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13. ABSTRACT ***(Maximum 200 Words)***

Increased expression of HGF and its receptor Met in breast cancer has been identified as a possible independent predictor of recurrence in breast cancer patients. In contrast, most non-malignant epithelial cells express Met but not HGF. Thus, the change in regulation of HGF expression may be a key step in breast cancer progression. In this project, Dr. Hung has demonstrated a co-operative effect of c-Src and Stat3 in the activation of HGF expression in carcinoma as well as epithelial cells. Changes in c-Src kinase activation affect Stat3 activity through its tyrosine phosphorylation and DNA binding activity to specific Stat3 consensus sites on the *HGF* promoter. These results suggest tumor specificity in the transcriptional regulation of HGF expression in carcinoma cells, and could thus lead to novel strategies for the design of low molecular weight antagonists to inhibit tumor metastasis.

Dr. Hung departed from this laboratory to take up a new position at the Sunnybrook Health Science Centre, University of Toronto. This PDF training award has recently been modified to extend the term, without any additional funds, from 1 July 01 to 1 July 02. All other terms and conditions of this award remain the same. There is no new progress since the last annual report was filed. We are aggressively recruiting for a new candidate for this PDF position.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....4

Reportable Outcomes.....4

Conclusions.....N/A

References.....N/A

Appendices.....5

INTRODUCTION AND BODY:

Increased expression of HGF and its receptor Met in breast cancer has been identified as a possible independent predictor of recurrence in breast cancer patients. In contrast, most non-malignant epithelial cells express Met but not HGF. Thus, the change in regulation of HGF expression may be a key step in breast cancer progression. In this project, Dr. Hung has demonstrated a co-operative effect of c-Src and Stat3 in the activation of HGF expression in carcinoma as well as epithelial cells. Changes in c-Src kinase activation affect Stat3 activity through its tyrosine phosphorylation and DNA binding activity to specific Stat3 consensus sites on the *HGF* promoter. These results suggest tumor specificity in the transcriptional regulation of HGF expression in carcinoma cells, and could thus lead to novel strategies for the design of low molecular weight antagonists to inhibit tumor metastasis.

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KEY RESEARCH ACCOMPLISHMENTS:

- Elevated expression of HGF and Met occurs in newly-established human breast carcinoma cell lines.
- Activation of a c-Src/Stat3 pathway (which occurs in most breast cancers) stimulates increased expression of HGF mRNA and protein breast in epithelial cells and carcinomas. This process may be a key step in early stage breast cancer.
- Information from these studies could lead to novel approaches to the therapy of breast cancer.

REPORTABLE OUTCOMES:

- Published two research papers and one review article in press (see Appendices).
- Applied for a grant from the Canadian Breast Cancer Research Initiative for continuation of this work (PI: Bruce Elliott, PhD). "Targeting HGF/Met signalling in breast cancer metastasis" C$384,000/3yr (2002-5). This grant is pending.

Appendices:

1) Assistance Agreement form for USAMRMC PDF Award # DAMD17-987-1-8330.

2) ***Publication***: c-Src kinase activity is required for HGF-induced motility and anchorage-independent growth of mammary carcinoma cells. N. Rahimi, W. Hung, E. Tremblay, R. Saulnier, and B. Elliott. J. Biol. Chem. 273:33714-21, 1998.

3) ***Publication***: Co-operative effect of c-Src tyrosine kinase and Stat3 in activation of HGF expression in mammary carcinoma cells. W. Hung and B. Elliott. J. Biol. Chem. 276:12395-03, 2001.

4) ***In press:*** The role of HGF/scatter factor in epithelial-mesenchymal transition and breast cancer. B. Elliott, W. Hng, A. Boag and A. Tuck. Can. J. Physiol. Pharm., 2001.

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Co-operative Effect of c-Src Tyrosine Kinase and Stat3 in Activation of Hepatocyte Growth Factor Expression in Mammary Carcinoma Cells*

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We have previously shown coexpression of hepatocyte growth factor (HGF) and its receptor Met in the invasive tumor front of human breast carcinomas. We have also demonstrated secretion of HGF, constitutive activation of Met, and increased invasion in a murine breast carcinoma cell line, SP1. These observations suggest the presence of an HGF autocrine loop in some breast carcinoma cells, which confers increased survival, growth, and invasiveness during tumor progression and metastasis. c-Src tyrosine kinase, which is critical in regulating the expression of many genes, is activated in SP1 carcinoma cells, as well as in most human breast cancers. We therefore examined the role of c-Src kinase in HGF expression in breast carcinoma cells. Expression of activated c-Src in SP1 cells increased transcription from the *HGF* promoter and expression of HGF mRNA and protein, while dominant negative c-Src had the opposite effect. Using deletion analysis, we showed that the region between −254 and −70 base pairs was required for c-Src responsiveness of the *HGF* promoter. This region contains two putative consensus sequences (at −110 and −149 base pairs) for the Stat3 transcription factor, which bind protein complexes containing Stat3 (but not Stat1, -5A, or -5B). Coexpression of activated c-Src and Stat3 synergistically induced strong *HGF* promoter activity in SP1 cells, as well as in a nonmalignant epithelial cell line, HC11 (HGF negative). c-Src kinase activity correspondingly increased the tyrosine 705 phosphorylation and DNA binding affinity of Stat3 (but not Stat1, -5A, or -5B). Collectively, our data indicate a cooperative effect of c-Src kinase and Stat3 in the activation of *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong repression of *HGF* expression in nonmalignant epithelium, and thereby promote tumorigenesis.

Scatter factor, also known as hepatocyte growth factor (HGF),[1] is a multifunctional cytokine. Through binding to its receptor (Met), HGF can induce cell survival (1), growth (2), differentiation (3), and motility (4). It has been shown that both HGF and Met are essential for embryo development. Disruption of HGF expression in mice results in lethality in early development (5), while deletion of Met causes underdevelopment of limb buds (6). During development of the mammary gland, HGF is expressed by stromal cells, whereas epithelial cells express Met, but not HGF (7). Paracrine stimulation of normal breast epithelium with HGF, in cooperation with other growth factors (*e.g.* neuregulin), promotes branching morphogenesis (8). The tissue-specific suppression of HGF expression in normal epithelial cells provides a tightly controlled regulation of mammary ductal morphogenesis (9).

In contrast to normal breast epithelium, HGF and Met are frequently overexpressed in breast carcinomas (10–12) as well as many other cancer types (10, 11, 13, 14). This high level of HGF and Met expression has been identified as a possible independent predictor of poor survival in breast cancer patients (11). Our laboratory has previously shown that invasive human carcinoma cells coexpress HGF and Met, particularly at the migrating tumor front (12). We have also found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met, but not HGF.[2] Furthermore, overexpression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (15, 16) or in transformed cell lines (17, 18) promotes tumorigenesis and metastasis. Together, these results suggest that establishment of an autocrine HGF loop and sustained activation of the Met signal transduction pathway in carcinoma cells may promote tumor progression. However, the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known.

A number of signaling molecules, such as c-Src (19), Grb2/Ras (17), and phosphatidylinositol 3-kinase (1), have been shown to be part of the HGF/Met signaling pathway. Activation of Met through binding of HGF causes autophosphorylation of two specific tyrosine residues in the cytoplasmic tail of the receptor tyrosine kinase (20). These phosphorylated tyrosine residues act as multifunctional docking sites that bind the SH2 domain of specific cytoplasmic signaling molecules and causes their activation. The c-Src nonreceptor tyrosine kinase is expressed in many cell types, and its activity is increased in response to HGF and binding to Met (19). Increased activation of the tyrosine kinase c-Src occurs in many human cancer cells, and c-Src plays a critical role in breast cancer. Overexpression

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[1] The abbreviations used are: HGF, hepatocyte growth factor; kb, kilobase pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); GUSB, β-galactosidase; PIPES, 1,4-piperazinediethanesulfonic acid.

[2] W. Hung, J. Gin, and B. Elliott, unpublished results.

of an activated form of c-Src in transgenic mice induces mammary hyperplasia (21). Furthermore, c-Src kinase is required in polyoma middle T-induced mammary tumorigenesis in transgenic mice (22). We have shown previously that c-Src kinase is constitutively activated in a mouse breast carcinoma cell line, SP1, which expresses both HGF and tyrosine-phosphorylated Met and which exhibits spontaneous invasion through matrigel (19, 23, 24). Furthermore, c-Src kinase activity is required for HGF-dependent cell motility and anchorage-independent growth of SP1 cells (19). Collectively, these findings indicate that c-Src kinase is an important requirement, but is not sufficient, for mammary tumorigenesis.

Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (25, 26) and parathyroid hormone-related peptide (27). We therefore hypothesized that elevated c-Src activity can promote increased HGF expression and the establishment of an HGF autocrine loop in SP1 cells. We observed that the c-Src tyrosine kinase inhibitor PP2 causes a 2-fold reduction in *HGF* transcription in SP1 cells. In addition, expression of a dominant negative mutant of c-Src (SRC-RF) in SP1 cells leads to similar levels of reduction in HGF mRNA and functional protein. Using deletion mutants of the *HGF* promoter, we have located a region (between −254 and −70) of the *HGF* promoter responsive to increased c-Src kinase activity in SP1 cells. This region contains two putative consensus binding sites for Stat3. Stat3 is a transcription factor originally described as the target of interferon receptors (28), but recent reports have indicated that Stat3 can be activated by c-Src kinase via platelet-derived growth factor (29) and HGF receptors (30), and is important in mammary differentiation (30). We therefore examined the role of Stat3 in c-Src-dependent regulation of *HGF* transcription. The results indicate that while expression of Stat3 alone increased *HGF* promoter activity, simultaneous expression of Stat3 and activated c-Src led to strong cooperative activation of *HGF* transcription in both nonmalignant epithelial and carcinoma cells. Expression of mutant c-Src kinases in breast carcinoma cells altered both the tyrosine phosphorylation status and DNA binding activity of Stat3. While activated c-Src induced Stat3 tyrosine phosphorylation and DNA binding activity, a dominant negative mutant of c-Src reduced tyrosine phosphorylation and DNA binding. Together these data suggest that c-Src kinase and Stat3 act cooperatively in the activation of HGF expression in breast carcinoma cells, and may be important in overriding the strong repression of HGF expression in nonmalignant epithelial cells.

MATERIALS AND METHODS

Antibodies and Reagents—Rabbit anti-c-Src IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody EC10 against chicken c-Src was a gift from Dr. S. Parsons. Rabbit anti-sheep IgG conjugated with horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Sheep anti-HGF IgG was a gift from Genentech (San Francisco, CA). Rabbit anti-HGF antibody was generated against recombinant glutathione *S*-transferase-HGF-(1–120) protein in our laboratory at Queen's University, this antibody recognizes only the N-terminal portion of HGF (data not shown). Anti-Stat1, -Stat3, -Stat5A, and -Stat5B and anti-phospho-Stat3 (Y705) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). c-Src family kinase inhibitor PP2 was obtained from Calbiochem (La Jolla, CA).

Plasmid Construction—c-Src expression plasmids were constructed by subcloning activated (Y527F) and dominant negative (K295R,Y527F) chicken *c-src* cDNAs (gift from Drs. J. Brugge and D. Shalloway) into the *Eco*RI site of DNA polymerase I (Klenow fragment)-treated pBabePuro plasmid to generate pBabe Y527F and pBabe Src-RF. A reporter construct containing the full-length *HGF* promoter region fused to luciferase (2.7 HGF-luc) was constructed by ligating the *Hin*dIII/*Xba*I fragment (treated with DNA polymerase I (Klenow fragment)) of 2.8 HGF-CAT (gift from Dr. R. Zarnegar) into the *Hin*dIII site of pGL2-Basic (Promega), also treated with DNA polymerase I (Klenow fragment). Further deletions were constructed by cutting 2.7 HGF-luc with *Sma*I, *Sac*I, and *Bgl*II, followed by re-ligation to generate 0.5 HGF-luc, 0.3 HGF-luc, and 0.1 HGF-luc, respectively. The 1.2 HGF-luc was constructed by ligating the 1.4-kb *Sal*I fragment from 2.7 HGF-luc into the *Xho*I site of pGL2-Basic. An internal deletion mutant 0.5Δ HGF-luc was constructed by digestion of 0.5 HGF-luc with *Pvu*II/*Bgl*II and treatment with DNA polymerase I (Klenow fragment) before religation. The Δ1 HGF-luc was constructed by ligating the *Sma*I fragment of 2.7 HGF-luc into the same site of 0.5Δ HGF-luc. The Δ2 HGF-luc was constructed by ligating the *Sma*I fragment of 2.7 HGF-luc into 0.8 HGF-luc. The ΔΔ HGF-luc was made by ligating the *Sma*I fragment of Δ2 HGF-luc into the same site of 0.5Δ HGF-luc. For normalization of transfection efficiency of each sample, pSG5βgal (a gift from Dr. M. Petkovich) or pCHCβgal (a gift from Dr. F. Kern) (31), which expresses β-galactosidase under the control of SV40 and cytomegalovirus promoters, respectively, was used.

Tissue Culture and Cell Lines—The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma, and expresses both HGF and Met. The characterization of the SP1 cell line has been described previously (19, 23, 24). Maintenance medium for SP1 cells was RPMI 1640 supplemented with 7% fetal bovine serum. HC11 is a mammary epithelial cell line (32) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, insulin (5 μg/ml), and epidermal growth factor (10 ng/ml).

Cell Transfection—All transfections were carried out with LipofectAMINE Plus reagent (Canadian Life Technology, Burlington, ON, Canada) according to manufacturer's instructions. Cells (15,000) were seeded in a 24-well plate and transfected with 0.4 μg of reporter plasmid, 0.1 μg of pSG5βgal, and up to 0.4 μg of expression plasmids (such as c-Src) as indicated. After 48 h, transfected cells were harvested and lysed. One-fifth of the cell lysate was used to assay for β-galactosidase activity, an equal amount of lysate was used for a luciferase assay using PharMingen Luciferase Substrates (BD PharMingen, Mississauga, ON). Luciferase activity was measured using a luminometer with wavelength at 562 nm. Luciferase activity of each sample was normalized to the corresponding β-galactosidase activity. For immunoprecipitation and *in vitro* c-Src kinase assays, 2.5×10^5 cells were seeded in a 100-mm tissue culture plate and transfected with 4 μg of reporter plasmid, 1 μg of pSG5-β-galactosidase, and up to 4 μg of expression plasmids as indicated. One-tenth of the cells was used for a luciferase assay, and the remaining cells were lysed and used for immunoprecipitation.

To obtain stably transfected cells, SP1 cells were plated at 70% confluence in 60-mm plates and transfected with 2 μg of plasmids expressing various mutants of c-Src. Puromycin (2 μg/ml, Sigma, Oakville, ON) was added to cells 24 h following transfection, and was maintained until all cells in the mock transfection were killed. Puromycin-resistant cells were then collected and used as pooled cell lines. Expression and activity of c-Src mutants in transfected cells were checked using Western blotting analysis and a c-Src kinase assay. Total c-Src protein was immunoprecipitated with an excess amount of anti-c-Src (pan) antibody to maximize the amount of antibody-protein complex formed. We have previously found that these c-Src mutants are quite effective, and that relatively small levels of expression can result in significant phenotypes (19).

RNA Isolation and RT-PCR—Cells grown to 80% confluence on a 100-mm dish were washed and lysed with TriZol reagent (Canadian Life Technology). Phase separation was achieved by addition of chloroform and centrifugation at top speed in a microcentrifuge for 10 min. Aqueous phase containing total RNA was removed to a new tube and precipitated with an equal volume of isopropyl alcohol for 10 min at room temperature. The RNA pellet was recovered by centrifugation and washed with 70% ethanol. After brief drying, the RNA pellet was resuspended in diethyl pyrocarbonate-treated water. RNA concentration was determined by spectrophotometry. An aliquot (1 μg) of total RNA was used for reverse transcription with avian myeloblastosis reverse transcriptase at 42 °C for 15 min. One-tenth of the reaction was used in PCR analysis with end-labeled oligonucleotides specific for *HGF* (5′-TGTCGCCATCCCCTATGCAG-3′ and 5′-GGAGTCACAAGTCTTCAACT-3′) and β-glucuronidase (*GUSB*) sequences, as previously described (33). The PCR reaction conditions were 2 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and a final cycle of 10 min at 72 °C. The reaction was then analyzed on a 2% agarose gel by electrophoresis. The bands corresponding to the *HGF* and *GUSB* products were excised and the amount of radioactivity was determined by scintillation counting.

Copper Affinity Column Chromatography—Conditioned media were collected and HGF was partially purified using copper (II) affinity column chromatography, as described previously (34). Cells were grown to 80% confluence. The cell monolayer was washed with fresh Dulbecco's modified Eagle's medium and incubated in serum-free Dulbecco's modified Eagle's medium for 24 h. Conditioned media were collected, and cell debris was removed by centrifugation. Conditioned medium (10 ml) from each cell line was then loaded onto a copper (II) affinity column. The copper (II) affinity column was prepared by chelating Cu^{2+} ions on a 1-ml HiTrap Chelating column (Amersham Pharmacia Biotech, Baie d'Urfe, PQ), and equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). The conditioned medium was recycled through the column 5 times to ensure binding of all HGF proteins, and the column was washed thoroughly with 15 volumes of equilibration buffer. HGF protein was eluted from the column with equilibration buffer containing 80 mM imidazole at a flow rate of 1 ml/min. Fractions of 1 ml each were collected; previous experiments have determined that essentially all HGF was eluted in fraction 2 (Ref. 34 and data not shown). The fraction containing HGF was concentrated by centrifugation with Microcon centrifugal filter devices (Millipore Corp., Bedford, MA) with a 10-kDa molecular mass cut off. The samples were analyzed on a denaturing 10% SDS-PAGE gel, followed by Western blotting with anti-HGF antibody.

Immunoprecipitation, Western Blotting Analysis, and c-Src Kinase Assay—Cells were grown to confluence and treated as indicated. After three washes with cold phosphate-buffered saline, cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation and protein concentrations were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). For immunoprecipitation, equal amounts of lysate were incubated with the indicated antibodies at 4 °C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE gel, and transferred to a nitrocellulose membrane. Western blotting analysis was performed as described previously (19).

In vitro c-Src kinase assays were performed as described previously (19). Briefly, each lysate was immunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. One-half of each immunoprecipitate was subject to SDS-PAGE under nondenaturing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity by incubating with 10 μl of reaction buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4), 1.4 μg of freshly prepared acid-denatured enolase (Sigma), and 10 μCi of [γ-^{32}P]ATP. After a 10-min incubation at 30 °C, reactions were terminated by the addition of 2 × SDS sample buffer, and samples were subjected to 8% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature. The gel was dried under vacuum. Autoradiograms were produced and analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotides and Probe Labeling—Oligonucleotides used for electrophoretic mobility shift assay binding were Stat3-110F (5′-GGGCTGTTGTTAAACAGT-3′), Stat3-110R (5′-AGAACTGTTTAACAACAG-3′), Stat3-149F (5′-GGGGTTGAGGAAAGGAAG-3′), and Stat3-149R (5′-CCCCTTCCTTTCCTCAAC-3′). Complementary oligonucleotides were annealed by boiling equal molar amounts of each oligonucleotide for 10 min and then cooling slowly to room temperature. The annealed oligonucleotides (20 pmol) were labeled by a filling-in reaction with Klenow enzyme and [α-^{32}P]dCTP.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described previously (35). Briefly, 10^7 cells were washed once with phosphate-buffered saline before resuspension in cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate). Cells were allowed to swell on ice for 10 min before lysis by brief vortexing. Nuclei were pelleted and resuspended in buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate). High salt extraction was performed by incubation on ice for 30 min in buffer C and centrifugation at 4 °C. The protein content of the supernatant (nuclear extract) was determined using a Bradford protein assay (Bio-Rad, Mississauga, ON).

Electrophoretic mobility shift assays were performed as described by Mohan *et al.* (36). Briefly the binding reaction was performed by incubating 5 μg of nuclear extracts with 0.1 pmol of ^{32}P-labeled oligonucleotide probe in the presence of binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol), 9% glycerol, and 4 μg of poly(dI-dC) (Amersham Pharmacia Biotech). Binding was allowed to proceed at room temperature for 10 min before analysis on 5% nondenaturing PAGE gel in Tris glycine buffer (40 mM Tris-HCl, pH 8.4, 266 mM glycine). When unlabeled oligonucleotides were added, 10-fold molar excess was included in the binding reaction. For supershifting experiments, nuclear extracts were incubated with 2 μg of the indicated antibody at room temperature for 20 min prior to the binding reaction. After electrophoresis, the gel was fixed in 7% acetic acid, 40% methanol for 30 min, and dried under vacuum. The gel was then exposed to a PhosphorImager screen, and analyzed using a Storm PhosphorImager.

RESULTS

Inhibition of Activity of c-Src Family Kinases Impairs HGF mRNA Expression—To study the regulation of HGF expression in breast carcinoma cells, we used the mouse mammary carcinoma cell line SP1, which coexpresses HGF and tyrosine-phosphorylated Met (23). Semi-quantitative RT-PCR was performed to determine the levels of HGF mRNA in SP1 cells. We first examined the dose-dependent effect of an inhibitor of c-Src family kinases, PP2 (37). Total RNA was isolated from SP1 cells treated with different concentrations of PP2 and used for cDNA synthesis by reverse transcription. Relative HGF mRNA levels were determined by RT-PCR with HGF-specific primers, and each sample was normalized to the expression of a housekeeping gene β-glucuronidase (*GUSB*) (33). The results showed that the PP2 inhibitor reduced HGF mRNA expression in a dose-dependent manner up to 40% of untreated cells (Fig. 1*A*). In addition, we examined the level of transcription of the *HGF* gene using a reporter plasmid. A plasmid containing a 2.7-kb fragment 5′ of the *HGF* transcriptional start site ligated to the firefly luciferase gene was transiently transfected into SP1 cells. Bell *et al.* (38) have previously shown that this 2.7-kb fragment of the *HGF* promoter contains all the necessary sequence to direct HGF expression and mimics the expression pattern of the endogenous *HGF* gene in transgenic mice. Following transfection, these cells were treated with different concentrations of the PP2 inhibitor under conditions used in Fig. 1*A*. After a 24-h incubation, the cells were lysed and luciferase activity in each sample was determined and compared with control cells. The results show a similar dose-dependent reduction of *HGF* transcription following PP2 treatment (Fig. 1*B*). These findings suggest that the activity of c-Src kinase family members is important in the regulation of *HGF* transcription and mRNA expression.

c-Src Kinase Activity Regulates HGF Expression at Both mRNA and Protein Levels—We further investigated the role of c-Src tyrosine kinase in HGF expression by transfecting chicken c-Src mutants (SRC-Y527F and SRC-RF) with altered kinase activity into SP1 cells. The SRC-Y527F mutant contains a phenylalanine substitution at tyrosine 527 which results in constitutive kinase activity (39, 40). The SRC-RF mutant contains a double substitution at tyrosine 527 to phenylalanine and at lysine 295 to arginine, which produces a dominant negative phenotype (26). We have previously shown that expression of a similar dominant negative form of murine c-Src in SP1 cells reduces endogenous c-Src kinase activity and also impairs anchorage-independent growth in soft agar (19). As predicted, expression of the dominant negative form of chicken c-Src (SRC-RF) also decreased total c-Src kinase activity in SP1 cells, when compared with untransfected cells (Fig. 2, *top panel*). In addition, expression of the activated form of c-Src (SRC-Y527F) dramatically increased total c-Src kinase activity in SP1 cells. Expression of the chicken c-Src mutants was detected by an antibody (EC10) specific for avian c-Src (Fig. 2, *bottom panel*).

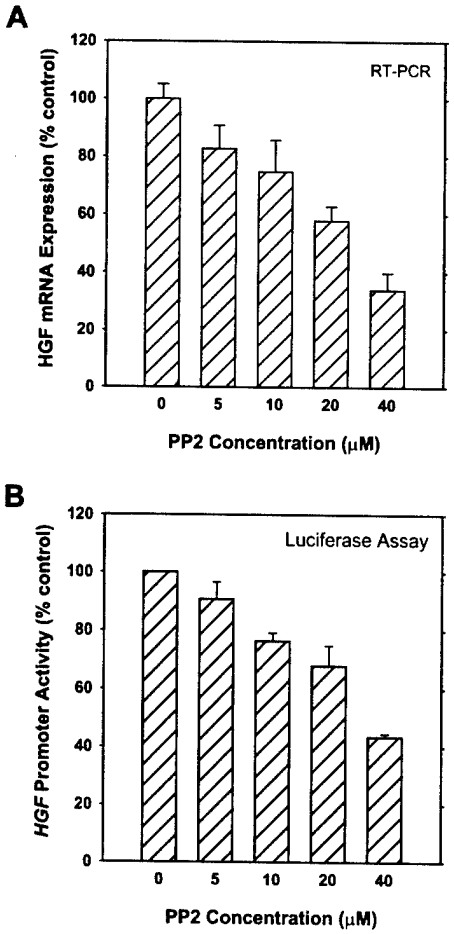

FIG. 1. **Treatment with the c-Src family kinase inhibitor PP2 decreases HGF mRNA level and transcription.** *Panel A*, prestarved SP1 cells were incubated with the Src family kinase inhibitor PP2 at the concentrations indicated. After 24 h, cells were lysed and total RNA was extracted. The amount of HGF mRNA in each sample was quantitated using RT-PCR with HGF-specific primers and primers for GUSB (see "Materials and Methods"). The amount of HGF mRNA was normalized to GUSB mRNA, and the level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments ± range. *Panel B*, SP1 cells were transfected with a reporter plasmid containing the 2.7-kb fragment of the *HGF* promoter driving expression of the luciferase gene (2.7 HGF-luc). A β-galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 24 h of incubation, PP2 was added at the concentrations indicated, and the cells were incubated for an additional 24 h, lysed, and assayed for luciferase activity. Luciferase activity of each sample was expressed as a percentage of control (untreated) cells. Values represent the mean ± S.D. of triplicate samples. The experiment was done twice with similar results.

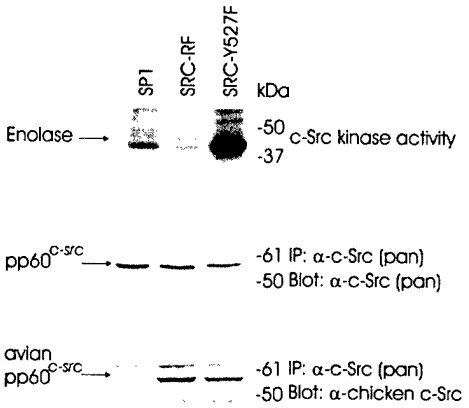

FIG. 2. **Ectopic expression of c-Src kinase mutants in SP1 cells.** SP1 cells were transfected with expression vectors containing activated c-Src (SRC-Y527F) or dominant negative c-Src (SRC-RF) or an empty expression vector (SP1). After 48 h, cells were lysed. Equal amounts of the cell lysates were immunoprecipitated with anti-c-Src (pan) antibody at excess antibody concentration. Half of the immunoprecipitates was used to detect c-Src kinase activity using enolase as a substrate (*top panel*). The other half was subjected to Western blotting with anti-Src (pan) antibody to confirm equal amounts of total c-Src protein in the immunoprecipitates (*middle panel*), and then reprobed with monoclonal anti-chicken c-Src (EC10) antibody to detect the relative level of ectopic expression of each c-Src mutant (*bottom panel*). The amount of chicken c-Src compared with total c-Src may be relatively low, and cannot be directly inferred from these results, since different antibodies and exposure times were used for each Western blot.

To assess the effect of c-Src kinase activity on HGF mRNA expression, RT-PCR analysis was carried out on RNA extracted from SP1 cells expressing the different c-Src mutants, or treated with the PP2 inhibitor (Fig. 3*A*). Expression of the dominant negative SRC-RF mutant or treatment with PP2 reduced the HGF mRNA level in SP1 cells by ~60%. Conversely, expression of the constitutively active SRC-Y527F mutant increased HGF mRNA expression by about 2-fold. In a parallel approach, the level of secreted HGF protein was compared in conditioned media collected from the same cells and under the same conditions described in Fig. 3*A*. Our laboratory has previously shown that HGF is a Cu(II)-binding protein, which can be purified with high recovery from conditioned media with copper (II) affinity chromatography (34) and analyzed on a denaturing SDS-PAGE gel (Fig. 3*B*). Using this method, we showed that expression of the dominant negative SRC-RF mutant or treatment with PP2 significantly decreased the amount of HGF protein secreted by SP1 cells. In contrast, expression of activated c-Src (SRC-Y527F) increased the amount of secreted HGF protein. Together these data suggest that HGF expression (both at the mRNA and protein levels) is regulated by c-Src kinase activity.

c-Src Kinase Activity Induces HGF Expression through a Specific cis-Acting Region on the HGF Promoter—To determine the effect of c-Src kinase mutants on *HGF* promoter activity, we constructed a series of reporter plasmids with the luciferase gene linked to different fragments of the 2.7-kb region 5′ of the *HGF* transcriptional start site (Fig. 4*B*). These reporter constructs were co-transfected into SP1 cells with a control vector, or vectors expressing the SRC-Y527F or SRC-RF mutants of chicken c-Src kinase, and luciferase activity of the transfected cells was compared (Fig. 4*A*). The results show that expression of activated c-Src increased up to 2-fold the activity of the 2.7-kb *HGF* promoter, whereas dominant negative c-Src had the opposite effect. Deletions of up to −538 bp (0.5Δ HGF) had no significant effect on the c-Src dependent activity of the *HGF* promoter, although some fluctuations in basal activity of the promoter were apparent. A further deletion of −273 bp (0.3 HGF-luc) significantly reduced the basal *HGF* promoter activity, while some c-Src dependent activity remained. The remaining c-Src kinase responsiveness was eliminated when all but 72 bp (0.1 HGF-luc) of the *HGF* promoter was removed. This suggests that a *cis*-acting element responsive to c-Src kinase activity is located within −273 and −70 bp of the *HGF* promoter. An internal deletion construct lacking the −70 to −254-bp region (named 0.5Δ HGF-luc) was used to confirm the c-Src responsiveness of this region. As predicted, the 0.5Δ HGF-luc reporter did not respond to expression of SRC-Y527F, although basal activity remained. A similar pattern of repression of the luciferase activity among all the *HGF* promoter deletion mutants used was seen when dominant negative c-Src (SRC-RF) was coexpressed with the HGF-luc constructs.

To confirm the importance of the regions of the promoter

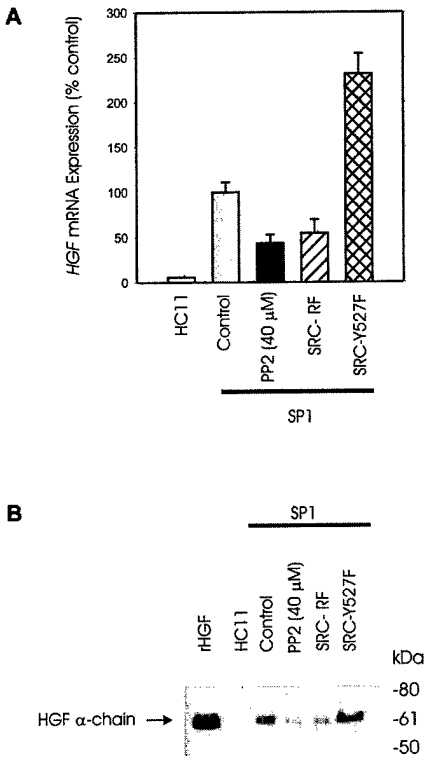

FIG. 3. **c-Src kinase activity modulates HGF mRNA and protein levels in SP1 cells.** *Panel A,* SP1 cells transfected with dominant negative Src (SRC-RF) or activated Src (SRC-Y527F) or empty vector (control) were prestarved overnight. PP2 (40 μM) was added to one plate of SP1 cells and incubated for an additional 24 h. A nonmalignant breast epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of HGF mRNA in each sample was quantitated using RT-PCR and normalized to GUSB mRNA as described in the legend to Fig. 1. The level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments ± range. *Panel B,* serum-free conditioned media were collected for 24 h from HC11 cells, PP2-treated SP1 cells, and SP1 cells transfected as in *Panel A*. HGF protein from the conditioned media was purified using copper (II) affinity chromatography (34). The fraction containing HGF protein was concentrated in Microcon concentrators and subjected to denaturing SDS-PAGE. Recombinant HGF (100 ng) was included in one lane as a control. After electrophoresis, the proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF antibody. Immunoreactive bands were revealed using Enhanced Chemiluminescence kit.

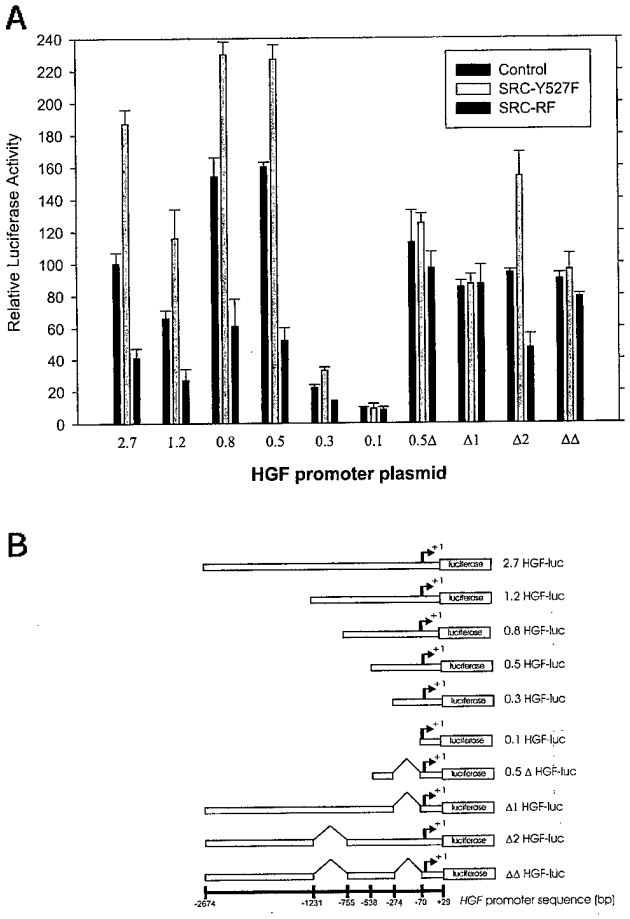

FIG. 4. **c-Src kinase responsiveness of *HGF* transcription requires the −254 to −70 bp region of the *HGF* promoter.** *Panel A,* the 2.7-kb HGF-luciferase reporter (2.7 HGF-luc), or reporter constructs containing various deletions of the HGF promoter (see *Panel B*), were co-transfected into SP1 cells with activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF), or an empty expression vector (control). Luciferase activity of each sample was determined, and normalized to the empty vector control value within each group as described in the legend to Fig. 1*B*. Values represent mean ± S.D. of triplicate samples. The experiments were done three times using two different preparations of plasmid DNA with similar results. *Panel B*, schematic representation of the wild-type *HGF* reporter construct and the corresponding internal deletion mutants used in *Panel A* is shown. The name of each construct refers to the full-length (2.7 kb) or truncated promoter sequences (1.2, 0.8, 0.5, 0.3, and 0.1 kb) upstream of the transcriptional start site (indicated by *arrow*). In addition, constructs containing the 0.5-kb sequence with an internal deletion of the region between −254 and −70 (0.5Δ), or the full-length sequence containing internal deletion of regions between −254 and −70 (Δ1), −1231 and −755 (Δ2), or both (ΔΔ) were used.

responsive to activated c-Src, several internal deletion mutants were constructed. Full-length reporter constructs missing −273 to −70 bp (Δ1), −1231 to −755 bp (Δ2), or both regions (ΔΔ) of the *HGF* promoter were transfected into SP1 cells in the presence or absence of the SRC-Y527F and SRC-RF mutants (Fig. 4*A*). As predicted, Δ1 and ΔΔ deletion mutants exhibited neither induction nor repression of *HGF* promoter activity when activated c-Src or dominant negative c-Src was expressed, respectively. In contrast, the Δ2 mutant showed strong induction of *HGF* promoter activity corresponding to expression of the activated SRC-Y527F mutant, and strong repression of *HGF* promoter activity when the SRC-RF mutant was expressed. This finding shows that only the region between *−254* and −70 bp of the *HGF* promoter is important for c-Src responsiveness of *HGF* expression in SP1 cells. We will refer to this region as the c-Src responsive region.

Stat3 Activates HGF Transcription in Cooperation with Activated c-Src—Examination of the c-Src responsive region of the *HGF* promoter revealed several Stat3-binding sites. This consensus sequence is highly conserved among mouse, rat, and human (100% identity), while this conservation is lost in regions upstream of −500 bp of the *HGF* promoter (41). Since Stat3 activation by Src induces specific gene expression and is required for cell transformation (42, 43), we examined whether expression of Stat3 in the presence or absence of the activated c-Src mutant (SRC-Y527F) has any effect on *HGF* promoter activity. A reporter plasmid containing the −2.7-kb full-length *HGF* promoter was co-transfected with a constant amount of the SRC-Y527F, and varying amounts of Stat3, expression plasmids. Expression of activated c-Src (SRC-Y527F) alone increased *HGF* transcription by about 2-fold (Fig. 5*A*). Likewise, expression of Stat3 alone increased *HGF* transcription by about 2-fold, and maintained a plateau value with even 0.05 μg of plasmid DNA. However, in cells coexpressing both the activated c-Src mutant and increasing amounts of Stat3, *HGF* transcription increased up to 5-fold. This result indicates that there is a cooperative effect between c-Src kinase activity and

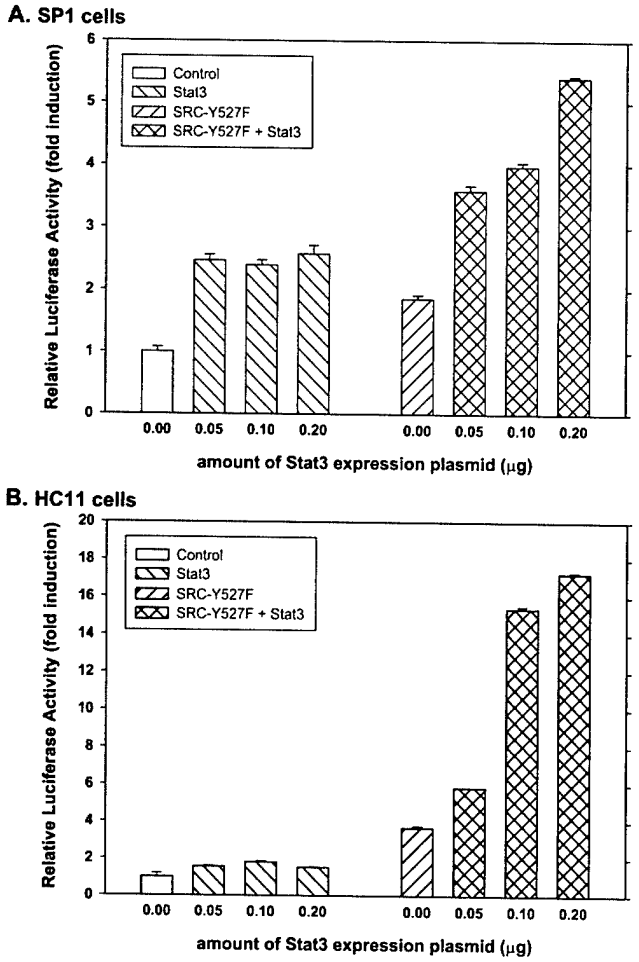

FIG. 5. **Stat3 induces *HGF* transcription in cooperation with activated c-Src.** SP1 carcinoma cells (*Panel A*) and HC11 mammary epithelial cells (*Panel B*) were co-transfected with the 2.7 HGF-luc reporter and activated c-Src (SRC-Y527F) or an empty vector (*control*), in combination with varying amounts of Stat3. Luciferase activity was determined and expressed as a percentage of that in control cells as described in the legend to Fig. 1*B*. Values represent the mean ± S.D. of triplicate samples. The experiment was done twice with similar results.

Stat3 protein in the regulation of *HGF* transcription.

The nonmalignant mammary epithelial cell line, HC11, shows at least a 15-fold lower level of *HGF* transcription and no detectable HGF protein, compared with SP1 carcinoma cells (data not shown). We therefore determined whether coexpression of c-Src and Stat3 can activate *HGF* transcription in HC11 cells. Expression of activated c-Src induced expression by about 4-fold (Fig. 5*B*). In contrast to SP1 cells, expression of Stat3 alone in HC11 cells did not significantly induce *HGF* transcription. However, when activated c-Src and Stat3 were coexpressed, *HGF* transcription was synergistically induced up to 17-fold. Similarly, two clones of HC11 cells, stably transfected with Stat3 followed by transient expression of activated c-Src, showed up to a 20-fold increase in *HGF* promoter activity (data not shown). Together, these results suggest that increased c-Src kinase activity and Stat3 expression can override the repression of *HGF* transcription in nonmalignant mammary epithelial cells.

To determine whether the c-Src responsive region of the *HGF* promoter is involved in the observed cooperative effect between c-Src and Stat3, the transcriptional activity of a mutant *HGF* reporter lacking the c-Src responsive region (Δ1 HGF-luc) was compared with that of the full-length (2.7 HGF-luc) *HGF* reporter. Each reporter construct was transfected into SP1 cells alone, or in combination with Stat3, and the activated c-Src (SRC-Y527F) mutant, expression plasmids. Expression of the activated c-Src mutant induced activation of the full-length *HGF* promoter, but not of the deletion mutant (Δ1 HGF-luc) (Fig. 6). Similarly, Stat3 expression increased the activity of the full-length *HGF* promoter, and only marginally affected that of the deletion mutant (Δ1 HGF-luc), this result suggests that Stat3 activates the *HGF* promoter. The level of induction due to Stat3 expression is even higher than that due to activated c-Src alone. This effect is probably due to a limiting amount of endogenous Stat3 in SP1 cells. When both Stat3 and activated c-Src were coexpressed, *HGF* promoter activity in the full-length construct was strongly induced, this effect was not seen in the deletion mutant (Δ1 HGF-luc). These results show a cooperative effect between Stat3 and activated c-Src in the induction of *HGF* transcription, and imply the presence of specific Stat3-binding sites on the *HGF* promoter.

c-Src Kinase Regulates Tyrosine 705 Phosphorylation and DNA Binding Activity of Stat3—Previous reports have found that c-Src activates Stat3 by inducing tyrosine phosphorylation of Stat3 and increasing its DNA binding affinity (42, 44). We therefore examined the effect of c-Src kinase activity on Stat3 tyrosine 705 phosphorylation in SP1 cells. We found that expression of activated c-Src induced Stat3-specific tyrosine 705 phosphorylation, while expression of dominant negative c-Src had the opposite effect (Fig. 7). c-Src kinase activity similarly affected the nuclear protein binding affinity of the Stat3 consensus sites on the *HGF* promoter (Fig. 8). We used electrophoretic mobility shift assays to examine the Stat3 consensus DNA binding affinity of nuclear protein extracts from cells expressing different mutants of c-Src. Radiolabeled oligonucleotide probes with DNA sequences corresponding to the two Stat3 consensus binding sites in the region between −254 to −70 of the *HGF* promoter were used to detect putative Stat3 binding (Fig. 8). Binding of probes corresponding to each Stat3 consensus site (−110 or −149) was detected in nuclear protein extracts of SP1 cells (*lane 1* in Fig. 8, *A* and *B*, respectively). These DNA binding activities were specific since the presence of the corresponding unlabeled probes abolished the binding (*second lane*), while a probe with an unrelated DNA sequence had no effect (*third lane*). In addition, when comparing *first, fourth*, and *seventh lanes* (Fig. 8), it is apparent that there was less specific DNA binding in nuclear extracts from SP1 cells expressing dominant negative c-Src than in control cells expressing no exogenous c-Src. Moreover, nuclear extracts from SP1 cells expressing activated c-Src had higher binding activity than that from untransfected cells. This finding indicates that the expression of dominant negative c-Src reduces nuclear protein binding to the Stat3 consensus sites, whereas activated c-Src has the opposite effect. Thus specific binding of nuclear protein to the Stat3 consensus sites correlates with phosphorylation at tyrosine 705 of Stat3 in these cells.

Although there is a strong indication of Stat3 being the transcription factor binding to the c-Src responsive region of the *HGF* promoter, other Stat proteins (such as Stat1, Stat5A, and Stat5B) can also bind to a Stat3 consensus site, albeit at lower levels (28, 45, 46). Therefore, antibodies against specific Stat proteins were used in supershift experiments to determine the composition of the DNA binding complex (Fig. 9). Nuclear extracts from SP1 cells were preincubated with antibodies against Stat1, Stat3, Stat5A, or Stat5B prior to the addition of the radiolabeled probe. Both −110 (Fig. 9*A*) and −149 (Fig. 9*B*) probes formed DNA-protein complexes when nuclear extracts were added. However, only anti-Stat3 antibody could efficiently bind to these complexes to form a supershift band. Antibodies to Stat1 (data not shown), Stat5A, or Stat5B did not retard the DNA-protein complex further, despite the fact that these tran-

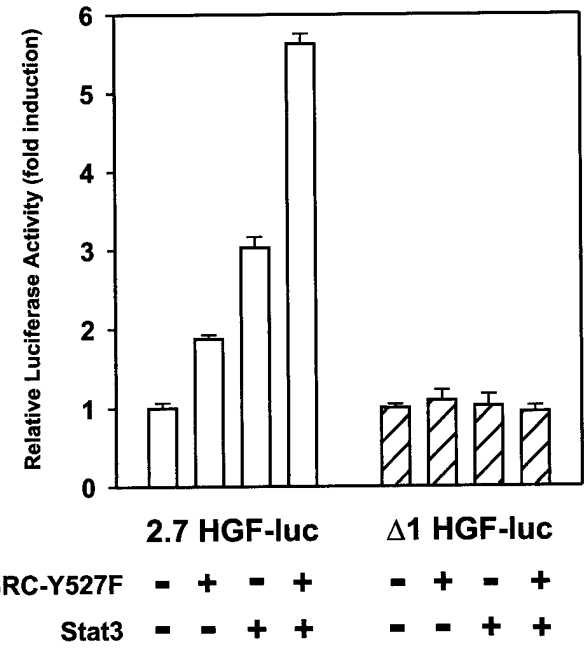

FIG. 6. **The cooperative effect of Stat3 and activated c-Src on induction of *HGF* transcription requires the c-Src responsive region of the HGF promoter.** SP1 cells were co-transfected with the 2.7 HGF-luciferase reporter, an internal deletion mutant (Δ1 HGF-luc), and a combination of activated Src (SRC-Y527F) and Stat3 as indicated. Transfections and luciferase assays were performed as described in the legend to Fig. 1*B*. Values represent the mean ± S.D. of triplicate samples. The experiment was done four times with similar results.

scription factors were present in SP1 cells (data not shown). This observation suggests that Stat3 is preferentially involved in the DNA-protein complexes which bind to the c-Src responsive elements in the *HGF* promoter.

DISCUSSION

During normal breast development, HGF is expressed primarily by mesenchymal cells, while its receptor Met is expressed by epithelial cells (7). However, HGF is expressed in regions of human invasive breast carcinoma, and in various breast carcinoma cell lines (11–13). During tumorigenesis HGF stimulates angiogenesis, invasion, and metastasis (47, 48). Our laboratory (1) and others (49) have shown that HGF can stimulate survival of carcinoma cells. Therefore, acquired HGF expression leading to an HGF autocrine loop in breast carcinoma cells may be an important step during mammary tumorigenesis. However, the regulation of HGF expression in breast carcinoma cells is not very well understood, although some studies have been done in fibroblasts (50–54). In the present study, we examined the role of c-Src kinase, which shows increased activity in human breast cancer (55), in controlling HGF expression in breast carcinoma cells.

We previously described a mammary breast carcinoma cell line, SP1, which expresses both HGF and activated Met (23). In SP1 cells, several downstream signaling molecules, such as phosphatidylinositol 3-kinase, phospholipase Cγ, and focal adhesion kinase, are constitutively phosphorylated on tyrosine residues in SP1 cells, consistent with the presence of an autocrine loop (1, 23). We have also found that c-Src tyrosine kinase in SP1 cells is constitutively active and is required for several HGF-dependent processes, such as cell motility and anchorage-independent growth (19).

In this report, we showed that inhibition of c-Src kinase activity in SP1 cells, through either the presence of c-Src kinase inhibitors or the expression of a dominant negative mutant of c-Src, caused a decrease in HGF mRNA and protein levels. Expression of an activated c-Src kinase had the reverse effect. This finding suggests that c-Src is important in regulating the basal level of *HGF* transcription in epithelial and carcinoma cells, and can induce elevated expression of *HGF*. However, since inhibition of c-Src kinase activity cannot completely eliminate HGF basal expression, other transcription factors may play roles in maintaining HGF basal expression. Indeed, in our system, the Sp1 transcription factor is essential in maintaining HGF basal level transcription, but has no effect on c-Src-induced HGF expression (data not shown). Furthermore, aggregates of SP1 cells expressing the activated form of c-Src, in which HGF protein level was high, showed spontaneous scattering when plated on plastic, compared with the parent cell line which required addition of exogenous HGF.[3] The higher level of endogenous HGF expression in SP1 cells expressing the activated form of c-Src may be sufficient to induce spontaneous scattering of these cells. Together, these findings suggest that c-Src kinase activity is important in regulating HGF expression.

By using deletion mutants of the *HGF* promoter, we mapped the c-Src responsive element to −254 to −70 bp. Since there is significant homology among the mouse, rat, and human *HGF* promoter sequences between −500 and +1 (41), the regulation of *HGF* expression by c-Src kinase through this element is probably conserved among these species. Previous studies in fibroblast cells have demonstrated several transcription factors which regulate *HGF* expression: C/EBP (−4 bp) (50), an epithelial cell-specific repressor (−16 bp) (9), Sp1/Sp3 (−318 bp) (52), estrogen receptor (−872 bp) (51), and chicken ovalbumin upstream promoter-transcription factor (−860 bp) (51). Transgenic mouse studies showed that 0.7 kb of the *HGF* promoter exhibited the same expression pattern as the full-length (2.7 kb) promoter (38). Although in our system we observed that Sp1/Sp3 maintain the basal level expression of HGF in breast carcinoma cells, these sites are not responsible for c-Src induced expression of HGF (data not shown). The C/EBP site appeared to have no transcriptional activity *in vivo* (38). Binding sites for estrogen receptor and chicken ovalbumin upstream promoter-transcription factor are likely to be involved in estrogen-induced expression of HGF since the upstream sequence between −2.7 and −0.7 kb has been shown to be necessary for maximal inducibility of the *HGF* promoter (such as after partial hepatectomy) (38). However, the c-Src responsive region (−254 to −70 bp) described here has not been previously reported.

In the c-Src responsive region of the *HGF* promoter there are two consensus binding sites for Stat3 (at −110 and −149), both of which are completely conserved among human, mouse, and rat. Our results showed that Stat3, in cooperation with c-Src kinase, can activate *HGF* promoter, this activation is completely dependent on the presence of these Stat3-binding sites and implies a role of Stat3 as a downstream effector of c-Src kinase. We therefore examined the mechanism by which c-Src regulates Stat3 activity in SP1 carcinoma cells. Stat3 has been shown to be regulated by both tyrosine and serine phosphorylations (56–58). Although there is no direct evidence that Stat3 is phosphorylated directly by c-Src, some reports suggest that c-Src and Stat3 interact physically (30, 59). Therefore, it is possible that c-Src regulates Stat3 through tyrosine phosphorylation. Our results showed that expression of a dominant negative form of c-Src reduced tyrosine phosphorylation of Stat3 and the expression of constitutively active c-Src mutant had the opposite effect. In addition, we found that the formation of a DNA-protein complex with the two Stat3-binding sites in the c-Src responsive elements was dependent on

[3] B. Elliott, unpublished results.

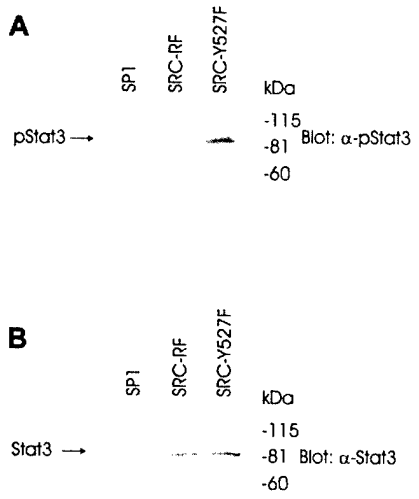

FIG. 7. **c-Src kinase activity regulates phosphorylation of tyrosine residue 705 of Stat3.** SP1 cells transfected with SRC-RF or SRC Y527F, or untransfected SP1 cells, were lysed. Equal amounts of proteins from each cell lysate were subjected to denaturing SDS-PAGE. The proteins were then transferred onto nitrocellulose and the blot was probed with antibody specific for phosphotyrosine 705 of Stat3 (*Panel A*). The blot was subsequently reprobed with anti-Stat3 (pan) antibody (*Panel B*).

the level of c-Src kinase activity in the cells. An apparently greater effect of activated c-Src on the binding activity of the −149 Stat3 site compared with the −110 Stat3 site was observed. This difference could potentially represent different binding affinities, or interaction with other transcription factors.

Stat2, -4, and -6 are not normally expressed in mammary tissues (60–63), and are therefore unlikely to be involved in the formation of DNA-protein complexes in SP1 cells. Both Stat1 and

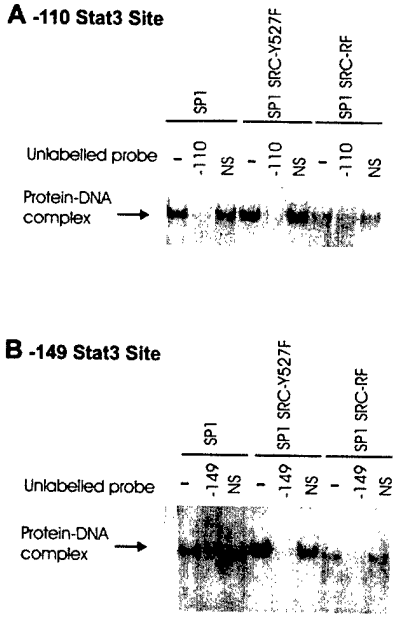

FIG. 8. **c-Src kinase activity regulates nuclear protein binding to the Stat3 consensus sites (at positions −110 and −149) of the *HGF* promoter.** Nuclear extracts were prepared from SP1 cells transfected with SRC-RF, SRC-Y527F, or untransfected cells. Equal amounts of each nuclear extract were used in binding studies with radiolabeled probes containing either the −110 (*Panel A*) or the −149 region (*Panel B*) of the *HGF* promoter. 10-fold molar excess of an unlabeled probe containing the −110, −149 or a nonspecific sequence (*NS*), respectively, was included in the binding reaction where indicated. The gel was fixed, dried, and analyzed using a Storm PhosphorImager as described under "Experimental Procedures." The *arrow* indicates the position of the protein-DNA complex.

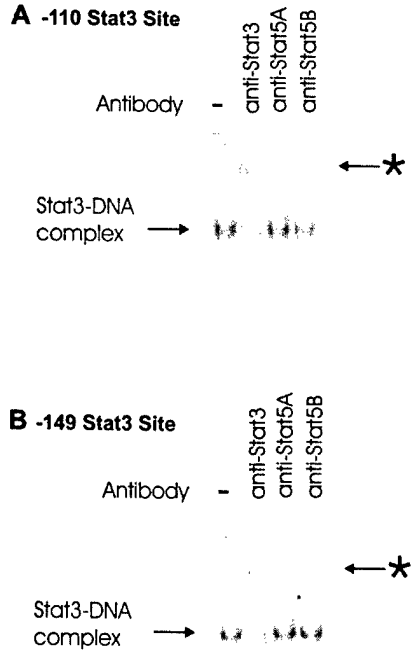

FIG. 9. **Stat3 forms part of the DNA-protein complex at both the −110 and −149 consensus sites.** Nuclear extracts were prepared from SP1 cells as described under "Experimental Procedures." For supershift assays, nuclear extracts were incubated with anti-Stat3, Stat5A, or Stat5B antibody on ice for 30 min prior to electrophoretic mobility shift assay analysis. After incubation with labeled −110 (*Panel A*) or −149 (*Panel B*) probes, the reaction was subjected to nondenaturing PAGE. The *asterisk* indicates the position of supershift band.

Stat3 have been shown to be activated by c-Src in fibroblast cells when they are stimulated with various growth factors (30, 45, 64), while Stat5 is expressed and activated during mammary development (60). Moreover, both Stat3 and Stat5 have been found to be constitutively active in cells transformed by v-Src, v-Abl, and other oncoproteins (42–44, 59, 65–67). Therefore, other Stat proteins cannot be ignored as part of the complex. Supershift studies with antibodies against specific Stat proteins allowed us to identify Stat3, and exclude Stat1, -5A, or -5B, as a component of the DNA-protein complex. Furthermore, since there is only one DNA complex formed with each probe and each probe can effectively abolish DNA-protein complex formation with the other (data not shown), the same DNA-binding protein(s) must be involved in binding to each of these regions. Since Stat3 protein binds as dimers to its binding sites, it is reasonable to assume that Stat3 dimers are binding to both sites in the c-Src responsive region. Together, these observations suggest that c-Src kinase may regulate Stat3-dependent transcriptional activation through direct or indirect tyrosine phosphorylation of Stat3, resulting in increased DNA binding ability.

In contrast to SP1 carcinoma cells, the nonmalignant mammary epithelial cell line, HC11, showed a very low level of *HGF* transcription with no detectable HGF protein. Furthermore, expression of activated c-Src (Y527F) had very little effect on *HGF* transcription in HC11 cells, possibly due to the presence of the epithelial cell type-specific repressor (9). However, coexpression of Stat3 and activated c-Src caused a strong synergistic induction of *HGF* transcription in HC11 cells, implying that the lack of c-Src kinase activity and the low level of activated Stat3 may be limiting for *HGF* transcription in HC11 cells. Increased activities of these proteins can possibly override the repression by the cell type-specific repressor and allow expression of HGF in epithelial cells. Interestingly, we found that fibroblast cells, which normally express HGF, also require c-Src kinase activity to regulate HGF expression, and that this

regulation of HGF is dependent on the same region of the *HGF* promoter as our breast carcinoma cell model (data not shown). These results suggest a similar regulation pattern between fibroblast cells that express HGF endogenously, and carcinoma cells, which acquire the ability to express HGF. It is possible that during epithelial-mesenchymal transition, epithelial cells acquire different genetic mutations leading to the activation of c-Src kinase. For example, increased expression and activity of HER2/Neu, an epidermal growth factor-like receptor tyrosine kinase, in breast carcinoma cells has been shown to activate c-Src kinase (64, 68). Activation of c-Src, in turn, may lead to a de-repression of HGF expression, giving these cells a growth advantage compared with nontransformed epithelial cells. This step may be an important initial step in tumorigenesis.

Here, we have reported that c-Src kinase and Stat3 act cooperatively in stimulating *HGF* gene expression in breast carcinoma cells, most likely via regulation of Stat3-dependent transcriptional activation of the *HGF* promoter. Although many reports have indicated that increased Src kinase activity (particularly through the expression of v-Src) can activate gene expression via Stat3, in this study we identify a target region (−254 to −70 bp) of the *HGF* promoter responsive to elevated activity of c-Src kinase in breast carcinoma cells. There is recent evidence suggesting that an HGF autocrine loop can provide selective survival and growth advantage to carcinoma cells and that overexpression of HGF can be a reliable indicator of poor survival of breast cancer patients (11). Our findings therefore provide an important link between breast cancer progression and HGF expression, and suggest that the c-Src/Stat3 pathway regulating HGF expression can be a potential target for therapy in breast cancer treatment.

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c-Src Kinase Activity Is Required for Hepatocyte Growth Factor-induced Motility and Anchorage-independent Growth of Mammary Carcinoma Cells*

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Overexpression and amplification of hepatocyte growth factor (HGF) receptor (Met) have been detected in many types of human cancers, suggesting a critical role for Met in growth and development of malignant cells. However, the molecular mechanism by which Met contributes to tumorigenesis is not well known. The tyrosine kinase c-Src has been implicated as a modulator of cell proliferation, spreading, and migration; these functions are also regulated by Met. To explore whether c-Src kinase is involved in HGF-induced cell growth, a mouse mammary carcinoma cell line (SP1) that co-expresses HGF and Met and a nonmalignant epithelial cell line (Mv1Lu) that expresses Met but not HGF were used. In this study, we have shown that c-Src kinase activity is constitutively elevated in SP1 cells and is induced in response to HGF in Mv1Lu cells. In addition, c-Src kinase associates with Met following stimulation with HGF. The enhanced activity of c-Src kinase also correlates with its ability to associate with Met. Expression of a dominant negative double mutant of c-Src (SRC-RF), lacking both kinase activity (K295R) and a regulatory tyrosine residue (Y527F), in SP1 cells significantly reduced c-Src kinase activity and strongly blocked HGF-induced motility and colony growth in soft agar. In contrast, expression of the dominant negative c-Src mutant had no effect on HGF-induced cell proliferation on plastic. Taken together, our data strongly suggest that HGF-induced association of c-Src with Met and c-Src activation play a critical role in HGF-induced cell motility and anchorage-independent growth of mammary carcinomas and further support the notion that the presence of paracrine and autocrine HGF loops contributes significantly to the transformed phenotype of carcinoma cells.

Evidence supports a role of hepatocyte growth factor (HGF)[1] and its receptor, the product of the *met* protooncogene, in both normal (1, 2) and malignant (3–5) epithelial cell development. In addition, a majority of human breast cancers show increased expression of HGF and Met (6–8), and this high level of HGF expression correlates with recurrence and poor patient survival (9). Met is also overexpressed in several other human cancers, including ovarian (10), melanoma (11), colon carcinomas (12), and osteosarcomas (13). Collectively, these observations suggest that activation of Met by overexpression, gene amplification, or establishment of an HGF autocrine loop may contribute to growth and development of mammary carcinomas. Previous studies demonstrated that co-expression of HGF and Met (4, 14), as well as expression of a constitutively active Met (Tpr-Met) in NIH-3T3 fibroblasts (15, 16) directly leads to cell transformation and tumorigenicity. However, the molecular mechanism by which HGF binding to its receptor elicits cell transformation is not fully understood.

A number of cytoplasmic signaling proteins, such as phosphatidylinositol (PI) 3-kinase, Grb2, Shc, Ras, and c-Src, have been shown to be involved in Met-dependent signal transduction pathways (17, 18). It is important to establish which of these signaling proteins regulate Met-dependent steps in tumor progression, because different signaling proteins may regulate various HGF-induced cellular functions, including mitogenic, motogenic, and morphogenic signals in target cells (18–22). The HGF-mediated signaling pathway is further complicated by the observation that the majority of SH2-containing cytoplasmic effectors bind to a single multifunctional docking site on the cytoplasmic domain of Met, whereas a second site is required for Grb2 binding (17, 18). Recent findings using a mutational approach demonstrated that different HGF-induced effects are regulated by these separate Met binding sites for cytoplasmic transducers (23–25) and that complementation in *trans* between these two binding sites is required for the invasive-metastatic phenotype (25). However, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required. Recently, we (26) and others (27) have demonstrated that PI 3-kinase activity is required for HGF-induced mitogenic (26) and motogenic functions (27). These findings strongly argue that PI 3-kinase may play an important role in HGF-mediated growth of mammary carcinomas.

The tyrosine kinase c-Src is activated in response to HGF (17, 18) and other growth factors such as platelet-derived growth factor (PDGF) (28–30), fibroblast growth factor (31),

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[1] The abbreviations used are: HGF, hepatocyte growth factor; PI, phosphatidylinositol; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; PDGF, platelet-derived growth factor.

and epidermal growth factor (32). c-Src kinase activity is known to modulate cell proliferation (33, 34), spreading (35, 36), and migration (36–38) in many cell types; these functions are also regulated by HGF (19–23). c-Src kinase activity is increased 4-fold in human breast cancer (39, 40) and is also elevated in Neu-induced mouse mammary carcinomas in transgenic mice (41, 42). Activation of c-Src tyrosine kinase in transgenic mice induces mammary epithelial hyperplasias and is required, but is not sufficient, for induction of mammary tumors in polyoma virus middle T-transgenic mice (42, 43). Altogether, these observations support the notion that increased c-Src kinase activity in mammary carcinomas plays an important role in mammary tumor growth and development. However, the role of c-Src kinase in HGF-induced functions in mammary carcinoma cells is not clearly known.

To analyze whether c-Src kinase is involved in HGF-induced mammary carcinoma cell growth, we used a mouse mammary carcinoma cell line, SP1, which expresses HGF and tyrosine-phosphorylated Met, thereby generating an autocrine HGF loop in these cells (44). Our current results demonstrate that c-Src kinase activity is elevated in SP1 cells, compared with nonmalignant Mv1Lu epithelial cells. The increased activity of c-Src kinase correlates with its ability to associate with tyrosine-phosphorylated Met. We therefore examined the effect of expressing a dominant negative mutant form of c-Src on c-Src kinase activity and HGF-induced cell motility and anchorage-independent growth of SP1 carcinoma cells. Taken together, our findings show that c-Src kinase activation plays a significant role in HGF-induced cell motility and anchorage-independent growth, characteristics of the transformed phenotype.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit anti-sheep IgG conjugated to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (Westgrove, PN). Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KT). Rabbit anti-c-Src IgG, anti-Met (mouse) IgG, and anti-PLC-γ1 IgG were obtained from Santa Cruz Biotechnology (San Diego, CA).

Tissue Culture and Cell Lines—Mv1Lu cells are members of a mink lung epithelial cell line obtained from ATCC (Rockville, MA). Maintenance medium for Mv1Lu cells was Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% FBS. The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma and expresses HGF and Met. The characteristics of the SP1 cell line have been described elsewhere (45, 46). Maintenance medium for SP1 cells was RPMI 1640 (Life Technologies, Inc.) supplemented with 7% FBS (Life Technologies, Inc.).

Cell Transfection—cDNAs encoding wild type *c-src* (SRC) and a dominant negative double mutant of *c-src* (SRC-RF) with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) ligated into the pRc/CMV plasmid (Invitrogen, San Diego, CA) carrying the neomycin resistance marker were obtained from Dr. J. Brugge (47). SP1 cells expressing the mutant c-Src and wild type c-Src were established using the stable transfection LipofectAMINE (Life Technologies, Inc.) method (48). Briefly, SP1 cells were grown to 80% confluence. The DNA (1 μg) was mixed with LipofectAMINE reagent (9 μl) in 200 μl of serum-free medium and was incubated for 15 min at room temperature. Before transfection, cells were washed once with 2 ml of serum-free medium. For each transfection, the mixed DNA and LipofectAMINE were combined with 0.8 ml of serum-free RPMI 1640 medium, and the cells were incubated with this transfection mixture. After 5 h of incubation, an equal volume of RPMI/14% FBS was added to the transfection medium, and incubation proceeded for an additional 24 h. For most experiments, pooled transfected cells selected with G418 (450 μg/ml) were used. In one experiment, SP1 cells were transfected with SRC-RF or SRC, and clones were isolated and tested for Src kinase activity and colony forming efficiency.

Cell Proliferation and Colony Growth Assay—Cell proliferation was carried out as described elsewhere (45). Briefly, SP1 carcinoma cells and Mv1Lu cells were plated at 10^4 cells/well in 24-well plates under the various conditions indicated. DNA synthesis was measured by adding 0.2 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Oakville, ON, Canada) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (ICN, Costa Mesa, CA), and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman, Mississauga, ON, Canada). Results are expressed as the mean cpm/well ± S.D. of triplicates.

Colony growth assays were performed as described previously (49). Briefly, a solution of 1.2% Bactoagar (Difco Lab) was mixed (1:1) with 2× RPMI 1640, supplemented with FBS at final concentrations of 7 or 1% alone or with HGF as indicated, and layered onto 60 × 15-mm tissue culture plates. SP1 cells (10^3/2.5 ml) were mixed in a 0.36% Bactoagar solution prepared in a similar way and layered (2.5 ml/plate) on top of the 0.6% Bactoagar layer. Plates were incubated at 37 °C in 5% CO_2 for 8–10 days. Colonies were fixed with methanol, stained with Giemsa, and counted manually. Results are expressed as mean number of colonies per dish ± S.D. of quadruplicates.

Cell Motility Assay—To measure cell motility, Transwell culture inserts (8-μm pore size) (Costar, Toronto, ON, Canada) were coated uniformly with gelatin (0.25% w/v, Sigma, Oakville, ON, Canada) on both sides for 2 min at room temperature (50). Membranes were washed twice with serum-free RPMI 1640 medium and inserted into a 24-well culture plate (Costar, Toronto, ON, Canada) with 1 ml of RPMI 1640 containing 0.5 mg/ml bovine serum albumin (Life Technologies, Inc.). Cells were grown to 50% confluence, serum-starved overnight, and harvested in 5 mM EDTA. Cells (2×10^4/100 μl) were plated in the insert and incubated for 6–8 h at 37 °C. Following the incubation, excess medium was removed, and cells were fixed in 1% paraformaldehyde (Sigma) for 15 min and stained with hematoxylin (Fisher, Oakville, ON, Canada). Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the under side of the membrane were counted using an inverted microscope with phase contrast illumination. Cell motility is expressed as the number of migrating cells per well. In a parallel study, a wounding assay was performed, as described previously (36). Briefly, monolayers of each cell type were "wounded" by scraping with an Eppendorf yellow tip, washed, and incubated alone or with HGF for varying times. Migration was assessed visually by the ability of cells to close the wounded area.

Immunoprecipitation and Western Blotting—Cells were grown to confluence and serum-starved for 24 h. Cells were rinsed with cold phosphate-buffered saline three times and lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4 °C. Protein concentration of supernatants was determined using a bicinchoninic acid protein assay (Pierce). Equal protein amounts from each cell lysate were incubated with the indicated antibodies at 4 °C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with 3% skimmed milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and probed for 1 h with the indicated antibodies. The membrane was washed three times for 5 min each with TBST buffer, incubated with horseradish peroxidase-labeled secondary anti-rabbit or anti-mouse antibodies for 15 min, and washed three times with TBST for 10 min each time. Immune complexes were detected using ECL (Amersham).

In Vitro c-Src Kinase Assay—In most experiments, an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described previously (51). Briefly, lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts from each cell lysate were immunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. The amount of anti-c-Src IgG was pre-determined to be in excess over c-Src protein, indicating that the majority of c-Src protein in cell lysates is immunoprecipitated (data not shown). One-half of each immunoprecipitate was subjected to SDS-PAGE under nonreducing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity, by incubating with 10 μl of reaction buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4), 1 μl of freshly prepared acid-denatured enolase (Sigma) (5 μg of enolase + 1 μl of 50 mM HCl incubated at 30 °C for 10 min then neutralized with 1 μl of 1 M PIPES, pH 7.0), and 10 μCi of [γ-^{32}P]ATP. After 10 min of incubation at 30 °C, reactions were terminated by the addition of 2× SDS sample buffer, and samples were subjected to 8% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature and drying for 2 h at

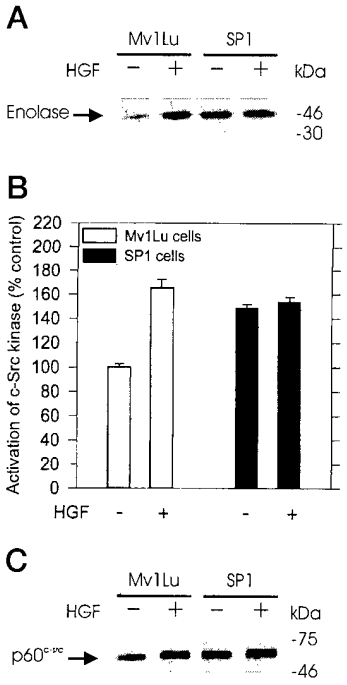

FIG. 1. **c-Src kinase activity is elevated in SP1 carcinoma cells compared with Mv1Lu epithelial cells.** Cell lysates were prepared from serum-starved Mv1Lu and SP1 cells treated without (−) or with (+) HGF (40 ng/ml) for 10 min and were immunoprecipitated with anti-c-Src IgG. Immunoprecipitates were subjected to an *in vitro* kinase assay using enolase as a substrate, and kinase activity was measured as described under "Experimental Procedures." *A*, autoradiogram showing ^{32}P-labeled enolase. *B*, quantitation of autoradiogram using PhosphorImager. Results are expressed as the percentage of cpm in untreated Mv1Lu cells (100%), normalized to the amount of c-Src protein in *C*. The means ± range of two experiments are shown. Similar results were obtained using the c-Src kinase family-specific cdc2 peptide as substrate (data not shown). *C*, Western blot analysis of immmunoprecipitates in *A*, probed with anti-c-Src IgG.

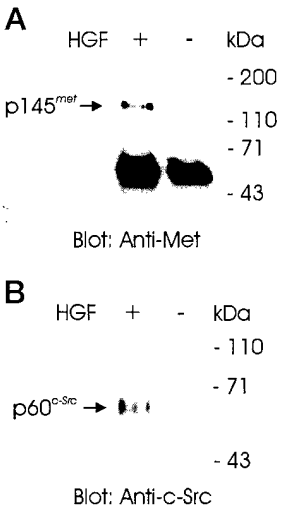

FIG. 2. **c-Src kinase binds to tyrosine-phosphorylated Met.** Cell lysates derived from serum-starved Mv1Lu cells treated without (−) or with (+) HGF (40 ng/ml) for 15 min were immunoprecipitated with anti-c-Src IgG (*A*) or anti-Met IgG (*B*). The immune complexes were separated by 8% SDS-PAGE and immunoblotted with anti-Met IgG (*A*) or anti-c-Src IgG (*B*). Protein molecular mass standards are shown on the *right*. This experiment was done twice with similar results.

80 °C under a vacuum. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In some experiments (see Fig. 3), c-Src kinase activity was assayed according to Cheng *et al.* (52) using the c-Src tyrosine kinase family-specific cdc2 peptide substrate. Anti-c-Src or anti-Met immunoprecipitates prepared as above were incubated with 40 μl of a reaction buffer (100 mM Tris-HCl, pH 7.0, 0.4 mM EGTA, 0.4 mM Na_3VO_4, 40 mM $Mg(OAc)_2$), 5 μl of cdc2 peptide (Life Technologies, Inc., 250 μM/assay), 5 μl of cold ATP (25 μM), and 2.5 μCi of [γ-^{32}P]ATP. A control consisting of immunoprecipitation with anti-Met IgG under more stringent conditions with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) where c-Src would not be co-precipitated was also carried out. After 15 min of incubation at 37 °C, reactions were terminated by the addition of 20 μl of 40% trichloroacetic acid and incubated for an additional 5 min. Aliquots subsequently were blotted on to p81 paper (Whatman, Fisher, Ottawa, ON, Canada). The p81 paper was washed three times (5 min/wash) with 0.75% phosphoric acid and once with acetone at room temperature, and the radiolabeled c-Src kinase substrate was counted in a liquid scintillation counter.

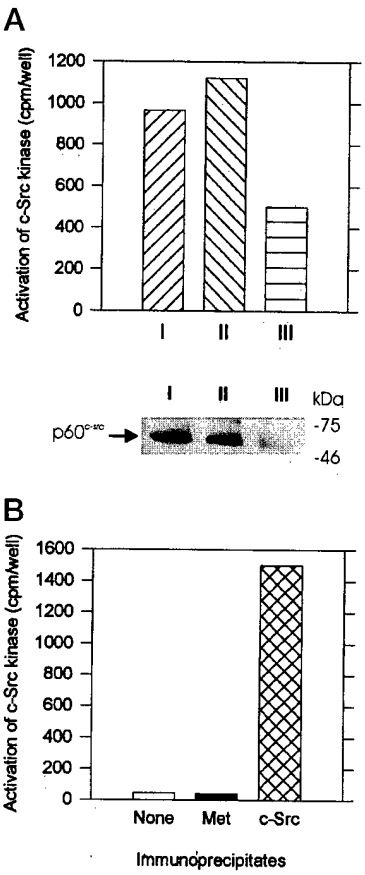

FIG. 3. **Detection of c-Src kinase activity in Met immunoprecipitates.** *A*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-c-Src antibody (*bar I*) or anti-Met antibody (*bar II*). The supernatant from immunoprecipitates of anti-Met antibody was subsequently immunoprecipitated with anti-c-Src antibody (*bar III*). *In vitro* c-Src kinase activity was determined as described under "Experimental Procedures" using the c-Src kinase family-specific cdc2 peptide substrate. The amount of radiolabeled cdc2 substrate was determined and plotted as c-Src kinase activity (cpm/well) (*top panel*). Half of each immunoprecipitate in the *top panel* was subjected to SDS-PAGE, and p60$^{c\text{-}src}$ protein in each sample was identified by immunoblotting with anti-c-Src antibody (*bottom panel*). *B*, equal amounts of cell lysates derived from serum-starved SP1 cells were immunoprecipitated with anti-Met antibody or anti-c-Src antibody under more stringent conditions with RIPA buffer to prevent co-precipitation of other proteins (see "Experimental Procedures"). The immunoprecipitates were used in an *in vitro* c-Src kinase assay with the c-Src kinase family-specific cdc2 substrate. As a control, a reaction containing no protein (*None*) was carried out concurrently. Results are plotted as c-Src kinase activity (cpm/well) as in *A*. Anti-Met immunoprecipitates under these more stringent conditions showed no significant phosphorylation of the cdc2 substrate.

In Vitro Met Kinase Assay—Cell lysates from SP1 and Mv1Lu cells were prepared, and equal protein amounts of each lysate were immunoprecipitated with anti-Met IgG as described above. Immunoprecipitates were washed twice with cold lysis buffer and once with cold kinase

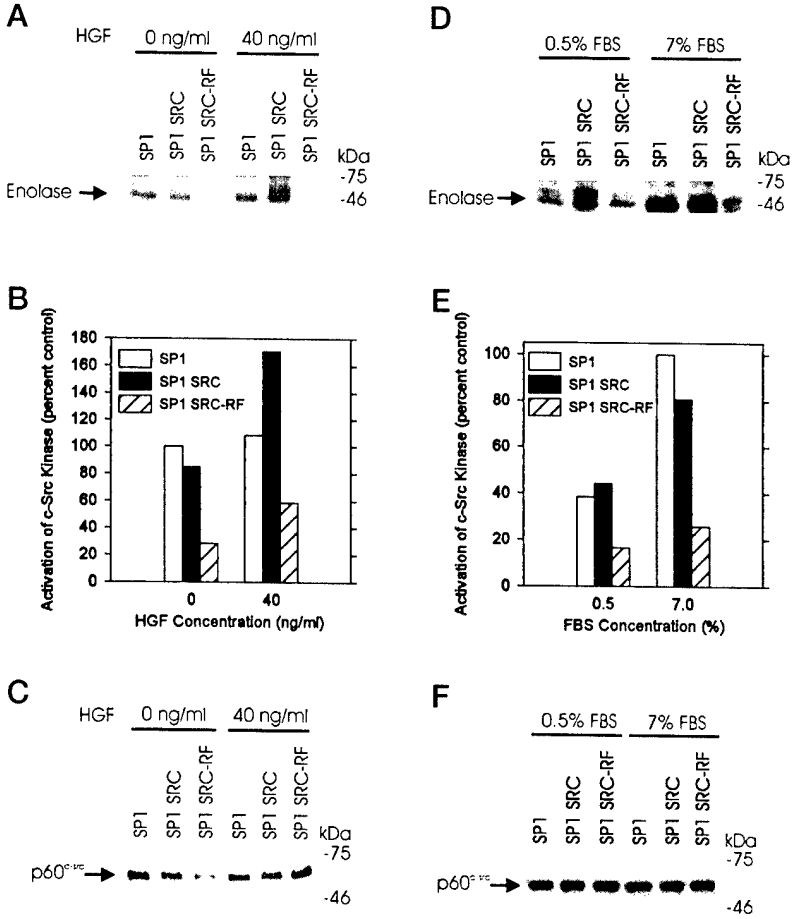

FIG. 4. **Effect of transfected dominant negative SRC-RF on Src kinase activity in SP1 cells.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were plated at 70% confluence and prestarved overnight. Cells in each group were then cultured alone, with HGF (40 ng/ml), or with 0.5 or 7% FBS, and an *in vitro* c-Src kinase assay using enolase as a substrate was performed as described under "Experimental Procedures." *A* and *D*, autoradiograms showing ^{32}P-labeled enolase. *B*, and *E*, quantitation of autoradiogram using densitometry. Results are normalized to amount of c-Src protein in *C* and *F*. *C*, and *F*, Western blot analysis of immmunoprecipitates in *A* and *D*, probed with anti-c-Src IgG. This result is representative of five experiments.

buffer (20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 10 μM Na_3VO_4). *In vitro* Met kinase activity was determined by incubating immunoprecipitates with 20 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP at 30 °C for 10 min. The reaction was stopped by addition of 2× SDS sample buffer containing 5% β-mercaptoethanol. Samples were boiled for 3 min and subjected to 7% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing and drying as described above. Autoradiograms were produced and quantitated using a Storm PhosphorImager (Molecular Dynamics).

RESULTS

Detection of Elevated c-Src Tyrosine Kinase Activity in SP1 Carcinoma Cells—SP1 carcinoma cells express HGF and tyrosine-phosphorylated Met, consistent with an HGF autocrine loop in these cells (44). To test the possibility that activation of c-Src kinase may be involved in Met-induced signaling pathways, we measured the kinase activity of c-Src in SP1 carcinoma cells and an HGF-sensitive epithelial cell line, Mv1Lu. c-Src kinase activity was measured by the capacity of c-Src immunoprecipitates from these cells to tyrosine phosphorylate the substrate, enolase. c-Src immunoprecipitates from serum-starved SP1 cells showed a pronounced elevated kinase activity, which increased only slightly following treatment with exogenous HGF (Fig. 1). In contrast, c-Src kinase activity in Mv1Lu cells was highly dependent on stimulation of cells with exogenous HGF (Fig. 1). The levels of c-Src kinase activity observed correlated with the constitutive tyrosine phosphorylation of Met (44) and *in vitro* Met kinase activity (data not shown) in SP1 cells, and the HGF-induced tyrosine phosphorylation of Met in Mv1Lu cells (Ref. 26 and data not shown).

Association of c-Src Kinase Protein and Activity with Activated Met—It is conceivable that the high level of c-Src kinase activity in SP1 cells, could have resulted from interaction of c-Src with activated Met due to an autocrine HGF loop in these cells (44). To test for interaction of c-Src kinase family proteins with activated *versus* nonactivated Met, we first examined the association of c-Src with Met in Mv1Lu cells that express Met but not HGF. Serum-starved Mv1Lu cells were incubated alone or with HGF, and cell lysates were immunoprecipitated with anti-Met IgG or anti-c-Src IgG. Protein precipitates were electrophoresed and subjected to Western blotting with anti-c-Src IgG or anti-Met IgG, respectively. As shown in Fig. 2 (*A* and *B*), an increased amount of c-Src protein was recovered from anti-Met immunoprecipitates and *vice versa* in cell lysates from HGF-treated Mv1Lu cells compared with untreated Mv1Lu cells. We also showed that association of c-Src kinase with Met occurred via the SH2 domain of c-Src and correlated with tyrosine phosphorylation of Met (data not shown). It should be noted that a trace amount of c-Src protein was detected in lysates of unstimulated cells immunoprecipitated with anti-Met IgG and blotted with anti-c-Src IgG, possibly due to incomplete starvation of these cells before HGF stimulation (Fig. 2*B*). Thus, stimulation with HGF causes increased association of c-Src protein with Met.

To determine whether elevated activity of c-Src kinase in SP1 cells correlates with its ability to associate with Met, serum-starved SP1 cells were immunoprecipitated with anti-Met IgG or anti-c-Src IgG, and immunoprecipitates were tested for the ability to tyrosine phosphorylate the c-Src kinase family-specific cdc2 peptide substrate (52). As shown in Fig. 3*A* (*bars I* and *II*), similar amounts of c-Src kinase protein and activity were recovered from immunoprecipitates of both anti-Met and anti-c-Src antibodies. In contrast, immunoprecipitates from anti-Met IgG under more stringent conditions with RIPA buffer where c-Src is not co-precipitated resulted in no signifi-

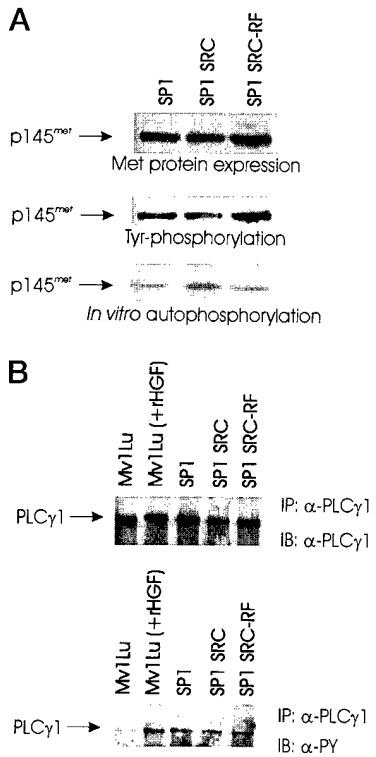

FIG. 5. **Expression of dominant negative mutant SRC-RF does not alter Met protein levels or activity and downstream signaling.** *A*, SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight and lysed as described in the legend to Fig. 1. Equal amounts of protein from each lysate were concentrated on Microcon 10 filters (Amicon Inc., Beverly, MA) and analyzed by Western blotting with anti-Met IgG (*top panel*). The blot was stripped and reprobed with anti-phosphotyrosine antibody (*middle panel*). Cell lysates were also immunoprecipitated with anti-Met IgG, and immunoprecipitates were subjected to an *in vitro* Met kinase assay as described under "Experimental Procedures." The autoradiogram depicting ^{32}P-labeling of Met is shown (*bottom panel*). Relative band intensities and amount of ^{32}P labeling was determined using a Storm PhosphorImager. The relative amount of Met tyrosine phosphorylation (1.0, 1.0, or 1.0) or of *in vitro* Met autophosphorylation (1.0, 1.1, or 1.0) was not significantly different among the three cell lines. *B*, serum-starved SP1 cells transfected with SRC-RF or SRC and untreated SP1 cells were lysed as described in the legend to Fig. 1. Prestarved Mv1Lu cells untreated or treated with HGF (40 ng/ml) for 10 min were used as negative and positive controls, respectively. Equal amounts of protein from each lysate were immunoprecipitated with anti-PCL-γ1 IgG. Immunoprecipitates were subjected to 7% SDS-PAGE and transferred to nitrocellulose. The blot was probed with anti-PCL-γ1 IgG (*top panel*) before being stripped and reprobed with anti-phosphotyrosine antibody (*bottom panel*). This experiment was done twice with similar results. *IP*, immunoprecipitation; *IB*, immunoblot.

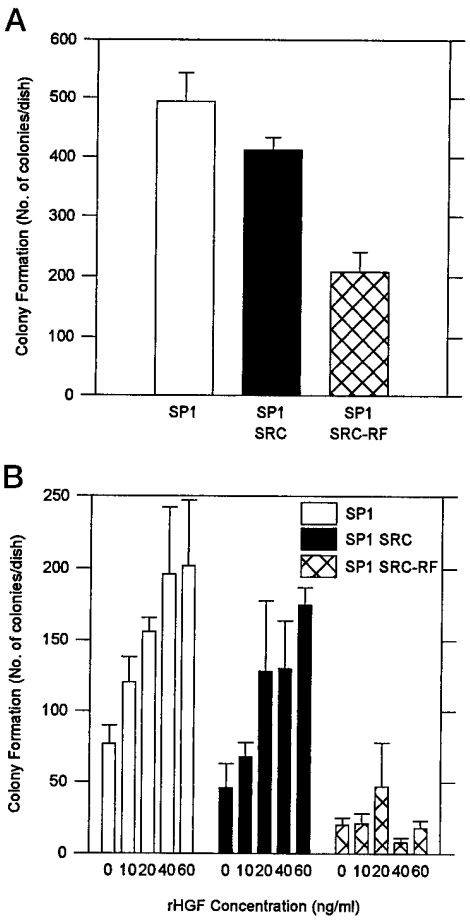

FIG. 6. **Effect of transfected dominant negative SRC-RF on growth of SP1 cells in agar.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were cultured (10^3 cells/dish) in 60-mm tissue culture plates in soft agar (0.36%) with RPMI 1640 medium supplemented with 7% FBS (*A*) or 1% FBS plus HGF at the concentrations indicated (*B*) as described previously (49). After 8 days, colonies were stained with Giemsa and counted visually. Results are expressed as the mean colony numbers ± S.D. of quadruplicate cultures. This experiment was done three times with similar results.

cant phosphorylation of cdc2 peptide (Fig. 3*B*), confirming the specificity of the cdc2 peptide as a substrate for c-Src (52). Thus a significant portion of c-Src kinase activity is associated with activated Met in SP1 cells. To further evaluate the contribution of c-Src association with Met, the supernatant from the immunoprecipitate of anti-Met IgG was immunoprecipitated for a second time with anti-c-Src IgG and subjected to the *in vitro* c-Src kinase assay. As shown in Fig. 3*A* (*bar III*), some c-Src kinase activity was detected in the Met-depleted SP1 cell lysate; however, it was with much lower activity, corresponding to the reduced amount of c-Src protein present. Immunoprecipitation of SP1 cell lysates with higher concentrations of anti-Met IgG and subsequently with anti-c-Src IgG showed a similar result (data not shown). These results demonstrate that the majority of c-Src kinase activity correlates with its ability to associate with Met in SP1 cells.

c-Src Kinase Activity Is Required for Colony Growth in Agar, but Not Cell Proliferation on Plastic—SP1 cells exhibit paracrine stimulation by HGF of colony growth in agar and proliferation on plastic (45, 49). To determine whether c-Src kinase activity is required for HGF-induced proliferation or colony growth in agar, an expression vector (SRC-RF) containing cDNA encoding a dominant negative double mutant of *c-src* with loss-of-function mutations in the kinase domain (K295R) and a regulatory tyrosine residue (Y527F) (47) was stably transfected into SP1 cells. A control consisted of cells transfected with the same vector expressing wild type *c-src* (SRC). Uncloned (pooled) transfected cells were selected in G418-containing medium and assessed for c-Src kinase activity and HGF-induced functions. For *in vitro* c-Src kinase assays, immunoprecipitation with anti-c-Src IgG was carried out at antibody excess, indicating that the majority of wild type c-Src protein was present in immunoprecipitates. The results showed that c-Src kinase activity was strongly reduced in SRC-RF transfected SP1 cells compared with SRC transfected or untransfected cells incubated alone, or following stimulation with 40 ng/ml HGF or 7% FBS (Fig. 4). However, tyrosine phosphorylation of Met or an unrelated signaling molecule PLC-γ1 and *in vitro* autophosphorylation of Met remained unaffected in SRC-RF- or SRC-transfected SP1 cells, compared with untransfected cells (Fig. 5). These results demonstrate

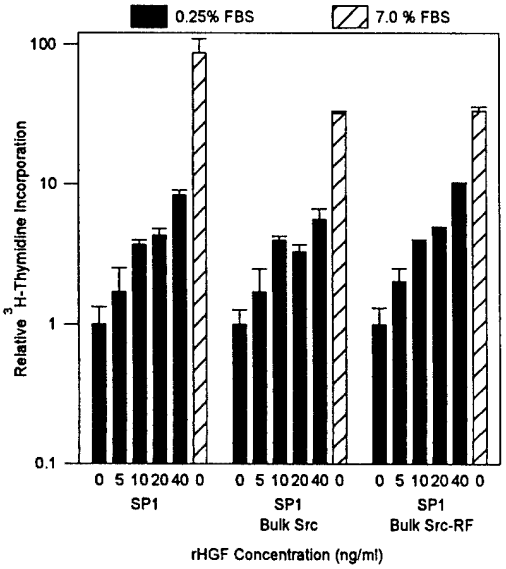

FIG. 7. **Effect of transfected dominant negative mutant SRC-RF on HGF-induced proliferation of SP1 cells on plastic.** Pooled SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were prestarved overnight, and each cell line was plated at 10^4 cells/well in 24-well plates in 0.25% FBS without or with HGF at the concentrations indicated. Controls consisted of cultures with 7% FBS. DNA synthesis was measured as described under "Experimental Procedures." Results are expressed as relative mean [^{3}H]thymidine incorporation compared with control (no HGF) (mean cpm/well ± S.D. of triplicates). This result is representative of four experiments.

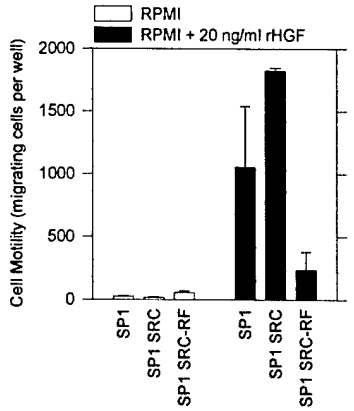

FIG. 8. **Effect of transfected dominant negative SRC-RF on HGF-induced cell motility.** SP1 cells transfected with SRC-RF or SRC or untreated SP1 cells were serum-starved overnight, and each cell line (2×10^4 cells) was plated into Transwell inserts (8-μm pore size) in 24-well plates in 0.5 mg/ml bovine serum albumin in RPMI without (*open bars*) or with (*closed bars*) HGF (20 ng/ml) as described under "Experimental Procedures." After 6–8 h of incubation at 37 °C, cells were fixed in 1% paraformaldehyde and stained with hematoxylin. Cells on the upper side of the membrane were removed by wiping with cotton. Cells on the underside were counted using an inverted microscope with phase contrast illumination. The results are expressed as the relative number of migrating cells/well (means ± range of two wells/point). This experiment was done twice with similar results. Similar results were obtained using a wound healing assay (data not shown).

specificity of the inhibitory effect of SRC-RF on c-Src kinase activity.

Expression of the dominant negative SRC-RF mutant in SP1 cells significantly inhibited FBS- and HGF-induced colony formation in soft agar, compared with SRC-transfected or untransfected SP1 cells (Fig. 6). Similarly, a subclone of SP1 cells expressing SRC-RF showed a marked reduction in colony formation, compared with a wild type SRC-transfected subclone or untransfected SP1 cells (data not shown). In contrast, SRC-RF-transfected SP1 cells showed no difference in HGF-induced or serum-induced proliferation on plastic, compared with SRC-transfected or untransfected SP1 cells (Fig. 7). Thus reduction of c-Src kinase activity in SRC-RF-transfected cells abrogated HGF- or serum-induced colony growth in soft agar but had no effect on cell proliferation on plastic.

c-Src Kinase Activity Is Required for HGF-induced Cell Motility—Because c-Src kinase activity has been shown to modulate cell motility in several cell types (36–38), we examined the role of c-Src kinase in HGF-induced cell motility in SP1 cells. Our results showed that HGF strongly stimulated motility of SP1 cells through collagen-coated porous membranes in a paracrine manner. HGF-induced motility was significantly reduced in SP1 cells transfected with dominant negative mutant SRC-RF, compared with SRC-transfected or untransfected cells (Figs. 8 and 9). Similar results were obtained using a wounding assay (data not shown). These results are consistent with a role of c-Src kinase in HGF-induced cell motility.

DISCUSSION

We (6) and others (7, 8) have previously shown that HGF and Met mRNA are strongly co-expressed in invasive carcinomas in human breast cancer. These findings suggest that signals transduced by activated Met confer survival and growth advantage to carcinoma cells during progression to metastasis. This concept is further supported by the observation that cells transfected with an activated version of *met* (*tpr-met*) acquire invasive and metastatic properties (15, 16). Unlike most other receptor tyrosine kinases, Met shows one high affinity binding site for the majority of SH2-containing cytoplasmic effectors, suggesting that these proteins bind Met in a competitive manner (23–25). Therefore, to study the role of specific SH2-containing cytoplasmic effectors in HGF receptor function, approaches to target individual cytoplasmic effectors are required.

To analyze downstream effector molecules in HGF-induced tumorigenic properties of mammary carcinoma cells, we have studied a mouse mammary carcinoma, SP1, which co-expresses HGF and Met (44). However, depending on culture conditions, both paracrine and autocrine effects of HGF have been observed in SP1 cells (44, 45, 49). In monolayer cultures, autocrine phosphorylation of Met at tyrosine in SP1 cells without addition of exogenous HGF was observed (44). In contrast, tyrosine phosphorylation of Met was reduced in suspended SP1 cells and can be restored by addition of exogenous HGF.[2] These observations suggest that the base level of Met activation may be influenced by extracellular environmental conditions, such as cell adhesion to various substrata (53), cell density effects on HGF expression and secretion (54), or proteolytic processing of pro-HGF to the biologically active form (55). In the present report, paracrine stimulation with exogenous HGF was required for optimal cell proliferation, motility, and colony growth in agar under serum-starved conditions. Previous studies showed that PI 3-kinase activity is elevated in SP1 cells and that its activity is required for HGF-induced proliferation in monolayer culture. Treatment of SP1 cells with wortmannin, a potent inhibitor of PI 3-kinase (56), or transfection of a dominant negative mutant of the p85 subunit of PI 3-kinase into these cells (26) inhibited HGF-induced cell proliferation in monolayer culture.

In the present report, we show that c-Src kinase activity is elevated in SP1 mammary carcinoma cells compared with nonmalignant Mv1Lu epithelial cells and is associated with Met. The elevated level of c-Src kinase activity in SP1 cells and its association with Met strongly suggest that this signaling mol-

[2] R. Saulnier and H. Qiao, unpublished observation.

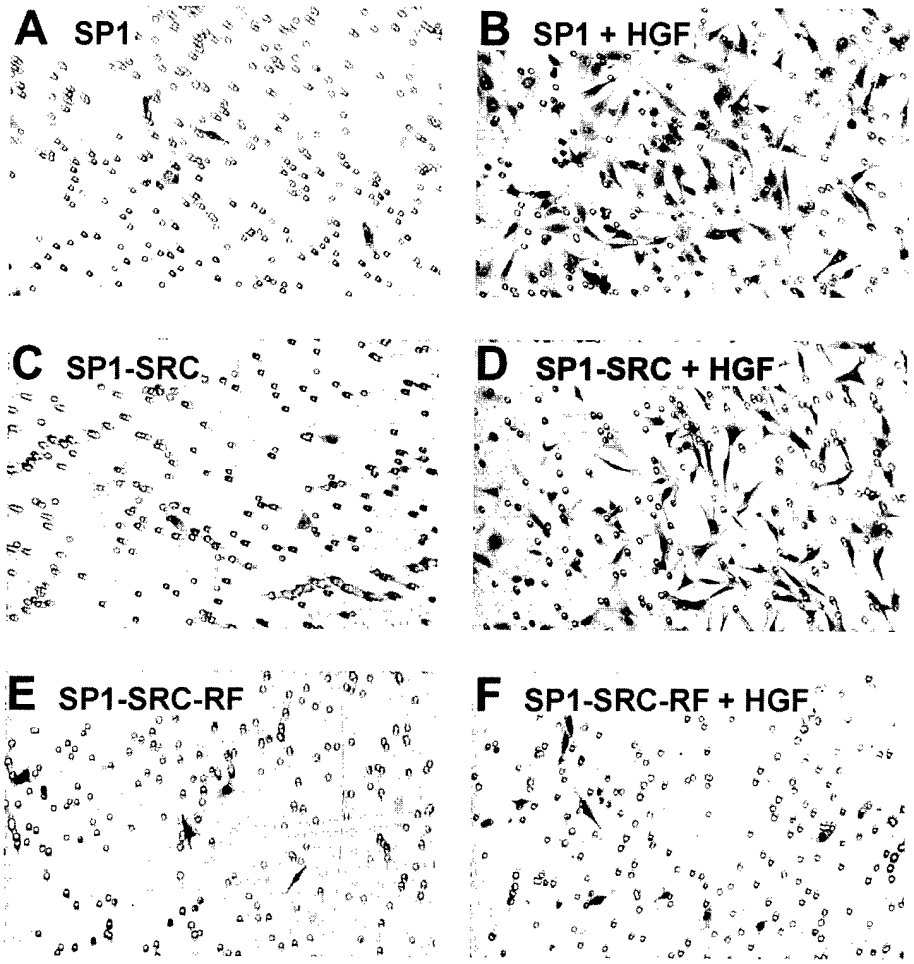

FIG. 9. **Photomicrographs of migrating SP1 cells transfected with SRC or SRC-RF or untransfected SP1 cells following HGF stimulation.** SP1 cells untransfected (*A* and *B*) and transfected with SRC (*C* and *D*) or SRC-RF (*E* and *F*) were serum-starved overnight and assessed for cell motility without (−) or with (+) HGF (20 ng/ml) as described in the legend to Fig. 8. After removing nonmigrating cells on the upper side of the membrane, membranes were mounted onto glass slides, and migrating cells were photographed using a Leitz microscope with phase contrast illumination. Photographs correspond to the groups shown in Fig. 8. Original magnification, 250×.

ecule may be involved in intracellular events triggered by HGF. This observation prompted us to test whether expression of a dominant negative mutant form of c-Src influences growth of SP1 cells. Expression of a dominant negative form of c-Src (SRC-RF) in SP1 cells showed no significant effect on HGF-induced cell proliferation on plastic but markedly inhibited HGF- or serum-induced colony growth in soft agar. Thus activation of c-Src kinase is essential for colony formation in agar by SP1 cells but appears not to be required for cell proliferation on plastic.

Other laboratories have reported variable effects of c-Src kinase on cell growth. In support of our observations, Demali and Kazlauskas (57) have shown that a mutant form of PDGF β-receptor that cannot bind or activate, c-Src, retains the ability to stimulate growth of fibroblasts on plastic or in agar in response to PDGF. In contrast, Courtneidge and co-workers (33, 58) have shown that microinjection of a kinase dead mutant c-Src or neutralizing antibodies that inhibit basal and stimulated c-Src kinase activity inhibited PDGF-dependent DNA synthesis in fibroblasts. Similarly, constitutive expression of c-Src mutants inhibited PDGF and epidermal growth factor-induced mitogenesis of mouse embryonal fibroblasts lacking c-Src (34). The apparent differences in the role of c-Src kinase in the above systems could be due to different levels of residual basal activity of c-Src kinase or the developmental and malignant status of the cells used. Our observation that anchorage-independent growth but not proliferation on plastic is inhibited in cells expressing dominant negative SRC-RF suggests that the reduced level of c-Src kinase activity in SRC-RF expressing SP1 cells is insufficient to support anchorage-independent growth, whereas proliferation on plastic remains unaffected. c-Src-independent signaling mechanisms may also promote HGF-induced proliferation of SP1 cells on plastic.

We have also shown that SP1 cells transfected with the dominant negative SRC-RF mutant showed reduced cell motility in response to HGF compared with SRC-transfected or untransfected SP1 cells. Thus c-Src kinase activity is required for HGF-induced cell motility in SP1 carcinoma cells, although complementary signaling molecules may also be involved. This observation reflects recent reports that c-Src kinase activity is required for epithelial cell scattering (38–40, 50) and organization of the cortical cytoskeleton (50). In addition, Richardson *et al.* (59) have shown that co-expression of c-Src in cells expressing the dominant negative C-terminal domain of focal adhesion kinase can reconstitute cell spreading and motility and induces tyrosine phosphorylation of paxillin. Together, these observations raise the possibility that HGF-induced c-Src kinase activity may regulate cell motility through the cytoskeletal complex. This possibility is currently being investigated.

There is now growing evidence that the c-Src family protein-tyrosine kinases are involved in signal transduction pathways that result in cell growth, adhesion, and differentiation. c-Src kinase activity is required for cell proliferation induced by platelet-derived growth factor, colony stimulating factor-1, and epidermal growth factor (51, 60), and increased c-Src kinase activity is associated with many cancers. These observations support the notion that increased c-Src kinase activity in mammary carcinomas may play an important role in mammary tumor growth and development. Our findings involving transfection of a dominant negative c-Src kinase-defective mutant into SP1 cells represent the first direct demonstration of a requirement for c-Src kinase activity in HGF-induced cell mo-

tility and anchorage-independent growth of carcinoma cells, although interactions with other signaling molecules may also be involved. These data strongly suggest that HGF-induced association of c-Src kinase with Met and its activation are important in growth and transformation of mammary carcinomas and further argue that paracrine and autocrine HGF loops play a significant role in the transformed phenotype of some mammary carcinomas.

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The role of hepatocyte growth factor/scatter factor in epithelial-mesenchymal transition and breast cancer

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The role of hepatocyte growth factor/scatter factor in epithelial-mesenchymal transition and breast cancer

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Abstract:

North American women have a 1 in 8 life time risk of developing breast cancer, and approximately 1 in 3 women with breast cancer will die of metastases. We, and others, have recently shown that high levels of expression of hepatocyte growth factor (HGF), and its receptor Met, are associated with invasive human breast cancer, and may be causally linked to metastasis. This high level of HGF and Met expression has been considered as a possible indicator of earlier recurrence and shortened survival in breast cancer patients. In contrast, HGF expression (but not Met) is strongly suppressed in normal breast epithelial cells. HGF and Met are therefore candidate targets for therapeutic intervention in the treatment of breast cancer. We have recently demonstrated that sustained or hyper-activation of c-Src and Stat3, which occurs in invasive breast cancer, can stimulate strong expression of HGF in carcinoma cells. In contrast, transient induction of Stat3 occurs in normal epithelium, and promotes mammary tubulogenesis. We hypothesize that increased autocrine HGF/Met signalling is a critical downstream function of c-Src/Stat3 activation in mammary tumorigenesis. Future studies will identify novel Stat3 consensus sites that regulate HGF promoter activity and HGF expression preferentially in carcinoma cells, and could lead to novel therapeutic drugs that specifically block HGF expression in mammary carcinoma cells, and which could be used in combined treatments to abrogate metastasis.

Keywords: HGF, Src/Stat3 signalling, epithelial-mesenchymal transition, breast cancer

Introduction to mammary gland development: In normal mammary gland development, branching tubulogenesis develops from a single mammary bud which undergoes elongation and side-branching at puberty to infiltrate the entire mammary fat pad (Medina and Daniel, 1996; Richert et al. 2000; Ronnov-Jessen et al. 1996). Stromal interactions are critical in mammary tubulogenesis (Woodward et al. 1998). The estrogen signal, a key initiator of this process, is thought to be delivered at least in part via the mammary stroma (Cunha and Hom, 1996), which secretes growth factors, e.g. hepatocyte growth factor (HGF) (Soriano et al. 1998; Rahimi et al. 1994), also known as scatter factor, and keratinocyte-derived growth factor (Bansal et al. 1997), that act in a paracrine manner on adjacent basal (progenitor) epithelial cells (Smith and Chepko, 2001). The basal cells at the stromal-epithelial interface secrete transforming growth factor-α (TGF-α) (Snedeker et al. 1991), which promotes autocrine activation of EGF receptor and basal cell proliferation. Metalloproteinases, e.g., MMP3 (Rudolph-Owen and Matrisian, 1998), are also required as the dividing basal cells migrate through the interstitial tissue of the fat pad. As the basal cells divide they move away from the stromal interface, and align to form a polarized luminal epithelium (keratins 8/18 positive), with clearly defined cadherin-based adherens junctions and desmosome-based tight junctions. Myoepithelial cells (keratins 5/14 positive), which are thought to be derived from basal cells (Petersen et al. 2001) and display smooth muscle-like characteristics, form an outer layer surrounding the luminal cells. TGF-β, produced in epithelial cells and adjacent stroma, promotes a differentiated epithelial phenotype and stimulates deposition of extracellular matrix (ECM) proteins and formation of a basement membrane sheath around the entire duct (Daniel et al. 1996). The regulation of mammary gland development is a complex cascade of intercellular interactions beyond the scope of this review, and has been extensively reviewed elsewhere (Robinson et al. 1999; Werb et al. 1996; Dunbar and Wysolmerski, 2001).

Epithelial-mesenchymal transition in breast cancer: North American women have a 1 in 8 risk of developing breast cancer in their lifetime (Lippman et al. 2001). Development of a malignant phenotype is a multi-step process, characterized by the loss of epithelial polarity, dispersion of cell-cell junctions, degradation of basement membrane, and increased cell migration and invasion (Boyer et al. 1996; Birchmeier et al. 1996). This process, referred to as epithelial-mesenchymal transition (EMT), is indicative of invasion by breast carcinoma cells, earlier recurrence, and shortened patient survival. EMT is caused by multiple genetic changes including activating mutations in receptor tyrosine kinases (RTKs, e.g. Her2/Neu (Chan et al. 1999)) and loss-of-function of adhesion molecules such as E-cadherin, β-catenin (Birchmeier et al. 1995), and adenomatous polyposis coli (APC) protein (Barth et al. 1997).

HGF and Met regulate both morphogenic and tumorigenic phenotypes: HGF is a potent inducer of EMT in many epithelial systems (Birchmeier et al. 1996), although other growth factors (e.g. FGF and EGF) may also be involved (Boyer et al. 1996). HGF is a multi-functional cytokine which stimulates morphogenesis, cell survival (Bowers et al. 2000; Qiao et al. 2000), mitogenesis, motility, invasion, and metastasis (Lamszus et al. 1997). In normal mammary development, HGF is produced by mesenchymal cells and, along with other growth factors (e.g., neuregulin (Yang et al. 1995)) and the stromal protein epimorphin (Hirai et al. 1998), stimulates tubulogenesis in a tightly controlled paracrine manner (Brinkmann et al. 1995). However, over-expression of HGF and Met occurs in many types of invasive cancers, including breast carcinomas (reviewed in Tuck et al. 1996). Furthermore, diverse tumorigenesis in a broad range of tissues occurs in transgenic mice over-expressing HGF or constitutively activated tpr-Met (Otsuka et al. 2000; Takayama et al. 1997; Liang et al. 1996). Ectopic over-expression of HGF and Met also causes transformation in a variety of epithelial cell lines (Fixman et al. 1995; Bellusci et al. 1994). Thus, a shift from transient activation of Met, to sustained high levels of Met

activation (Marshall, 1995), and co-operativity with other receptor tyrosine kinases (RTKs), e.g., Her2/Neu (Andrechek and Muller, 2000), can cause a switch in the HGF response from morphogenesis to tumorigenesis.

Recent studies have identified high levels of HGF and Met expression in breast carcinoma cells as a possible independent predictor of recurrence and shortened survival in breast cancer patients (Yamashita et al. 1994; Jin et al. 1997; Ghoussoub et al. 1998; Toi et al. 1998). Sustained activation of HGF/Met signalling is associated with dissociation of cadherin-based adherens junctions, followed by loss of cadherin expression (Zschiesche et al. 1997). The modulation of adherens junctions by HGF/Met involves phosphorylation of β-catenin, leading to its reduced affinity for the E-cadherin complex and subsequent degradation (Hiscox and Jiang, 1999; Huber et al. 1996). In normal breast epithelium and ductal carcinoma *in situ* (DCIS), strong expression of E-cadherin with accentuation at cell-cell contacts is evident (Fig. 1 B,D). In contrast, decreased expression of E-cadherin frequently occurs in invasive ductal carcinoma (IDC), while Met expression is relatively consistent throughout, and most intense in IDC (Figure 1 A-F). Although HGF and Met mRNA are differentially expressed in normal stromal and epithelial cells, respectively (Brinkmann et al, 1995), it was not clear what cell types produce HGF and Met in breast carcinomas. Using *in situ* hybridization (ISH) we have shown that invasive human breast carcinoma cells frequently show strong HGF mRNA expression particularly at the migrating tumor front, compared to weak expression in more central regions of invasive tumor (Figure 2A,B). Met mRNA showed a similar gradient of strong expression at the stromal interface, to weak expression in the central regions of the tumor (Tuck et al. 1996). In contrast, HGF mRNA is weakly expressed in normal epithelium (Fig. 2C), although some epithelial expression of HGF mRNA in certain instances (such as hyperplasia) was evident (Tuck et al. 1996; Wang et al. 1994). Jin et al. (1997) have reported a similar gradient of HGF and Met expression from normal

breast/benign hyperplasias (lowest), to DCIS (higher), to invasive carcinoma (highest). Together, these findings raise the possibility that high level of HGF expression and sustained activation of Met (referred to as an HGF/Met autocrine loop) in breast carcinoma cells can promote increased EMT, invasion, and metastasis. Support for this contention was provided by Vande Woude (Cao et al. 2001), who showed that an anti-HGF neutralizing antibody combination can inhibit growth of human glioblastoma xenografts dependent on an HGF/Met autocrine loop. Further studies are needed to test the effect of HGF neutralizing antibodies on growth and metastasis of breast carcinoma cells.

c-Src kinase is a key regulator of EMT in breast carcinoma cells: The non-receptor tyrosine kinase, c-Src, is expressed in many cell types, and is required for normal mammary ductal development (W.J. Muller et al. person. comm.). Hyper-activation of c-Src occurs in many human cancers (Ottenhoff-Kalff et al. 1992), due to a variety of mechanisms including increased RTK (e.g. Her2/Neu (Muthuswamy and Muller, 1995)) or integrin signalling (Bjorge et al. 2000), dephosphorylation of a negative regulatory tyrosineY529 (Bjorge et al. 2000), or mutation (Irby et al. 1999). c-Src plays a critical role in breast cancer; however activation of c-Src alone is not sufficient for mammary tumorigenesis in transgenic mice (Guy et al. 1994), indicating that c-Src must interact with other signalling pathways. c-Src is recruited to focal adhesions and cell-cell contacts (McLean et al. 2000), and is known to modulate cell proliferation, spreading, and migration (reviewed in Rahimi et al. 1998). Activation of c-Src causes increased turnover of focal adhesions on ECM substratum, and is required for scattering of carcinoma cells (Figure 3, and Owens et al. (2000)). Activated c-Src can also induce expression of many genes, including cellular growth factors such as vascular endothelial growth factor (Mukhopadhyay et al. 1995), WAF1/CIP1 and cyclin D1 (Sinibaldi et al. 2000).

c-Src kinase is required for HGF expression in breast carcinoma cells: Since c-Src is

activated in most human and mouse breast carcinomas, we examined the role of c-Src in HGF expression in a mouse breast carcinoma cell line SP1, which co-expresses HGF and activated Met. We showed that HGF mRNA and protein levels increased in tumor cells expressing a constitutively activated c-Src mutant, while a dominant negative (DN) c-Src mutant or an inhibitor of c-Src family kinases (PP2) (Hung and Elliott, 2001), had the opposite effect (Figure 4A). These data suggest that HGF expression (both mRNA and protein levels) is regulated by c-Src kinase activity. To determine the effect of c-Src kinase mutants on *HGF* promoter activity, we constructed a reporter plasmid (HGF-luc) with the luciferase gene linked to the 2.7 kb region 5' of the *HGF* transcriptional start site (Figure 4B). The 2.7 kb 5' upstream segment of the promoter has been shown previously to be sufficient to support *HGF* transcriptional activity in transgenic mice (Bell et al. 1998). Co-transfection of the HGF-luc construct with an activated c-Src mutant increased *HGF* promoter activity, whereas a DN c-Src mutant had the opposite effect. Thus *HGF* transcription is strongly responsive to c-Src. Using a deletion analysis of the *HGF* promoter, we identified a novel region between -254 and -70 bp which is required for c-Src responsiveness of *HGF* promoter activity in carcinoma cells (Figures 5,6). Deletion of this region had no effect on basal activity of the full-length HGF promoter, suggesting that the c-Src responsive effect is distinct from other regulatory factors.

Organization of the *HGF* promoter: Previous studies in fibroblast cells have demonstrated several transcription factors which regulate *HGF* promoter function (Figure 6) (reviewed in Liu et al. (1994b)). These include C/EBP (binding to a site at -9 to -4 bp) (Jiang and Zarnegar, 1997) which mediates inducibility by various cytokines, Sp1/Sp3 (binding at -318 to -308 bp) (Jiang et al. 1997b) which is required for maintenance of basal transcription, and Il-6-RE binding protein (BP) (binding at -211 to -206 bp and -188 to -182 bp). In addition, estrogen receptor (ER) (binding at -872 to -860 bp) mediates estrogen-inducibility of the promoter (Jiang

et al. 1997a). The nuclear orphan receptor, chicken ovalbumin upstream promoter-transcription factor (COUP-TF), also binds with high avidity at this site; however ER can compete with COUP-TF binding and thereby reverse the suppressive effect of COUP-TF on *HGF* transcription. Other repressor factors include AP2 (binding at -230 bp to -260 bp) (Jiang et al. 2000) and TGF-β inhibitory (TIE) BP (binding at -364 to -355 bp) (Liu et al. 1994b). Members of the upstream stimulator factor (USF) and nuclear factor-1 (NF-1) transcription factors also bind strongly to the AP2 site (Jiang et al. 2000); NF-1 family factors suppress, while USF factors activate *HGF* promoter function. A putative AP1 consensus site is located 13 bp downstream of the TIE BP site, and binding of the respective factors to these adjacent sites could further modulate *HGF* transcription (Liu et al. 1994b). Thus, multiple transcription factors link *HGF* promoter activity with many growth factors, cytokines and steroid hormones. A negative regulatory site (at -16 to +4 bp) is required for suppression of *HGF* transcription in normal epithelial cells, and suppresses induction of *HGF* transcription by most paracrine activators (Liu et al. 1994a). The exact mechanism of this strong suppressive effect on *HGF* transcription is not known, however it likely plays a key role in maintaining the differentiated state of mammary epithelial cells.

Interestingly, the c-Src responsive region of the *HGF* promoter contains two putative consensus sequences for the signal transducer and activator of transcription-3 factor, Stat3 (at -149 kb and -110 kb), as well as one putative Stat5 consensus site (at -96 to -87 kb) (Figure 6) (Hung and Elliott, 2001). This region is distinct from the negative regulatory site involved in cell-type specific transcriptional repression of *HGF* promoter activity in epithelial cells, or sites involved in cytokine-, and estrogen-, responsive promoter activity.

Role of Stats in normal and malignant breast development: The Stat family proteins, originally identified as transcription factors mediating most cytokine signalling through Jaks, have recently been shown to be downstream of a wide variety of growth factor RTKs as well as

some non-receptor tyrosine kinases (e.g. v-Src and Abl) (Bowman et al. 2000; Garcia and Jove, 1998). Stat5a knockout (KO) mice are phenotypically normal, however the mammary glands in these mice fail to develop during pregnancy or to lactate (Liu et al. 1997). In contrast, Stat3 KO mice die *in utero* (Takeda et al. 1997), whereas conditional KO of Stat3 in the mammary gland inhibits epithelial apoptosis and delays involution (Chapman et al. 2000; Chapman et al. 1999). The reciprocal effect of Stat5 and Stat3 on lactation and involution suggests complex roles of these Stats in the regulation of apoptosis and development in the mammary gland.

Previous reports have shown sustained high level of Stat3 activity in many human cancers including breast cancer (Bowman et al. 2000); in contrast normal cells exhibit rapid transient induction of Stat3 activity. In addition, inhibition of Stat3 activity in human tumor cells results in cell death and/or growth arrest preferentially in malignant cells (Catlett-Falcone et al. 1999; Niu et al. 2001b). Evidence indicates that activation of Stat3 stimulates production of soluble factors that suppress pro-apoptotic molecules (e.g. TRAIL (Niu et al. 1999a)) and induce anti-apoptotic molecules (eg Bcl-XL (Grandis et al. 2000)) in some tissues including lymphoid and myeloid cells, and many cancers including breast carcinomas and myelomas. The mechanism that shifts the Stat3 phenotype from pro-apoptotic to apoptotic in the mammary gland is not known.

Co-operative effect of c-Src and Stat3 in stimulating *HGF* transcription and EMT:

Since Stat3 activation by c-Src induces specific gene expression and is required for cell transformation, we examined whether c-Src interacts with Stat3 in the regulation of *HGF* transcription. A strong co-operative effect of c-Src and Stat3 in the activation of *HGF* transcription was observed in both breast carcinoma (SP1) and epithelial (HC11) cells (Figure 7). The c-Src/Stat3 co-operativity in the induction of *HGF* transcription required the c-Src responsive region of the *HGF* promoter (Hung and Elliott, 2001). We also showed that c-Src kinase activity increased tryrosine 705 phosphorylation and DNA binding affinity of Stat3 (but

not Stat1, 5A, or 5B) to the putative consensus sites (at -110 and -149 bp). In addition, co-expression of activated c-Src and Stat3 in mammary epithelial cells showed marked cell scattering and loss of cell-cell contacts, compared to cells expressing activated c-Src, or Stat3, or vector alone (B. Elliott et al. manuscript in preparation). Our data indicate that hyper-activation of a novel c-Src/Stat3 pathway induces *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong repression of *HGF* transcription in normal epithelium, thereby promoting HGF protein expression, autocrine activation of Met, and mammary tumorigenesis.

HGF/Met autocrine loops as a potential target for the treatment of breast cancer metastasis: As discussed in this review, increased expression of HGF, and sustained autocrine activation of Met, are important steps in breast cancer invasion. The ability of tumor cells to grow and invade at distant sites is also believed to be important in metastasis. HGF and Met are therefore candidate targets for therapeutic intervention in the prevention and/or treatment of breast cancer metastasis. Previous technologies involving anti-HGF blocking antibodies have proven difficult in inhibiting Met *in vivo*, particularly in carcinoma cells expressing both HGF and Met. Moreover, a systemic block of HGF or Met may cause severe damage to normal tissues, since the HGF/Met pathway is vital for normal epithelial development (Brinkmann et al. 1995). Our approach is therefore to identify tumor-associated regulatory molecules that modulate HGF expression preferentially in carcinoma cells. We have recently demonstrated that activation of two regulatory molecules, designated c-Src and Stat3, induces strong transcriptional activity of the *HGF* promoter in breast carcinoma cells; this effect involves an *HGF* promoter region which is distinct from cytokine-, or estrogen-, dependent regulation of *HGF* transcription

in fibrobasts (Figure 6). Sustained hyper-activation of c-Src and Stat3 has also been demonstrated to occur in invasive human breast cancer, and is required for mammary tumorigenesis. In contrast, transient induction of Stat3 occurs in normal epithelial cells, and promotes mammary tubulogenesis. Thus, the sustained high level of c-Src/Stat3 activity in carcinoma cells may be sufficient to induce HGF expression and autocrine activation of Met, compared to normal epithelial cells. We are therefore currently investigating whether activation of an HGF/Met autocrine loop is causally linked to the oncogenic effects of c-Src and Stat3. Approaches are in progress to identify the mechanisms by which c-Src and Stat3 regulate HGF expression in carcinoma cells, and to determine whether blocking (by mutation) c-Src/Stat3-dependent HGF expression can inhibit or prevent the development of mammary tumors and metastasis. This study suggests tumor specificity in the transcriptional regulation of HGF expression in carcinoma cells, and could thus lead to novel strategies for the design of low molecular weight antagonists (Gambarotta et al. 1996; Epling-Burnette et al. 2001) to inhibit *HGF* gene expression preferentially in breast cancer (Figure 8). Drugs that specifically target induction of HGF (ligand) expression in cancer cells could be used in combination with other treatments that target growth factor receptors (e.g., the use of herceptin for treatment of Her2/Neu-positive breast cancer (Slamon et al. 2001)). These novel treatment strategies provide an exciting new direction for inhibitor design in the treatment of breast cancer metastasis.

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Figure Legends:

Figure 1) Immunoperoxidase localization of Met and E-cadherin in normal ducts (A and B), DCIS (C and D), and IDC (E and F) from human breast cancer tissues: A, C, E) Staining with anti-Met antibody. B, D, F) Staining with anti-E-cadherin antibody. Staining for Met was relatively consistent in all epithelial regions (normal and malignant), but was more intense in IDC than in benign ducts or DCIS. In contrast, E-cadherin staining was strong with accentuation at cell-cell contacts in normal epithelium and DCIS, whereas E-cadherin staining was frequently reduced or absent in IDC. The scale bar refers to the lower magnification in each panel. A 4-fold higher magnification is shown in the insets.

Figure 2) *In situ* hybridization (ISH) analysis of HGF mRNA expression in IDC and normal ducts from human breast cancer tissues: ISH analysis of HGF mRNA expression in IDC (A,B) and normal ducts (C) from human breast cancer tissues was carried out as described previously (Tuck et al. 1996). Strong positivity for HGF mRNA was observed at the advancing front of invasive breast carcinomas (A [arrowhead],B). In contrast, central regions of invasive tumors frequently showed less intense expression of HGF mRNA (right hand side of panel A). Normal ducts showed weak expression of HGF mRNA, although variable sometimes intense staining was seen in the peri-epithelial stromal cells (C [arrowheads]). Scale bars are indicated.

Figure 3) c-Src kinase activity is required for HGF-induced dispersion of breast carcinoma cells: SP1 breast carcinoma cells were transfected with activated c-Src (C,D), or DN c-Src (E,F), or vector alone (control) (A,B). Each cell line was allowed to form aggregates in suspension rotating cultures overnight in RPMI with 0.5 mg/ml BSA. Cell aggregates were washed and plated on plastic with (B,D,F), or without (A,C,E), HGF (30 ng/ml) for 6 h. The representative

photographs show that activated c-Src induced spontaneous dispersion of aggregates, compared to control cells which required stimulation with HGF for dispersion. In contrast, DN c-Src inhibited aggregate dispersion even in the presence of HGF.

Figure 4) c-Src modulates HGF mRNA expression and *HGF* promoter activity: A) SP1 cells transfected with DN c-Src, or activated c-Src, or vector alone (control) (see Figure 3), were serum starved overnight. A nonmalignant mammary epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of HGF mRNA in each sample was determined using RT-PCR, normalized to β-glucuronidase mRNA, and expressed as the percentage of control. Expression of DN c-Src reduced the level of HGF mRNA, whereas expression of activated c-Src increased the level of HGF mRNA by 2.5-fold. B) A reporter construct consisting of the 2.7 kb 5' *HGF* promoter region upstream of the transcriptional start site ligated to the luciferase gene (2.7 HGF-luc), or vector alone, was co-transfected transiently into SP1 cells with DN c-Src or activated c-Src or vector alone. Luciferase activity of each sample was determined, and normalized to the empty vector control value within each group, as described previously (Hung and Elliott, 2001). Expression of the DN c-Src inhibited *HGF* promoter activity, whereas expression of activated c-Src increased *HGF* promoter activity by 2-fold (Modified from Hung and Elliott (2001).

Figure 5) Identification of the c-Src kinase responsive region (-254 to -70 bp) of the *HGF* promoter: The 2.7 HGF-luc reporter, or HGF-luc reporter constructs containing various deletions of the *HGF* promoter were co-transfected into SP1 cells with activated c-Src, DN c-Src or empty vector. Luciferase activity of each sample was determined and normalized within each group, as described in Figure 4. Basal activity in each group is expressed as a percentage of that of the full length promoter (2.7 HGF-luc). c-Src responsive activity of each HGF-luc promoter

construct is calculated relative to the basal level in each group, and is expressed as a percentage of that of the 2.7 HGF-luc reporter. The fold-increase in c-Src responsive *HGF* promoter activity is also indicated. The results show that deletions up to -538 bp (0.5 HGF-luc) had no significant effect on the c-Src responsiveness of the *HGF* promoter. A further deletion up to -273 bp (0.3 HGF-luc) significantly reduced the basal activity of the promoter, while some c-Src responsiveness remained. The remaining c-Src responsiveness was eliminated when all but 72 bp (0.1 HGF-luc) of the *HGF* promoter remained. Internal deletion constructs (0.5Δ HGF-luc and 2.7Δ HGF-luc) lacking the region between -254 and -70 bp of the *HGF* promoter did not respond to expression of activated c-Src, although basal activity remained. Detailed results are published elsewhere (Hung and Elliott, 2001).

Figure 6) Proposed organization of the *HGF* promoter: The relative positions of various putative (indicated by "?") and established consensus sites in the 2.7 kb 5' region of the *HGF* promoter upstream of the transcriptional start site are shown (see text for details). The c-Src responsive region is enlarged, and the Stat3 consensus sites at positions -149 and -110 are indicated by "*". The negative regulatory region (-16 to +11) responsible for suppression of *HGF* transcription in epithelial cells is shown by " ⊖ ". β-cat, β-catenin.

Figure 7) Co-operative effect of activated c-Src and Stat3 in activation of *HGF* transcription in breast carcinoma and epithelial cells: A) SP1 cells were co-transfected with the 2.7 HGF-luc reporter, an internal deletion mutant (Δ1HGF) of the 2.7 HGF-luc reporter construct (Figure 5), and combinations of activated c-Src and Stat3 as indicated. After 48 h, luciferase assays were performed as described in Figures 3 and 4. The results show a strong co-operative effect of activated c-Src and Stat3 on *HGF* transcription, which is dependent on the c-Src responsive region of the *HGF* promoter. B) HC11 mammary epithelial cells were transfected transiently

with the 2.7 HGF-luc reporter and activated c-Src or an empty vector (control), in combination with varying amounts of Stat3. After 48 h, luciferase assays were performed, and results are expressed as in Figures 3 and 4. The results show very low levels of *HGF* transcription in HC11 cells expressing Stat3, or activated c-Src, or empty vector alone. However, co-expression of activated c-Src and Stat3 induced *HGF* transcription up to 17-fold. (Reprinted with permission from Hung and Elliott (2001)).

Figure 8) Model of regulation of HGF expression in EMT and breast carcinomas: Paracrine stimulation by HGF of mammary epithelial cells results in tightly controlled transient activation of c-Src and Stat3, and is required for ductal morphogenesis in mammary gland development. Sustained hyper-activation of c-Src and Stat3 occurs in breast cancer due to a variety of mechanisms, such as increased stimulation by upstream transducer molecules (e.g., RTKs, integrins) or mutations in regulatory oncogenes. Sustained high level of c-Src/Stat3 activation induces *HGF* transcription, and HGF protein expression in epithelial cells which express Met, resulting in activation of an HGF/Met autocrine loop. These combined events promote EMT, mammary tumorigenesis, and metastasis. Thus, blocking c-Src/Stat3-dependent *HGF* transcriptional activity, by mutation or specific inhibitors, would be expected to preferentially neutralize autocrine HGF/Met activation in breast carcinoma cells.

Figure 1

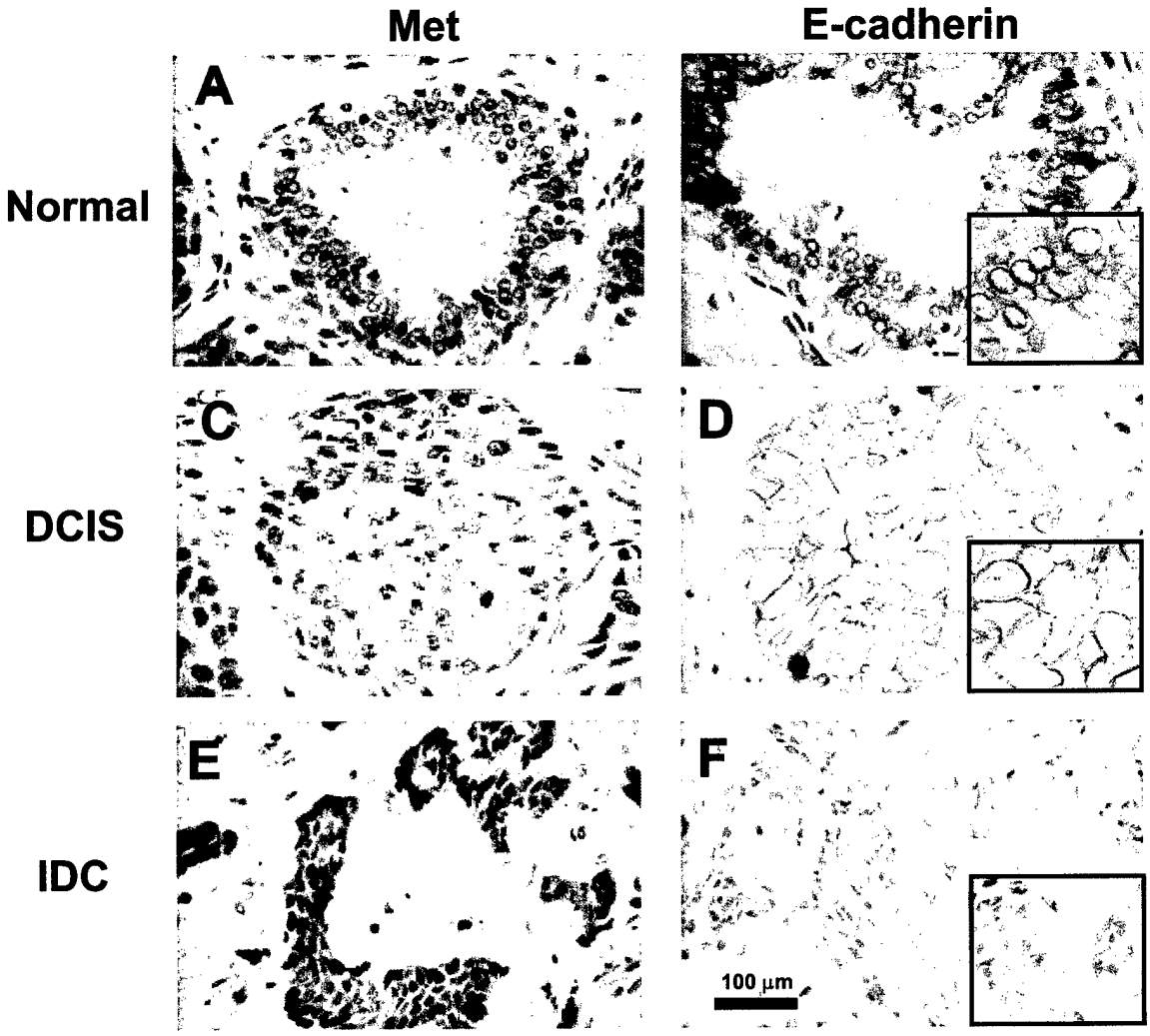


Figure 2

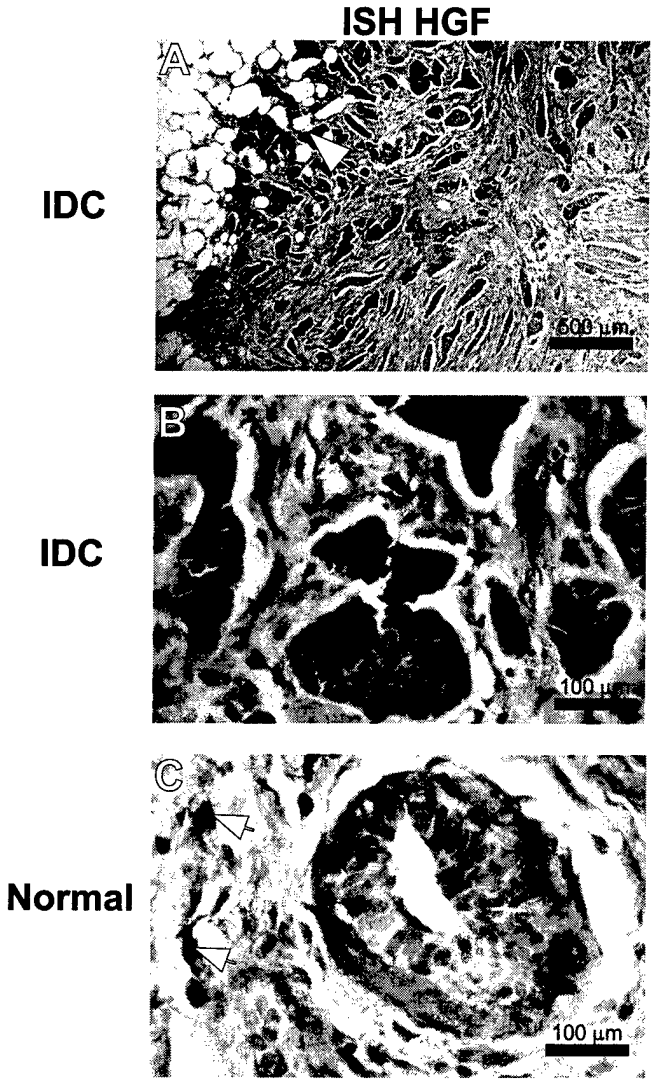


Figure 3

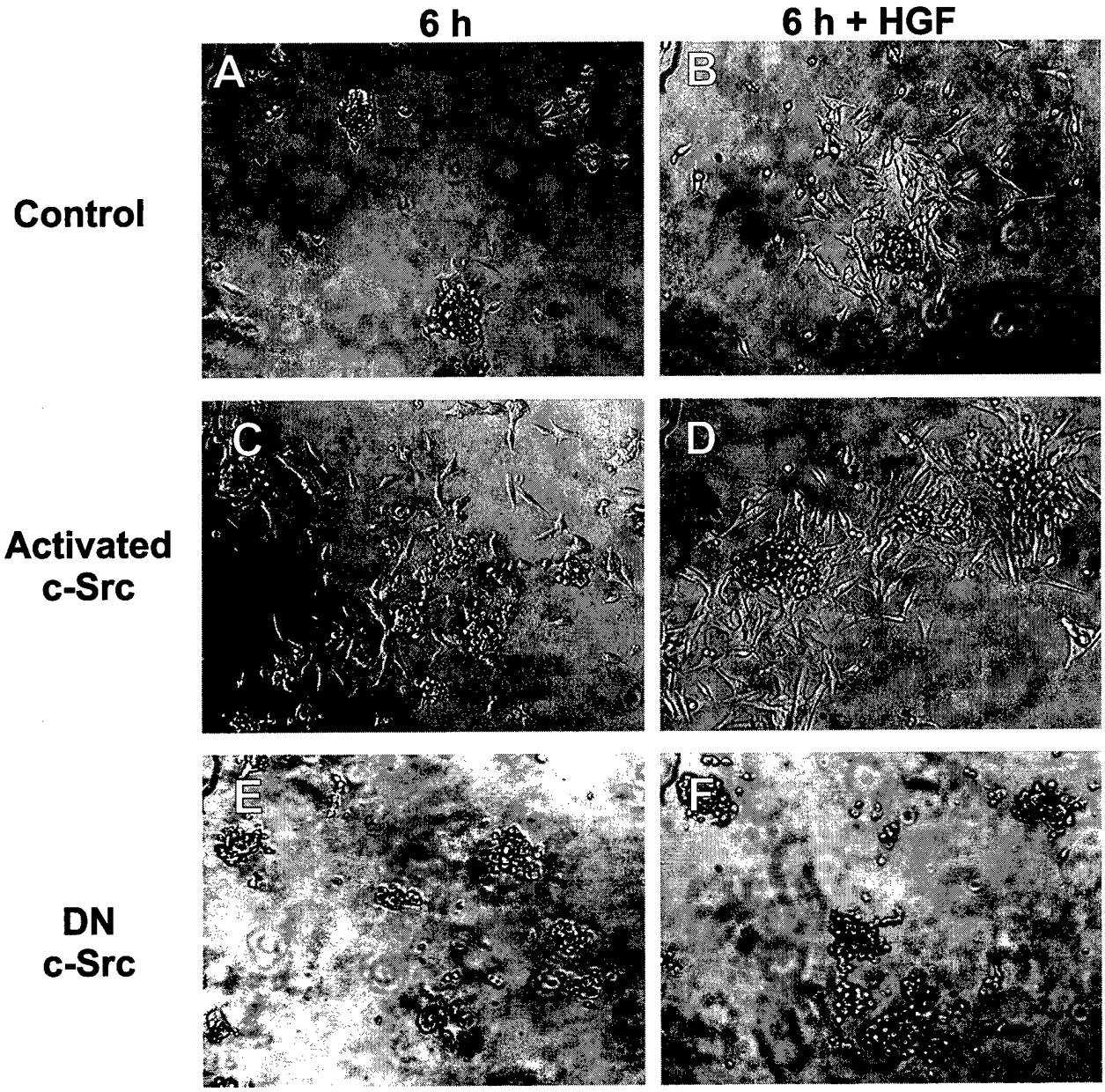


Figure 4

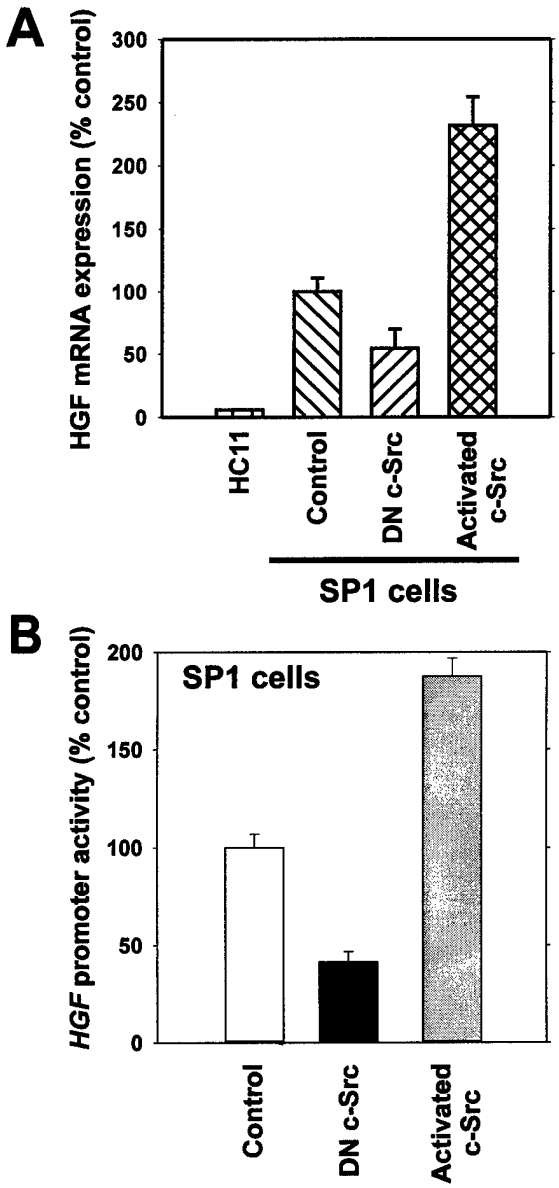
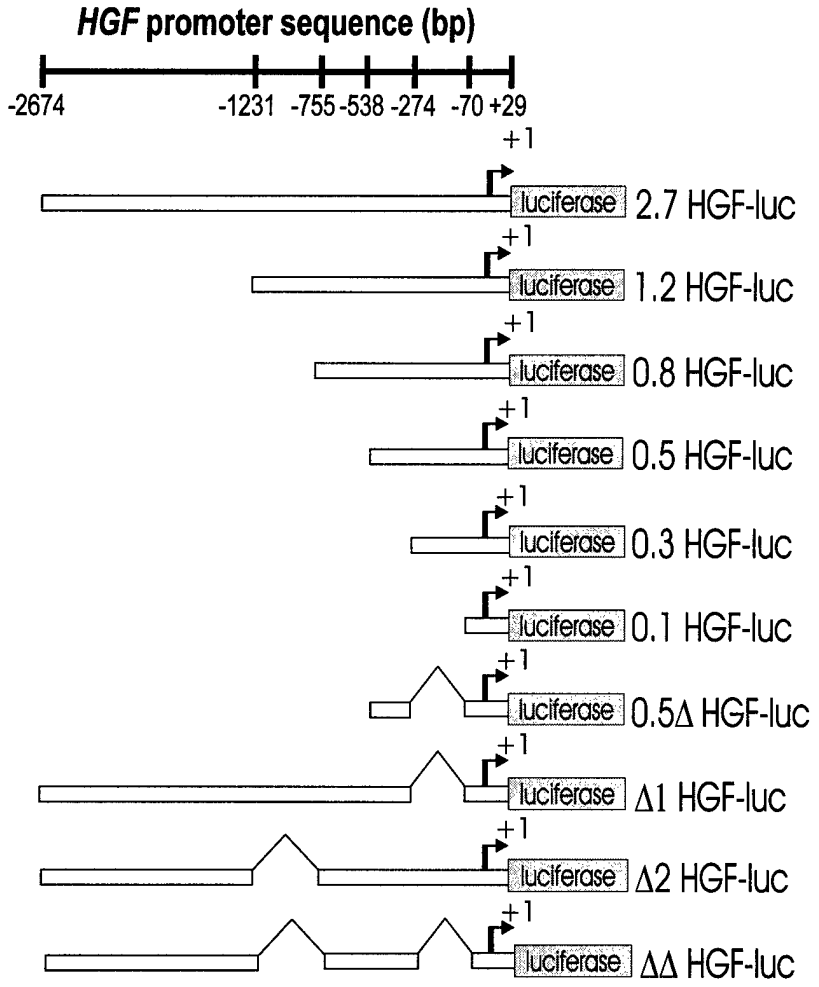

A
HGF mRNA expression (% control)
300
250
200
150
100
50
0
HC11
Control
DN c-Src
Activated c-Src
SP1 cells
B
HGF promoter activity (% control)
200
150
100
50
0
SP1 cells
Control
DN c-Src
Activated c-Src

Figure 5



***HGF* promoter activity**

Basal (%)	c-Src responsive (%)	Fold-increase
100 ± 7	100 ±11	1.9
66 ± 5	87 ± 20	1.8
154 ± 12	57 ± 6	1.5
160 ± 3	48 ± 3	1.4
22 ± 2	58 ± 9	1.5
9 ± 1	0 ± 0	1.0
113 ± 20	12 ± 3	1.1
85 ± 4	3 ± 0	1.0
94 ± 2	73 ± 9	1.6
90 ± 4	8 ±1	1.0

Figure 6

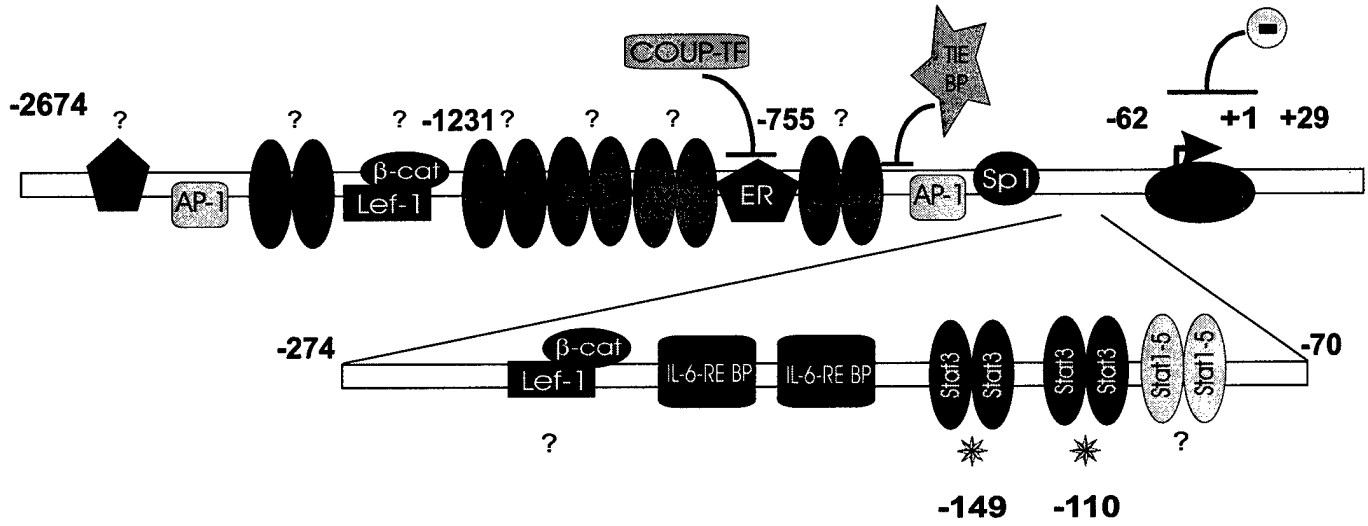

COUP-TF
TIE BP
-2674
?
?
?
-1231
?
?
?
-755
?
-62
+1
+29
β-cat
Lef-1
AP-1
ER
AP-1
Sp1
-274
β-cat
Lef-1
IL-6-RE BP
IL-6-RE BP
Stat3
Stat3
Stat3
Stat3
Stat1-5
Stat1-5
-70
?
?
-149
-110

Figure 7

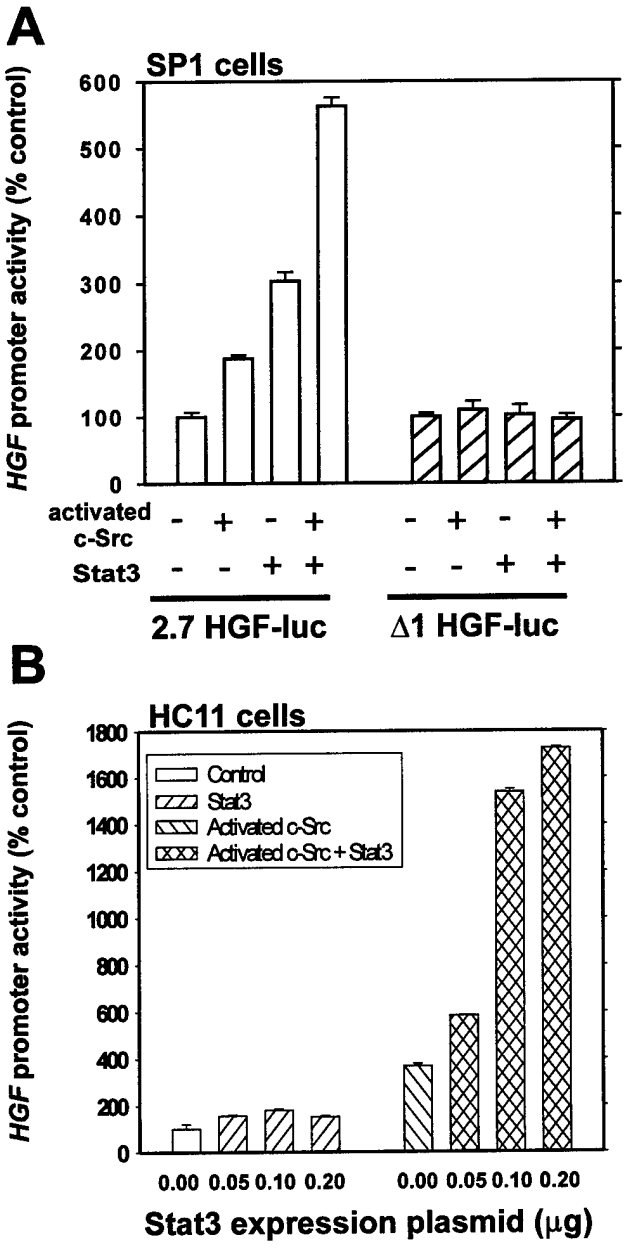
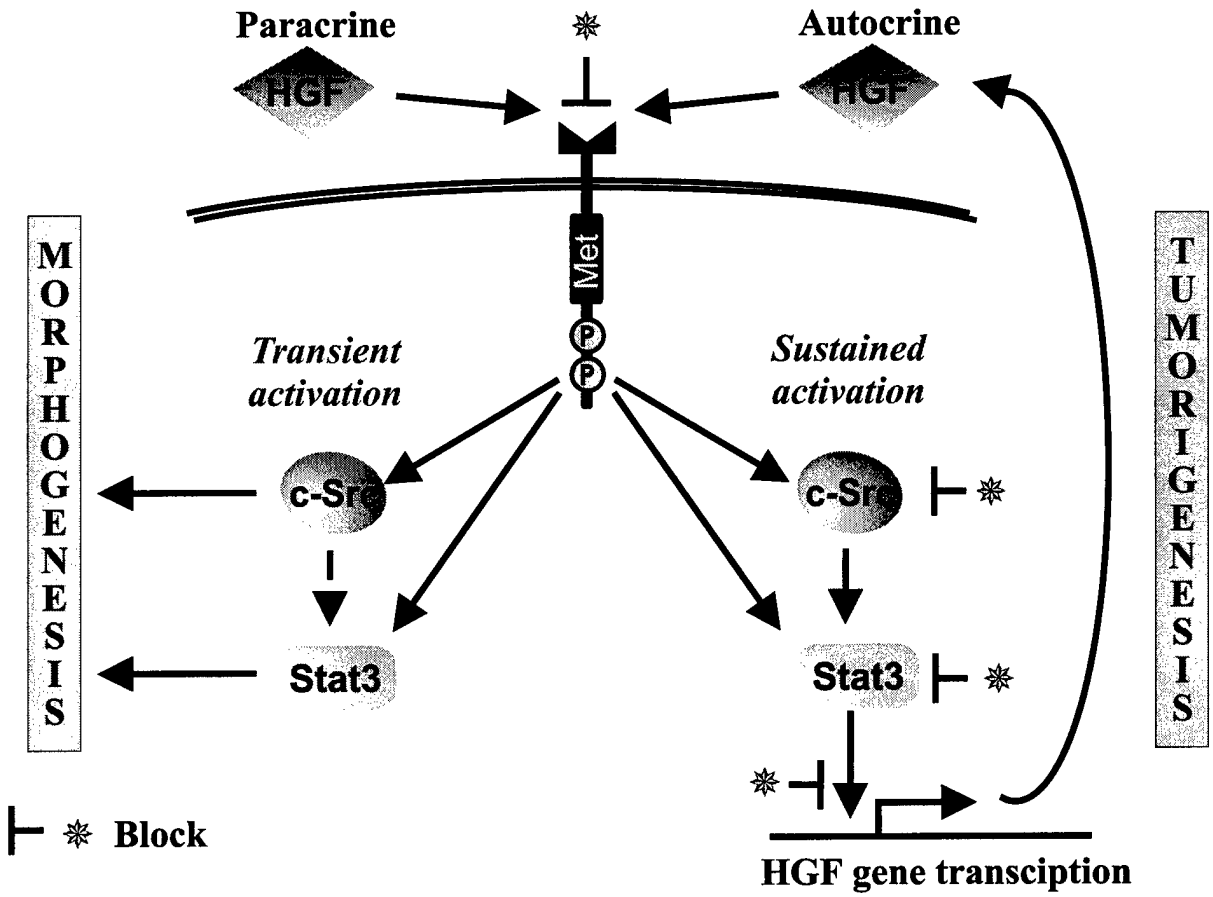

A
SP1 cells
HGF promoter activity (% control)
600
500
400
300
200
100
0
activated c-Src
Stat3
- + - + - + - +
- - + + - - + +
2.7 HGF-luc
Δ1 HGF-luc
B
HC11 cells
HGF promoter activity (% control)
1800
1600
1400
1200
1000
800
600
400
200
0
Control
Stat3
Activated c-Src
Activated c-Src + Stat3
0.00 0.05 0.10 0.20
0.00 0.05 0.10 0.20
Stat3 expression plasmid (μg)

Figure 8


Paracrine
HGF
Autocrine
HGF
Met
P
P
Transient activation
Sustained activation
MORPHOGENESIS
TUMORIGENESIS
c-Src
c-Src
Stat3
Stat3
Block
HGF gene transciption