

# Src Family Kinases Mediate Epidermal Growth Factor Receptor Ligand Cleavage, Proliferation, and Invasion of Head and Neck Cancer Cells

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## ABSTRACT

Head and neck squamous cell carcinomas (HNSCCs) are characterized by up-regulation of the epidermal growth factor receptor (EGFR). We previously reported that a gastrin-releasing peptide/gastrin-releasing peptide receptor (GRP/GRPR) autocrine growth pathway is activated early in HNSCC carcinogenesis. GRP can induce rapid phosphorylation of EGFR and p42/44 mitogen-activated protein kinase (MAPK) activation in part via extracellular release of transforming growth factor  $\alpha$  (TGF- $\alpha$ ) by matrix metalloproteinases (MMPs). It has been reported that Src family kinases are activated by G-protein-coupled receptors (GPCRs), followed by downstream EGFR and MAPK activation. To further elucidate the mechanism of activation of EGFR by GRP in HNSCC, we investigated the role of Src family kinases. Blockade of Src family kinases using an Src-specific tyrosine kinase inhibitor A-419259 decreased GRP-induced EGFR phosphorylation and MAPK activation. GRP also failed to induce MAPK activation in dominant-negative c-Src-transfected HNSCC cells. Invasion and growth assays showed that c-Src was required for GRP-induced proliferation or invasion of HNSCC cells. In addition to TGF- $\alpha$  release, GRP induced amphiregulin, but not EGF, secretion into HNSCC cell culture medium, an effect that was blocked by the MMP inhibitor marimastat. TGF- $\alpha$  and amphiregulin secretion by GRP stimulation also was inhibited by blockade of Src family kinases. These results suggest that Src family kinases contribute to GRP-mediated EGFR growth and invasion pathways by facilitating cleavage and release of TGF- $\alpha$  and amphiregulin in HNSCC.

## INTRODUCTION

Overexpression of epidermal growth factor receptor (EGFR) has been reported in a variety of human cancers such as those derived from the breast, lung, colon, prostate, brain, ovarian, and head and neck (1–4). This increased expression of EGFR generally is accompanied by up-regulation of EGFR ligands, implicating an autocrine regulatory pathway (5, 6). Although promising results in the preclinical setting have been observed by targeting EGFR expression or activation in tumor cells using EGFR monoclonal antibodies or EGFR tyrosine kinase inhibitors, limited antitumor effects have been observed when these agents have been administered as monotherapy to cancer patients (7, 8). These cumulative results suggest that alternative routes of EGFR activation and/or EGFR-independent pathways contribute to tumor growth.

Cumulative evidence shows that EGFR can be activated by G-protein-coupled receptors (GPCRs) in diverse cell types, including fibroblasts, smooth muscle cells, and tumor cells (9–13). The primary mechanism of GPCR-mediated EGFR activation involves proteolytic release of EGFR ligand(s) (10, 12–14). However, the specific EGFR ligand released by GPCRs appears to be cell type and GPCR ligand

specific. Heparin-binding EGF-like growth factor (HB-EGF) has been shown to be the primary EGFR ligand involved in the EGFR activation by GPCRs in COS-7, HEK-293, and breast cancer cells (10, 14), whereas transforming growth factor  $\alpha$  (TGF- $\alpha$ ) has been implicated in colon epithelial cells and head and neck squamous cell carcinomas (HNSCCs; refs. 11, 12, 15). Amphiregulin also has been shown to play a role in GPCR-EGFR transactivation by lysophosphatidic acid (LPA) or carbachol treatment of HNSCC (13). We previously reported that gastrin-releasing peptide (GRP) and its receptor (GRPR) participate in an autocrine growth pathway in HNSCC, in which GRPR levels in the primary tumor are correlated with survival (16). In contrast, the biological significance of the other GPCR ligands studied to date in EGFR activation in HNSCC remains undetermined.

In addition to the release of EGFR ligands, Src family kinases have been shown to contribute to EGFR activation by GPCRs in several systems. G proteins transduce signals from GPCRs to EGFR and mitogen-activated protein kinase (MAPK) via unknown intermediate molecules. Emerging evidence has shown that Src family kinases are associated with G proteins and are directly activated by G $\alpha$  subunits in fibroblasts (17). Activation of c-Src kinase also has been shown to be an early event in G $\beta\gamma$ -mediated MAPK activation by GPCRs in fibroblasts (18). It has been reported that Src family kinases are activated by GPCR ligands, followed by downstream activation of EGFR and MAPK in the colon cancer cell line Caco-2, RGM1 gastric epithelial cells, COS-7 fibroblasts, and GT1-7 neuronal cells (11, 18, 19). Src family kinases directly associate with EGFR and mediate phosphorylation of tyrosine residues Y845 and Y1101 on the EGFR (20, 21). The precise mechanism of GPCR-mediated EGFR activation by Src family kinases is incompletely understood. The present study was undertaken to test the hypothesis that Src kinases mediate EGFR activation by GRP via cleavage of EGFR proligands. Here we show that blockade of Src kinases using either pharmacologic inhibitors or dominant-negative mutants decreased GRP-induced EGFR phosphorylation and MAPK activation by inhibiting release of TGF- $\alpha$  and amphiregulin into HNSCC cell culture medium. Additional investigation showed the importance of Src family kinases in mediating GRP-induced growth and invasion in HNSCC cells. These results show a novel role for Src kinases in mediating EGFR proligand release in response to GPCR ligands.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Human GRP was obtained from Sigma-Aldrich Corporation (St. Louis, MO). Human recombinant EGF was obtained from Oncogene Research Products (San Diego, CA). Antibodies against EGFR (monoclonal antibody) were obtained from the Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY). The Src-specific tyrosine kinase inhibitor A-419259 was a gift from Abbott Bioresearch Center (Worcester, MA). Antibodies against p42/44 MAPK and phosphorylated p42/44 MAPK were from New England Biolabs (Beverly, MA). Amphiregulin-neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). The EGFR-blocking antibody C225 was a gift from Imclone Systems Incorporated (New York, NY). Antibody against the activation loop of Src (PY418) was purchased from Biosource International (Camarillo, CA). The

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matrix metalloproteinase (MMP) inhibitor marimastat was obtained from British Biotech (Oxford, United Kingdom). The TGF- $\alpha$  ELISA kit was purchased from Oncogene Research Products, and the amphiregulin and EGF ELISA kits were purchased from R&D Systems.

**Cell Culture.** All of the HNSCC cell lines (1483, PCI-37a) were of human origin. Cells were maintained in DMEM with 12% heat-inactivated FCS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO<sub>2</sub>. Primary cell cultures were generated from murine embryonic fibroblasts derived from EGFR knockout mice (Jackson Laboratories, Bar Harbor, ME) and their corresponding wild-type littermates (CD1 background) obtained at age E16.5 days as described previously (15).

**Expression and Purification of Glutathione S-Transferase Downstream of Kinase (GST-Dok) Substrate.** To create a substrate for *in vitro* Src kinase assays, we expressed a portion of the p62 Ras GTPase-activating protein (GAP)-associated protein Dok as a GST fusion protein (22). The coding region of murine Dok residues Ser 309 to Leu 429 was amplified by PCR and cloned downstream and in-frame of GST in the baculovirus transfer vector pVL-GST (23). This region of Dok contains multiple consensus sequences for tyrosine phosphorylation. The resulting pVL-GST-Dok vector was used to create a recombinant baculovirus using Baculogold DNA and the manufacturer's protocol (BD Pharmingen, San Diego, CA). The GST-Dok fusion protein was expressed in Sf-9 insect cells and purified in one step using glutathione-agarose beads. The protein was eluted from the beads with free glutathione and dialyzed against 50 mmol/L HEPES buffer (pH 7.4) containing 10% glycerol. The final protein ran as a single band of the expected molecular mass following analysis by SDS-PAGE and Coomassie staining.

**In vitro Kinase Assays.** After treatment with GRP or EGF, cells were washed three times with cold PBS, lysed with lysis buffer [10 mmol/L Tris HCl (pH 7.6), 50 mmol/L Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 50 mmol/L NaF, 1 mmol/L NaV<sub>3</sub>O<sub>4</sub>, 1% Triton X-100, and 1× protease inhibitor mixture tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases; Roche, Basel, Switzerland], scraped off the plate, and passed through a 26.5-gauge needle three or four times. The lysate then was centrifuged at 4°C, 14,000 rpm for 10 minutes. Supernatant was collected for protein quantitation. Protein quantitation was performed using the Protein Assay Solution (Bio-Rad Laboratories, Hercules, CA) and BSA of known concentration as the standard. Anti-c-Src and c-Yes antisera (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate Src family proteins. Forty microliters of protein agarose G beads (Invitrogen) were added to the lysate and incubated overnight at 4°C with gentle agitation. The beads were collected by centrifugation at 4°C, 14,000 rpm for 1 minute. The beads were resuspended and washed with lysis buffer three times. Immunoprecipitates were washed twice with kinase buffer [50 mmol/L HEPES (pH 7.4) and 10 mmol/L MgCl<sub>2</sub>]. Kinase buffer containing 1  $\mu$ g of the tyrosine kinase substrates GST-Dok and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP (3,000 Ci/mmol; PerkinElmer, Boston, MA) was added, and the reactions were incubated for 15 minutes at 30°C. Reactions were stopped by adding SDS-PAGE sample buffer and heating to 95°C for 5 minutes. Radiolabeled substrates were visualized by autoradiography.

**Transfection of HNSCC Cells with Dominant-Negative c-Src.** HNSCC 1483 cells were transfected with a pUSEamp vector (Upstate Biotechnology) containing mutant Src (K296R/528F) cDNA using LipofectAMINE (Life Technologies, Rockville, MD) according to the manufacturer's recommendations. Stably transfected clones were selected for resistance to the neomycin analog G418 (800  $\mu$ g/mL; Life Technologies) as described previously (22). Dominant-negative c-Src was generated by mutation of K296 to R, which rendered the kinase domain incapable of binding ATP.

**Cell Treatments.** HNSCC cells were plated at a density of 2 × 10<sup>5</sup> cells/mL in 10-cm<sup>2</sup> plates. Twenty-four hours after plating, cells were serum starved for 72 hours in serum-free DMEM. During serum starvation the media were changed every 24 hours. For the experiments with inhibitors, cells were pretreated with 6  $\mu$ g/mL of C225, 20  $\mu$ mol/L marimastat, 100 nmol/L A-419259, or 15  $\mu$ g/mL amphiregulin-neutralizing antibody. Control wells were treated with vehicle (water) or DMSO for 2 hours before the addition of growth factors. After pretreatment when required, 400 nmol/L GRP or 10 ng/mL EGF were added to the cells. At selected time points after growth factor stimulation, cells were washed three times with cold PBS, lysed with lysis buffer [10 mmol/L Tris HCl (pH 7.6), 50 mmol/L Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 50 mmol/L NaF, 1 mmol/L NaV<sub>3</sub>O<sub>4</sub>, 1% Triton X-100, and 1× protease inhibitor mixture tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases;

Roche], scraped off the plate, and passed through a 26.5-gauge needle three or four times. The lysate then was centrifuged at 4°C, 14,000 rpm for 10 minutes. Supernatant was collected for protein quantitation.

**Western Blot Analysis and Immunoprecipitation.** For immunoprecipitation, 100  $\mu$ g of total protein were incubated for 2 hours at 4°C with 3  $\mu$ g of anti-EGFR antibody (Upstate Biotechnology) or with 7.5  $\mu$ L of anti-c-Src and c-Yes antisera (Santa Cruz Biotechnology) with gentle agitation. Forty microliters of protein agarose G beads (Invitrogen) were added to the lysate and incubated overnight at 4°C with gentle agitation. The beads were collected by centrifugation at 4°C, 14,000 rpm for 1 minute. The beads were resuspended and washed with lysis buffer three times. The beads were resuspended in 20  $\mu$ L of 2× loading dye and boiled for 5 minutes at 95°C, followed by Western blot analysis. The immunoprecipitated proteins then were resolved on an 8% SDS-PAGE gel. After being transferred onto a Protran membrane, the membrane was blocked in 5% milk and blotted with the antiphosphotyrosine antibody PY99 (Santa Cruz Biotechnology) at 1:1,000 in Blotto solution [0.6% dry milk powder, 0.9% NaCl, 0.5% Tween 20, and 50 mmol/L Tris (pH 7.4)]. After washing three times with Blotto solution, the membrane then was incubated with the secondary antibody (goat anti-rabbit/mouse IgG-horseradish peroxidase conjugate; Bio-Rad Laboratories) for 1 hour and washed three times for 10 minutes. The membrane was developed with Luminol Reagent (Santa Cruz Biotechnology) by autoradiography. Blots were stripped in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 15 minutes at room temperature, blocked for 1 hour, and reprobbed with EGFR antibody (Transduction Laboratories) at 1:1,000 or with anti-c-Src and c-Yes antisera (Santa Cruz Biotechnology) for 2 hours.

**Invasion of HNSCC Cells Defective in c-Src.** The ability of HNSCC cells to invade on GRP stimulation in the presence and absence of c-Src was measured using Matrigel-coated modified Boyden inserts with a pore size of 8  $\mu$ m (Becton Dickinson/Biocoat, Bedford, MA). Cells were plated at a density of 2 × 10<sup>4</sup> cells/well in DMEM with or without GRP (400 nmol/L)/5% serum in the insert. The lower well contained 10% fetal bovine serum with or without 400 nmol/L GRP. After 48 hours of treatment at 37°C in a 5% CO<sub>2</sub> incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific, Hampton, NH) according to the manufacturer's instructions. Invading cells in four representative fields were counted using light microscopy at 400× magnification. Mean  $\pm$  SE was calculated from two independent experiments.

**ELISA Assay.** HNSCC cells were plated at a density of 2 × 10<sup>5</sup> cells/mL in 10-cm<sup>2</sup> plates. Twenty-four hours after plating, cells were serum starved for 72 hours in serum-free DMEM. During serum starvation the media were changed every 24 hours. Cells were pretreated with 20  $\mu$ mol/L marimastat or 100 nmol/L A-419259 for 2 hours at 37°C with 5% CO<sub>2</sub>. Cells were treated with 400 nmol/L GRP for 10 minutes. Cell culture media were collected, and cells were subjected to centrifugation at 1300 rpm for 10 minutes. Pellets were discarded, and the supernatant was tested for levels of TGF- $\alpha$ , amphiregulin, or EGF as per the manufacturer's instructions.

**Proliferation Assays.** Cells were plated at low density (2 × 10<sup>4</sup> cells) and allowed to seed overnight in a 24-well plate. Cells then were treated with GRP (400 nmol/L) or the control vehicle (water) in serum-free DMEM. After 3 days, cell number was determined by counting. Serum-containing DMEM also was used to treat cells for 3 days as a positive control or cell growth.

**Statistics.** All of the group differences were tested with the exact Wilcoxon test. The *P* values from multiple comparisons within the same experiment were adjusted with the Bonferroni procedure.

## RESULTS

### GRP Induces Src Family Kinase Activation in HNSCC Cells.

We previously have shown that GRP stimulates p42/44 MAPK activation via EGFR phosphorylation (15). Additional investigation showed that EGFR ligand induced phosphorylation of Src family kinases, including c-Src, Lyn, Fyn, and c-Yes, in HNSCC cells (24). Src family kinases previously have been reported to mediate EGFR activation by GPCRs in fibroblasts (25, 26). To determine whether GRP could mediate activation of Src family kinases, we treated HNSCC (1483) cells with GRP or EGF. The c-Src and c-Yes immu-

nonprecipitates were incubated *in vitro* with [ $\gamma$ - $^{32}$ P] ATP and an  $M_r$  40,000 GST-Dok (22) fusion protein as substrate. As shown in Fig. 1A, c-Src immunoprecipitates exhibited strong autophosphorylation and substrate phosphorylation *in vitro* on EGF or GRP treatment. However, c-Yes immunoprecipitates showed strong autophosphorylation and substrate phosphorylation on EGF treatment but not on GRP treatment as shown in Fig. 1B. Control blots indicated that equivalent amounts of Src family proteins were present in the immunoprecipitates. These results suggest that c-Src kinase can be activated in HNSCC cells by GRP and EGFR ligands.

**EGFR Activity Is Required for Maximum GRP-Induced Activation of Src Family Kinases.** EGF and GRP can activate Src family kinases. Although EGF activates Src family kinases by direct interaction between Src and EGFR (24), the mechanism of c-Src kinase activation by GRP remains unclear. To investigate the role of EGFR in the activation of Src family kinases by GRP, we generated cells from EGFR knockout mice and their wild-type littermates. As shown in Fig. 2A, EGFR expression was absent in EGFR knockout cells. Src family proteins such as c-Src and c-Yes also were expressed in EGFR wild-type cells and EGFR knockout cells. On treatment with EGF or GRP, as expected, EGF stimulated activation of Src family kinases in EGFR wild-type cells but not in EGFR knockout cells. GRP induced activation of Src family kinases in EGFR wild-type cells and in EGFR knockout cells but to a lesser degree in the EGFR-deficient cells (Fig. 2B), suggesting that EGFR activity is required for maximum activation of Src family kinase by GRP.

**Src Family Kinases Regulate EGFR and MAPK Activation by GRP in HNSCC Cells.** Src family kinases have been implicated in the cross-talk between EGFR and GPCRs (9, 11). However, the mechanism of how Src family kinases are involved in this process remains to be elucidated. To examine whether Src family kinases mediate EGFR activation by GRP in HNSCC, we treated HNSCC cells (1483) with the Src family kinase inhibitor A-419259, followed

by GRP stimulation (Fig. 3A). The small molecule inhibitor A-419259 has been shown to inhibit Src-family kinases while displaying no inhibition of EGFR kinase activity in HNSCC cells (24, 27). As shown in Fig. 3A, GRP significantly induced EGFR phosphorylation within 10 minutes, an effect that was nearly completely blocked by pretreatment with A-419259 (Fig. 3A;  $P = 0.004$ ). Similar results were found in another HNSCC cell line, PCI-37a (data not shown). In addition to A-419259, we also used two additional Src family kinase inhibitors, PP2 and PD0180970 (28, 29), and similar results were observed (data not shown).

We previously reported that MAPK activation by GRP occurs via EGFR phosphorylation in HNSCC (15). We also showed that blockade of EGFR activity decreased MAPK activation by GRP (15). To investigate the role of Src family kinases in GRP-mediated MAPK activation, HNSCC cells (1483) were treated with A-419259, followed by GRP or EGF treatment. As shown in Fig. 3B, blockade of Src family kinases with A-419259 (Fig. 3B;  $P = 0.0011$ ) inhibited MAPK activation by GRP. In contrast, activation of MAPK by EGF was not affected by Src family kinase blockade. Similar results were obtained with the Src family kinase inhibitors PP2 and PD0180970 (data not shown). These findings indicate that Src family kinases mediate MAPK activation by GRP but not by direct activation of EGFR by EGFR ligand.

Additional investigation using dominant-negative c-Src-transfected HNSCC cells previously shown to exhibit reduced c-Src activation (24) showed that GRP was able to stimulate MAPK activation in vector-transfected control cells (Neo 1483) but not in dominant-negative c-Src-transfected cells (DN c-Src 1483; Fig. 3C). MAPK activation by EGF was intact in dominant-negative c-Src-transfected cells, indicating that MAPK activation by GRP, but not EGF, requires c-Src activity. These results suggest that Src family kinases mediate EGFR and MAPK activation by GRP in HNSCC cells.

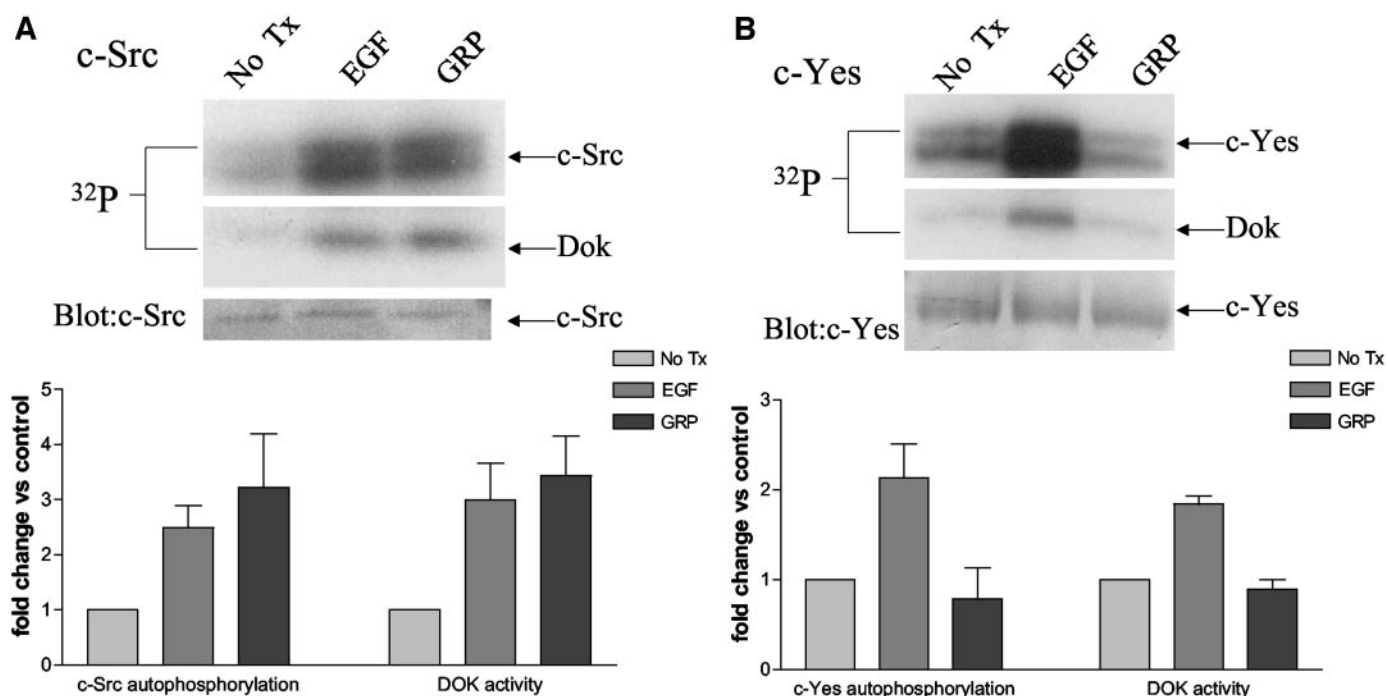


Fig. 1. Stimulation of Src family kinase activity by GRP. Representative HNSCC cells were serum starved for 72 hours, stimulated with GRP (400 nmol/L) or EGF (10 ng/mL) for 10 minutes, followed by immunoprecipitation with antisera against (A) c-Src and (B) c-Yes. Immunoprecipitates were washed and resuspended in 20  $\mu$ L of kinase buffer containing [ $\gamma$ - $^{32}$ P]ATP and a GST-Dok fusion protein as substrate. Following incubation, Src family proteins and Dok were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, followed by autoradiography. Arrows indicate the position of autophosphorylated Src family proteins and Dok (*top and middle*). The membranes were probed with anti-c-Src and c-Yes to ensure equivalent recovery of Src family proteins in the immunoprecipitates (*bottom*). Cumulative results are shown from two independent experiments.



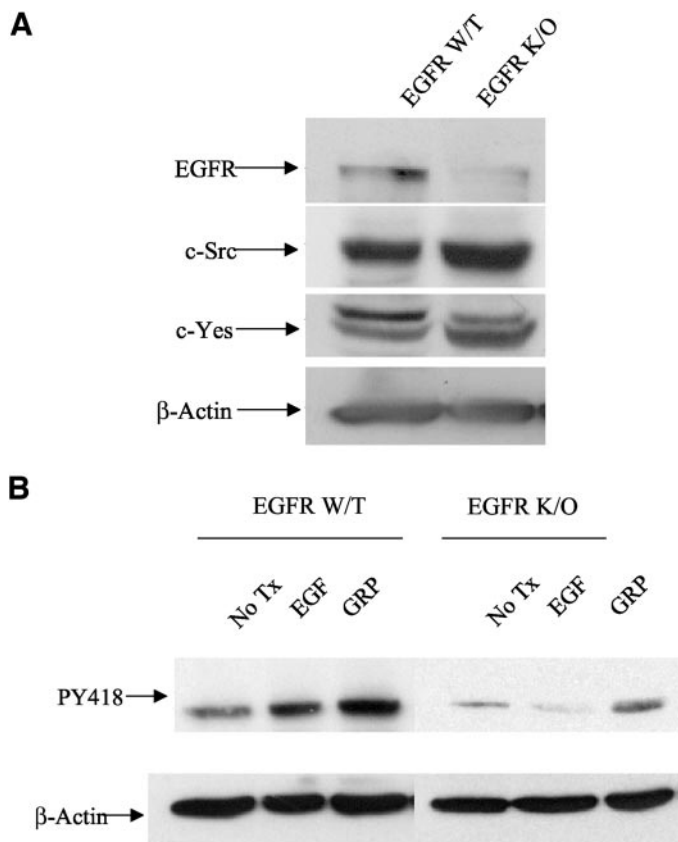


Fig. 2. EGFR activity is required for maximum activation of Src family kinases by GRP. *A*, Cell lysates derived from EGFR knockout mice (*K/O*) or their wild-type littermates (*W/T*) were subjected to Western blot analysis for EGFR, c-Src, and c-Yes.  $\beta$ -Actin was used as a control for loading. *B*, Cells derived from EGFR knockout mice (*K/O*) or their wild-type littermates (*W/T*) were treated with recombinant EGF (10 ng/mL) or GRP (400 nmol/L) for 10 minutes, followed by immunoblotting for PY418 or  $\beta$ -actin (as a control for loading).

**Amphiregulin, but not EGF, Is Cleaved by GRP Stimulation in HNSCC Cells.** Previous reports have shown that activation of EGFR by GPCRs can involve intracellular and extracellular pathways (10, 11). It was found that extracellular release of EGFR ligands from the plasma membrane was mediated by MMP activity, which is required for EGFR activation by GPCRs (10). We previously reported that TGF- $\alpha$ , but not HB-EGF, was implicated in the activation of EGFR by GRP in HNSCC (15). To determine whether other EGFR ligands were involved in the activation of EGFR by GRP, ELISAs were performed to examine amphiregulin and EGF release. As shown in Fig. 4A, GRP induced amphiregulin secretion into HNSCC cell culture medium. Pretreatment of HNSCC cells with the MMP inhibitor marimastat abrogated amphiregulin release. In contrast, GRP treatment did not induce EGF release into HNSCC cell culture medium (Fig. 4B;  $P = 0.343$ ). These results indicate that in addition to TGF- $\alpha$ , amphiregulin is cleaved by GRP stimulation in HNSCC cells. To confirm that amphiregulin binding to EGFR mediated GRP-induced EGFR activation, HNSCC cells were treated with an amphiregulin-neutralizing antibody or an EGFR-blocking antibody (C225), followed by GRP treatment. As shown in Fig. 4C, GRP-mediated EGFR phosphorylation was abrogated by blockade of amphiregulin or EGFR ligand binding.

**Src Family Kinases Mediate GRP-Induced Ligand Release into HNSCC Cell Culture Medium.** Our cumulative results suggest that amphiregulin and TGF- $\alpha$  are two specific EGFR proligands cleaved by GRP treatment in HNSCC cells. Src family kinases also mediate EGFR and MAPK activation by GRP. Therefore, we hypothesized

that Src family kinases contribute to EGFR and MAPK activation by GRP through EGFR proligand cleavage. To test this hypothesis, we treated HNSCC cells with the Src inhibitor A-419259, followed by GRP treatment and TGF- $\alpha$  or amphiregulin determinations in cell culture medium. As shown in Fig. 5A and B, secretion of amphiregulin or TGF- $\alpha$  following GRP treatment was abrogated by blockade of Src family kinases (Fig. 5A,  $P = 0.0143$ ; Fig. 5B,  $P = 0.0011$ ). To determine whether c-Src activity contributes to GRP-mediated MAPK activation by regulating EGFR ligand release, we treated vector-transfected and dominant-negative c-Src-transfected HNSCC cells with GRP, followed by determination of amphiregulin levels in cell culture medium. We found that GRP induced amphiregulin release in vector-transfected HNSCC cells but failed to induce the ligand release in dominant-negative c-Src-transfected cells (data not shown). These results indicate a novel role for Src family kinases in mediating EGFR proligand cleavage following treatment with a GPCR ligand.

**HNSCC Cell Proliferation and Invasion by GRP Depends on c-Src Activity.** We previously reported that GRP induces HNSCC cell proliferation in a dose-dependent manner (16). Additional investigation showed that the GRP-mediated mitogenic effects in HNSCC cells occurred via an MAP-ERK kinase (MEK)/MAPK signaling pathway (15). To elucidate the role of c-Src activity on the proliferation of HNSCC cells by GRP, we treated dominant-negative c-Src-transfected HNSCC cells with GRP, followed by cell count determinations using vital dye exclusion. As shown in Fig. 6A, GRP induced proliferation of vector-transfected control HNSCC cells (Neo;  $P = 0.014$ ), whereas no mitogenic effects were observed in dominant-negative c-Src-transfected cells. To confirm whether cells expressing mutant c-Src were capable of proliferating, these cells were treated with 5% serum, followed by cell count determinations (Fig. 6A). These results suggested that c-Src activity is required for GRP-induced HNSCC cell proliferation.

Previous studies showed that the GPCR ligand LPA induced tumor cell migration and invasion (13, 30). We examined whether GRP treatment could increase HNSCC cell invasion. We also determined whether HNSCC cell invasion by GRP is mediated by c-Src activity. As shown in Fig. 6B, GRP induced HNSCC invasion in vector-transfected control cells (Neo), an effect that was abrogated in dominant-negative c-Src-transfected cells. To determine whether dominant-negative c-Src-transfected cells could invade, these cells were treated with 5% serum, followed by invasion determinations. These results suggest that c-Src activity is necessary for GRP-induced HNSCC cell invasion.

## DISCUSSION

Studies to date have shown that EGFR activation by GPCRs represents a paradigm of potential cross-talk between tyrosine kinase receptors and GPCRs (31). Although the biological significance of the initial studies performed in fibroblasts was undetermined, subsequent investigations in cancer cells have shown that activation of EGFR by GPCR ligands leads to downstream MAPK activation, tumor cell invasion, and DNA synthesis (13, 32). We previously reported that HNSCC cell lines and tissues express increased levels of GRP and GRPR when the levels of GRPR in the primary tumor were correlated with patient survival (16). Additional investigation showed that blockade of GRP using the neutralizing antibody 2A11 inhibited HNSCC growth *in vitro* and *in vivo*, thus implicating an autocrine regulatory pathway involving this GPCR ligand and receptor in HNSCC (16). The importance of EGFR up-regulation in HNSCC carcinogenesis has been well documented (5, 6, 33, 34). Additional investigation showed that treatment of HNSCC cells with GRP led to rapid phosphorylation of EGFR and MAPK activation with resultant cell proliferation (15).

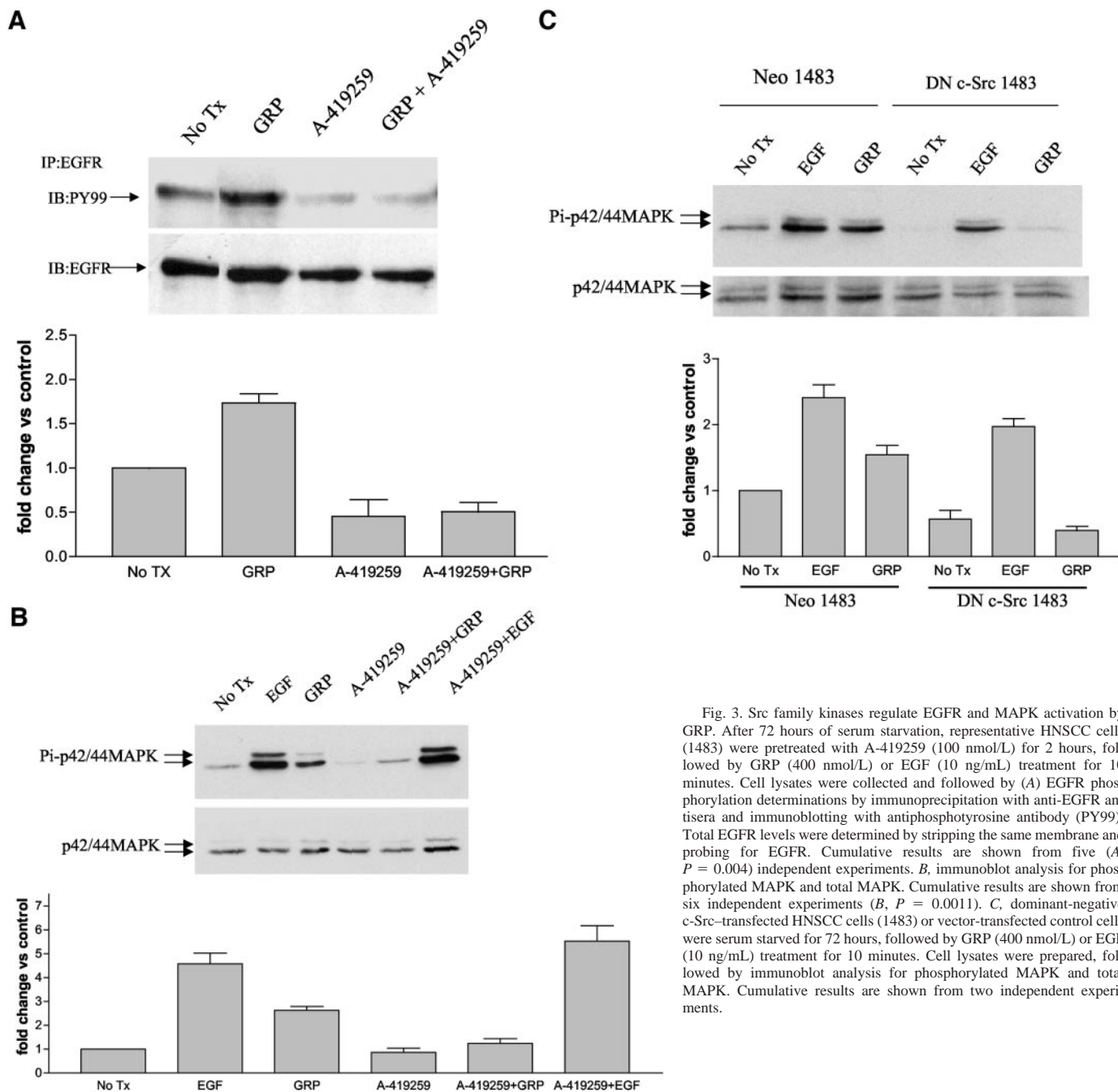


Fig. 3. Src family kinases regulate EGFR and MAPK activation by GRP. After 72 hours of serum starvation, representative HNSCC cells (1483) were pretreated with A-419259 (100 nmol/L) for 2 hours, followed by GRP (400 nmol/L) or EGF (10 ng/mL) treatment for 10 minutes. Cell lysates were collected and followed by (A) EGFR phosphorylation determinations by immunoprecipitation with anti-EGFR antiserum and immunoblotting with antiphosphotyrosine antibody (PY99). Total EGFR levels were determined by stripping the same membrane and probing for EGFR. Cumulative results are shown from five (A,  $P = 0.004$ ) independent experiments. B, immunoblot analysis for phosphorylated MAPK and total MAPK. Cumulative results are shown from six independent experiments (B,  $P = 0.0011$ ). C, dominant-negative c-Src-transfected HNSCC cells (1483) or vector-transfected control cells were serum starved for 72 hours, followed by GRP (400 nmol/L) or EGF (10 ng/mL) treatment for 10 minutes. Cell lysates were prepared, followed by immunoblot analysis for phosphorylated MAPK and total MAPK. Cumulative results are shown from two independent experiments.

Others have reported that treatment of HNSCC cells with LPA, bradykinin, carbachol, or thrombin induced rapid EGFR phosphorylation and MAPK activation with resulting cell invasion, thus suggesting that a variety of GPCR ligands may lead to EGFR transactivation and regulate behaviors critical to tumor progression (13, 32). These cumulative results implicate EGFR as a therapeutic target and support the potential significance of this growth factor receptor as an integration point for convergent signaling pathways in HNSCC cells. Although a variety of potential intracellular and extracellular pathways have been described in the context of GPCR-mediated EGFR activation, studies to date suggest that the specific mechanisms are likely cell type specific. The results of the present study underscore the importance of cleavage of the EGFR ligands, TGF- $\alpha$ , and amphiregulin in this process in HNSCC cells and show a novel role for Src family kinases.

Src family kinases previously have been implicated in the cross-talk between EGFR and GPCRs (14, 19, 35). Activation of Src family kinases, including Fyn, c-Yes, and c-Src, have been reported to be an early event following thrombin treatment in lung fibroblast CCL39 cells (36). A variety of GPCR ligands, including bombesin, bradykinin, and vasopressin, can rapidly activate Src family kinases in Swiss mouse fibroblast cells (37). In addition to fibroblasts, it has been shown that c-Src is activated by prostaglandin  $E_2$  treatment in colon cancer cells in conjunction with EGFR activation (11). Because GRP is the "mammalian counterpart" of bombesin (15), our findings of increased phosphorylation of c-Src by GRP in HNSCC cells are consistent with these earlier studies. In all of the cell lines examined, activation of c-Src by GRP was more pronounced than the other Src family kinases and was higher following treatment with this GPCR ligand compared with EGF. Interestingly, our preliminary experi-

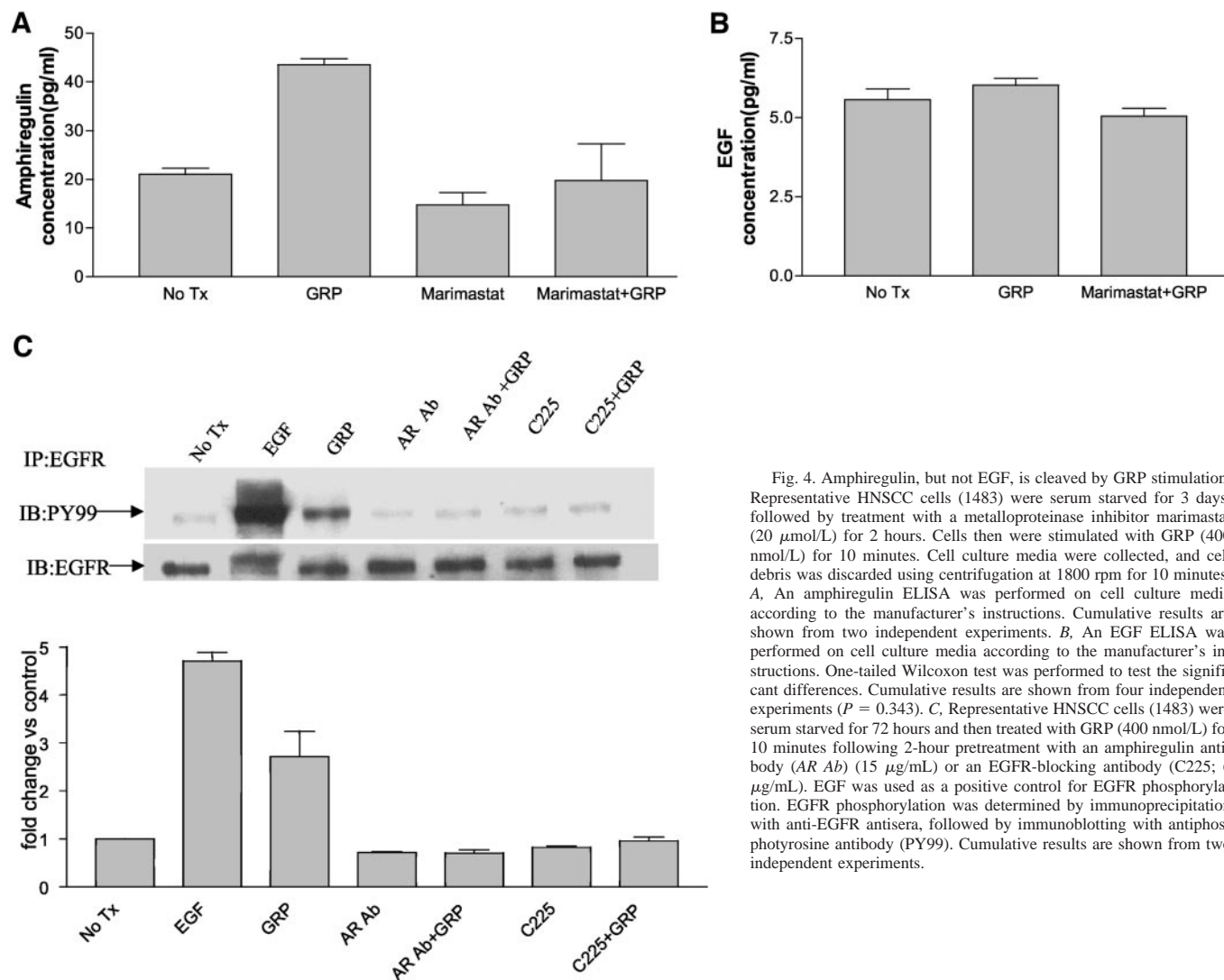


Fig. 4. Amphiregulin, but not EGF, is cleaved by GRP stimulation. Representative HNSCC cells (1483) were serum starved for 3 days, followed by treatment with a metalloproteinase inhibitor marimastat (20  $\mu\text{mol/L}$ ) for 2 hours. Cells then were stimulated with GRP (400 nmol/L) for 10 minutes. Cell culture media were collected, and cell debris was discarded using centrifugation at 1800 rpm for 10 minutes. **A**, An amphiregulin ELISA was performed on cell culture media according to the manufacturer's instructions. Cumulative results are shown from two independent experiments. **B**, An EGF ELISA was performed on cell culture media according to the manufacturer's instructions. One-tailed Wilcoxon test was performed to test the significant differences. Cumulative results are shown from four independent experiments ( $P = 0.343$ ). **C**, Representative HNSCC cells (1483) were serum starved for 72 hours and then treated with GRP (400 nmol/L) for 10 minutes following 2-hour pretreatment with an amphiregulin antibody (AR Ab) (15  $\mu\text{g/mL}$ ) or an EGFR-blocking antibody (C225; 6  $\mu\text{g/mL}$ ). EGF was used as a positive control for EGFR phosphorylation. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antisera, followed by immunoblotting with antiphosphotyrosine antibody (PY99). Cumulative results are shown from two independent experiments.

ments also suggest that GRP can modestly up-regulate Lyn and Fyn activation. Furthermore, experiments in murine EGFR knockout cells showed that EGFR was required for maximum induction of Src kinase activation by GRP. These results suggest that activation of Src family kinases by GRP is upstream of EGFR. Activation of Src family kinases has been implicated either upstream or downstream of EGFR phosphorylation, depending on the cellular context (9, 24, 35). In contrast, EGFR phosphorylation is required for EGFR ligand-induced activation of Src family kinases (24).

Previous studies have shown that Src family kinases can be activated by GPCRs (35, 36). However, the specific Src family kinase(s) involved in EGFR and MAPK activation by GPCRs in human cancer cells remains incompletely understood. To elucidate the role of specific Src family kinases in EGFR phosphorylation and MAPK activation by GRP, we used several approaches, including three different pharmacologic inhibitors and generation of HNSCC cells expressing mutant c-Src. Compared with vector-transfected control cells, dominant-negative c-Src-transfected cells were found to specifically express lower levels of c-Src but not Fyn, Lyn, or c-Yes (24). Dominant-negative c-Src-transfected cells also were growth inhibited compared with vector-transfected controls (24). In the present study, GRP stimulated MAPK activation in vector-transfected control HNSCC cells but not in dominant-negative c-Src-transfected HNSCC cells, indicating that c-Src may play a critical role in MAPK activation by GRP.

In contrast, we found that MAPK activation by EGF was not affected by decreased c-Src activity in HNSCC cells, suggesting that c-Src modulates activation of MAPK by GRP but not EGF. In addition to MAPK activation, c-Src also was found to play a critical role in GRP-mediated HNSCC proliferation and invasion, indicating the importance of c-Src in GPCR-mediated EGFR activation and HNSCC progression.

EGFR ligand cleavage by the MMP and a disintegrin and metalloprotease (ADAM) families of metalloproteinases from the plasma membrane has been shown to be involved in EGFR activation by GPCRs (14, 38, 39). However, the precise EGFR ligand(s) and cleavage enzymes that participate in this process appear to depend on the specific different biological system being investigated (39). Evidence to date has indicated the involvement of several EGFR ligands, including amphiregulin, HB-EGF, and TGF- $\alpha$  (10–15). However, there are no reports showing that more than one EGFR ligand may participate in EGFR activation by a single GPCR ligand in a specific tumor system. We previously reported that TGF- $\alpha$  but not HB-EGF is involved in EGFR activation by GRP in HNSCC (15). In the present study, we further show that amphiregulin release into HNSCC cell line supernatants also is induced by GRP. The concentration of amphiregulin in the cell culture media following GRP treatment is 8- to 10-fold higher compared with TGF- $\alpha$  levels. Whereas TGF- $\alpha$  has been extensively studied as an autocrine growth factor in HNSCC,

there are few reports on the role of amphiregulin in this tumor system. Our findings suggest that EGFR activation by GPCRs may involve cleavage of more than one EGFR proligand and its subsequent release from the plasma membrane.

Several studies have attempted to identify the specific cleavage enzyme involved in GPCR-mediated release of EGFR ligand (10, 14, 39). MMP-2 and MMP-9 have been shown to be involved in the release of HB-EGF and subsequent EGFR activation induced by estradiol ( $E_2$ ) in breast cancer cells (14). Phorbol-12-myristate-13-acetate-induced activation of ADAM-9 was reported in HB-EGF shedding in Vero cells (40). A recent report showed that TNF- $\alpha$  converting enzyme (TACE)/ADAM-17 is involved in cleavage of proamphiregulin and downstream EGFR and MAPK activation in HNSCC cells (13). Zymography results showed that MMP-2 and MMP-9 are not involved in the release of EGFR ligands induced by GRP in HNSCC cells (data not shown). Further studies are required to identify the precise cleavage enzyme involved in the GRP-induced EGFR ligand release.

The role of Src family kinases in mediating metalloproteinase activity and shedding of EGFR ligands has not been reported previously. We show here that several Src family kinases can be activated by GRP, with subsequent phosphorylation of EGFR and downstream MAPK activation. Emerging evidence suggests that Src family kinases may mediate metalloproteinase activity (14).  $E_2$  has been shown to induce MMP-2 and MMP-9 activity in MCF-7 cells, in which the effect was attenuated by the Src family kinase inhibitor PP2 or a dominant-negative c-Src construct (14). Although MMP-2 and MMP-9 do not appear to be involved in GRP-mediated EGFR ligand cleavage in HNSCC cells, TACE/ADAM-17 has been shown to mediate cleavage of amphiregulin by several GPCR ligands. A phys-

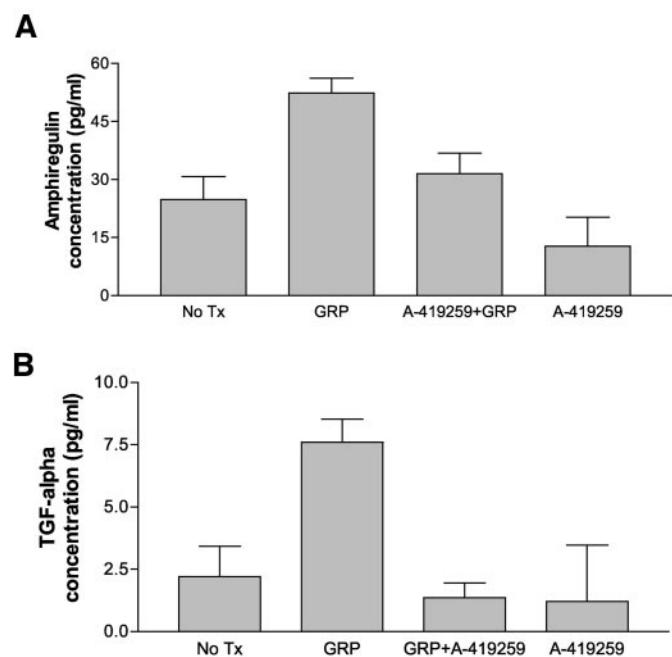


Fig. 5. Src family kinases regulate GRP-induced TGF- $\alpha$  and amphiregulin release into cell line supernatants. Representative HNSCC cells (1483) were serum starved for 3 days, followed by treatment with the Src-selective inhibitor A-419259 for 2 hours. Vector-transfected and dominant-negative c-Src-transfected HNSCC cells were plated and serum starved for 2 days. These inhibitor-treated cells or serum-starved cells were treated with GRP (400 nmol/L) for 10 minutes. Supernatants were collected, and cell debris was discarded using centrifugation at 1800 rpm for 10 minutes. (A) Amphiregulin or (B) TGF- $\alpha$  ELISA assays were performed according to the manufacturer's instructions. One-tailed Wilcoxon test was performed to test the significant differences. Cumulative results are shown from four independent experiments (A,  $P = 0.0143$ ) and six independent experiments (B,  $P = 0.0011$ ).

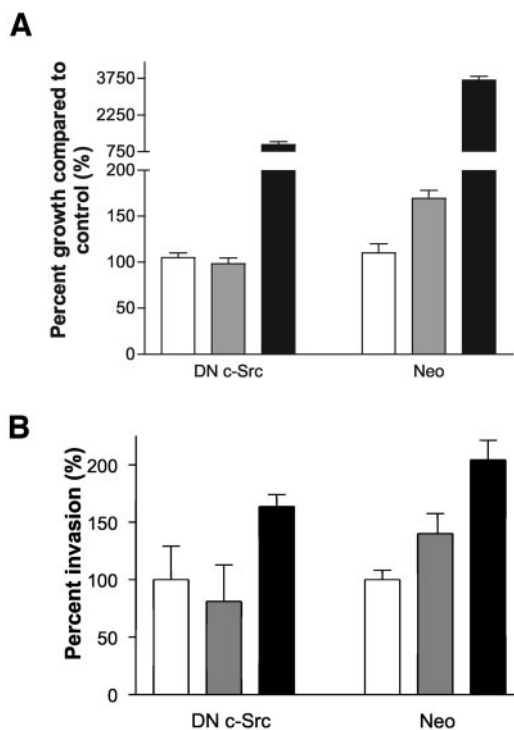


Fig. 6. GRP-induced cell growth and invasion depend on c-Src activity. A, Dominant-negative c-Src-transfected HNSCC cells (DN c-Src) or vector-transfected control cells (Neo) were plated at a density of  $2.5 \times 10^4$  cells in a 24-well plate. After 3 days of serum starvation, GRP (400 nmol/L) or 5% serum was added to the cells. After 24 hours, the percentage change in cell growth (compared with no treatment) was determined by cell counting via vital dye exclusion. Cumulative results are shown from four independent experiments ( $P = 0.014$ ). B, Cells were plated at a density of  $2 \times 10^4$  cells/well in DMEM with or without GRP (400 nmol/L)/5% serum in the insert. The lower well contained 10% FBS with or without 400 nmol/L GRP. After 48 hours of treatment at 37°C in a 5%  $CO_2$  incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific) according to the manufacturer's instructions. Invading cells in four representative fields were counted using light microscopy at 400 $\times$  magnification. Cumulative results were calculated from two independent experiments.  $\square$ , no Tx;  $\blacksquare$ , GRP;  $\blacksquare$ , serum.

ical association between ADAM and Src family proteins has been reported previously (41, 42). It also has been shown that Src family kinases induced ADAM-mediated release of L1 adhesion molecule from human tumor cells (43). These cumulative findings show a novel role for Src family kinases in mediating the release of EGFR ligands induced by GRP and contributing to HNSCC progression.

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