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13. ABSTRACT (Maximum 200 words) 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 <i>in vitro</i> 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 <i>in vitro</i> . 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 EGF signal transduction may play a significant role in the development of many kinds of cancers.				
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INTRODUCTION:

Mammalian cell proliferation and differentiation are controlled by cytokines, growth factors and other polypeptide ligands which 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 ref. 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 MAP kinase (mitogen-activated kinase) family. The MAP kinases may translocate to the nucleus and phosphorylate transcription factors such as 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 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-MAP kinases, this direct signal transduction is featured by direct interactions of SH2-containing transcriptional factor STATs with tyrosine phosphorylated receptor complexes.

The JAK (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 EGF (epidermal growth factor), PDGF (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 JAK kinases (14, 23-26). Our recent work has further shown that FGF (fibroblast growth factor) receptor tyrosine kinase, focal adhesion kinase (FAK), and many other tyrosine kinases can also directly activate STAT proteins (27; and Xie and Fu et al., unpublished). Therefore, STAT proteins may play important roles generally in downstream direct signal transduction of a variety of protein tyrosine kinases (PTKs). 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.

BODY:

Experimental methods:

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 (DMEM, Gibco BRL) containing 5 % bovine calf serum, PC12 rat adrenal pheochromocytoma cells (ATCC, CRL-1721) in DMEM containing 10 % fetal bovine serum, 10 % horse serum, and 1 mM L-glutamine, HepG2 human hepatoma cells (ATCC, HB-8065) in DMEM containing 10 % fetal bovine serum, T-47D human breast ductal carcinoma cells (ATCC, HTB-133) in RPMI 1640 containing 10 % fetal bovine serum and 10 μ g/ml insulin, WiDr human colon adenocarcinoma cells (ATCC, CCL-218) in Eagle's Minimum Essential Medium (MEM, Gibco BRL) containing 10 % fetal bovine serum, HT-29 human colon adenocarcinoma cells (ATCC, HTB-38) in McCoy's 5A medium (Gibco BRL) containing 10 % fetal bovine serum, and SW1116 human colon adenocarcinoma cells (ATCC, CCL-233) in Leibovitz's L-15 (Gibco BRL) containing 10 % fetal bovine serum. THP-1 human monocyte cells (ATCC, TIB-202) were grown in suspension in RPMI 1640 containing 10 % fetal bovine serum, 2 mM L-glutamine, and 50 μ M 2-mercaptoethanol.

***In vivo* activation of STAT proteins by cytokines**

Human recombinant EGF was purchased from Gibco BRL, IFN- γ from Genentech, and murine NGF from Boehringer Mannheim. The cells were grown to 70 % confluent and serum-starved for 17 hours prior stimulation by cytokines. Fifteen minutes after 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 modification by lysis of cells in 20 mM Hepes buffer (pH7.9) containing 0.5 % (v/v) NP-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 (PMSF), and leupeptin, pepstatin, and aprotinin (1 μ g/ml each). Whole cell extracts were immediately subjected to electromobility shift assay.

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, rinsed twice with ice-cold PBS, scraped from the dishes, and pelleted. The cells were lysed in three cell-pellet volumes of 20 mM Hepes buffer (pH7.9) containing 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 125 nM okadaic acid, 0.4 mM ammonium molybdate, 0.5 mM PMSF, and leupeptin, pepstatin, and aprotinin (1 μ g/ml each) by a Dounce homogenizer. The lysate was microcentrifuged for 5 minutes at 3,000g at 4 $^{\circ}$ C. The supernatant was adjusted to 120 mM NaCl and microcentrifuged at 10,000g for 30 minutes at 4 $^{\circ}$ 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 lysis buffer containing 150 mM NaCl and glycerol, and microcentrifuged at 10,000g for 30 minutes at 4 $^{\circ}$ C. The pellet was saved as a membrane fraction. Both of the cytosol and membrane fraction were frozen on dry ice and kept at -80 $^{\circ}$ C until use for *in vitro* activation.

***In vitro* activation of STAT proteins by EGF**

The *in vitro* activation was performed as described (35) with the following modifications. The membrane pellet was thawed on ice and resuspended in 20 mM Tris buffer (pH7.4) containing, 0.25 % (v/v), 1 mM EDTA, 10 % glycerol, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 20 mM sodium fluoride, 0.5 mM PMSF, and

leupeptin, pepstatin, and aprotinin (1 $\mu\text{g}/\text{ml}$ each) (buffer A). Three microliters of the membrane fraction were mixed with 2 μl of the cytosol fraction and 0.5 μl of either 100 $\mu\text{g}/\text{ml}$ EGF (EGF +) or PBS (EGF -). The mixture was incubated on ice for 15 minutes. The reaction was started by addition of 2.5 μl of 50 mM Hepes buffer (pH7.4) containing 20 mM MgCl_2 , 10 mM MnCl_2 , and 10 mM ATP. After incubation at 30 $^{\circ}\text{C}$ 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 dependency of STAT protein activation on the cytosol fraction (STAT protein) concentration.

Electromobility shift assay (EMSA)

The sample after *in vitro* activation (3 μl) (1 μl for the whole cell extract) was mixed with 15 μl of 13 mM Hepes buffer (pH7.9) containing 50 mM NaCl, 0.1 mM EDTA, 8 % glycerol, 0.5 mM dithiothreitol, 66.7 $\mu\text{g}/\text{ml}$ poly(deoxyinosine-deoxycytidine), and 33.3 $\mu\text{g}/\text{ml}$ salmon sperm DNA. After incubation on ice for 30 minutes, 1 μl of double-stranded oligodeoxynucleotide M67-SIE probe, end-labeled with [γ - ^{32}P]ATP, was added. For the supershift assay, 1 μl of each anti-serum (1:5) was added after addition of the end-labeled DNA probe. The mixture was then incubated at room temperature for 30 minutes. The DNA-protein complexes were separated on non-denaturing acrylamide gel (5 %) in 0.5 % Tris-borate EDTA and detected by autoradiography. Antibodies against STAT2, STAT3 or STAT4 used in the supershift of STAT complexes were previously described (13, 32); 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 (pH8.0) containing 0.1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 0.1 % (v/v) NP-40, and 6 M guanidine hydrochloride, followed by incubation at room temperature for 15 min. The solution was dialyzed against 180 volumes of 20 mM Tris buffer (pH7.4) 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 dialytic 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 dialyzate was microcentrifuged at 10,000g for 30 min at 4 C. The supernatant was saved as a solubilized membrane fraction (10,000g pellet, solubilized) and kept at 4 C until use.

Column chromatographic procedures

The gel filtration Sephadex G25 (Pharmacia) and the anion exchanger DEAE-Sephacel (Pharmacia) columns were used for partial purification of the inhibitor. One milliliters of the solubilized membrane fraction (4 mg protein) was loaded onto the column of Sepadex G25 (1 x 13 cm) previously equilibrated with 20 mM Tris buffer (pH7.4) containing 0.5 mM EDTA, 10 % glycerol, 0.05 % Triton X-100, 20 mM sodium fluoride, 0.1 mM sodium vanadate, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM PMSF, and 2 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-Sephacel (0.4 x 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 microliters 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, we found that no obvious inducible STAT binding activities were observed after the similar EGF treatment (Fig. 1) in some other cells.

Using a high-affinity STAT interactive element, M67-SIE (sis-inducible element) (29,30), as a probe in the electrophoresis mobility shift assay (EMSA), three inducible 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 homodimer, STAT1/STAT3 heterodimer, and STAT1 homodimer respectively (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 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 nerve growth factor (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), showing that STAT proteins may 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). These results further show that STAT proteins may not usually be activated by receptor tyrosine kinases in PC12 cells in response to either EGF or NGF. Another possibility is that STAT proteins can only be activated under certain conditions; however, these cultured cells such as PC12 and HeLa do not provide these conditions.

Activation of STAT proteins with EGF treatment in an in vitro System Using Different Cell Fractions

One possible explanation for observed differences 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 are 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 Materials and Methods 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 from either of A431 or PC12 cells were required for activation of STAT protein in the *in vitro* system. Addition of EGF to this *in vitro* reaction

was essential for induction of the STAT activity (Fig. 2A, odd numbered lanes); and the membrane fraction and/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 fractions from PC12 cells were 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), indicating that there was no deficiency in STAT proteins in PC12 cells. The identities of these SIFs were confirmed by specific antibodies against different STAT proteins: STAT1 antibody supershifted SIF-C, and partially SIF-B; STAT3 antibody supershifted SIF-A and SIF-B, further indicating that these *in vitro* -generated SIFs are consistent with those generated *in vivo*: SIF-C is a STAT1 homodimer, SIF-B is 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 complex was mainly observed in reactions involving A431 membrane and cytosol fractions due to the fact that less amount of A431 cytosol (5 μ g) was used in this reaction (Fig. 2A, lane 1, and Fig. 2B, lane 1). These results showed 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 existence of specific inhibitor(s). As described above, using the *in vitro* STAT activation system, we further 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-described *in vitro* activation system and determined the optimal conditions for the inhibitor assay. We first fixed the protein concentrations of the A431 membrane and the cytosol fractions to a relatively low degree. And then, the reaction time period was determined to give a linear dependency of the STAT protein activation on the cytosol fraction (STAT protein) concentration (see Material and Methods for details). 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; similar results were shown in Fig. 2B). 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 μ g protein of 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 fraction 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 vivo* results (Fig. 1), EGF did not induce STAT activation in 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 the cytosol fractions (Fig. 4A) or the A431 membrane and the HeLa cytosol fractions (Fig. 4B), we found that an inhibitor activity also associated with the HeLa membrane fraction, showing further that this inhibitor may be responsible for failure of STAT activation in response to EGF treatment in both PC12 and HeLa cells.

We have also found that 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 fact that this inhibitor may specifically block STAT activation by EGF but not that by interferons.

The Inhibitor Exerts its Effect during EGF-Induced STAT Activation

There are at least two possibilities as to how this inhibitor could block EGF-induced STAT activation. First, the inhibitor may block initial activation of STAT by the EGF receptor. For instance, the inhibitor may specifically block the interaction of STAT with the EGF receptor. Second, the inhibitor may interact directly with the STAT complex to disrupt the complex or prevent it from binding the SIE. In the latter case, this inhibitor should block the SIE-binding of the STAT complex even after it is formed.

To distinguish between these two possibilities, we conducted the following experiment. The PC12 or the HeLa membrane fraction, containing the inhibitor(s), was added to the reaction mixture either before or after the *in vitro* STAT activation reaction by EGF. We assume that if this inhibitor blocked only the initiation of STAT activation by the EGF receptor, the inhibitor would not work if it was added after the reaction had been completed. On the other hand, if the inhibitor could directly block the SIE-binding of the STAT complex, it should work even when the STAT complex had been formed after initial reaction.

As shown in Fig. 5, the inhibitor fractions from HeLa or PC12 membrane but not buffer A, reduced the levels of activated STAT1 (SIF-C) (comparing lanes 3 with 4, and lanes 6 with 7) when these fractions were added before initiation of the reaction by EGF. However, when these fractions were added after the reactions had been completed, no inhibitory effects were observed (lanes 5 and 8). These results showed that the inhibitor may block initial event of STAT activation by EGF receptor. One possibility is that it may block direct interactions of STAT with EGF receptor.

Partial Purification of the inhibitor

One of the major concerns about this inhibitor is whether it is a protein factor. To further characterize this inhibitor we have tried to purify this inhibitor from the membrane fraction.

Since this factor is membrane-associated, the first step was to separate this factor from the nuclear and cytosol fractions, and then to solublize the membrane pellet. We have found that this inhibitor was not solublized in either 0.25% Triton or 1.2% sodium cholate (data not shown), but was resolublized with high salt (6M guanidine hydrochloride). The soluble components were then dialyzed and renatured. In this step we not only separated membrane proteins from the large amount of nuclear and cytosol proteins, but also eliminated large quantities of insoluble membrane proteins and other components. The inhibitor activity was substantially enriched with much less non-specific proteins (Fig. 6A, lane 5). The second step in purification was the gel filtration with Sephadex G25. Interestingly, the inhibitor activity was co-eluted with the protein fractions from 4 to 9, as judged by a Coomassie staining (Fig. 6B), indicating that the inhibitor is likely a protein factor. After the gel filtration, the loading materials were greatly diluted, thus the inhibitory

activity shown in lanes 4-9 was rather weak. In the third step of the purification, this diluted inhibitor activity was significantly enriched in fraction 5 from a DEAE-Sephacel column (Fig. 6C, lane 12). With this three step purification, we estimated that this inhibitor was purified over 1,000 fold (see Table 1). The protein nature of this inhibitor was further confirmed by observed protease sensitivity of this factor (data not shown).

The Inhibitor may Exist 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 may show 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, in addition to PC12 and HeLa cells, many of cancer cell lines contain inhibitor activity in their membrane fractions which could inhibit the STAT activation *in vitro* (Fig. 7). Consistent with these *in vitro* activation results, EGF treatment of these cells *in vivo* did not generate detectable STAT activation (data not shown). Similarly 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 shows 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 C-terminal region (Chin et al., unpublished results). 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. The A431 cell membrane contains an abnormally high level of EGF receptor protein, then 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 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-MAP kinase and PI3 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-MAP kinase, PI3 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 his colleagues 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 loss of STAT activation in response to EGF may cause oncogenesis and autonomous growth of hepatocytes.

Although JAK 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 FGF receptor kinase and focal adhesion kinase (FAK) (27; and Xie and Fu et al., unpublished). 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 (our unpublished data). Therefore, similar inhibitors may also exist in insulin-induced STAT activation. Recently, several laboratories have reported identification and cloning of specific inhibitors for the JAK kinases (39-41). These JAK kinase inhibitors are believed to be involved in negative feedback regulation of the JAK-STAT pathway. Our work presented in this report may shed new light on the negative regulation of the receptor PTK-initiated STAT pathways.

CONCLUSIONS:

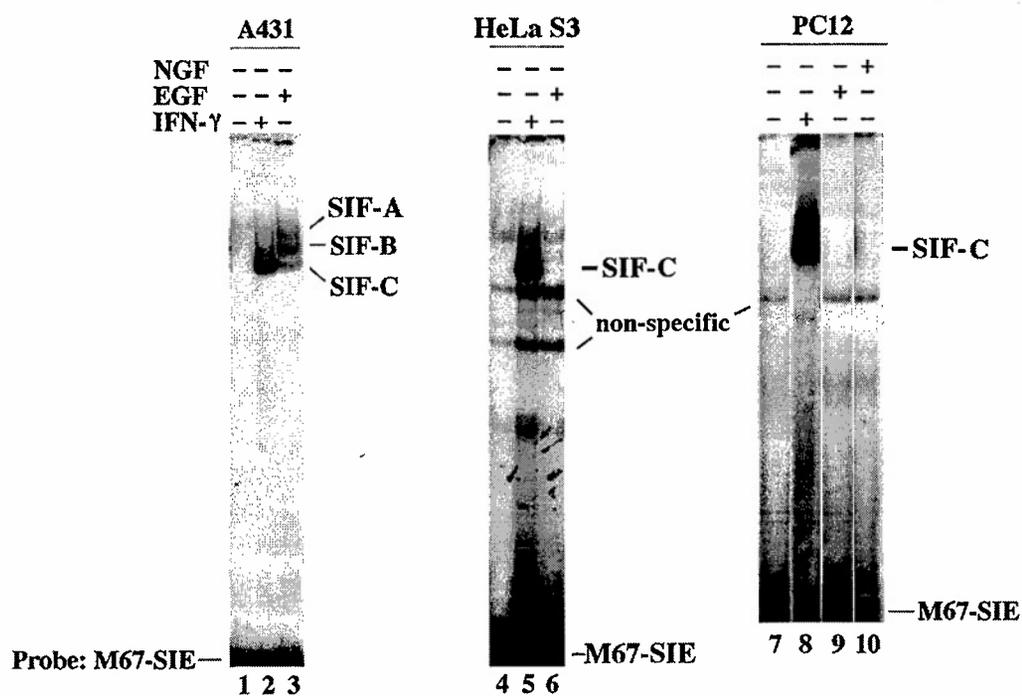
Many growth factors, including epidermal growth factor (EGF), can activate the STAT signaling pathway. Here we have identified a potential inhibitory factor (s) that is membrane associated and can block STAT activation induced by EGF. STAT activation by EGF is conditional due to presence of this inhibitor. In many cancer cell lines including those of breast cancers, EGF can not activate the STAT pathway and lose the negative control of cell growth. Failure in STAT activation during the EGF signal transduction may lead to uncontrolled proliferation of the cells. Cell proliferation may be controlled by altering the inhibitor activity.

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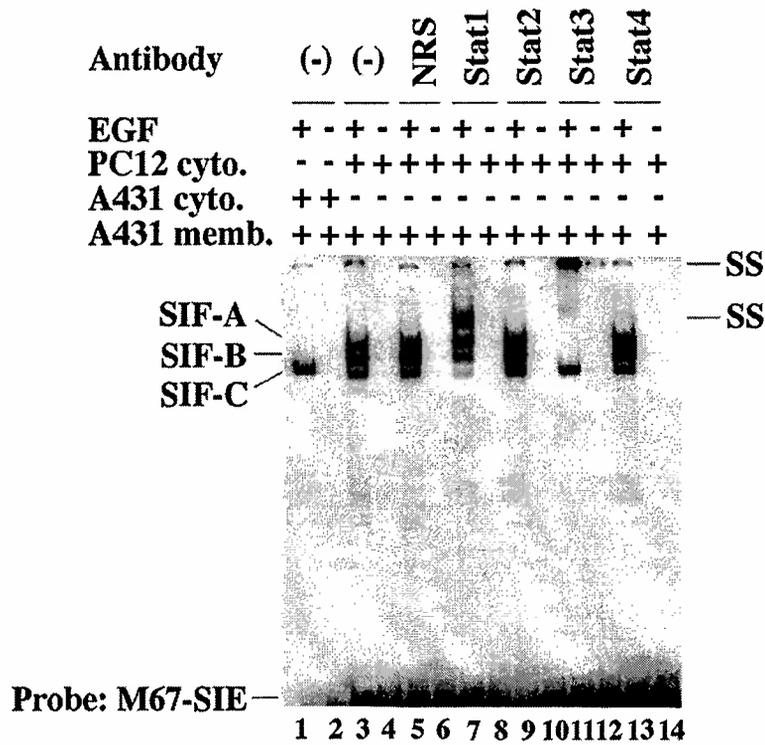
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Figure 1



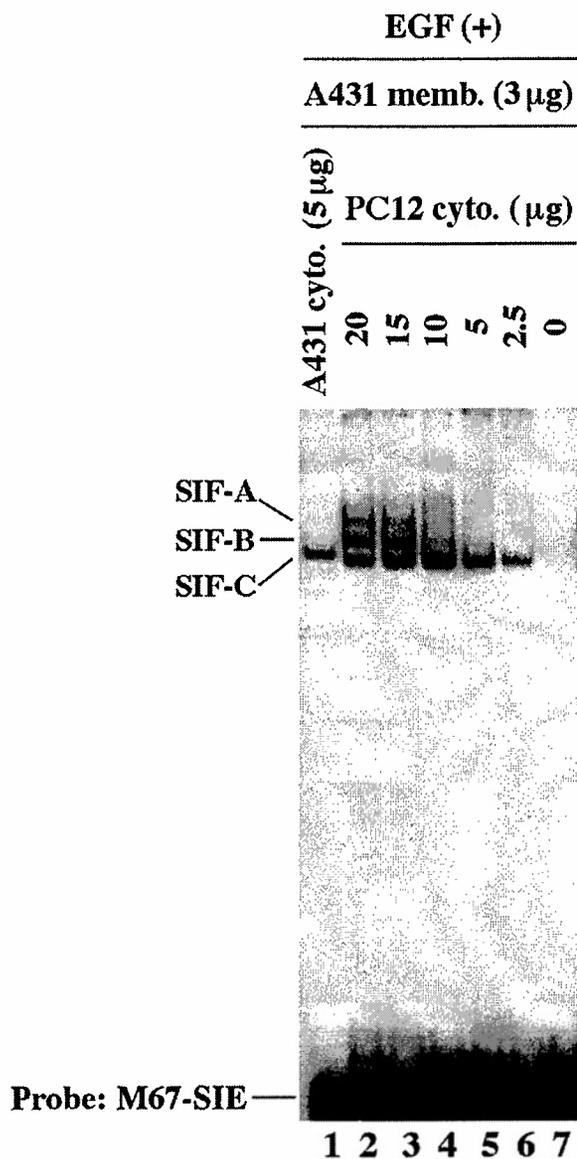
In vitro activation of STAT proteins by EGF is observed in A431 cells but not in many other cell lines. A431, HeLa S3, and PC12 cells were deprived of serum for 17 h and then stimulated with 1 ng/ml IFN- γ , 100 ng/ml EGF, or 50 ng/ml NGF for 15 min. One microliter of the whole cell extract was subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. Three types of the STAT protein-SIE complexes (SIF-A, B, and C) were observed. SIF-A: STAT3 homodimer; SIF-B: STAT1/STAT3 heterodimer; SIF-C: STAT1 homodimer.

Figure 2A



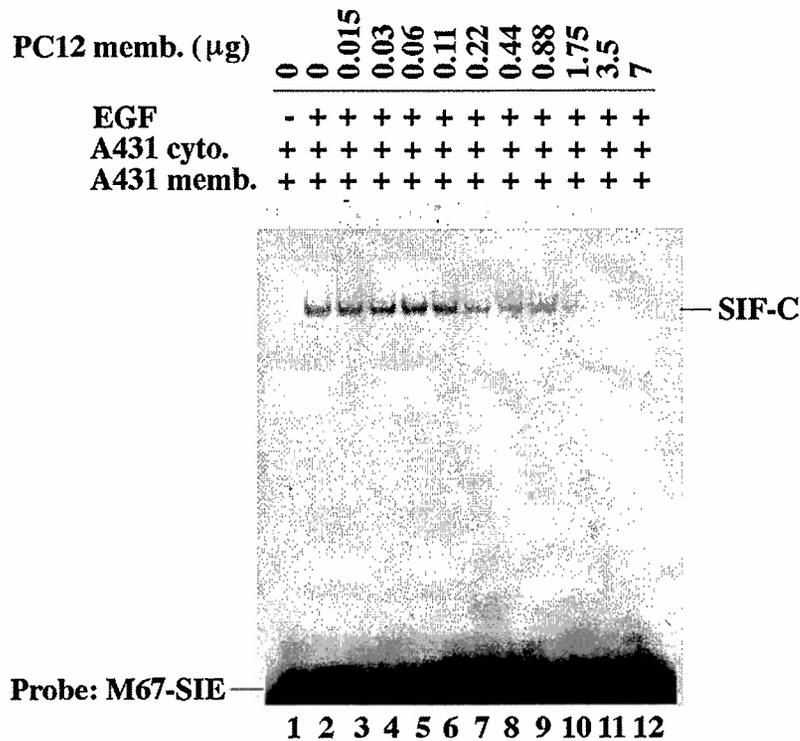
In vitro activation and supershift of STAT proteins. The STAT proteins in either A431 (5 ug protein) or PC12 (20 ug) cytosol fraction were activated by the A431 membrane fraction (3 ug) in vitro (30 min reaction). Three microliters of the whole cell extract was subjected to EMSA using ³²P-end-labeled M67-SIE as a probe. Three types of the STAT protein-SIE complexes (SIF-A, B, and C) were observed and supershifted by antisera against STAT proteins.

Figure 2B



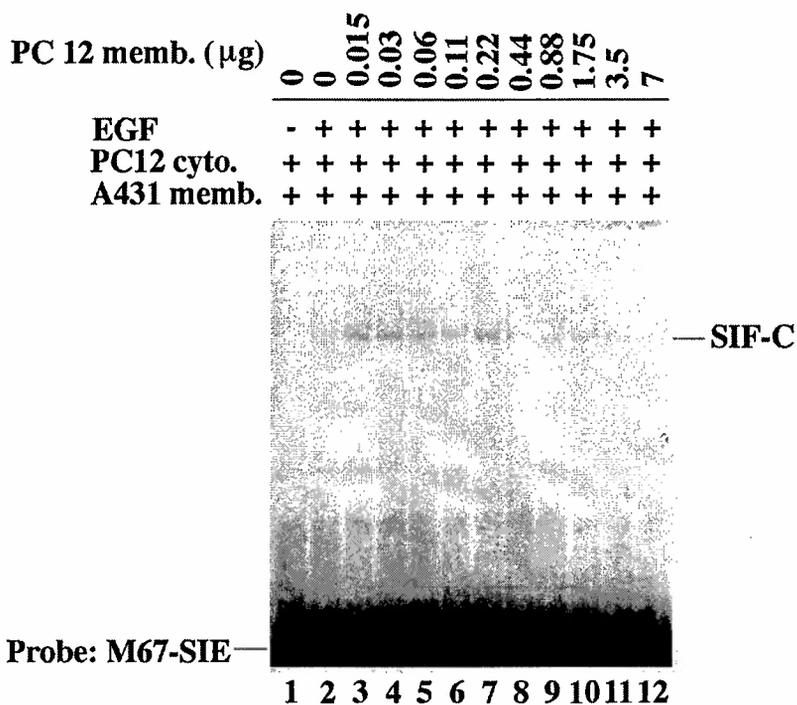
Protein concentration-dependency of SIFs formation. The STAT proteins in various protein concentrations of the PC12 or A431 cytosol fraction (5 μ g protein) were activated by the A431 membrane fraction (3 μ g) in vitro (30-min reaction). Three microliters of the in vitro-activated sample were subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. Three types of the STAT protein-SIE complexes (SIF-A, B, and C) were formed depending on the protein concentration of the cytosol fraction.

Figure 3A



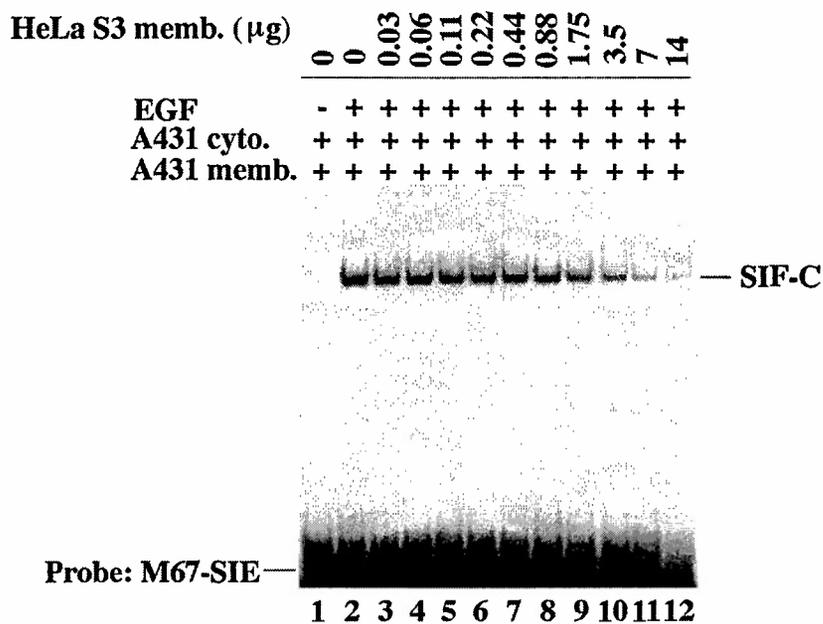
Titration of the PC12 membrane fraction to inhibit the in vitro activation of STAT proteins in the A431 cytosol by the A431 membrane. The STAT proteins in the A431 cytosol fraction (2 μ g protein) were activated in vitro by the A431 membrane fraction (0.3 μ g) in the presence of various protein concentrations of the PC12 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32 P-end-labeled M67-SIE as a probe.

Figure 3B



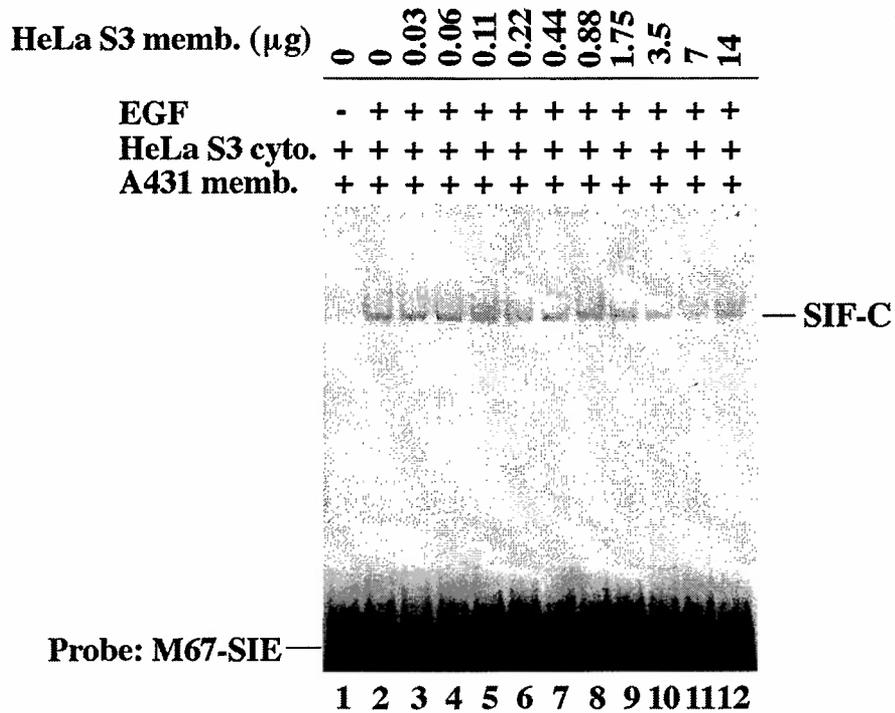
Titration of the PC12 membrane fraction to inhibit the in vitro activation of STAT proteins in the PC12 cytosol by the A431 membrane. The STAT proteins in the PC12 cytosol fraction (2 μg protein) were activated in vitro by the A431 membrane fraction (0.3 μg) in the presence of various protein concentrations of the PC12 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ^{32}P -end-labeled M67-SIE as a probe.

Figure 4A



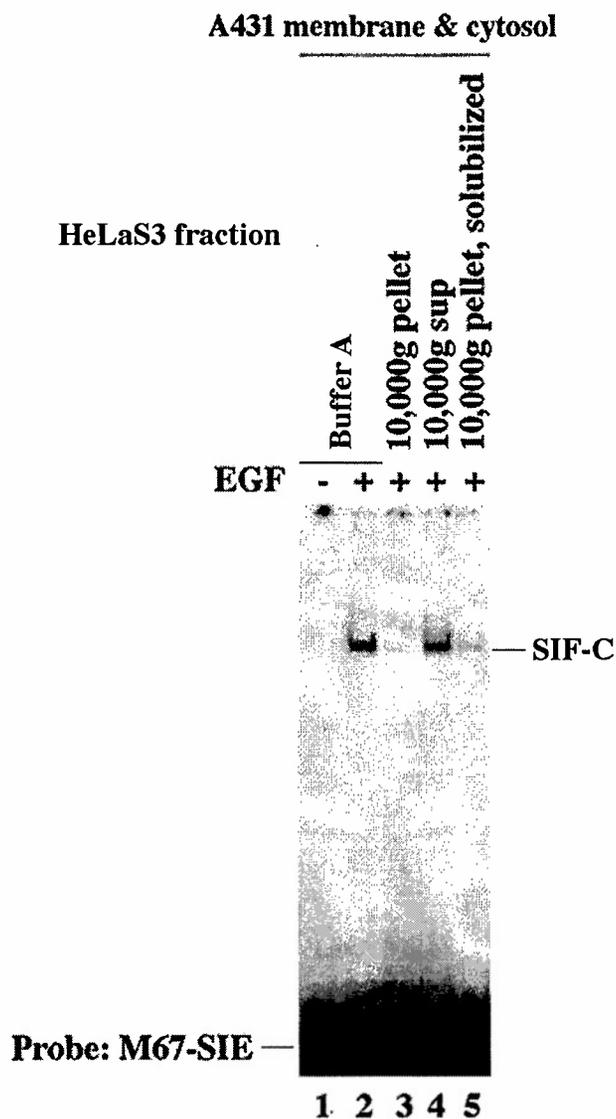
Titration of the HeLa S3 membrane fraction to inhibit the in vitro activation of STAT proteins in the A431 cytosol by the A431 membrane. The STAT proteins in the A431 cytosol fraction (2 μ g protein) were activated in vitro by the A431 membrane fraction (0.3 μ g) in the presence of various protein concentrations of the HeLa S3 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32 P-end-labeled M67-SIE as a probe.

Figure 4B



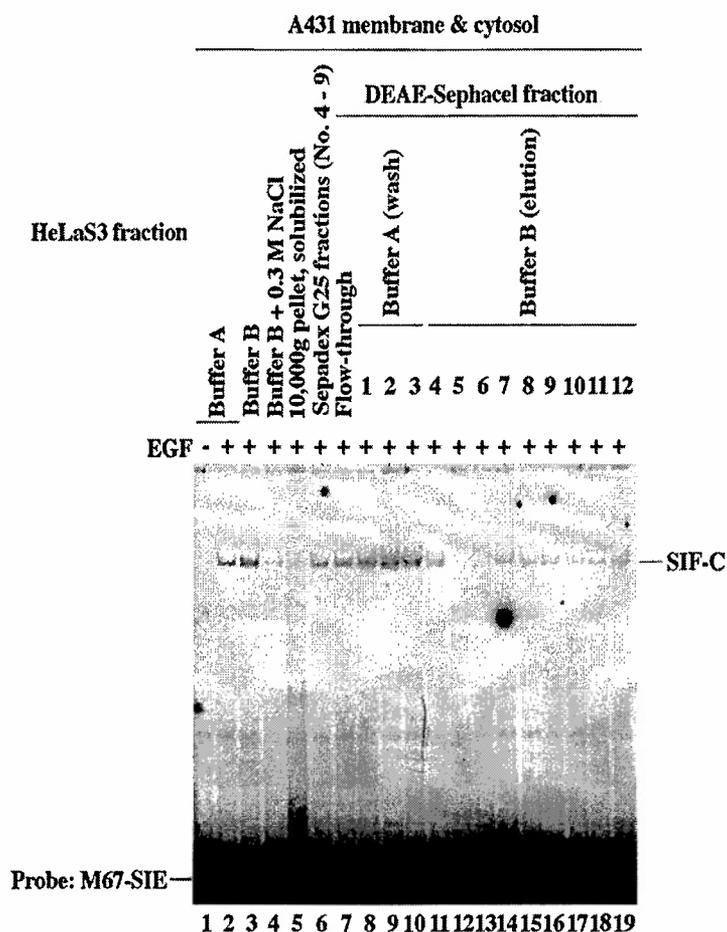
Titration of the HeLa S3 membrane fraction to inhibit the in vitro activation of STAT proteins in the HeLa S3 cytosol by the A431 membrane. The STAT proteins in the HeLa S3 cytosol fraction (2 ug protein) were activated in vitro by the A431 membrane fraction (0.3 ug) in the presence of various protein concentrations of the HeLa S3 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32P-end-labeled M67-SIE as a probe.

Figure 6A



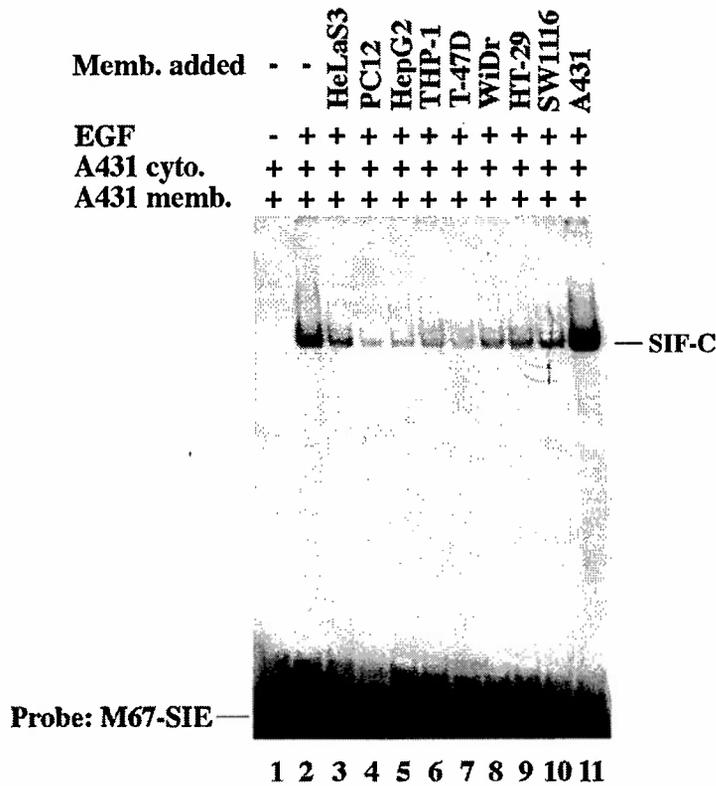
Solubilization of the STAT inhibitor from the membrane fraction: The STAT proteins in the A431 cytosol fraction (2 ug protein) were activated in vitro by the A431 membrane fraction (0.3 ug) in the presence of HeLa S3 fractions (40 ug). The 10,000g pellet refers to the membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ³²P-end-labeled M67-SIE as a probe.

Figure 6C



Separation of the STAT inhibitor activity by DEAE-Sephacel column chromatography: Two microliters of each fraction eluted from DEAE-Sephacel was subjected to the STAT inhibitor assay as described in Experimental methods. Two microgram protein of the A431 cytosol fraction (containing the inactive STAT proteins) and 0.3 ug 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 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.

Figure 7



STAT inhibitor activity in various cell lines: The STAT proteins in the A431 cytosol fraction (5.5 ug protein) were activated in vitro by the A431 membrane fraction (0.44 ug) in the presence of the HeLa S3, PC12, HepG2, THP-1, T-47D, WiDr, HT-29, SW1116, or A431 membrane fraction (6.2 ug). Three microliters of the in vitro-activated sample were subjected to EMSA using 32P-end-labeled M67-SIE as a probe. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed.

APPENDICES

Curriculum Vitae

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1992 - 1995 Research Assistant, Department of Biochemistry, Dr. Ryo Kido's Laboratory, Wakayama Medical School, Wakayama, Japan

1995-present Postdoctoral Associate, Fellow, Department of Pathology, Dr. Xin-Yuan Fu's Laboratory, Yale University School of Medicine, New Haven, CT

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1997-2000 US Army Breast Cancer Research Fellowship Award

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- Iwamoto, Y., Tsubaki, M., Hiwatashi, A. and Ichikawa, Y. (1988) Crystallization of cytochrome P-450_{SCC} from bovine adrenocortical mitochondria. FEBS Lett., 233: 31-36.
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Nakamura, Y., Funasako, M. and Saijo, N. (1996) $1\alpha,25$ -dihydroxyvitamin D₃ and all-trans-retinoic acid inhibited the growth of a non-small lung cancer cell line. AntiCancer Research, 16, 2653-2660.

Chin, Y.E., Kitagawa, M., Su, W.S., You, Z., Iwamoto, Y. and Fu, X. (1996) Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21^{WAF/CIP1} mediated by STAT1. Science, 272, 719-722.

Iwamoto, Y., Chin E.Y. and Fu, X.(1998) Identification of a membrane-associated inhibitor(s) of EGF-induced STAT activation. J.Biol.Chem., in press..

Maeda, H., Iwamoto, Y., Tone, S., Kadoya, A., Minatogawa, Y. and Kido, R. (1998) Characterization of human indoleamine 2,3-dioxygenase expressed in *E.coli*, to be submitted.

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

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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)¹ 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).² 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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¹ 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.

² B. Xie and X.-Y. Fu, unpublished data.

bovine serum; T-47D human breast ductal carcinoma cells (ATCC, HTB-133) in RPMI 1640 containing 10% fetal bovine serum and 10 μ g/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 monocyte cells (ATCC, TIB-202) were grown in suspension in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, and 50 μ M 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 μ g/ml each). Whole cell extracts were immediately subjected to electrophoresis shift assay.

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 EGTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 125 nM okadaic acid, 0.4 mM ammonium molybdate, 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin (1 μ g/ml each) by a Dounce homogenizer. The lysate was microcentrifuged for 5 min at 3,000 \times g at 4 $^{\circ}$ C. The supernatant was adjusted to 120 mM NaCl and microcentrifuged at 10,000 \times g for 30 min at 4 $^{\circ}$ 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 lysis buffer containing 150 mM NaCl and 8% glycerol and microcentrifuged at 10,000 \times g for 30 min at 4 $^{\circ}$ 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 Vitro Activation of STAT Proteins by EGF—The *in vitro* 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 μ g/ml each) (buffer A). Three μ l of the membrane fraction was mixed with 2 μ l of the cytosol fraction and 0.5 μ l of either 100 μ g/ml EGF (EGF+) or phosphate-buffered saline (EGF-). The mixture was incubated on ice for 15 min. The reaction was started by the addition of 2.5 μ l of 50 mM Hepes buffer (pH 7.4) containing 20 mM MgCl₂, 10 mM MnCl₂, and 10 mM ATP. After incubation at 30 $^{\circ}$ C for the indicated time, the reaction was stopped by putting the tubes on dry ice. For the STAT inhibitor assay, 2 μ l 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 after *in vitro* activation (3 μ l) (1 μ l for the whole cell extract) was mixed with 15 μ l of 13 mM Hepes buffer (pH 7.9) containing 50 mM NaCl, 0.1 mM EDTA, 8% glycerol, 0.5 mM dithiothreitol, 66.7 μ g/ml poly(deoxyinosine-deoxycytidine), and 33.3 μ g/ml salmon sperm DNA. After incubation on ice for 30 min, 1 μ l of double-stranded oligodeoxynucleotide M67-SIE probe end-labeled with [γ -³²P]ATP was added. For the supershift assay, 1 μ l 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

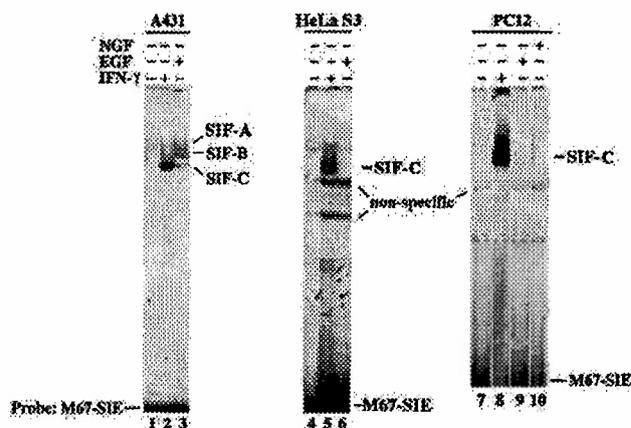


Fig. 1. *In vivo* activation of STAT proteins by EGF is observed in A431 cells but not in many other cell lines. A431, HeLa S3, and PC12 cells were deprived of serum for 17 h and then stimulated with 1 ng/ml IFN- γ , 100 ng/ml EGF, or 50 ng/ml NGF for 15 min. One μ l of the whole cell extract was subjected to EMSA using ³²P-end-labeled M67-SIE as a probe. Three types of the STAT protein-SIE complexes (SIF-A, B, and C) were observed. SIF-A, STAT3 homodimer; SIF-B, STAT1/STAT3 heterodimer; SIF-C, STAT1 homodimer (for details, see Refs. 30–32).

dithiothreitol, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, and 6 M 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 $^{\circ}$ C, and dialysis was continued for additional 2 h. The dialytic buffer was changed, followed by dialysis at 4 $^{\circ}$ C for 2 h; and the dialytic buffer was replaced again, and the final cycle of dialysis was continued at 4 $^{\circ}$ C overnight. The dialysate was microcentrifuged at 10,000 \times g for 30 min at 4 $^{\circ}$ C. The supernatant was saved as a solubilized membrane fraction (10,000 \times g pellet, solubilized) and kept at 4 $^{\circ}$ C until use.

Column Chromatographic Procedures—The gel filtration Sephadex G-25 (Amersham Pharmacia Biotech) and the anion exchanger DEAE-Sephacel (Amersham) columns were used for partial purification of the inhibitor. One ml of the solubilized membrane fraction (4 mg of protein) was loaded onto the column of Sephadex G-25 (1 \times 13 cm) previously equilibrated with 20 mM Tris buffer (pH 7.4) containing 0.5 mM EDTA, 10% glycerol, 0.05% Triton X-100, 20 mM sodium fluoride, 0.1 mM sodium vanadate, 1 μ g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM 2 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-Sephacel (0.4 \times 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 μ l 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 homodimer, STAT1/STAT3 heterodimer, and STAT1 homodimer, respectively (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*. SIF-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 μ g) was used in this

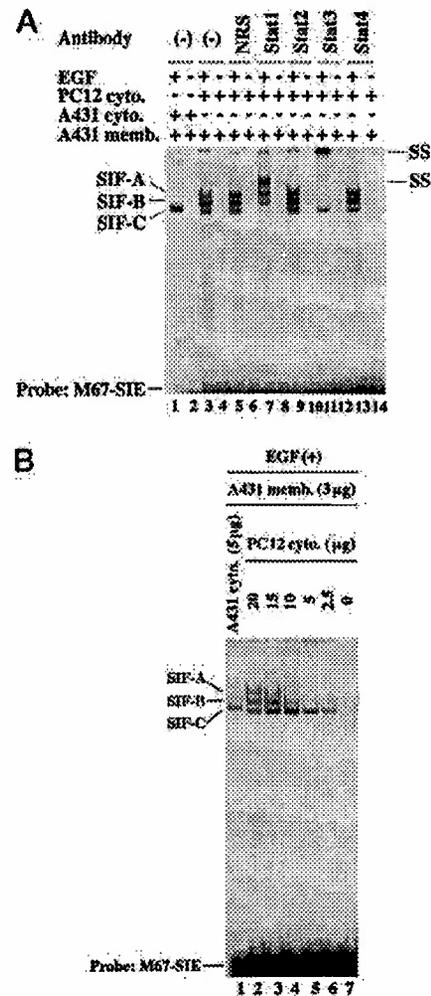


FIG. 2. A, *in vitro* activation and supershift of STAT proteins. The STAT proteins in either A431 (5 μ g protein) or PC12 (20 μ g) cytosol (cyto.) fraction were activated by the A431 membrane (memb.) fraction (3 μ g) *in vitro* (30-min reaction). Three μ l of the *in vitro* activated sample were subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. Three types of protein-SIE complexes (SIF-A, B, and C) were observed and supershifted (SS) by antisera against STAT proteins. NRS, nonrelated rabbit serum. B, protein concentration dependence of SIFs formation. The STAT proteins in various protein concentrations of the PC12 cytosol or the A431 cytosol fraction (5 μ g protein) were activated by the A431 membrane (3 μ g) *in vitro* (30-min reaction). Three μ l of the *in vitro* activated sample were subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. Three types of protein-SIE complexes (SIF-A, B, and C) were formed depending on the protein concentration of the cytosol fraction.

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-

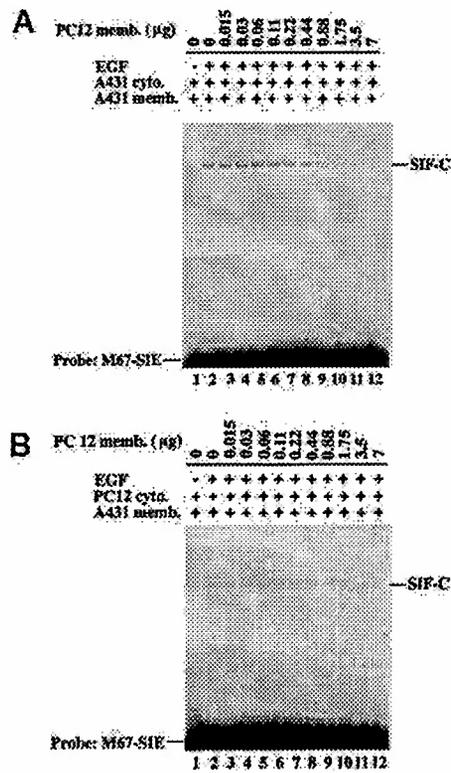


FIG. 3. *A*, titration of the PC12 membrane (*memb.*) fraction to inhibit the *in vitro* activation of STAT proteins in the A431 cytosol (*cyto.*) by the A431 membrane. The STAT proteins in the A431 cytosol fraction (2 μg of protein) were activated *in vitro* by the A431 membrane fraction (0.3 μg) in the presence of various protein concentrations of the PC12 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ³²P-end-labeled M67-SIE as a probe. *B*, titration of the PC12 membrane to inhibit the *in vitro* activation of STAT proteins in the PC12 cytosol by the A431 membrane. The STAT proteins in the PC12 cytosol fraction (2 μg of protein) were activated *in vitro* by the A431 membrane fraction (0.3 μg) in the presence of various protein concentrations of the PC12 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ³²P-end-labeled M67-SIE as a probe.

scribed *in vitro* activation system and determined the optimal conditions for the inhibitor assay. We first fixed the protein concentration of the A431 membrane to a relatively low degree. Then, the reaction time was determined to give a linear dependence of the STAT protein activation on the cytosol fraction (STAT protein) concentration (see "Experimental Procedure" for details). 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. 3*A*, lanes 1–2; similar results were shown in Fig. 2*B*). 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. 3*A*, 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

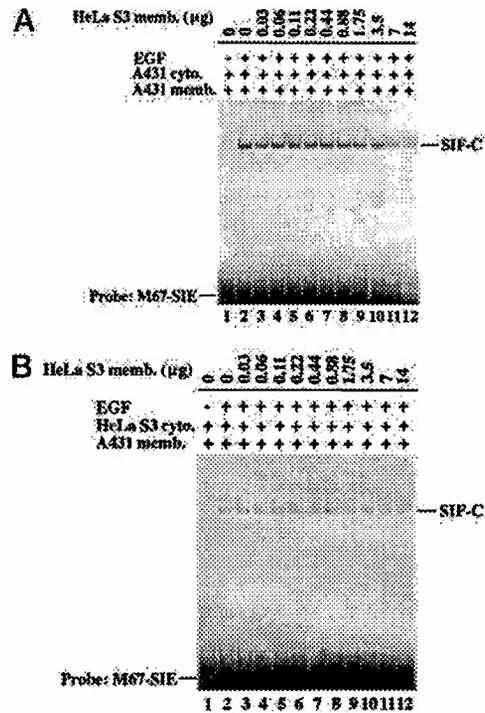


FIG. 4. *A*, titration of the HeLa S3 membrane to inhibit the *in vitro* activation of STAT proteins in the A431 cytosol by the A431 membrane. The STAT proteins in the A431 cytosol fraction (2 μg of protein) were activated *in vitro* by the A431 membrane (*memb.*) fraction (0.3 μg) in the presence of various protein concentrations of the HeLa S3 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ³²P-end-labeled M67-SIE as a probe. *B*, titration of the HeLa S3 membrane to inhibit the *in vitro* activation of STAT proteins in the HeLa S3 cytosol by the A431 membrane. The STAT proteins in the HeLa S3 cytosol fraction (2 μg of protein) were activated *in vitro* by the A431 membrane fraction (0.3 μg) in the presence of various protein concentrations of the HeLa S3 membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using ³²P-end-labeled M67-SIE as a probe.

the A431 cytosol was used for STAT activation (Fig. 3*B*), 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 vivo* 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. 4*A*) or the A431 membrane and HeLa cytosol fractions (Fig. 4*B*), 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.

The Inhibitor Exerts Its Effect during EGF-induced STAT Activation—There are at least two possibilities how this inhib-

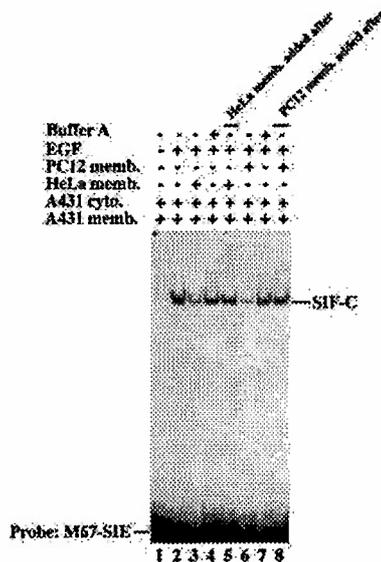


FIG. 5. The inhibitor in HeLa S3 or PC12 membrane inhibits the *in vitro* activation of the STAT1 but not the binding of the activated STAT1 with SIE. Lanes 1–3 and 6, the STAT proteins in the A431 cytosol (cyto.) fraction (5.5 μ g of protein) were activated *in vitro* by the A431 membrane (memb.) fraction (0.44 μ g) in the absence or the presence of the HeLa S3 or the PC12 membrane fraction (6.2 μ g). Three μ l of the *in vitro* activated sample was subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. Lanes 4 and 5 and 7 and 8, two μ l of buffer A, the HeLa S3, or the PC12 fraction (6.2 μ g) was mixed with the *in vitro* activated sample (10 μ l), and 3 μ l of the mixture was subjected to EMSA. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed.

itor could block EGF-induced STAT activation. First, the inhibitor may block initial activation of STAT by the EGF receptor. For instance, the inhibitor may specifically block the interaction of STAT with the EGF receptor. Second, the inhibitor may interact directly with the STAT complex to disrupt the complex or prevent it from binding the SIE. In the latter case, this inhibitor should block the SIE-binding of the STAT complex even after it is formed.

To distinguish between these two possibilities, we conducted the following experiment. The PC12 or the HeLa membrane fraction containing the inhibitor(s) was added to the reaction mixture either before or after the *in vitro* STAT activation reaction by EGF. We assume that if this inhibitor blocked only the initiation of STAT activation by the EGF receptor, the inhibitor would not work upon its addition after completion of the reaction. On the other hand, if the inhibitor could directly block the SIE binding of the STAT complex, it should work even when the STAT complex had been formed after initial reaction.

As shown in Fig. 5, the inhibitor fractions from the HeLa or the PC12 membrane but not buffer A reduced the levels of activated STAT1 (SIF-C) (comparing lanes 3 with 4, and lanes 6 with 7) when these fractions were added before initiation of the reaction by EGF. However, when these fractions were added after the reactions had been completed, no inhibitory effects were observed (lanes 5 and 8). These results suggested that the inhibitor may block the initial event of STAT activation by EGF receptor. One possibility is that it may block direct interactions of STAT with EGF receptor (see "Discussion").

Partial Purification of the Inhibitor from the HeLa Cell Membrane—One of the major concerns about this inhibitor is whether it is a protein. To further characterize the inhibitor, we have tried to purify it from the membrane fraction.

Since the factor is membrane-associated, the first step was to solubilize it from the membrane. Both 0.25% Triton and 1.2% (w/v) sodium cholate failed in solubilization of the inhibitor (data not shown). Then we tried to use 6 M guanidine hydro-

chloride. After treatment with 6 M guanidine hydrochloride followed by extensive dialysis to remove it, the inhibitor was found to be successfully solubilized and renatured. A large amount of other proteins was precipitated during dialysis and removed by centrifugation. This inhibitor seems to be a protein because it did not pass through the dialytic membrane. After this step, the inhibitor activity was substantially enriched (Fig. 6A, lane 5). The second step was gel filtration with Sephadex G-25. As expected, the inhibitor activity was co-eluted with the protein fraction, as judged by SDS-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue R-250 staining (data not shown), further indicating that the inhibitor is likely to be a protein factor. After gel filtration, the loaded solution was greatly diluted; thus the inhibitor activity of the eluate, with the fraction 6 as a peak, was rather weak (Fig. 6B, lane 11). In the third step of purification, the diluted inhibitor activity was significantly enriched in the fraction 5 from the DEAE-Sephacel column (Fig. 6C, lane 12). This three-step procedure led to more than 1,000-fold purification of the inhibitor. The protein nature of this inhibitor was further confirmed by its protease sensitivity (data not shown).

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 may 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 vivo* 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

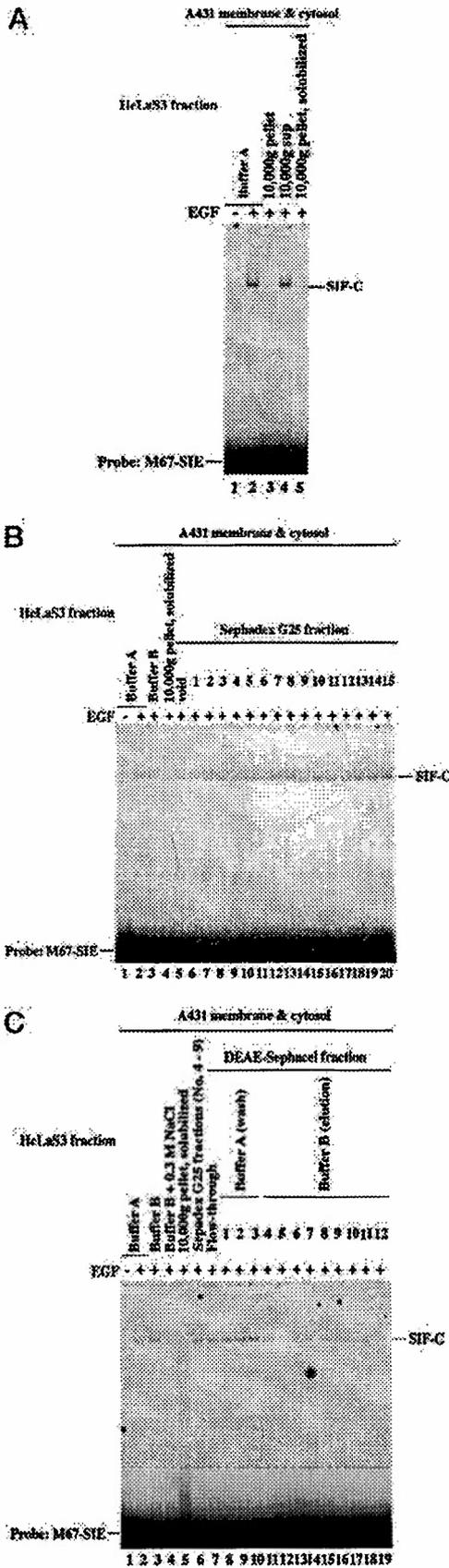


FIG. 6. Partial purification of the inhibitor. A, solubilization of the STAT inhibitor from the membrane fraction. The STAT proteins in the A431 cytosol fraction (2 μ g of protein) were activated *in vitro* by the A431 membrane fraction (0.3 μ g) in the presence of HeLa S3 fractions (40 μ g). The 10,000g pellet refers to the membrane fraction. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed on EMSA using 32 P-end-labeled M67-SIE as a probe. *sup*, supernatant. B,

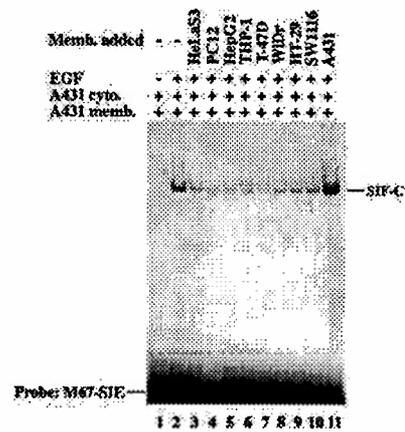


FIG. 7. STAT1 inhibitor activity in various cell lines. The STAT proteins in the A431 cytosol fraction (5.5 μ g of protein) were activated *in vitro* by the A431 membrane (*memb.*) fraction (0.44 μ g) in the presence of the HeLa S3, PC12, HepG2, THP-1, T-47D, WiDr, HT-29, SW1116, or A431 membrane fraction (6.2 μ g). Three μ l of the *in vitro* activated sample were subjected to EMSA using 32 P-end-labeled M67-SIE as a probe. The formation of the STAT1 homodimer-SIE complex (SIF-C) was observed.

C-terminal region.³ 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

³ Y. E. Chin, X. Peng, and X.-Y. Fu, unpublished results.

separation of the STAT inhibitor activity by Sephadex G-25 gel filtration column chromatography. After Sephadex G-25 gel filtration, two μ l of each fraction was subjected to the STAT inhibitor assay as described under "Experimental Procedures." Two μ g of protein of the A431 cytosol fraction (containing the inactive STAT proteins) and 0.3 μ g 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 32 P-end-labeled M67-SIE as a probe. C, separation of the STAT inhibitor activity by DEAE-Sephacel column chromatography. Two μ l of each fraction eluted from DEAE-Sephacel was subjected to the STAT inhibitor assay as described under "Experimental Procedures." Two μ g of protein of the A431 cytosol fraction (containing the inactive STAT proteins) and 0.3 μ g 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 32 P-end-labeled M67-SIE as a probe.

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).² 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.

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