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Regulation of luteinizing hormone-releasing hormone receptor binding by heterologous and autologous receptor-stimulated tyrosine phosphorylation

(oncogenes and anti-oncogenes/pancreatic cancer/epidermal growth factor/somatostatin/tyrosine kinase and phosphatase)

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ABSTRACT **Pancreatic cancers overexpress tyrosine kinase and luteinizing hormone-releasing hormone (LH-RH) receptor (LH-RHR)-mediated tyrosine phosphatase. LH-RHR is a 60-kDa protein. One of the substrates of epidermal growth factor (EGF)-stimulated tyrosine kinase activity and LH-RH- and somatostatin-stimulated tyrosine phosphatase activity is also a 60-kDa protein. This suggests the possibility that LH-RHR regulation by tyrosine phosphatase and tyrosine kinase is mediated by (de)phosphorylation of existing LH-RHR. To test this hypothesis, membranes of MIA PaCa-2 cells, a human dedifferentiated pancreatic cancer cell line, were incubated without hormone (control) or with 0.1 μM EGF or somatostatin analogue RC-160 for 1 hr at 4°C to phosphorylate the 60-kDa protein. Competition binding experiments with I^{125}-labeled [D-Trp^6]LH-RH by displacement with a nonradioactive ligand showed that the LH-RH binding in 69% of the points was increased by EGF and 85% was decreased by RC-160 compared with controls ($n = 61$; both significant, $P < 0.001$). The specific binding was altered, increasing 50–150% after preincubation with EGF and decreasing 60–70% after RC-160. No change was seen in the binding affinity constant after pretreatment with EGF or RC-160. This shows that phosphorylation regulates binding of LH-RH and may explain the up-regulation by EGF and down-regulation by RC-160 and by LH-RH of the LH-RH response.**

Two lines of evidence suggest that the class of tyrosine phosphatases may be as important in cancer as the class of tyrosine kinases (1–3). These findings are based on the existence of a broad family of tyrosine phosphatase genes with receptor-like structures (4–8) and the demonstration that two hormone receptors stimulate tyrosine phosphatase activity (9–11, 36). This has led to a rather unsuccessful attempt to implicate these genes as antioncogenes (tumor suppressor genes), or "emerogenes" (3, 12, 13). Investigators assumed that just as the expression of oncogenes in tissue heralds the development of cancer (14–17), the loss of expression of emerogenes should be associated with cancer development (12, 13, 18). Researchers have found an increase in tyrosine kinase (19) but have not been able to find a statistical decrease in tyrosine phosphatase activity associated with cancer, though isolated instances of such a decrease have been noted (20).

The investigation of the luteinizing hormone-releasing hormone (LH-RH) receptor (LH-RHR), which after activation by hormone binding, stimulates tyrosine phosphatase, confirms the paradoxical expression of this antigrowth signal in cancers. Pancreatic cancers and a range of other solid tumors that overexpress tyrosine kinase-associated oncogenes (15, 21) have been found to overexpress LH-RHR in comparison with normal pancreas (11, 22–24, 36). One would expect a loss of an antigrowth signal in rapidly growing cancers instead of its overexpression. This unexpected finding suggests that tyrosine phosphatases, or more specifically the LH-RHR, might be involved in a negative feedback regulatory loop limiting excess growth of cells. A hypothesis that could explain the emergent expression of a growth-inhibiting tyrosine phosphatase in cancers is as follows: first, the growth signal, tyrosine-phosphorylated proteins activated by tyrosine kinases, promotes tyrosine phosphatase activity; and then, this tyrosine phosphatase activity inactivates the growth signal, limiting excess growth. Inactivation of this signal reduces the stimulation for promotion of the tyrosine phosphatase.

If this hypothesis is true, certain responses can be predicted. One is that treatment of cancers with tyrosine phosphatase stimulants should reduce the number of LH-RHR in the treated cancer. This prediction was confirmed. Treatment of responsive cancers with [D-Trp^6]LH-RH or somatostatin analogue RC-160, both tyrosine phosphatase stimulants, down-regulated LH-RHR (23). Though LH-RH analogues could be predicted to down-regulate their own receptors by promoting ligand-induced receptor internalization, no such prediction could be made for somatostatin analogues. In addition, it could be predicted that in cancers expressing epidermal growth factor (EGF) receptor, EGF would up regulate LH-RHR. This has also been confirmed in preliminary studies (25). Therefore, this evidence supports the hypothesis that the substrate of tyrosine phosphatase promotes the activity of the receptor.

There are several possible mechanisms for regulation of the LH-RHR. These mechanisms include receptor internalization, degradation, or synthesis. Another possible mechanism capable of producing such regulation is activation of the receptor. The LH-RHR has been reported to be a 60-kDa protein (26–28) that can form a larger polymeric complex (29). One substrate of EGF-stimulated tyrosine kinase and RC-160- and [D-Trp^6]LH-RH-stimulated tyrosine phosphatase is a 60-kDa protein (36). This protein appears to be present in cells that respond to LH-RH analogues and not in unresponsive cells (36). This suggests the possibility that the LH-RHR can be phosphorylated and autoregulated by such phosphorylation, in analogy to the EGF receptor (17, 30). This study was performed to test this hypothesis.

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Abbreviations: LH-RH, luteinizing hormone-releasing hormone; LH-RHR, luteinizing hormone-releasing hormone receptor; EGF, epidermal growth factor.

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MATERIALS AND METHODS

Peptides. LH-RH agonist [D-Trp^6]LH-RH and somatostatin agonist D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2 (RC-160) were synthesized in our laboratory by solid-phase methods and repurified by HPLC. ^{125}I-labeled [D-Trp^6]LH-RH was generated by the chloramine-T method as described (31, 32).

Buffers. The homogenization buffer used for membrane receptor assays consisted of 0.3 M sucrose, 0.25 mM Tris·HCl, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 mM monothioglycerol, and aprotinin at 10,000 kallikrein inactivator units/liter (pH 7.5). For binding of ^{125}I-labeled [D-Trp^6]LH-RH, the buffer was composed of 25 mM Tris·HCl, 1 mM dithiothreitol, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin (pH 7.5).

Cell Culture and Membrane Preparation. MIA PaCa2, a dedifferentiated human pancreatic cancer cell line, was obtained from the American Type Culture Collection. The MIA PaCa2 cell cultures were maintained at 37°C in humidified 95% air/5% CO_2 and grown in a monolayer on a plastic tissue flask containing Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum. The cells were used for experiments on the first day of confluence. Membranes were prepared as described (36). The final membrane pellet was resuspended in receptor binding assay buffer. The protein concentration was adjusted to 50 μg per 50 μl and then divided into aliquots and stored at −70°C. Protein concentration was determined by using the Bio-Rad protein assay kit as described (33).

Receptor Binding of Peptides. The binding assay for [D-Trp^6]LH-RH was conducted as described by Fekete *et al.* (33), but both competition binding and saturation binding were performed. Binding reactions were performed in 12 × 75 mm polypropylene conical culture tubes at 4°C. The polypropylene culture tubes were preincubated with 1% bovine serum albumin for 30 min at room temperature. The tubes were then washed with distilled water three times and dried. The MIA PaCa2 cell membranes were divided into three groups and preincubated at 4°C for 30 min in the following media: (*i*) 0.1 μM EGF/60 μM ATP, (*ii*) 0.1 μM RC-160/60 μM ATP, and (*iii*) 60 μM ATP. The protein concentration was 1000 μg/ml. Each group contained eight polypropylene tubes. The receptor binding procedure was performed with membranes from each group. The first step was the only difference between the saturation binding studies and the displacement studies. In the saturation binding studies, 50 μl of receptor binding assay buffer was pipetted into each tube, and 50 μl of ^{125}I-labeled [D-Trp^6]LH-RH (containing 100,000, 40,000, 20,000, 10,000, 5,000, 2,500, 1,000, or 500 cpm) was added and mixed with a Vortex mixer. In the competition studies, 50 μl of receptor binding assay buffer was pipetted into each tube, and 50 μl of 40,000-cpm ^{125}I-labeled [D-Trp^6]LH-RH with nonradioactive [D-Trp^6]LH-RH (5 fmol, 15 fmol, 50 fmol, 150 fmol, 500 fmol, 1.5 pmol, 5 pmol, 15 pmol, 50 pmol, or 150 pmol) was added and mixed with a Vortex. Next, 50 μl of membranes from each preincubated group was added and mixed with a Vortex. The tubes were then incubated at 4°C for 60 min. The receptor binding assay was terminated by rapid filtration through glass fiber filters (Whatman GF/F) that had been prewetted in assay buffer and previously soaked for 3 hr in 0.5% polyethylenimine solution. A PHD (Cambridge Technology, Cambridge, MA) cell harvester system with 10-sec washing time was used. The radioactivity of glass fiber filters was counted in an automatic γ counter in 12 × 75 mm borosilicate culture tubes. Each assay point was performed in duplicate. The results of saturation binding and competition binding were comparable in the overlapping concentration range, so that the data could be combined.

RESULTS

[D-Trp^6]LH-RH binding assays were performed in six separate trials. Fig. 1 shows a Scatchard plot of one such trial. High- and low-affinity binding sites for [D-Trp^6]LH-RH were found in controls and in membranes treated with EGF or RC-160. The high-affinity binding sites had a K_d of the order of 10^{-10} M, and the low-affinity binding sites had a K_d in the 10^{-7} M range. The maximum binding capability of the high-affinity system of control membranes was in the range of 3 pmol of [D-Trp^6]LH-RH per mg of membrane protein. The low-affinity system had a maximum binding about 2 orders of magnitude higher.

When membranes were preincubated in the presence of hormones or analogues of hormones, their binding of

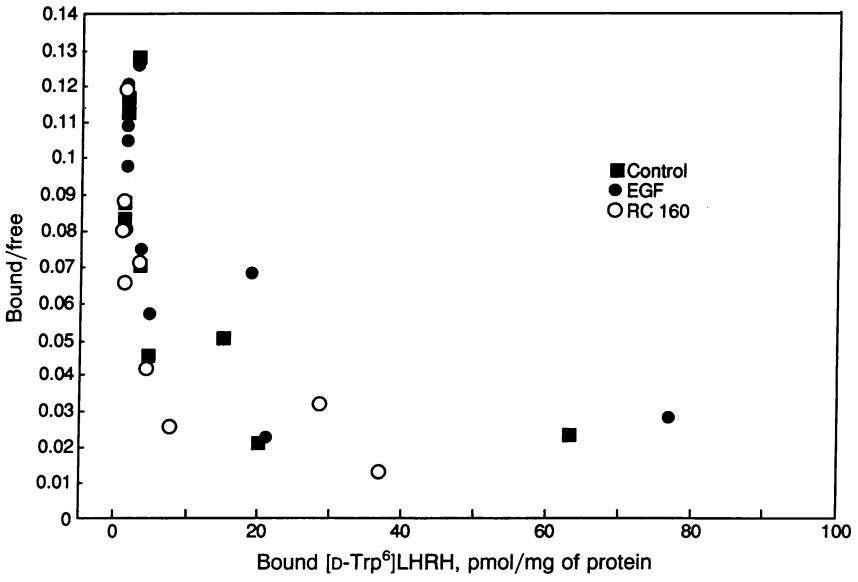

FIG. 1. Scatchard plot of one experiment of [D-Trp^6]LH-RH binding. The ratio of bound [D-Trp^6]LH-RH to free analogue is plotted against the bound analogue (pmol of [D-Trp^6]LH-RH per mg of membrane protein). ●, Membranes pretreated with 100 μM EGF; ○, membranes pretreated with 100 μM RC-160. Note the steep first compartment indicating a high-affinity system. Also note that most of the EGF-treated samples lie above the curve formed by the control points, and most of the RC-160-treated samples lie below the curve.

[D-Trp^6]LH-RH changed. Pretreatment with EGF increased the binding at most points, and RC-160 pretreatment decreased it (Fig. 1). In six different trials, 61 sets of membranes were incubated at various concentrations of [D-Trp^6]LH-RH; each set included one control, one EGF-pretreated sample, and one RC-160-pretreated sample. In these sets, [D-Trp^6]LH-RH binding was greater in 69% of the pairs after EGF treatment compared with control binding but was smaller in 85% of the pairs after RC-160 treatment compared with control binding (both values are significant at the $P < 0.001$ by the nonparametric sign test). Total binding on average was increased from control values of 7.9 pmol of [D-Trp^6]LH-RH per mg of membrane protein in controls to 12.0 pmol by EGF pretreatment and was decreased to 2.5 pmol by RC-160 pretreatment ($P < 0.05$ and 0.01, respectively) (see Table 1). Under conditions of low free ligand ([D-Trp^6]LH-RH < 0.2 nM), the contribution of high-affinity site binding could be maximized. We observed similar effects under these conditions, with controls binding 1.0 pmol of [D-Trp^6]LH-RH per mg of membrane protein, EGF pretreated membranes binding 2.39 pmol, and RC-160 pretreated membrane binding 0.32 pmol ($P < 0.05$ for both) (see Table 1). The number of points differed in these three groups of membranes in this comparison because it was based on free [D-Trp^6]LH-RH. Although the same amount of analogue was added to each set of three tubes (i.e., control membranes and those preincubated with EGF or RC-160), more analogue was bound by EGF-pretreated membranes than by controls, and more by controls than by RC-160-pretreated membranes. Therefore, the amount of free ligand was opposite, with more in RC-160-pretreated membranes than in controls than in EGF-pretreated membranes. For this reason, more points fell below the limit of 0.2 nM free ligand among EGF-pretreated membrane samples than in controls or RC-160-pretreated membrane samples. In individual Scatchard plots in which nonspecific binding was subtracted, no change was seen in the high-affinity binding constants after treatment. None were significantly different from 0.1 nM: 0.10 nM for controls, 0.11 nM for EGF-pretreated membranes, and 0.13 nM for RC-160-pretreated membranes.

This data can also be expressed in terms of the change in binding of samples after pretreatment with EGF or RC-160 compared with control samples plotted against the free concentration of [D-Trp^6]LH-RH. The results of pretreatment with EGF are shown in Fig. 2*A*. Values above 1 indicate an increase in binding because of EGF pretreatment that is greater than 1 fmol/mg of protein, and values below −1 indicate a decrease greater than that value. It can be seen that many more points show an increase in binding than a decrease, as indicated by the results in the above paragraph. Fig. 2*B* is a similar plot showing the effects of pretreatment with RC-160. In this case, many more points showed decreased binding than increased binding. It can be seen in both of these graphs that at higher concentrations of free ligand, the resulting change in binding becomes more uniform and reaches a plateau. This indicates that the change in binding produced by hormone pretreatment occurs in the high-affinity binding system, as shown by saturation at high concentrations of ligand.

Table 1. Binding of [D-Trp^6]LH-RH

Hormone treatment	[D-Trp^6]LH-RH binding, pmol/mg of membrane protein					
	All trials			Low free [D-Trp^6]LH-RH		
	Mean	SEM	*n*	Mean	SEM	*n*
EGF	12.0	4.9	61	2.39	1.18	20
Control	7.9	2.9	61	1.00	0.41	16
RC-160	2.5	2.3	61	0.32	0.22	6

By matched pair analysis, the binding of [D-Trp^6]LH-RH was greater in EGF-pretreated membranes than in control membranes, where the binding was greater than in RC-160-pretreated membranes as follows: for all trials, EGF > control ($P < 0.05$) and control > RC-160 ($P < 0.01$); for low free [D-Trp^6]LH-RH, EGF > control ($P < 0.05$) and control > RC-160 ($P < 0.05$).

DISCUSSION

This work shows that the number of LH-RHRs, as measured by conventional binding assay, can vary in a cell-free system in the absence of protein synthesis. It is well accepted that a hormone can regulate its own receptors *in situ* through receptor internalization and receptor synthesis. However, it has not been demonstrated that the apparent receptor number (i.e., the measure B_{max}) can be modulated in a cell-free system in the absence of protein synthesis.

[D-Trp^6]LH-RH binding is modulated by phosphorylation. Increased phosphorylation of a 60-kDa membrane protein, a molecular size consistent with the LH-RHR itself (26–28), was promoted by EGF (36) and causes increased LH-RH analogue binding. Decreased phosphorylation of this same protein promoted by the somatostatin analogue RC-160 caused a decrease in LH-RH analogue binding. Changes in the high-affinity binding account for alterations in binding produced by preincubation of membranes with EGF or the somatostatin analogue. The basic binding maximum is a little higher but of a similar order of magnitude as reported elsewhere (33, 34). The higher binding is consistent with the fact that in our work pure pancreatic cancer cell membranes were used from a cell culture line known to be responsive to EGF (10). In addition, the affinity constant was somewhat lower (i.e., binding was of higher affinity) but of a similar order as previously reported (33, 34). This supports the view that the LH-RHRs investigated by us are similar to LH-RHRs reported by others. Pretreatment of isolated membranes with EGF increased the maximum binding of [D-Trp^6]LH-RH without altering the affinity, which classically would be considered as evidence for increased receptor number. Similarly, preincubation of isolated membranes with RC-160 decreased the B_{max} of [D-Trp^6]LH-RH without altering the K_d, which would normally be viewed as proof for receptor destruction. The fact that no protein synthesis is occurring in a membrane preparation rules out the possibility that the increased receptor number is due to receptor synthesis. Though it is possible that RC-160 could promote degradation of the LH-RHR, no evidence exists for this. Since EGF and RC-160 both affect tyrosine phosphorylation of specific protein residues, increasing or decreasing it, respectively, it seems reasonable to explain the receptor modulation in terms of this effect.

The proposal that phosphorylation of receptors could regulate the binding of ligand to LH-RHRs is not without precedent. Allosteric enzymes offer a good model with which the receptors can be compared. A typical allosteric enzyme has a separate regulator and catalytic site. A receptor also has a ligand-binding site and an enzymatic site. The two sites of the allosteric enzyme are separated by a significant distance, as in the case of the two sites of the receptor. Binding of regulator or ligand activates the enzymatic site of both proteins. The typical allosteric enzyme also functions as a complex of multiple subunits. Likewise, the LH-RHR appears to function as a polymer (29). These facts all support the similarity of the two systems. With allosteric enzymes, binding of a substrate is known to favor binding of the regulator. We have observed a similar phenomenon with the LH-RHR. LH-RHR functions as a tyrosine phosphatase (11, 36), making the phosphate a substrate for this receptor/enzyme. In our study, we have shown that the binding of phosphate to the LH-RHR can potentiate the ability of

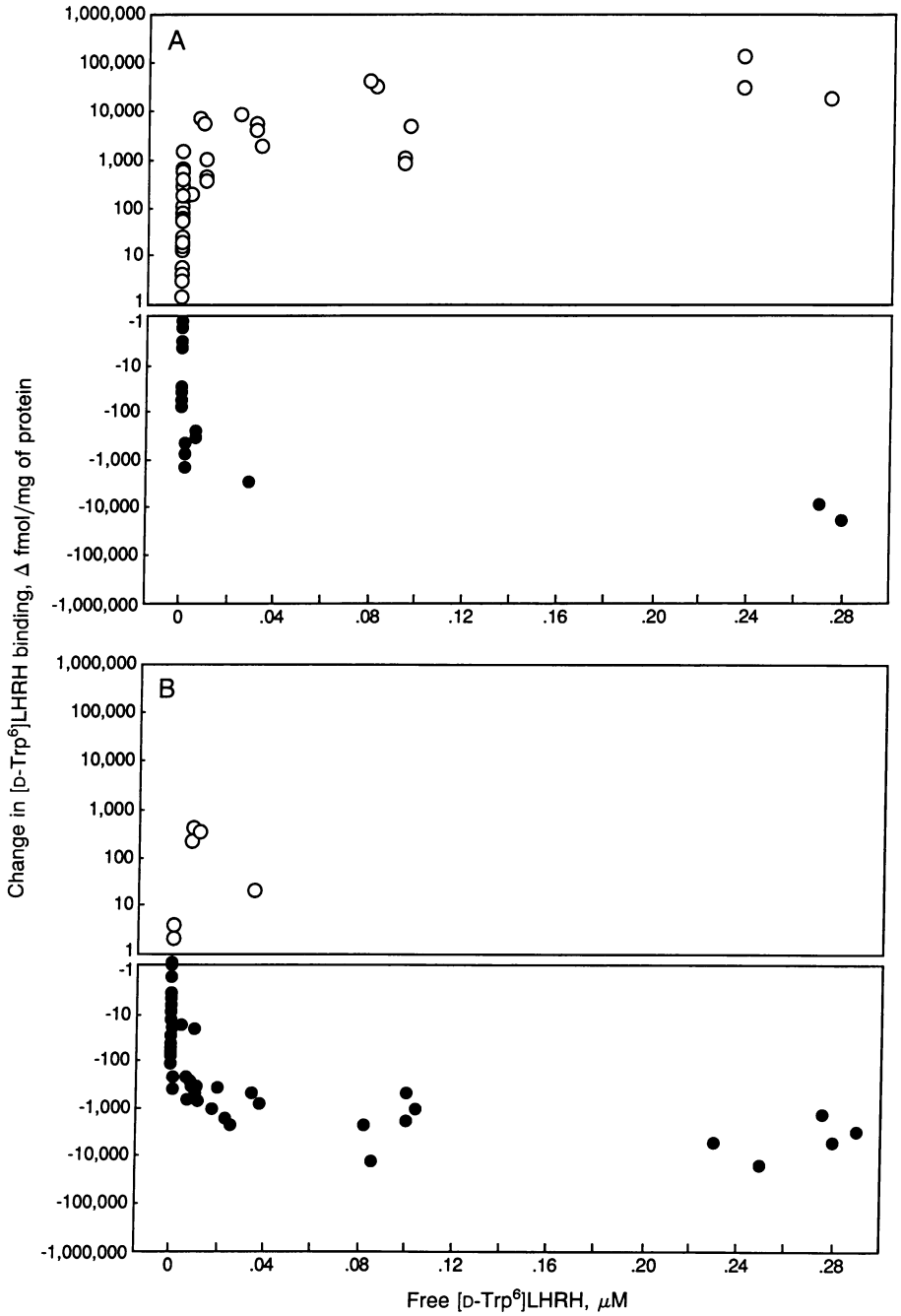

FIG. 2. Change in [D-Trp^6]LH-RH binding upon hormone or analogue pretreatment. The change in analogue binding (maximal binding capacity) (binding of treated sample − binding of control sample) is plotted on a log scale as the abscissa in units of fmol/mg of membrane protein. The scale is broken between 1 fmol/mg of protein to −1 fmol/mg of protein. These changes were considered sufficiently small to drop from the plot. The scale then ranges from 1 fmol to 1 nmol in both a positive direction and a negative direction. The *x* axis represents the free [D-Trp^6]LH-RH in μM. ○, Increases in binding; ●, decreases in binding. (*A*) Comparison after EGF pretreatment. (*B*) Comparison after RC-160 pretreatment. Note that most points are increased after EGF pretreatment, and most are decreased after RC-160 pretreatment. Also note that at high concentrations of analogue (>0.1 μM free ligand), both changes appear to become saturated at the pmol/mg of protein range.

LH-RHR to bind LH-RH. EGF and somatostatin analogue RC-160 regulate the phosphorylation of the 60-kDa protein, the presumptive LH-RHR, in addition to regulating the binding of [D-Trp^6]LH-RH by this receptor. Such control of the regulatory or ligand-binding site by substrate binding at the enzymatic site appears, therefore, comparable to the allosteric enzyme model.

The regulation of LH-RHR by tyrosine phosphorylation creates an autoregulatory negative feedback loop for growth control. Increased phosphorylation promoted in the presence of a growth factor or oncogene such as the EGF receptor or its ligands would stimulate growth. At the same time, however, this phosphorylation increases the number of functional LH-RHRs and their potential for dephosphorylation and deactivation of the growth message. At the same time, the dephosphorylation will also inactivate the LH-RHR.

This demonstration that one receptor system involved in regulation of phosphorylation can in turn be regulated itself by phosphorylation has significant implications for other receptor systems. Heterologous receptor regulation—i.e., regulation of binding of one ligand by another ligand—has long been noted but not explained in the context of any cohesive model. Many receptors are involved in phosphorylation and dephosphorylation, and any or all of these

receptors could be similarly regulated. Our work demonstrates that RC-160, an analogue of somatostatin, stimulates tyrosine phosphatase and can regulate LH-RHR function. Somatostatin also affects many hormonal signals, including secretions promoted by a wide variety of stimulatory agents, many of which alter cellular phosphorylation. The antisecretagogue effects of somatostatin could be also mediated by tyrosine phosphatase activity. Since many neuroendocrine ligands affect phosphorylation, their receptors might be similarly regulated. An example of a hormone that promotes phosphorylation is insulin. The effects of insulin can be inhibited by microinjection of protein-tyrosine phosphatase into the target cell (35), which is consistent with its regulation by phosphorylation. Heterologous and homologous receptor modulation should be reassessed with this potential regulatory mechanism in mind.

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