pp60^c-src Is a Positive Regulator of Growth Factor-induced Cell Scattering in a Rat Bladder Carcinoma Cell Line

Jean-Michel Rodier, Ana Maria Vallés, Monique Denoyelle, Jean Paul Thiery, and Brigitte Boyer
UMR 144 CNRS, Compartimentation et Dynamique Cellulaires, Institut Curie Section de Recherche, 75005 Paris, France

Abstract. The NBT-II rat carcinoma cell line exhibits two mutually exclusive responses to FGF-1 and EGF, entering mitosis at cell confluency while undergoing an epithelium-to-mesenchyme transition (EMT) when cultured at subconfluency. EMT is characterized by acquisition of cell motility, modifications of cell morphology, and cell dissociation correlating with the loss of desmosomes from cellular cortex. The pleiotropic effects of EGF and FGF-1 on NBT-II cells suggest that multiple signaling pathways may be activated. We demonstrate here that growth factor activation is linked to at least two intracellular signaling pathways. One pathway leading to EMT involves an early and sustained stimulation of pp60^c-src kinase activity, which is not observed during the growth factor-induced entry into the cell cycle. Overexpression of normal c-src causes a subpopulation of cells to undergo spontaneous EMT and sensitizes the rest of the population to the scattering activity of EGF and FGF-1 without affecting their mitogenic responsiveness. Addition of cholera toxin, a cAMP-elevating agent, severely perturbs growth factor induction of EMT without altering pp60^c-src activation, therefore demonstrating that cAMP blockade takes place downstream or independently of pp60^c-src. On the other hand, overexpression of a mutated, constitutively activated form of pp60^c-src does not block cell dispersion while strongly inhibiting growth factor-induced entry into cell division. Moreover, stable transfection of a dominant negative mutant of c-src inhibits the scattering response without affecting mitogenesis induced by the growth factors. Altogether, these results suggest a role for pp60^c-src in epithelial cell scattering and indicate that pp60^c-src might contribute unequally to the two separate biological activities engendered by a single signal.

Scattering of epithelial cells has a central role in the control of embryogenesis, and in the process of carcinoma cell dispersion. The search for inducer molecules of epithelial cell scattering has led to the discovery of scatter factor (SF) that has been recognized to also behave as a mitogenic factor (54). Other proteins with scatter activity were first described as mitogens: for example, EGF, one of the first characterized growth factors is able to induce keratinocyte migration (2) and membrane ruffling of mammary carcinoma cells (4, 11). We previously demonstrated that several growth factors (FGF-1, EGF, TGF-α), all of which bind to tyrosine kinase receptors, are endowed with two distinct activities toward NBT-II cells. On subconfluent cultures, these growth factors generate a scattering response, characterized by several properties: cells lose their epithelial features, they become fibroblastic, and they dissociate and start moving individually (7, 18, 49). The entire spectrum of changes has been termed epithelium-to-mesenchyme transition (EMT) to refer to similar phenomena identified during morphogenetic processes in embryos. The dispersing effect of FGF-1 or EGF is strictly restricted to subconfluent cultures of NBT-II cells and cannot be observed in confluent cultures. In sharp contrast, the same growth factors stimulate the entry into cell division of G0-arrested confluent cultures, and have no mitogenic effect on sparse cultures (50). The scattering and mitogenic functions of EGF or FGF-1 are therefore distinct and can be distinguished on the basis of cell culture conditions. The question thus arises as to how and when the signaling pathways triggered by the growth factors, leading to either cell division or dispersion, diverge in the cell. In that respect, we have recently demonstrated that the two pathways can be distinguished on the basis of their sensitivity to increased levels of intracellular cAMP, suggesting the existence of a branching point (6). We thus tested whether signaling molecules known to interact, directly or indirectly, with FGF-1 and EGF receptors could be differentially involved in the two pathways elicited by FGF-1 and EGF. Among the signaling molecules known to bind to activated EGF and FGF-1 receptors, and to be involved in the signaling pathways generated by growth factors, pp60^c-src was particularly interesting for three main reasons, related to its plausible role in growth factor in-

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duced changes in cell morphology; (a) Activated src has been shown to be involved in epithelial plasticity (52, 53) and in the loss of intercellular adhesion (50). The role of activated src in the destabilization of epithelial junctions could be related to its ability to phosphorylate several cytoskeleton-based components of adherent junctions (12, 26, 56). (b) In epithelial cells, pp60src is primarily found in intercellular contacts (45) where it is colocalized with various cytoskeletal components described as cellular targets of pp60src tyrosine kinase activity. (c) While its role during the early phases of mitogenesis remains elusive, pp60src has been shown to be activated during the G2-M transition, suggesting that its function could be related to the morphological changes arising during cell mitosis (17).

The kinase activity of pp60src is regulated by various phosphorylation events whose effects are either positive or negative. One major site of phosphorylation is tyrosine 527. When phosphorylated by a specific enzyme, termed csk, tyr-527 interacts with the SH2 domain of pp60src thereby masking the catalytic domain. Consequently, substituting tyr-527 with phenylalanine converts pp60src into a constitutively active, oncogenic-like molecule (38, 39).

We therefore tested pp60src activation and implication in the two responses (i.e., EMT and entry into cell division) induced by FGF-1 and EGF. By expressing high levels of wild-type pp60src in NBT-II cells, we demonstrated that pp60src activity distinguishes the two pathways triggered by the growth factors. Low level expression of tyr-527 pp60src in NBT-II cells abolished the growth factor-induced mitogenesis without inhibiting EMT, indicating that unregulated pp60src activity differentially affected the two growth factor-mediated responses of NBT-II cells. Finally, the growth factor-induced scattering response of cells expressing a dominant-negative mutant of c-src was impaired while its mitogenic response was not affected, confirming the fact that c-src is positively involved in cytokine-mediated EMT.

**Materials and Methods**

**Reagents**

The pp60src monoclonal antibody Mab327 was purchased from Oncogene Science Inc. (Uniondale, NY). Monoclonal antibodies against desmoplakin (anti-DP antibodies) were kindly provided by Pr. W. W. Franke (German Cancer Research Center, Heidelberg, Germany). Texas Red-coupled goat anti-mouse IgG were obtained from Immunotech (Mar- seille, France). Human recombinant FGF-1 was the generous gift of Dr. M. Jaye (Rhône-Poulenc Rorer Central Research Inc., King of Prussia, PA). Heparin (Choay laboratories, Paris, France) was added to stabilize its biological activity (19).

Cholera toxin and receptor grade mouse EGF were purchased from Sigma (St. Louis, MO). 125I-labeled rabbit anti-mouse IgG and [3H]methyl-thymidine (1 mCi/ml) were obtained from Amersham International (Buckinghamshire, UK). Tissue culture reagents were as previously described (7, 46, 49). All chemicals were reagent grade.

**Cell Culture**

The NBT-II cell line obtained from Professor Marc M. Mareel (University Hospital, Gent, Belgium) was established from a rat bladder carcinoma chemically induced by Toyoshima et al. (43) and is grown in monolayer culture in DME supplemented with 10% FCS. Twice a week, cells were passaged by gentle trypsinization and replating at 1:10 dilution. When needed (see Results), cells were grown to high density, forming monolayers of tightly packed cells, that completely covered the surface of the dish. This condition defined confluent cultures. Alternatively, cells were grown to ~30% confluency and formed clusters of closely apposed epithelial cells. This condition defined subconfluent cultures.

**Recombinant Plasmids**

pHBAP1-1-neo plasmid (23) containing the human beta-actin promoter was a gift of Dr. Gunning (Children's Medical Research Foundation, Camperdown, New South Wales, Australia). The pAT-c-src and pUC-c-src were the generous gift of Dr. G. Calothy (Institut Curie, Centre Universitaire d'Orsay, France). The pAT plasmid contains the 1600 bp of the normal c-src cDNA. The pUC plasmid contains a mutated form of the same DNA: tyrosine 527 of the corresponding protein is replaced by a phenylalanine, so that it cannot be phosphorylated, leading to a permanently activated protein. Each of these cDNAs was inserted in the pHBAB1-1-neo expression vector at the polynucleotide site. Control experiments were performed with the pHBAP1-1-neo vector.

The srcKPSGT vector coding for the kinase inactive form of chicken c-src (47), was kindly provided by Dr. S. A. Courtneidge (EMBL, Heidelberg, Germany).

**Transfection**

NBT-II cells were transfected by the calcium-phosphate coprecipitation technique according to Graham and Van Der Eb (21). Selection of transfected cells was initiated 24 h later by adding active Geneticin (G418 sul fate; Gibco BRL, Gaithersburg, MD) at 400 μg/ml. After 2-3 wk, resistant colonies were individually picked and expanded in selective medium for the first two passages and then passed in standard medium.

**Immunoblot Analysis**

Cells were washed, lysed in a lysis buffer consisting of 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 60 mM Tris-HCl, pH 6.8, and boiled for 3 min. Samples were separated by SDS-PAGE and the proteins in the gel were electrophoretically transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were incubated overnight at 4°C with 1 μg/ml mouse monoclonal phosphotyrosine antibodies 4G10. Blots were extensively washed with PBS-0.1% Tween 20 and then incubated for 1 h with ~10^6 cpm/ml 125I-labeled rabbit anti-mouse antibody. After several washes, blots were exposed to X-OMAT AR x-ray films (Eastman Kodak, Rochester, NY). Alternatively, blots were incubated with a dilution of horseradish-peroxidase anti-mouse antibodies and the labeled bands were visualized with the ECL reagent (Pharmacia, Uppsala, Sweden).

**Cell Motility Assays**

Time-lapse video microscopy was done as described previously (46). Briefly, 5 × 10⁴ cells were seeded on glass coverslips and preincubated for 2 d before the eventual addition of growth factor at 30 ng/ml. Recordings were done over a 24-h period, after which tracks of 20 randomly selected cells were traced. The total distance of migration was estimated with a map measurer. Motility was expressed as μm/h.

"Wound healing" assay was performed as described (7). Subconfluent cell monolayers were scratched with a Gilson pipette yellow tip, and rinsed with standard medium to remove all cellular debris before overnight incubation with different concentrations of EGF and FGF-1. Cell cultures were subsequently fixed and photographed under a phase contrast microscope (Nikon, Tokyo, Japan).

**Immunofluorescent Staining of Cells**

Cells preincubated for 48 h on glass coverslips were stimulated with increasing concentrations of FGF-1 or EGF before processing for immunofluorescence studies. Briefly, cell cultures were fixed with methanol/acetone at −20°C, rehydrated in PBS before 1-h incubation with a dilution of anti-DP or anti-vimentin antibody followed by 1-h incubation with Texas Red-coupled goat anti-mouse IgG. Cells were mounted in Moviol (Hoechst, Frankfurt, Germany) and viewed with a Zeiss microscope equipped with epifluorescence. When appropriate (see Results), the percentages of DP-negative cells were counted. Cells were considered DP-negative when DP immunoreactive "dots" were totally absent from the cell periphery and appeared instead in the cytoplasm. For each measurement, at least 500 cells were counted.
Mitogenic Assay

Cells grown to confluency in 24-well plates were serum-starved for 24 h before addition of various concentrations of growth factors into the medium. Incubation was continued for 24 h. [3H]methyl-thymidine (specific activity, 42 Ci/mmol) was added at 1 μCi/well during the last 4 h of the experiment. Plates were rinsed with ice-cold PBS and the insoluble material was precipitated with 10% trichloroacetic acid for 20 min at 4°C before solubilization in 0.1 N NaOH, and radioactivity was quantitated by liquid scintillation spectrometry of acid-insoluble material. Experiments were conducted in triplicate cultures. Results are expressed as the means ± SE of the radioactivities counted in control and growth factor-treated cultures.

Immunoprecipitation of pp60c-src and Kinase Assay

Cells were incubated with 0.2 mCi/ml [35S]methionine in methionine-free medium for 15 h at 37°C. After washing, cells were lysed in lysis buffer consisting of PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml apro tinin, 50 mM sodium fluoride, and 10 mM sodium orthovanadate. After centrifugation, supernatants were pre cleared for 30 min with protein A-Sepharose and then incubated for 2 h with 1.5 μg Mab327 preadsorbed on protein A-Sepharose beads. Bound material was extensively washed with lysis buffer and analyzed by SDS-PAGE before autoradiography.

Kinase assays were done according to Piwnica-Worms et al. (38) with minor modifications. Briefly, pp60c-src immunoprecipitated from unla beled cell extracts was immobilized on Mab327-protein A-Sepharose beads, as described above. Beads were extensively washed with lysis buffer, then with water before incubation in 45 μl kinase buffer (10 mM Tris, pH 7.4, 7 mM MgCl2, 0.5 mM ATP, 2 μCi [γ-32P]-ATP or [γ-32P]- ATP, as indicated in the text). Samples were divided into three parts. The first one, used as a negative control, was incubated in the absence of enolase for 8 min at 30°C. Four mg acid-denatured enolase were added in the two other aliquots and incubations were carried out for 4 and 8 min at 30°C. In some experiments (see Results), the kinase reactions were conducted for 8 min with the totality of each immunoprecipitation product. Concentrated SDS-containing sample buffer was added to stop the reaction and the samples were then boiled before electrophoresis on SDS-PAGE.

Quantitation of the radioactivity incorporated into enolase was determined by analyzing the resulting fluorograms in a Bio com spectrophotometer equipped with a scanning densitometer and the RAG program.

EGF and FGF-1 Receptor Assay

Growth factors were labeled with 125I by a modification of the chloramine T method (25) as described (37). The affinity and number of receptors were determined according to Scatchard (41) using the LIGAND program. Briefly, subconfluent cultures were rinsed twice with binding buffer (20 mM Hepes, 0.9 mM CaCl2, 0.8 mM MgCl2, 3 mM KCl, 0.14 M NaCl, 1 mg/ml BSA, pH 7.2) and incubated with various concentrations of radiolabeled growth factors (from 0.03 to 30 ng/ml) for 4 h at 4°C. The specificity of the binding was tested by the addition of a large excess of unlabeled ligand. The cells were washed extensively with binding buffer before incubation in solubilizing buffer (20 mM Hepes, 1% Triton X-100, 10% glycerol, 1 mg/ml BSA, pH 7.2) overnight at 4°C. Cell lysates were harvested and counted on a gamma counter.

Results

pp60c-src Is Activated during EMT

On confluent cultures, FGF-1 and EGF are strictly mitogenic while addition of these growth factors to sparse colonies of NBT-II cells induces cell dissociation and motility (49). The two culture conditions were therefore used throughout this study to differentiate the two biological activities of the growth factors. Subconfluent and confluent cultures of NBT-II cells were stimulated for various periods of time with either FGF-1 or EGF. Cells were lysed and equivalent amounts of extracted proteins were subjected to immunoprecipitation with Mab 327 before processing for in vitro tyrosine kinase assay of pp60c-src.

The kinase activity of pp60c-src measured on enolase as an exogenous substrate was substantially increased after exposure of sparse cultures to 100 ng/ml EGF (~3.5-fold over the basal level) (Fig. 1 A). A similar sustained increase in pp60c-src kinase activity was observed after addi-
tion of 100 ng/ml FGF-1 (~2.7 times higher than the basal level) (Fig.1 B). In sharp contrast, the kinase activity of pp60\(^{c-src}\) immunoprecipitated from confluent cultures remained at basal levels during the entire period of growth factor stimulation (Fig. 1, C and D). Quantitative measurements of the radioactivity incorporated into enolase confirmed the complete absence of kinase activation under these conditions. This experiment revealed therefore a clear difference in the activation of pp60\(^{c-src}\) during the early phase of EMT as opposed to mitogenesis. Since the two functions of growth factors were tested by using two different culture conditions, we were concerned that the activation of pp60\(^{c-src}\) observed in sparse cultures was not related to the scattering activity itself. To test this hypothesis, we measured pp60\(^{c-src}\) activation in sparse G0-arrested cultures of NBT-II cells stimulated to enter the cell cycle by addition of FCS, which is strictly mitogenic and does not promote cell scattering. Under these conditions, a weak and transient activation of pp60\(^{c-src}\) was occasionally observed (Fig. 1 E). Although we cannot exclude the mitogenic stimulus delivered by FCS follows a pathway different from that triggered by FGF-1 and EGF, these data suggested that pp60\(^{c-src}\) activation was related to the scattering activity of EGF and FGF-1 rather than to culture conditions.

We had previously shown that elevation of intracellular levels of cAMP antagonized the FGF-1-induced EMT (6). It was therefore interesting to test whether pp60\(^{c-src}\) activation was affected by cAMP augmentation. As shown in Fig. 1 F, addition of cholaetoxin to cell cultures before induction of EMT did not block the increase in pp60\(^{c-src}\) kinase activity. This result indicated that, under experimental conditions in which EMT was inhibited, pp60\(^{c-src}\) activation was not sufficient to induce cell scattering and, more importantly, it suggested that cAMP acted independently or downstream of pp60\(^{c-src}\) stimulation.

To address the question as to whether pp60\(^{c-src}\) activation plays a direct, positive or negative role in EMT, as opposed to mitogenesis, we generated clones of the NBT-II cell line that overexpressed normal and activated forms of pp60\(^{c-src}\), and analyzed their responses to FGF-1 and EGF.

**Biochemical Analysis of pp60\(^{c-src}\) Overexpressing Cells**

After transfection of plasmids containing the cDNA encoding either wild-type or tyr-527-mutated c-src, clones were selected on the basis of steady-state amounts of pp60\(^{c-src}\) protein, as estimated by immunoblot analysis of cell extracts (data not shown). For each transfection, ~10 positive clones were isolated and of these, 2 representative clones were further analyzed. c1 and c2 were mock-transfected and were subsequently used as controls. w1 and w2 are two clones overexpressing wild-type c-src, while m1 and m2 are two clones expressing mutated c-src. The levels of pp60\(^{c-src}\) were evaluated by immunoprecipitating extracts of \(^{35}\)S-labeled cells with Mab 327 (Fig. 2 A). The antibody recognized two molecular species, one running with an apparent MW identical to that of pp60\(^{c-src}\) while the other had a lower MW. This band could correspond to degradation products of pp60\(^{c-src}\) or, alternatively, to molecules coprecipitating with pp60\(^{c-src}\). w1 and w2 expressed respectively, ~8 and 15 times more pp60\(^{c-src}\) than control cells, without any obvious effect on cell transformation (see below). The total levels of pp60\(^{c-src}\) in m1 and m2 clones were three- and twofold over the endogenous level found in c1 and c2 cells. For these experiments, a modest expression of the constitutively activated pp60\(^{c-src}\) was necessary to reduce its transforming potential (38, 39, 52). The levels of in vivo tyrosine phosphorylation were then evaluated in c-src transfectants. As shown in Fig. 2 B, tyrosine phosphorylation levels, estimated by immunoblotting cell extracts containing similar amounts of proteins with anti-phosphotyrosine monoclonal antibodies, were increased in all src transfected clones, as compared to control cells. These data suggested that the enzymatic function of pp60\(^{c-src}\) was elevated in the transfected cells. Accordingly, when the kinase activities of endogenous and transfected pp60\(^{c-src}\) were compared in a kinase assay by using enolase as an exogenous substrate (Fig. 2 C), both wild-type and mutated pp60\(^{c-src}\)-transfectants displayed a much higher kinase activity than control cells. These results indicated that the transfected cDNAs encoded functional enzymes. Moreover, the ratio of pp60\(^{c-src}\) kinase activity to its level of expression in m1 and m2 clones compared with that of c1 and c2 control clones was consistent with the expression of a constitutively active pp60\(^{c-src}\) in m1 and m2 clones. Immunoprecipitation of pp60\(^{c-src}\) from extracts of \([\gamma\text{-}^{32}\text{P}]\)-labeled cells revealed that the amount of radioactivity incorporated into endogenous and transfected pp60\(^{c-src}\) paralleled the levels of pp60\(^{c-src}\) expression but not the levels of pp60\(^{c-src}\) kinase activity (Fig. 2, compare A to C and D).

**Figure 2.** Analysis of pp60\(^{c-src}\) expression in transfected cells. (A) Immunoprecipitation of pp60\(^{c-src}\) from equivalent amounts of proteins extracted from \([\text{\textit{\textsuperscript{35}}S}]\)methionine-labeled cells. (B) Pattern of phosphotyrosine-containing proteins revealed by immunoblotting of cell extracts with a monoclonal phospho-tyrosine antibody. Cell extracts from equivalent number of cells were separated on SDS-PAGE and immunoblotted with phosphotyrosine antibodies. (C) Detection of pp60\(^{c-src}\) kinase activity in transfected cells. Equivalent amounts of proteins from cell lysates were immunoprecipitated with Mab 327. The kinase activity of pp60\(^{c-src}\) was assayed on enolase as an exogenous substrate as described in Fig. 1. Samples were analyzed by 10% SDS-PAGE and autoradiography. (D) Analysis of pp60\(^{c-src}\) phosphorylation in transfected clones. Subconfluent cultures were incubated with \([\gamma\text{-}^{32}\text{P}]\) and lysed. pp60\(^{c-src}\), immunoprecipitated from equivalent amounts of \([\gamma\text{-}^{32}\text{P}]\)-labeled proteins, was visualized by SDS-PAGE and autoradiography. En, enolase.
These results suggested that pp60^src phosphorylation in the transfected cells did not strictly correlate with its own kinase activity.

**Growth Properties of pp60^src Overexpressing Cells**

An interesting feature of NBT-II cell response to EGF and FGF-1 is the absolute requirement of high cell density for these factors to elicit a mitogenic response. EGF and FGF-1 can neither support cell proliferation of unsynchronized sparse cell cultures nor induce entry into the cell cycle of G0-arrested subconfluent cultures. The lack of mitogenic activity is correlated with the scattering effect of these growth factors tested under these conditions. We therefore determined whether src transfected clones would exhibit the same growth properties.

Whether propagated in medium containing or not various concentrations of FGF-1, EGF, or FCS, the pp60^src transfected clones had doubling times similar to those of control cells (data not shown). Particularly, all transfected clones were dependent on the presence of FCS for their growth, as previously shown for NBT-II untransfected cells (49), indicating that transfection of c-src was not sufficient to induce cells to proliferate upon EGF or FGF-1 stimulation.

In contrast, FGF-1 (49) and EGF (unpublished data; this work) are able to induce DNA synthesis of confluent, G0-arrested cell cultures of NBT-II cells. We therefore examined the effect of FGF-1 and EGF in promoting the entry into cell division of serum-starved, confluent cultures of transfected cells (Fig. 3). Both FGF-1 and EGF were potent inducers of DNA synthesis in confluent cultures of control and wild type c-src-transfected clones. The dose-response curves were similar in the two types of clones, and resembled those obtained with the parental cell line (49). In sharp contrast, ml and m2 clones did not enter cell division after stimulation with FGF-1 and EGF. A large range of concentrations (0.01 to 30 ng/ml) was tested without any observable effect on DNA synthesis. [3H]methylthymidine addition at different times (from 4 to 20 h) after growth factor-stimulation did not abolish the lack of growth factor-induced DNA synthesis, therefore excluding the possibility that the absence of response was due to the accelerated progression of these clones from G0 to the S-phase. Furthermore, serum-starved ml and m2 cells had basal levels of DNA synthesis comparable to those of control cells and they were able to incorporate [3H]methyl-thymidine after stimulation with 10% FCS (data not shown), thus ruling out the possibility that these clones were intrinsically incapable of leaving the G0 phase or that they were not G0-arrested.

Since the mitogenic responses induced by FGF-1 and EGF in clones transfected with tyr-527-mutated c-src were abolished, we sought to determine whether the differences in the biological responses to the growth factors did not arise from modifications in the number and/or affinity of growth factor receptors. We therefore carried out a Scatchard analysis of the binding of radiolabeled FGF-1 and EGF at the cell surface of cl, w1 and ml (Table I). All transfectants expressed high-affinity binding sites for EGF and FGF-1. Thus, the unresponsiveness of ml cells to the mitogenic effects of FGF-1 and EGF cannot be regarded as a consequence of the loss of binding sites for these growth factors.

**pp60^src Overexpression Modifies the Morphological Characteristics of NBT-II Cells**

As illustrated in Fig. 4, mock-transfected clones grew as pavement-like islands of polygonal, tightly interconnected cells. These cells exhibited morphological characteristics that were indistinguishable from those of the parent cell line. Even when grown in small clusters, cells were linked by desmosomes, as inferred from desmoplakin (DP) immunolocalization at the cell periphery and most of them (~98%) were devoid of vimentin expression (data not shown).

The majority of w1 and w2 cells assumed a similar morphology. However, dispersed, fibroblast-like cells were observed at the periphery of cell clusters. These scattered...
Table 1. Scatchard Analysis of EGF and FGF-1-specific Binding to Transfected Cells

|       | EGF     | FGF-1   |
|-------|---------|---------|
| c 1   | $R = 2.6 \times 10^4$ | $R = 0.5 \times 10^4$ |
|       | $K_d = 0.19 \text{ nM}$ | $K_d = 0.025 \text{ nM}$ |
| w 1   | $R = 0.75 \times 10^4$ | $R = 3.9 \times 10^4$ |
|       | $K_d = 0.24 \text{ nM}$ | $K_d = 0.24 \text{ nM}$ |
| m 1   | $R = 1.3 \times 10^4$ | $R = 1.0 \times 10^4$ |
|       | $K_d = 0.36 \text{ nM}$ | $K_d = 0.15 \text{ nM}$ |

Results represent the mean value of duplicate samples. Cultures of transfected cells were incubated for 4 h at 4°C with decreasing concentrations of radiolabeled growth factors. The amounts of bound and free $^{125}$I-EGF and $^{125}$I-FGF-1 calculated according to the method of Scatchard were used to determine the number of binding sites per cell ($R$) and the dissociation constant ($K_d$).

Cells were dissociated and had lost their desmosomal interconnections, as judged from the absence of DP immunoreactivity from cell cortex (Fig. 4). They also expressed vimentin, a marker of mesenchymal cells that is expressed by NBT-II cells having undergone EMT changes (7). Cell heterogeneity was reproducibly observed, in all clones derived from WT c-src transfection, and was never seen in control neo-transfected clones. It was therefore unlikely that it resulted from the selection procedure of the transfectants. Thus, by different criteria, some transfected cells in WT clones resembled those generated by growth factor-induced EMT-like changes. It was therefore important to determine whether the spontaneous cell scattering observed in wild-type src transfected clones was a reversible process, as previously demonstrated for growth factor-initiated EMT (7). Since earlier studies demonstrated that elevation of intracellular levels of cAMP triggers the reversion of growth factor-induced EMT (6), we examined the effect of cholera toxin, a cAMP-elevating agent, on the characteristics of w2 cells. Addition of 1 µg/ml cholera toxin into the culture medium of w2 cells induced the disappearance of scattered, fibroblastic-like cells, as con-

Figure 4. Phenotypic characteristics of transfected cells. (A and B) Phase-contrast micrographs of c2 (A) and w2 (B) cell cultures under standard conditions. Note the appearance of fibroblastic-like cells at the periphery of a cell colony in B. (C–F) Sparse cultures of w2 cells were incubated for 2 d in standard medium without (C and D) or with cholera toxin at 1 µg/ml (E and F) before fixation and processing for immunofluorescence labeling with anti-DP (C and E) and anti-vimentin (D and F) antibodies. (G and H) Phase-contrast micrographs of m1 cell cultures maintained for 2 d in the absence (G) or presence (H) of 1 µg/ml cholera toxin. Note the compaction of cell colonies and the disappearance of isolated cells induced by cholera toxin in H. Bars: (A, B, G, and H) 50 µm; (C–F) 20 µm.
firmed by immunofluorescence data showing that the vast majority of cells were DP-positive and devoid of vimentin staining (Fig. 4). Although m1 and m2 cells remained in cohesive structures, they were more elongated than control clones (Fig. 4). As already noticed for w1 and w2 cells, some of them escaped from cell collectives. As shown for w2 cells, addition of cholera toxin at 1 μg/ml into the medium prevented cells from scattering out of cell colonies, suggesting that cell dispersion of m2 cells is reversed by the agents inducing the reversion of growth factor-mediated EMT.

**pp60^src Overexpression Renders Cells More Sensitive to Growth Factor Induction of EMT**

The present findings indicated that src transfection was not sufficient to induce spontaneous EMT of the totality of the transfected cell population. However, the occasional presence of scattered cells suggested the possibility that src might be positively involved in EMT-like changes. To examine this point, we carried out a series of experiments to determine whether the transfected cells were more receptive to the scattering activity of EGF and FGF-1. Three criteria, previously applied to FGF-induced EMT of the parental NBT-II cell line (7) were studied. Cell dissociation was estimated by the disappearance of desmosomes from the cell periphery, as visualized by the loss of cortical DP immunoreactivity. Acquisition of a mesenchymal differentiation was correlated with the expression of intermediate filaments of the vimentin type. Acquisition of cell motility was monitored by video microcinematography and wound healing assays.

We first analyzed DP and vimentin immunostaining in transfected cells exposed to optimal concentrations of FGF-1, to determine whether they were capable of undergoing EMT. Under standard conditions of culture, DP im-

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**Figure 5. c-src transfectants undergo EMT-like changes upon exposure to FGF-1.** w1 (A-D) and m2 cells (E-H) were cultured under standard conditions (A, C, E, and G) or exposed to 30 ng/ml FGF-1 plus heparin for 18 h (B and F) or 3 d (D and H). Cells were fixed and processed for immunofluorescence microscopy with a mAb against DP (A, B, E, and F) or vimentin (C, D, G, and H). Bar, 20 μm.
FGF-1 also induced a modulation of DP immunoreactivity staining was visualized. DP antibody. Cells were considered as DP-positive when cortical presence of FGF-1 at the indicated concentration. Cells were FGF-1 in transfected cells. Subconfluent cultures of c2 (*), wl clones, indicating that FGF-1 thus induced DP modulation and vimentin expression in all clones, indicating that c-src-transfected cells were able to undergo EMT upon exposure to FGF-1. Identical results were obtained with EGF stimulation (data not shown).

We then determined the dose response curves of the scattering responses as a function of the concentration of FGF-1. For that purpose, the number of cells having lost their cortical DP immunolabeling was estimated as a function of the concentration of FGF-1 added into the culture medium for 16 h. As shown in Fig. 6, w1 cells were more sensitive than c2 cells to the dissociating effect of FGF-1, as inferred from the higher number of cells having lost cortical DP immunostaining at 3.5 and 6 ng/ml. FGF-1 also induced a modulation of DP immunoreactivity in m2 cells, a significant percentage of these cells retained cortical DP immunostaining at 12.5 ng/ml FGF-1, a concentration that was sufficient to induce DP internalization in more than 80% c2 and w1 cells.

To assay for growth factor-induced cell movement, video-

**Figure 6.** Decrease of cortical DP immunoreactivity induced by FGF-1 in transfected cells. Subconfluent cultures of c2 (*), w1 (open circles) and m2 (black circles) cells were incubated in the presence of FGF-1 at the indicated concentration. Cells were fixed and processed for immunofluorescence with a monoclonal DP antibody. Cells were considered as DP-positive when cortical staining was visualized.
Figure 7. Effects of FGF-1 on motility of transfected cells. Areas free of cells (wounds) made in near-confluent cultures of cl (A–C), w1 (D–F), and m1 (G–I) cells were examined after 24 h of culture in standard medium (A, D, and G) or in the presence of FGF-1 at 3 ng/ml (B, E, and H) or 10 ng/ml (C, F, and I). At 3 ng/ml FGF-1, the wound produced in w1 cell culture is repaired (E), whereas it is not totally repopulated by cl and m1 cells (B and H). Bar, 200 μm.

Extracts of subconfluent and confluent cultures of w2 cells were similar, and their basal kinase activities measured on the incorporation of [γ-32P] into enolase were equivalent (data not shown). Therefore, the differences in the ability of pp60c-src molecules immunoprecipitated from subconfluent versus confluent cultures to be activated by FGF-1 and EGF cannot be accounted for by differences in the amount or basal activity of pp60c-src under the two culture conditions.

Transfection of Kinase-defective Interfering Mutant of c-src

To confirm the involvement of Src in the growth-factor induced EMT, a kinase-negative mutant of c-src was introduced by transfection into NBT-II cells. The mutated enzyme that carries a mutation in the ATP-binding site, cannot hydrolyze ATP and therefore cannot exert its catalytic activity. However, the binding sites for target proteins are preserved and therefore the modified Src can assume a dominant negative role. After transfection, we obtained several clones overexpressing Src K-10 to 20-fold over the endogenous, active protein (data not shown). Three clones (K-1, K-13, K-14) were selected for further analyses. Their responsiveness to scattering factors was monitored by using the criteria described in the preceding paragraphs. Growth factor-induced cell dissociation was estimated by the number of cells having lost DP immunoreactivity from the cell periphery. When compared to control cells, K-1 and K-13 cells were notably impaired in their ability to break their desmosomal connections in response to scatter factors (Table II). K-14 cells that expressed lower levels of the dominant negative construct were less inhib-
Table II. Growth Factor-induced EMT of Src Kinase-defective Clones

| Clone   | % Inhibition of DP modulation | % Inhibition of vimentin expression | Speed of locomotion |
|---------|-------------------------------|-------------------------------------|---------------------|
| Control | 0                             | 0                                   | 20 ± 4              |
| K-1     | 59 ± 8                         | 65 ± 11                             | 4 ± 1               |
| K-13    | 66 ± 9                         | ND                                  | ND                  |
| K-14    | 24 ± 4                         | ND                                  | 5 ± 2               |

Control cells and cells overexpressing the kinase-defective form of Src were stimulated with 30 ng/ml EGF. The number of cells having lost their cortical DP immunoreactivity was estimated after overnight incubation. The number of cells expressing vimentin was evaluated by immunofluorescence labeling after three days in the presence of FGF-1. The speed of locomotion in the inducing medium was estimated as described in Materials and Methods. Similar results obtained with FGF-1. ND, not determined.

Discussion

The epithelial NBT-II cell line is a convenient model to study the mechanisms whereby a given growth factor can assume two opposite functions, triggering either entry into cell division or morphological transformations referred to as EMT. The choice between the two adverse cellular responses to growth factor stimulation is restrained by the cell status: in sparse cultures of NBT-II cells, growth factors such as FGF-1 and EGF induce EMT while confluent cultures stimulated by the same growth factors are driven into the mitogenic pathway.

Our previous studies indicated that the type and the number of growth factor-receptors expressed at the surface of cells cultured at high versus low cell density do not account for the divergence between the two responses (40, 50). The two different functions are rather related to two distinct signaling pathways elicited by the growth factors in sparse versus confluent cultures (6). In a search of a protein that could signal differentially for an EMT response as opposed to mitogenesis, we had to consider that both EGF and FGF-1 can trigger EMT, and therefore that the signaling molecule should belong to the transduction pathways of either factor. For that reason, phosphatidylinositol 3-kinase, which is not activated by FGFR-1 and 4 (48) was an unlikely candidate. In contrast, activated FGF-R and EGFR have been shown to bind PLC-γ (32, 34). However, PLC-γ does not seem to play a role in FGF-1-induced chemotaxis (13) and mitogenesis (35). pp60 src is another common denominator of several growth factor-induced signaling pathways (15, 29, 31, 57). Moreover, the role of pp60 src in epithelial remodeling (52, 53) and in cell dissociation and dispersion (3, 24, 33) has been clearly established. It was therefore tempting to determine whether pp60 src played a role in the growth factor-induced EMT.

We therefore studied pp60 src activation under conditions promoting either cell dispersion or entry into cell division. In addition, we examined the effects of overexpression of a wild type or a constitutively activated form of c-src on the characteristics of FGF-1- and EGF-induced responses.

In NBT-II cells, a robust and sustained activation of pp60 src was observed in subconfluent cultures stimulated by FGF-1 and EGF. Growth factor-induced pp60 src activation was evidenced on growing and G0-arrested cells undergoing EMT (data not shown), suggesting that pp60 src activation was not related to G0-G1 transition. The duration of pp60 src activation in subconfluent cultures stimulated by either factor, as opposed to the weak and occasional activation of pp60 src elicited by a mitogenic signal (see below) is reminiscent of the sustained activation of MAP kinase in PC12 cells under conditions leading to cell differentiation but not to mitogenesis (16, 44).

In contrast to what was observed in subconfluent cultures, pp60 src was not activated between 15 and 90 min of FGF-1 and EGF stimulation in G0-arrested confluent cells. Although an early and very transient activation of...
pp60<sup>src</sup>, occurring before 15 min of stimulation cannot be ruled out, pp60<sup>src</sup> activation does not seem to be rapidly downregulated in most cases (20, 36, 57). In contrast to the lack of any observable pp60<sup>src</sup> stimulation during the G0-G1 transition of NBT-II cells, pp60<sup>src</sup> activation has been detected within minutes after addition of PDGF, CSF-1 and FGF-1 under mitogenic conditions (15, 29, 57). The apparent discrepancy between these results and ours might reside in the fact that the involvement of pp60<sup>src</sup> activity during the first hour of mitogenesis was demonstrated in normal fibroblasts, which could regulate G0-G1 transition by means different from those of epithelial transformed cells, such as NBT-II cell line. Activation of pp60<sup>src</sup> in confluent cultures could also happen at later time points (after 90-min stimulation), when the cell cycle has further progressed. In that respect, pp60<sup>src</sup> activation was observed in G2-M transition (1, 9, 17, 42) and might also be important during late G1 (5).

Stimulation of mitogenesis by FCS in subconfluent G0-arrested cultures does not induce the sustained activation of pp60<sup>src</sup> seen after stimulation with scatter factors. This suggested to us that, in NBT-II cells, pp60<sup>src</sup> activation could be implicated in cell dispersion rather than mitogenesis. To strengthen this notion, we examined the consequences of pp60<sup>src</sup> overexpression on the cellular responses to FGF-1 and EGF. Firstly, we found that overexpression of pp60<sup>src</sup> was clearly involved in spontaneous EMT-like modifications, as inferred from the significant percentage of cells that acquired fibroblastic-like features. However, the majority of cells remained epithelial, suggesting that pp60<sup>src</sup> overexpression was not sufficient to induce EMT. Interestingly, in pp60<sup>src</sup> overexpressors, cells with epithelial characteristics had the tendency to segregate from the population of fibroblastic-like cells, which were confined to the periphery of multicellular groups. We previously demonstrated that cells located at the edges of cell colonies are endowed with a positional information that sensitizes them to the dispersing effect of FGF-1 (50). Therefore, the observation that pp60<sup>src</sup> overexpressors segregate out of cell collectives suggested to us that pp60<sup>src</sup> kinase activity might be one key regulator of EMT, permitting the scattering of cells already committed, by their location, to the EMT pathway.

Moreover, the majority of cells transfected with the activated c-src gene retained some epithelial features, such as desmosomal junctions and the absence of vimentin expression, but acquired the elongated shape characteristic of mesenchymal cells. These observations strengthen the notion that an increase of pp60<sup>src</sup> kinase activity is not sufficient to induce the entire range of modifications referred to as EMT. Our results are in good agreement with those of Warren and Nelson (52, 53), showing that pp60<sup>src</sup> overexpression and low levels of pp60<sup>src</sup> expression in epithelial cells induce an epithelial remodeling rather than a complete loss of the epithelial phenotype.

The elevation of intracellular levels of cAMP was previously shown to induce the reversion of FGF-1-stimulated fibroblastic cells toward an epithelial phenotype (6). Since cAMP elevation had the same effect on spontaneously fibroblastic c-src transfectants, these cells were possibly resulting from a process of EMT. This result also indicated that cAMP effect takes place downstream of pp60<sup>src</sup> activation in cell scattering. As it has recently been demonstrated that cAMP negative effects on mitogenesis result from the inhibition of raf-1 activation (8, 14, 56), it would be interesting to know whether cAMP acts at the same level in NBT-II cells.

The involvement of pp60<sup>src</sup> activity in growth factor-induced epithelial cell dispersion was confirmed by further analyses of c-src overexpressors. Overexpression of non-mutated c-src gene, which correlated with increased levels of basal pp60<sup>src</sup> activity, rendered NBT-II cells more sensitive to the scattering activity of either EGF or FGF-1, as inferred from the displacement of the dose-response curves of cell dissociation and wound repair. It is noteworthy that w2 clone expressed twice more pp60<sup>src</sup> than w1, yet exhibiting a sensitivity to the scatter factors similar to that of w1. This result suggested that some other cellular components may have been limiting for the conveyance of the EMT signal. In contrast, FGF-1 and EGF inductions of cell mitogenesis were not modified. Accordingly, pp60<sup>src</sup> kinase activity was stimulated under conditions promoting cell dispersion but not mitogenesis.

Although less responsive than controls, cells transfected with the mutated c-src gene still exhibited a scattering response toward FGF-1 and EGF, while their mitogenic response was completely abrogated. We interpret these data as indicating that unregulated pp60<sup>src</sup> activity blocks the mitogenic but not the scattering response. However, it should be pointed out that if v-src overexpression was demonstrated to inhibit the mitogenic response of murine fibroblasts to EGF, overexpression of normal c-src in these cells resulted in an augmentation of EGF-responding cells (30). Therefore, although the effects of pp60<sup>src</sup> are thought to reflect only the unregulated kinase activity of the protein, it is plausible that pp60<sup>src</sup> and pp60<sup>src</sup> may interfere with distinct pathways. Nevertheless, the persistence of the scattering response together with the abrogation of the mitogenic responsiveness to FGF-1 and EGF in cells expressing an activated pp60<sup>src</sup> unambiguously indicated that the two responses were differentially affected by an unregulated increase in pp60<sup>src</sup> activity. The weak upregulation of the kinase activity of the mutant pp60<sup>src</sup> upon stimulation of EMT by FGF-1 or EGF presumably results from the fact that the high levels of basal kinase activity might either mask the augmentation by growth factors or already represent near-maximal enzymatic activities.

Our results argue for a key role of pp60<sup>src</sup> in the regulation of growth factor-induced epithelial cell scattering. These conclusions are also supported by the capacity of src interfering mutants to severely affect growth factor-induced EMT. In sharp contrast, cytokine-mediated entry into S phase of confluent cultures was not inhibited by the expression of SrcK<sup>-</sup>, confirming that Src might correspond to a diverging point between the two biological responses engendered by EGF and FGF-1 in NBT-II cells. They suggest furthermore that pp60<sup>src</sup> activation is an early event in the signaling pathway leading to EMT but not to mitogenesis. On the other hand, fibroblast locomotaxis towards PDGF requires the association of both PLC-γ and PI-3 kinase with the activated receptor, while Ras-GAP exhibits migration-suppressing activity (28, 55). Consistently, the autophosphorylation sites of EGF receptor are essential
for EGF-stimulated fibroblast movement (10) and binding of scatter factor to its cognate receptors activates Ras (22). Further experiments are therefore required to dissect the pathway involving pp60src, and particularly to examine whether pp60src regulates or acts in concert with some elements of the Ras pathway, as suggested in other models (27).

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