Matrix Metalloproteinases 2 and 9 Mediate Epidermal Growth Factor Receptor Transactivation by Gonadotropin-releasing Hormone*

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Rapid engagement of the extracellular signal-regulated kinase (ERK) cascade via the Gq coupled GnRH receptor (GnRHR) is mediated by transactivation of the epidermal growth factor receptor (EGFR). Here we show that the cross-talk between GnRHR and EGFR in gonadotrope cells is accomplished via gelatinases A and B (matrix metalloproteinases (MMPs) 2 and 9), identifying gelatinases as the first distinct members of the MMP family mediating EGFR transactivation by G protein-coupled receptors. Using a specific MMP2 and MMP9 inhibitor, Ro28-2653, GnRH-dependent EGFR transactivation was abrogated. Proving the specificity of the effect, transient transfection of αT3-1 cells with ribozymes directed against MMP2 or MMP9 specifically blocked EGFR tyrosine phosphorylation in response to GnRH stimulation. GnRH challenge of αT3-1 cells furthered the release of active MMP2 and MMP9 and increased their gelatinolytic activities within 5 min. Rapid release of activated MMP2 or MMP9 was inhibited by ribozyme-targeted down-regulation of MT1-MMP or MMP9, respectively. We found that GnRH-induced Src, Ras, and ERK activation were also gelatinase-dependent. Thus, gelatinase-induced EGFR transactivation was required to engage the extracellular-signal regulated kinase cascade. Activation of c-Jun N-terminal kinase and p38 MAPK by GnRH was unaffected by EGFR or gelatinase inhibition that, however, suppressed GnRH induction of c-Jun and c-Fos. Our findings suggest a novel role for gelatinases in the endocrine regulation of pituitary gonadotropes.

While the effect of receptor/G protein systems in sensory transduction and maintenance of cell homeostasis have been appreciated for quite some time, it has only recently been fully realized that heptahelical receptors play an important role in cellular growth, differentiation, and even transformation (1, 2). An intensive cross-talk between different classes of receptors, e.g. GPCRs and RTKs such as platelet-derived growth factor receptor or EGFR, give rise to a wide range of cellular responses (3). Transactivation of the EGFR has been shown to be a common phenomenon of GPCR-dependent signal transduction (4). Our mechanistic understanding of this kind of receptor cross-talk as well as its biological importance, however, are still rather vague.

An attractive model providing a mechanistic explanation for EGFR activation via GPCRs is based on regulated proteolytic release of local EGF-like ligands from transmembrane precursors (5). Secreted agonists are then thought to activate vicinal EGFRs after binding to the extracellular receptor domain (4). EGFR-related growth factors like amphiregulin, transforming growth factor-α, betacellulin, HB-EGF, and epiregulin are synthesized as membrane spanning pro-growth factors and are released through proteolytic processing. Enzymes that have been implicated in ectodomain shedding of growth factors mainly belong to two families: matrix metalloproteinases (MMPs) and metalloprotease-disintegrin proteins (MDCs/ADAMs) (3, 6).

MMPs are a group of zinc- and calcium-dependent enzymes that regulate cell-matrix composition by degrading components of the extracellular matrix. MMPs are synthesized as zymogens with a signal sequence and a propeptide segment being removed during activation. After activation, MMPs are secreted into the extracellular medium, except for the membrane type (MT)-MMPs, which are tethered to the cell surface by a hydrophobic transmembrane domain (7). Because GPCR-induced EGFR transactivation is sensitive to pretreatment of cells with the broad-spectrum MMP inhibitor batimatstat, a cardinal role of MMPs in the receptor cross-talk has been invoked (5). However, the identity of the participating MMPs, their activation mechanism, as well as their physiological relevance for GPCR-initiated signaling processes still remain elusive.

ADAMs are transmembrane proteins characterized by a zinc-dependent metalloproteinase, an adhesion domain, a fusion domain, and an intracellular signaling domain (8). Recently, ADAM9 MDC9/meltrin-γ has been identified as the protease responsible for the shedding of HB-EGF precursor in response to PKC activation (9). However, GPCR-stimulated HB-EGF

factor receptor; ERK, extracellular signal-regulated kinase; FGF-BP, fibroblast growth factor-binding protein; GnRH, gonadotropin-releasing hormone; HB-EGF, heparin-binding epidermal growth factor; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MT1-MMP, membrane type MMP 1; PKC, protein kinase C; Rz, ribozyme; TIMP, tissue inhibitor of matrix metalloproteinase; MT, membrane type; GST, glutathione S-transferase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PEI, polyethylami-
cleavage cannot be suppressed by dominant-negative forms of ADAM9, indicating that yet another protease mediates EGFR activation in response to GPCR signaling.

In the pituitary gland, the decapetide GnRH interacts with a heptahelical receptor to regulate the synthesis and secretion of the gonadotropins leutinizing hormone and follicle stimulating hormone and to secure the maintenance of the gonadotropic phenotype (10). In transfected COS-7 cells and αT3-1 cells derived from the gonadotrope lineage, the GnRH initiates multiple signaling pathways by exclusively coupling to G_{q/11} proteins (11). In αT3-1 cells, all four subfamilies of MAPKs, i.e. ERKs, JNK/SAPK, p38 MAPK, and big MAPK (BMK1/ERK5) (12), are activated upon GnRH stimulation (13), and ERK activity is required for GnRH-induced expression of the common α-subunit gene (14) through a pathway critically involving PKC (15, 16). We have recently shown that GnRHR/G_{q/11}-coupling leads to rapid PKC-dependent ERK activation requiring functional Ras. Additionally, the kinetics and maximal response of ERK activation critically depends on EGFR transactivation (17). However, the exact mechanism underlying GnRH-induced EGFR activation as well as its consequences for transcriptional responses in gonadotropes remain unknown.

Here we dissect the signal transduction pathway underlying GnRH-induced EGFR activation in αT3-1 and LβT2 cells. By applying a pharmacological approach with a set of specific MMP inhibitors as well as ribosome-mediated down-regulation of MMP gene expression, we show that MMP2 and MMP9 (gelatinases A and B, respectively) become activated within 5 min after GnRH challenge leading to EGFR transactivation. We provide initial evidence that MT1-MMP is involved in this process in αT3-1 cells. Furthermore, gelatinase-evoked EGFR activation is of cell physiological relevance because it is indispensable for the induction of immediate early response genes c-fos and c-jun by GnRH. Thus, apart from defining a role of gelatinases for the endocrine regulation of gonadotropic cells, we describe a novel signaling concept, namely the activation of gelatinases by G_{q/11}-coupled receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**

The cell culture supply was purchased from PAA (Coelbe, Germany). Ro28-2653 and batimatostat were provided by H.-W. Krell, Roche Diagnostics GmbH, Pharma Research (Penzberg, Germany). AG1478, PP2, phorbol ester, and gelatin were from Calbiochem (San Diego, CA). Anti-phosphotyrosine antibody (4G10) was supplied from Upstate Biotechnology (Wolwater Mill South, United Kingdom). Anti-phospho-EGFR (V30), and anti-phospho-Src (Y418) antibodies were from BIOSOURCE International (Camarillo, CA). Anti-EGFR- (sc-03), anti-Src- (Sc2), anti-c-Fos- (4), and anti-ERK1/2 antibodies (K23) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP2 (SA-103) and anti-MMP9 antibodies (SA-106) were purchased from Biomol (Plymouth Meeting, PA). Anti-MT1-MMP antibody was from Chemicon International (Temecula, CA). Anti-Ras antibody (pan-ras) was from Oncogene Research Products (San Diego, CA). Anti-c-Jun antibody was from Transduction Laboratories (Lexington, KY). Anti-phospho-ERK antibody was obtained from New England Biolabs (Beverly, MA). Anti-phospho-JNK antibody was from Promega (Mannheim, Germany). Neutralizing anti-HB-EGF antibody was obtained from R&D Systems (Minneapolis, MN). Glutathione-Sepharose 4B, nitrocellulose membranes, and the enhanced chemiluminescence system (ECL) were from Amersham Biosciences. RNA transfection was performed using jetPEI (polyethylenimine) from Polytransfection (Illkirch, France). Ribozymes were purchased from Genet-Oligos (Paris, France). All other reagents were obtained from Sigma.

**Methods**

**Tissue Culture**—αT3-1 cells and LβT2 cells were kind gifts of P. L. Mellon (18, 19). PC-3 cells were from the American Type Culture Collection. αT3-1 cells were grown in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml glutamine. LβT2 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml l-glutamine. Gelatinase activity was visualized as clear areas in a Coomassie Blue-stained gel. Gelatinolytic activity was visualized as clear areas in a Coomassie Blue-stained gel. Gelatinolytic activity was visualized as clear areas in a Coomassie Blue-stained gel. Gelatinolytic activity was visualized as clear areas in a Coomassie Blue-stained gel.

To identify distinct proteolytic enzymes involved in GnRH-mediated EGFR transactivation, we first chose a pharmacological approach. Because members of the gelatinase subfamily of MMPs (MMP2 and MMP9) are expressed in pituitary tumors and normal pituitary glands (24–26), we initially focused on a potential involvement...
of MMP2 and MMP9 in GnRH-dependent signal transduction in gonadotropes.

Prior to GnRH challenge, αT3-1 cells were incubated with Ro28-2653, a novel pyrimidine-2,4,6-trion-based MMP inhibitor characterized by a high selectivity for MMP2 and MMP9 (27–29). Subsequent to agonist stimulation, the EGFR was immunoprecipitated and Western blots of immunoprecipitates were probed with an anti-phosphotyrosine antibody. As shown
GnRH transactivated the EGFR in a Ro28-2653-sensitive manner implicating a role for gelatinases in the cross-talk between GnRHR and EGFR. The unspecific MMP inhibitor batimastat (BB-94) had only a minor inhibitory effect on GnRH-mediated EGFR activation in aT3-1 cells at the concentration tested (10 μM). We have previously shown that GnRH-induced ERK activation in aT3-1 cells is preceded by EGFR transactivation (17). Because ERK activation in these cells is dependent on Src activity, we chose antibodies specific for EGFR\textsuperscript{Tyr845} to detect activated EGFR. Tyrosine 845 within the catalytic domain of the EGFR has been shown to be phosphorylated by Src and is involved in regulating receptor function (30). GnRH challenge led to phosphorylation of tyrosine 845 of the EGFR, which was inhibited by preincubation of the cells

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**Fig. 2.** GnRH-mediated gelatinase activation is inhibited by Ro28-2653 as well as targeting gelatinases by ribozymes and MT1-MMP is activated in a GnRH-dependent manner in aT3-1 cells. A, serum-starved aT3-1 cells were preincubated with 10 μM Ro28-2653 for 30 min and subsequently stimulated with 1 μM GnRH for 5 min. Culture media were collected and concentrated. Western blots of samples containing 15 μg of protein were probed with an anti-MMP2 antibody (upper panel) or an antibody directed against MMP9 (lower panel). B, aT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12), or FGF-BP (Rz-FGF-BP). After serum depletion, cells were stimulated with 1 μM GnRH for 5 min. Culture media were collected and concentrated. Western blots of samples containing 15 μg of protein were probed with an anti-MMP2 (upper panel) or an anti-MMP9 (lower panel) antibody. C, serum-starved aT3-1 cells were preincubated with 10 μM Ro28-2653 for 30 min and subsequently stimulated with 1 μM GnRH for 5 min. Culture media were collected and concentrated. Samples containing 15 μg of protein were subjected to gelatin zymography. Molecular weight markers are indicated at the right. D, serum-starved aT3-1 cells were stimulated with 1 μM GnRH for 5 min. Western blots of cell lysates (upper panel) and concentrated culture medium containing 15 μg of protein (lower panel) were probed with an anti-MT1-MMP antibody. E, aT3-1 cells were transfected with ribozymes directed against MT1-MMP (Rz-MT1-MMP). After serum depletion, cells were stimulated with 1 μM GnRH for 5 min. After collection and concentration of culture media, Western blots of samples containing 15 μg of protein were probed with an anti-MMP2 antibody. F, upper panel, aT3-1 cells were transiently transfected with ribozyme Rz-MT1-MMP. After serum starvation, cells were stimulated with 1 μM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phospho-EGFR antibody. Lower panel, blots were reprobed with an anti-EGFR antibody.
with Ro28-2653 (Fig. 1B). Batimastat treatment had only a less pronounced effect (Fig. 1B). Ro28-2653 or BB-94 treatment of unstimulated cells did not reduce the basal phosphorylation status of the EGFR. GnRH-induced tyrosine phosphorylation of EGFR was almost completely prevented by pretreatment of cells with 1,10-phenanthroline, a chelator of bivalent ions such as zinc (data not shown), thus lending additional support to the involvement of zinc-dependent endopeptidases. As a control, the effect of Ro28-2653 and batimastat on EGF-mediated EGFR activation in L929 cells was examined. As expected, EGF increased EGFR phosphorylation in a Ro28-2653- and batimastat-independent way (data not shown). Batimastat has been reported to ablate bombesin-induced EGFR transactivation in PC-3 prostate cancer cells (5). To control for the activity of the batimastat solution used, PC-3 cells were pretreated with batimastat and subsequently stimulated with bombesin. Batimastat abrogated bombesin-induced EGFR phosphorylation (data not shown) demonstrating that the observed weak inhibitory property of batimastat (Fig. 1, A and B) was a characteristic feature of αT3-1 cells. These results indicate a predominant role of gelatinases for GPCR-induced EGFR tyrosine phosphorylation in gonadotropic cells.

To unequivocally define the role of gelatinases for the activation of EGFR by GnRH, an independent specific ribozyme-based approach was applied. αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), and, as controls, against MMP12 (Rz-MMP12) or an unrelated fibroblast growth factor-binding protein (Rz-FGF-BP). MMP12, human macrophage elastase belonging to the MMP subgroup of stromelysins, was targeted because Ro28-2653 inhibits MMP12 apart from MMP2 and MMP9 (28). Beforehand, the kinetics of ribozyme-induced down-regulation of MMP2 and MMP9 expression in αT3-1 cells were studied. Cells were incubated with ribozymes for 6 h, 12 h, 18 h, and 24 h. Incubation of αT3-1 cells with ribozymes for 6 h resulted in maximal suppression of GnRH-mediated EGFR phosphorylation, whereas longer incubation times had no further inhibitory effect (data not shown). Basal values did not change significantly in cells transfected with either ribozyme compared with non-transfected cells (Fig. 1C). The most prominent inhibitory effect was observed when αT3-1 cells were transfected with ribozyme Rz-MMP2 (Fig. 1C). GnRH-mediated EGFR activation was also substantially blocked in cells transfected with ribozymes directed against MMP9. However, GPCR-induced EGFR phosphorylation was nearly unaffected in cells transfected with ribozyme Rz-MMP12. As a negative control, αT3-1 cells were transiently transfected with a ribozyme directed against FGF-BP, because
this binding protein is not involved in the receptor cross-talk under study. As expected, cells transiently expressing Rz-FGF-BP were not affected with regard to GnRH-mediated EGFR tyrosine phosphorylation (Fig. 1C).

We further extended our study to another gonadotropic cell line, L929 cells. L929 cells express both the α- and β-subunit of luteinizing hormone as well as GnRHR (18). L929 cells were pretreated with the gelatinase inhibitor Ro28-2653 or the EGFR-specific tyrosphostin AG1478 prior to GnRH treatment and activation of EGFR was analyzed with an antibody recognizing phosphorylation of tyrosine 845. Pretreatment of cells with Ro28-2653 resulted in a decline of EGFR phosphorylation to basal levels. Furthermore, phosphorylation of EGFR was completely blocked by AG1478 pretreatment (Fig. 1D).

To investigate whether gelatinases are activated in a GnRH-dependent manner, conditioned αT3-1 cell culture supernatants were concentrated, resolved by SDS-PAGE, and probed with anti-MMP2- or anti-MMP9 antibodies. A 5-min GnRH challenge resulted in enhanced release of MMP2 (Fig. 2, A and B, upper panels). Preincubation of αT3-1 cells with Ro28-2653 decreased the MMP2 contents of the medium as did transfection with ribozymes targeting MMP2 (Fig. 2, A and B, upper panels). On the contrary, transfection of cells with Rz-MMP9, Rz-MMP12, or Rz-FGF-BP had no impact on MMP2 levels (Fig. 2B, upper panel). GnRH stimulation also led to an increase in MMP9 protein levels in the medium (Fig. 2, A and B, lower panels). The release of MMP9 was counteracted by preincubation of cells with Ro28-2653 (Fig. 2A, lower panel) or transfection with Rz-MMP9 (Fig. 2B, lower panel). Targeting MMP2 via Rz-MMP2 entailed a decrease in MMP9 release (Fig. 2B, lower panel) potentially indicating a role for MMP2 in MMP9 activation (31). MMP12 or FGF-BP do not govern MMP9 activation because down-regulation of these proteins did not inhibit MMP9 activation (Fig. 2B, lower panel).

To directly show that the enzymatic activity of gelatinases is subject to hormonal regulation in αT3-1 cells, cell culture media were analyzed by gelatin zymography (Fig. 2C). Under all conditions tested, we observed a gelatinolytic 72-kDa band corresponding to the proform of MMP2. A additional band migrating with a molecular mass of ~66 kDa was detected after GnRH stimulation of cells and represents the active form of MMP2. Activated MMP2 was not demonstrable in conditioned media of cells pretreated with Ro28-2653. A gelatinolytic band with a molecular mass of ~92 kDa corresponding to pro-MMP9 was noted in all samples. Yet, release of activated MMP9, which has a molecular mass of ~68 kDa, subsequent to GnRH treatment could not be readily observed (Fig. 2C).

MT1-MMP plays a prominent role in the activation of gelatinases. MT1-MMP is anchored to the plasma membrane tethering secreted pro-MMP2 in a complex also containing TIMP2. As a result of activating stimuli, pro-MMP2 is cleaved and activated by MT1-MMP (7, 32). Along these lines, αT3-1 cells were stimulated with GnRH and MT1-MMP was detected in cell lysates as well as concentrated conditioned medium (Fig. 2D). As shown in Fig. 2D, upper panel, a 5-min stimulation of αT3-1 cells with GnRH resulted in a decrease of MT1-MMP in cell lysates, whereas agonist challenge led to secretion of MT1-MMP into the cell medium (Fig. 2D, lower panel).

To further show that MT1-MMP is not only rapidly activated by GnRH stimulation but also is responsible for MMP2 activation (7), MT1-MMP was targeted with Rz-MT1-MMP and the protein level of MMP2 was detected in conditioned medium of αT3-1 cells 5 min after GnRH challenge (Fig. 2E). Rz-MT1-MMP transfection reduced GnRH-mediated MMP2 levels in conditioned medium of αT3-1 cells (Fig. 2E). Finally, the involvement of MT1-MMP in GnRH-mediated EGFR phosphorylation was investigated. αT3-1 cells were transfected with ribozyme Rz-MT1-MMP and the phosphorylation status of EGFR was analyzed after GnRH challenge. Induction of EGFR phosphorylation by GnRH was markedly suppressed in cells transfected with Rz-MT1-MMP (Fig. 2F).

ADAM10, ADAM12, and ADAM17/TACE belonging to the disintegrin family of proteases have been shown to participate in RTK transactivation (33–35). Experiments using a specific ADAM17/TACE inhibitor revealed ADAM17/TACE as not being involved in the cross-talk between the GnRHR and the EGFR in gonadotropic cells (data not shown). Reverse transcriptase-PCR of total αT3-1 RNA using primer pairs for ADAM10 and ADAM12 revealed ADAM10 being expressed in this gonadotropic cell line. Because down-regulation of ADAM10 by ribozyme targeting did not have any influence on GnRH-mediated EGFR phosphorylation (data not shown), we conclude that ADAM10 does not participate in the GnRHR/EGFR cross-talk in αT3-1 cells.

In summary, our data show that the cross-talk between GnRHR and EGFR proceeds via MT1-MMP and gelatinases. Furthermore, we provide evidence that MT1-MMP is involved in MMP2 activation in αT3-1 cells.

GnRH-mediated Src Activation in αT3-1 Cells Depends on Gelatinase Activity—We have recently shown that inhibition of Src activity suppresses GnRH-mediated GFP loading of Ras and subsequent ERK activation in αT3-1 and transfected COS-7 cells (17). To directly address the role of Src kinases in GnRH-induced EGFR transactivation in αT3-1 cells, Src kinase activity was blocked by the specific Src inhibitor PP2. As shown in Fig. 3A, preincubation of cells with PP2 resulted in diminished GnRH-mediated EGFR tyrosine phosphorylation. Likewise, pretreatment of cells with AG1478 abrogated EGFR

![Fig. 4.](image-url)
with an anti-ERK1/2 antibody. Lower panels, blots were reprobed with an anti-ERK1/2 antibody. A, upper panels, serum-starved αT3-1 cells were pretreated with 10 μM Ro28-2653, 10 μM BB-94, or 100 nM AG1478 for 30 min and stimulated with 1 μM GnRH (A) or 1 μM TPA (B) for 5 min. Western blots of cell lysates were probed with an anti-phospho-ERK antibody. Lower panels, blots were reprobed with an anti-ERK1/2 antibody. C, upper panel, αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12), or FGF-BP (Rz-FGF-BP). Serum-depleted cells were stimulated with 1 μM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phospho-ERK antibody. Lower panel, blots were reprobed with an anti-ERK1/2 antibody. D, upper panel, serum-starved LβT2 cells were pretreated with 10 μM Ro28-2653 or 100 nM AG1478 for 30 min and subsequently stimulated with 1 μM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phospho-ERK antibody. Lower panel, blots were reprobed with an anti-ERK1/2 antibody.
ing to ERK can be fully mimicked by PKC activation induced by short term TPA pretreatment. TPA-elicited Ras activity was substantially diminished in cells preincubated with Ro28-2653 (Fig. 4B). Likewise, inhibition of EGFR tyrosine kinase activity by AG1478 reduced the amount of precipitated Ras proteins to basal levels. On the contrary, pretreatment of cells with batinastat did not adversely affect TPA- mediated GTP loading of Ras. To summarize, our data demonstrate that MMP2 and MMP9 mediate GnRH activation of EGFR resulting in the engagement of a Ras signaling pathway in αT3-1 cells.

GnRH-induced ERK Activation Depends on Gelatinase Activity in Gonadotropic Cells—To further assess whether gelatinase-mediated EGFR transactivation is a necessary intermediate step to engage signaling pathways downstream of Ras, we examined the role of MMP2 and MMP9 in GnRH-mediated ERK activation. Gonadotropic αT3-1 cells were incubated with Ro28-2653, batimastat, or AG1478 prior to GnRH or TPA challenge. Activated ERK1 and ERK2 were detected by Western blotting with a phosphorylation-specific anti-p42/p44 (ERK1/ERK2) antibody (Fig. 5, A and B). GnRH-elicited ERK activation was almost completely blocked after pretreatment of cells with Ro28-2653, whereas 10 μM batimastat had no inhibitory consequence (Fig. 5A). EGFR tyrosine kinase activity is required for GnRH-dependent engagement of the ERK cascade, because ERK phosphorylation was found to be substantially reduced upon preincubation of cells with AG1478 (Fig. 5A). According to our previous findings in transfected COS-7 cells that stimulation of ERK activity by GnRH is mediated by PKC, short term TPA challenge of αT3-1 cells resulted in ERK phosphorylation (Fig. 5B). TPA-elicited ERK activation was reduced to basal levels in Ro28-2653-treated cells (Fig. 5B). Incubation of cells with the broad-spectrum MMP inhibitor batimastat did not yield an inhibitory effect on TPA-induced ERK phosphorylation (Fig. 5B). However, inhibition of EGFR tyrosine kinase activity by AG1478 suppressed ERK activation (Fig. 5B).

To strengthen the notion that gelatinases are specifically involved in GnRH-induced ERK activation in αT3-1 cells, the expression of distinct MMPs was targeted by transfection of ribozymes and agonist-induced ERK activation was monitored (Fig. 5C). Cells transfected with Rz-MMP2 or Rz-MMP9 displayed a significant decline in GnRH-induced ERK phosphorylation, whereas transient expression of Rz-MMP12 or the unrelated Rz-FGF-BP did not interfere with GnRH-dependent ERK activation. In summary, these data provide strong support for the concept that GnRH-mediated ERK activation is because of gelatinase-sensitive EGFR transactivation in αT3-1 cells. In LβT2 cells, GnRH-elicited ERK phosphorylation was blocked to basal levels in cells pretreated with Ro28-2653 as well as AG1478 (Fig. 5D).

HB-EGF Shedding Is Involved in GnRH-mediated ERK Activation—Each of the known EGF-like mammalian gene products is synthesized as a transmembrane precursor protein subject to proteolytic cleavage of its ectodomain to release a soluble growth factor. To identify the pertinent EGFR ligand involved in EGFR transactivation in gonadotropes, αT3-1 cells were treated with neutralizing antibodies against HB-EGF. Scavenging of soluble HB-EGF with neutralizing antibodies completely prevented GnRH-elicited EGFR tyrosine phosphorylation (Fig. 6A). To test whether liberated HB-EGF serves to induce signaling pathways downstream of the EGFR, lysates of αT3-1 cells preincubated with anti-HB-EGF antibodies prior to GnRH challenge were probed with anti-phospho-ERK antibodies (Fig. 6B). In line with our data obtained when monitoring EGFR phosphorylation, trapping of HB-EGF strongly inhibited GnRH-mediated ERK activation. On the contrary, EGFR phosphorylation and ERK activation induced by exogenously added EGF were not affected by preincubation of cells with anti-HB-EGF (data not shown).

EGFR Tyrosine Kinase and MMP Activities Are Not Required to Mediate GnRH-induced Activation of JNK and p38 MAPK—Apart from engaging the ERK cascade, GnRH also stimulates the activities of all three stress-related MAPKs, i.e., JNK, p38 MAPK, and BMK/ERK5 (13). We have previously shown that inhibition of EGFR by AG1478 retarded the onset of agonist-dependent ERK activation in αT3-1 cells (17). To extend this experimental approach to the JNK and p38 MAPK cascades, we monitored the time course of MAPK activation subsequent to GnRH challenge in the presence of AG1478 as well as Ro28-2653 and batimastat. In congruence with previous observations (36), an increase in the phosphorylation status of JNK1 and -2 was noted 5 min after GnRH challenge (Fig. 7A). JNK activation reached a maximum after 30 min and remained at a comparably high level throughout the observation period. The tyrosine kinase activity of the EGFR appears not to be required for GnRH signaling to JNK, because preincubation of cells with AG1478 had no inhibitory impact (Fig. 7B). Along these lines, application of Ro28-2653 (Fig. 7C) or batimastat (Fig. 7D) left GnRH-induced JNK phosphorylation completely unaffected. EGFR as well as gelatinases are not required for GnRH-mediated p38 MAPK activation because pretreatment of cells with AG1478, the gelatinase inhibitor, or batimastat did not show any inhibitory effect on p38 MAPK phosphorylation (data not shown).

The Induction of c-Fos and c-Jun Depends on Gelatinase Activity and EGFR Transactivation—GnRH stimulation of αT3-1 cells results in increased mRNA levels for the immediate early genes c-fos and c-jun (37) as well as elevated c-Fos and c-Jun protein levels (38). The timing of c-Fos and c-Jun induction upon GnRH challenge, however, is discussed controver-
starved

B–D, serum-starved αT3-1 cells were stimulated with 1 μM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti- phospho-JNK antibody.

Western blots of cell lysates were probed with anti-phospho-JNK antibody. A 60-min GnRH challenge resulted in a strong induction of c-Fos and c-Jun proteins.

To address the issue as to whether gelatinase activation and subsequent EGFR transactivation are of cellular physiological relevance or whether the latter events are dispensable for the complex GnRH action on gonadotropic cells, c-Fos and c-Jun induction in the presence of various pharmacological inhibitors was examined by stimulating αT3-1 cells with GnRH for 60 min. EGFR tyrosine kinase activity was blocked by AG1478, and cells were further preincubated with the gelatinase inhibitor Ro28-2653, batimastat, and the Src kinase inhibitor PP2. After GnRH challenge, Western blots were performed with antibodies recognizing c-Fos and c-Jun proteins (Fig. 8, C and D). Pretreatment of cells with AG1478, Ro28-2653, or PP2 profoundly counteracted the induction of c-Fos and c-Jun. Incubation of cells with batimastat, however, had no effect on c-Jun and c-Fos protein levels.

To confirm the role of MMP2 and MMP9 in GnRH-mediated c-Fos and c-Jun induction by an independent experimental approach, αT3-1 cells were transfected with ribozymes directed against MMP2, MMP9, MMP12, or FGF-BP. Cells treated with ribozyme Rz-MMP2 were characterized by a substantial suppression of c-Fos and c-Jun protein levels in response to GnRH challenge (Fig. 8, E and F). Likewise, Rz-MMP9-transfected cells displayed reduced c-Fos and c-Jun protein levels, although the inhibitory effect of the transfected ribozyme was less obvious when compared with Rz-MMP2-mediated gene silencing. Transfection of αT3-1 cells with ribozymes directed against MMP12 or FGF-BP did not affect GnRH-mediated gene induction. Taken together, our findings highlight a cardinal contribution of MMP2 and MMP9 and subsequent EGFR transactivation to GnRH-elicted gene induction in gonadotropic cells (Fig. 9).

**DISCUSSION**

Previously, we have shown that GnRH-elicted EGFR transactivation in αT3-1 cells is required for Ras activation and accelerates the kinetics of GnRH-induced ERK activity (17). In the present study we identify the first distinct members of the MMP family, namely MMP2 and MMP9, as being responsible for GnRH-mediated EGFR transactivation. Our experiments support a concept in which EGFR transactivation is of physiological relevance in gonadotropic cells because gelatinase activation and subsequent EGFR transactivation are necessary intermediate steps required for the induction of transcription factors c-Jun and c-Fos (Fig. 9).

Using non-selective MMP inhibitors, it was shown that GPCR stimulation resulted in cleavage of EGFR ligand precursors and production of diffusible growth factors that then activate the EGFR (6). Lyposphosphatidic acid and carbachol-induced EGFR activation in COS-7 cells as well as bombesin-mediated EGFR phosphorylation in PC-3 cells was found to be batimastat-sensitive (5). Prostaglandin E2-mediated activation of EGFR in gastric epithelial and colon cancer cells was inhibited by another broad-spectrum metalloproteinase inhibitor, GM6001 (39). Whereas the principal involvement of MMPs in the EGFR transactivation process appears to be a rather undisputed issue, the identity of the proteolytic enzymes and their regulation via GPCR ligands still remains elusive. Recently, three members of the ADAM family of proteases, i.e., ADAM 10, ADAM12, and ADAM17/TACE, have been claimed responsible for shedding of EGFR-like ligands resulting in EGFR transactivation (33–35). So far, it has not been possible to conclusively ascribe a defined role in EGFR/GPCR cross-talk to distinct members of the MMP family.

The expression of gelatinases MMP2 and MMP9 can be demonstrated in a variety of human tumors and a major impact of these enzymes on the acquisition of a malignant phenotype during multiple stages of tumor progression has been emphasized (40). For instance, up-regulation of MMP2 and MMP9 has been correlated with increased invasiveness of different tumors including pituitary adenomas and carcinomas (25). Elevated levels of gelatinases as well as of the membrane-spanning MT1-MMP and the endogenous MMP inhibitors TIMP1 and TIMP2 were noted in pituitary adenomas (25). Furthermore, MMP9 expression in pituitary tumors was related to biological behavior (26) and the incidence of MMP9 secretion was found to be significantly higher in invasive pituitary adenomas (41) and carcinomas (26) as compared with non-invasive tumor species. In light of these observations, we first concentrated on a specific gelatinase inhibitor, Ro28-2653, to dissect the signaling pathway linking the GnRHR to the ERK family of MAPKs in gonadotropic cells. Inhibition of MMP2 and MMP9 activities with Ro28-2653 profoundly suppressed GnRH-induced mitogenic signaling events in αT3-1 cells including EGFR phosphorylation, phosphorylation of Src kinases, GTP loading of Ras, stimulation of ERK activity, and induction of transcription factors like c-Fos and c-Jun.

To unequivocally identify MMP2 and MMP9 as the relevant proteolytic enzymes involved in the cross-communication between GnRHR and EGFR in αT3-1 cells, we opted for a novel

**FIG. 7.** AG1478, Ro28-2653, or BB-94 do not effect GnRH-induced JNK activation in αT3-1 cells. A, serum-starved αT3-1 cells were stimulated with 1 μM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti-phospho-JNK antibody. B–D, serum-starved αT3-1 cells were stimulated with 1 μM GnRH for the indicated time periods. Prior to GnRH challenge, cells were incubated with 100 nm AG1478 (B), 10 μM Ro28-2653 (C), or 10 μM BB-94 (D) for 30 min. Western blots of cell lysates were probed with an anti-phospho-JNK antibody.
ribozyme approach to specifically ablate MMP2 and MMP9 expression. A low molecular weight PEI was used as a transfection reagent to stabilize and introduce unmodified bioactive all-RNA ribozymes into cells (23). We recently showed in our laboratory that PEI-complexed ribozymes are bioactive intracellularly as demonstrated by efficient down-regulation of mRNA and protein levels of various gene products in vitro and in vivo (23). GnRH-induced EGFR phosphorylation was mitigated in cells transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12), or FGF-BP (Rz-FGF-BP). Serum-depleted cells were stimulated with 1 μM GnRH for 60 min. Western blots of cell lysates were probed with an anti-c-Fos antibody (E) or an anti-c-Jun antibody (F).

MMP2 is set apart from other MMPs in that its proform is not activated by enzymatic digestion with exogenous proteinases, but requires the activity of MT-MMPs. The activation of pro-MMP2 is potentiated by the formation of a trimolecular complex, consisting of MT1-MMP, TIMP2, and pro-MMP2 thought to cause a sufficient local concentration of pro-MMP2 allowing activation by TIMP2-free MT1-MMP and ensuing autocatalytic processing to the fully active form (46, 47). MMP2 released from the cell surface is then assumed to convert pro-MMP9 into its active form (32). It is an attractive hypothesis to

![Fig. 8](https://example.com/fig8.png)

**Fig. 8.** Induction of c-Fos and c-Jun in αT3-1 cells depends on gelatinase-triggered EGFR transactivation. A and B, serum-starved αT3-1 cells were stimulated with 1 μM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti-c-Fos antibody (A) or an anti-c-Jun antibody (B). C and D, serum-starved αT3-1 cells were pretreated with 10 μM Ro28-2633, 10 μM BB-94, 1 μM PP2, or 100 nM AG1478 for 30 min and subsequently treated with 1 μM GnRH for 60 min. Western blots of cell lysates were probed with an anti-c-Fos antibody (C) or an anti-c-Jun antibody (D). E and F, αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12), or FGF-BP (Rz-FGF-BP). Serum-depleted cells were stimulated with 1 μM GnRH for 60 min. Western blots of cell lysates were probed with an anti-c-Fos antibody (E) or an anti-c-Jun antibody (F).
assume that the agonist-occupied GnRH regulates MT1-MMP function by an intracellular mechanism employing PKC.

The gelatinase inhibitor Ro28-2653 exemplifies a new class of pyrimidine-2,4,6-trione MMP inhibitors. Batimastat is a hydroxamic acid derivative exhibiting broad-spectrum inhibitory activity and was the first metalloproteinase inhibitor to enter clinical testing (48). As opposed to Ro28-2653, batimastat at 10 μM displayed only minor inhibitory effects on metalloproteinase-mediated mitogenic signaling in gonadotropes. An optimized specificity of Ro28-2653 for gelatinases in vivo may offer one explanation for these discrepancies. Both batimastat and Ro28-2653 chelate the zinc ions in the catalytic core of metalloproteinases, but Ro28-2653 additionally forms four hydrogen bonds yielding a nearly perfect fit toward MMP2 and MMP9 (28). Furthermore, batimastat is characterized by an exceptionally poor water solubility, thus favoring the access to cells of more soluble compounds such as Ro28-2653. Third, gelatinases that belong to the most abundant MMPs in a variety of tissues may in fact represent the vast majority of MMPs secreted by αT3-1 cells.

To address the issue of specificity of the pharmacological approach of gelatinase inhibition, we turned our attention to other members of the MAPK family known to be activated by GnRH (13): p38 MAPK and JNK are primarily known to be stimulated by environmental stress and inflammatory cytokines (49). Our data show that gelatinase-triggered EGFR activation is not involved in GnRH-induced p38 MAPK or JNK activation in αT3-1 cells. Furthermore, the pharmacological tools used allow for selective targeting of GnRH-dependent ERK activation without unspecifically affecting other MAPK cascades.

So far, the physiological consequences of GnRH-mediated EGFR transactivation have not been investigated. Agonist occupation of the GnRH receptor in αT3-1 cells results in MAPK activation and increased mRNA and protein levels of the immediate early gene products c-Jun and c-fos (36, 37) as well as in transcriptional activation of the common α-subunit gene (14) through a pathway involving PKC (15, 16). In congruence with our data, c-Fos induction has been reported to occur 60 min after agonist challenge in gonadotropic αT3-1 cells (37) and LpT2 cells (50). In the present study, we show that GnRH-mediated EGFR activation is an indispensable step in the induction of c-Fos and c-Jun and that MMP2 and MMP9 play a pivotal role in GnRH-dependent gene induction.

The results presented herein define a new role for gelatinases in the endocrine regulation of the pituitary. We show for the first time that gelatinases are activated via an inside-out signaling mechanism by G\(_{\alpha}\)11-coupled receptors and thus we identify hitherto unknown signaling components of the EGFR transactivation cascade. Apart from their matrix-degrading abilities, MMP2 and MMP9 seem to be essential for GnRH-induced EGFR transactivation and gene induction in gonadotropes. Considering that gelatinases are also abundantly expressed in many human tumors, our results may shed new light on the mechanism by which agonists acting on other G\(_{\alpha}\)11-coupled receptors can promote tumor growth.

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