Activation of Translation in Pituitary Gonadotrope Cells by Gonadotropin-Releasing Hormone

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The neuropeptide GnRH is a central regulator of mammalian reproductive function produced by a dispersed population of hypothalamic neurosecretory neurons. The principal action of GnRH is to regulate release of the gonadotropins, LH and FSH, by the gonadotrope cells of the anterior pituitary. Using a cultured cell model of mouse pituitary gonadotrope cells, αT3–1 cells, we present evidence that GnRH stimulation of αT3–1 cells results in an increase in cap-dependent mRNA translation. GnRH receptor activation results in increased protein synthesis through a regulator of mRNA translation initiation, eukaryotic translation initiation factor 4E-binding protein, known as 4EBP or PHAS (protein, heat, and acid stable). Although the GnRH receptor is a member of the rhodopsin-like family of G protein-linked receptors, we show that activation of translation proceeds through a signaling pathway previously described for receptor tyrosine kinases. Stimulation of translation by GnRH is protein kinase C and Ras dependent and sensitive to rapamycin. Furthermore, GnRH may also regulate the cell cycle in αT3–1 cells. The activation of a signaling pathway that regulates both protein synthesis and cell cycle suggests that GnRH may have a significant role in the maintenance of the pituitary gonadotrope population in addition to directing the release of gonadotropins. (Molecular Endocrinology 14: 1811–1819, 2000)

INTRODUCTION

The anterior pituitary consists of several subpopulations of cells identified by their production of specific hormones and responses to specific releasing factors. The gonadotrope cells, which produce the heterodimeric glycoprotein gonadotropins LH and FSH, are responsive to the decapptide releasing factor GnRH. Stimulation of gonadotrope cells by pulsatile GnRH from the hypothalamus leads to the increased production and release of LH and FSH. Both the pulse frequency and amplitude differentially regulate the synthesis of gonadotropin mRNA, and the release of gonadotropins by gonadotrope cells (1–4).

The GnRH receptor expressed in pituitary gonadotropes has been cloned from a number of species (5). The receptor is a unique member of the rhodopsin family of G protein-linked seven-transmembrane domain receptors. The receptor lacks the intracellular carboxyl-terminal tail and contains numerous sequence differences in otherwise highly conserved regions. Ligand binding to the GnRH receptor causes activation of the G proteins, Gq and G11, although some evidence exists for the additional activation of Gi/G0 (6–8). Protein kinase C (PKC) activity is increased by GnRH receptor activation. Additionally, GnRH receptor activation leads to stimulation of the mitogen-activated protein kinase (MAPK) pathway (9). This signaling cascade results in increased transcription of the glycoprotein hormone α-subunit and LH β-subunit genes (10). Previous studies have demonstrated that GnRH signaling involves activation of the GTPase Ras (9), but subsequent studies have shown that this may not be necessary for transcriptional activation via the MAPK signaling cascade (11).

The αT3–1 cultured pituitary gonadotrope cell line expresses the GnRH receptor and is responsive to GnRH stimulation. This cell line has been a valuable tool in dissecting the transcriptional regulatory regions of the α-subunit glycoprotein hormone gene (α-GSU) that are necessary for pituitary-specific transcription (12). It has been shown that the mouse and human genes are transcriptionally responsive to GnRH stimulation in αT3–1 cells (13). However, some evidence suggests that the increase in α-GSU expression may involve a translational component in addition to the well described transcriptional component. First, although the MAPK cascade mediates increased transcription of the mouse gene in response to GnRH stimulation (9), the human gene, which is not transcriptionally stimulated by MAPK (14), is also responsive to GnRH in αT3–1 cells. This suggests that other factors may participate in the GnRH response. Second, in
transient transfection studies of αT3–1 cells with an α-subunit promoter-driven reporter gene, GnRH-stimulated reporter enzyme activity peaks within 3 h of GnRH stimulation, whereas increase of endogenous α-subunit mRNA does not reach maximal levels until 12 h (10). This observation suggests that the transcriptional response is delayed with respect to the increase of reporter gene enzyme activity and may be a result of increased mRNA stability, increased translation, or both.

Using the αT3–1 cell line as a model system, we investigated the ability of GnRH receptor activation to modify translation. In this study, we demonstrate that GnRH-stimulated synthesis of the α-subunit is Ras dependent. Further, we show GnRH stimulation results in an increase in both phosphorylation of the translation-regulatory factor 4EBP by the kinase mammalian target of rapamycin (mTOR) and an increase in cap-dependent translation. These data suggest that GnRH stimulation of αT3–1 cells is partly exerted through a general increase in cap-dependent translation. We also show that GnRH stimulates translation through activation of PKC, Ras, and the mTOR kinase, leading to the direct phosphorylation of the translational regulatory factor 4EBP.

RESULTS

GnRH Stimulates Glycoprotein α-Subunit Synthesis

Previous reports have indicated that α-GSU gene expression can be increased by GnRH stimulation through activation of the MAPK pathway. Although Ras may participate in the transduction of signals from the GnRH receptor to MAPK, it is not obligatory and may not be necessary for stimulation of transcriptional activity. To demonstrate that GnRH stimulation of αT3–1 cells does indeed lead to a Ras-dependent increase in α-GSU protein synthesis, serum-starved αT3–1 cells were microinjected with nonimmune IgG (Fig. 1, A and B) or with anti-Ras IgG (Fig. 1, C and D) and subsequently stimulated with GnRH analog (GnRHa). After 24 h of incubation, cells were fixed and processed for immunohistochemical identification of α-GSU. Samples were then examined by fluorescence microscopy for the presence of the injection marker cascade blue (Fig. 1, A and C) or for the presence of α-subunit (Fig. 1, B and D). Injection with anti-Ras IgG blocked the GnRH-dependent increase in subunit synthesis seen in control injected cells (Fig. 1, A and B). A summary of five independent experiments is presented graphically in Fig. 1E. This observation suggests that synthesis of α-GSU protein may be partly dependent on Ras activity. To test this, we examined the ability of Ras alone to increase α-GSU protein in the absence of GnRH stimulation. Microinjection of control nonspecific IgG had no effect on α-GSU synthesis in serum-starved αT3–1 cells (Fig. 2, A and B), whereas injection of purified Ras protein caused an increase in α-subunit protein synthesis in the absence of GnRH stimulation (Fig. 2, C and D). A summary of five independent determinations is presented graphically in Fig. 2E. The observation that Ras activation alone can recapitulate the GnRH stimulation of α-GSU protein synthesis strongly suggests the involvement of Ras in GnRH receptor signal transduction. The further observation that GnRH action can be blocked by blocking Ras activation provides strong evidence that under this paradigm Ras is a component of the GnRH signaling cascade leading to increased expression in α-GSU protein in αT3–1 cells.

GnRH Stimulates Cap-Dependent Translation

Observations by others that α-GSU promoter-driven reporter gene activity peaks well before the endogenous levels of α-GSU mRNA, and that the stabilization effect of GnRH treatment on α-GSU mRNA is itself
delayed by several hours (10), suggests that mRNA synthesis and degradation rates, although decreased by GnRH stimulation, may not fully account for the GnRH-stimulated increase in $\alpha$-GSU protein synthesis. The contribution of increased translation rates to GnRH-stimulated $\alpha$-GSU synthesis has not been addressed. To test the hypothesis that GnRH stimulation of gonadotrope cells includes a translational component, we constructed a bicistronic reporter gene that would distinguish between changes in transcriptional and translational activity (Fig. 3A). Similar reporter plasmids have been used to demonstrate the regulation of cap-dependent translation by insulin (15), which acts through a receptor tyrosine kinase. The cytomegalovirus (CMV) immediate early enhancer and promoter direct synthesis of the bicistronic transcript. The resultant mRNA contains a firefly luciferase coding sequence followed by an internal ribosomal entry site derived from the 5'-untranslated region of encephalomyocarditis virus (EMCV) (16) and a second reading frame encoding the $\beta$-galactosidase gene. Transcription of the reporter plasmid in transfected cells produces a mRNA template for cap-dependent translation of the luciferase reporter, and for cap-independent translation of the $\beta$-galactosidase gene directed by the EMCV 5'-untranslated region. Measurement of the ratio of reporter gene activity provides a direct measurement of cap-dependent vs. cap-independent translation, independent of transcriptional effects. Previous studies have shown that ratios are consistent independent of reporter gene order, or overall reporter gene composition, indicating that reporter enzyme activity is not altered (15). The bicistronic reporter gene was transfected into the pituitary gonadotrope-derived $\alpha$T3-1 cells, which were then serum starved for 12 h. Subsequently, cells were stimulated with GnRH or insulin for 8 h and assayed for reporter gene activity. Comparison of the luciferase to $\beta$-galactosidase ratio showed that both GnRH and insulin increased the ratio of luciferase to $\beta$-galactosi-
dase activity (Fig. 3B). In contrast, GnRH had no significant effect on NIH/3T3 cells, which do not express the GnRH receptor, indicating that the effect of GnRH on translation was specific to the αT3–1 cells. These data demonstrate that both GnRHa and insulin increase cap-dependent translation in αT3–1 cells. It has been demonstrated that epidermal growth factor (EGF) stimulation, a factor that also activates translation in a manner similar to insulin, increases GnRH signaling intensity or facilitates GnRH signal transduction (17). To test whether EGF affects GnRH signal transduction resulting in translational stimulation, αT3–1 cells transfected as above were stimulated with GnRH analog, EGF, or both. The results shown in Fig. 3C indicate that cap-dependent translation is stimulated by EGF but that GnRH and EGF together do not exhibit synergistic action. No significant increase in cap-dependent translation was observed with dual stimulation of GnRHa and insulin (data not shown). These data strongly suggest that GnRH stimulation of αT3–1 cells results in an increase in cap-dependent translation, and that GnRH, insulin, and EGF stimulation may function through common signaling intermediates.

Rapamycin Inhibits GnRH-Stimulated Translation

The majority of eukaryotic mRNAs bear a 5′-cap structure consisting of m7GpppN that is recognized by a complex of proteins known as the cap-binding complex. A component of this complex, eIF-4E, recognizes this cap structure and is essential for the interaction of mRNA with the cap-binding complex and the subsequent initiation of translation (18). The activity of eIF-4E is repressed by a family of binding proteins, known as 4EHP or PHAS (protein, heat, and acid stable) (15, 19) that interfere with eIF-4E activation of the cap-binding complex and thereby inhibit translation initiation. The repressor activity of 4EHP is regulated by phosphorylation in response to receptor tyrosine kinase activity. It has been shown that 4EHP is regulated through the pathway involving a rapamycin-sensitive kinase (20–22) and that 4EHP is directly phosphorylated by mTOR (23). Although stimulation of the MAPK pathway results in mTOR activation, it is not clear at which point the MAPK and mTOR cascades diverge. Activation of mTOR is not dependent on MAPK activity, as the inhibitor of MAPK activation, PD098059, does not inhibit activation of mTOR or phosphorylation of 4EHP (22, 24). To determine whether GnRH signaling involves mTOR, activation of cap-dependent translation by GnRHa was analyzed for sensitivity to rapamycin. Pretreatment of αT3–1 cells with rapamycin before stimulation with GnRHa resulted in attenuation of the activation to 51% that of untreated cells (Fig. 4A). Further evidence that GnRH regulates translation via phosphorylation of 4EHP is obtained by Western blot analysis of 4EHP in protein extracts isolated from αT3–1 cells that have been stimulated with GnRHa alone or in the presence of rapamycin (Fig. 4B). In untreated cells, 41% of total 4EHP detected was present in the nonbinding γ-form, with the remainder present in the β-form. This is consistent with observations by others of the phosphorylated state of wild-type 4EHP in other cell systems (25). Stimulation of αT3–1 cells with GnRHa caused 90% of the total detected 4EHP to be found in the non-eIF4E-binding γ-form after 15 min of stimulation. Rapamycin treatment causes the conversion of 4EHP to the α- and β-inhibitory forms, and this conversion is not overcome by GnRH treatment. These data confirm that GnRH stimulation of cap-dependent translation involves regulation of 4EHP and subsequent derepression of translation through the eIF-4E initiation pathway.

GnRH Stimulation of Translation Is Ras Dependent

Insulin, EGF, and other hormones acting through receptor tyrosine kinases activate the MAPK cascade
through recruitment and activation of the ubiquitous GTPase Ras, either by the adapter protein complex of Grb2/Sos1 or by the activation of phospholipase C-γ. The MAPK cascade is directly activated by Ras, but Ras also activates other GTPases and protein kinases. Previous studies have demonstrated that GnRH ligand binding leads to activation of Ras, but that this is not necessary for activation of MAPK (11).

To examine the potential role of Ras in GnRH regulation of translation, αT3-1 cells were cotransfected with the bicistronic reporter plasmid and an expression vector encoding the dominant-negative mutant Ras A15 (26). If Ras is a component of the signaling pathway leading to translational stimulation by GnRH, the presence of dominant negative Ras would be expected to impair the ability of GnRH stimulation to cause an increase in cap-dependent translation. Indeed, as shown by the results presented in Fig. 5A, the presence of dominant-negative Ras (A15) limited GnRH stimulation of translation to approximately 50% of that observed in cells cotransfected with a null expression vector (−). The ability of dominant-negative Ras to attenuate GnRH stimulation of cap-dependent translation indicates that Ras may participate in the GnRH signaling cascade leading to the regulation of 4EBP.

PKC Activity Is Necessary for GnRH Action

The stimulation of the MAPK cascade by GnRH signaling is dependent on protein tyrosine kinase activity (11). However, at least two mechanisms are possible by which tyrosine kinase activity can be induced by a G protein-coupled receptor. The first is through activation of Ras via Gβγ-dependent PI 3-kinase activity (27). The second involves a mechanism dependent on PKC activity and intracellular calcium (8). To differentiate these mechanisms, αT3-1 cells cotransfected with the bicistronic reporter gene and the dominant-negative mutant rasA15 expression vector were stimulated with GnRHα or the PKC activator phorbol myristate-acetate (PMA). Treatment with PMA stimulated cap-dependent translation in αT3-1 cells. Additionally, the action of PMA was inhibited by the presence of dominant-negative RasA15, similar to the inhibition of GnRH action (Fig. 5A). As a further demonstration of the involvement of PKC in the activation of translation by GnRH, stimulation of translation in αT3-1 cells by GnRH was also attenuated by the PKC inhibitor bis-indolylmaleimide (Bis-IM). The histogram indicates the relative induction of luciferase to β-galactosidase activity of the treated cells normalized to the untreated control. Error bars indicate the SEM of at least three experiments. Asterisks indicate a significant difference (P < 0.05) between treated null and treated Ras A15 cotransfected cells as determined by ANOVA and Fisher’s PLSD.

DISCUSSION

The regulation of protein synthesis by endocrine factors such as insulin, acting through receptor tyrosine kinases, is well described. However, until recently (28),
similar actions have not been described for hormones that utilize G protein-coupled receptors. Here we present evidence that the G protein-linked GnRH receptor regulates translation by a mechanism involving PKC, Ras, the rapamycin-sensitive mTOR kinase, and the translational inhibitor 4EBP. We demonstrate this using a novel reporter gene that distinguishes between regulated, cap-dependent translation and unregulated cap-independent translation. We show that the stimulation of translation is similar to that observed by insulin and EGF stimulation. We also show that the effect on translation can be attenuated by the PKC inhibitor bis-indolylmaleimide and can be mimicked by direct stimulation of PKC activity with phorbol ester. Additionally, the involvement of Ras in this aspect of GnRH signaling is demonstrated by the ability of the dominant-negative mutant ras A15 to attenuate the GnRH response, as well as the ability of anti-Ras IgG to attenuate the response in microinjected αT3–1 cells treated with GnRH analog.

The GnRH-stimulated increase in translational capacity of αT3–1 cells occurs concurrently with previously reported changes in α-GSU mRNA stability. It has been shown that α-GSU mRNA half-life is increased in αT3–1 cells with GnRH treatment from 1.2 to 8 h (10). However, in untreated cultured rat pituitary cells the half-life of mRNA is about 6 h (29). It is possible that factors in addition to GnRH contribute to α-GSU mRNA stability. However, the ability of GnRH to modify α-GSU mRNA stability in vitro suggests that this mechanism is physiologically relevant in vivo. Although our initial observation of the effect of GnRH stimulation on protein synthesis in αT3–1 cells was made monitoring α-GSU protein synthesis, the mechanism characterized in this report is more general in its effect. In addition to specific transcriptional activation, a more general activation of cap-dependent translation would serve to amplify stimulatory response signals rapidly. The translational effects of stimulation are long lasting and have been measured as much as 20 h after stimulation (15). Therefore, GnRH can modulate transcriptional, posttranscriptional, and translational mechanisms to effect changes in target cell metabolism that enhance hormone biosynthesis.

The data presented provide evidence that GnRH receptor activation leads to signaling targets normally associated with growth factor receptor activation and results in activation of translation in a manner similar to insulin receptor and EGF receptor activation. GH-releasing hormone was shown to stimulate translation in GH3 cells through a calcium-dependent pathway resulting in regulation of eIF2, not 4EBP, as described here for GnRH (30, 31). The involvement of the eIF2 pathway in GnRH stimulation of translation has not been investigated. A significant difference between translational regulation by GnRH vs. that by insulin via 4EBP is the potential role of Ras. Previous studies examining the regulation of 4EBP by the insulin receptor have not implicated Ras in the signaling cascade regulating translation, although Ras is a downstream component of insulin receptor signaling (32). Our results show that Ras has a role in the regulation of translation by GnRH receptor activation. The MAPK cascade can independently phosphorylate eIF4E, as can PKC, and this stimulates cap-dependent translation (33). It is not likely that our observations are solely a result of direct regulation of eIF4E by PKC because the effect was attenuated by dominant negative Ras and rapamycin. Additionally, the MEK inhibitor PD098059, which prevents MAPK activation and subsequent phosphorylation of eIF4E, did not inhibit GnRHa-induced translational stimulation (M. A. Lawson, unpublished observations). Roles for other signaling pathways regulating translation cannot be ruled out because the inhibitors tested were not capable of completely abolishing the stimulatory effect of GnRH, consistent with the participation of multiple signal cascades in translation regulation.

The demonstration that GnRH influences cell metabolism through stimulation of cap-dependent translation strongly suggests that the gonadotrope population can be dynamically regulated by GnRH stimulation, as can the amount of hormone synthesized and released in response to stimulation. Activation of translation in response to releasing factor stimulation is a rapid and simple mechanism to replenish protein levels before an increase in mRNA synthesis. In concert with the reported increased α-GSU mRNA stability in response to GnRH stimulation, significant increase in protein synthetic capacity can be attained. The demonstration that GnRH increases protein synthesis in gonadotrope cells through a transduction pathway normally associated with growth factor activation provides evidence that releasing factors may have a significant role in the maintenance of pituitary cell subpopulations. Others have reported that GnRH does regulate cell cycle in αT3–1 cells (34). The αT3–1 cell line represents an immature, proliferating gonadotrope cell that expresses the definitive marker GnRH receptor and steroidogenic factor-1 genes, but not the LH and FSH β-subunit genes expressed in fully mature gonadotropes (35). The possibility that GnRH could act through an insulin-like signaling mechanism as reported here in a cultured cell model system has important implications for the role of GnRH in the development of tissues expressing the GnRH receptor. GnRH-expressing neurons can be detected as early as embryonic day 11.5 and are established in the hypothalamus before the development of the gonadotrope population (36). It can be postulated that GnRH expression is a gonadotrope-specific signal that affects the proliferation of GnRH receptor-expressing cell types. Precedence can be found in the parallel GH-releasing hormone (GHRH)/somatotrope endocrine axis. Overexpression of human GHRH in transgenic mice leads to pituitary somatotropes hyperplasia (37). Mutation of the GHRH receptor in the little mouse leads to a paucity of somatotrope in the anterior pituitary (38), and evidence exists that receptor function is necessary for normal development of the
somatotrope cell population (39). These observations suggest that GHRH receptor signaling is necessary for proper proliferation of the somatotrope population. More strikingly, it has also been observed that CRF and EGF can serve as mitogenic factors for the corticotrope population (40). By analogy, similar action can be postulated for the regulation of the gonadotropes. At least one model system suggests that this is indeed possible. The hypogonadal hpg (41) mouse bears a deletion in the Gnrh gene that renders it nonfunctional (42). Pituitary function can be recovered by transplantation of tissue containing Gnrh neurons or by implantation of immortalized Gnrh-secreting cells (43). Although the increase in circulating gonadotropin content has been documented (44), the effect on cell number in the pituitary has not been examined. Future studies will examine the potential role of Gnrh in gonadotrope cell proliferation more closely.

In summary, activation of the Gnrh receptor stimulates cap-dependent translation through the phosphorylation of the translational regulatory factor 4ebp. This action suggests that Gnrh signaling regulates both transcriptional and translational activity in the target cell population. Use of a regulatory pathway associated with growth factor signaling also suggests that Gnrh signaling may play a role in the maintenance of cell types expressing the Gnrh receptor and provide a specific mechanism for controlling the activity of Gnrh-responsive cell populations.

MATERIALS AND METHODS

Plasmids

The bicistronic reporter plasmid pgL3-EMC5-β was constructed in the reporter gene vector pgL3 Basic (Promega Corp., Madison, WI). The plasmid contains the 664-bp ClaI fragment of pCDNAI (Invitrogen, San Diego, CA) containing the cytomegalovirus immediate early enhancer and promoter inserted into the Nhel site of the multiple cloning site. The 692-bp CglI to NcoI fragment from the plasmid pTM1 (45) containing the 5′-untranslated region and internal ribosomal entry site of EMCV, the 3.2-kb NcoI to BamHI fragment of pSDKlacZ containing the coding sequence of β-galactosidase, and the SV40 T-antigen splice and polyadenylation sites were also inserted after the luciferase coding region. The resultant plasmid contained the luciferase coding region following by the EMCV 5′-untranslated region and internal ribosomal entry site of EMCV, the 5′-untranslated region and internal ribosomal entry site of EMCV, the 3.2-kb NcoI to BamHI fragment of pSDKlacZ containing the coding sequence of β-galactosidase, and the SV40 T-antigen splice and polyadenylation sites were also inserted after the luciferase coding region. The resultant plasmid contained the luciferase coding region following by the EMCV 5′-untranslated region and internal ribosomal entry site of EMCV, the 3.2-kb NcoI to BamHI fragment of pSDKlacZ containing the coding sequence of β-galactosidase, and the SV40 T-antigen splice and polyadenylation sites were also inserted after the luciferase coding region.

Cell Culture and Transfection

The pituitary gonadotrope cell line αT3-1 (12) and NIH/3T3 cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS, 4.5 mg/ml glucose, 100 μg/ml of penicillin, and 0.1 mg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO₂. Cells were transfected with 3 μg of reporter plasmid DNA in 35-mm dishes or plates by the calcium phosphate method (46) for 4–6 h, washed twice with PBS, and incubated in fresh serum-free medium for 12–16 h. Transfected cells were then treated with 5 nM (im-Bzl-D-His⁶, Pro⁹-gal) GnRH analog (GnRHa, kindly provided by Jean Rivier), insulin (80 nM), or EGF (50 μg/ml) for 8 h. Transfected cells were pretreated with the inhibitors bis-indolylmaleimide or rapamycin (Calbiochem, La Jolla, CA) at 100 nM or 20 nM, respectively, for 30 min before stimulation with GnRHa. In cotransfection experiments, 1 μg of reporter plasmid DNA was used with two molar equivalents of empty vector or Ras A15 expression plasmid. Constant DNA concentration was maintained by supplementation with nonspecific plasmid DNA. Cells were harvested by scraping into 1 ml of 150 mM NaCl, 1 mM EDTA, and 40 mM Tris-Cl (pH 7.4 at 25 C). Harvested cells were pelleted in a 5415C centrifuge (Eppendorf, Madison, WI) and resuspended in 50 μl of 100 mM potassium phosphate (pH 8.0) at 25 C, 0.2% Triton X-100. The resultant extracts were clarified by further centrifugation for 5 min and assayed immediately for luciferase activity (Analytical Luminescence Laboratory, Ann Arbor, MI) and β-galactosidase activity (Tropix, Inc., Bedford, MA) using a MicroLumat 96P luminometer (EG&G Berthold, Gaithersburg, MD). Results are reported as a mean of at least three experiments. Error is reported as SEM.

Western Blot Analysis

Twenty-four hours after plating, αT3-1 cells were washed twice with PBS and placed in serum-free medium for 12–16 h. Control and rapamycin-pretreated cells were then stimulated with GnRHa at 5 nM for 15 min and immediately harvested in ice-cold buffer as described above. After pelleting, cells were lysed in a buffer of 50 mM Tris-Cl (pH 7.4 at 25 C), 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 mM β-glycerophosphate, 1 mM EGTA, 50 mM NaF, 10 mM Na₃PO₄, 0.1 mM Na₂VO₃, and 50 mM okadaic acid and subjected to three cycles of freeze-thaw. Extracts were clarified by centrifugation and assayed for protein content by the method of Bradford (47). For each sample, 50 μg of protein were boiled in Laemmli sample buffer and run on a 15% denaturing polyacrylamide gel. Protein was blotted to Immobilon-P membrane (Millipore Corp., Bedford, MA) by semidry transfer. Detection of 4EBP was performed using rabbit antiserum (15) diluted 1:4000 and enhanced chemiluminescence (Amer sham Pharmacia Biotech, Arlington Heights, IL) with biotinylated secondary antibody and horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories, Inc., Burlingame, CA). Blots were visualized by exposure to BioMax film and by storage phosphorimaging on a Molecular Imager GS-525 (Molecular Dynamics, Inc., Sunnyvale, CA). Stored images were analyzed with Molecular Analyst 1.5 software (Bio-Rad Laboratories, Inc., Hercules, CA).

Microinjection and DNA Synthesis Assays

Trypsinized αT3-1 cells were seeded on glass coverslips at 75% confluence and starved for 24 h in serum-free DMEM (Life Technologies, Inc.). For microinjection, the culture medium was replaced with serum-free DMEM containing vehicle or GnRHa analog (3 nM) and incubated a further 24 h. Cells were injected with 0.5X Tris-Borate buffer containing either 10 μg/ml normal guinea pig IgG, or 5 μg/ml rabbit anti-rat Ras IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 5 μg normal guinea pig IgG (26), or 3 μg/ml purified bacterially expressed wild-type H-Ras protein and 7 μg/ml normal guinea pig IgG. Injected cells were marked by the inclusion of cascade blue in the injection buffer. After incubation for 24 h, Cells were fixed and stained as described previously (48). Briefly, after 15–30 min in PBS containing 3.7% formaldehyde, processed for immunohistochemistry using rabbit antirat α-glycoprotein hormone subunit IgG, and visualized by incubation with flu-
orescein-conjugated goat-antirabbit IgG secondary antibody. Results are reported as percentage of injected cells staining for $\alpha$-GSU. Between 3S and 152 injected cells were counted per experiment. Error is reported as SEM proportion by the method of Fisher. Significance is reported at $P < 0.05$.

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