those produced by RC-160 (Fig. 3). From 8 weeks of DMBA application onward, the decrease in net phosphorylation in response to LH-RH analogue became significantly greater with tumor progression in a continuous manner (weeks 8–12, $P = 0.03$). The y -axis intercept of the phosphorylation line from week 0 to week 8 was not different in the presence or absence of [D-Trp⁶]LH-RH, suggesting no LH-RH-sensitive phosphatase receptors existed in membranes derived from normal tissues.

EGF activation of somatostatin- and LH-RH-sensitive receptors.

Since EGF has been shown to activate latent LH-RH receptors by radioreceptor binding assay (Liebow et al. 1991), we examined the ability of analogues of somatostatin and LH-RH to reduce net phosphorylation in the presence and absence of EGF in the *in vitro* phosphorylation mixture. Figure 4 shows the results with somatostatin analogue RC-160. These results confirm

Fig. 4 Decreases (%) in phosphorylation in the presence (open bars) and absence (solid bars) of 0.1 μ M EGF promoted by 0.1 μ M RC-160 in mucosal sample preparations as a function of the time after DMBA application (weeks). Note the decreased level of phosphorylation in the presence of RC-160 after EGF addition compared to phosphorylation, in the absence of EGF

Fig. 5 Decreases (%) in phosphorylation in the presence (open bars) and absence (solid bars) of 0.1 μ M EGF promoted by 0.1 μ M [D-Trp⁶]LH-RH in mucosal sample preparations as a function of the time after DMBA application (weeks). Note the decreased level of phosphorylation in the presence of [D-Trp⁶]LH-RH after EGF addition compared to phosphorylation in the absence of EGF

the observations recorded in Fig. 3 in terms of the ability of RC-160 to reduce net phosphorylation in the absence of EGF: (a) there was no significant decrease in phosphorylation in the presence of RC-160 over the first 6 weeks; (b) from week 6 to week 12 there was a significant decrease in phosphorylation ($P < 0.0001$); (c) all the measurements throughout the first 6 weeks showed slight decreases in phosphorylation in response to RC-160, and the week-0 assay time y -axis intercept for the RC-160-induced reduction in phosphorylation was positive ($P = 0.02$). In the presence of EGF, decreases in phosphorylation promoted by RC-160 were significantly greater than those produced by RC-160 in the absence of EGF ($P = 0.02$). The decrease in phosphorylation in the presence of EGF was shown to increase over the entire observation period in comparison to decreases in phosphorylation only from week 6 to week 12 after DMBA application in the absence of EGF ($P < 0.0001$). This suggests that EGF up-regulated the somatostatin-receptor-mediated response. It is possible, however, that the increased response to RC-160 in the presence of EGF is due to additional phosphorylated substrates that are available for regulation by somatostatin-stimulated phosphatase receptors. This seems unlikely for two reasons. First, the decrease in phosphorylation is represented as the percentage of total available substrate (Fig. 4) so this should nullify the decrease in phosphorylation by a rise in substrate concentration. Second, the time course of increased response to RC-160 is very different from that of the augmentation in kinase activity.

Figure 5 shows the ability of [D-Trp⁶]LH-RH to decrease phosphorylation in the presence or absence of

EGF. These results again confirm the observations shown in Fig. 3 in terms of [D-Trp⁶]LH-RH ability to reduce phosphorylation in the absence of EGF: (a) there was no decrease in [D-Trp⁶]LH-RH-stimulated phosphorylation over the first 8 weeks; (b) from week 8 to week 12 there was a significant decrease in phosphorylation (for week-12 samples, [D-Trp⁶]LH-RH-reduced phosphorylation was significantly greater than in untreated tissue samples from week 0; $P < 0.05$); (c) the measurements during the first 8 weeks showed no response to [D-Trp⁶]LH-RH in phosphorylation. In the presence of EGF, [D-Trp⁶]LH-RH stimulated a significant decrease in phosphorylation from week 0 ($P < 0.0001$), as in the case of reduced phosphorylation in response to RC-160. In addition, [D-Trp⁶]LH-RH induced a significant reduction in phosphorylation in the presence of EGF even in the untreated, normal membranes ($P < 0.05$). At all assay times throughout the first 8 weeks, there was in fact no detectable [D-Trp⁶]LH-RH response in the absence of EGF, but a significant response in its presence.

Discussion

In this investigation, we examined alterations in constitutive and ligand-induced phosphorylation in mucosal membranes derived from tissues in various stages of malignant transformation. Liebow and Kamer (1992) have postulated a model describing growth regulation as a function of tyrosine phosphorylation and dephosphorylation. According to this model, tyrosine kinase activation is a primary event. This is followed by tyrosine phosphatase activation.

The objective of this study was to assess the ability of tissue undergoing transformation to respond to various ligands. The response to somatostatin, LH-RH and their analogues that we were interested in is the inhibition of growth of transformed tissue. This response could be measured in transforming tissue directly by growth studies, or estimated by measuring either the binding of an appropriate ligand to membranes or the ability of the ligand to reduce net phosphorylation in these membranes. The use of a ligand's ability to stimulate phosphatase, or to reduce the amount of net phosphorylation in the membranes, as an estimate of its ability to inhibit growth, has been shown to be appropriate by several studies (Hierowski et al. 1985; Lee et al. 1991; Liebow et al. 1986, 1989, 1990).

Measuring the growth response to various analogues, though the most valid technique, has the most difficulties. In vivo measurement of growth retardation is impractical. In vitro results require the removal of cells and cell or tissue culture of these

extracted cells. This can alter the characteristics of the cells. In addition, these assays are labor-intensive and not very precise.

Binding of somatostatin, LH-RH, or their analogues, to their receptors cannot simply be equated to tyrosine phosphatase activation or growth inhibition. One phenomenon that could dissociate ligand binding and tyrosine phosphatase activation is the existence of a cascade of events between ligand binding and phosphatase stimulation. Interruption of any step in the cascade would block the process. The nature of a particular bound ligand activating the effector portion of the receptor may differ dramatically, since some ligands can act as agonists and others as antagonists (Liebow et al. 1990; Keri et al. 1993). If an enzyme molecule independent of the receptor serves as the phosphatase, then its absence would make hormone binding ineffective in stimulating phosphatase activity. Similarly, even if all the systems cited are functional, if there is no phosphorylated substrate on which the enzyme works, no dephosphorylation would be seen in response to hormone addition (Wang et al. 1993). It also has been found that multiple forms of somatostatin receptors exist, with significant homology shared on the extracellular portion of the molecule and little homology on the intracellular portion (Rohrer et al. 1993). One or more of these receptors appears to be associated with tyrosine phosphatase activation, and others could have different transduction pathways (Buscail et al. 1994). All this evidence taken together, supports the view that the best predictor of the ability of a particular ligand to inhibit growth of a target tissue is the measurement of decreased phosphorylation in response to that particular ligand in that tissue. The measure of altered phosphorylation in response to a ligand would include changes in the ability of the tissue to bind the hormones and also changes in the receptor's ability to transduce its signal (Lee et al. 1991).

In the isolated membrane system we could, therefore, use changes in net phosphorylation in response to addition of these ligands as estimates of enzymatic activity. The difference between net phosphorylation in the presence and absence of exogenously added 0.1 μ M EGF in the incubate was used as an estimate of EGF-activated kinase. Hormonally-induced phosphatase activity was estimated by the difference of phosphorylation in the absence and presence of 0.1 μ M RC-160 or [D-Trp⁶]LH-RH. This estimate of course would not only measure increased phosphatase activity, but also decreased kinase activity. Both of these activities (i.e., increased phosphatase and decreased kinase), however, should contribute to growth inhibition and therefore need not be separated in this study. This activity was evaluated in the absence or presence of EGF to determine EGF activation of hormone-dependent phosphatase. The decrease in

protein phosphorylation is dependent on both the amount of active phosphatase and the amount of phosphorylated substrate. Since the amount of phosphorylated substrate varies with the state of tumor progression, the results are presented both as the absolute decrease in phosphorylation and as a percentage decrease in phosphorylation. The second value partially accounts for variations in substrate availability.

In this study, the existence and amplification of the kinase message transduction system was demonstrated during the process of malignant transformation. Constitutive kinase activity increased during this transformation. EGF-stimulated kinase activity did not change during transformation. Because of the increase in constitutive activity, however, total kinase activity increased significantly during the course of malignant transformation. Thus, in this model, kinase activity is increasing in a linear and continuous manner, from the very start of tissue transformation, independent of EGF-stimulated kinase.

We estimated the ability of somatostatin and LH-RH receptors in membranes to stimulate phosphatase functionally by measuring the decrease in total phosphorylation in the presence and absence of the appropriate analogue. There are a number of methods that could have been used to determine the alterations in these receptor complexes. Binding of labeled analogues could have provided the number of sites on the membrane that were available to bind ligand actively. This method, however, would not have determined whether these sites are functional or involved with phosphatase stimulation. Five different somatostatin cDNA have already been identified, and only one or two of these are involved in phosphatase stimulation (Buscail et al. 1994). Consequently, radioreceptor binding would not provide an adequate picture.

As a more definitive measure of receptor response, membrane products could have been separated on gels after ^{32}P labeling. The dephosphorylation of individual membrane proteins then could be assessed by various methods. This has already been done on selected membrane samples, and the specificity of substrate dephosphorylation seems to be qualitatively, though not quantitatively, identical to the substrate phosphorylation stimulated by EGF (Lee et al. 1991). In addition, the dephosphorylation stimulated by somatostatin is vanadate-sensitive, and effective only for tyrosine, not serine, histone substrates (Hierowski et al. 1985). Somatostatin-stimulated dephosphorylation also shares substrate specificity with LH-RH-stimulated activity (Lee et al. 1991). The technique of gel separation, however, is very time-consuming and difficult to quantify accurately.

Phosphatase activity could be more accurately measured by prelabeling a substrate on tyrosine residues and incubating this substrate with membranes and ligands. This would have the advantage over

evaluating net phosphorylation in that it would only measure increased phosphatase activity, not decreased kinase activity. However, this method would also not be sensitive to alterations in the content of available cellular proteins that serve as substrates for the kinases and phosphatases. There is evidence, for instance from the decreased initial rate of the kinase reaction, that somatostatin- and LH-RH-receptor complexes can in fact decrease kinase activity, probably through dephosphorylating the EGF receptor. This activity, however, is also an index of somatostatin and LH-RH receptor activities, and contributes to limiting cellular growth rates. The differences in net alterations of phosphorylation in the presence and absence of ligand, therefore, represent a reasonable index of not only available receptors, but also their contribution to growth control.

Analogues of somatostatin and LH-RH, such as RC-160 and $[\text{D-Trp}^6]\text{LH-RH}$, have many biological actions. It has been established that the various actions of somatostatin and LH-RH and their analogues in experimental animals and in man are mediated through specific, high-affinity receptors (Schally 1988; Schally et al. 1990, 1993). Cell lines and experimental tumor models having receptors for either hormone respond to their respective analogues (Schally 1988; Schally et al. 1990, 1993). Through these investigations, it has become evident that not only normal tissues, but also various malignant tissues, contain somatostatin and LH-RH receptors. In this study, somatostatin analogue RC-160 significantly decreased the level of phosphorylation in cancerous tissues by 33%, but had only minimal effects in normal tissues. $[\text{D-Trp}^6]\text{LH-RH}$ significantly decreased the level of phosphorylation by 12% in cancerous tissues, but not in normal tissues. These results suggest that there is an expression of somatostatin- and LH-RH-sensitive receptor-transduction complexes for phosphatase activity on the membranes of cancerous tissues that is not observed in normal tissues.

In vitro incubation of membranes with EGF enhanced the phosphatase response of these membranes to somatostatin and LH-RH analogues. In the presence of EGF, somatostatin analogue significantly decreased the phosphorylation in normal tissues by 8% and in cancerous tissues by 46%, compared to 5% and 33%, respectively, in the absence of EGF. $[\text{D-Trp}^6]\text{LH-RH}$ reduced phosphorylation in cancerous tissues by 39% in the presence of EGF, compared to 12% in its absence. The decreased phosphorylation in response to RC-160 and $[\text{D-Trp}^6]\text{LH-RH}$ after in vitro incubation with EGF suggests that EGF activates latent receptor/phosphatase transduction complexes. These observations are consistent with the activation of LH-RH receptor by EGF-stimulated phosphorylation (Liebow et al. 1991).

Since it was demonstrated that there were significant differences in RC-160 and $[\text{D-Trp}^6]\text{LH-RH}$ -induced

phosphatase expression in normal and cancerous tissues, the time course of expression was investigated. Examination of the first 6-week period of DMBA application to oral mucosal tissue showed no significant decrease in phosphorylation that could be mediated by somatostatin receptors. Similarly, there was no significant increase in $[D-Trp^6]LH-RH$ reduction in phosphorylation in tissues during weeks 0–8 after DMBA application. After 6 weeks, in the premalignant tissues, a somatostatin-sensitive reduction in phosphorylation was first observed. This response increased in a continuous manner through the development of invasive cancer. The decrease in phosphorylation in response to $[D-Trp^6]LH-RH$ increased in a similar manner starting at week 8 after DMBA application.

Previously it was demonstrated that one class of somatostatin and LH-RH receptors, after activation by hormonal analogue binding, stimulates tyrosine phosphatase activity leading to the dephosphorylation of substrates previously phosphorylated by tyrosine kinases (Hierowski et al. 1985; Lee et al. 1991; Liebow et al. 1986). In this study, somatostatin analogue RC-160 reduced phosphorylation. This response increased in linear fashion in tissues after 6 weeks of DMBA application. These results confirm the observations of RC-160-stimulated phosphatase activity during the same period. $[D-Trp^6]LH-RH$ induced a similar reduction in phosphorylation. There was no significant response to $[D-Trp^6]LH-RH$ during the first 8 weeks after DMBA application. But from week 8 to week 12, there was a significant response consistent with the expression of functional LH-RH-sensitive phosphatases.

Examination of the ability of RC-160 to reduce phosphorylation in tissues during the first 6 weeks of DMBA treatment in the absence of EGF (Fig. 3) showed a y-axis intercept that was significantly less than values for control samples without RC-160. This might provide evidence for some somatostatin-sensitive phosphatase receptors in normal tissues. In the presence of EGF, however, the reduction in phosphorylation promoted by somatostatin analogue RC-160 increased significantly over controls and this increase occurred at earlier times than when EGF was absent. These results confirm that some latent somatostatin-sensitive phosphatase receptors may exist in normal tissues and are activated by EGF-stimulated phosphorylation. Likewise, tissues not treated with DMBA but exposed to $[D-Trp^6]LH-RH$ in the presence of EGF showed significant decreases in phosphorylation. These results suggest an activation of latent LH-RH-sensitive phosphatase receptors by EGF in accord with previous studies (Liebow et al. 1991).

The ability of LH-RH to reduce phosphorylation appears to be more dependent upon EGF-mediated activation than is somatostatin. LH-RH analogue was incapable of altering phosphorylation in normal tissues without in vitro activation by EGF. Examination of

y-axis intercepts of the phosphorylation plots during weeks 0–8 in the presence or absence of $[D-Trp^6]LH-RH$ demonstrates no significant difference. Some somatostatin-sensitive receptors, however, exist in normal tissues. One explanation for this is that both somatostatin and LH-RH receptors are activated and inactivated by phosphorylation/dephosphorylation, but LH-RH-sensitive receptors may require higher concentrations of phosphorylation for activation than do somatostatin-sensitive receptors.

Cancer cells expressing increased tyrosine kinase activity may utilize several mechanisms in an attempt to limit the accumulation of excess phosphorylated growth message. These could include (a) greater expression of constitutive tyrosine phosphatases, (b) activation of latent tyrosine phosphatases, (c) increased synthesis of tyrosine phosphatase receptors, and (d) increasing secretion of hormones that activate tyrosine phosphatases. Constitutive tyrosine phosphatases may exist, which work together with tyrosine kinases to balance production of phosphorylated product. The excess tyrosine kinase activity in some cancer cells increases the accumulation of phosphorylated products. Not only are growth messages phosphorylated, but latent tyrosine phosphatase receptors could be activated through phosphorylation as well (Liebow et al. 1991). Evidence exists that phosphorylation can involve rapid receptor activation independent of synthesis of new receptors. EGF-induced in vitro phosphorylation caused increases in LH-RH and somatostatin-sensitive phosphatases in this experiment. The relationship between increased synthesis and increased activation of these receptors is not yet clear, but both appear to be altered during the process of carcinogenesis.

Our findings may have several important biological implications: (a) somatostatin and LH-RH can alter phosphorylation in premalignant tissues and this might serve as an important marker of transformation; (b) the responses to somatostatin and LH-RH appear at different times during transformation (i.e., constitutive tyrosine kinase activity increases from the first DMBA treatment, while somatostatin and LH-RH responses appear later, but they all increase prior to cancer development); (c) the cellular responses to somatostatin and LH-RH are regulated by EGF stimulation; (d) the model of Liebow and Kamer (1992) is supported where tyrosine kinase activation is a primary process in cellular transformation with phosphatase activation lagging behind.

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