Dephosphorylation of Cancer Protein by Tyrosine Phosphatases in Response to Analogs of Luteinizing Hormone-releasing Hormone and Somatostatin

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Abstract. *Protein phosphorylation/dephosphorylation of tyrosine residues is an important regulatory mechanism in cell growth and differentiation. Previously it has been reported that RC-160, an octapeptide analog of somatostatin, and [D-Trp^6]LHRH, an agonist of luteinizing hormone-releasing hormone (LHRH), stimulate receptor-mediated activity of tyrosine phosphatases (PTP) and reverse growth promotion of the tyrosine kinase (PTK) class of oncogenes in tumor cells. The effect of RC-160 and [D-Trp^6]LHRH on protein phosphorylation was further examined in surgical specimens of human carcinomas. Protein extracts of human ovarian, liver, breast and prostate tumor samples were preincubated with epidermal growth factor (EGF) (10^{-7} M) with or without [D-Trp^6]LHRH or RC-160 (10^{-6} M) at 25˚C for 2 h, followed by incubation for 10 min with [γ-^{32}p]ATP. SDS-PAGE, Western blotting, autoradiography and densitometry were then used to quantify the phosphorylation level of individual protein bands. It was found that EGF enhanced, and [D-Trp^6]LHRH and RC-160 reduced phosphorylation of a prominent 300-kDa band. Two proteins (65 and 60 kDa), involved in growth control in tumor cell lines, were also identified in this study. The homology of substrate phosphorylation between induced PTK and PTP in the presence of hormones provided evidence that these substrates might be identical or related in tumors. These findings, along with the previous cell culture results, suggest that many solid tumors may respond to treatment with analogues of somatostatin and LHRH. Collectively, the results further support the hypothesis that these 60-, 65- and 300-kDa protein substrates may be involved in growth-message transduction.*

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It has been known for many years that phosphorylation is an important step in regulating cell function. Many growth factors, such as the family of epidermal growth factor (EGF), transmit their growth signals to the cell by promoting the phosphorylation of specific tyrosine residues of various protein substrates. The expression of tyrosine kinases (PTKs), including several membrane-associated oncogene products, is elevated in tumor cells (1). The relationship between this increased phosphorylation and cancer development has been well documented (2-4). Any error increasing the phosphorylation signal stimulates cell growth and may lead to cellular transformation. This additional phosphorylation could be caused by augmentation of any step in the signal-transduction cascade (5, 6). To maintain the balance of the phosphorylation signal in cells, there must be a system for removal of the phosphate from the phosphotyrosyl protein to turn off growth. While the involvement of PTKs in the cellular signal pathway has been intensively investigated and the research extensively reviewed, relatively little is known with respect to the precise nature of protein tyrosine

phosphatase (PTP) (7). Nevertheless, the discovery of the tyrosine PTP family and the examination of the tyrosine phosphatases themselves led to the prediction that PTPs can be regulated (7, 8). A great deal of evidence is accumulating in support of the regulatory role of PTP activity (9). These observations on PTP have led to the conclusion that carcinomas should, on average, produce less PTP activity while exhibiting greater PTK activity.

Inhibition of cellular protein kinases and activation of protein phosphatase activities have been associated with growth inhibition in various tumor cells. It has been shown that two hormones, somatostatin and luteinizing hormone-releasing hormone (LHRH), stimulate the activity of protein tyrosine phosphatase in a broad range of tumor cells with potency equivalent to the growth inhibition (10-14). It was reported that the octapeptide RC-160, an analog of somatostatin and [D-Trp^6]LHRH, an agonist of LHRH, both peptide analogs of hypothalamic hormones, inhibit the growth of various tumors (10, 15, 16). We have previously reported that [D-Trp^6]LHRH reduced total protein phosphorylation of HCPC and MiaPaCa-2 cellular proteins and inhibited growth of these cell lines, while EGF acted in the opposite way (11). In addition, *in vivo* treatment of experimental animals with agonist [D-Trp^6]LHRH, the LHRH antagonist cetrorelix, or the somatostatin analog RC-160, also resulted in the enhancement of apoptosis in MXT breast carcinomas in mice, Dunning prostate carcinomas in rats, and nitrosamine-induced pancreatic carcinomas in hamsters (17). However, Grundker *et al*. (18) have shown, in EFO-21 and EFO-27 ovarian cancer cells, that *in vitro* treatment with [D-Trp^6]LHRH induced NFκB activation and inhibited apoptosis. Although this effect is mainly mediated by an indirect mechanism involving the reduction of hormone secretion, it has been demonstrated that various tumors possess specific receptors for LHRH and somatostatin (10, 15, 16). Also [D-Trp^6]LHRH receptor-binding activated a specific PTP in MiaPaCa-2 and A431 tumor cells. [D-Trp^6]LHRH may function, at least in part, by inhibiting tyrosine kinase activity of EGF receptor (EGFR), thereby mediating growth suppression (11, 12). In addition, [D-Trp^6]LHRH reduced the levels of basal and EGF stimulated total kinase activity, particularly phosphorylation of certain proteins including the 170-, 65- and 60-kDa proteins (11, 12). The similarity in substrate specificity suggests that the (de)phosphorylation of these proteins may play an important role in the regulation of cell growth.

The relationship between the efficacy of tyrosine phosphatase activity and growth inhibition suggests that net phosphorylation of one or more signal molecules may well be the determining factor for growth control. This phenomenon was also observed in our parallel studies using tyrosine kinase inhibitors (19) in carcinoma cells. It was, therefore, of interest to examine the response of solid-tumor proteins treated with somatostatin or [D-Trp^6]LHRH in terms of phosphorylation. Finding important protein substrate(s) for PTK and PTP could differentiate the control of cell growth in normal and tumor cells.

It is important to identify not only the ligands that activate the tyrosine phosphatases, but also the related substrates specific for these activated tyrosine phosphatases. For a protein to be regulated by phosphorylation, the maximal activities of PTK and PTP acting on a particular protein substrate must be in balance (20). To facilitate the analysis of protein substrate phosphorylation and dephosphorylation, it is useful to have reagents that activate or inhibit tyrosine kinase. In our previous studies, we provided evidence that [D-Trp^6]LHRH could activate the enzyme tyrosine phosphatase in tumor cells, which in turn dephosphorylates certain phosphotyrosyl proteins stimulated by EGFR tyrosine kinase activity (11, 12).

In the present study, whether or not the changes in protein substrate phosphorylation that occur in response to [D-Trp^6]LHRH and RC-160 in cell cultures parallel those in surgical specimens of human carcinomas was investigated. The rationale for the previous *in vitro* study was that, given the existence of endogenous molecules such as receptors for LHRH and the somatostatin, the protein tyrosine phosphatase activity could be stimulated by the treatment with LHRH or RC-160. Conversely, EGF receptor tyrosine kinase activity could also be stimulated by the addition of EGF (11, 12). Experiments in animal models showed that [D-Trp^6]LHRH and RC-160 can be of benefit in treatment of prostate, breast and pancreatic carcinomas (17). In order to elucidate whether the substrates dephosphorylated by the tyrosine phosphatase activated by [D-Trp^6]LHRH and RC-160 are the same as those phosphorylated by EGF-stimulated tyrosine kinase in surgical specimens of human tumors, breast, prostate, liver and ovarian carcinomas, with demonstrated similarity in substrate specificity suggesting antagonistic activity between the EGF and [D-Trp^6]LHRH ligands (11), were examined.

Materials and Methods

Analog and chemicals. The LHRH agonist [D-Trp^6]LHRH and somatostatin analog RC-160, were synthesized by our group using solid-phase methods (21) and purified by HPLC. The EGF was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The [γ-^{32}P]ATP was obtained from Amersham Bioscience (Buckinghamshire, UK). Unless otherwise indicated, all the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of tumor proteins. Liver, ovarian, prostate and breast cancer samples were collected from patients after surgical resection at the National Taiwan University Hospital (Taipei, Taiwan, PRC) in accordance with established guidelines. Informed consent was obtained from patients before collection of specimens. All the patients involved in this study were under the authorization of the

Human Subject and Utilization Committee of the Institute of Biological Chemistry, Academia Sinica and School of Medicine, National Taiwan University. The tumor tissues were minced in PBS buffer (pH 7.2) containing 0.25% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF) (3 ml/g tissue), and homogenized with Polytron (Kinematic, Luzern, Switzerland). The homogenate was maintained at 4˚C for 4 h. The soluble proteins were separated from the pellets by centrifugation at 5000 g for 30 min at 4˚C. The supernatants were then precipitated with 70% ammonium sulfate. After dialysis against 0.02 N Hepes buffer (pH 7.2), the crude tumor protein concentration was determined using the method of Bradford (22) and adjusted to 100 μg/50 μl. The samples were then divided into 200 μl aliquots and stored at –70˚C for future study.

Preparation of ascites proteins. Human ovarian cancer ascites was first precipitated with 70% ammonium sulfate. After dialysis against 0.02 N Hepes buffer (pH 7.2), the ascites protein concentration was determined (as above) and adjusted to 100 μg/50 μl. The samples were then divided into 200 μl aliquots and stored at –70˚C for future study.

Hormone-induced phosphorylation/dephosphorylation. The tumor proteins extracted from natural occurring tumors were incubated in the presence of 100 nM EGF, 100 nM RC-160 or 100 nM [D-Trp^6]LHRH or in the absence of these substances individually or in combination (EGF + RC-160 or EGF + [D-Trp^6]LHRH) at the same levels. After this hormone-induced phosphorylation/dephospho-rylation, the incorporated proteins were characterized using 4-18% SDS-PAGE, Western blotting and autoradiographic analysis. To determine whether the phosphorylation/dephosphorylation of the specific protein substrates occurred in response to the EGF or somatostatin/LHRH analogues, autoradiography was used to quantify the incorporation of ^{32}P label.

Total kinase activity assay. The total kinase activity assays were performed as originally described by Cohen *et al*. (23) with some modification (11). Briefly, the reaction mixtures in a final volume of 80 μl containing 50 μg of cellular protein in 20 mM Hepes buffer (pH 7.4) and 1.0 mM $MnCl_2$, with 100 nM of [D-Trp^6]LHRH, RC-160, or EGF alone, or a combination of 100 nM EGF and [D-Trp^6]LHRH or RC-160, respectively, were preincubated at room temperature for 2 h and then chilled at 4˚C for 10 min. The reaction was initiated by the addition of [γ-^{32}P]ATP (6-12×10^6 cpm).

Gel electrophoresis, Western blotting and autoradiography. The kinase assay reaction mixtures (as described above) were also subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to examine further changes in tumor protein phosphorylation in response to [D-Trp^6]LHRH, RC-160 and EGF. The kinase reactions were terminated by the addition of 50 μl sample treatment solution (0.05 M Tris-Cl [pH 6.9], 2% SDS, 20% glycerol, 0.1% bromophenol) followed by boiling for 3 min. The reaction products were subsequently subjected to electrophoresis using SDS-PAGE (3-18% gradient) according to the method of Laemmli (24). The proteins were then electrophoretically transferred to nitrocellulose membranes as described by Towbin *et al*. (25) and then autoradiographed using Kodak X-Omat AR film (Kodak, Rochester, NY, USA) between two intensifying screens at –70˚C. The intensity of the ^{32}P-labeled protein bands were determined using a densitometer (Vilber Lourmat, Marne la Vallée, France).

Results

Phosphorylation of tumor proteins by EGF and dephosphorylation by analog of somatostatin and LHRH. The tumor homogenates were evaluated to determine the decrease in total phosphorylation induced by [D-Trp^6]LHRH or RC-160, as well as the extent of any EGF-induced promotion.

In the ovarian carcinoma samples, RC-160 and [D-Trp^6]LHRH stimulated the dephosphorylation of two major protein bands at 60 and 80 kDa (Figure 1A). The former produced 38% and 72% decreases in phosphorylation of the 80 and 60-kDa bands (lane a) respectively, while the latter yielded 88% and 90% decreases (lane d), as compared to the control. EGF alone (lane c) slightly increased the phosphorylation of these two proteins (16% and 10%). RC-160 and [D-Trp^6]LHRH also surprisingly enhanced the phosphorylation of a 10-kDa protein (202% and 108%, respectively). This protein remains to be further characterized.

To further document the phosphorylation of specific protein substrates in response to EGF, RC-160 and [D-Trp^6]LHRH, tumor proteins extracted from prostate, breast and liver carcinomas were also investigated (results summarized in Table I). Figure 1B shows the pattern of liver tumor protein phosphorylation generated by EGF-induced tyrosine kinase, and RC-160 and [D-Trp^6]LHRH-activated tyrosine phosphatase activity. EGF increased the phosphorylation level of the 300-, 65-, 48- and 45-kDa proteins by 15%, 8%, 21% and 10%, respectively (lane c). In this same extract, RC-160 promoted dephosphorylation of the 300-, 65-, 48- and 45-kDa protein bands (lane a) by 30%, 20%, 44% and 10%, respectively, whereas the dephosphorylation by [D-Trp^6]LHRH was 20%, 25%, 67% and 70% (lane d). EGF plus RC-160 (lane b) reduced phosphorylation of the 300- and 48-kDa proteins by 32% and 60%, respectively, whereas EGF plus [D-Trp^6]LHRH (lane e) reduced them by 24% and 56%, respectively.

With the proteins extracted from a breast carcinoma, EGF (lane c) stimulated phosphorylation of the 300-, 65-, 60-, 48- and 40-kDa proteins by 8%, 15%, 10%, 22% and 12%, respectively (Figure 1C; Table I). [D-Trp^6]LHRH (lane d) effectively promoted dephosphorylation of the 300- and 60-kDa proteins by 24% and 12%, respectively. Phosphorylation in two of the proteins (48 and 40 kDa) decreased by 32% and 23%, respectively. [D-Trp^6]LHRH also neutralized EGF-induced phosphorylation (lane e). However, RC-160 (lane a) did not alter the phosphorylation of the 300-kDa protein, but stimulated detectable decreases in phosphorylation of the 48 and 40-kDa forms (17% and 12%, respectively).

With the prostate carcinoma protein, both RC-160 and [D-Trp^6]LHRH greatly promoted dephosphorylation of the 300-kDa protein (85% and 83%, respectively; Figure 1D,

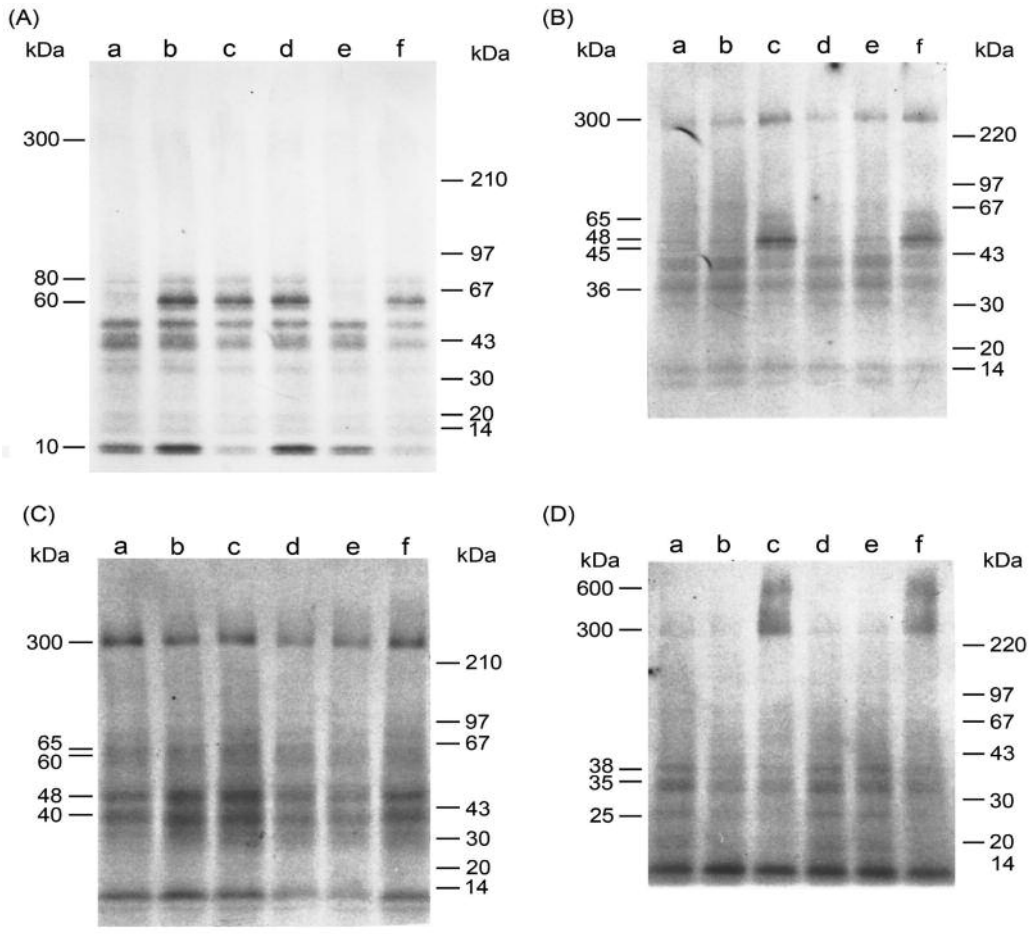

Figure 1. *Effect of [D-Trp^6]LHRH and RC-160 on basal and EGF-induced tumor protein phosphorylation. Representative autoradiograms of tumor proteins after phosphorylation/dephosphorylation induced by peptide analogues are shown. Tumor proteins (50 μg) (Panels: A, ovarian; B, liver; C, breast; D, prostate) were incubated with various hormones and/or analogues in the presence of [γ-^{32}P]ATP. Lanes: a, 100 nM RC-160; b, 100 nM RC-160 + 100 nM EGF; c, 100 nM EGF; d, 100 nM [D-Trp^6]LHRH; e, 100 nM [D-Trp^6]LHRH + 100 nM EGF; f, control (medium alone). The reaction mixtures were subjected to SDS-PAGE, immunoblotting and autoradiography.*

lanes a and d). In addition, a 600-kDa protein responded to RC-160 and [D-Trp^6]LHRH treatment. This protein was not detected in the other tumors and was completely dephosphorylated in response to the treatment with RC-160 and [D-Trp^6]LHRH. Moreover, in contrast, RC-160 and [D-Trp^6]LHRH caused slight phosphorylation increases in the 38-, 35- and 25-kDa protein bands (lanes a and d). The percentage changes are summarized in Table I. RC-160 and [D-Trp^6]LHRH also suppressed the EGF-induced protein phosphorylation (lanes b and e) in the prostate sample.

An ascites sample from the ovarian cancer patient (Table I) was also investigated. Surprisingly, RC-160 and [D-Trp^6]LHRH markedly promoted tyrosine phosphatase activity, and phosphorylation of the 300-kDa band was completely eliminated in response to the treatment with RC-160 and [D-Trp^6]LHRH (Figure 2, lanes a, d). Interestingly, this 300-kDa protein was detected in the liver, breast and prostate tumors, but not in the ovarian carcinoma. RC-160 and [D-Trp^6]LHRH both promoted the dephosphorylation of the 65-kDa protein (8% and 30%, respectively). EGF slightly increased phosphorylation of the 300 and 65-kDa proteins (43% and 22%, respectively; lane c). Surprisingly, RC-160, [D-Trp^6]LHRH and EGF stimulated the 35-kDa protein phosphorylation by 40%, 21% and 36%, respectively. Both RC-160 and [D-Trp^6]LHRH also suppressed the EGF-induced protein phosphorylation in these tumor proteins.

Discussion

RC-160 and [D-Trp^6]LHRH were shown to be effective stimulants of tyrosine phosphatase that especially promoted the dephosphorylation of the proteins which

Table I. *Results of densitometry scan for autoradiograms of individual protein bands in ovarian, liver, prostate and breast carcinoma membranes.*

Tumor type	Protein size (kDa)	Phosphorylation change *vs.* control (%)		
		+ RC-160	+ EGF	+ [D-Trp^6]LHRH
Ovarian	80	–38	+16	–88
	60	–72	+10	–90
	10	+202	–	+108
Liver	300	–30	+15	–20
	65	–20	+8	–25
	48	–44	+21	–67
	45	–10	+10	–70
	35	+15	–	+20
Breast	300	–	+8	–24
	65	–	+15	–
	60	–	+10	–12
	48	–17	+22	–32
	40	–12	+12	–23
Prostate	600	XX	–	XX
	300	–85	+10	–83
	38	+28	–	+25
	35	+42	–	+27
	25	+23	–	+18
Ascites	300	XX	+43	XX
	65	–8	+22	–30
	35	+40	+36	+21

Percentage change in optical density compared to control. –: No measurable change in phosphorylation. XX: Complete dephosphorylation.

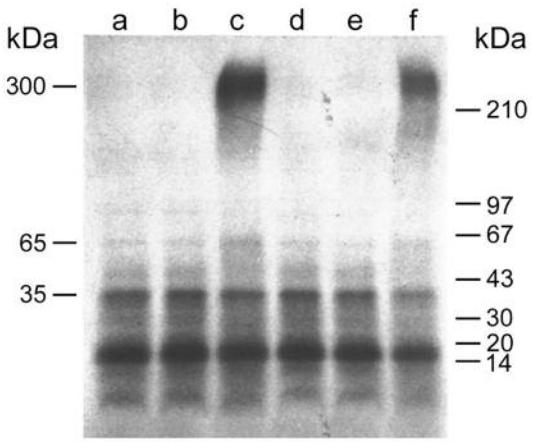

Figure 2. *Effect of [D-Trp^6]LHRH and RC-160 on basal and EGF-induced ovarian tumor ascites protein phosphorylation. A representative autoradiogram of ascites proteins after hormone-induced phosphorylation/dephosphorylation is shown. Ascites proteins (50 μg) were incubated with various hormones and/or analogs in the presence of [γ-^{32}P]ATP. Lanes: a, 100 nM RC-160; b, 100 nM RC-160 + 100 nM EGF; c, 100 nM EGF; d, 100 nM [D-Trp^6]LHRH; e, 100 nM [D-Trp^6]LHRH + 100 nM EGF; f, control (medium alone). The reaction mixtures were subjected to SDS-PAGE, immunoblotting and autoradiography.*

were phosphorylated by EGF (Table I). Previously, our results in cell culture showed that phosphorylation/dephosphorylation of the 60-, 65- and 170-kDa proteins appeared to predict the ability of EGF to stimulate the growth, and LHRH and somatostatin to inhibit growth; those tumor cells which did not exhibit phosphorylation/dephosphorylation in these bands did not alter growth in response to EGF and LHRH or somatostatin. In the present study, the intensity of the phosphorylation of the 60-kDa (ovarian, breast), 65-kDa (liver, breast, ascites) and 300-kDa (liver, breast, prostate, ascites) proteins decreased in the presence of RC-160 and [D-Trp^6]LHRH and increased in the presence of EGF, except for the 300-kDa variant in the breast cancer sample which did not exhibit any detectable change in phosphorylation (Figure 1). The analogs of RC-160 and [D-Trp^6]LHRH appeared to biochemically reverse the specific phosphorylation process initiated by EGF. Our previous findings using a cell-model system suggested that 170-, 65- and 60-kDa protein substrates are involved in growth-message transduction (10, 11, 19). The failure to detect a clearly phosphorylated 170-kDa band under the present conditions could be explained by the fact that the amount of 170-kDa protein present in the tumor extract was not sufficient to yield measurable phosphorylation (Figure 1). However, the 65- and 60-kDa proteins, found in most of the studied carcinomas, coupled with our previous cell culture results suggested that many solid tumors would respond to treatment with hormone analogs.

The presence of a 300-kDa phosphoprotein in carcinoma *in vivo*, but not *in vitro*, could be explained by the fact that it was either a soluble or cytosolic protein discarded in the cell culture experiment during membrane preparation, or a protein secreted by cells surrounding the cancer *in situ*, or a product of cancer cells produced in the body but not in cell cultures. Which of these explanations might account for the presence of this relatively large protein remains to be established, however, it appears reasonable to suggest that this protein substrate may be of clinical importance.

EGF promoted phosphorylation of the 48-, 45-, 40-, 35- and 10-kDa protein. In most cases, these proteins were the same as those dephosphorylated by RC-160 and [D-Trp^6]LHRH. Although, in most solid carcinomas, the predominant biochemical response to RC-160 and [D-Trp^6]LHRH is through tyrosine phosphatase, some tyrosine kinase activity has been identified in solid tumors in the presence of these two analogs. This study demonstrated that both RC-160 and [D-Trp^6]LHRH stimulated phosphorylation of proteins of 25-38 kDa in the prostate carcinomas, indicating the existence of endogenous kinases in tumors, and that these

kinases are not affected or regulated by RC-160 or [D-Trp^6]LHRH and that tyrosine kinase activity may be related to the transduction of a message separate from that used for growth control. Although this hypothesis requires further testing, it is worth noting that the tyrosine phosphatase and tyrosine kinase activities do not negate each other in prostate carcinoma because they affect different substrates. In addition, we previously showed that the LHRH receptor is found in carcinomas, but not in most normal tissues. This is surprising since the LHRH receptor gene can serve as an anti-oncogene, or emerogene. The product of an anti-oncogene slows growth; its loss would transform a normal cell into a malignant one. Why then should an anticancer gene be expressed only in carcinoma and not in normal tissues? Perhaps the answer to this apparent dilemma is that although the tyrosine phosphatase genes are expressed, only the tyrosine kinase gene is overexpressed. To better substantiate this hypothesis, we are currently performing immunohistochemical analysis of the parity of normal and tumor specimens using a variety of anti-PTP antibodies. In tumor tissues, relatively high PTP expression might be expected, however, lower or weaker PTP expression could be observed in normal tissues. This hypothesis of exclusive overexpression of the tyrosine kinase gene remains to be validated.

The present findings were significant in several ways, showing that the protein substrates assay can be performed on surgical samples, phosphorylation of the protein substrate in a cell culture system parallels that naturally occurring in tumors and the assay can be run on ascites fluids.

In conclusion, analogs of somatostatin and LHRH stimulate tyrosine phosphatase activity in a variety of solid tumors. The tyrosine phosphatase activity seems to have similar substrate specificity to the tyrosine kinase activity stimulated by EGF. This tyrosine phosphatase activity could reverse the growth stimulation induced by EGF. The body of evidence presented in this study suggests that the 300-, 65- and 60-kDa protein substrates may be useful as tumor markers. Further, based on these results, it appears reasonable to suggest that these protein substrates may be of clinical importance.

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