Src and Ras are involved in separate pathways in epithelial cell scattering

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We have demonstrated previously that Src controls the epidermal growth factor (EGF)-induced dispersion of NBT-II carcinoma epithelial cells. Here we show that while only Src and Yes were expressed and activated by EGF, microinjected kinase-inactive mutants of Src (SrcK⁻) and Fyn (FynK⁻) were able to exert a dominant-negative effect on the scattering response. Both SH2 and SH3 domains of FynK⁻ were required for inhibition of cell scattering. Expression of dominant-negative N17Ras also abrogated EGF-induced dispersion, showing that Ras is another regulator of cell dispersion. Expression of SrcK⁻ did not alter EGF-evoked Shc tyrosine phosphorylation, Shc-Grb2 complex formation and MAPK activation, three elements of the Ras pathway. Furthermore, the expression of Jun-Fos and Slug rescued the block induced by N17Ras but not by SrcK⁻, showing that Src kinases and Ras operate in separate pathways. In addition, actinomycin D inhibition of RNA synthesis repressed the ability of the activated mutant L61Ras but not that of F527Src to induce epithelial cell scattering. Since tyrosine phosphorylation of cytoskeleton-associated proteins pp125FAK and cortactin were abolished in EGF-stimulated SrcK⁻ cells, we concluded that, in contrast to Ras, Src kinases may control epithelial cell dispersion in the absence of gene expression and by directly regulating the organization of the cortical cytoskeleton. *Keywords:* epithelium–mesenchyme transition/growth factors/Ras/signaling pathway/Src protein tyrosine kinase

Introduction

Epithelial cells are able, in specific circumstances, to modulate their phenotype extensively. For example, their phenotype is altered dramatically during the epithelium–mesenchyme transition (EMT) identified during morphogenetic processes in embryos. EMT is characterized by the individualization of cells emerging and dissociating from epithelial structures. Such cell dissociation involves alterations in cell–cell adhesion systems. Depending on the species, this process is observed during gastrulation, neural crest cell emigration from the neural tube and organogenesis (reviewed in Duband *et al.*, 1995; Hay, 1995; Viebahn, 1995). Scattering of epithelial cells has

also been identified in adult tissues and is likely to play an important role in the process of carcinoma cell invasion and metastasis. Cells of epithelial origin can be induced to scatter by treatment with scatter factor/hepatocyte growth factor (HGF/SF) (Weidner *et al.*, 1990), and in some cases by other growth factors: members of the fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor (TGF) families have also been reported to possess potential scattering activities (Blay and Brown, 1985; Barrandon and Green, 1987; Geimer and Bade, 1991; Miettinen *et al.*, 1994). Depending on the cellular context, these scatter factors also act as growth factors, using specific signal transduction pathways in each case.

We previously have characterized a rat bladder carcinoma cell line, NBT-II, that is induced to scatter after stimulation by various growth factors (FGF-1, EGF, TGF- α) (Gavrilovic *et al.*, 1990; Vallés *et al.*, 1990; B.Boyer, unpublished data), all of which bind to tyrosine kinase receptors. Under these conditions, cell dissociation can be monitored by the loss of desmosomes from the cell periphery, which correlates with the acquisition of active cell migration and scattering (Boyer *et al.*, 1989). By using this cell line, we recently have shown that the cytoplasmic protein tyrosine kinase Src is a positive regulator of growth factor-induced epithelial cell dispersion (Rodier *et al.*, 1995). Src belongs to a family of non-receptor tyrosine kinases that comprises nine members, three of which, Src, Fyn and Yes, are expressed in a broad range of tissues. These enzymes have amino-terminal fatty acids which link them to the cytoplasmic face of membranes, they also have one SH3 and one SH2 domain, a catalytic domain and carboxy-terminal regulatory sequences. These kinases have a pivotal role in the regulation of a variety of biological processes that are associated with changes in cell morphology, including malignant transformation (Parsons and Weber, 1989), epithelial plasticity (Warren *et al.*, 1988) and modulation of intercellular adhesion (Volberg *et al.*, 1991). In addition, Src family kinases are required in EGF-, platelet-derived growth factor (PDGF)- and colony-stimulating factor 1 (CSF-1)-induced mitogenesis and cell division in fibroblasts (Luttrell *et al.*, 1988; Twanley-Stein *et al.*, 1993; Roche *et al.*, 1995a).

The function of each individual member has been unraveled by genetic analysis in mice, which revealed the partial overlapping function among the Src family during development (Imamoto *et al.*, 1994; Lowell *et al.*, 1994; Thomas *et al.*, 1995). Loss-of-function mutations in the *src* gene family did not produce the expected perturbations in mouse embryogenesis, whereas combinations of double null mutants led to increased lethality. Consistent with these data are experiments demonstrating that a Src-specific neutralizing antibody inhibited cell division in

fibroblasts expressing only Src but not in fibroblasts expressing several members of the Src family (Roche *et al.*, 1995b). On the other hand, genetic crosses between *csk*⁻ and *fyn*⁻ or *src*⁻ mutant mice suggested that Src might have both specific and redundant functions in regulation of the cytoskeleton (Thomas *et al.*, 1995).

The mechanisms whereby Src kinases exert their functions are still debated. Three hypotheses have emerged. Src activity may affect specific substrates which are mainly cytoskeletal-based components, and molecules localized in cell-cell and cell-substrate adhesion sites (Kellie *et al.*, 1991; Wu *et al.*, 1991; Clark and Brugge, 1993; Okamura and Resh, 1995), the tyrosine phosphorylation of which could in turn modify the cellular architecture. Src activity may interact with the Ras signaling pathway. For example, activated Src phosphorylates Shc, an early element of the Ras cascade (McGlade *et al.*, 1992; Nagai *et al.*, 1995). Finally, Src may also generate a unique pathway that culminates in the activation of specific transcription factors. In that respect, it has been shown recently that Src family tyrosine kinases control the entry into S-phase of fibroblasts by inducing Myc expression independently of Ras activity (Barone and Courtneidge, 1995). The three proposed roles of Src in cell signaling are not mutually exclusive and might depend on the cellular context and on the response in which Src is activated.

In this study, we investigated the role of Src kinase activity during epithelial cell scattering and its relationship to both the Ras pathway and transcription factors potentially involved in this response.

Results

Functional overlap among Src family kinases

Since we previously had demonstrated the involvement of c-Src in EGF-mediated NBT-II cell scattering, we asked whether other members of the Src family were also implicated in this process. First, Src, Fyn and Yes kinase activities were measured in both unstimulated and EGF-stimulated NBT-II cells. After immunoprecipitation with antibodies specific to each protein, *in vitro* activity was assessed by using acid-denatured enolase as an exogenous substrate. As shown in Figure 1A, Yes was expressed in NBT-II cells and activated 1.9-fold after 15 min EGF stimulation. This was similar to the increase of Src activity measured under the same conditions (2.2 times higher than the basal level). In contrast, Fyn activity was undetectable, indicating that the kinase is not expressed in these cells. The levels of Src or Yes in unstimulated and stimulated cells were similar, demonstrating the specificity of the observed increase in kinase activity in EGF-stimulated cells (data not shown).

We next tested whether a dominant-negative mutant form of Fyn (FynK⁻) was able to suppress the function of the endogenous Src family members. Desmoplakin (DP) immunolocalization was used as a marker of cell scattering. In epithelial NBT-II cells, DP is concentrated in cell-cell contacts, from which it disappears when EGF-induced cell scattering occurs. An expression vector coding for FynK⁻ was microinjected into the nuclei of NBT-II cells seeded on coverslips prior to EGF stimulation. After overnight incubation, cells were fixed and double immunostained for Fyn overexpression and DP immunoreactivity. Statistical

Fig. 1. Src family kinases share redundant functions in cell scattering and require both SH2 and SH3 domains of the molecule.

(A) Activation of Src and Yes kinases during EGF-induced cell scattering. NBT-II cell cultures were incubated with (+) or without (-) 20 ng/ml EGF for 15 min. After cell lysis, immunoprecipitation of Src, Fyn and Yes was performed, followed by an *in vitro* kinase assay using denatured enolase as a substrate. Gels were treated with KOH before autoradiography. The upper band corresponds to the autophosphorylated Src family kinase and the lower band to phosphorylated enolase as indicated. All antibodies used recognize rat species. (B) Effect of mutated forms of Src and Fyn expression on EGF-induced cell scattering. NBT-II cells seeded on coverslips were microinjected into the nucleus with 100 ng/ml of the indicated expression vectors. Four hours later, EGF (20 ng/ml) was added (+) or not (-) to the cells, and 18 h later they were fixed and immunostained for both ectopic protein and desmosome expression as described in Materials and methods. For each experiment, several coverslips were analyzed and the percentage of desmosome-negative cells calculated. The results have been averaged from several experiments ($n > 3$) and from at least 200 microinjected cells. The means and SEM are shown.

analysis of data obtained from several independent experiments are shown in Figure 1B. In the absence of stimulation, only 10% of cells dissociated spontaneously, as shown by DP expression. EGF treatment induced >85% cells to dissociate, and this response was blocked by FynK⁻ in >80% of the cells. The block was specific since expression of wild-type Fyn did not affect the EGF-induced response. The inhibiting activity of FynK⁻ was

Fig. 2. Ras activity is required during EGF-induced cell scattering. NBT-II cells were microinjected as described in the legend to Figure 1 with the indicated constructs. After incubation, cells were immunostained for N17Ras and L61Ras expression as well as for desmosome breakdown. The percentage of desmosome-negative cells was calculated. Results from several ($n > 3$) experiments have been averaged. The means and SEM are shown.

almost as efficient as that of SrcK⁻. Since Fyn is not expressed in NBT-II cells, these results indicate that the dominant-negative function is carried by domains common to at least Src and Fyn.

We next analyzed whether the SH2 and SH3 domains of the molecule were necessary for the scattering function. Plasmids encoding Fyn lacking the kinase domain (FynΔK), Fyn lacking the kinase domain and the SH2 domain (FynΔKΔSH2) or Fyn lacking the kinase domain and the SH3 domain (FynΔKΔSH3) were microinjected into cells (Figure 1B). Neither FynΔKΔSH2 nor FynΔKΔSH3 suppressed the scattering activity of EGF. In contrast, FynΔK had the same blocking activity as FynK⁻ and SrcK⁻, indicating that the lack of dominant-negative effects of FynΔKΔSH2 and FynΔKΔSH3 did not result from the absence of the kinase domain. None of the constructs had any effect on cell dissociation in the absence of EGF, showing that the cell response was not due to ectopic protein expression. Taken together, these results show that both SH2 and SH3 domains of Src kinases are required for the molecule to exert its role in cell scattering.

EGF-induced cell scattering requires Ras activity

The Ras pathway has been implicated in regulation of cell scattering and motility (Graziani *et al.*, 1993; Hartmann *et al.*, 1994). We therefore tested whether Ras was also involved in EGF-induced cell scattering in NBT-II cells. Constructs encoding a dominant-negative (N17Ras) or activated (L61Ras) form of Ras were microinjected in NBT-II cells. As shown in Figure 2, constitutively activated Ras (L61Ras) induced cell dissociation even in the absence of EGF. Conversely, a dominant-negative form of Ras (N17Ras) blocked desmosome internalization in EGF-stimulated cells. Altogether, these data confirm a positive role for Ras in EGF-induced cell dispersion.

The Ras pathway is unaffected in SrcK⁻ cells

We next asked whether Src kinase activity interacts with the Ras pathway. Several steps of the Ras cascade were

Fig. 3. SrcK⁻ expression does not alter EGF-evoked MAPK activation. (A) p42/44MAPK is activated during EGF treatment of NBT-II and SrcK⁻ cells. Parental (left panel) and SrcK⁻-transfected NBT-II cells (right panel) were stimulated with 20 ng/ml EGF for the indicated times in minutes. p42/44MAPK was isolated from cell lysates by immunoprecipitation, and an *in vitro* kinase assay was performed using MBP as a substrate. Radioactivity incorporated into MBP was visualized by autoradiography. The experiment was repeated with three different transfected cell lines with similar results. (B) JNK1 is weakly activated by EGF in NBT-II and SrcK⁻ cells. Cultures of parental (left panel) and SrcK⁻-transfected NBT-II cells (right panel) were stimulated with 20 ng/ml EGF for the indicated times. JNK1 was immunopurified from cell lysates and JNK1 activity was measured in a kinase assay using ATF-2 as a substrate.

examined in NBT-II cells stably transfected with a *src* gene encoding a kinase-inactive Src mutant enzyme (SrcK⁻ cells). We previously demonstrated that SrcK⁻ cells are defective in their ability to scatter in response to EGF (Rodier *et al.*, 1995). The effect of SrcK⁻ expression on EGF-induced p42/p44 MAPK and Jun kinase (JNK) 1 activation, two downstream elements of the Ras pathway, was first examined. *In vitro* p42/p44 MAPK and JNK1 activities were assayed using myelin basic protein (MBP) and ATF-2, respectively. EGF stimulated the up-regulated MAP kinase activity, resulting in a dramatic increase of MBP phosphorylation within minutes of stimulation (Figure 3A). No significant differences in MAP kinase activation were detected in SrcK⁻ cells when compared with NBT-II parental cells. Also, a weak but reproducible increase of EGF-induced JNK1 activity was observed, but again no difference was detected when comparing both cell types (Figure 3B). These data suggest that Ras-dependent MAP kinase and JNK1 activations are not linked directly with Src signaling. Furthermore, the fact that JNK1 was weakly activated during EGF treatment suggests that it may not be of pivotal importance for NBT-II cell scattering.

We next examined the interaction of Src kinases with early elements of the Ras cascade. The adaptor protein Shc becomes tyrosine phosphorylated following growth factor stimulation and associates with the Grb2-SOS complex leading to Ras activation. Shc was also shown to be phosphorylated by activated Src kinases (McGlade *et al.*, 1992; Nagai *et al.*, 1995), and was therefore a good candidate for linking Src and Ras activity. As shown in Figure 4, EGF induced a strong and rapid tyrosine phosphorylation of both p46 and p52 Shc isoforms which correlated with an increase in Shc-Grb2 complex formation. No differences were observed when comparing parental and SrcK⁻ NBT-II cell lines. Collectively, our data point to a divergence of the Src kinases and Ras pathways at the immediate post-receptor stage which could

Fig. 4. SrcK⁻ expression does not affect early steps of Ras activation. Parental (left panels) and SrcK⁻-transfected cells (right panels) were stimulated with 20 ng/ml EGF for the indicated times in minutes. Shc was immunoprecipitated from cell lysates and subjected to immunoblot analyses with anti-phosphotyrosine (A), anti-Grb2 (B) and anti-Shc antibodies (C). Similar results were obtained from two other SrcK⁻-transfected cell lines.

be accomplished by differential activation of effector molecules.

The downstream effectors of Ras are not involved in Src kinase signaling cascade

To confirm the functional independence between Src and Ras signals during the scattering response, we determined whether they share the same effector molecules. Among transcription factors potentially implicated in EGF-induced Src- or Ras-mediated cell scattering, three were chosen for these studies: Myc, a downstream effector of Src kinases in the fibroblast mitogenic response (Barone and Courtneidge, 1995), the Jun-Fos complex, a downstream effector of Ras which has been shown to induce a scattering response in epithelial cells (Reichman *et al.*, 1992; Fialka *et al.*, 1996), and Slug, a transcription factor of the *snail* gene family which has been implicated in embryonic EMTs (Nieto *et al.*, 1994). Microinjection of Jun-Fos- or of Slug-encoding plasmids led to a significant restoration of the EGF-induced scattering response in N17Ras-expressing cells (Figure 5A). On the other hand, Myc expression did not affect the N17Ras block, indicating that the rescue effects were specific to the transcription factor tested. In sharp contrast, none of these nuclear proteins was able to rescue the block induced by SrcK⁻ (Figure 5B). Similar results were obtained when microinjected cells were identified by the ectopic expression of the transcription factors, showing that the absence of cell scattering was not due to the lack of construct expression (data not shown). Therefore Jun-Fos and Slug may correspond to downstream elements of the Ras but not of the Src pathway, demonstrating that Src kinases and Ras generate separate signaling cascades for cell scattering.

Fig. 5. The block to EGF-induced cell scattering elicited by N17Ras, but not SrcK⁻, is released by Jun-Fos and Slug expression. NBT-II cells seeded onto coverslips were microinjected with N17Ras- (A) or SrcK⁻ (B) encoding plasmids, in the presence or absence of Jun-Fos, Slug or Myc constructs as shown, and stimulated (+) or not (-) with EGF (20 ng/ml). Cells were fixed and processed for immunofluorescence labeling 18 h later. Co-detection of Src or Ras and DP was essentially as described in Materials and methods. For each lane, >200 cells were analyzed and the percentage of desmosome-negative cells calculated. Results from at least three independent experiments have been averaged. The means and SEM are shown.

Inhibition of RNA synthesis does not affect F527Src-mediated cell scattering

Since none of the transcription factors tested was linked to the Src pathway, we next asked whether Src kinases require DNA transcription to transmit the scattering response. To address this question, cells were treated with actinomycin D for 30 min, leading to irreversible inhibition of RNA synthesis from chromosomal DNA (Yung *et al.*, 1990). Four hours after the removal of the drug, cells were injected with plasmids encoding an active form of Src (F527Src) or Ras (L61Ras). Under these conditions, transcription from plasmid DNA was only partially inhibited, resulting in ectopic protein expression in the absence of cellular gene expression. After overnight incubation, cells were fixed and processed for immunofluorescence. In the absence of actinomycin D, transient expression of activated L61Ras and F527Src induced cell dispersion as inferred from DP immunostaining (Figure 6, left panel). In addition, microinjected cells were distant

Fig. 6. Actinomycin D inhibits L61Ras-, but not F527Src-mediated cell dispersion. Left panel: NBT-II cells were seeded on coverslips and treated with actinomycin D for 30 min. After removal of the drug, cells were incubated for 18 h in the presence of 20 ng/ml EGF (A). Alternatively, cells were treated (D, E, H and I) or not (B, C, F and G) with actinomycin D for 30 min prior to microinjection with F527Src (B, C, D and E) or L61Ras constructs (F, G, H and I) and further incubated for 18 h. Cells were fixed and immunostained with anti-DP antibody (A). Microinjected cultures were double immunolabeled for the expression of DP (B and D) and Src (C and E) or DP (F and H) and Ras (G and I). Note that actinomycin D treatment prevents the EGF-induced disappearance of DP from the cellular cortex (A) and cell scattering in L61Ras-injected cells (H) whereas it does not affect dissociation of F527Src-expressing cells (D). Arrows point to injected cells. Bar, 10 μ m. Right panel: three independent experiments have been analyzed and the results averaged. For each lane, at least 50 cells have been counted. The mean and SEM are shown.

from the epithelial islands, indicating that these cells had effectively migrated from the epithelial structures. Therefore, activated forms of Ras or Src may be sufficient to ensure cell scattering. Actinomycin D treatment blocked cell dispersion induced by both EGF stimulation and L61Ras expression (Figure 6, right panel), showing that RNA synthesis had to take place for cell scattering to occur. In sharp contrast, actinomycin D treatment did not affect cell scattering induced by F527Src expression. Therefore, activated Src may function in cell scattering at specific times without the need for gene expression. This result is consistent with the idea that the Src pathway does not lead to the activation of specific transcription factors for cell scattering.

We next asked whether the observed Src signal was specific to NBT-II cells or more general to other epithelial cells. The kidney epithelial cell line MDCK scatters after

SF/HGF treatment and was used for subsequent studies. As shown in Figure 7A, SF induced a Src kinase activation, suggesting that Src may also be involved in MDCK cell scattering. The increase in activity was detected after 8 min of stimulation and peaked at 30 min (3.6-fold the basal level) similarly to what was observed in both EGF- and FGF-1-stimulated NBT-II cells (Rodier *et al.*, 1995). Accordingly, transient expression of F527Src in MDCK cells induced >90% of the cells to scatter, similar to results obtained by transient expression of L61Ras and by SF stimulation (Figure 7B). Actinomycin D treatment completely blocked both SF- and oncogenic L61Ras-dependent scattering of MDCK cells but had no effect on F527Src-dependent cell dispersion (Figure 7B). Therefore, a common feature of epithelial cell scattering may be the involvement of Src kinases acting in a pathway independent of the induction of gene expression.

Fig. 7. SF-induced MDCK cell scattering stimulates Src activity which exerts its functions independently of RNA synthesis. (A) Src kinase activity is stimulated by SF. MDCK cells were stimulated with 20 ng/ml SF for the indicated times in minutes prior to cell lysis. Src from cell extracts was immunoprecipitated and subjected to an *in vitro* kinase assay using enolase as substrate (upper panel). Gels were treated with KOH before autoradiography. In the lower panel, the immunoprecipitated proteins were immunoblotted with anti-Src antibodies. (B) Actinomycin D (Act) does not perturb F527Src-mediated cell scattering. MDCK cells were seeded on coverslips and treated with actinomycin D for 30 min prior to microinjection with F527Src or L61Ras. Eighteen hours later, cells were fixed and processed for double immunofluorescence labeling of Src and DP or Ras and DP. For each lane, >50 cells were analyzed. Results from independent experiments have been averaged. The mean and SEM are shown.

L61Ras induces Src-dependent cell scattering

According to the results described above, Src and Ras were required independently for EGF-induced cell dispersion while either oncogenic-like Src or Ras alone was sufficient to induce cell scattering. These data suggested that, in contrast to their cellular counterparts, oncogenic-like Ras and Src may be connected in a common signaling cascade. To examine this hypothesis, functional inter-

Src and Ras act independently in cell scattering

Fig. 8. V12Ras induces Src-dependent cell scattering. NBT-II cells seeded on coverslips were microinjected with the indicated constructs. After incubation, cells were immunostained for Ras and Src expression as well as for desmosome breakdown. The percentage of DP-negative cells was calculated. Results from three independent experiments have been averaged. The means and SEM are shown.

actions between activated Src and Ras were investigated by overexpressing dominant-negative and activated mutants of Src and Ras in NBT-II cells. As shown in Figure 8, SrcK⁻ blocked the ability of L61Ras to induce cell dispersion, whereas inhibition of Ras activity had no effect on F527Src-induced cell scattering. These data proved the requirement for Src in Ras-mediated cell scattering. They also demonstrated a sequential sequence of Ras and Src actions, with L61Ras acting first to induce Src activation.

Src activity affects cytoskeleton-based components

Since none of the transcription factors tested appeared to be downstream of Src in the scattering response, it was essential to ensure that Src had effective targets. Several cytoskeleton-associated proteins are *in vivo* substrates of oncogenic Src, including the actin-binding protein cortactin and the cytoplasmic tyrosine kinase FAK. Phosphorylation of cortactin correlates with association with Src, and phosphorylation of FAK correlates with an increase in its kinase activity. As shown in Figure 9, a strong association of cortactin with Src was detected in NBT-II cells within minutes of EGF stimulation and persisted up to 90 min. This association coincided with a robust increase in tyrosine phosphorylation of cortactin. In contrast, both molecular events were abolished in SrcK⁻ cells (Figure 9), under conditions where cortactin levels were not affected by SrcK⁻ expression (data not shown). Similarly, FAK tyrosine phosphorylation underwent a marked increase after EGF stimulation (Figure 9) that correlated with an increase in its enzymatic activity as assessed by its ability to autophosphorylate *in vitro*. Again, both molecular events were strongly inhibited in EGF-stimulated SrcK⁻ cells. Therefore, both cortactin and FAK phosphorylation may be involved for Src kinases to transmit a scattering signal. Interestingly, although p120Cas and paxillin have been found to be Src substrates *in vivo*, they were not tyrosine phosphorylated in EGF-stimulated NBT-II cells (data not shown).

Fig. 9. SrcK⁻ expression abrogates EGF-induced tyrosine phosphorylation of cortactin and FAK. Extracts of parental (left panels) and SrcK⁻-transfected cells (right panels) stimulated with EGF for the indicated times in minutes were immunoprecipitated with anti-cortactin antibody and immunocomplexes were immunoblotted with anti-phosphotyrosine antibody (A). Alternatively, cell extracts were immunoprecipitated with monoclonal anti-Src antibody and immunoblotted with anti-cortactin antibody (B). (C) Cell extracts were immunoprecipitated with anti-FAK antibody followed by an *in vitro* autophosphorylation assay. Gels were treated with KOH before autoradiography.

Discussion

We investigated the mechanisms whereby Src controls growth factor-induced cell scattering. Bladder carcinoma NBT-II cells were used in this study because of their striking scattering response after growth factor stimulation. We first observed that, in addition to Src, Yes, another ubiquitous Src kinase, is expressed in these cells and becomes activated during EGF-induced cell dispersion, indicating that both enzymes may be involved in this process. Expression of a dominant-negative form of Src (SrcK⁻) abolished cell scattering, as demonstrated by both transient (this study) and stable expression experiments (Rodier *et al.*, 1995), suggesting that either Yes is not important or that there is a functional redundancy among Src family members. The latter hypothesis was confirmed by results that show that FynK⁻ expression blocks the scattering response in NBT-II cells which do not express Fyn. A redundancy of function has been already shown in the mitogenic response of fibroblasts induced by several growth factors, during cell division as well as during mouse embryogenesis (Lowell *et al.*, 1994; Roche *et al.*, 1995a; Thomas *et al.*, 1995). Therefore, Src and Yes may also have redundant functions during cell scattering, probably by phosphorylating common substrates important for signaling.

Testing of deletion mutants of Fyn by microinjection pointed to regions of the kinases important for signaling, i.e. the catalytic domain, the SH2 and the SH3 domains. Deletion of either the SH2 or SH3 domain abolished the interfering function of FynΔK, suggesting that both domains are required for efficient signaling. How do they act? In a model in which the exogenously overexpressed kinase-defective mutant competes with the endogenous Src kinases for binding sites, one could postulate that in the absence of either domain, the kinase-defective mutant has a low affinity for its binding partners, preventing it from being an efficient competitor for the endogenous

molecules. Alternatively, SH2 and SH3 domains may be involved simultaneously for efficient binding of target molecules, as recently shown for the cytoskeleton-associated protein p130Cas or the RNA-binding protein Sam68 (Nakamoto *et al.*, 1995). In either case, the requirement for SH2 and SH3 domains for Src function is not surprising. For example, PDGF-induced entry into S-phase of quiescent fibroblasts needs the presence of intact SH2 (Twanley-Stein *et al.*, 1993) and SH3 domains (Broome and Hunter, 1996; Erpel *et al.*, 1996).

Since the Ras pathway has been implicated in SF signaling (Graziani *et al.*, 1993), we examined whether Ras was another regulator of EGF-induced NBT-II cell scattering. We observed that the oncogenic form of Ras was sufficient to induce cell dispersion in the absence of growth factor, suggesting that it is a key regulator of cell scattering. Consistently, Ras is also necessary for the EGF response, as shown by the N17Ras microinjection experiments. Therefore, since both Src kinases and Ras are required for the EGF-induced cell scattering, none of them may be sufficient alone for efficient EGF signaling. In NBT-II cells, EGF-induced Ras activation is likely to result, at least in part, from Shc tyrosine phosphorylation followed by Shc-Grb2 association, initiating the Ras cascade (see Figure 4). Accordingly, overexpression of Shc amplifies the scattering of MDCK cells in response to SF (Pellicci *et al.*, 1995). Downstream elements of the Ras pathway in NBT-II cells may lead primarily to MAPK activation. Indeed, a robust EGF-induced activation of p44MAPK was observed, which contrasted with the very weak activation of JNK 1. This result is consistent with data demonstrating a strict specificity of activation of the different MAPKs by individual external stimuli (Brunet and Pouyssegur, 1996).

The Ras cascade may end in the nucleus, since microinjections of Jun-Fos or Slug constructs were able to rescue the block caused by the expression of N17Ras. A

role for Jun–Fos in the initiation of cell scattering is also supported by observations that the expression of activated Jun in mammary epithelial cells causes the disruption of intercellular junctions (Fialka *et al.*, 1996) while activation of c-Fos induces epithelial–fibroblastoid conversion (Reichman *et al.*, 1992).

The mechanism by which Src kinases exert their function for epithelial cell scattering was next examined. Two hypotheses were considered: Src kinases may function by regulation of other signaling molecules leading to gene expression, or by the reorganization of cytoskeleton and the modulation of cellular junctions. A link between Src and Ras activities has been established previously, since the serine/threonine kinase MAPK is regulated upstream by Ras and an Src-dependence of the MAPK activation has been shown for G-coupled receptors (Divik *et al.*, 1996; Schieffer *et al.*, 1996; Wan *et al.*, 1996). Src kinases may also up-regulate the Ras activity via phosphorylation of Shc (Li *et al.*, 1996). Moreover, activated forms of Src may up-regulate the serine/threonine kinase Raf-1, an effector of Ras, and normal Src has also been shown to control Ras activation and Ras–Raf-1 complex formation (Schieffer *et al.*, 1996). However, no interaction between Src kinases and the first elements of the Ras pathway was observed in EGF-stimulated NBT-II cells. EGF-induced MAP kinase activation of Shc phosphorylation and Shc–Grb2 association were not affected in SrcK[−] cells, indicating that Shc was not a direct substrate of Src. Shc might rather associate directly with the activated EGF receptors, which do not bind Src (B. Boyer, unpublished observations). Finally, neither Fos–Jun nor Slug expression, two nuclear events of the Ras cascade, rescued the EGF scattering response in cells expressing SrcK[−]. Collectively, these data clearly show that Src kinases and Ras are involved in separate pathways for the cell scattering response. Interestingly, Myc overexpression was not able to rescue SrcK[−] cells. In contrast, constitutive expression of Myc overcame the SrcK[−]-mediated block of DNA synthesis in PDGF-stimulated fibroblasts (Barone and Courtneidge, 1995). The apparent discrepancy between these results and ours may reflect the specific commitment of Myc in mitogenic responses (Haltmeier and Rohrer, 1990). Alternatively, it may illustrate differences of signals operating in normal fibroblasts versus transformed epithelial cells. In that context, it is noteworthy that tumor-derived epithelial cells express much higher levels of c-Myc than transformed fibroblasts (Martel *et al.*, 1995).

The fact that Src did not control any of the transcription factors tested suggested to us that the 'Src pathway' involved in epithelial cell dispersion is different from the one involved in fibroblast S-phase entry and may not lead to changes in gene expression. Cytoskeleton organization and cellular adhesion may rather be regulated by Src-dependent modifications of target proteins localized in the vicinity of the kinase. To strengthen this notion, actinomycin D was used to block RNA synthesis in cells in which activated F527Src and L61Ras were transiently expressed or stimulated by EGF. As expected, under those conditions, both EGF- and L61Ras-induced cell scattering was inhibited whereas cell dispersion elicited by F527Src was not perturbed. This effect was not specific to the epithelial cell line used since similar results were obtained

with the epithelial cell line MDCK. Therefore, F527Src may trigger cell scattering in the absence of transcription.

In addition to being required for EGF-induced cell dispersion, Src appeared to be a downstream element of L61Ras-induced cell scattering. Accordingly, Ras-regulated fibroblast cell locomotion is impaired in Src(−/−) mice (Hall *et al.*, 1996) while, in contrast, v-Src-dependent cell differentiation is controlled by Ras activity (Kremer *et al.*, 1991). These data suggest that Src generates a unique pathway for cell scattering and motility, regardless of its mode of activation (i.e. growth factors or oncoproteins).

The fact that Src can induce cell scattering in the absence of gene transcription is in apparent contradiction to the common need for gene expression for SF-, EGF- and L61Ras-induced responses. However, although both oncogenic Src and Ras induced desmosome breakdown in the absence of growth factor, F527Src did not have the same striking scattering effect as L61Ras. The response was restricted to sparse colonies and, more specifically, to the border cells. In contrast, L61Ras induced a strong scattering response that was not restricted to sparse colonies. One explanation would be that gene expression is required for efficient cell scattering except for cells present at the borders of the colonies (maybe because of differences in gene expression). This would explain why F527Src triggers scattering of border cells even in the presence of actinomycin D and not of cells within the colony. Nevertheless, these data strongly suggest that the Src pathway does not need gene expression for cell scattering. In that case, what are the effectors of Src kinases? It is likely that Src kinase activities primarily regulate tyrosine phosphorylation of cytoskeleton-based components important for cell scattering. Our data suggest that Src kinases regulate tyrosine phosphorylation of both cortactin and FAK after EGF stimulation. Interestingly, other known Src substrates, including paxillin and p120Cas, were not tyrosine phosphorylated after EGF stimulation, although expressed in NBT-II cells. This indicates that Src kinases may select their substrates. Whether cortactin and FAK effectively control cell scattering needs to be clarified further.

In summary, our data strongly suggest that Ras and Src kinases define separate pathways, both of which are required for growth factor-induced epithelial cell scattering. In addition, whereas the Ras pathway may end in the nucleus by AP-1 complex activation, Src kinases may transmit their signal in the absence of gene expression by phosphorylating a restricted range of substrates, all of which are cytoskeleton-based components.

Materials and methods

Constructs and antibodies

cDNAs containing the complete coding sequence of the kinase-active and -inactive forms of Src and Fyn, as well as FynΔKASH2 and FynΔKASH3 and the inactive form of Ras (N17Ras), have been described elsewhere (Twanley-Stein *et al.*, 1993; Barone and Courtneidge, 1995). cDNA encoding the active form of Ras (L61Ras) was kindly provided by Dr R. Brambilla (EMBL, Heidelberg). Affinity-purified anti-Src family (anti-cst1), anti-Fyn and anti-Yes polyclonal antibodies have been described elsewhere (Kypta *et al.*, 1990; Roche *et al.*, 1995a). Anti-Src mAb 327 was purchased from Oncogene Science and monoclonal anti-human focal adhesion kinase (pp125FAK) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Monoclonal anti-p42/44

MAPK, anti-Myc (9E10) and rabbit anti-Fos antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-Shc and anti-cortactin antibodies were from Upstate Biotechnology Inc., mouse anti-Grb2 antibody was from Transduction Laboratories (Lexington, KY) and rabbit anti- β -galactosidase antibody was from Rockland (Gilbertsville, PA). Monoclonal rat anti-Ras antibody (Y13 259) was kindly provided by Dr J. Downward (ICRF, London), 4G10 monoclonal anti-phosphotyrosine was the generous gift of Dr B. Druker (Harvard Medical School, Boston, MA) and anti-JNK 1 antibody was kindly provided by Dr B. Derijard (Centre de Biochimie du CNRS, Nice, France). Texas red-conjugated anti-mouse antibody was purchased from Jackson Laboratories (West Grove, PA); fluorescein isothiocyanate (FITC)-coupled anti-rabbit antibody, biotinylated anti-rat antibody and FITC-conjugated streptavidin were obtained from Amersham International (Buckinghamshire, UK).

Cell culture, microinjection and immunofluorescence

NBT-II and MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Three different SrcK⁻-transfected clones derived from the NBT-II cell line (Rodier *et al.*, 1995) were routinely cultured in DMEM containing 10% FCS and geneticin at 400 μ g/ml (G418 sulfate; Gibco BRL, Gaithersburg, MD). Cells were injected with 100 ng/ml plasmids in the nucleus 2 days after seeding. At least 100 cells were injected on each coverslip. Cells were incubated at 37°C for 4 h prior to stimulation with EGF (Sigma, St Louis, MO) or SF (R & D Systems, Minneapolis, MN). After overnight incubation, microinjected or growth factor-treated cells on coverslips were rinsed in phosphate-buffered saline (PBS) before being fixed for 5 min at -20°C in methanol. Coverslips were dried and the cells were double-labeled as follows: SrcK⁻, SrcK⁺ and Src Δ K-injected cells were incubated with a mixture of polyclonal anti-cst1 antibody (1:100) and anti-DP antibody (1:50) for 60 min, then washed extensively with PBS before a 60 min incubation with FITC-conjugated anti-rabbit antibody (1:100) and Texas red-conjugated anti-mouse antibody (1:100). After the final washes in PBS, coverslips were rinsed briefly in H₂O and ethanol, air-dried and mounted in Moviol. Coverslips were examined by a Zeiss Axiophot microscope. Images were recorded on T-Max 3200ASA film. Negatives were scanned using a Nikon scanner and images were treated by Adobe Photoshop software.

Fyn Δ KASH2- and Fyn Δ KASH3-injected cells were visualized with rabbit anti- β -galactosidase antibody (1:1000) and FITC-conjugated anti-rabbit antibody (1:100). L61Ras- and N17Ras-injected cells were recognized by the rat monoclonal anti-Ras antibody (1:50), followed by biotinylated anti-rat antibody (1:100) and FITC-conjugated streptavidin (1:100).

Myc-injected cells were visualized with 9E10 antibody (1:30) followed by FITC-coupled anti-mouse antibody (1:100). Fos-injected cells were recognized by anti-Fos antibody (1:50) followed by FITC-coupled anti-rabbit antibody.

Biochemistry

The activity of Src family kinases was assayed as described in Rodier *et al.* (1995).

JNK 1 and MAP kinase assays were performed essentially as described (Derijard *et al.*, 1994). After EGF stimulation, cells were lysed in Triton X-100-containing buffer. The extracts containing equivalent amounts of proteins were pre-cleared with protein A-Sepharose, then incubated for 1 h with antibody specific to the kinases pre-bound to protein A-Sepharose beads. Immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer (25 mM HEPES, pH 7.5, 25 mM MgCl₂, 25 mM β -glycerophosphate, 0.1 mM Na orthovanadate) before 20 min incubation at 30°C in kinase buffer containing 6 μ g of MBP (for MAP kinase assay) and 3 μ g of ATF-2 (for JNK 1 assay) as substrate, 50 μ M ATP and 10 μ Ci [γ -³²P]ATP. The reactions were terminated with Laemmli sample buffer and the products were resolved by SDS-PAGE. FAK kinase activity was assayed as follows: after EGF stimulation, cells were lysed in a buffer consisting of 10 mM Tris pH 7.5, 50 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 50 mM sodium fluoride and 10 mM Na orthovanadate. Five hundred μ g of proteins were immunoprecipitated with 4 μ g of anti-FAK antibody pre-bound to protein G-Sepharose beads. Immunoprecipitates were washed three times in lysis buffer and once in kinase buffer (20 mM HEPES pH 7.5, 5 mM MnCl₂, 5 mM MgCl₂, 1 mM dithiothreitol, 10 μ Ci of [γ -³²P]ATP) before a 30 min incubation at 25°C in 30 μ l of kinase buffer. The products of the reaction were resolved by SDS-PAGE. The gels were treated with 1 M KOH at 56°C for 30 min before autoradiography.

Immunoprecipitations of Shc and cortactin were performed according to the manufacturer's instructions and immunoblotting was done as described previously (Rodier *et al.*, 1995).

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