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# Modulation of Hepatic Acute Phase Gene Expression by Epidermal Growth Factor and Src Protein Tyrosine Kinases in Murine and Human Hepatic Cells

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As part of systemic inflammatory reactions, interleukin 6 (IL-6) induces acute phase protein (APP) genes through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Epidermal growth factor (EGF), which contributes to the regenerative process after liver injury and also activates STATs, does not induce but attenuates IL-6–stimulated expression of several APP genes in primary mouse hepatocytes. The APP-modifying action of EGF receptor (EGFR) was characterized in HepG2 cells. Although EGF less effectively engages STAT proteins in these cells, it reduces expression of fibrinogen and haptoglobin, but stimulates production of $\alpha_1$-antichymotrypsin and induces transcription through the $\alpha_1$-antichymotrypsin and C-reactive protein promoter. The stimulatory EGFR signal is insensitive to inhibition of JAKs and appears to involve Src kinases and STAT proteins as shown by inhibition through overexpression of C-terminal Src kinase (Csk) and transdominant negative STAT3, respectively. A mediator role of Src is supported by the ability of c-Src and v-Src to activate STATs and induce transcription through APP promoters. Src kinases have been observed in association with the IL-6 receptor; however, inhibition of Src kinases by Csk enhances IL-6–induced transcription. The Csk effect is attributed to prevention of Src kinases from phosphorylating gp130 at the docking site for the signal-moderating protein tyrosine phosphatase SHP-2. The inhibitory EGFR signal on APP expression correlates with the activation of Erk1 and Erk2. The study shows a dual signaling function for EGFR and suggests that the ratio of receptor-activated STATs and Erks influence the level of stimulated or inhibited expression of individual APPs. (HEPATOLOGY 1999;30: 682-697.)

The increased production of acute phase plasma proteins (APP) by the liver in response to inflammation at extrahepatic sites is proposed to be mediated primarily by the concerted action of interleukin-1 (IL-1)– and IL-6–type cytokines and glucocorticoids.[1,2] However, the level of expression and cytokine regulation of individual APPs appears also to be additionally modulated by the action of endocrine hormones[3,4] and growth factors.[5-7] Few studies indicated a modest inhibitory effect of epidermal growth factor (EGF) on APPs in cultured hepatic cells.[8,9] However, EGF action on liver cells, in the context of inflammatory mediators as predicted to be present during acute phase response *in vivo*, during liver regeneration after partial hepatectomy, or during intrahepatic inflammation,[10-12] has not been determined. In particular, the suppressed execution of the hepatic acute phase response in regenerating liver[13] suggests an inhibitory effect of the proliferative signals delivered by growth factors, including EGF. Because under conditions of liver injury and regeneration, factors that promote hepatocyte growth and cytokines, which induce an acute phase response, temporally coexist in the liver, we hypothesize that growth factor receptor signals attenuate the action of IL-6 cytokines in a dominant fashion. The goal of this study was to charter the signaling pathways engaged by EGF that influence the liver cell response to IL-6 cytokines and affect expression of APPs.

Transcriptional induction of many APP genes depends on IL-6. The related cytokines, IL-11, leukemia inhibitory factor, and oncostatin M, can in part reproduce the effects of IL-6.[1] The functional redundancy of these cytokines is explained by the involvement of the common receptor subunit, gp130, in the signal transduction process by the respective cytokine receptor complex.[14] The activation of the DNA binding activity of signal transducer and activator of transcription (STAT)3 is the hallmark of gp130-dependent signaling[14,15] and is mediated by the receptor-associated Janus kinases, JAK1, JAK2, and TYK2.[16] The binding of activated STAT3 to STAT recognition sites within promoter regions of APP genes is suggested to be instrumental in controlling transcriptional induction.[17]

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Abbreviations: APP, acute phase protein; IL, interleukin; EGF(R), epidermal growth factor (receptor); STAT, signal transducer and activator of transcription; PTK, protein tyrosine kinase; MAPK, mitogen-activated protein kinase; Csk, C-terminal Src kinase; SSI-1, STAT induced STAT inhibitor-1(=SOCS1); G-CSF(R), granulocyte-colony stimulating factor (receptor); SHP-2, SH2 containing protein tyrosine phosphatase; MUP, major urinary protein; CAT, chloramphenicol acetyl transferase; HPX, hemopexin; α-FB, α-fibrinogen; ACH, $\alpha_1$-antichymotrypsin; HP, haptoglobin; CRP, C-reactive protein; C/EBP, CAAT/enhancer binding protein; EMSA, electrophoretic mobility shift assay; SDS, sodium dodecyl sulfate; mRNA, messenger RNA; SIE, *sis*-inducible element; AGP, $\alpha_1$ acid glycoprotein.

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JAKs are not the only kinases that are associated with signal transduction by gp130. Additional, cytoplasmic protein tyrosine kinases (PTK) are found in complex with gp130, which, depending on the cell type, include Src kinases, (Lyn,[18] c-Src, c-Yes,[19,20] Fyn,[21] and Hck[22]), Fes,[23] and Tec.[24] Several of these kinases also participate in the signal transduction by other hematopoietin receptors and by protein tyrosine kinase receptors for growth factors including EGF.[25-28] These kinases have generally been implicated in the control of cellular architecture and proliferation by engaging cytoskeletal elements and the Ras-mitogen-activated protein kinase (MAPK) pathways. Moreover, by manipulating their expression or using oncogenic variants, the kinases were also recognized to activate STAT proteins.[29-34] Although the link of receptor and Src PTKs to STAT activation would suggest potential regulation of STAT-responsive genes, the contribution of these kinases to the signaling process that affects, either positively or negatively, the expression of APPs in hepatic cells has not been assessed. This is particularly evident in the example of the response of liver cells to EGF. *In vivo* administration of EGF produces an immediate activation of STAT3 in liver in a fashion similar to IL-6.[35] However, a corresponding induction of the STAT3-responsive APP gene has not been reported. This report describes the various modes through which epidermal growth factor receptor (EGFR), as well as the suggested signal-communicating Src kinases, influence expression and IL-6 induction of APPs in cultured hepatic cells.

## MATERIALS AND METHODS

*Plasmids.* The DNAs encoding v-Src (coding region from pGDvSrc Schmitt-Ruppin A Rous sarcoma virus oncogene[36,37]), human C-terminal Src kinase (Csk)[38] (2.4 kb *Sma* I fragment, provided by Dr. D.O. Morgan, University of California, San Francisco, CA), rat STAT1α, STAT3, STAT5,[39] STAT3$_\Delta$55C,[40] mouse STAT4,[41] and human STAT6[42] were subcloned as *Not* I fragments into pDC vector.[43] The full-length STAT-induced STAT-inhibitor-1 (SSI-1 or SOCS1) gene[44] was amplified by polymerase chain reaction with a primer pair (forward primer: CTCGAGTAGGATGGTAGCAC; reverse primer: TGTAAACATGGAGAGGTAGGA), based on the published sequence using murine genomic DNA as a template. The polymerase chain reaction was performed in the following conditions: 94°C, 1 minute; 56°C, 1 minute; 72°C, 2 minutes. The amplified product was purified, verified by sequencing, and subsequently cloned into the expression vector, pcDNA3.1/His (InVitrogen, Carlsbad, CA). The following vectors have been described: FJ vector, containing avian c-Src[45] (provided by Dr. J.V. Jung, Harvard Medical School, Southborough, MA), pSV containing human EGFR,[46] pDC containing the human chimeric receptor granulocyte colony stimulatory factor receptor (G-CSFR)-gp130-FLAG or G-CSFR-gp130(Y2F)-FLAG (containing the mutated SH-2 domain containing protein tyrosine phosphatase (SHP)-2 binding site in which tyrosine 759 is substituted by phenylalanine, =Y2F)[47,48] and pIE-MUP containing the murine major urinary protein (MUP) gene.[49] The expression vector for the constitutively active, double mutant MEK-1 (S218D/S222D) with hematoglutinin antigen tag was purchased from Upstate Biotechnology, Lake Placid, NY.

Gene induction was measured with the following chloramphenicol acetyl transferase (CAT) reporter gene constructs: pHPX(5xIL-6RE)-CAT (containing 5 tandem copies of the STAT3-specific IL-6RE of the rat hemopexin [HPX] gene in pCAT[50]), p$_\alpha$FB(6900)-CAT (containing the 6,900-bp promoter of the rat $_\alpha$ fibrinogen [$_\alpha$-FB] gene[51]), pACH(3700)-CAT (containing the 3,700-bp promoter of the human $_{\alpha1}$-antichymotrypin [ACH] gene[52]), pHP(4200)-CAT (containing the 4,200-bp 5′ flanking region from position of rat haptoglobin [HP] gene[53]), and pCRP-CAT constructs (containing the promoter region of human C-reactive protein [CRP] gene of 565, 219,[54] 123, 85, or 50 bp length[55]). The 123-bp promoter constructs with site-directed mutations of the binding sites for STAT3 (STAT3mut) and/or CAAT/enhancer binding protein (C/EBP) (C/EBPmut) were also included as described.[55] pTIMP-1-CAT containing the AP-1–responsive human tissue inhibitor of metalloproteinase (TIMP)-1 promoter (−62 to +47) was provided by Dr. C. Richards, McMaster University, Hamilton, Ontario, Canada.[56]

*Cells and Transfection.* Mouse hepatocytes were prepared by *in situ* retrograde perfusion of the liver of 10-week-old C57BL/6J male mice with collagenase.[3,57] The parenchymal cells were collected by differential centrifugation and showed viability >90%. The cells were plated into culture dishes coated with collagen (Vitrogen 100; Collagen Biomaterials, Palo Alto, CA) ($1 \times 10^5$ cells/cm$^2$) in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 4.5 g/mL glucose, penicillin, streptomycin, and gentamicin. No hormones (*e.g.*, insulin, EGF, dexamethasone) were added to avoid modulation of the cytokine responsiveness of the cells. The hepatocytes that adhered after a 1-hour incubation were cultured overnight (16 hours) before use for hormonal treatment. Human HepG2 cells[58] were also maintained in Dulbecco's modified Eagle medium but containing 1 mg/mL glucose. Because the present cultures of HepG2 cells (termed "standard culture") have a low to nondetectable level of EGFR, a clonal line (termed "86-6") with the highest level of expressed EGFR was selected from the 78th passage of HepG2 cells obtained from Dr. B. Knowles in 1986. Analysis of RNAs (reverse-transcription polymerase chain reaction and Northern blot hybridization) and proteins (Western blot and activity neutralizing antibodies) indicated that none of the HepG2 cell lines, whether transfected or not, expressed detectable levels of endogenous IL-6.

HepG2 cells were transfected with the calcium phosphate method,[59] using a final DNA concentration of 20 µg/mL of precipitation mixture. In standard transfection protocols, a 1-mL mixture contained 10 to 15 µg of CAT reporter gene construct, 0 to 5 µg of kinase expression vector, 0 to 3 µg of STAT expression vector, 0 to 5 µg of expression vector for receptor subunits, and 1 µg of pIE-MUP as an internal marker.[47,49,60] Empty expression vector was added to bring the DNA to the identical concentration within the experimental series. From the mixture, aliquots representing 1/10 volume of the culture medium were added to HepG2 cell cultures. The concentration of expression vectors for EGFR and STATs was increased to 5 µg/mL and 10 µg/mL, respectively, to gain a level of protein expression detectable in the transfected culture by immunoblotting and electrophoretic mobility shift assay (EMSA) using whole cell lysates. The cells were incubated overnight in the presence of calcium phosphate precipitates at 35.5°C in a 2.5% $CO_2$/air atmosphere. Where necessary, the cell cultures were subdivided and plated into 6-well cluster plates. After 24-hour recovery, the cells were used for determining the activation of STAT proteins by EMSA, the levels of expressed proteins by Western blotting, or the activation of CAT gene expression by CAT enzyme assay.

Cell cultures intended for STAT and protein analysis were maintained for 6 hours in serum-free medium and, where necessary, were treated for 15 minutes with 100 ng/mL of G-CSF, oncostatin M (Immunex Corp., Seattle, WA), IL-6 (Genetics Institute, Cambridge, MA), or EGF (Collaborative Research, Inc., San Jose, CA) or 500 ng/mL porcine insulin (Sigma, St. Louis, MO). The cells were washed with phosphate-buffered saline and scraped off the dish in phosphate-buffered saline containing a cocktail of protease and phosphatase inhibitors. Part of the cell suspension was used to prepare whole cell[61] or nuclear extracts,[39] and part was dissolved in boiling sodium dodecyl sulphate (SDS) sample buffer.

Cells used for CAT and APP gene regulation were treated for 24 hours with serum-free medium alone or medium containing 100 ng/mL of cytokines, 1 µmol/L dexamethasone, 500 ng/mL insulin, or 10 ng/mL IL-1β (Immunex). CAT activity was determined in serially diluted cell extracts to ensure measurement in the linear range of the assay. The CAT value for each culture (percent conversion of chloramphenicol to acetylated products per hour) was normalized to the amount (pg) of MUP secreted by the same culture during the 24-hour treatment period (derived from the cotransfected marker pIE-MUP and determined by immunoelectrophoresis) and termed normalized CAT activity. Fold stimulation was calculated relative to the value of control-treated culture in each series (defined as 1.0).

Changes in APP messenger RNA (mRNA) were determined by Northern blot hybridization[62] and signal quantitated by phosphorimaging (Molecular Dynamics, Sunnyvale, CA). The amounts of APP secreted into the culture medium were measured by immunoelectrophoresis using standardized conditions.[63] The area under the precipitation peaks was integrated by using the NIH Image program version 1.61 (Bethesda, MD), and values were normalized to equal cell number ($1 \times 10^5$) and expressed in arbitrary immunoelectrophoretic units.

*EMSA.* Aliquots of whole cell extracts containing 5 µg of protein were applied to EMSA as described.[61] $^{32}$P end-labeled double-stranded oligonucleotides, representing the 23-bp high affinity *sis*-inducible element (SIE)m67,[61] served as substrates for STAT1, STAT3, and STAT4; the 40-bp TB-2[64] as a substrate for STAT5; and the 23-bp gamma activated sequence of the Fc-receptor II gene as a substrate for STAT6.[42] The DNA protein complexes formed by endogenous STAT proteins were identified by antibody-mediated supershift using anti-STAT1 and anti-STAT3 from Santa Cruz Biotechnology (Santa Cruz, CA). Relative differences in the radioactive signals associated with gel shift complexes were quantitated by phosphorimaging.

*Immunoprecipitation and Western Blot.* Cells were lysed in modified RIPA buffer (50 mmol/L Tris, pH 7.4, 50 to 150 mmol/L NaCl, 1% Brij96, 0.25% sodium deoxycholate, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 µg/mL leupeptin and aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10% glycerol). After centrifugation at 15,000*g* for 10 minutes, the soluble fraction was used for immunoprecipitation with anti-FLAG antibody (M2) from Eastman Kodak (Rochester, NY), anti–SHP-2 antibodies, or goat anti-human EGFR from Upstate Biotechnology. The immune complexes were recovered with protein G-Sepharose (Amersham Pharmacia, Piscataway, NJ) by incubating for 16 hours, and then were washed with modified RIPA buffer. Immunoprecipitated proteins or aliquots of SDS cell lysates containing 30 µg of protein were separated in SDS-polyacrylamide gels. Proteins were transferred to Immobilon P-membrane (Millipore, Marlborough, MA) and reacted with rabbit or goat anti-mammalian p60Src (Santa Cruz Biotechnology), anti-avian p60Src (Upstate Biotechnology), anti-SHP2 (Santa Cruz Biotechnology), anti-STAT3 (Transduction Laboratories Biotechnology, Lexington, KY), anti-phosphotyrosine(705)-STAT3, anti-phospho-Erk (New England Biolabs, Beverly, MA), or anti-phosphotyrosine PY20 (Transduction Laboratories) antibodies. The immune complexes were visualized by enhanced chemiluminescence reaction (Amersham).

*Statistical Analysis.* The quantitative data from experimental series, representing normalized CAT activities, fold stimulation of CAT expression, or production of plasma protein (immunoelectrophoretic units), are shown as mean ± SD. Differences between control and experimental treatments were evaluated by Student's *t* test. A *P* value of less than .05 was considered statistically significant.

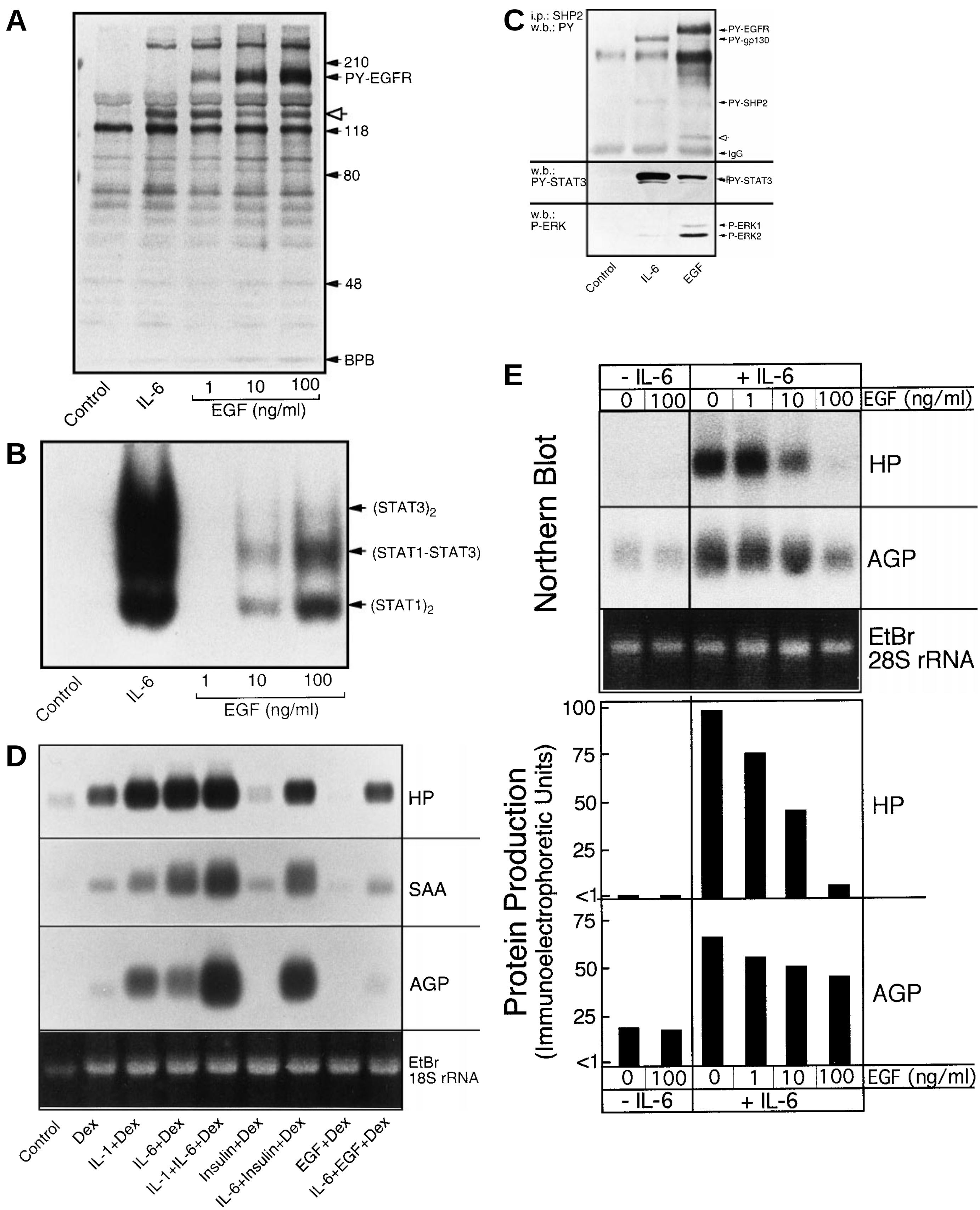
## RESULTS

*EGF Modulated APP Expression in Mouse Hepatocytes.* To assess EGF effects on expression and cytokine regulation of APPs in normal hepatocytes, the EGF response of primary cultures of adult mouse hepatocytes was determined. The parenchymal cells from several independent preparations responded in a comparable dose-dependent manner to EGF treatment by a prominent tyrosine phosphorylation of EGFR (results of a representative experiment are shown in Fig. 1A). The same cells also showed enhanced tyrosine phosphorylation of few proteins after IL-6 treatment. The engagement of the STAT pathway by both factors was apparent from the pattern of SIE-binding activity of STAT1 and STAT3 (Fig. 1B) and by the immunodetectable phospho-STAT3 (Fig. 1C). The signaling capability of EGFR and IL-6R in hepatocytes to other shared pathways was also apparent by similar recruitment of the protein tyrosine phosphatase SHP-2 (coimmunoprecipitation of the receptors with SHP-2 in Fig. 1C) and by phosphorylation of Erk1 and Erk2 (Fig. 1C). The results clearly indicated that EGF has a greater effect on the MAPK pathway than on the STAT pathway, whereas the reverse was seen with IL-6.

Analysis of the cellular RNAs from hepatocytes treated for 24 hours with a combination of cytokines showed the characteristic regulation of APP genes with prominent induction of HP, serum-amyloid A, and $\alpha_1$ acid glycoprotein (AGP) (Fig. 1D). Despite the activation of STATs, EGF did not induce mRNA for these APPs, even in the presence of dexamethasone that normally enhances induction of APPs by cytokines.[1] In fact, EGF produced a 2- to 5-fold reduction in the expression of the gene products. The inhibitory action of EGF exceeded that of insulin, a hormone that does not appreciably activate STATs and reduces IL-6 action on type 2 APPs such as HP, but enhances cytokine-regulated expression of type 1 APP such as AGP (Fig. 1D[4,5]). The inhibitory activity of EGF was particularly evident in the presence of IL-6, was dose dependent but not critically influenced by dexamethasone, and was similarly manifested at the level of mRNA and secreted protein (Fig. 1E).

*EGF Response in HepG2 Cells.* Because primary hepatocyte cultures do not consist of homogenous cell populations, are subject to phenotypic changes, and are not as readily amenable to experimental manipulation by DNA transfection as are hepatoma cells, we determined whether HepG2 cells, one of the most commonly used cell lines for studying APP gene regulation, reproduce an EGF response like mouse hepato-

FIG. 1. EGF response of primary mouse hepatocytes. (A and B) Cultures of adult male mouse hepatocytes were treated for 15 minutes with IL-6 (100 ng/mL) or increasing concentrations of EGF as indicated. (A) Cell lysates were subjected to Western blot analysis for antiphosphotyrosine reactive proteins. The positions of the molecular size markers (kd) and phosphotyrosine-EGFR (*PY-EGFR*) are marked at the right. The *open arrow* indicates the IL-6– and EGF-responsive phosphotyrosine protein of approximately 130 kd, probably representing a member of the SIRP family.[87] (B) Whole cell extracts of the cells in A were subjected to EMSA using SIE as substrate. Position of the SIE complexes containing STAT1 and/or STAT3 are marked. (C) Cell lysate from hepatocytes, treated for 15 minutes with medium alone, IL-6 (100 ng/mL), or EGF (100 ng/mL) were immunoprecipitated with anti–SHP-2 antibodies, and Western blotted (*upper panel*) using anti-phosphotyrosine antibodies. Aliquots of the total cell extract were also Western blotted using anti–phosphotyrosine-STAT3 (*middle panel)* and anti–phospho-Erk (*lower panel*). The *open arrow* indicates an EGF-stimulated phosphotyrosine protein comigrating with $pp60^{c\text{-}Src}$. (D) Hepatocytes treated for 24 hours with the factors indicated at the bottom (1 µmol/L dexamethasone, 10 ng/mL IL-1, 100 ng/mL IL-6 or EGF, and 500 ng/mL insulin) were extracted, and total cellular RNA were analyzed on Northern blots for mRNA to HP, serum-amyloid A, and AGP. Autoradiograms after 24-hour exposure are shown. The ethidium bromide (EtBr) staining of the separated 18S rRNA is shown in the bottom panel. (E) Mouse hepatocytes from a separate preparation were treated for 24 hours in the presence or absence of 100 ng/mL IL-6 and the indicated doses of EGF. Total cellular RNAs were analyzed for mRNA to HP and AGP and medium for the amounts of the secreted proteins.


A
210
PY-EGFR
118
80
48
BPB
Control
IL-6
1
10
100
EGF (ng/ml)
B
(STAT3)2
(STAT1-STAT3)
(STAT1)2
Control
IL-6
1
10
100
EGF (ng/ml)
C
i.p.: SHP2
w.b.: PY
PY-EGFR
PY-gp130
PY-SHP2
IgG
w.b.:
PY-STAT3
PY-STAT3
w.b.:
P-ERK
P-ERK1
P-ERK2
Control
IL-6
EGF
D
HP
SAA
AGP
EtBr
18S rRNA
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Dex
IL-1+Dex
IL-6+Dex
IL-1+IL-6+Dex
Insulin+Dex
IL-6+Insulin+Dex
EGF+Dex
IL-6+EGF+Dex
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EGF (ng/ml)
Northern Blot
HP
AGP
EtBr
28S rRNA
Protein Production
(Immunoelectrophoretic Units)
100
75
50
25
<1
HP
75
50
25
<1
AGP
0
100
0
1
10
100
EGF (ng/ml)
- IL-6
+ IL-6

cytes. Of note is that HepG2 cells and mouse hepatocytes have species-specific differences in the profile of APPs, but also have a common set of IL-6–regulated APPs including HP, FB, and AGP.[1] In this study, we purposely analyzed, in each cell system, several APPs that represent common (HP, AGP) and species-specific APPs (mouse serum-amyloid A, human CRP, and ACH) to ensure that we were assessing general and specific features of EGF action.

Several lines of long-term HepG2 cell cultures showed very low amounts of immuno-detectable EGFR protein and EGF-inducible receptor tyrosine phosphorylation. However, a clonal screening of early passage HepG2 cells identified a line (86-6) that expressed appreciable amounts of EGFR protein and responded to EGF with a robust EGFR phosphorylation (Fig. 2A) and Erk activation (Fig. 2B). Despite the obvious EGF signaling in this HepG2 cell line, no significant activation of STAT proteins by EGF treatment was detectable at the level of STAT3 phosphorylation (Fig. 2A) or STAT1/3 binding to SIE (Fig. 2B). This difference of 86-6 cells to mouse hepatocytes is probably in part because of the several-fold lower level of immunodetectable EGFR protein and EGF response, as measured by tyrosine phoshorylation of EGFR (Fig. 2C). Nevertheless, the 86-6 clone, like the standard HepG2 cell lines, showed the characteristically strong activation of STAT3 by IL-6–type cytokines that was not significantly modified by EGF (Fig. 2A, bottom panel). Analysis of secreted APPs indicated 2 notable EGF effects. EGF treatment for 24 hours increased the production of ACH by approximately 3-fold, but slightly reduced the IL-6–induced ACH expression (Fig. 2D, left panel). In contrast, EGF treatment caused 3- to 4-fold reduction of basal production of FB and HP, and a 2-fold reduction of IL-6 stimulated secretion of the 2 proteins (Fig. 2D, center and right panel). These results show that EGF exerts 2 opposing activities on APP synthesis, which are clearly distinct from those of IL-6. The data in Figs. 1 and 2 also suggest that EGFR signaling generally counteracts IL-6.

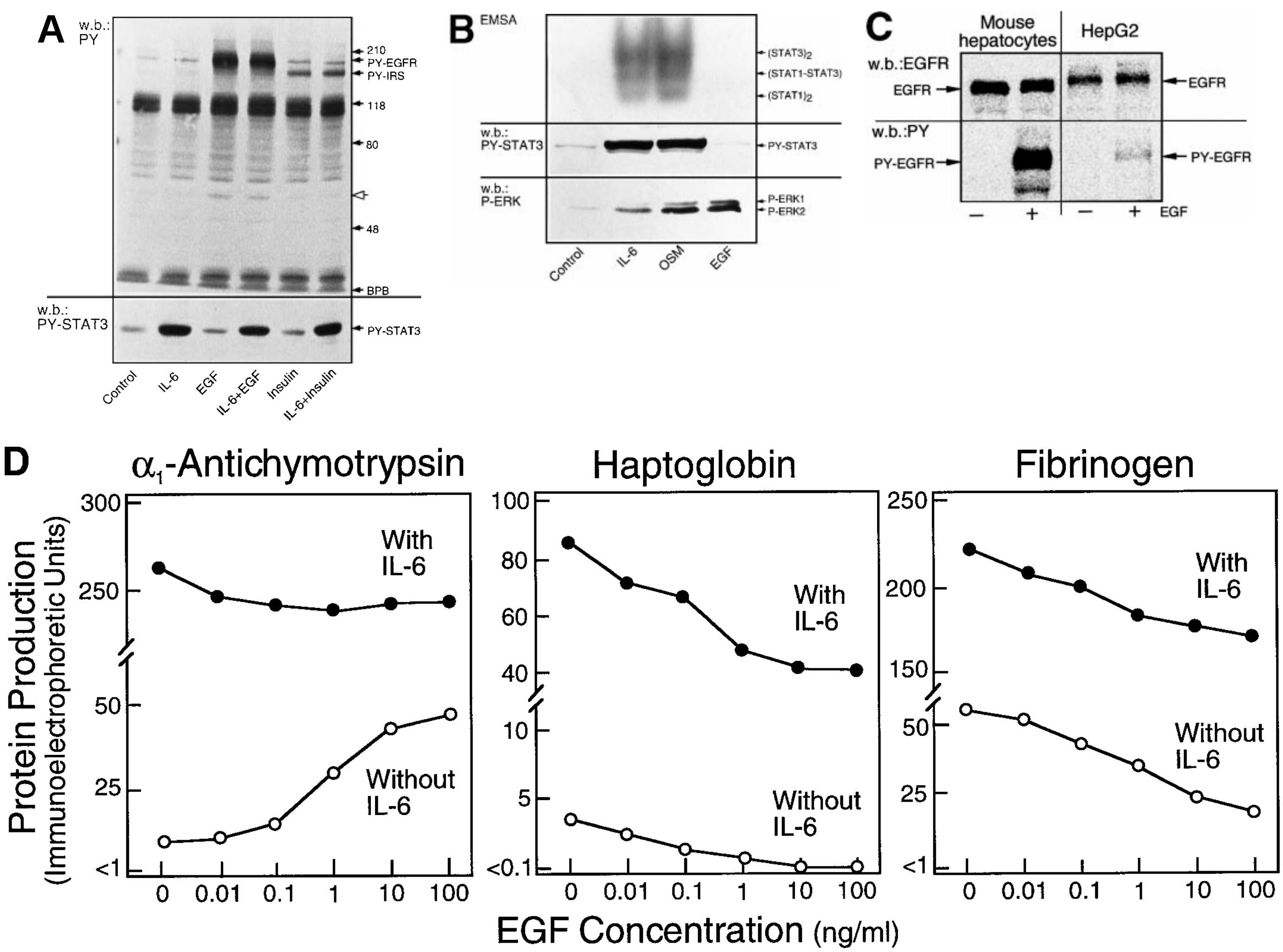



FIG. 2. EGF response of clone 86-6 of HepG2 cells. (A) Cultures from the 86-6 clone of HepG2 cells were treated for 15 minutes with the factors indicated. Total cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibodies (*upper panel*) or anti–phosphotyrosine-STAT3 (*lower panel*). Position of the major phosphoproteins and size markers are indicated. The *open arrow* marks phosphoproteins of approximately 60 kd that are primarily observed in EGF-treated cells and comigrate with pp60$^{c\text{-}Src}$. (B) Whole cell extracts from clone 86-6 of HepG2 cells treated as indicated were analyzed by EMSA for SIE binding activity (*upper panel*) and by Western blotting for phosphotyrosine STAT3 (*middle panel*) and phospho-Erk (*lower panel*). (C) Equal amounts of lysates from HepG2 cells and mouse hepatocytes, treated for 15 minutes with or without 100 ng/mL EGF, were analyzed on the same gel for immunodetectable tyrosine phosphorylated protein and EGFR. (D) Cultures from clone 86-6 of HepG2 cells were treated for 24 hours with increasing concentrations of EGF in the presence or absence of IL-6 (100 ng/mL). Aliquots of the culture media were analyzed for secreted human $\alpha_1$-antichymotrypsin, haptoglobin, and fibrinogen.

*Influence of EGFR and STAT Expression Levels on Signaling Function.* To define more clearly the signaling capabilities of EGFR in HepG2 cells, we took advantage of the standard HepG2 cell lines that express barely detectable EGFR. In these cells, we determined the profile of EGF responses as a function of EGFR concentration established by transfection of an EGFR expression vector. A prominent increase of ligand-activated EGFR signal was detected in the transfected cell cultures with a maximal signal achieved with 5 μg vector/mL (Fig. 3A). Considering that approximately 5% of the cells in the culture represented transfected cells, we estimated the level of EGFR protein in these cells to be at least equal to, if not several-fold higher than that in primary hepatocytes. To visualize the preference of EGFR for STAT isoforms, we cointroduced expression vectors for the various STAT isoforms. We observed a strong activation of STAT1, a lesser one for STAT3 and STAT5B (Fig. 3B), and none for STAT4 or STAT6 (data not shown). This STAT activation pattern by EGF established in transiently transfected HepG2 cells is qualitatively in agreement with that determined in mouse hepatocytes (Figs. 1B and 3B).

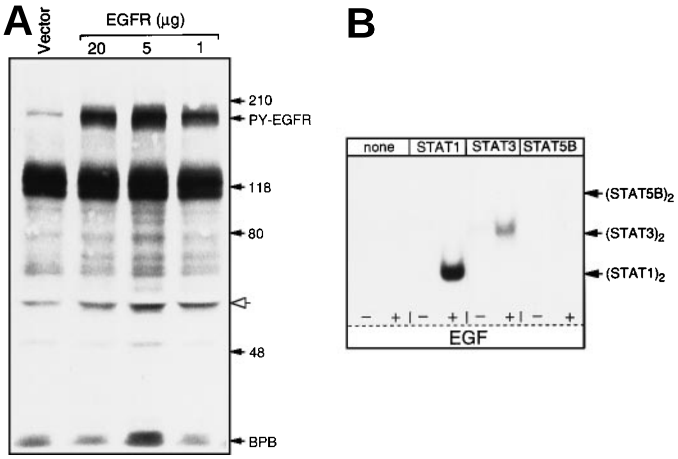
The reconstitution of a hepatocyte-like STAT regulation by EGF in HepG2 cells through the transfection approach provided us with a means to assess whether the EGF-regulated STAT proteins are relevant for APP gene regulation. Previous analyses of several APP gene promoters have indicated the presence of STAT binding sites and their inducibility by STAT3.[39,40,50,52,55] At present, no APP gene has been identified that is significantly inducible by STAT1. Hence, in the following experiments we limited our attention to STAT3. HepG2 cells, transfected with the representative CAT reporter gene constructs containing the promoter of ACH, CRP, HP, or FB together with increasing amounts of EGFR expression vector, showed 2 types of EGF responses (Fig. 3C). The ACH and CRP promoters responded by a receptor dose-dependent increase in expression, whereas the HP and FB promoters responded by a decrease. The decrease of the FB construct was particularly convincing because of the fact that this promoter yielded a high basal CAT expression in HepG2. Coactivated EGFR signals reduced the generally prominent induction of the same constructs by IL-6, with the exception of the CRP promoter. The maximal reduction of reporter gene expression and activation by IL-6 was of similar magnitude as the EGF-mediated reduction of APP secretion in clone 86-6 HepG2 cells (Fig. 2D) but somewhat less than that noted for HP in mouse hepatocytes (Fig. 1E). In the presence of coexpressed STAT3, whereby skewing the EGFR signaling towards the STATs pathway (Fig. 3B), the stimulatory effect of EGFR on ACH and CRP promoters was enhanced, and the inhibitory action on the HP and FB promoter was overcome (Fig. 3C). Using the transdominant negative $STAT3_{\Delta}55C$ instead of wild-type STAT3, the EGF-mediated stimulatory, but not inhibitory action on the reporter genes, was suppressed (Fig. 3C), supporting a signaling role of STAT3 as part of EGFR action. To identify the mechanism of the 2 EGFR signaling pathways, we attempted to characterize first the mode of signal initiation by EGFR that leads to activation of STAT3 and APP induction, and then to characterize the signaling pathway causing suppression of certain APPs.

*Signal Initiation by EGFR Mediated by Csk-Sensitive Pathway.* In as much as the intrinsic PTK activity of EGFR is critical for the overall signal initiation process, receptor-recruited Src kinases are implicated to participate in signal propagation.[26,28,65,66] Because association of Src kinases has similarly been detected with IL-6R cytokine receptors and the ability of Src kinases to engage STATs has been noted before, we wanted to determine the extent to which JAK and Src kinases act as mediators of EGF and IL-6–regulated APPs. First, we determined the kinase specificity of STAT3 activation by gp130 and EGFR. To follow the gp130-dependent action specifically in transfected cells for optimal comparison with EGFR action, we used the chimeric G-CSFR-gp130 receptor that permitted a dimerization of the cytoplasmic gp130 domain, and thus initiation of signaling under the control of the extracellular domain of G-CSFR, which was analogous to the dimerization of gp130 in the ligand-activated IL-6R complex.[14] The engagement of JAKs or Src kinase by the receptor was assessed by their inhibition through coexpressed JAK inhibitor, SSI-1 (or SOCS1), and the Src-kinase inhibitor, Csk, respectively.

Because high concentrations of SSI-1 suppressed many cellular functions, we determined the lowest concentration of SSI-1 expression vector that inhibited gp130 inducible transcription of the cotransfected STAT3-specific pHPX(5xIL-6RE)-CAT construct by 90% through endogenous IL-6R or transfected G-CSFR-gp130 (Fig. 4A, lanes 1-6). G-CSFR-gp130–transfected cells also showed that SSI-1–suppressed G-CSF–sensitive STAT3 activation (Fig. 4A, EMSA lanes 5 and 6). In contrast, activation of STAT3 and induction of reporter gene expression by EGF were insignificantly affected by SSI-1 (Fig. 4A, lanes 7 and 8), suggesting that JAKs have no obligatory signal transducing role in EGFR action. This is in agreement with the report that EGFR can activate STAT proteins independently of JAKs.[31]

Inhibition of Src kinases by overexpression of Csk produced a contrasting result. STAT3 activation and CAT gene regulation by a gp130 was not reduced; in fact, CAT expression was enhanced (lanes 10 and 11). Signaling to STAT3 and the reporter gene by EGFR, however, was mostly prevented by Csk (lanes 12 and 13), suggesting that a Csk-sensitive pathway communicates between EGFR, STAT3, and IL-6RE. To assess whether Src kinases were found in association with EGFR and therefore could account for signal communication as reported for EGFR in other cell systems,[28,65,66] we determined by coimmunoprecipitation the complex of c-Src with endogenous EGFR in clone 86-6 HepG2 cells (Fig. 4B) and in mouse hepatocytes (Fig. 4C). Immunoprecipitation of EGFR led to the recovery of low level of c-Src, which appeared to be enhanced tyrosine phosphorylated after EGF treatment. Because of the low amount of c-Src immunoprecipitated with EGFR, an EGF-sensitive increase of kinase activity of the EGFR-bound c-Src could not be determined. Moreover, the available anti–c-Src antibodies were unable to yield convincing coimmunoprecipitation of the EGFR precluding a complementary experimental verification of the composition of the signaling-competent EGFR–c-Src complex.

Because we detected the Csk sensitivity of EGFR signaling in EGFR-transfected cells, we needed to determine whether a corresponding Csk sensitivity applied to endogenous EGFR in hepatic cells, *i.e.*, clone 86-6 HepG2 cells. The specificity of Csk action was defined by the regulation of the 4 representative APP-CAT reporter constructs (Fig. 5). Csk essentially abolished induction of the ACH and CRP-CAT constructs by EGF but enhanced induction by IL-6. Similar analyses with the HP and $_{\alpha}$-FB constructs indicated that EGF-mediated suppression of these constructs was not relieved by Csk but



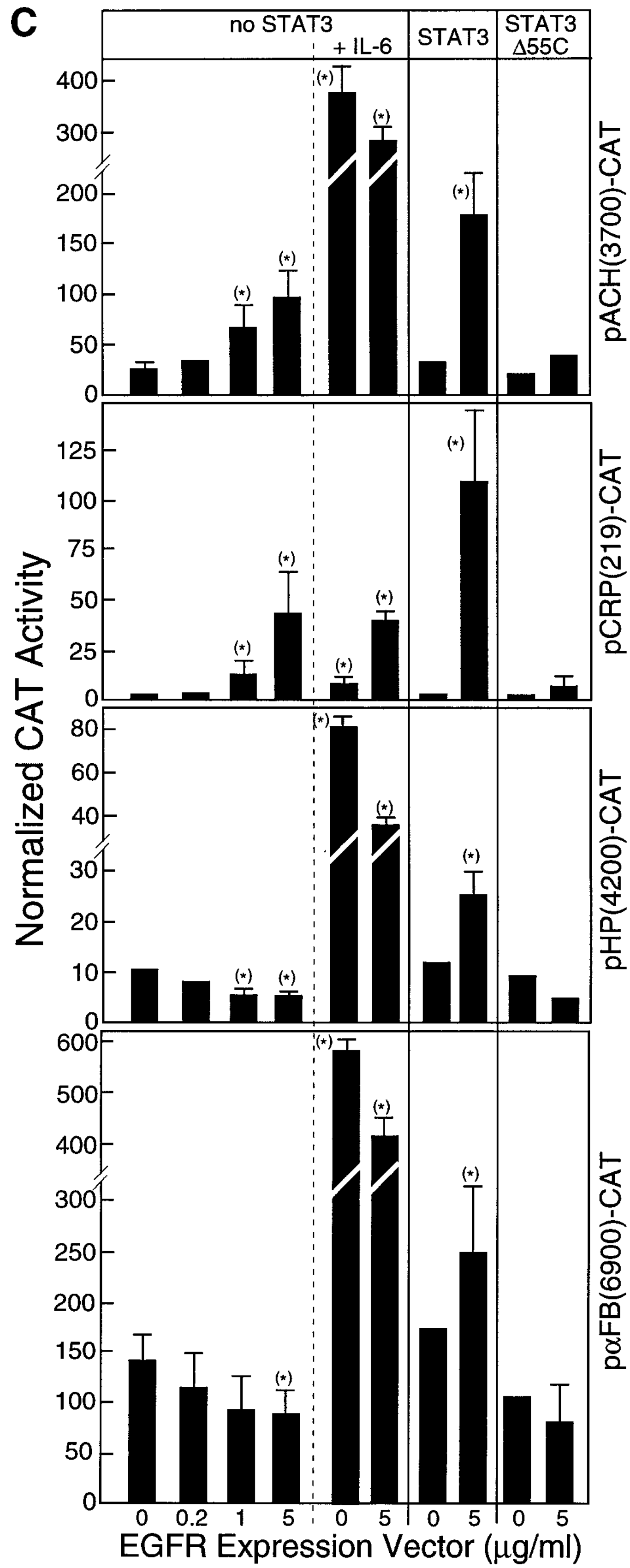




FIG. 3. STAT activation on gene regulation by EGFR. (A) HepG2 cells were transfected with the expression vector for EGFR and then treated with EGF for 15 minutes. Total cell lysates were analyzed by Western blotting using anti-phosphotyrosine antibodies. The *open arrow* indicates position of EGF-induced phosphorylated proteins at 60 kd. (B) HepG2 cells were transfected with expression vectors for EGFR (5 µg/mL) and STAT proteins (10 µg/mL) as indicated at the top. Subcultures were treated for 15 minutes with ligands and equal amounts of cell extract processed for determining the activity of the STAT proteins by EMSA. (C) HepG2 cells were transfected with the CAT reporter gene constructs (12 µg/mL) and expression vector for EGFR (0-5 µg/mL), STAT3 (3 µg/mL) and STAT3Δ55C (3 µg/mL) as indicated. All cultures were treated with 100 ng/mL EGF for 24 hours. Several cultures were also treated in addition with 100 ng/mL IL-6. CAT activity normalized to the transfection marker. Mean ± SD from three separate experiments are shown. (*) $P < .05$.

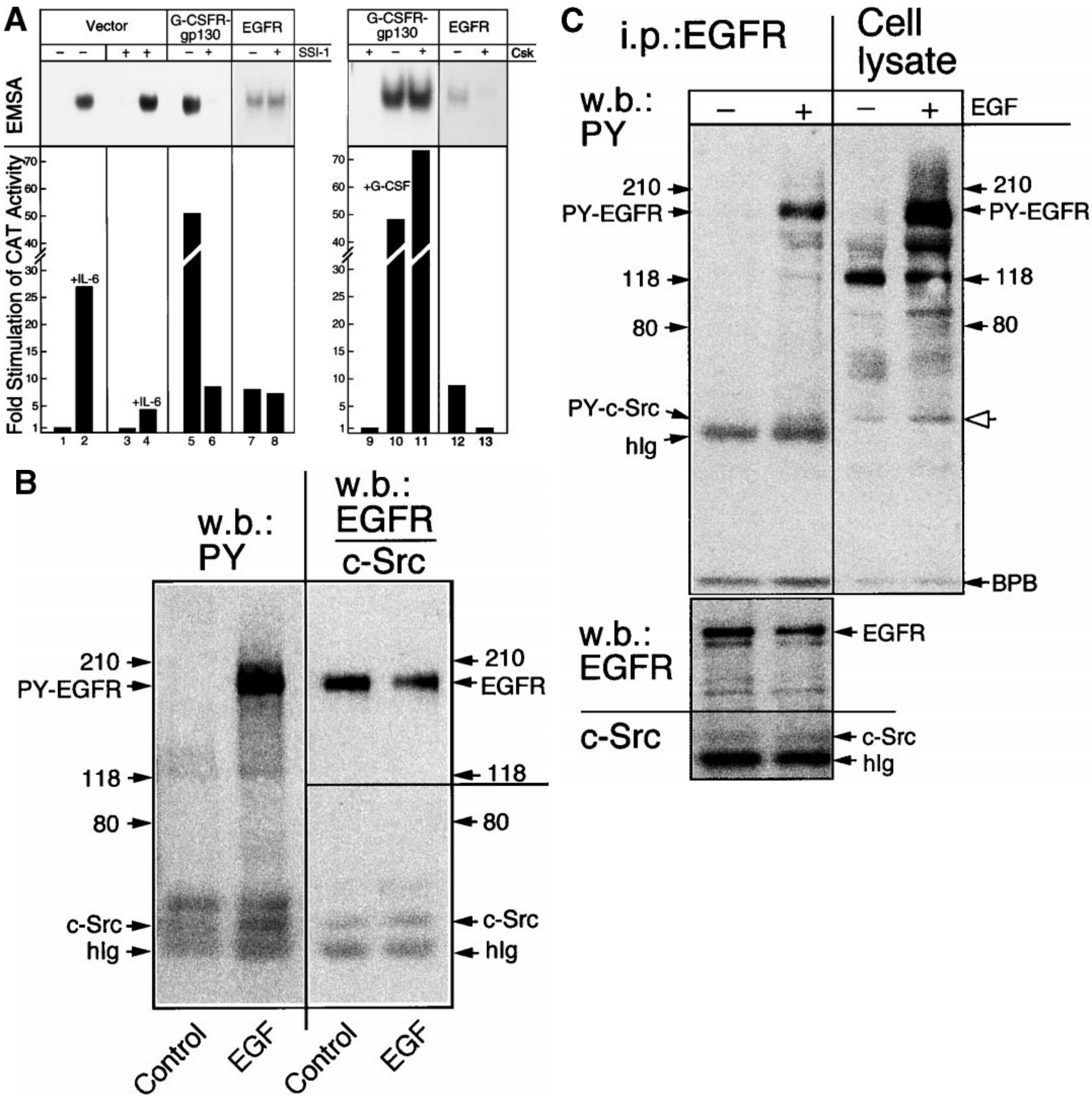



FIG. 4. Effect of SSI-1 and Csk on signaling by gp130 and EGFR and association of c-Src with EGFR. (A) HepG2 cells were transfected with combinations of pHPX(5xIL-6RE)-CAT (14 µg/mL) and expression vectors for STAT3 (3 µg/mL), alone or together with the expression vectors for G-CSFR-gp130 (0.5 µg/mL), EGFR (1 µg/mL), and SSI-1 (0.5 µg/mL) (*left panel*) or Csk (3 µg/mL) (*right panel*). Cultures were treated for 24 hours with serum-free medium alone (lanes 1, 3, 7, and 9), medium containing 100 ng/mL of IL-6 (lanes 2 and 4), G-CSF (lanes 5, 6, 10, and 11), or EGF (lanes 7, 8, 12, and 13). Cell monolayers were then scraped off the culture well and divided into two. From one part, whole cell extracts were prepared for use in EMSA with SIE as probe, and from the other part, extracts were prepared to determine CAT activity. Normalized CAT activity values were expressed relative to the values of the untreated cultures. (B) HepG2 cells of clone 86-6 were treated for 5 minutes with serum-free medium or medium containing EGF. Whole cell extracts were reacted with goat anti-EGFR antibodies. Immunoprecipitates were divided in two equal parts and separated on two identical SDS gels. One Western blot was reacted with anti-phosphotyrosine antibodies (*left panel*). The other Western blot (*right panel*) was cut into two sections along the 100-kd line. The upper part with ≥100 kd proteins was reacted with monoclonal anti-EGFR antibodies and the lower part with <100 kd protein was reacted with rabbit anti-human c-Src antibodies. (C) Mouse hepatocytes were treated as the HepG2 cells in (B) and anti-EGFR precipitates (i.p.: EGFR), and total cell lysates were analyzed on Western blot (w.b.) for tyrosine phosphorylated proteins, EGFR, and c-Src as indicated.

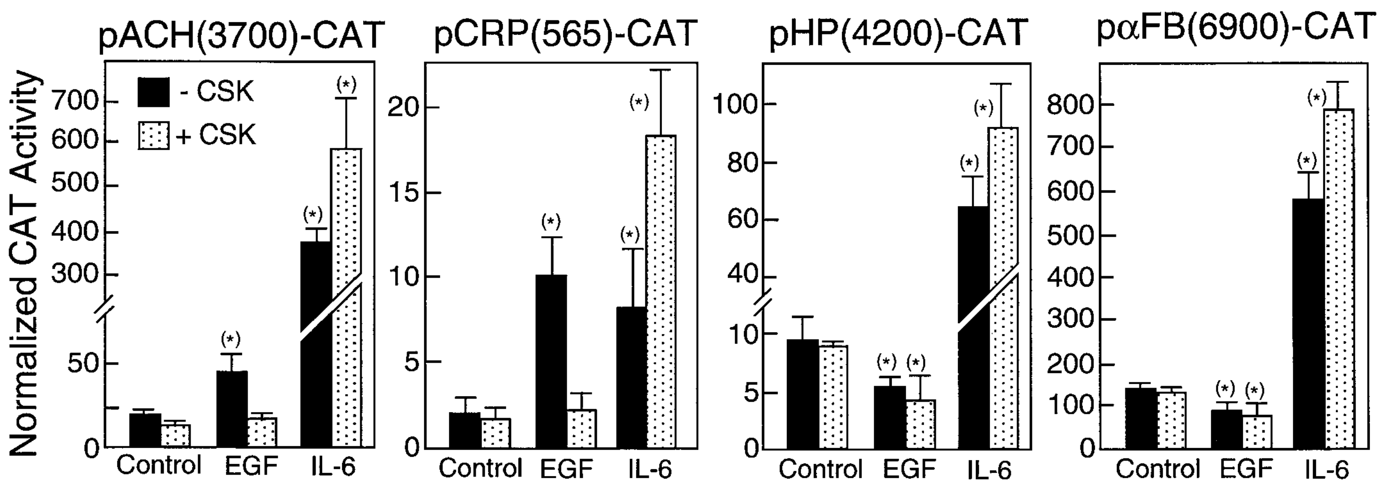



FIG. 5. Induction of transcription by EGF and effects of Csk. Cultures of the clone 86-6 of HepG2 cells were transfected with the indicated APP-CAT reporter gene constructs in the presence or absence of the expression vector for Csk (5 μg/mL)as indicated. The normalized CAT activities represent (mean ± SD of 3-5 separate analyses). (*)$P < .05$.

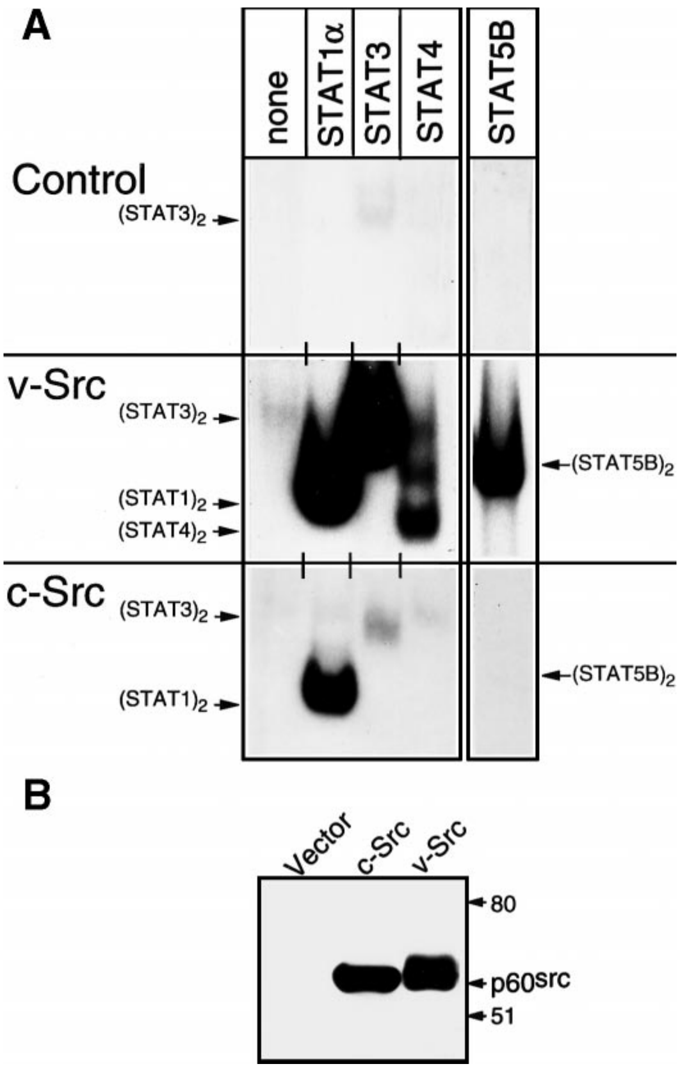
that induction of the genes by IL-6 was increased. These results suggested that EGFR engages endogenous Csk-sensitive kinase(s) for signaling to enhance expression of some APPs, and that IL-6R signaling is subject to a negative autoregulatory process that also involves a Csk-sensitive kinase(s). In the following set of experiments, we address first whether Src kinase could fulfill, in principle, the role as mediator of EGFR signal and activation of APPs, and then we attempt to explain the unprecedented Csk-dependent effect on the signaling process by gp130.

*Signaling by Src Kinases.* The ability of Src kinases in engaging STATs and inducing APP gene constructs was shown by expressing in HepG2 cells the constitutively active v-Src and the regulated c-Src. Overexpression of the latter kinase was assumed to be required to out-titrate the endogenous inhibitory mechanisms and thus gain an indication of the kinase's signaling capabilities. Gel shift analyses of the kinase-transfected but otherwise untreated cell cultures showed a low but detectable increase of DNA-binding activity of the endogenous STAT3 (Fig. 6A, lane marked "none"). The low STAT signal was attributed to the low percentage of transfected cells in the culture.[40] The detection of STAT activation was enhanced by coexpression of individual STAT proteins (Fig. 6A, lanes marked with STAT1α, STAT3, STAT4, and STAT5B; data on STAT6 not shown). Both kinases activated prominently STAT1, somewhat variably STAT3 and STAT5B, and not detectably STAT6. v-Src also activated STAT4. The STAT activation profile for c-Src was strikingly similar to that established for EGFR (compare Fig. 6A with Fig. 3B). The relative patterns of activated STAT proteins, as shown for whole cell extracts in Figure 6A, were also observed for nuclear extracts (data not shown), indicating that the transfected kinases did not detectably modify the nuclear translocation of STATs. A comparable expression of v-Src and c-Src protein in transfected cells was confirmed by Western analysis (Fig. 6B).

Based on the STAT activation patterns, a STAT3-dependent induction of APP promoters was anticipated. Indeed, transfection of the kinases together with the reporter gene constructs indicated a stimulatory action, which, as shown for the representative example of the pHPX-IL-6RE-CAT (Fig. 6C) was roughly proportional to the kinases' ability to activate STAT3. v-Src was highly effective and elicited an induction of most constructs tested that equaled that mediated by IL-6 (Fig. 6D). Only at a higher dose of transfected expression vectors did c-Src stimulate the reporter genes. As predicted, the gene induction by the kinases was enhanced in the presence of transfected STAT3 (Fig. 6C) and reduced in the presence of the transdominant negative $STAT3_{\Delta}55C$ (data not shown).

A unique and exceptionally prominent Src kinase-mediated induction was presented by the CRP promoter (Fig. 7). This preferential regulation of the CRP construct by Src kinases may also explain the appreciable stimulation of the same construct by endogenous EGFR in clone 86-6 (Figs. 5 and 7) and transfected EGFR (Fig. 3C). The 219-bp CRP promoter, containing the minimal IL-6 inducible region,[55] displayed a very low basal expression but produced a transcriptional activity in the presence of coexpressed Src kinases that was 10- to 100-fold higher than the activity achieved by treatment with IL-6 (Fig. 7). The promoter analysis indicated that the same array of regulatory elements within the CRP promoter cooperates in generating the Src, EGF, and IL-6 response. The region from position −157 to −219, including the binding sites for hepatocyte nuclear factor 1/3 and C/EBP,[55,67] was required for the maximal induction and the single STAT3 binding site approximately −110 and C/EBP site-60 were critical for inducibility. In separate experiments (data not presented), we established that the Src action is not reproducible by the C/EBP or the hepatocyte nuclear factor 1/3 binding sites alone. Reporter gene constructs with a minimal SV40 promoter under the control of oligomers of the C/EBP or hepatocyte nuclear factor 1/3 binding site sequences proved insensitive to overexpressed v-Src.

*Src Kinase Phosphorylates gp130 at the SHP-2 Binding Site.* Thus far, the experimental data explain the stimulatory action of EGFR to include a Csk-sensitive pathway that likely involves Src kinase and STAT3. What remained to be determined is why the Csk does not reduce but enhances the gp130 action (see Fig. 5). One possibility is that activation of gp130 signaling is associated with a modification of gp130 by



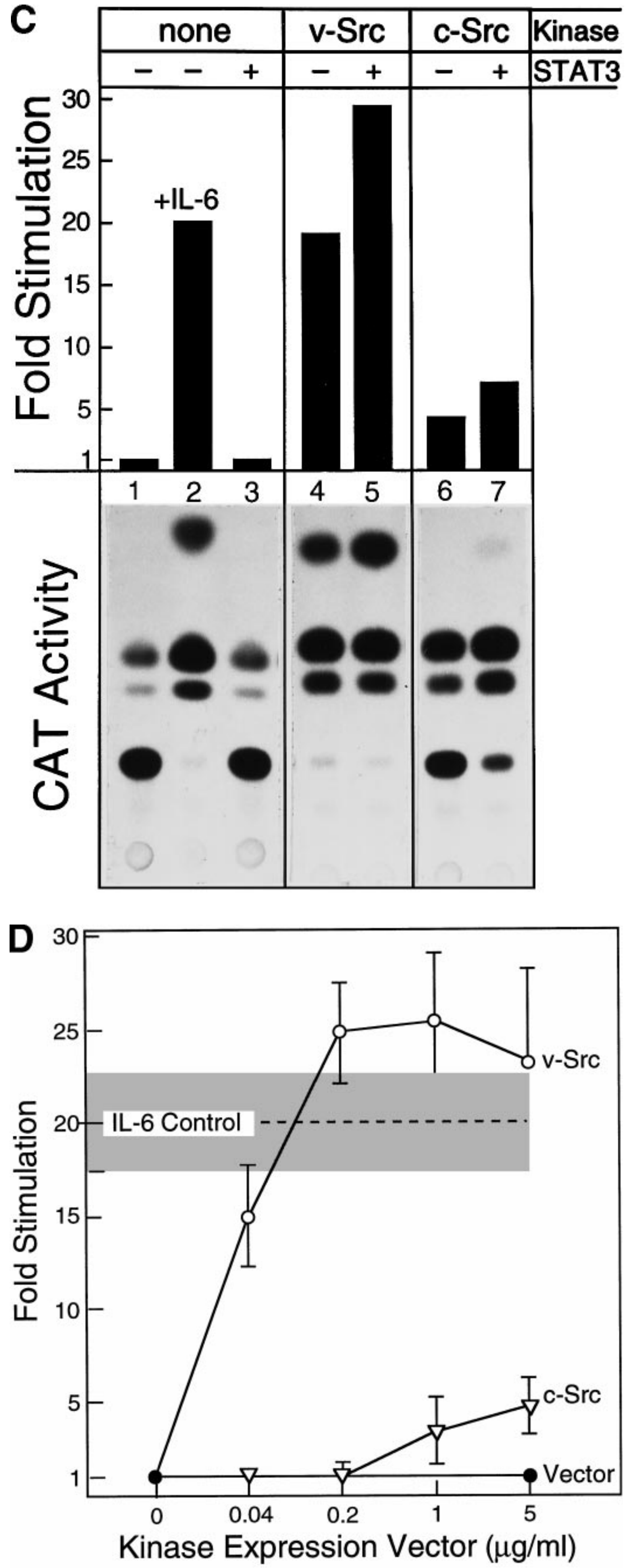




FIG. 6. Expression and signaling of Src kinases in transfected HepG2 cells. (A) HepG2 cells were transfected with expression vectors for v-Src or c-Src (5 μg/mL) alone or together with expression vectors for the STAT proteins (10 μg/mL) listed at the top. After 36 hours, cell extracts were prepared and equal amounts of the extracts were subjected to EMSA using SIE as binding substrate for lanes marked with "none," STAT1α, −3 and −4; and TB-2 for lane STAT5B. All gel shift patterns were exposed for 24 hours and the regions containing the DNA-STAT protein complexes are reproduced. (B) Aliquots of whole cell lysates from HepG2 cells from (A) were analyzed by Western blotting for the expression of the immunoreactive avian $p60^{Src}$ protein. Positions of the kinases and molecular size markers (kd) are shown on the right. (C) HepG2 cells were transfected with pHPX(5xIL-6RE)-CAT alone (*none*) or together with expression vectors for v-Src (0.1 μg/mL), c-Src (5 μg/mL), and STAT3 (3 μg/mL) as indicated at the top. Subcultures were treated for 24 hours with serum-free medium, except one control culture that was treated with IL-6 (lane 2). CAT activities in one representative enzyme assay are reproduced in the lower panel, and normalized values for the relative CAT activities are shown in the upper panel. (D) HepG2 cells were transfected with pHPX(5xIL-6RE)-CAT and increasing amounts of expression vector for v-Src and c-Src. Each experimental series included vector controls but were treated with IL-6. Changes in CAT activity relative to untreated controls are calculated (mean ± SD; N = 3-6).

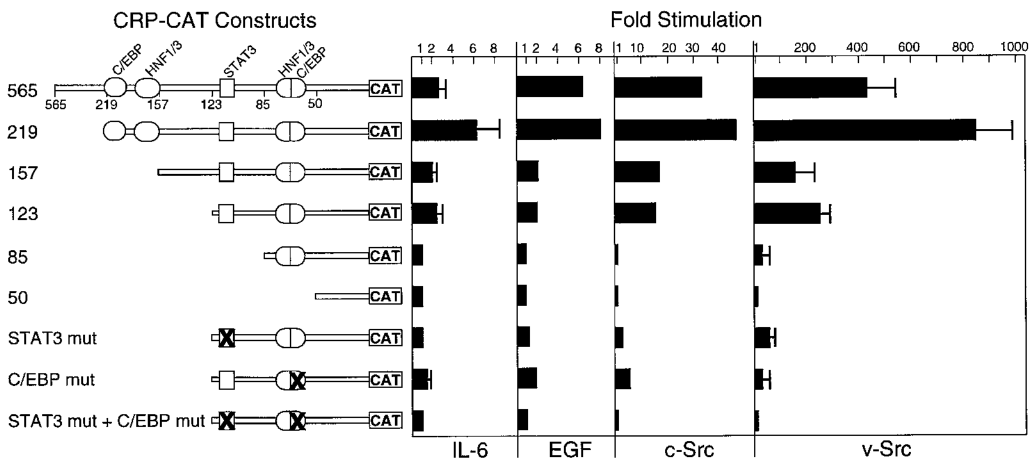



FIG. 7. Regulation through CRP promoter. The CRP-CAT constructs listed on the left (schematic presentation of the transcription factor binding sites according to Zhang et al.[55] and Li and Goldman[67]) were transfected alone into clone 86-6 of HepG2 cells or together with expression vector for v-Src (0.1 µg/mL) or cSrc (5 µg/mL). The relative increase in CAT activities mediated by treatment with IL-6 or EGF, or by expression of Src was determined in 2-8 independent experiments (where appropriate, means ± SD are shown).

a Src kinase and this modification in turn attenuates signaling. Recent functional characterization of the cytoplasmic domains of gp130[48,68] has indicated that tyrosine 759 (here referred to as Y2, representing the second of the 6 tyrosine residues in the cytoplasmic domain[68]) is a site, that when phosphorylated, serves as a binding motif for the protein tyrosine phosphatase SHP-2. Recruitment of SHP-2 to gp130 is believed to contribute to down-modulated signaling by gp130, in part by the phosphatase action.[48,69] To show that Csk-sensitive Src kinases, in principle, could target gp130 by modifying the Y2 site, we determined the effects of Csk on signaling mediated by the cytoplasmic domain of gp130 that contained the wild-type or the tyrosine to phenylalanine mutant (Y2F) SHP-2 binding site. We introduced into HepG2 cells the expression of the chimeric G-CSFR-gp130 receptors that carried the cytoplasmic domain of gp130 with or without the Y2F mutation. In the presence of Csk, the wild-type chimera mediated an increased induction of the reporter construct (Fig. 8A, lanes 2 and 5) that was similar to the Csk effect on the endogenous IL-6R (lanes 3 and 6). As expected, the Y2F mutant chimera produced a higher transcriptional response to G-CSF treatment than the wild-type chimera in the absence of cotransfected Csk (Fig. 8A, lanes 2 and 8). This response was not further increased by Csk (Fig. 4A, lanes 8 and 11). These results are consistent with the model that Src-related kinases, which are activated as a result of the action of gp130, phosphorylate gp130 tyrosine 759 that recruits SHP-2, which then reduces the overall signaling activity of gp130. Inhibition of these gp130-modifying kinase(s) by Csk produces an increased transcriptional response that is equivalent to that obtained by the Y2F mutant.

To determine whether Src kinases indeed have the potential to mediate the phosphorylation of gp130, we asked whether v-Src, as an experimental representative of Src kinase activity, could modify specifically gp130 at the Y2 site. The assay entailed expressing G-CSFR-gp130 (wild type) or G-CSFR-gp130(Y2F) in the presence or absence of v-Src, followed by determining the level of phosphorylation and SHP-2 association of the receptor immunoprecipitated through its C-terminal FLAG epitope (Fig. 8B). As expected, in the absence of v-Src, the wild-type and mutant receptors produced a G-CSF–induced tyrosine phosphorylation of the receptors, but only the wild-type receptor associated with SHP-2. In contrast, v-Src produced primarily a phosphorylation of the wild-type receptor and only a minimal signal with the Y2F mutant receptor, highlighting a Y2 specificity of v-Src action. The phosphorylation of the wild type, but not of the mutant receptor, led to the presence of SHP-2 in the immunoprecipitate.

*Erks Are Involved in Mediating the Inhibitory Activity of EGFR.* Inasmuch as the stimulatory activity of EGFR through Src/STAT pathway was experimentally separable from the inhibitory activity and was readily identifiable by the transfection studies (Figs. 3C and 5), it was the inhibitory activity of EGFR that appeared to play a more important role in determining the expression level of a number of APP genes as suggested by the mouse hepatocyte's response (Figs. 1D and 1E). Because expression of IL-6–responsive APPs has been noted to be reduced in growth-stimulated (or insulin-treated) hepatoma cells and enhanced in serum-deprived hepatoma cells,[70] a contribution of the EGF-stimulated MAPK pathway to the inhibitory process was suspected. Supporting evidence was obtained by the observation that the EGF-mediated reduction of FB production in clone 86-6 cells was prevented by treatment with the MEK1/2 inhibitor PD98059 (Fig. 9A). Separate Western blot analyses, using cell extracts from HepG2 cells treated with EGF in the presence of PD98059 (data not shown) indicated that greater than 90% suppressed appearance of phosphorylated Erk. Unfortunately, mouse hepatocytes proved to be resistant to the same inhibitor treatment (as high as 100 µmol/L PD98059), which prevented a similar assessment in these cells of the contribution of EGF-stimulated Erks to APP expression.

A complementary approach to measure the influence of activated Erks on APP expression was to introduce constitutively active MEK1 into HepG2 cells. The cotransfected marker APP-CAT reporter gene constructs showed an exceptionally strong 8-fold inhibition of $_{\alpha}$-FB promoter activity, and a 3-fold inhibition of HP promoter activity by MEK-1 action (Fig. 9B). No inhibition, but minor stimulation of the ACH promoter was detected. The ability of the MEK-1 to elicit in HepG2 cells a robust gene induction through a promoter containing a conventional AP-1 site was shown by the approximately 10-fold stimulation of the TIMP-1-CAT construct (Fig. 9B). In separate experiments (data not shown) we determined that the effect of MEK-1 activity persisted in IL-6–treated cells attesting to the dominant mode of MAPK pathway signals. Collectively, these results indicate an inhibitory action of the activated Erks that is APP gene specific and, to some extent, is comparable with that noted for EGF. Hence,

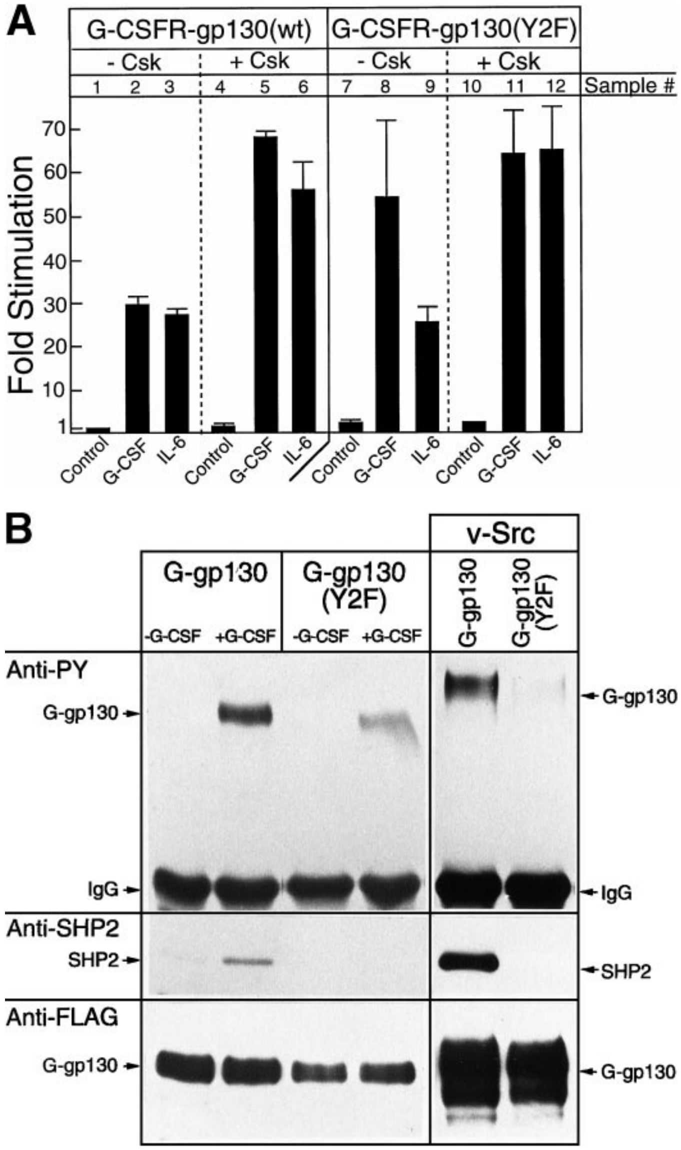



FIG. 8. Phosphorylation of the SHP-2 binding site of gp130 and effect on signaling. (A) HepG2 cells were transfected with pHPX(5xIL-6RE)-CAT and the expression vector for G-CSFR-gp130 wild type (wt) or G-CSFR-gp130(Y2F) mutant (0.5 µg/mL) and for Csk (5 µg/mL) as indicated. Subcultures were treated with medium alone (*control*), or medium with G-CSF or IL-6. The change in CAT activities was calculated relative to the untreated controls (sample 1). (B) In separate experiments, cells were transfected with the expression vectors for G-CSFR-gp130 wild type (G-gp130) or G-CSFR-gp130(Y2F) (G-gp130(Y2F)) (4 µg/mL) alone or combined with vector for vSrc (1 µg/mL). Subcultures without cotransfected PTKs were treated for 10 minutes with or without G-CSF before extraction. The PTK-transfected cells were treated with serum-free medium only. Cell extracts were reacted with anti-FLAG and the recovered immunocomplexes analyzed on Western blots by sequential reaction with anti-phosphotyrosine (PY), anti–SHP-2 and anti-FLAG antibodies. The chemiluminescence patterns of the indicated protein bands are reproduced.

the MAPK pathway engaged by EGFR signaling appears to exert a regulatory function that in part opposes that of the activated STAT pathway. The ratio of Erk to STAT3 activated by EGFR, by IL-6R, or by the combination of the two appears to determine the degree of inhibited or stimulated expression of the various APP genes.

## DISCUSSION

The major finding of this study was that EGF modulates in hepatic cells the expression and IL-6 induction of specific APP genes through both inhibitory and stimulatory mechanisms and is summarized in Fig. 10. The data suggest that the stimulatory pathway of EGFR may involve Csk-sensitive Src kinases that have the ability to activate STAT proteins and induce APP genes independently of JAKs. The inhibitory pathway of EGFR that is not critically dependent on Src operates in part through Erks and may mechanistically relate to the reduced APP expression in growth-stimulated hepatic cells or in regenerating liver.

The data provide an intriguing scenario of growth factor control of APP gene expression: (1) EGFR, but not the insulin receptor,[70] activates STATs that then contribute to enhanced expression of certain APP genes; (2) the same EGFR, similar to the insulin receptor, also signals toward SHP-2,[71] and other linkers such as SHC,[72] GRB-2,[73] and Cbl[74] to MAPKs, which may assist in the reduced basal expression of a number of APPs and reduced manifestation of the IL-6 effects; and (3) although the growth factor receptors appear to engage a Csk-sensitive mechanism for its stimulatory pathway, the Csk-sensitive pathway exerts a rather moderating action on the JAK-dependent gp130 signaling. In the latter case, it seems possible that Src kinases, as part of the IL-6R signaling establish an auto-regulatory loop, and, through activation by EGFR or other PTK receptor, attenuate *in trans* the IL-6 responsiveness of liver cells. Unexpectedly, hepatic cells, *i.e.*, HepG2 cells, possess an efficient signaling pathway that is engaged by activated Src kinases to elicit an APP induction similar to the action of JAKs. The differences in regulation of specific APP genes such as CRP by Src kinase and IL-6 (Fig. 7), also highlight that the mode of transcriptional control by the two activation procedures is not identical and may involve sets of transcription factors that only in part include common components. Clearly, a conclusion drawn from the response established in tissue culture models is significantly influenced by the experimental cell system. In particular, hepatoma cells, although convenient for molecular manipulation, are incomplete representatives of parenchymal cells *in vivo*. One highlight of this fact is that an expression of EGFR comparable with normal liver cells is not found in established hepatoma cell lines, including HepG2.

*The Role of EGFR-Activated STATs.* The previous observations that liver cells, which express EGFR, respond to EGF by activation of STAT proteins has been compared with the seemingly similar action of IL-6 mediating the hepatic acute phase response.[35] The fact that the EGF failed to elicit an IL-6–similar induction of APP gene expression[9] remained unexplained. Our results showed that EGFR effectively signals through pathways with opposing activities. EGFR, like other PTK receptors,[75,76] activated either by their intrinsic PTK activity or through recruited Src kinase[65,66,77] the DNA-binding activity of STAT1 and STAT3.[30-32] Hence, a corresponding induction of transcription through STAT-responsive regulatory elements, such as an APP gene element, is anticipated. Indeed, we could experimentally show this link by choosing appropriate gene sequences integrated into the transfected reporter gene construct (Figs. 5 and 7) and by testing these as a function of EGFR (Fig. 3C) or STAT protein expression (Figs. 3B, 3C, and 4A). The involvement of the same pathway in controlling expression of the chromosomal

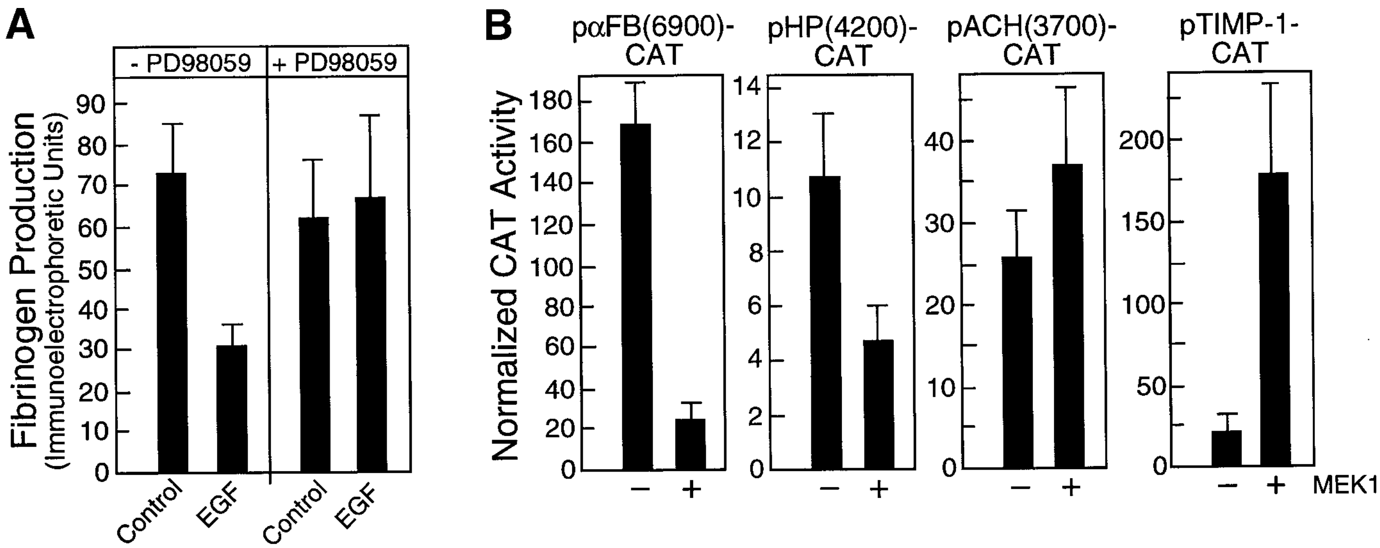



FIG. 9. Effect of activated Erk pathway on APP expression. (A) Clone 86-6 of HepG2 cells were treated for 24 hours with serum-free medium alone or containing EGF in the absence or presence of 10 μmol/L PD98059. The immunodetectable fibrinogen in the culture medium was quantitated (mean ± SD; N = 3-6). (B) HepG2 cells were transfected with the indicated APP-CAT reporter gene constructs (15 μg/mL) with or without expression vector for the constitutively active MEK-1 (1 μg/mL). (In separate dose analyses, maximal MEK effects were obtained with MEK expression vector ≥1 μg/mL.) After culturing the cells for 24 hours in serum-free media, the level of CAT activity was determined (mean ± SD; N = 3).

APP genes (Figs. 1D, IE, or 2D) is assumed but not directly shown. Particularly puzzling was the observation that, despite the detectable STAT activation (Fig. 1B),[35] the expression of the prominent STAT3-sensitive APP genes, such as HP or FB, is not induced, but reduced. The explanation lies in the fact that attention was restricted to the activation of STATs. Not anticipated was the more prominent inhibitory action of, among others, the coactivated MAPK branch of EGF signaling. The ratio of STAT to Erk appears to be a more appropriate predictor for the type of APP regulation and can be expected not only for the action of EGF but also for other growth factors and all the IL-6 cytokines.

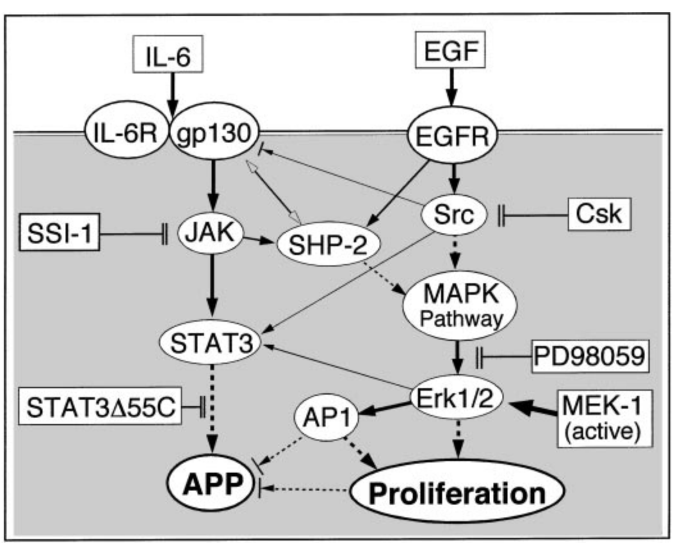



FIG. 10. Communication between EGF and IL-6 signal transduction in liver cells. The schematic presentation outlines a restricted set of the primary pathways that have been characterized in this study and that include the key elements noted in oval frames: IL-6–dependent link of IL-6R/gp130 via JAKs to STAT3 and APP gene and via JAKs to SHP-2 and MAPK (Erk1/2); and EGF-dependent link of EGFR via Src and SHP-2 to Erk1/2, via Src to STAT3 and APP, and via Src to the SHP-2 binding site in gp130. The experimental probing of these pathways was achieved by the reagents listed in rectangular frames: Stimulation by IL-6 and EGF added to cells, inhibition by transfected SSI-1 (JAKs), STAT3Δ55C (STAT3), and Csk (Src PTKs), and constitutive activation by the double mutant MEK-1 (Erk1/2).

APP gene induction, although sensitive to STATs, likely requires additional regulatory factors that may include C/EBP isoforms,[48,54,55,67,78] glucocorticoid receptor, AP-1, SP-1, and others,[56,78-81] some of which may not be similarly active in EGF-treated cells as in IL-6–treated cells. Evidently, the set of EGF-regulated factors can also yield an expression of a specific APP gene, which is higher than the expression after IL-6 treatments, as seen in the example of the CRP promoter response (Fig. 7). The target factors, other than STAT3, that are responsible for this enhanced CRP promoter activity remain to be identified.

*Role of Src Kinases as Activators of APP.* Several Src kinases had been reported to be associated with activated IL-6 cytokine receptors in various cell types.[18-22] Our experiment failed to show that representatives of these kinases play, besides JAKs, a critical role in the induction of APP genes by IL-6 (Figs. 4A, 5, and 8). The contributions of Src kinase were assessed by overexpressed Csk, an approach that relied on the promise that Csk maintains Src kinase in an inhibited state.[26,38,82] The observations that Csk enhances the IL-6 effect (Figs. 5 and 8) and that v-Src phosphorylates gp130 at its SHP-2 binding site (Fig. 8B), led to the proposal that Src kinase may act as attenuator of gp130 signaling through assisting in the recruitment of SHP-2. Whether the Csk-enhanced IL-6 effects are just the consequence of preventing SHP-2 recruitment is uncertain. Indirect routes of Src kinase action, such as by interference with components of IL-6 signaling transduction are conceivable.[14,83]

The two observations, that EGFR signaled independently of JAKs (Fig. 4A)[29,32,34] and that the limited gene induction through APP promoter by EGF (Figs. 3C and 5) was abolished by Csk, pointed to a mediator role of Src kinases.

The finding that expression of specific APP genes, such as human ACH and CRP, was positively responsive to Src kinase signals, suggests that this Src regulatory pathway may also be relevant in liver during normal physiological challenges and may contribute to the particular prominence of CRP gene induction during inflammation.[1]

*EGF-Mediated Inhibition of APP Expression.* A striking effect of EGF in mouse hepatocytes (Figs. 1D and 1E) and to a lesser extent in HepG2 cells (Fig. 2D) is the reduced expression of certain APP genes and reduced activation by IL-6. The biochemistry of this EGF-dependent inhibition is unclear. EGFR, like receptors for other growth factors, including insulin, activates through a variety of routes the MAPK pathway resulting invariably in the increased phosphorylation and activity of Erk1 and Erk2. The MAPK system has been suggested to exert a moderating influence basal expression of APPs (Fig. 9) and a signaling mechanism that would ultimately affect APP genes, such as phosphorylation of STAT3[84] or C/EBPβ.[14] Whether the rather transient regulation of MAPK activity significantly attenuates the induction of the APP gene, which depends on long-term treatment (hours to days) with cytokines, is not readily identifiable. Evidently, the presence of a constitutively activated MAPK pathway, as achieved by transfection of mutated MEK-1 (Fig. 9) or oncogenic ras (not presented), is effective in generating a profound inhibition. Activation of Erk1 and 2 is also part of the signaling reaction by IL-6 cytokines, in particular oncostatin M (Fig. 2B), yet these cytokines manage, because of the more prominent activation of STAT3, to achieve a high level of APP induction.

The experiments performed thus far cannot, however, rule out that persistent proliferative stimulus, including enhanced Erk activity caused by growth factors such as EGF/TGFα, may condition hepatic cells for a lower cytokine response. In fact, enhanced proliferation, either in serum- or insulin-treated hepatoma cells or, physiologically more relevant, in liver during the regenerative process,[11,12] has been shown to exert a prominent suppressive effect on APP gene expression and induction.[13,85] Although activation of STAT3 in the liver after hepatectomy is essentially the same as seen in normal liver cells during acute phase response,[86] its APP-inducing effects are suppressed. Whether the inhibited APP induction is mediated by enhanced AP-1 components such as JunB[11,78] or the one specifically observed in regenerating liver[86] remains to be proven.

Taken together, the results on EGFR and Src kinases have broadened our view on the control circuitry affecting APP genes in liver cells. The data emphasize that cytokine-activated pathways are sensitive to significant interference by factors that are not customarily associated with APP induction, but play a pivotal role in liver growth control.

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