c-Src Is Activated by the Epidermal Growth Factor Receptor in a Pathway That Mediates JNK and ERK Activation by Gonadotropin-releasing Hormone in COS7 Cells*

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Key participants in G protein-coupled receptor (GPCR) signaling are the mitogen-activated protein kinase (MAPK) signaling cascades. The mechanisms involved are not well understood. Here we show that c-Src is activated by the epidermal growth factor receptor (EGFR) and that this activation is mediated by the EGF receptor, c-Src and phosphatidylinositol 3-kinase (PI3K). ERK activation by the EGFR involves a sequential activation of the EGFR, which activates Ras either directly or via c-Src, and PI3K. The PI3K signaling may initiate additional, albeit minor, pathways that contribute to the activation of MAPK cascades. Interestingly, c-Src plays a central role in the activation of MAPK signaling cascades (reviewed in Ref. 25). G protein-coupled receptors (GPCRs) are the largest group of membrane receptors that transmit signals from a diverse array of external stimuli including neurotransmitters, hormones, phospholipids, and mitogens. Each of these extracellular agents binds to a specific GPCR that consequently interacts with a G protein to induce downstream signaling. The G protein-coupled receptors include the G12(Gq), G13(Gz), G11, and G10 (12). c-Src is a component of three of these G proteins and is activated by the dissociation of the G protein to free c-Src. In addition, c-Src is activated by other signaling mechanisms to the G proteins, mainly via Gq, which serves as a key regulator of the reproductive hormones (13), or can lead to MAPK activation by a specific guanine nucleotide exchange factor (14). Interestingly, calcium/calmodulin-dependent protein kinase II (CaMKII, Ref. 15) as well as several protein-tyrosine kinases (PTKs) such as Pyk2, FAK, BTK, and c-Src have been implicated in Gαq/PLC signaling but their mechanism of action is not yet fully understood (9). Gαq operates primarily by stimulation of PTKs (16), Ras-GAP, and PLCβ, which directly regulate Ras (17). Go signals can be transmitted via transactivation of receptor-tyrosine kinases (RTKs, Ref. 18) or association and activation of Rap-GAP (19).

Beside the Gα subunits that seem to be responsible for most of the GPCRs signaling, additional signaling mechanisms toward MAPKs involve the dissociated βγ dimer (20) as well as other signaling molecules. The dissociated βγ dimer may operate via several pathways including activation of receptor-tyrosine kinases (21), direct activation of the protein serine/threonine kinase KRAS (22), and direct interaction and activation of PI3K. Finally, it has recently been shown that the receptors may activate signaling proteins including dynamin (23, 24), β-arrestins (25–27), and c-Src (28), which transmit the signals downstream to the activation of MAPK cascades. Interestingly, c-Src plays a central role in most GPCR-mediated signaling cascade mechanisms that are involved, suggesting that it is a major signal transducer of most ligands examined.

Gonadotropin-releasing hormone (GnRH) serves as a key peptide hormone that regulates the secretion of reproductive hormones (29). GnRH receptor (GnRHR), which is coupled to G protein subunits, as well as the dissociated G protein to free c-Src. In addition, c-Src is activated by other signaling mechanisms to the G proteins, mainly via Gq, which serves as a key regulator of the reproductive hormones (13), or can lead to MAPK activation by a specific guanine nucleotide exchange factor (14). Interestingly, calcium/calmodulin-dependent protein kinase II (CaMKII, Ref. 15) as well as several protein-tyrosine kinases (PTKs) such as Pyk2, FAK, BTK, and c-Src have been implicated in Gαq/PLC signaling but their mechanism of action is not yet fully understood (9). Gαq operates primarily by stimulation of PTKs (16), Ras-GAP, and PLCβ, which directly regulate Ras (17). Go signals can be transmitted via transactivation of receptor-tyrosine kinases (RTKs, Ref. 18) or association and activation of Rap-GAP (19).

This article has been withdrawn by the authors. Lanes 1-4 and lanes 7-10 of the G-JNK immunoblot in Fig. 1B were duplicated. In Fig. 2A, the DP-JNK immunoblot on the left were inappropriately manipulated. In Fig. 2A, the G-JNK immunoblot in the middle row were duplicated. In Fig. 3B, lanes 2 and 7 of the phosphorylated MBP panel were duplicated. In Fig. 3A, lane 1 of the Ras-GTP panel was inappropriately manipulated. Lanes 7 and 11 were duplicated. Because the original data are no longer available, in the interest of maintaining accuracy in the published scientific literature, the authors wish to withdraw this article. However, the authors have full confidence in the findings and conclusions of this paper.

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¶ The abbreviations used are: GPCR, G protein-coupled receptor; Cak, C-terminal Src kinase; CM, conditioned medium; Dn, dominant negative; DP, doubly phosphorylated; ERK, extracellular signal-regulated kinase; Ib, heparin binding; FAK, focal adhesion kinase; FRNK, truncated FAK; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GnRHa, GnRH analog ([D-Trp6]-GnRH); JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PTK, protein-tyrosine kinase; RTK, receptor-tyrosine kinase; RIPA, radioimmune precipitation assay buffer.

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regulator of the reproductive system. It acts via a specific GPCR (GnRHR) and triggers the synthesis of the common α- and β-chains of the gonadotropins, which in turn, control the function of the gonads and induce steroidogenesis (reviewed in Refs. 29 and 30). In the pituitary-derived cT3-1 cells, it was shown that GnRHR transmits its signals primarily via Gq phospholipases, PKCs, and Ca2+, culminating in the activation of several MAPK cascades (reviewed in Refs. 9 and 31). Studies from our laboratories have shown that the signaling of GnRH in αT3-1 cells involves a direct activation of Raf-1 by PKC, and this step is partially dependent on a second pathway consisting of Ras activation downstream of dynamin and c-Src (32–34). The activation of JNK in these cells is also mediated primarily by PKC that further induces the sequential activation of c-Src and CDC42/RAC (35). Interestingly, few additional signaling pathways that can lead from GnRHR to MAPKs were identified in αT3-1 cells as well. Grosse et al. (36) showed that GnRHR signals to ERK by activating EGF receptors whereas Mulvaney et al. (37, 38) showed that ERK is activated in αT3-1 via calcium influx through L-type calcium channels and that JNK activation is PKC-independent but mediated by elevated intracellular calcium. On the other hand, Vasiliev et al. (39) showed that in L6/T4 cells short-term incubation with GnRH leads to induction of LHβ transcription, whereas continuous long-term incubation leads to repression of the LHβ transcription. We recently showed that in L6/T2, ERK, and JNK are involved in the expression of the LHβ subunit promoter (40). GnRHR was found to utilize as yet additional distinct intracellular signaling pathways to activate MAPKs. These include PKA (41), independent Gβγ subunits of the G12/13 proteins (42), and PDGFR receptor (36, 43, 44). Collectively, these results indicate that GnRHR can utilize several signaling pathways depending on cell types and under different conditions to execute a single intracellular effect. Therefore, GnRHR represents a novel model to study signaling transduction and the activation of MAPK cascades.

In the current study we used cocultures of GnRHR-expressing COS7 cells and T3-1 cells and found that both ERK and JNK were activated with similar kinetics to that found in αT3-1 cells, which implicates a mechanism that mediates this activation is src-dependent. Thus, in the GnRHR-expressing COS7 cells, GnRHR transmits its signals to MAPKs mainly by activating the EGF receptor, although a minor contribution was detected also for the dissociated Gβγ and β-arrestin. JNK activation by GnRHR in these cells is fully dependent on a sequential activation of c-Src and PI3K, which operate mainly downstream of the EGF receptor, but can be activated mildly also by Gβγ. On the other hand, ERK activation by GnRHR in these cells is fully mediated by the EGF receptor, which activates Ras directly or via c-Src. This activation is not dependent on the secretion of heparin binding (Hb)-EGF as shown for other GPCRs (45). Thus, in transfected COS7 cells GnRHR elicits a unique signaling system in that it places c-Src downstream of EGF receptor in the pathway that leads from GPCR to MAPK cascades.

**MATERIALS AND METHODS**

**Stimulants, Inhibitors, Antibodies, and Miscellaneous Reagents**—[o-Trp]-GnRH, a stable GnRH analog (GnRH-a), Genistein (PTK inhibitor), enolase, and protein AG-Sepharose, were obtained from Sigma Chemical. GF109203X, PD98059, SB203580, wortmannin, AG1478, Genistein, PP1, pertussis toxin, anti-pan-ras monoclonal antibody, and TPA were purchased from Calbiochem. Polyclonal anti-ERK (C16), anti-c-Src, anti-phosphooyrinosine (pY90), anti-phospho ERK (Tyr-1173), anti-EGRF, and anti-Hb-EGF (C-18) antibodies were from Santa Cruz Biotechnology, Inc. Monoclonal anti-diphospho (DP)-ERK and JNK antibodies (active-ERK and JNK), polyclonal anti-phospho S473 protein kinase B (PKB) antibody, and polyclonal anti-general ERK, JNK and PKB antibodies were from Sigma, Israel (Rehovot, Israel). Polyclonal anti-phospho-(Tyr-416)-Src antibody (active-anti Src) was purchased from New England Biolabs.

**Plasmids**—GnRHR (mouse) was cloned into pCDNA1 using the BamHI/XhoI sites. Human FAK and N-terminally truncated FAK (DN-FAK, FRNK, human) were cloned in pCDNA1 using the BamHI/XhoI sites. N-17 Ras in pCDNA1 and Csk-pRK5 were prepared as previously described (46). Mammalian expression vectors containing wild-type ras, dominant negative ras (K44A-dynamin), β-arrestin2 in pCMV5 and dominant negative β-arrestin (V54D-β-arrestin2) in pCDNA3 were a gift from Dr. M. Caron, Duke University. CD8-tagged β-AR was from Dr. Zvi Vogel (Weizmann Institute of Science). Dn-EGF receptor (KT21A) was provided by Dr. Y. Yarden (Weizmann Institute of Science).

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inhibitors (15 min, 37°C). Following stimulation, the cells were lysed in Ral buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 200 mM NaCl, 2.5 mM MgCl₂, and 1% Nonidet P-40) and centrifuged at 14,000 g for 10 min. The supernatant was subjected to immunoprecipitation with anti-PKB C-terminal antibody using protein A-agarose or protein G-agarose (20 µl). PKB activity was determined by the phosphorylation of histone H2B as described for MBP phosphorylation by ERK above.

c-Src Activity—Cell lysates (400–500 µg of protein in Buffer H containing 1% Triton X-100) were incubated with anti-c-Src-antibody coupled to protein A-Sepharose and mixed at 4°C. The immunocomplexes were washed once with RIPA, twice with 0.5 M LiCl in 0.1 M Tris-HCl, pH 8.0, and once with Buffer A. The washed immunoprecipitates were resuspended in a kinase assay buffer and the c-Src activity was determined using acid-denatured enolase (3 mM) as substrate in the presence of 20 µM [γ-32P]-ATP (8,000 cpm/pmol). The enzymatic reactions were terminated by the addition of sample buffer. The samples were then subjected to SDS-PAGE and autoradiography. Alternatively, the harvested fraction were separated by SDS-PAGE and subjected to Western blot analysis with anti-active c-Src antibody and anti-general c-Src antibody.

**FIG. 1.** Effect of various inhibitors on JNK1 activation by GnRH-a. A, phosphorylation of JNK. A plasmid containing mouse GnRHR was transfected into COS7 cells using the DE-dextran method. One plate was transfected with vector alone as control (Vec). This procedure routinely yielded >80% transfected cells as judged by control transfecion of plasmid containing green fluorescent protein. To ensure similar level of expression of the GnRHR in all plates, 16 h after transfection, the transfected cells were combined and split into smaller plates (6 cm) for an additional 10 h. The cells were serum-starved (Dulbecco’s modified Eagle’s medium + 0.1% fetal calf serum, 18 h) and then were either pretreated (15 min) with 200 µM Genistein (Gen), 3 µM GF109203X (GF), 25 nM wortmannin (Wort), 5 µM AG1478, or 5 µM PP1, or left untreated. GnRH-a (10⁻⁷ M, 10 min) was added to the pretreated, as well as to the untreated cells (5, 10, 30, and 60 min), or the cells were left untreated as a control (Vec). Phosphorylation of JNK1 was determined by Western blot with anti-DP-JNK antibody (α-DP-JNK). Both JNK1 and JNK2 were detected, but the results here represent only JNK1. The total amount of JNK1 was detected with the anti-JNK antibody (α-G-JNK). The results shown in the bar graphs are an average of two experiments. B, activation of JNK. The GnRHR-transfected COS7 cells were treated as in A. Activity toward GST-c-Jun-(1–91) was determined (Jun Phos.) as described under “Materials and Methods.” The site of p-GST-c-Jun-(1–91) is indicated. The total amount of JNK1 was detected with anti-JNK antibody (α-G-JNK). The results in the bar graph below are an average of two experiments.
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**FIG. 2.** Effect of various signaling components on JNK1 activation by GnRH. A, involvement of signaling components. COS7 cells were cotransfected with plasmid containing mouse GnRHR together with each of the following plasmids: K721A-EGF receptor (Dn-EGFR); CD8-tagged β-ARK (β-scaV); N-17 Ras (Dn-Ras); Csk-pIkK1 (Csk); β-arrestin2 (Arr); V54D-β-arrestin2 (Dn-Arr); dynamin (Dyn); K44A-dynamin (Dn-Dyn); human FAK (FAK); and N-terminally truncated FAK (Dn-FAK). Two days after transfection, the cells were serum-starved for 16 h and then either treated with GnRH-a (10⁻⁷ M; 10 min, +) or left untreated (−). Activated JNK1 was determined with anti-DP-JNK antibody (α-DP-JNK). The amount of total JNK1 was detected with anti-JNK antibody (α-JNK). The results shown in the bar graph represent percent activation of JNK1 obtained in the GnRH-a-stimulated cells that were cotransfected with GnRHR and vector control in each experiment. The results are the average of two experiments. B, GnRHR expression in transfected COS7 cells. The amount of transfected GnRHR was detected by an anti-GnRHR antibody.

**JNK1 Activation by GnRH-a—**GnRH signaling by GPCRs is mediated via several signaling pathways that vary among cell types and stimuli. GnRHR has been proven as a good tool in the study of GPCR signaling mechanism toward MAPK cascades (9). To determine the cell type specificity of GnRH signaling and to study the effect of various signaling inhibitors on this activation we used COS7 cells that do not express endogenous GnRHR. These cells were transfected with a plasmid containing mouse GnRHR that yielded a considerable amount of expression of the GnRHR in most cells as judged by Western blot analysis with anti-GnRHR antibody and expression of an unrelated green fluorescent protein (data not shown). To ensure similar level of expression of the GnRHR in all plates of any experiment, the transfected cells were combined and cut into smaller plates. The cells were serum-starved for 16 h prior to the stimulation, pretreated with various pharmacological inhibitors, and then stimulated with 10⁻⁷ M GnRH-a. The high yield of transfection in COS7 cells allowed detection of endogenous MAPK activation, without a significant background from non-transfected cells. Thus, when examined with anti-DP-JNK antibody, a gradual change in recognition by the antibody was observed in two endogenous bands at molecular masses of 48 and 54 kDa, which corresponded to JNK1 and JNK2, respectively. Since the relative amount of staining of the 48 kDa JNK1 was stronger than that of the 54 kDa JNK2, we demonstrate here only the results of JNK1. Thus, expression of GnRHR in the COS7 without an addition of GnRH-a did not change the activity of JNK1. However, elevation in JNK1 phosphorylation was detected already 5 min after stimulation with GnRH-a, peaked at 10–30 min after stimulation and declined thereafter (Fig. 1A). Pretreatment of the cells with the PTK inhibitor, Genistein as well as inhibitors of PI3K (wortmannin), EGF receptor (AG1478), and c-Src (PP1) but not the PKC inhibitor, GF109203X, significantly reduced the activation of JNK1 by GnRH-a. Similar results to those obtained with the anti-DP-JNK antibody were observed when the endogenous JNK activity was measured by an in vitro kinase assay (Fig. 1B). Again, Genistein, wortmannin, AG1478, and PP1 significantly prevented JNK activation by GnRH-a, while GF109203X had no inhibitory effect. This pattern of inhibition is markedly different from that obtained in αT3-1 cells where stimulation of JNK activity with GnRH-a was inhibited by GF109203X but not by wortmannin or AG1478 (Ref. 35 and data not shown), indicating that the pathway that leads to JNK activation by GnRH may differ in different cell lines.

**Involvement of EGF Receptor, βγ Dimer, c-Src, and β-Arrestin in JNK1 Phosphorylation by GnRH-a—**To study the possible involvement of additional signaling components in the GnRHR to JNK pathway and to confirm the involvement of components that were identified by the inhibitors above, we coexpressed GnRHR together with interfering mutants of various signaling components into COS7 cells. Serum starvation and treatment with GnRH-a were followed as described above. As expected from the inhibition with AG1478 and PP1, the dominant negative form of the EGF receptor as well as Csk, which inhibits the activity of c-Src, nearly abolished the activation of JNK1 by GnRH-a (Fig. 2A). β-Arrestin, which can serve as a mediator of signaling of GPCRs toward MAPKs (3, 26), seemed to play a minor role in the GnRHR-JNK signaling.
Although the wild-type β-arrestin had no significant effect on JNK1 activation by GnRH-a, the dominant negative form of this protein inhibited this activation by ~30%. Similar inhibition was exerted by CD8-tagged β-ARK, which acts as a scavenger for the dissociated βγ dimer (48). On the other hand, dominant negative Ras, as well as the wild type and the dominant negative forms of FAK and dynamin, did not seem to influence the studied pathway. Taken together, these results suggest a major role for EGF receptor, c-Src, and PI3K, and a minor role for β-arrestin and βγ dimer, in the pathway that links the GnRHR to JNK in the transfected COS7 cells. Other known signaling components such as PKC, FAK, dynamin, and Ras do not seem to be involved in this process. As seen in Fig. 2B, the amount of transfected GnRHR was roughly similar in all experiments and to the amount of the receptor in aT3-1 cells. This similarity was consistent in all the experiments.
(data not shown) indicating that the amount of receptors in the transfected COS7 cells was not too high, which make the results more reliable.

**ERK Activation by GnRH-a in COS7 Cells**—We then studied the mechanism of ERK activation by GnRH in transfected COS7 cells. As above, the cells were serum-starved and treated, and anti-DP-ERK antibody was used to detect the phosphorylation of ERK in its activation loop. As with JNK1, the expression of GnRHR in COS7 cells did not change the level of the regulatory phosphorylation of endogenous ERK1 and 2 (Fig. 3A). Addition of GnRH-a to the transfected cells resulted in a substantial phosphorylation of both ERK1 and ERK2, which peaked at 5 min after treatment, remained high for additional 25 min and declined 30 min later. Pretreatment of the cells with the PTK inhibitor Genistein, AG1478, and to some extent also with PPI, inhibited the GnRH-a-induced phosphorylation of ERK. On the other hand, treatment with the PI3K inhibitor wortmannin and the PFK inhibitor GF109203X had no inhibitory effect. Similar results were observed also when the endogenous ERK activity toward MBP was measured by an in vitro kinase assay (Fig. 3B). Again, ERK was transiently activated by GnRH-a with a peak at 5–10 min after stimulation. Genistein, and AG1478 completely prevented the GnRH-a-induced ERK activation, PPI inhibited this activation by ~35%, while GF109203X and wortmannin had no detectable inhibitory effect. As with JNK1, this pattern of inhibition is different from that obtained in T3-1 cells, where stimulation of ERK by GnRH-a was inhibited by GF109203X, but not by AG1478 (33). Thus, the pathway that leads to ERK activation by GnRH-a differs in the different cell lines. Moreover, the sensitivity of ERK activation to inhibitors in the transfected COS7 cells was different from the sensitivity of JNK to the same inhibitors.
This indicates that the pathway that leads to ERK activation by GnRH is different, at least in part, from the one leading to the JNK cascade.

To study the possible involvement of additional signaling components in the GnRHR to ERK pathway, we coexpressed the GnRHR together with interfering mutants of various signaling components in COS7 cells. As expected from the study using pharmacological inhibitors, the dominant negative form of EGF receptor significantly attenuated the phosphorylation of ERK1 and ERK2 upon GnRH-a treatment (Fig. 4). In addition, the expression of dominant negative Ras, which acts upstream of the ERK cascade in many systems (5) also caused a substantial reduction in GnRH-a-stimulated ERK phosphorylation. Csk, that inhibits c-Src activity, reduced the GnRH-stimulated phosphorylation of ERK by ~40%. As observed for JNK1, the dominant negative form of β-arrestin inhibited this activation by ~30%. On the other hand, the Gβγ scavenger CD8-β-ARK, as well as the wild type and the dominant negative forms of dynamin and FAK had no effect. Taken together, the results suggest a role for the EGF receptor, Ras, c-Src, and possibly also β-arrestin in the pathway that links the GnRHR to ERK in transfected COS7 cells. Other known signaling components such as PKC, FAK, dynamin, PI3K, and dissociated Gα do not seem to be involved in this process.

Ras Activation by GnRH-a Is Mediated by EGF Receptor c-Src—One of the most important mediators of signals to the ERK cascade is the small GTP-binding protein Ras. Therefore we used the Raf-RBD pull-down assay (49) in order to detect the Ras activation by GnRH. As expected, Ras was activated within 2 min after GnRH-a addition to the transfected COS7 cells, the activity peaked at 5 min and decreased slightly 5 min later. Interestingly, when the cells were cotransfected with dominant negative EGF receptor or preincubated with the EGF receptor inhibitor AG4178, both basal activity and GnRH-a-stimulated activity were almost completely abolished (Fig. 5A). On the other hand, cotransfection with Csk or preincubation with the c-Src inhibitor PP1 also caused reduction (50 ± 10%, Fig. 5B) in Ras activation, but their effect was significantly lower than that of the inhibitors of the EGF receptor. Therefore, it is reasonable to assume that EGF receptor is the main stimulator of Ras, where c-Src may mediate part of the EGF receptor-induced signal and the well established Grb-Sos pathway probably mediates the other part of the signal (9).

Role of EGF Receptor in the Mediation of GnRH Signals—Our results show that EGF receptor plays a central role in the transmission of GnRHR-initiated signals toward JNK and ERK. Another signaling component that is involved in this process is c-Src that seems to be a central player in the pathway that leads to JNK activation and participates to some extent also in the GnRHR-ERK pathway. It became important to study the activation of these two components and the interplay between them. Using both anti-pEGF receptor and anti-PY antibodies we showed that the EGF receptor was rapidly activated by GnRH (Fig. 6, A and B), and remained active for more than 60 min. This activation was not affected by inhibitors of c-Src, indicating that this PTK is not located upstream of the

Fig. 6. EGF receptor transactivation by GnRH. A and B, COS7 cells that were transiently transfected and serum-starved as described, were stimulated with GnRH-a (10^-7 M) for the indicated times and tested for their enhanced tyrosine phosphorylation using anti-phospho-EGF receptor (A) and anti-PY (B) antibodies. C, detection of Hb-EGF shedding in COS7 cells expressing GnRHR. Conditioned medium (CM) was collected and incubated with heparin-agarose to precipitate the Hb-EGF ectodomain. sHb-EGF secreted in the culture medium was detected by Western blot analysis using goat anti-Hb-EGF antibody. D, ERK activation in non-GnRHR-expressing COS7 cells treated with CM from GnRHR-treated GnRHR-expressing cells: COS7 cells were transfected as described, serum-starved for 16 h, and treated with GnRH-a (10^-7 M) for 5 and 10 min. CM from transfected cells (lanes 1–6) was collected and transferred to non-transfected COS7 cells (lanes 7–12) that did not express the GnRHR and further incubated for the indicated times. Non-transfected cells stimulated (5 min) with CM from untreated transfected cells or cells transfected with vector alone were used as controls (lanes 7 and 8). Phosphorylation of ERK was determined with the appropriate anti-DP and anti-general ERK antibodies.

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EGF receptor as suggested in several other systems (50, 51). A main mechanism for GPCR-induced activation of EGF receptor seems to be through activation of a membrane proteinase (e.g., MMP9), which in turn releases the membrane-bound Hb-EGF to activate the receptor (52). We examined the amount of Hb-EGF released to the medium upon activation of the GnRHR-transfected COS7 cells and found that it could not be detected in the first day after GnRH-a addition, but appeared in later time points after stimulation (Fig. 6C). In order to verify the lack of Hb-EGF, we used CM of GnRH activated COS7 cells to activate the MAPK in non-transfected COS7 cells as described by Pierce et al. (45). Indeed, the CM used did not induce any activation of ERK under any of the conditioned medium (Fig. 6D), indicating that the activation of EGF receptor is not mediated by Hb-EGF.

Activation of c-Src by GnRH-a Is Mediated Mainly by EGF Receptor—It became clear from the results above that c-Src is not localized upstream of the EGF receptor. We then undertook to find whether it may act downstream of this RTK in our system. Thus, addition of either GnRH-a or EGF to these cells triggered a sustained activation of c-Src (Fig. 7A) that was apparent already 2 min upon treatment. Moreover, addition of the EGF receptor inhibitor—AG1478, or cotransfection of the dominant negative GnRHR together with the EGF receptor inhibited most of the activation of c-Src by GnRH-a (Fig. 7B). Inhibition was also detected for EGF, but neither with the dominant negative forms of dynamin or β-arrestin (Fig. 7, C and D), nor with the PKC inhibitor GF109203X (data not shown). As activation of c-Src is mediated by a unique mechanism in which the EGF receptor, and not c-Src, is a signal via the dissociated G/Gp, PKC, and β-arrestin, the activation of c-Src by GnRH in transfected cells, and β-arrestin do not seem to participate in the activation of the GnRHR-expressing COS7 cells.

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**A**

Stim. (min) 0 2 5 10 15 p-Src

EGF GnRH

**B**

GnRH (min) 0 2 5 10 15 p-src

Vec Dn-EGFR Dn-Dyn Dn-Arr

**C**

GnRH (min) 0 10 10 0 10 0 10 0 p-Src

β scav Dn-Dyn Dn-Arr

**D**

c-Src activity (% of GnRH)

AG1478 Dr-EGFR P-Src DM

Fig. 7. Mechanism of c-Src activation by GnRH. A, COS7 cells were transfected with a plasmid containing mouse GnRHR. Thirty-two hours after transfection, the cells were serum-starved for 16 h, and one plate was pretreated with PP1 (5 μM, 15 min). Then the cells were stimulated either with EGF (50 ng/ml) or GnRH-a (10−7 M) for the indicated times. Phosphorylation of c-Src on Tyr-416 (activated c-Src) was detected with anti-phospho-Src antibody (p-Src). The amount of c-Src did not change throughout the experiment as detected by anti-c-Src antibody (Src). These results were reproduced twice. B, COS7 cells were cotransfected with mouse GnRHR together with either K721A-EGF receptor (Dn-EGFR) or with a vector control. One plate was transfected with vector alone (Vec). Thirty-two hours after transfection, the cells were serum-starved for 16 h after which one plate was pretreated with AG1478 (5 μM, 15 min). Then the cells were stimulated with GnRH-a (10−7 M for the indicated times) and c-Src activity toward denatured enolase was determined as described under “Materials and Methods.” The amount of immunoprecipitated c-Src was determined using anti-c-Src antibody (lower panel). The results were reproduced three times. C, COS7 cells were cotransfected with plasmid containing mouse GnRHR together with plasmids containing either CD8-tagged βARK (β ARK2); K44A-dynamin (Dn-Dyn); V54D-β-arrestin2 (Dn-Arr), or no insertion as control. Two days after transfection, the cells were serum-starved for 16 h and then either treated with GnRH-a (10−7 M; 10 min) or left untreated (0). Activation of c-Src was determined by Western blot analysis using anti-phospho c-Src antibody (p-Src) or anti-general Src antibody (Src). These results were reproduced twice. D, the amount of activated c-Src was determined by densitometry and plotted as a bar graph of percent activation from that of GnRH-a-stimulated cells that were cotransfected with GnRHR and vector control in each experiment. These are averages and S.E. of three experiments.
above that c-Src activation by GnRH in the transfected COS7 cells is mediated mainly by the EGF receptor, and is complemented by the dissociated /H9252/H9253 dimmer.

**Involvement of Gαi in GnRH-a-mediated Signaling**—Our results clearly show that EGF receptor is the main upstream mediator of GnRH signaling; however, it is not clear how this receptor is activated by the GnRHR. It is well established that in /H9251 T3-1 cells, GnRHR transmits its signal via G/H9251q and PLC/H9252 (9). However, in the GnRHR-transfected COS7 cells we did not find any role for PKC in the activation of MAPKs, suggesting the involvement of other G proteins. To test the possible involvement of other Ga isoforms we used pertussis toxin that serves as a selective inhibitor of Gαi (54). The serum-starved GnRHR-transfected COS7 cells were pretreated with the toxin for 5 h, after which they were stimulated with GnRH-a and the phosphorylation of JNK, ERK, c-Src, and EGF receptor was

**Fig. 8. Mechanism of activation of PI3K/PKB by GnRH-a.** A, COS7 cells were transfected with a plasmid containing mouse GnRHR. Thirty-two hours after transfection, the cells were serum-starved for 16 h, after which the plates were pretreated with either 200 μM Genistein (Gen), 3 μM GF109203X (GF), or 25 μM wortmannin (Wort). Then the cells were either stimulated with GnRH-a (10⁻⁷ M for the indicated times) or with the nonselective activator peroxovanadate (200 μM H₂O₂, 100 μM vanadate; VOOH). Cellular extracts of these cells were subjected to a Western blot analysis using either anti-phospho-PKB antibody (Ser-473; p-PKB) or anti-PKB antibody (PKB). These results were reproduced twice. B, COS7 cells were treated as in A and then subjected to immunoprecipitation with anti-PKB antibody followed by an in vitro phosphorylation reaction with histone H2B as a substrate (p-H2B). The phosphorylation was detected by autoradiography, and the amount of immunoprecipitated PKB was detected with anti-PKB antibody (PKB). These results were reproduced twice. C, COS7 cells were cotransfected with plasmid containing mouse GnRHR together with plasmid containing either Csk; K721A-EGF receptor (Dn-EGFR), or no insertion as vector control. Thirty-two hours after transfection, the cells were serum-starved for 16 h after which two plates were pretreated with the c-Src inhibitor PP1 (5 μM, 15 min). Then the cells were stimulated with GnRH-a (10⁻⁷ M for the indicated times) and phosphorylation of PKB was detected with either anti-phosphorylated PKB antibody (p-PKB) or with anti-PKB antibody (PKB). These results were reproduced three times. D, COS7 cells were cotransfected with plasmid containing mouse GnRHR together with plasmids containing either CD8-tagged ARK (scav); K44A-dynamin (Dn-Dyn); or V54D-β-arrestin2 (Dn-Arr); or no insertion as vector control. Two days after transfection, the cells were serum-starved for 16 h and then either treated with GnRH-a (10⁻⁷ M; 30 min) or left untreated (0). Activation of PKB was determined by Western blot analysis as in C. The results were reproduced twice. E, activation of PKB was determined by densitometry and plotted as a bar graph of percent activation from that of GnRH-a-stimulated COS7 cells that were cotransfected with GnRHR and vector control in each experiment. These are averages and S.E. of three experiments.
examined using the appropriate antibodies. We found that the GnRH-a-stimulated phosphorylation of all these signaling components was significantly inhibited by the pertussis toxin (Fig. 9). No effect of the pertussis toxin on the amount of the MAPKs, c-Src, or EGF receptor or the viability of the transfected COS7 cells was observed in the course of this experiment. Therefore, the results suggest that Goi is a main intermediate in the GnRHR to MAPKs signaling pathway in COS7 cells, although additional components may be involved in the pathway to JNK. These results are best explained by a model in which ERK activation is mediated mainly by the EGF receptor and Ras, with some involvement of c-Src but not of PI3K, while JNK activation is mediated by Goi, EGF receptor, and PI3K (Fig. 10). β-arrestin appears to be involved in the activation of both cascades and to operate via an independent pathway. On the other hand, other signaling molecules such as FAK, dynamin,
and PKC do not seem to be involved in the GnRH-MAPK signaling in GnRHR-expressing COS7 cells.

**DISCUSSION**

The receptor for GnRH serves as a good model in GPCR signaling toward MAPKs (9). Here we describe signaling processes that lead from GnRHR activation to MAPK pathways in GnRHR-transfected COS7 cells. This was identified in these cells differ significantly from the signaling mechanisms identified in the pituitary-derived αT3-1 cells. The mechanisms involved in MAPK activation by GnRH reported (32) for other GPCRs activation of the MAPK cascades or in cooperation with additional signaling components (33). The three pathways do not seem to contradict, but rather to complement each other to form a signaling network, which is essential to achieve the full GnRH effect on MAPKs. Furthermore, it is tempting to suggest that changes in the frequency of GnRH pulses or in the condition of the cells might recruit selective signaling components, and thus change their relative contribution to the MAPK activation. Notably, Gαi and PKC are central components in the signaling network initiated by GnRHR in the αT3-1 cells (9). However, these components do not seem to play a role in the GnRHR-transfected COS7 cells, where Gαi and EGF receptor seem to play the major role, with a smaller contribution by β-arrestin and to some extent also the dissociated βy dimers (Fig. 10).

Interestingly, under different experimental systems, GnRHR was found to utilize as yet additional distinct intracellular signaling pathways to activate MAPKs. Thus, in GGH3 cells, ERK activity is mediated by PKA as well as by PKC (41); in Caov-3 cells, GnRHR operates via Gαi as well as by independent Gβγ of the Gα/α3 proteins (42), in L/β2T2 cells GnRH activation of JNK is not dependent on PKC (37), and in immortalized GT1-7 neurons it signals through PKC that transactivate EGF receptor (43, 44). Collectively, these results indicate that GnRH can utilize several signaling pathways in different cell types and under different conditions to execute a single intracellular effect.

Additional variability in the mechanism of GnRHR signaling was detected even within a particular cell line. We demonstrate here the involvement of Gαi, EGF receptor, and to some extent also of c-Src and β-arrestin in the activation of ERK. On the other hand, Grosse et al. (36), provided evidence for the involvement of Gαi and PKC in the same pathway and the same cells. Grosse et al. (36) also showed that GnRH signals to ERK is mediated by EGF receptor in αT3-1 cells, while we found no role for EGF receptor in this process in the same cells (33). Moreover, Mulvany and Roberson (38) showed that JNK activation in αT3-1 cells is mediated by elevated intracellular Ca²⁺, whereas our results (35) show that PKC plays an important role for GnRH-MAPK signaling to JNK in these cells. The discrepancies between the results are correct for the same reasons. We modified under different growth conditions to execute a single intracellular effect under varying experimental conditions.

We show here the involvement of both Gαi and EGF receptor in the transduction of GnRH signals. These two components seem to be important in the signaling of many GPCRs (9). The mechanism by which Gαi induces the activation of the EGF receptor seems to vary in different systems. This activation was shown to be mediated by c-Src, by the GPCR itself, by the dissociated Gβγ, by the release of Hb-EGF, and more (45). The system examined here seems to be unique in that the EGF receptor is activated in a mechanism that does not involve any of the above mechanisms and is probably mediated directly by the dissociated Gαi, upon the engagement of the GnRHR with its ligand.

In αT3-1 cells for more than three months in culture modulates their signaling properties in that the signaling is dependent on PKC for the activation of ERK (data not shown). We also found that appropriate serum-starvation for a minimal time of 14 h is necessary to obtain reproducible signaling results in both cells, probably because this time is required for a complete removal of MAPK phosphatases (data not shown). Thus, GnRH may utilize more than one pathway to transmit its signals in different cells and under different conditions. The pathways used are probably dependent on the repertoire of signaling components that are available to the receptor under each growing condition. Despite of the different signaling pathways, the downstream processes that are activated, such as the MAPK cascades or even gene expression, are remarkably similar. This can be explained by the centrality of the MAPK cascades that can receive input from various sources, and direct them to their right destination under varying experimental conditions.

Fig. 10. Schematic representation of GnRH signaling toward the MAPK cascades in transfected COS7 cells. Broken lines indicate an indirect or weak activation, and a solid line indicates a direct activation. Arr, β-arrestin.

Note: The text is not shown due to the overlay of the watermark.
Mechanisms of JNK and ERK Activation by GnRH Receptor

To the activation of JNK and ERK by GnRH in several cellular systems. We have previously shown that in α2β1 cells, c-Src is activated via a mechanism that is partially (~70%) dependent on PKC (35), but may involve also dynamin (33). However, this is clearly not the situation here, since the selective PKC inhibitor GF109203X had no influence on the activation of c-Src, ERK or JNK upon GnRH stimulation. Instead, the activation of c-Src observed here is mainly dependent on the activation of the EGF receptor (Fig. 7). In addition, the dissociated Gβγ slightly contributes to c-Src activation similar to other systems (2), but the receptor itself, β-arrestin or dynamin does not influence this process. To our knowledge this is the first demonstration of a direct c-Src activation by RTKs upon stimulation by GPCRs. Hence, c-Src activation by GPCRs can occur not only before but also through transactivation of RTKs in different cellular systems.

PI3K is another key player in GPCR signaling (67), and it has been recently implicated in the control of cell growth, survival, and malignant transformation (68). Several mechanisms of PI3K activation by GPCRs have been elucidated over the past few years (9). These include activation of β-arrestin-mediated signals shown here. PI3K is another key player in GPCR signaling (67), and it may be activated by a PKC-dependent mechanism (61), via a dissociated βγ dimer (62), or by a direct interaction with the α subunit of G proteins (63). Interestingly, activation of c-Src may be mediated also by its recruitment of this protein kinase to the GPCRs themselves, and this can occur by a direct interaction of the c-Src with proline-rich motifs in the GPCR (64), or by an interaction with the scaffold protein β-arrestin, which often interacts with GPCRs (65, 66). These interactions usually occur specifically through the SH3 domain of c-Src, and often lead to the c-Src activation.

C-Src is involved in the activation of MAPKs by GnRH in several cellular systems. We have previously shown that in α2β1 cells, c-Src is activated via a mechanism that is partially (~70%) dependent on PKC (35), but may involve also dynamin (33). However, this is clearly not the situation here, since the selective PKC inhibitor GF109203X had no influence on the activation of c-Src, ERK or JNK upon GnRH stimulation. Instead, the activation of c-Src observed here is mainly dependent on the activation of the EGF receptor (Fig. 7). In addition, the dissociated Gβγ slightly contributes to c-Src activation similar to other systems (2), but the receptor itself, β-arrestin or dynamin does not influence this process. To our knowledge this is the first demonstration of a direct c-Src activation by RTKs upon stimulation by GPCRs. Hence, c-Src activation by GPCRs can occur not only before but also through transactivation of RTKs in different cellular systems.

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c-Src Is Activated by the Epidermal Growth Factor Receptor in a Pathway That Mediates JNK and ERK Activation by Gonadotropin-releasing Hormone in COS7 Cells

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