Identification of a Membrane-associated Inhibitor(s) of Epidermal Growth Factor-induced Signal Transducer and Activator of Transcription Activation*

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Yoshiki Iwamoto‡, Yue E. Chin, Xianbu Peng, and Xin-Yuan Fu§

From the Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520

Many growth factors, including epidermal growth factor (EGF), can activate the signal transducer and activator of transcription (STAT) signaling pathway. Here, we demonstrate that STAT activation by EGF treatment is conditional. EGF activates STAT1 and STAT3 in A431 but not in HeLa and PC12 cells. Using a reconstituted in vitro STAT activation system, we have identified and partially purified a potential inhibitor(s) that is membrane-associated and can block STAT activation induced by EGF in vitro. However, this inhibitor has no effect on STAT complexes after they are formed. We have further shown that this inhibitor(s) also exists in many other cancer cell lines, suggesting that blocking the STAT activation during growth factor signal transduction may play a significant role in the development of many kinds of cancers.

Mammalian cell proliferation and differentiation are controlled by cytokines, growth factors, and other polypeptide ligands that may initiate a number of different but interactive signaling pathways. It is well established that cytokines and growth factors can activate a mitogenic pathway involving a protein kinase cascade (reviewed in Refs. 1–3). This cascade links the receptor-associated tyrosine kinase to the Ras protein, then to the downstream serine/threonine kinases, such as the members of the mitogen-activated protein kinase family. The mitogen-activated protein kinases may translocate to the nucleus and phosphorylate transcription factors such as ternary complex factor (TCF) (2, 4).

Parallel to the kinase cascade signaling pathway, a direct signaling pathway from receptors to transcription factors was first revealed in the interferon (IFN) system and then in signal transduction of many other cytokines (reviewed in Refs. 5–7). Expression of IFN-α-induced genes is mediated by a transcriptional complex termed ISGF3 (interferon-stimulated gene factor 3) (8, 9). Purification of ISGF3 led to cloning of p91 and p113 subunits of ISGF3 (10, 11), which were later named as the first two members of the STAT (signal transducer and activator of transcription) family of proteins (5, 12).

STAT proteins contain a conserved SH2 domain and a SH3-like domain (13). The SH2 domain has been shown to mediate the interactions of signaling proteins with the phosphorylated receptor/tyrosine kinases (reviewed in Ref. 3). It was shown that STATs are transiently associated with the tyrosine kinase(s) complex through the SH2 domain after IFN-α treatment (13) and with other cytokine receptors in response to their ligands (14–16). In contrast to the conventional second messenger mechanism and the signaling cascade by Ras-mitogen-activated protein kinases, this direct signal transduction is featured by direct interactions of SH2-containing transcriptional factor STATs with tyrosine-phosphorylated receptor complexes.

The Janus kinase family of tyrosine kinases was initially recognized as activators of STAT proteins in response to many cytokines (17–22). However, a variety of tyrosine kinases, including epidermal growth factor (EGF), platelet-derived growth factor, and insulin receptor tyrosine kinases as well as the Src family of kinases may activate STAT proteins directly and independently of Janus kinases (14, 23–26). Our recent work has further shown that fibroblast growth factor receptor tyrosine kinase, focal adhesion kinase, and many other tyrosine kinases can also directly activate STAT proteins (Ref. 27). Therefore, STAT proteins may play important roles generally in downstream direct signal transduction of a variety of protein-tyrosine kinases. More than six members of the STAT family have been identified, and these STAT proteins mediate signal transduction and gene expression in response to most cytokines and growth factors (28).

Many receptor tyrosine kinases, the EGF receptor kinase in particular, have the potential of activating STAT proteins. However, we have identified the inhibitor(s) that can specifically block STAT activation in response to EGF. We present evidence here suggesting that this inhibitor is associated with the membrane and exists in many cell lines. A working model of conditional activation of STAT proteins by receptor tyrosine kinases is proposed. The possible implications of this conditional STAT activation in cell proliferation and cancer development are further discussed.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture Cells—**A431 human epidermoid carcinoma cells (ATCC, CRL-1555) and HeLa S3 human epitheloid carcinoma cells (ATCC, CCL-2.2) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% bovine calf serum; PC12 rat adrenal pheochromocytoma cells (ATCC, CRL-1721) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 10% horse serum, and 1 mM L-glutamine; HepG2 human hepatoma cells (ATCC, HB-8065) in Dulbecco’s modified Eagle’s medium containing 10% fetal

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§ A recipient of a National Institutes of Health career development award. To whom correspondence should be addressed: Dept. of Pathology, Yale University School of Medicine, 310 Cedar St., New Haven, CT 06520. Tel.: 203-737-1246; Fax: 203-785-7303; E-mail: xin-yuan.fu@yale.edu.

The abbreviations used are: IFN, interferon; STAT, signal transducer and activator of transcription; EGF, epidermal growth factor; NGF, nerve growth factor; EMSA, electromobility shift assay; SIF, sis-inducible factor; SIE, sis-inducible element.

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bovine serum; T-47D human breast ductal carcinoma cells (ATCC, HTB-133) in RPMI 1640 containing 10% fetal bovine serum and 10 μg/ml insulin; WiDr human colon adenocarcinoma cells (ATCC, CCL-218) in Eagle’s minimum essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum; HT-29 human colon adenocarcinoma cells (ATCC, HTB-38) in McCoy’s 5A medium (Life Technologies, Inc.) containing 10% fetal bovine serum; and SW1116 human colon adenocarcinoma cells (ATCC, CCL-233) in Leibovitz’s L-15 (Life Technologies, Inc.) containing 10% fetal bovine serum. THP-1 human mono
cyte cells (ATCC, TIB-202) were grown in suspension in RPMI 1640 containing 10% fetal bovine serum, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol.

In Vivo Activation of STAT Proteins by Cytokines—Human recombinant EGF was purchased from Life Technologies, Inc., IFN-γ from Genentech, and murine nerve growth factor from Boehringer Mannheim. The cells were grown to 70% confluent and serum-starved for 17 h before stimulation by cytokines. Fifteen min after the addition of 100 ng/ml EGF, 1 ng/ml IFN-γ, or 50 ng/ml NGF, the cells were put on ice, and whole cell extracts were prepared as described (32) with some modifications by lysing cells in 20 mM Hepes buffer (pH 7.9) containing 0.5% (v/v) Nonidet P-40, 15% (v/v) glycerol, 300 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 10 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin (1 μg/ml each). Whole cell extracts were immediately subjected to electrophoretic analysis.

Preparation of Membrane and Cytosol Fractions—The membrane and cytosol fractions were prepared as described (35) with the following modifications. The cells were grown to confluent without stimulation with either EGF or any other cytokines, rinsed twice with ice-cold phosphate-buffered saline, scraped from the dishes, and pelleted. The cells were lysed in 3-cell-pellet volumes of 20 mM Hepes buffer (pH 7.9) containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 125 mM okadaic acid, 0.4 mM ammonium molybdate, 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin (1 μg/ml each) by a Dounce homogenizer. The lysate was microcentrifuged for 5 min at 3,000 × g at 4 °C. The supernatant was adjusted to 120 mM NaCl and microcentrifuged at 10,000 × g for 30 min at 4 °C. Glycerol was added to the supernatants to 10%. The resulting sample was saved as a cytosol fraction. The high speed pellet was resuspended in the lysing buffer containing 150 mM NaCl and 8% glycerol and microcentrifuged at 10,000 × g for 30 min at 4 °C. The pellet was saved as a membrane fraction. Both of the cytosol and membrane fractions were frozen on dry ice and kept at −80 °C until use for in vitro activation.

In Vivo Activation of STAT Proteins by EGF—The in vivo activation was performed as described (35) with the following modifications. The membrane pellet was thawed on ice and resuspended in 20 mM Tris buffer (pH 7.4) containing 0.25% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 20 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin (1 μg/ml each) (buffer A). Three μl of the membrane fraction and 2 μl of the cytosol fraction (containing 100 μg/ml EGF (EGF +) or phosphate-buffered saline (EGF −)) was mixed with 10 μl of the membrane fraction (10,000 × g) and the mixture was incubated on ice for 15 min. The reaction was started by the addition of 2.5 μl of 50 mM Hepes buffer (pH 7.4) containing 20 mM MgCl₂, 10 mM MnCl₂, and 10 mM ATP. After incubation at 30 °C for the indicated time, the reaction was stopped by putting the tubes on dry ice. For the STAT inhibitor assay, 2 μl of the inhibitor (membrane) fraction was added to the reaction mixture, and the reaction time was determined to exhibit a linear dependence of STAT protein activation on the cytosol fraction (STAT protein) concentration.

Electromobility Shift Assay (EMSA)—The sample, in vitro activation (3 μl) (1 μl for the whole cell extract) was mixed with 15 μl of 13 mM Hepes buffer (pH 7.9) containing 50 mM NaCl, 0.1 mM EDTA, 8% glycerol, 0.5 mM dithiothreitol, 0.7 μl/g DNA poly(dCTP-dGTP-dATP-dTTP), and 33.3 μg/ml salmon sperm DNA. After incubation on ice for 30 min, 1 μl of double-stranded oligonucleotide M67-SIE probe end-labeled with 32P-γ-ATP was added. For the supershift assay, 1 μl of each anti-serum (1:5) was added after the addition of the end-labeled DNA probe. The mixture was then incubated at room temperature for 30 min. The DNA-protein complexes were separated on nondenaturing acrylamide gel (5%) in 0.5% Tris-borate EDTA and detected by autoradiography. The antibodies against STAT2, STAT3, or STAT4 used in the supershift of STAT complexes were previously described (13, 32). The antibody against STAT1 was purchased from Santa Cruz Biotech.

Solubilization of the Inhibitor from the Membrane Fraction—The membrane fraction prepared as described above was dissolved in 2 volumes of 50 mM Tris buffer (pH 8.0) containing 0.1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, and 6 μg guanidine hydrochloride, followed by incubation at room temperature for 15 min. The resulting sample was dialyzed against 180 volumes of 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 20 mM sodium fluoride, and 1 mM dithiothreitol at room temperature for 30 min. Then, the dialysis equipment was moved to the cold room at 4 °C, and dialysis was continued for additional 2 h. The dialyzed buffer was changed, followed by dialysis at 4 °C for 2 h; and the dialytic buffer was replaced again, and the final cycle of dialysis was continued at 4 °C overnight. The dialysate was microcentrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was saved as a solubilized membrane fraction (10,000 × g pellet, solubilized) and kept at 4 °C until use.

Column Chromatographic Procedures—The gel filtration Sephadex G-25 (Amersham Pharmacia Biotech) and the anion exchanger DEAE-Sepharose (Amersham) columns were used for partial purification of the inhibitor. One ml of the solubilized membrane fraction (4 mg of protein) was loaded on the column of Sephadex G-25 (1 × 13 cm) previously equilibrated with 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 10% glycerol, 0.05% Triton X-100, 20 mM sodium fluoride, 0.1 mM sodium vanadate, 1 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol (buffer B). Proteins were eluted by gravity, and 1-ml fractions were collected in 1.5-ml microcentrifuge tubes. The 4–9 fraction was applied to the column of DEAE-Sepharose (0.4 × 8 cm) previously equilibrated with buffer B. After washing the column with three-column-volumes of buffer B, elution was carried out with buffer B containing 0.3 M NaCl, and 1 ml fractions were collected. Two μl of each fraction was subjected to the STAT inhibitor assay as described above.

RESULTS

Activation of STAT Proteins with EGF Treatment in A431 but Not in HeLa S3 and PC12 Cells—A431 cells, which express large amount of EGF receptors, have been used widely in studies of EGF and the EGF receptor in the past decade. It is known that STAT proteins can be strongly activated in A431 cells in response to EGF. However, no obvious inducible STAT activation was observed after the EGF treatment in some other cells (Fig. 1).

Using a high affinity STAT-interactive element, M67-SIE (sis-inducible element) (29, 30), as a probe in the EMSA, three inducible STAT-DNA complexes, SIF-A, SIF-B, and SIF-C, were specifically observed in the extracts from EGF-treated A431 cells (Fig. 1, lane 3). SIF-A, -B, and -C are the STAT3 heterodimer; SIF-C, STAT1 homodimer (for details, see Refs. 31–33). However, under similar conditions, treatment of HeLa S3 and PC12 cells with EGF was unable to induce STAT activation (Fig. 1, lanes 6 and 9). In
contrast, STAT1 activation was strongly generated in all of these cells after IFN-γ treatment (Fig. 1, lanes 2, 5, and 8), indicating that there is probably no defect in STAT proteins themselves, but the activation of STAT proteins may be specifically defective in the EGF signaling both in HeLa and PC12 cells.

PC12 cell line is often used as a model system to study induction of neuronal differentiation in response to NGF (1). We further examined whether STAT proteins can be activated in this process. No detectable STAT activity was observed with the M67-SIE probe in NGF-treated PC12 cells (Fig. 1, lane 10), suggesting that STAT proteins do not act as signal transducers for NGF in PC12 cells. However, it appears that the Trk family of receptor tyrosine kinases, which are receptors for neural trophins like NGF, have the potential to activate STAT proteins in vitro (14). It further suggests that the reason STAT proteins are not activated in PC12 cells in response to either EGF or NGF may be because the PC12 cells do not provide the suitable conditions to have STAT proteins activated by receptor tyrosine kinases.

**Activation of STAT Proteins with EGF Treatment in an in Vitro System Using Different Cell Fractions—**One possible explanation for observed difference in the EGF-induced STAT activation among these cell lines is that a specific inhibitor(s) may exist in PC12 and HeLa cells. To examine this possibility and to identify the inhibitor biochemically, we employed an *in vitro* cell-free STAT activation system previously developed (34, 35). In this reconstituted system, isolated cell fractions instead of intact cells are used to activate STAT proteins in response to cytokines *in vitro*. This system is optimal when the membrane fraction from A431 cells is used to induce STAT activation in response to EGF (35) (Fig. 2).

In this *in vitro* activation system, the EGF receptor was provided by an enriched membrane fraction of untreated A431 cells (see the "Experimental Procedures" section for technical details), whereas unactivated STAT proteins were provided by a cytosol fraction from untreated cells. In the presence of ATP and under proper buffer conditions, the A431 membrane fraction and the cytosol fraction of various cells were incubated together to activate STAT proteins in response to added EGF (Fig. 2A). Both the membrane fraction of A431 cells and the cytosol fraction of either A431 or PC12 cells were required for STAT activation in the *in vitro* system. The addition of EGF to this *in vitro* reaction was essential for induction of the STAT activity (Fig. 2A, odd numbered lanes), and either the membrane fraction or the cytosol fraction alone was not sufficient (see below).

Intriguingly, although no STAT activation was induced *in vivo* in PC12 cells (Fig. 1), the cytosol fraction from PC12 cells was competent for producing activated STAT complexes (SIF-A, -B, and -C) when the A431 membrane fraction and EGF were provided as activators (Fig. 2A, lane 3). The identities of these SIFs were confirmed by specific antibodies against different STAT proteins. STAT1 antibody supershifted SIF-C and partially supershifted SIF-B; STAT3 antibody supershifted SIF-A and SIF-B. It indicates that these *in vitro* generated SIFs are consistent with those generated *in vivo*. STAT-C is a STAT1 homodimer, SIF-B is a STAT1/STAT3 heterodimer, and SIF-A is a STAT3 homodimer (see Ref. 31). Furthermore, STAT3 complexes were only formed when higher concentrations of cytosol fractions were added, whereas with reduced amounts of cytosol fractions of PC12 cells, STAT3-containing complexes (SIF-A and -B) were not formed (Fig. 2B, lanes 2–6). Consistently, a STAT1 homodimer complex was mainly observed in reactions involving A431 membrane and cytosol fractions since less amount of A431 cytosol (5 μg) was used in this reaction (Fig. 2A, lane 1, and Fig. 2B, lane 1). These results suggested that the STAT1 homodimer may be much more stable than the STAT3 homodimer. Additionally, the A431 membrane fraction alone was not sufficient for generating any STAT complex (Fig. 2B, lane 7).

These *in vitro* experiments provided critical conditions and practical assays for biochemically analyzing the possible inhibitory activity (see below).

**Existence of an Inhibitor(s) of EGF-induced STAT Activation in the Membrane Fractions of PC12 and HeLa but not A431 Cells—**It is possible that the failure of STAT activation by EGF in PC12 and HeLa cells is due to the existence of specific inhibitor(s). As described above, using the *in vitro* STAT activation system, we found that the cytosol fraction of PC12 cells contained functional STAT proteins. Therefore, the possible inhibitor(s) may be associated with the membrane fraction(s) of PC12 and HeLa cells.

To examine the possible inhibitor, we used the above-de-
Inhibitor(s) of EGF-STAT Signaling

 Activation—There are at least two possibilities how this inhibitory effect is achieved. First, the inhibition may be due to the activity of an inhibitor(s) that is associated with the membrane. Second, the formation of an inhibitor complex with the STAT proteins may prevent their activation. Under these conditions, observed STAT activation is considered to be most sensitive to inhibitory effect of possible inhibitor(s).

Under these optimal conditions, the in vitro reaction generated only the SIF-C (STAT1) complex in response to EGF (Fig. 3A, lanes 1–2). To analyze whether the membrane fraction from PC12 cells contains the possible inhibitor, various protein concentrations of the PC12 membrane fraction were added to the reactions (Fig. 3A, lane 3–12). As expected, STAT1 activation was inhibited in proportion to added membrane concentrations. In particular, the STAT1 activation was completely inhibited when 7 μg of protein of the PC12 membrane fraction was added. These results indicate the existence of an inhibitor(s) of STAT activation in the PC12 cell membrane fraction. This inhibitory effect was not due to dilution, since adding the same volume of buffer did not inhibit STAT activation (data not shown). Furthermore, under the same conditions, the PC12 membrane fraction alone with the cytosol fraction did not induce STAT activation in response to EGF (data not shown). Similar results were obtained in the experiments in which the PC12 cytosol instead of the A431 cytosol was used for STAT activation (Fig. 3B), further indicating that the inhibitor did not exist in the PC12 cytosol fraction. These results further demonstrate that the failure of STAT activation in PC12 cells in response to EGF may be due to the activity of an inhibitor(s) that is associated with the membrane.

As indicated by in vitro results (Fig. 1), EGF did not induce STAT activation in either HeLa or PC12 cells. We suspect that the same inhibitor activity may also exist in the HeLa cell membrane fraction. To examine this possibility, the membrane fraction from HeLa cells was subjected to the similar analysis (Fig. 4). Using the in vitro assay systems either with the A431 membrane and cytosol fractions (Fig. 4A) or the A431 membrane and HeLa cytosol fractions (Fig. 4B), we found that an inhibitor activity also associated with the HeLa membrane. It further suggests that the same inhibitor may be responsible for failure of STAT activation by EGF both in PC12 and HeLa cells.

Larner and co-worker (34) have shown previously that STAT proteins can be activated by interferon treatment in an in vitro system similar to what we used in the present study. We have also reproduced their result indicating the HeLa membrane is capable of activating STAT proteins in response to interferons in the in vitro system (data not shown). This result is consistent with the suggestion that this inhibitor may specifically block STAT activation by EGF but not that by interferons.
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The inhibitor in HeLa S3 or PC12 membrane inhibits the in vitro activation of the STAT1 but not the binding of the activated STAT1 with SIE. Lanes 1–3 and 6, the STAT proteins in the A431 cytosol (cyto.) fraction (5.5 µg of protein) were activated in vitro by the A431 membrane (membr.) fraction (0.44 µg) in the absence or the presence of the HeLa S3 or PC12 membrane fraction (6.2 µg). Three µl of the in vitro activated sample was subjected to EMSA using [32P]-end-labeled M67-SIE as a probe. Lanes 4 and 5 and 7 and 8, two µl of buffer A, the HeLa S3, or the PC12 fraction (6.2 µg) was mixed with the in vitro activated sample (10 µl), and 3 µl of the mixture was subjected to EMSA. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed.

The Inhibitor Exists in a Number of Cell Lines—As we have shown, STAT proteins are strongly activated in A431 cells in response to EGF (Fig. 1). Furthermore, the A431 membrane fraction is potent for the in vitro STAT activation. These observations suggest that the inhibitor is not present or present at a lower level in A431 cells. Alternatively, the EGF receptor is overexpressed in A431 cells, and the receptors may outnumber inhibitors to activate STAT proteins. In contrast, STAT proteins are not activated in response to EGF in HeLa and PC12 cells due to existence of possible inhibitor(s).

We further analyzed a number of other cancer cell lines to examine whether they have the same inhibitor activity in their membrane fractions. We found that, besides PC12 and HeLa cells, many other cancer cell lines contain inhibitor activity in their membrane fractions that could inhibit the STAT activation in vitro (Fig. 7). Consistent with the in vitro activation results, EGF treatment of these cells in vitro did not generate detectable STAT activation (data not shown). Similar to PC12 and HeLa cells, however, STAT1 was activated in all these cells in response to IFN-γ treatment (data not shown). These observations indicate that the signal pathway from EGF receptor to STAT proteins was specifically blocked in many cancer cell lines. The identified inhibitor(s) in the membrane fraction of these cells may be responsible for this specific inhibition of signal transduction.

DISCUSSION

In this report we have presented data suggesting that STAT proteins are activated in response to EGF in A431 cells but not in many other cells. This conditional activation of STAT proteins may be due to the existence of specific inhibitor that is associated with the membrane fraction.

We have previously shown that STAT proteins may directly interact with the EGF receptor and that the SH2 domain of STAT1 was essential for this receptor–STAT interaction (14). These observations suggest that STAT proteins bind directly to specific phosphotyrosine sites in the EGF receptor. This conclusion was further supported by recent results that a STAT3 isoform can directly interact with the EGF receptor (26) and that the EGF receptor kinase is capable of phosphorylating STAT proteins at the critical C-terminal tyrosine site (tyrosine 701 in STAT1) in vitro (23, 26). Our recent data show that the short tyrosine-phosphorylated peptides analogous to the C terminus of the EGF receptor competed with EGF receptor and inhibited the in vitro STAT activation by EGF, indicating that the EGF receptor interacts with the STAT protein through its

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It is possible that STAT-EGF receptor interaction is a target for the inhibitor. However, we do not know whether the inhibitor that we have identified in this report works through this mechanism.

Furthermore, many adapter proteins and potential substrates of the EGF receptor may also bind to the EGF receptor directly. Thus, it is possible that STAT proteins have to compete with other substrates of the EGF receptor for binding. The inhibitor may be one of these other substrates or adapter proteins. If this is the case, the presence or the absence of this STAT-binding competitor could work as a switch for STAT activation by the EGF receptor. Another intriguing possibility is that this inhibitor may be a phosphatase that could be specifically activated by EGF receptor and that it could dephosphorylate STAT during receptor activation.

One important question is what is the physiological relevance of our findings that STAT proteins are only conditionally activated in many cancer cells in response to EGF. Because the A431 cell membrane contains an abnormally high level of EGF receptor protein, STAT activation by EGF in A431 cells could be unusual. Additionally, we have mostly presented the biochemical data showing this inhibitor activity. What is in vivo implication of the STAT activation by growth factors?

We have recently demonstrated that STAT activation by EGF can cause induction of cyclin-dependent kinase inhibitor separation of the STAT inhibitor activity by Sephadex G-25 gel filtration column chromatography. After Sephadex G-25 gel filtration, two µl of each fraction was subjected to the STAT inhibitor assay as described under “Experimental Procedures.” Two µg of protein of the A431 cytosol fraction (containing the inactive STAT proteins) and 0.3 µg of the A431 membrane fraction were incubated with each fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32P-end-labeled M67-SIE as a probe. C, separation of the STAT inhibitor activity by DEAE-Sephacel column chromatography. Two µl of each fraction eluted from DEAE-Sephacel was subjected to the STAT inhibitor assay as described under “Experimental Procedures.” Two µg of protein of the A431 cytosol fraction (containing the inactive STAT proteins) and 0.3 µg of the A431 membrane fraction were incubated with each fraction. The effect of high salt on the formation of the STAT proteins-SIE complexes was also tested by the addition of buffer B containing 0.3 M NaCl instead of the eluate. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32P-end-labeled M67-SIE as a probe.

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p21 and the apoptosis mediator caspase 1 (interleukin-converting enzyme (ICE)) in A431 and MDA-MB-468 cells, resulting in growth arrest and apoptosis of these cells (30, 36). Thus, the STAT signaling pathway may play a negative role in the control of cell proliferation. This negative regulation may be required for maintaining homeostasis of the cell in balance with other positive signaling pathways such as Ras-mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. Thus, loss of the STAT activation by EGF in many cancer cells may be a cause of overproliferation.

We propose a working model that EGF may normally activate several different signaling pathways including the Ras-mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and EGF receptor-STAT pathways. These pathways are functionally complementary to each other to mediate growth or differentiation in vivo. However, induction of specific inhibitors for STAT activation might block the negative regulation and disrupt cell homeostasis. The positive signals would make the cell over-proliferate, losing balance with the negative signals generated by STAT proteins. Therefore, appearance or activation of the STAT inhibitor could be a cause of the uncontrolled cell growth and cell transformation.

There is the evidence supporting this model. For instance, S. Cohen and coworkers have shown that STAT proteins are specifically activated in liver cells by intraperitoneal administration of EGF in vivo (37). However, STAT proteins are not activated by EGF either in intact hepatoma-derived HepG2 cells or in the in vitro activation system using cellular fractions of HepG2 cells, most likely due to the presence of the inhibitor (Fig. 7). These observations suggest that loss of STAT activation in response to EGF may cause oncogenesis and autonomous growth of hepatocytes.

Although Janus kinase tyrosine kinases are first identified as activators of STAT proteins, a variety of other tyrosine kinases are capable of activating STAT in vivo and in vitro. We have recently shown that STAT can be activated by fibroblast growth factor receptor kinase and focal adhesion kinase (Ref. 27). More interestingly, insulin receptor kinase is capable of activating STAT proteins in vitro (14) and in vivo in liver cells (38); however, insulin failed to induce STAT activation in hepatoma-derived HepG2 cells.4 Therefore, similar inhibitors may also exist in insulin-induced STAT activation. Recently, several laboratories have reported identification and cloning of specific inhibitors for the Janus kinases (39–41). These Janus kinase inhibitors are believed to be involved in negative feedback regulation of the Janus kinase-STAT pathway. Our work presented in this report may shed new light on the negative regulation of the receptor protein-tyrosine kinase-initiated STAT pathways.

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4 Y. Iwamoto, Y. E. Chin, X. Peng, and X.-Y. Fu, unpublished data.