

Ras mediates Src but not epidermal growth factor-receptor tyrosine kinase signaling pathways in GH₄ neuroendocrine cells

(transcription control/prolactin promoter/*c-Fos* promoter/pituitary cells)

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ABSTRACT p21Ras has been implicated as a critical signaling component in mediating the effects of many growth factor receptor/tyrosine kinases on cell growth and differentiation. However, the precise functional role of Ras in establishing a cell-specific transcriptional response to a ubiquitous growth factor remains unclear. We have utilized a transient cotransfection model system in epidermal growth factor (EGF)-responsive cultured GH₄ rat pituitary neuroendocrine cells to investigate the role of Ras in coupling EGF receptor (EGF-R) and v-Src tyrosine kinase signals to the activation of a cell-specific promoter for the rat (*r*) prolactin (PRL) gene. A significant dose- and time-dependent EGF stimulation of the transfected rPRL promoter was obtained. A similar degree of activation of the rPRL promoter was obtained by cotransfection of a plasmid encoding v-Src. Cotransfection of a construct encoding the dominant-negative Ras, N17Ras, produced almost complete inhibition of v-Src-induced rPRL promoter activity, while EGF-stimulated rPRL promoter activity was unaffected. Similarly, EGF activation of a *c-Fos* promoter was unaffected by N17Ras, while v-Src activation was blocked. Hence, using transcription regulation as a functional assay, we show that Ras is not required for the EGF-mediated control of the rPRL and *c-Fos* promoters, whereas Ras is critical in mediating the v-Src effects to these two promoters. These observations emphasize that, despite current biochemical data linking the EGF-R and Ras pathways, the functional significance of such an interaction should be analyzed in a biologically relevant manner and may differ as a function of cell type.

Protein tyrosine kinases such as the epidermal growth factor receptor (EGF-R) and the nonreceptor kinase pp60^{c-Src} appear to trigger a cascade of intracellular serine/threonine phosphorylations that form a signaling pathway transducing the initial message from the cell membrane to the nucleus (1–3). Activation of the EGF-R is known to produce a mitogenic response in several cell types (1–3). However, in other cell types, including GH₃/GH₄ rat pituitary cells (utilized in these studies) (4, 5), EGF appears to enhance the expression of cell-specific gene products, promoting differentiation and cessation of cellular growth. The critical components that allow a growth factor signaling event, initiated at the level of the membrane, to result in either cellular proliferation or cessation of growth and differentiation are not well defined. However, such differences in the action of EGF could be dependent on cell-specific differences in the relative functional role of various signal transducers. Several proteins have been identified as substrates of the EGF-R tyrosine kinase, including Ras/GAP, phospholipase C- γ 1 (PLC- γ 1), phosphatidylinositol 3-kinase (PI 3-kinase), Shc, Grb2/mSos, and the p91 transcription factor (1–3, 6–9). These proteins each contain a Src homology 2 (SH2) domain

that interacts with discrete and distinct tyrosine phosphorylated regions of the EGF-R cytoplasmic tail (2, 3). From recently published reports (7, 8), the adaptor protein Grb2, in a preformed complex with the guanine nucleotide exchange factor, Sos, is thought to bind to the EGF-R in a ligand-dependent manner promoting the exchange of GDP for GTP on p21Ras, thus stimulating p21Ras activity. p21Ras appears to function as a critical switch, converting the initial EGF-R into an amplified cascade of activated, serine/threonine kinases (2). A similar mechanism of p21Ras activation has been inferred for v-Src (10–12), which is a constitutively activated, oncogenic form of the nonreceptor tyrosine kinase pp60^{c-Src}. The identification of Vav, a hematopoietic-specific Grb2 protein, underscores the possibility that cell-specific responses to otherwise ubiquitous signaling pathways may be mediated by distinct isoforms of these adaptor molecules (13). Thus distinct cell types may differ in their relative utilization of these initial signaling components or their downstream effectors.

The GH₃/GH₄ rat pituitary cells are well-differentiated, EGF-responsive neuroendocrine clonal cell lines that maintain lactotroph-specific functions, thus providing a model system to analyze tyrosine kinase signaling mechanisms in a highly specialized cell type (4, 5). Previous studies have shown that significant numbers (34,000 sites per cell) of high-affinity ($K_d = 1$ nM) EGF-Rs are present on GH₃ cells (4, 5). Interestingly, chronic EGF exposure appears to induce a phenotypic switch, manifested by alterations in morphology, a decrease in growth rate, and an alteration of the relative transcription of the growth hormone and prolactin (PRL) genes, resulting in a PRL predominance (4, 5). Indeed, EGF results in a significant and rapid increase in PRL gene transcription rates, resulting in increased steady-state PRL mRNA and protein levels (4, 5, 14). Thus EGF results in a nonmitogenic response and induces a more differentiated lactotroph cell type in GH₃ cells. To investigate the molecular mechanisms dictating basal, hormone-regulated, and pituitary-specific transcription of the rat PRL (rPRL) gene, we have developed a transient transfection system whereby a rPRL promoter-luciferase reporter (pA3PRL $_{luc}$) construct is introduced into GH₃ or GH₄ pituitary cells by electroporation (15–18). We have previously demonstrated that oncogenic V12 Ras specifically and significantly increases rPRL promoter activity in a Raf and mitogen-activated protein MAP kinase-dependent manner in GH₄ cells (15, 16). Hence these putative constituents of the EGF signaling pathway, distal to p21Ras, are effective in this pituitary cell system, and the activation of a cell-specific gene promoter can be used as a

Abbreviations: rPRL, rat prolactin; EGF, epidermal growth factor; EGF-R, EGF receptor; PLC, phospholipase C; PI 3-kinase, phosphatidylinositol 3-kinase; SH2, Src homology 2; FCS, fetal calf serum; luc, luciferase; MAP kinase, mitogen-activated protein kinase.

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functional read-out. However, the functional role of p21Ras in mediating specific EGF-dependent cellular responses and the molecular mechanisms by which ubiquitous signaling pathways are harnessed in a cell-specific manner are yet to be elucidated. In the studies described here, we have utilized this system to investigate whether p21Ras serves to couple the EGF-R and v-Src tyrosine kinase signals to the nuclear transcription machinery in the highly specialized GH₄ rat pituitary cell.

MATERIALS AND METHODS

All experiments were performed with 5 μ g of either the pA3PRL_{luc} reporter construct as described (15–18) or the pc-fosTK_{luc} construct (provided by G. Gill, University of California, San Diego). The pc-fosTK_{luc} construct contains two copies of the –357 to –276 region of the c-Fos enhancer that confers serum and EGF responsiveness, ligated upstream of the thymidine kinase promoter (–200 to +70) and the firefly luc reporter gene (19, 20). The plasmid pZCR17N codes for a dominant-negative Ras (N17Ras) (11). The v-Src construct has been described (21). In all electroporations either 300 ng of a pCMV β -gal or 5 μ g of a pSV β -gal plasmid was included as an internal control of transfection efficiency and as an indicator of the specificity of the response to EGF and the various effector DNA constructs (15, 16, 18). Non-specific promoter effects were controlled for using constructs with the same promoter regions either devoid of insert or containing an irrelevant insert. Finally, the total amount of DNA was kept constant with pGEM7 (Promega) plasmid DNA. All plasmid DNAs were purified and quantitated as described (15–18).

GH₄ rat pituitary cells were grown and harvested as described (15–18). Aliquots containing $\approx 7 \times 10^6$ cells were added to plasmid DNA and transfected by electroporation (15–18). After electroporation, cells were plated in 3 ml of Dulbecco's modified Eagle's medium (DMEM) with 0.6% fetal calf serum (FCS) and, unless otherwise indicated, incubated a total of 24 hr posttransfection. EGF was added at a final concentration of 25 nM to the existing medium for the last 6 hr of incubation. Cells were harvested and reporter enzyme activities were assayed as described (18).

Data are presented as the mean relative light units (total light units produced by luc normalized to total β -galactosidase activity) or as fold activation calculated from the relative light units. Although all experiments were repeated two to six times, with each transfection performed in triplicate within any given experiment, in some figures the results of a single representative experiment (mean of three transfections) \pm SD are depicted. Where possible, data are depicted as the mean of several experiments \pm SEM. For statistical analysis, *P* values were calculated by the Student's *t* test. Additionally, several preparations of a given plasmid construct were employed over the course of these studies.

RESULTS

Characterization and Optimization of the rPRL Promoter Response to EGF. The basal rPRL promoter activity is enhanced in complete medium (10% FCS) compared to serum-poor medium (0.6% FCS). However, addition of 25 nM EGF resulted in a similar level of rPRL promoter stimulation in either medium (data not shown). To ensure that any variability in the serum concentrations of hormones and growth factors, including EGF, would not confound our results, all experiments were performed in medium containing only 0.6% FCS. The data in Fig. 1A show that an EGF dose-dependent response of pA3PRL_{luc} expression was obtained, with as little as 0.1 nM EGF eliciting a 10-fold response, and the maximal effect noted at 10–25 nM EGF.

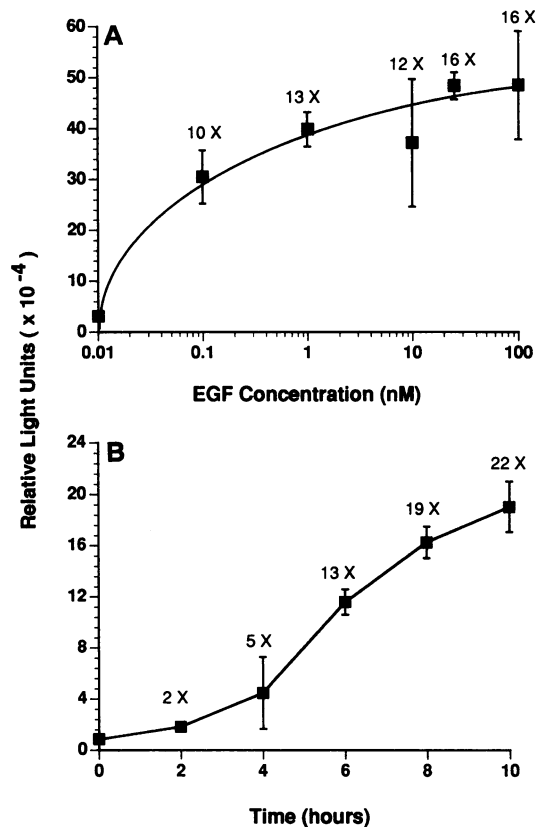


FIG. 1. Optimization of EGF-stimulated rPRL promoter activity. (A) Dose dependence. GH₄ cells were transfected with pA3PRL_{luc} and pCMV β -gal and then incubated in 0.6% FCS/DMEM at 37°C for 18 hr. EGF was added, at the indicated concentrations, for an additional 6 hr. Numbers above the graph indicate the fold increase in pA3PRL_{luc} expression for each EGF dose relative to the activity in untreated cells. (B) EGF time course. GH₄ cells were transfected and incubated as described in A in the presence of 25 nM EGF for the indicated times. The total posttransfection incubation period for each time point was 24 hr; the times indicated refer to the period of EGF exposure. Numbers above the graph indicate the EGF fold increase in pA3PRL_{luc} expression relative to basal activity at the 0 time point. Data in A and B are presented as the mean relative light units for three transfections \pm the SD.

The half-maximal response was estimated to be about 50–100 pM EGF, which is very similar to that previously reported for these cells (100 pM) (4, 5). EGF activation of the rPRL promoter was selective since neither the cytomegalovirus promoter nor the simian virus 40 promoter in the internal controls was stimulated by EGF at any of the treatment times or doses used (data not shown). The time course study shows a detectable response to 25 nM EGF at the earliest time point (2 hr), with a linear increase between 4 and 10 hr of EGF treatment (Fig. 1B). The rapidity of the response of the rPRL-luc reporter gene to EGF is consistent with previously published data indicating that EGF (i) maximally activates rPRL gene transcription rates in a transcription run-on assay within 30 min (14), (ii) increases steady-state mRNA levels within 3 hr (14), and (iii) increases PRL peptide hormone production by 25 hr (5). The 6-hr time point was chosen for all subsequent experiments, as it was in the linear response range and consistently produced a substantial rPRL promoter activation at a relatively early time point, thus more likely avoiding secondary effects of EGF. Of note, the EGF-mediated fold activation in the dose-response and time course studies was more exuberant than usually obtained, possibly due to subtle differences in overall cell health, such as cell passage number or the quality of medium or FCS.

Nevertheless, we have consistently obtained about a 5-fold effect, in agreement with previous reports of EGF induction of both endogenous PRL gene transcription and of exogenously transfected rPRL gene-reporter constructs (14, 22).

Effect of N17Ras on EGF Activation of the rPRL Promoter. Previously published reports have shown that EGF stimulates the association of the EGF-R with Grb2/Sos or induces GTP loading of p21Ras (7, 8, 23, 24). However, most of these studies fail to document that any EGF-mediated cellular response, such as transcription activation or mitogenesis, is actually Ras-dependent. Indeed, only the report by Cai *et al.* (23) shows that a dominant-negative N17Ras blocks the EGF-mediated transcriptional activation of the c-Fos promoter. Therefore, we designed studies to determine the precise role of p21Ras in mediating EGF effects on GH₄ rat pituitary tumor cells, using rPRL promoter activation as the functional assay. This approach also has the advantage that the EGF/Ras coupling is examined in a more specialized cell type that undergoes a nonmitogenic, differentiating response to EGF (4, 5). Thus, we used an expression vector encoding a dominant-negative Ras (pZCR17N) to determine if Ras activation was necessary for EGF-induced stimulation of the rPRL promoter (11, 25, 26). The N17Ras construct has been employed extensively to define the role of Ras; it is considered the method of choice to block c-Ras-mediated signaling in recombinant DNA approaches (11, 23, 25, 26). Fig. 2 shows the effect of increasing amounts of pZCR17N, from 0 to 20 μ g, transfected along with the pA3PRL luc reporter construct into GH₄ pituitary cells. The results show a 5.2 ± 1.3 -fold EGF response of the rPRL promoter in the absence of N17Ras and a 4.5 ± 0.5 -, 3.7 ± 1.0 -, 4.1 ± 1.1 -, and 4.1 ± 0.7 -fold response for N17Ras concentrations of 2, 5, 10, and 20 μ g, respectively (Fig. 2). The slight decrease in the EGF effect at 5 μ g of N17Ras DNA was not statistically significant ($P > 0.10$). Indeed, the N17Ras construct failed to produce any significant inhibition of the 5-fold activation of rPRL promoter by EGF, even at the highest amount of added N17Ras DNA (20 μ g). While N17Ras failed to block the EGF activation of the rPRL promoter, these increasing amounts of pZCR17N DNA reduced basal rPRL promoter activity in a dose-dependent manner (data not shown), as we have previously reported (15). This inhibitory effect on basal promoter activity was not evident using the pSV β -gal internal

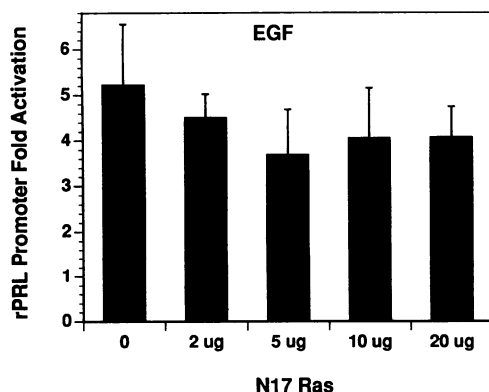


FIG. 2. Effect of N17Ras on EGF-stimulated rPRL promoter activity. GH₄ cells were transfected with pA3PRL luc and pCMV β -gal with varying amounts of the pZCRN17Ras construct, as shown. EGF was added to the cell medium for the last 6 hr and cells were harvested 24 hr after transfection. Data are shown as the mean fold EGF stimulation of pA3PRL luc over basal activity at each amount of pZCRN17Ras DNA \pm SEM for 9–30 transfections (3–10 separate experiments). Using Student's *t* test, there is no difference in EGF response for the control (0 μ g) vs. the 5 μ g N17Ras values, at $P > 0.1$.

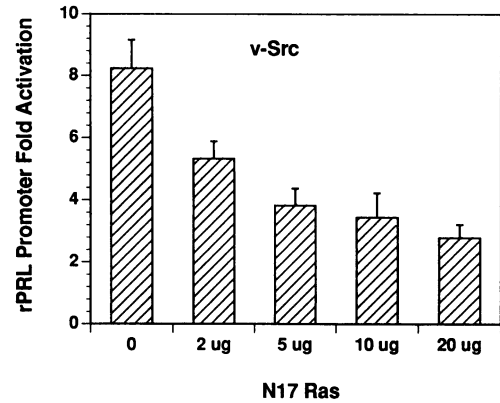


FIG. 3. Effect of N17Ras on v-Src-stimulated rPRL promoter activity. GH₄ cells were transfected with 5 μ g of v-Src and varying amounts of pZCRN17Ras constructs along with the pA3PRL luc and pCMV β -gal reporter constructs and harvested 24 hr later. Data are presented as fold activation of the rPRL promoter calculated from the relative light units obtained in the presence of v-Src divided by the relative light units in the absence of v-Src for each N17Ras condition. Data at 0 and 10 μ g of N17Ras are presented as mean \pm SEM for 15 transfections (five separate experiments). Data at 2, 5, and 20 μ g of N17Ras are the mean \pm SD of 3 transfections obtained in a single experiment. Using the Student's *t* test, P values were < 0.01 at 2 μ g, < 0.005 at 5 μ g, and < 0.002 at 10 and 20 μ g of N17Ras, as compared to the control (0 μ g of N17Ras).

control vector, indicating that the simian virus 40 early promoter is not affected (data not shown).

The results of the above experiments, however, could be due to a pZCR17N plasmid, which did not function appropriately in these cells. To exclude the possibility that an insufficient amount of N17Ras was present at the usual 24-hr harvest time, either due to an early and transient expression of N17Ras protein or due to a delay in its production, pA3PRL luc was transfected with or without pZCR17N DNA and both were allowed to express for varying amounts of time (specifically, 2, 12, 18, and 42 hr), after which 25 nM EGF was added for a constant period of 6 hr. EGF produced significant activation of rPRL promoter activity at all time points (4- to 11-fold); however, the N17Ras construct failed to inhibit EGF activation of the rPRL promoter at any of these post-transfection time points (data not shown), including the 24- and 48-hr time points used in previously published studies (12, 23). If the efficiency of the EGF-R tyrosine kinase coupling to p21Ras is modulated by growth factors in the serum, it is conceivable that the lack of an N17Ras effect in our system was due to the use of serum-poor medium (0.6% FCS). However, in medium containing 10% FCS/DMEM posttransfection, the EGF response (4.12 ± 1.97 -fold) was not inhibited by cotransfection of 10 μ g of the N17Ras construct (5.63 ± 0.38 -fold). Finally, enhanced basal rPRL promoter activity produced by transfection of a plasmid encoding c-Ras (2.3-fold with 10 μ g) was inhibited by 65% with cotransfection of 10 and 20 μ g of pZCR17N (data not shown).

Effect of N17Ras on v-Src Activation of the rPRL Promoter. Although these data indicate that the N17Ras DNA functions appropriately, we sought another means of verifying the effectiveness of the N17Ras construct. v-Src is a constitutively active tyrosine kinase and, like v-Ras, produces transformation in several cell types (10–12). Moreover, v-Src activity is Ras-dependent, since its biological effects can be inhibited by coexpression of N17Ras or microinjection of Ras antibodies (10, 12, 23). In the rat pituitary model system used here, cotransfection of a v-Src plasmid construct resulted in an 8.2-fold stimulation of rPRL promoter activity, as shown in Fig. 3. Addition of increasing amounts of the N17Ras DNA

produced a dose-dependent inhibition of the v-Src activation of the rPRL promoter, from 8.2-fold in the absence of N17Ras to 5.2-, 3.8-, 3.5-, and 3-fold at 2, 5, 10, and 20 μg doses of N17Ras DNA, respectively. These data verify that the N17Ras expression vector is functional in these cells and is able to block v-Src tyrosine kinase signaling. Furthermore, this dominant-negative form of Ras is capable of inhibiting endogenous Ras activation and/or downstream effects of Ras on rPRL promoter activity (data not shown). Clearly, however, expression of N17Ras does not significantly alter the EGF-mediated activation of the rPRL promoter (Fig. 2).

Effect of N17Ras on EGF and v-Src Activation of the c-Fos/Thymidine Kinase Heterologous Promoter. We next chose to determine the effect of the N17Ras construct on another EGF- and v-Src-responsive promoter: pc-fosTKluc (19, 20). The pc-fosTKluc construct has been well characterized and shown to be activated by serum, by several growth factors (including EGF), and by a number of oncogenes (including v-Src, Ha-Ras and v-Raf) (19, 20). As observed with the rPRL promoter, both EGF and v-Src activated the pc-fosTKluc reporter construct (Fig. 4). Three-fold activation was produced by 25 nM EGF exposure for 6 hr, and cotransfection of increasing amounts of N17Ras DNA, up to 20 μg , did not significantly interfere with the EGF-mediated effect ($P > 0.10$) (Fig. 4A). In contrast, v-Src produced a 7-fold increase in pc-fosTKluc expression, which

was inhibited by N17Ras DNA in a dose-dependent manner. Indeed, as little as 2 μg of N17Ras DNA resulted in a 68% inhibition of the v-Src response ($P < 0.002$), with almost complete inhibition at 10 μg of N17Ras DNA (Fig. 4B). Thus, for both the tissue-specific rPRL promoter and the ubiquitous c-Fos promoter, EGF activation appears to be independent of Ras, whereas the v-Src-mediated activation of both of these promoters is Ras-dependent. This would imply that the Ras-independent transduction of the EGF signal is not specific to the rPRL promoter but rather may represent a cell-specific property of the EGF signal transduction pathway in the GH₄ rat pituitary cell line.

DISCUSSION

Previous studies have used primarily biochemical approaches to show that p21Ras is involved in the EGF signaling pathway (7, 8, 23, 24); however, there has been a striking paucity of functional data documenting the physiological relevance of such biochemical associations. In this report, we have focused on the functional role of Ras in mediating receptor and cytoplasmic tyrosine kinase signaling to the transcription machinery in a highly specialized rat pituitary lactotroph cell. The data show that in GH₄ rat pituitary cells, the EGF-mediated regulation of the rPRL and c-Fos promoters is not transmitted via Ras, whereas the v-Src-mediated activation of these two promoters is Ras-dependent. These results are in contrast to the data obtained in fibroblast systems, wherein p21Ras appears to be a critical component of the EGF signaling cascade (8, 23, 24).

The observation that the EGF response is independent of p21Ras, whereas the v-Src response requires it, suggests that there are differences in the signaling components used by these two tyrosine kinases. Previous reports have shown that activation of the EGF-R tyrosine kinase or Src tyrosine kinase allows several SH2-containing proteins to become associated with specific phosphotyrosines of the activated tyrosine kinase (1–3). Each SH2 protein, such as Shc, PLC- γ 1, PI 3-kinase, Ras/GAP, or p91, then initiates a specific signaling cascade, which subsequently appears to be transduced to distinct intracellular compartments (1–3). However, the relative functional importance of each factor and signaling pathway to specific cellular processes, such as gene transcription in highly differentiated cells, remains unclear. Indeed, it is tempting to speculate that the lack of a p21Ras requirement for the EGF transcriptional effect may be due to either the nonmitogenic response of EGF on GH₄ cells or the fact that these are highly differentiated cells with specialized functions (4, 5). Nevertheless, since the v-Src tyrosine kinase pathway does act via p21Ras, it is clear that the coupling mechanism between p21Ras and the v-Src tyrosine kinase is intact in GH₄ rat pituitary cells. Furthermore, the downstream signaling components of the Ras pathway, including Raf and MAP kinases, are functional and actually activate rPRL promoter activity in GH₄ cells, as we have previously documented (15, 16, 18). Thus, the difference in the EGF-R vs. v-Src tyrosine kinase pathways is most likely due to differential utilization of distinct SH2 proteins, such as Grb2/Sos, PLC- γ 1, PI 3-kinase, p91, or Shc, to initiate a Ras-independent vs. a Ras-dependent signaling cascade, respectively (1–3).

Likely components of a Ras-independent signaling cascade include PI 3-kinase, the p91 transcription factor, or PLC- γ 1. Although it is possible that PI 3-kinase could mediate the EGF transcriptional effects reported here, its role in transcription regulation has not yet been defined and currently the best documented function of PI 3-kinase is that it appears to be involved in growth factor-mediated mitogenesis (1, 6). Alternatively, a Ras-independent, EGF-induced tyrosine phosphorylation and activation of a latent cytoplasmic tran-

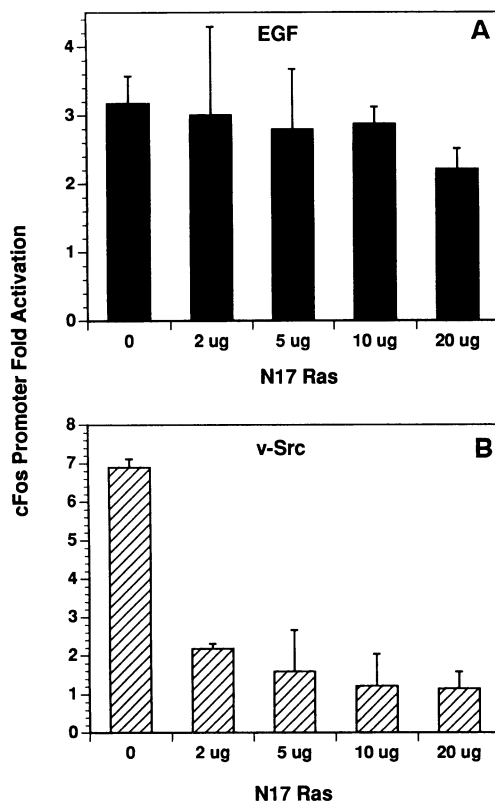


FIG. 4. Effect of N17Ras on EGF and v-Src-stimulated c-FosTK promoter activity. GH₄ cells were transfected with 5 μg of the pc-fosTKluc reporter construct, 5 μg of pSV β -gal, and the indicated amounts of the pZCRN17Ras construct. (A) Cells were treated with 25 nM EGF for the last 6 hr of the 24-hr posttransfection incubation time. Data are presented as the mean \pm SEM for 6–15 transfections (two to five separate experiments). There was no difference in EGF response for the control vs. the 20 μg N17Ras values, at $P > 0.1$. (B) The transfection included 5 μg of the v-Src construct. These cells were also incubated for 24 hr posttransfection but were not exposed to EGF. Data are presented as the mean fold activation \pm SD for three transfections. Comparing the control to the 2 μg values, the data are significant to $P < 0.002$.

scription factor (such as p91) could have a key role in regulating the rPRL promoter (9). The p91 factor appears to be a mediator of growth factor- and interferon-induced gene transcription that binds to a highly conserved DNA sequence with 5'-TTC(NNN)G(T/A)A-3' representing the core motif (9, 27). The EGF response element has been previously mapped to the -79 to -30 region of the rPRL promoter and it colocalizes with the most proximal pituitary-specific site (22). This element, termed footprint I (FP I), spans the -67 to -40 rPRL promoter region, binds the POU-homeodomain transcription factor GHF-1/Pit-1, and appears to be critical in mediating multiple hormonal responses (22, 28). Of note, the 3' end of the rPRL FP I site, from -51 to -44, contains the sequence 5'-TTCATGAA-3', which is similar to a putative p91 binding site (9, 27). However, this site in the rPRL promoter contains a 2-bp spacing between the dyad, rather than the conserved 3-bp spacing reported to date for p91 binding (9, 27). Furthermore, EGF treatment of GH₄ rat pituitary cells has failed to reveal any hormonal induction of a DNA-binding activity to the FP I site (9, 22). Given that the role of GHF-1/Pit-1 appears to be critical for mediating these various hormonal responses, it is likely that the role of the putative p91 binding site within FP I, with regard to the rPRL EGF response, is modulatory at best.

A Ras-independent signaling pathway that has been biochemically well-characterized for the EGF-R is mediated via PLC- γ 1 (1-3). Association of PLC- γ 1 with the activated EGF-R results in tyrosine phosphorylation and subsequent activation of PLC- γ 1, giving rise to inositol phosphates and diacylglycerol, which control calcium- and protein kinase C (PKC)-mediated cellular responses (1-3). It is tempting to speculate that the EGF activation of the rPRL promoter noted here is mediated via PLC- γ 1. Consistent with this interpretation are recent preliminary data indicating that the EGF response of the rPRL promoter is inhibited in a dose-dependent manner by either staurosporine or calphostin C (unpublished data). Furthermore, agents that activate PKC (e.g., phorbol esters) mimic many of the effects of EGF on GH₃/GH₄ pituitary cells (29). Specifically, phorbol esters also decrease GH₄ cellular proliferation, mediate a flat cellular phenotype, and activate PRL gene expression (29). Phorbol esters stimulate the rPRL promoter via the -79 to -30 FP I element (22, 28), and the EGF response also colocalizes to the FP I site (22, 28).

These observations raise the possibility that in GH₃/GH₄ rat pituitary cells, the EGF-R tyrosine kinase is primarily coupled to PKC activation (most likely via PLC- γ 1), whereas the cytoplasmic v-Src tyrosine kinase is primarily coupled to p21Ras (presumably via Shc and/or Grb2). We have previously shown that the rPRL promoter can be activated by oncogenic Ras and Raf in a MAP kinase- and ets-dependent manner (16). Furthermore, the Ras/Raf and, therefore, the v-Src, response has been mapped to the -255 to -212 region of the rPRL promoter (unpublished data), which is far upstream from the FP I region comprising the EGF/PKC response site (22), further distinguishing these two signaling pathways. Finally, we have shown that there is a phorbol ester dose-dependent inhibition of the Ras induction of the rPRL promoter (18), indicating that the PKC and Ras pathways are antagonistic; a similar result is obtained with EGF, which also blunts the Ras response (unpublished data). After this manuscript was submitted, Ohmichi *et al.* (30) reported that EGF treatment of GH₃ pituitary cells increased Ras-GTP and activated both Raf and MAP kinases; however, the authors did not show that any of the constituents of the Ras/Raf/MAP kinase pathway was necessary for a specific, EGF-mediated biological response. While it remains possible that the EGF-Ras-Raf-MAP kinase pathway may be critical for other effects of EGF, such as PRL hormone secretion,

changes in cell shape, or alterations in cellular proliferation rates, the role of these signaling components in EGF-mediated transcription regulation of the rPRL and c-Fos promoters is insignificant. Thus, despite these advances in our understanding of signal transduction mechanisms in the GH₄ pituitary tumor cell, the precise molecular mechanisms that give rise to distinct signaling pathways for two different tyrosine kinases, EGF-R and v-Src, remain to be elucidated. Taken together, these data emphasize that the functional downstream components by which a given tyrosine kinase signal, EGF-R or v-Src, is transduced may be completely different and that such differences may be dictated by the cell's developmental history.

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