

## EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes

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**Andl, Claudia D., Takaaki Mizushima, Kenji Oyama, Mark Bowser, Hiroshi Nakagawa, and Anil K. Rustgi.** EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. *Am J Physiol Gastrointest Liver Physiol* 287: G1227–G1237, 2004. First published July 29, 2004; doi:10.1152/ajpgi.00253.2004.—The epidermal growth factor receptor (EGFR) activates several signaling cascades in response to epidermal growth factor stimulation. One of these signaling events involves tyrosine phosphorylation of signal transducer and activator of transcription (STAT), whereas another involves activation of the phosphatidylinositol 3-OH kinase pathway. Two possibilities for STAT activation exist: a janus kinase (JAK)-dependent and a JAK-independent mechanism. Herein, we demonstrate that EGFR overexpression in primary esophageal keratinocytes activates STAT in a JAK-dependent fashion with the functional consequence of enhanced cell migration, which can be abolished by use of a JAK-specific inhibitor, AG-490. We determined the mechanisms underlying the signal transduction pathway responsible for increased cell migration. Stimulation of EGFR induces Tyr701 phosphorylation of STAT1 and initiates complex formation of STAT1 and STAT3 with JAK1 and JAK2. Thereafter, the STATs translocate to the nucleus within 15 min. In addition, we found that activation of this signaling pathway results in matrix metalloproteinase-1 (MMP-1) activity. By contrast, Akt activation does not impact the EGFR-STATs-JAKs complex formation and nuclear translocation of the STATs with subsequent MMP-1 activity, although Akt activation may contribute to cell migration through an independent mechanism. Taken together, we find that the recruitment of the STAT-JAK complex by EGFR is responsible for keratinocyte migration that, in turn, might be mediated by MMP-1 activation.

epidermal growth factor receptor; Akt; cell migration

ACTIVATION OF EPIDERMAL GROWTH FACTOR (EGF) receptor (EGFR) is involved in diverse cell functions, including regulation of proliferation, differentiation, cell survival, and motility, but also in processes that are crucial to cancer progression, including angiogenesis and metastatic spread. These cellular processes are mediated by a gamut of key molecules in overlapping and distinct signal transduction pathways.

Activation of signal transducer and activator of transcription (STAT) occurs when tyrosine residues are phosphorylated by janus kinase (JAK) that initiates dimer formation of STATs (29). The dimers are then translocated into the nucleus, where they are active in gene transcription. However, STAT activation can occur independently of JAKs, for example, as mediated directly by EGFR kinase activity (10). Truncation of the EGFR leads to loss of STAT activation, and specific docking sites for STAT1 and STAT3 in the cytoplasmic domain of

EGFR have been identified as allowing positive and negative regulation of STAT activity (39).

EGFR overexpression is found frequently in esophageal, head and neck, and breast cancers. Induction of STAT signaling has been shown to result from EGFR overexpression in tumors of different origin. STAT3 activation has been shown to increase cell proliferation in vitro and tumor growth rates in vivo (21). This increased proliferation can be abolished by use of dominant-negative mutants of STAT3 and anti-sense oligonucleotides; however, suppression of STAT1 with the same approach has no effect in other systems (15). In certain contexts, STAT1 has been postulated to be a potential tumor suppressor gene because of its growth suppressor functions (5). The effect of STAT1 on cell proliferation and tumorigenesis still requires reconciliation, and the particular cell type and tissue-specific context may be critical.

The phosphatidylinositol-3 kinase (PI3-kinase) pathway can also induce activation of STATs through phosphorylation on Ser727 in response to interferon (IFN)- $\gamma$  stimulation. After stimulation of the IFN receptor, PI3-kinase and Akt are activated. STAT is recruited by the activated JAKs and is phosphorylated on tyrosine, while a kinase downstream of Akt phosphorylates Ser727 of STAT (28).

Akt has been known also to be a downstream target of EGFR signaling. In addition, Akt is an essential element of the PI3-kinase pathway regulating chemotaxis and motility. Initially, recruitment and activation of PI3-kinase at the leading edge of migrating cells results in localized accumulation of phosphatidylinositol 4,5-trisphosphate (PIP<sub>3</sub>) (25). PIP<sub>3</sub> serves as a docking site for a subclass of PH domain-containing proteins. Dissection of this pathway in *Dictyostelium amoebae* identified Akt as an effector required for cell motility (11). Experiments in human squamous cancer cell lines support the important role Akt plays in cell motility, revealing that constitutively active Akt induces epithelial-mesenchymal transition leading to a loss of cell adhesion and increased motility and invasiveness (16). Furthermore, use of dominant-negative mutants of Akt reverses Akt-dependent increased cell motility in fibroblasts (18). That Akt not only increases cell motility but also promotes cancer cell invasion has been shown by recruitment of PI3-kinase and Akt to the leading edge of cells, where Akt kinase activity increases matrix metalloproteinase (MMP)-9 production (22). These observations are corroborated by the finding that Akt activates MMP-2 (30).

This led us to investigate the involvement of the PI3-kinase-Akt pathway in promoting cell migration and, possibly, regu-

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lating STAT activity. This line of investigation has uncovered that the PI3-kinase-AKT pathway contributes partially to cell migration and that the main contribution is through EGFR-mediated recruitment of JAK1/2 to phosphorylate STAT1 and STAT3. In turn, STAT1 and STAT3 form complexes and are translocated to the nucleus with possible direct activation of MMP-1 activity as a critical modulator of cell migration. As a result, we have focused on the specific functional role of JAK1/2 and STAT1 and STAT3 in mediating EGFR effects on cell migration.

## MATERIALS AND METHODS

**Cell lines.** Primary esophageal keratinocytes, designated as EPC2, from normal human esophagus were established and infected with filtered (0.45- $\mu$ m pore size) retroviral supernatant from an overnight culture of Phoenix-Ampho cells, producing the pFB-neo retroviruses encoding EGFR or green fluorescent protein (GFP) as described (1). EPC2 cells were grown at 37°C and 5% CO<sub>2</sub> with keratinocyte serum-free media, with 40  $\mu$ g/ml bovine pituitary extract, 1.0 ng/ml EGF, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

When cells were starved, the media used was keratinocyte basic medium (KBM) and supplemented with 0.5  $\mu$ g/ml hydrocortisone and 0.09 mM calcium chloride (BioWhittaker, Walkersville, MD). Cells were starved for 48 h without EGF and then stimulated with 10 ng/ml EGF for designated time periods at 37°C, washed three times with ice-cold PBS, lysed, and centrifuged for 15 min at 4°C.

Pharmacological inhibitors were added during culture in the following concentrations: 100 nM AG-1478, an EGFR inhibitor; 50  $\mu$ M AG-490, a JAK inhibitor; and the PI3-kinase inhibitors LY-294002 at 10  $\mu$ M and wortmannin at 50 nM, all purchased from Calbiochem (La Jolla, CA); and 50  $\mu$ M Fludara, a STAT1 inhibitor (Berlex, CA).

**Antibodies.** An antibody against EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), as were antibodies against JAK1 and JAK2. Antibodies against phospho-EGFR (Y1173), Akt1, phospho-STAT1 (Ser727), JAK1, and JAK2 were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies specific for Akt phosphorylated at Ser473, phospho-Tyr701 STAT1, and phospho-Tyr705 STAT3 were obtained from Cell Signaling (Beverly, MA). Antibodies against STAT1, STAT3, and PI3-kinase were obtained from Transduction Laboratories (Lexington, KY). Anti-mouse and -rabbit horseradish peroxidase-conjugated antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**Immunoprecipitation.** Preconfluent cells, starved in KBM and stimulated with EGF (10 ng/ml) for 15 min with or without inhibitors, were washed with PBS and incubated with 700  $\mu$ l of lysis buffer [1% Triton X-100, 1% Nonidet P-40, 50 mM Tris, pH 8, and proteinase inhibitors 2  $\mu$ g/ml Aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM Na-pyrophosphate] for 30 min on ice. Seventy microliters of 4% BSA and 140  $\mu$ l of 1.5 M NaCl were added to the extracts, which were then preabsorbed with 10  $\mu$ l of rProteinG agarose (GIBCO, Gaithersburg, MD) for 1 h at 4°C. Preabsorbed extracts were incubated with antibodies against STAT1, STAT3, JAK1, and JAK2. After a 1-h incubation at 4°C, the antigen-antibody complex was incubated with 10  $\mu$ l of rProteinG agarose for 1 h at 4°C. The precipitates were washed three times with 1 ml of wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) and boiled with 100  $\mu$ l of lithium dodecyl sulfate (LDS) buffer (Invitrogen, Carlsbad, CA) containing DTT for 10 min. Supernatants were used for Western blot analysis as described in *Western blot analysis*. Experiments were performed in triplicate.

**Nuclear and cytoplasmic extracts.** To generate whole cell lysates, preconfluent cells, starved in KBM and stimulated with 10 ng/ml EGF for the indicated time points, were washed with PBS and lysed in harvest buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose,

0.1 mM EDTA, 0.5% Triton X-100, 100 mM DTT). Cytoplasmic/nuclear extracts were prepared by dousing cells in *buffer A* (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) and sedimentation of the nuclei at 14,000 rpm for 15 min. The supernatant containing the cytoplasmic/membrane extract was removed, and the pellet was resuspended in *buffer C* (10 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT) to extract nuclear proteins.

**Western blot analysis.** Subconfluent cells were lysed in lysis buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, and a protease inhibitor mixture tablet, Roche Molecular Biochemicals, Indianapolis, IN). All inhibitors were added to the culture 12 h before harvesting to maintain the conditions employed for the migration assays. Protein concentration was determined by protein assay (Bio-Rad, Hercules, CA). The solution was subsequently solubilized in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) containing 50 mM DTT. Total protein samples (10  $\mu$ g) were separated on a 4–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked in 5% nonfat milk (Bio-Rad) in TBS (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) for 1 h at room temperature. Membranes were probed with primary antibody diluted 1:1,000 in 5% milk in TBS overnight at 4°C, washed three times in TBS-Tween 20, incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:3,000 in TBS for 1 h at room temperature, and then washed three times in TBS. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus, Amersham Pharmacia Biotech) and was exposed to Kodak-X-Omat LS film (Kodak, New York, NY). Experiments were repeated twice.

**Immunofluorescence.** EPC2-GFP and EPC2-EGFR cells, cultured in chamber slides (Nalge Nunc, Naperville, IL) in KBM starvation medium with and without inhibitors, were stimulated with 10 ng/ml EGF at the indicated time points. Cells were fixed in 4% paraformaldehyde (Fisher) for 10 min at room temperature. After fixation, cells were treated with 0.1% Triton X-100 in PBS without calcium and magnesium for 10 min. Objects were washed in PBS and blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) for 1 h. Incubation with primary antibodies was overnight at 4°C. After being washed with PBS, cells were incubated with Texas Red-conjugated secondary antibody (Molecular Probes, Eugene, OR) or Cy2-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h. Stained cells were examined with a Nikon microscope and imaged with a digital camera at indicated magnifications.

**Cell migration assay.** Cell migration assays were performed using 24-well inserts (Falcon cell culture inserts, 8- $\mu$ m pore size, BD, Franklin Lakes, NJ) according to the manufacturer's instructions. In brief, the lower chamber was filled with 0.6 ml of KBM containing 0.5  $\mu$ g/ml hydrocortisone with 10 ng/ml EGF, whereas a 0.5-ml cell suspension in KBM under serum-starving conditions was plated in the upper chamber in duplicate or triplicate wells and incubated at 37°C for 12 h. For inhibition studies, inhibitors were added during the time of incubation at the concentrations indicated above. Then, cells attached to the upper side of the membrane were removed gently with a cotton swab and rinsed. Cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with reagents from the Diff Quik staining set (Dade Behring, Newark, DE). Membranes were cut out and photographed such that migrated cells could be counted. There was no evidence of cell death. All experiments were performed at least three times in triplicate. Student's *t*-tests were performed, and  $P < 0.01$  was considered statistically significant.

**Collagen dissolution assay.** MMP-1 activity was measured by its ability to dissolve type 1 collagen as described previously (2). In brief, six-well cell culture plates were coated with 300  $\mu$ g/ml collagen type 1 (BD),  $6 \times 10^5$  cells were applied to the center of the collagen matrix

and incubated for 3 h before the medium was added, and plates were incubated for 2–6 days. After removal of cells with 0.25% Trypsin (GIBCO Life Technologies, Grand Island, NY), the collagen gel was stained with 0.2% Coomassie for 30 min. All experiments were performed at least three times in triplicate.

**Real-time confocal microscopy.** EPC2-GFP and EPC2-EGFR cells infected with GFP-Grp1 PH adenovirus (gift of M. Birnbaum, University of Pennsylvania) were maintained in the TC3 open dish system, which consists of individual 0.15-mm-thick tissue culture dishes optimized for use with oil-immersion microscope objectives, an objective heater, and a stage warmer to maintain the living cells at 37°C throughout the experiment (Bioptechs, Pittsburgh, PA). Images were acquired as described previously (38) using an Ultraview LCI Nipkow disc confocal microscope (Perkin-Elmer) attached to a Nikon model TE300 inverted microscope fitted with a  $\times 60$  oil-immersion objective. The GFP-Grp1 PH was visualized using the 488-nm line of an Argon laser, and the combination set of dichroic mirror (488 nm) and emission barrier filter (cutoff at 510 nm) was optimized to collect the GFP signal (Yokogawa). Images were collected at 7.5-s intervals before and after the addition of 10 ng/ml EGF. Recruitment of GFP-Grp1 PH to the plasma membrane was measured in response to EGF stimulation by GFP-Grp1 PH translocation and in vivo PIP<sub>3</sub> production.

## RESULTS

**JAK inhibitor AG-490 abolishes migration of EPC2-EGFR cells.** We wanted to investigate which signaling pathway, PI3-kinase-Akt and/or JAK-STAT, may mediate the enhanced cell migration in EGFR-overexpressing cells (EPC2-EGFR). We used canonical PI3-kinase-Akt and JAK-STAT pathway inhibitors to determine their effects on cell migration in EPC2-EGFR cells. LY-294002 and wortmannin are two widely used inhibitors of PI3-kinase. AG-490 is a JAK-specific inhibitor, with a potential preference for JAK2 (26). Fludara is a reported STAT1-specific inhibitor (12). The transwell-migration assay is a useful tool that demonstrated increased migration of EPC2-EGFR cells compared with GFP-expressing control cells (Fig. 1). However, because GFP control cells also express EGFR, it is expected that the inhibitors will have some effect on GFP control cells. In this assay, the different inhibitors were added during the total incubation time to test their effects on cell migration of EPC2-EGFR cells. The JAK inhibitor was the most potent, essentially abolishing migration. Importantly, addition of AG-490 had no effect on the viability of the cells. They were not growth arrested and did not exhibit signs of apoptosis (data not shown). Inhibitors of the PI3-kinase pathway only reduced migration of EPC-EGFR cells by ~50% (Fig. 1), with different specificity between LY-294002 and wortmannin. Fludara was not able to abolish migration to the same extent as the JAK inhibitor but reduced cell migration to about one-third (Fig. 1). This led us to postulate that the JAK-STAT pathway may be preferred in regulating the increased cell migration of EPC-EGFR cells.

**PI3-kinase and JAK inhibitors suppress activity of downstream molecules in EPC-EGFR cells.** To test the ability of the various inhibitors to efficiently block PI3-kinase-Akt and JAK-STAT signaling, we performed Western blot analysis using cell extracts from EPC-EGFR cells after incubation with inhibitors for PI3-kinase, JAK, and STAT1 (Fig. 2). Our laboratory has demonstrated previously (1) that EGFR overexpression induces phosphorylation of STAT1 after stimulation with EGF when compared with GFP control cells. Akt phosphory-

lation on Ser473 is elevated in EGFR-overexpressing cells, even in the absence of EGF, compared with GFP control cells that show no baseline phosphorylation before EGF stimulation. The use of the two different inhibitors of PI3-kinase activity, 10  $\mu$ M LY-294002 and 50 nM wortmannin, prevents phosphorylation of Akt on Ser473 in EPC-EGFR cells, which should render Akt inactive. In addition, Fludara, a putative STAT1-specific inhibitor decreases Akt Ser473 phosphorylation in a manner that is unknown, whereas total Akt levels remain constant under the different conditions tested. Use of 50  $\mu$ M AG-490, the JAK inhibitor, prevents JAK phosphorylation and phosphorylation of STAT1 on Tyr701 after EGF stimulation. Total STAT1, JAK1, and JAK2 levels were used as internal controls and remain essentially unchanged. Tyrosine phosphorylation of STAT is believed to be mediated by tyrosine receptor kinases or JAKs, whereas serine phosphorylation is induced by serine/threonine kinases such as Akt in INF- $\gamma$  signaling. STAT1 is activated through phosphorylation on Ser727 in response to EGF stimulation. LY-294002 and wortmannin prevent phosphorylation of STAT1 on Ser727. In addition, wortmannin specifically suppresses EGF-induced phosphorylation of STAT1 on Tyr701. Fludara, although described to be a STAT1-specific inhibitor (12) in lymphocytes and used at the same concentration (50  $\mu$ M) in our study, does not appear to be efficient in the suppression of STAT1 phosphorylation in our cell system, although it reduced cell migration in the transwell assay. With higher concentrations of Fludara, we observed a dose-dependent decrease in STAT1 phosphorylation and further inhibition of cell migration (data not shown), although it did not reach the same effectiveness as described for lymphocytes.

**Increased activity of PI3-kinase at the cell membrane in EPC-EGFR cells as measured by PIP<sub>3</sub> production.** In experiments with *Dictyostelium* and fibroblasts, it has been described that PI3-kinase localizes to the leading edge where it recruits

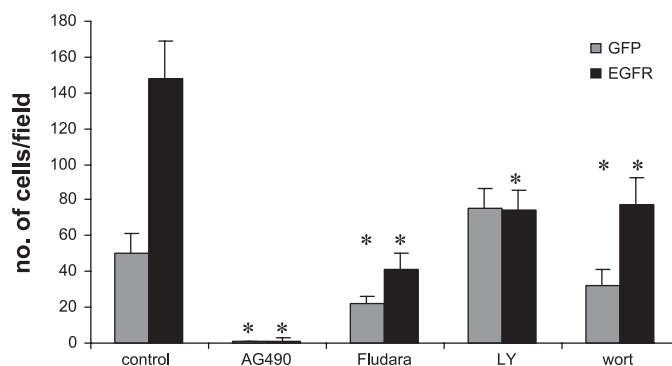


Fig. 1. Janus kinase (JAK) inhibition abolishes cell migration. Migration assays performed in transwell plates demonstrated that AG-490, a JAK-specific inhibitor, abolishes migration of endothelial progenitor cell (EPC)-epithelial growth factor receptor (EGFR) cells as well as EPC-green fluorescent protein (GFP) control cells. Fludara, a signal transducer and activator transcript (STAT) 1 inhibitor, partially reduces migration in both cell lines. At the same time, inhibitors of the phosphatidylinositol 3-kinase (PI3-kinase) pathway, LY-294002 and wortmannin, decreased migration by 50%. Inhibitors were present during the 12-h incubation time at concentrations described in MATERIALS AND METHODS. Student's *t*-test was carried out comparing EPC-EGFR cells treated with inhibitors vs. EPC-EGFR cells without inhibitors. Similar comparisons were done with EPC-GFP control cells treated with inhibitors vs. EPC-GFP cells without inhibitor. \*Significantly different from control ( $P < 0.001$ ).

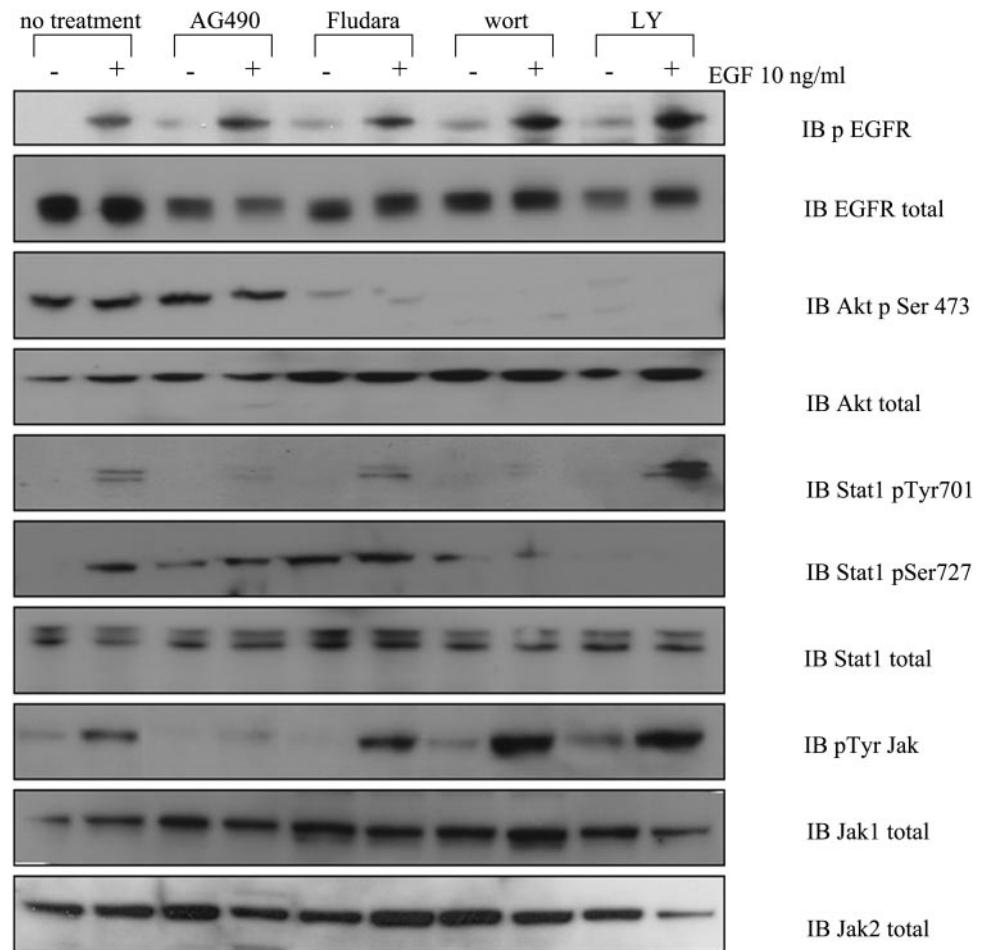


Fig. 2. Effects on EPC-EGFR cells with PI3-kinase and JAK/STAT inhibitors. Western blot analysis of EPC-EGFR cells treated without inhibitor and with inhibitors for 12 h at concentrations described in experimental procedures against the PI3-kinase-Akt and JAK-STAT pathways were performed to test their effectiveness in suppressing activation of these targets. Phosphorylation of EGFR was not inhibited by any of the inhibitors used. Wortmannin and LY-294002 inhibitors prevent Akt phosphorylation at Ser473 as does Fludara, a STAT1-specific inhibitor, whereas total Akt levels remain unchanged. AG-490, a JAK-specific inhibitor, and wortmannin decrease STAT1 phosphorylation at Tyr701, whereas wortmannin and LY-294002 prevent STAT1 phosphorylation on Ser727. Fludara seems to have minimal effect on Tyr phosphorylation of STAT1. Total STAT1 expression remains constant, and total JAK1 and JAK2 remain essentially unchanged. After the membrane was probed with phosphotyrosine antibody to determine the phosphorylation status of JAK, the membrane was stripped of signal, and total JAK levels were determined.

Akt resulting in an amplification of the signal to migrate (11, 25). Furthermore, a role for JAK and STAT1 for these processes in *Drosophila* could be demonstrated (3, 33). During oocyte migration, STAT3 could be found directly at the cell membrane where it colocalizes with PI3-kinase and Akt (20). We wanted to investigate PI3-kinase activity in our cell system with real-time confocal microscopy to determine whether the PI3-kinase-Akt pathway could be regulating STAT activity or whether there is even evidence for cross talk between the PI3-kinase-Akt and JAK-STAT pathways.

Activation of PI3-kinase generates increased 3'-phosphoinositides at the cell membrane. PIP<sub>3</sub> and phosphatidylinositol 4,5-diphosphate bind to the PH domain of Akt and recruit it to the plasma membrane. Use of an adenovirus encoding for the grp1 PH domain and GFP enables us to measure PI3-kinase activity in EGFR-overexpressing EPC cells compared with parental cells (Fig. 3A). After transduction with 10 MOI adenovirus expressing the GFPgrp1PH fusion protein (Ad5GFP grp1PH), we starved the cells overnight and stimulated them with 10 ng/ml EGF. Before stimulation, the GFP signal can be detected in the cytoplasm and the nucleus, the latter due to a cryptic nuclear localization signal. After stimulation with EGF, the fusion protein is translocated to a greater extent to the cell membrane in EGFR-overexpressing cells (white arrows in Fig. 3B, and supplemental data at <http://ajpgi.physiology.org/cgi/content/full/00253.2004/DC1>).

*Inhibition of the JAK-STAT pathway abolishes MMP-1 activity.* As previously shown by our laboratory (1), EGFR overexpression in primary esophageal keratinocytes induces upregulation of MMP-1 mRNA and enhanced secretion of MMP-1 compared with GFP-expressing control cells. This effect is specific to MMP-1 and not other MMPs. To link MMP-1 overexpression in response to EGFR activation and enhanced cell migration, we performed migration assays in the presence of two different MMP inhibitors, designated MMP inhibitor-II and MMP inhibitor-III (data not shown). The MMP inhibitors inhibit a broad spectrum of MMPs with different specificities and IC<sub>50</sub>. MMP inhibitor-III (suppresses MMP-1, MMP-2, MMP-3, MMP-7, and MMP-13) appears more effective in suppressing MMP-1-mediated migration than MMP inhibitor-II (suppresses MMP-1, MMP-3, MMP-7, and MMP-9). Using different concentrations of MMP inhibitor-III, we could demonstrate a dose-dependent decrease in migration, although the level of reduced migration was modest (data not shown). It is conceivable that the functional effects of MMP-1 activation may have been initiated already or progressed sufficiently, such that pharmacological inhibition is only partial.

MMP-1 is a collagenase, and its specific activity can be detected by plating cells on a type I collagen matrix (2). However, MMP-1 cannot be detected using zymography. To test our hypothesis that increased migration of EPC-EGFR cells is due to MMP-1 activity, we used the collagen dissolu-

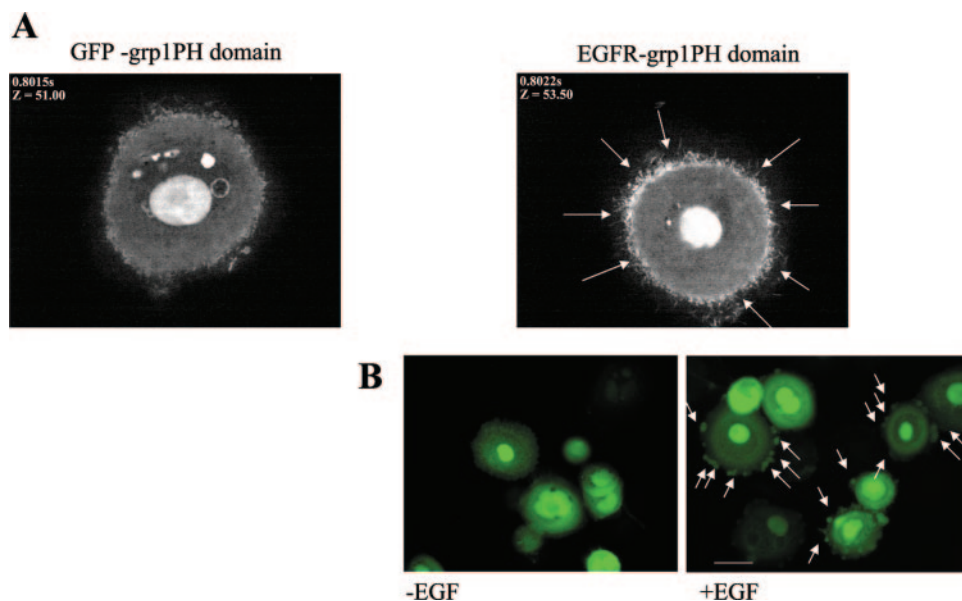


Fig. 3. PI3-kinase/Akt activity at the cell membrane in EPC-EGFR cells. Still photographs of real-time confocal microscopy demonstrate stronger PIP<sub>3</sub> production at the cell membrane of EGFR-overexpressing cells compared with parental or control cells (A) infected with the GFP-grp1 virus. After EGF stimulation, the GFP signal increases at the site of PI3-kinase activity (B; arrows).

tion assay that enables us to assay MMP-1 activity. Analysis of cells treated with inhibitors of different key pathways during their growth on collagen matrices permits us to gain insights into which signaling pathway may regulate MMP-1 activity. After 5 days, the cells were removed by trypsin treatment and the collagen was stained with Coomassie blue. In wells where MMP-1 digested the collagen, white halos can be observed, whereas in wells in which addition of the inhibitors prevented collagen dissolution by MMP-1, the white halos were absent. The addition of AG-490, the JAK inhibitor, prevents the dissolution of collagen, confirming that MMP-1 activity is completely abolished by the JAK inhibitor (Fig. 4). At the same time, use of the EGFR inhibitor AG-1478 and the STAT1 inhibitor Fludara suppresses collagen dissolution by MMP-1. EPC-EGFR cells without inhibitor and EPC-EGFR cells growing in the presence of PI3-kinase inhibitors reveal MMP-1

activity as detected by the presence of collagen dissolution (Fig. 4, small black arrows). EPC-GFP cells do not exhibit MMP-1 activity, demonstrating that MMP-1 activity is induced specifically by EGFR overexpression. The suppression of MMP-1 activity with JAK and STAT inhibitors leads us to believe that the JAK-STAT pathway is crucial in regulating MMP-1 activity and that the PI3-kinase-Akt pathway has little or no effect on MMP-1-mediated cell migration.

*EGFR activates STAT through complex formation with JAK1 and JAK2.* We hypothesized that EGFR leads to the activation of STAT1, possibly mediated by JAKs, and thereby induces a signaling cascade that results in MMP-1 activation. To answer the question of whether EGFR interacts with STAT or whether JAKs are involved in the activation of STAT, we performed immunoprecipitations with antibodies against STAT1, STAT3, JAK1, and JAK2. In IFN- $\gamma$  signaling, acti-

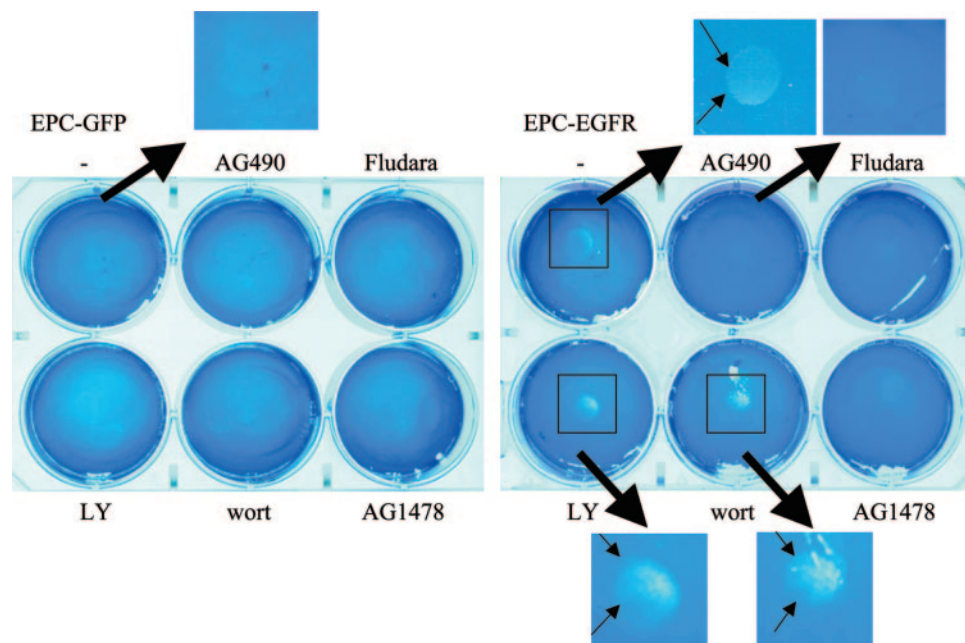


Fig. 4. JAK and STAT are necessary for matrix metalloproteinase (MMP)-1 activity. EPC-GFP and EPC-EGFR cells are grown on collagen matrices in the presence and absence of inhibitors. EPC-GFP cells have no MMP-1 activity. EPC-EGFR cells demonstrate MMP-1 activity, as detected by collagen dissolution or white halos (small black arrows), which is not suppressed by PI3-kinase inhibitors. AG-490, the JAK inhibitor, Fludara, and AG-1478, the EGFR inhibitor, block collagen dissolution mediated by MMP-1. wort, Wortmannin.

vation of JAK1 and JAK2 is known to phosphorylate STAT1 as well as STAT3 on tyrosine residues. We can show here that EGFR forms a complex with STAT1 and STAT3 in EPC-EGFR cells after stimulation with EGF (Fig. 5A, arrow). The enhanced signal found for STAT1 and STAT3 after complex formation induced by EGF stimulation could imply that there is heterodimerization. Phosphorylation of STAT1 on Tyr701 and STAT3 on Tyr705 appears necessary, since the interaction is only observed in EPC-EGFR cells after EGF stimulation.

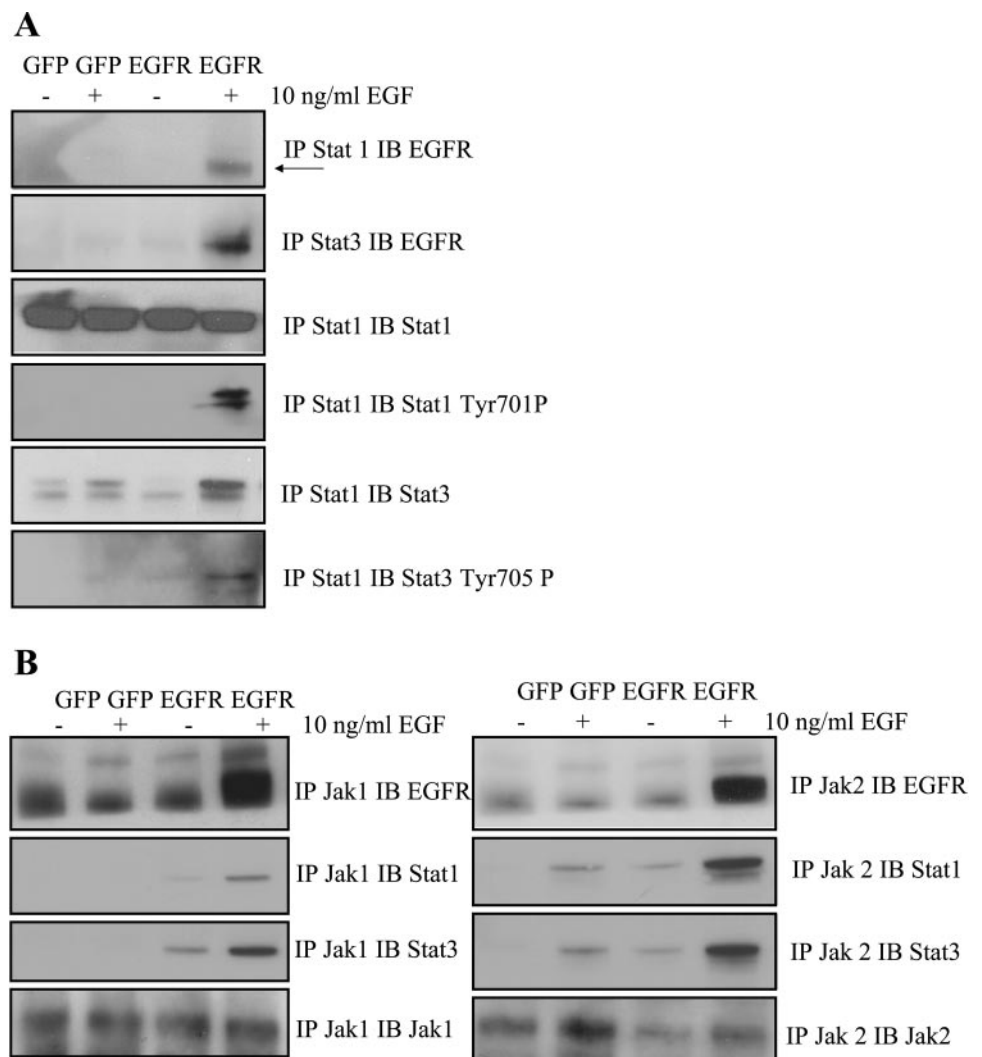
Immunoprecipitation with antibodies against JAK1 and JAK2 demonstrates that complex formation of JAK1 with STAT1 and STAT3 occurs exclusively in EPC-EGFR-overexpressing cells after stimulation with EGF. Although EPC-EGFR is present in a complex with JAK1 and JAK2 in GFP control cells and before EGF stimulation, the recruitment of STAT1 and STAT3 to this complex is only observed after EGF stimulation in EPC-EGFR cells (Fig. 5B).

**Inhibition of JAK activity suppresses EGFR-JAK-STAT complex formation.** To prove that the observed complex formation is indeed initiated by EGFR and mediated by the JAK-STAT pathway, we performed coimmunoprecipitations in the presence of EGFR, JAK, and STAT1 inhibitors (Fig. 6). The presence of JAK inhibitor AG-490 prevents complex

formation of EGFR and STAT1/STAT3. In addition, there is loss of complex formation after use of the EGFR inhibitor AG-1478, whereas the STAT1 inhibitor Fludara only reduces complex formation partially (Fig. 6A). This shows that JAK is essential in the recruitment of STATs to the EGFR-induced complex formation, but inhibition of EGFR prevents complex formation of STAT1 and STAT3. The JAK inhibitor and the EGFR inhibitor prevent JAK1 and JAK2 tyrosine-phosphorylation, demonstrating that EGFR activity is necessary to activate JAK1 and JAK2 (Fig. 6B), whereas JAK phosphorylation, in turn, is necessary to recruit STATs to the complex.

**pSTAT1 and pSTAT3 translocate to the nucleus after EGF stimulation.** Phosphorylation of STATs induces dimer formation and initiates their translocation to the nucleus. To analyze the kinetics of this event after EGF stimulation in our cell system, we utilized cytoplasmic/nuclear extractions of EPC-GFP and EPC-EGFR cells to document the translocation of the STAT dimer (Fig. 7A). Even before stimulation with EGF, EPC-EGFR cells have some basally activated STAT1 and STAT3 in the cytoplasmic fraction when compared with EPC-GFP cells that show no signal in Western blot analysis with antibodies against pSTAT1 and pSTAT3 (Fig. 7A). After 5 min of EGF stimulation, the total pool of activated STAT1 and

Fig. 5. A: complex formation of EGFR with STAT1 and STAT3 after stimulation in EPC-EGFR cells. Immunoprecipitation with antibodies against STAT1 and STAT3 demonstrates the presence of EGFR in a complex with STAT1 and STAT3 and interaction between STAT1 and STAT3 only after EGF stimulation of EPC-EGFR cells. This complex formation occurs as STAT1 is phosphorylated on Tyr701 and STAT3 is phosphorylated on Tyr705 after EGF stimulation of EPC-EGFR cells. B: JAK1 and JAK2 are found in the EGFR-STAT complex after EGF stimulation in EPC-EGFR cells. Immunoprecipitation with JAK1 and JAK2 antibodies shows weak complex formation with EGFR in EPC-GFP cells as well as EPC-EGFR cells. However, the complex formation is enhanced after stimulation with EGF of EPC-EGFR cells, and STAT1 and STAT3 are increasingly coprecipitated in EGF stimulated EPC-EGFR cells. Total JAK1 and JAK2 levels remain essentially unchanged. IP, immunoprecipitation.



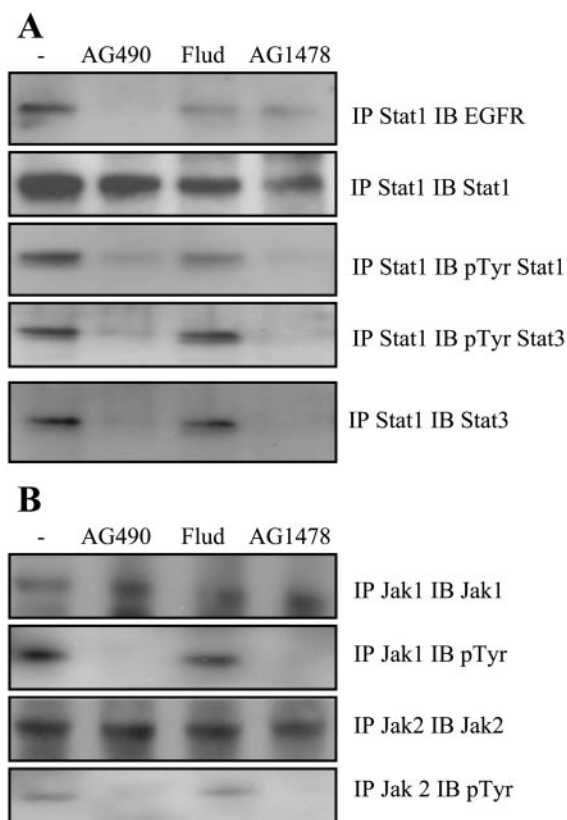


Fig. 6. *A*: inhibition of complex formation with AG-490. Coimmunoprecipitation with antibodies against STAT demonstrates that, in the presence of the JAK inhibitor AG-490 and the EGFR inhibitor AG-1478, complex formation is abolished, whereas Fludara, the inhibitor for STAT1, reduces the complex formation. *B*: inhibition of JAK1 and JAK2 tyrosine phosphorylation with AG-490 and AG-1478. Immunoprecipitations of JAK1 and JAK2 show comparable levels of JAK1 and JAK2 with the different inhibitors. Tyrosine phosphorylation is inhibited by the Jak inhibitor AG-490 and by the EGFR inhibitor AG-1478 but not by Fludara, the STAT1 inhibitor.

STAT3 has translocated from the cytoplasm to the nucleus in EPC-EGFR cells, whereas EPC-GFP cells have only a very weak signal at this time point. The delayed translocation of pSTAT1 and pSTAT3 in EPC-GFP cells results in a strong signal in the nuclear fraction at 30 min. Although phosphorylated forms of STAT1 and STAT3 can be detected in the nucleus of EPC-EGFR cells 5 min after stimulation, the strongest signals are detected after 15 min, and there is sustained activity up to 30 min (Figure 7B), indicating that EGFR overexpression facilitates and hastens the nuclear translocation of pSTAT1 and pSTAT3.

To further dissect the kinetics of the STAT1/STAT3 translocation in EGFR-overexpressing cells, we extracted cytoplasmic/nuclear fractions at time points of 5, 15, 30, and 60 min (Fig. 7B). At 5 min, STAT1 and STAT3 are phosphorylated at their respective tyrosine residues, and translocation to the nucleus has already occurred. Translocation is complete after 15 min when the strongest signal for activated STAT1 and STAT3 can be detected in the nucleus after 30 min of EGF stimulation. At 60 min, the signal is very weak and the activation cycle appears to have been completed. The parallel kinetics of pSTAT1 and pSTAT3 and the data obtained with the coimmunoprecipitation of STAT1 and STAT3 in the pres-

ence of EGFR lead us to conclude that STAT1 and STAT3 could form heterodimers.

To analyze the translocation of STATs in situ, EPC-GFP and EPC-EGFR cells were starved overnight and stimulated with EGF for the same time periods as indicated above. Cells were fixed in paraformaldehyde, and double immunofluorescence was performed to localize total STAT1, STAT3, and phosphorylated forms of STATs (Fig. 8). Phosphorylated STATs are detected by use of a Texas Red-conjugated antibody, and thus appear as red signals, whereas total STATs are detected with a green signal due to a Cy2-conjugated antibody. Before EGF stimulation, anti-pSTAT1 and anti-pSTAT3 yield a diffuse signal in GFP cells with little signal in the nucleus. EPC-EGFR cells exhibit strong staining for the phosphorylated forms at the cell membrane and in the cytoplasm with some staining in the nucleus (data not shown). In accordance with the cytoplasmic/nuclear extraction data, pSTAT1 and pSTAT3 can be localized almost exclusively to the nucleus at 15 min after EGF stimulation (Fig. 8). Stainings for total STAT1 and total STAT3 demonstrate similar levels in the cytoplasm and the nucleus.

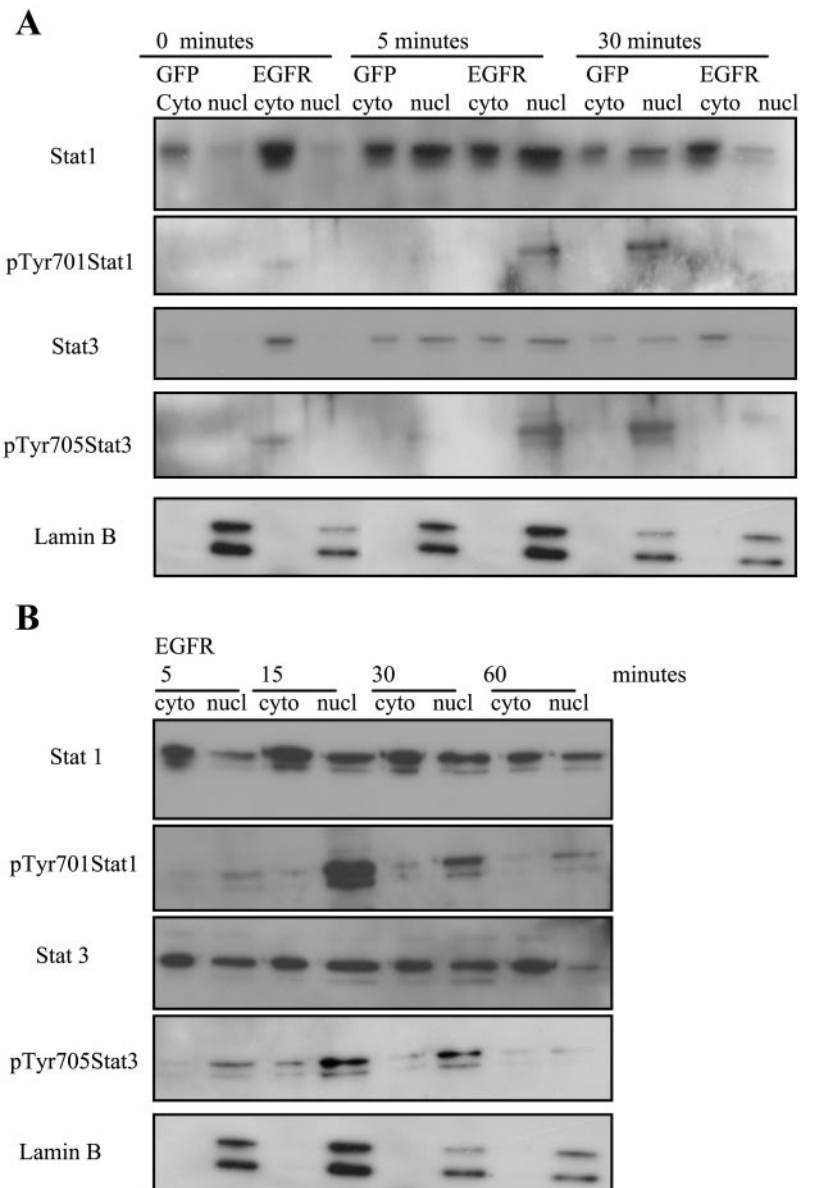
## DISCUSSION

Cell migration is a critical cellular function that is important for normal cellular homeostasis but necessary for tumor cells to invade through the extracellular matrix. Insights into underlying molecular mechanisms have been gained through investigation of lower organisms.

The PI3-kinase pathway has recently been implicated in cell migration in *Drosophila melanogaster* and *Dictyostelium amoebae*. In this context, cell migration is a response to chemotactic signals. Exposure to a chemotactic gradient induces activation of PI3-kinase and the accumulation of its lipid product PIP<sub>3</sub> to the leading edge (13, 32, 37). Our observation that Akt phosphorylation occurs in EPC-EGFR cells at a high basal level without EGF stimulation, compared with GFP control cells, led us to investigate the role of this particular pathway in our model system. Real-time confocal microscopy confirmed the enhanced PI3-kinase activity and demonstrated increased PIP<sub>3</sub> production in EGFR-overexpressing cells compared with parental cells, as well as spiking and blebbing at the cell membrane after EGF stimulation (see supplemental data at <http://ajpgi.physiology.org/cgi/content/full/00253.2004/DC1>). However, experiments using inhibitors against the PI3-kinase-Akt pathway led us to conclude that the PI3-kinase pathway, although active in our cell system, has only a partial effect on cell migration and no effect on MMP-1 activity. Furthermore, in mammalian cells, the activation of the PI3-kinase-AKT pathway leads to remodeling of the cytoskeleton, which in turn influences cell migration through mechanisms independent of MMP-1 activation (11).

Although we found STAT1 activation by Akt through phosphorylation on Ser727 in response to EGF stimulation as observed also in the IFN- $\gamma$  pathway (28), this activation did not appear to be necessary for MMP-1 activation and modulation of cell migration. This led us to focus upon the interaction of EGFR with the JAK-STAT pathway. Observations by other groups (9, 39) that the EGFR cytoplasmic tail contains docking sites for STAT1 and can directly regulate STAT1 activity prompted us to postulate that there is direct interaction of EGFR with STAT1 and STAT3. In addition, experiments with

Fig. 7. *A*: cytoplasmic fractions contain more STAT1/3 in EPC-EGFR-overexpressing cells compared with EPC-GFP cells. Western blot analysis of cytoplasmic (cyto) and nuclear (nucl) fractions from EPC-GFP and EPC-EGFR cells were performed using anti-STAT1 and anti-STAT3 antibodies to detect total STAT, and anti-phospho-STAT1 and anti-phospho-STAT3 antibodies to analyze activated STAT1 and STAT3 in their respective fractions. At *time 0*, STAT1 and STAT3 are found in the cytoplasm with almost no proteins detected in the nuclear fraction. EPC-EGFR cells not only have more STAT1 and STAT3 in the cytoplasmic fraction before stimulation with EGF than GFP cells, but also pSTAT1 and pSTAT3 antibodies demonstrate that the STATs are activated. After 5 min of EGF stimulation, the amount of total STAT1 and STAT3 in the cytoplasmic fraction is equal to the nuclear fraction, but a shift of activated STATs from the cytoplasmic to the nuclear fraction occurred in EPC-EGFR cells. After 30 min of EGF stimulation, pSTAT1 and pSTAT3 can now be detected in the nuclear fraction of EPC-GFP cells, demonstrating a delayed response to EGF stimulation. Lamin B serves as a control for fractionation of the cytoplasmic and nuclear extracts. *B*: phosphorylated STAT dimers translocate to the nucleus after EGF stimulation. Western blot analysis of cytoplasmic and nuclear fractions of EPC-EGFR cells were stimulated with EGF for 5, 15, 30, and 60 min. Although the total STAT1 and STAT3 amount stays constant from 5 to 30 min after stimulation and is equal in both fractions, their levels decrease at 60 min in the nuclear fraction. Activated STAT1 and STAT3 are detected by anti-phospho-STAT1 and anti-phospho-STAT3 antibodies and are translocated to the nucleus 15 min after stimulation and with sustained activity for 30 min after EGF stimulation. Thereafter, the signal for the activated STATs decreases.



EGFR-overexpressing cells defective in JAK1 and JAK2 have revealed that EGFR-induced phosphorylation activates JAKs but that JAKs are not necessary for STAT activation as measured by c-fos induction (23). Instead, only a kinase-dead mutant of EGFR abolished STAT activation consistent with a JAK-independent pathway in which the intrinsic kinase domain of the EGFR is crucial (23). Earlier *in vitro* kinase studies using EGFR, JAK1, JAK2, and STAT1 purified from insect cells demonstrated STAT1 phosphorylation on Tyr701 by JAK1 and JAK2 but not by a catalytic inactive mutant of JAK2. However, EGFR alone could also activate STAT1 and its DNA-binding activity *in vitro* (31). Tyrosine phosphorylation of JAK1 is not necessary for STAT activation by EGFR and amphiregulin (10). By contrast, our data demonstrate the novel finding that JAKs are essential to mediate interaction of EGFR with STAT1 and STAT3. Complex formation of JAKs with STAT1 and STAT3 initiates a signaling cascade that involves tyrosine phosphorylation of STAT1 and possible

dimerization with STAT3, leading to translocation into the nucleus after 15 min.

Given the fact that STAT1 plays a role in growth restraint (7), whereas STAT3 is frequently constitutively activated in a variety of cancers in response to EGFR and is sufficient for cellular transformation (4, 8), questions have been raised by the frequent coactivation of STAT1 and STAT3 by the same ligand and the often observed STAT1-STAT3 heterodimer. EGFR-dependent activation of STAT3, but not STAT1, has been described for squamous cell carcinoma (15). By contrast, we observed coactivation of STAT1 and STAT3 as a consequence of EGFR-JAK complex formation with subsequent translocation of STAT1/STAT3 from the cytoplasm to the cell nucleus. These findings may point to a balancing effect of STAT1 and STAT3 on each other (6). Indeed, the interplay between STAT1 and STAT3 that is fostered by EGFR and the JAKs may modulate cues to either reside in the native cellular environment or initiate cellular migration. Examination of

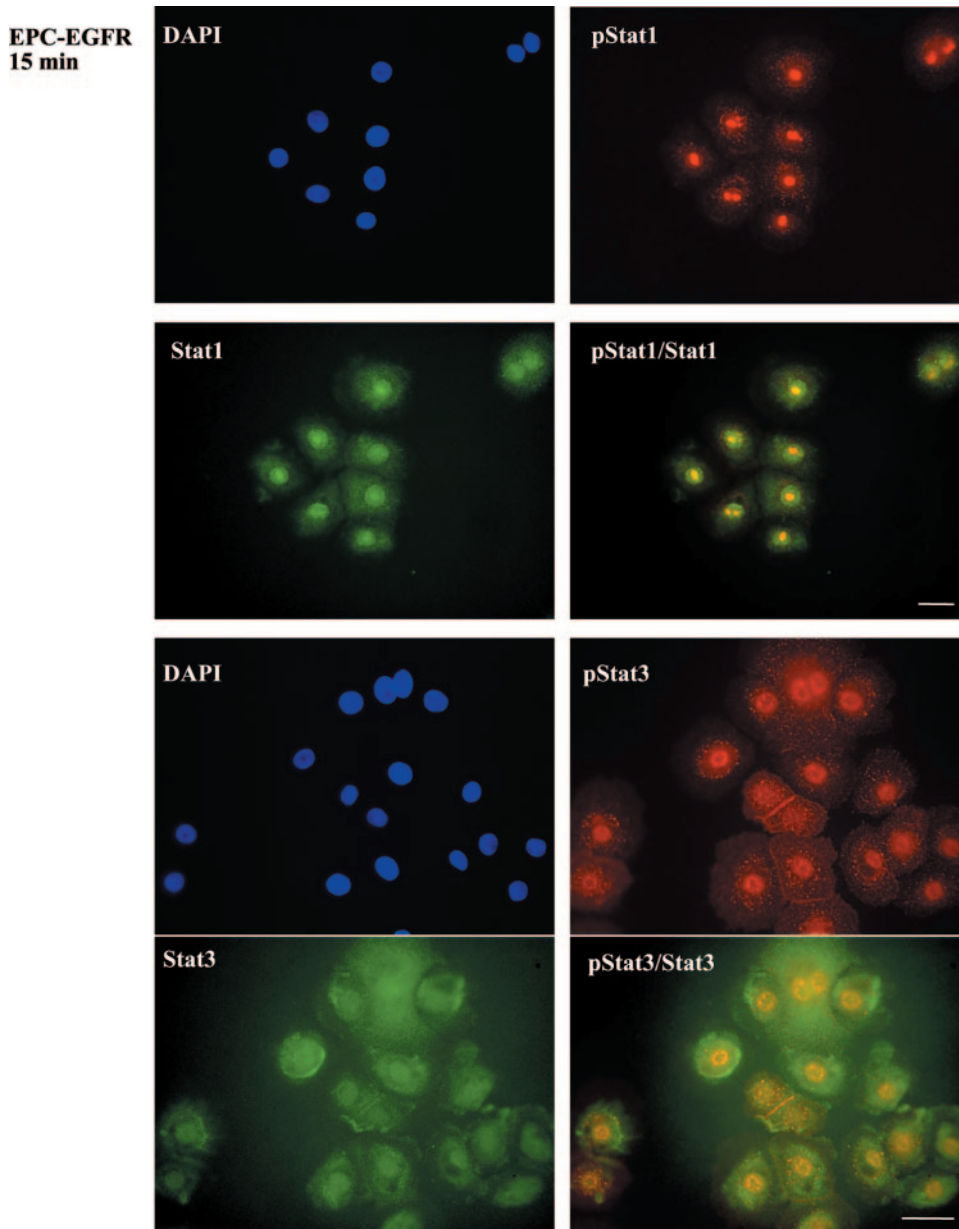


Fig. 8. Double immunofluorescence of EPC-EGFR cells fixed with paraformaldehyde 15 min after EGF stimulation. Cells were stained with antibodies against total STAT1 and STAT3 (green), and the activated forms were detected with antibodies against phospho-STAT1 and phospho-STAT3 (red). After 15 min of EGF stimulation, the activated forms of pSTAT1 and pSTAT3 are localized to the nucleus in EPC-EGFR cells, whereas total STAT1 and STAT3 are equally distributed between the cytoplasm and nucleus. 4,6-diamidino-2-phenylindole (DAPI; blue) serves as a counter stain.

downstream genes regulated by each STAT will be important in evaluating such a possibility.

A role of STATs in cell migration is described in lower organisms such as *Drosophila* (14), namely border cell migration during oogenesis (3), and *Dictyostelium* (20, 27). However, how the STATs are involved in cell migration in mammalian cell migration remains to be elucidated. STAT1 has been identified as a downstream target of focal adhesion kinase, and inactivation of focal adhesion kinase abolished STAT1 activation, correlating with decreased migration (40). In addition, depletion of STAT1 resulted in enhanced cell adhesion and a decrease in cell migration. Phosphorylation of STAT3 in response to VEGFR stimulation results in nuclear translocation and induction of endothelial cell migration (41). A dominant-negative mutant of STAT3 not only abolished nuclear translocation but inhibited endothelial migration completely (41).

The STAT COOH terminus contains an autonomously functioning transcriptional activation domain, and alternatively spliced isoforms of STAT1, STAT3, and STAT4 lacking this domain have attenuated transcriptional activity. Factors shown to bind to STATs include CBP/p300, c-Jun, MCM5, and BRCA-1 (19). STATs also interact with a wide variety of other factors, such as nuclear factor- $\kappa$ B, surfactant protein-1, and SMAD-1. Analysis of the rabbit MMP-1 promoter identified a STAT-binding site in close proximity to the TATA box (36) that upregulates MMP-1 transcription as a result of v-src signaling through STAT. This may lead to activation of the human MMP-1 promoter by STATs.

In mammalian cells, MMPs modulate cell migration through complicated interactions with components of the extracellular matrix, which provides a platform for tumor cell invasion. Unlike classical oncogenes, MMPs are generally not upregulated by gene amplification or activating mutations. The only

two reported alterations in cancer cells are translocation of *MMP23* gene in neuroblastoma (17) and amplification of the *MMP24* gene (24). Thus increased MMP expression in tumors is likely due to transcriptional induction rather than genetic alterations. Transcription of MMP-1 and MMP-13 has been demonstrated to be repressed by the p53 tumor suppressor gene (34, 35). Targeting extracellular factors or their cognate cell-surface receptors, signal transduction pathways, and nuclear factors that activate expression of these genes can inhibit MMP gene transcription. A greater understanding of regulatory mechanisms that control MMP transcription and activation provides new avenues for therapeutic intervention. To that end, abrogation of STAT activation may be a meaningful venue in which to abrogate MMP-1-mediated cell migration and tumor cell invasion.

In summary, we demonstrate in a novel fashion that JAK1 and JAK2 are necessary for activation of STAT1 and STAT3 in response to EGFR overexpression and that this signaling pathway has a novel regulatory function for cell migration and is correlated with MMP-1 activity, whereas the PI3-kinase-Akt pathway is not involved directly and may modulate migration through independent mechanisms.

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