

v-Src accelerates spontaneous motility via phosphoinositide 3-kinase, phospholipase C and phospholipase D, but abrogates chemotaxis in Rat-1 and MDCK cells

Anna Platek¹, Marcel Mettlen¹, Isabelle Camby², Robert Kiss², Mustapha Amyere¹ and Pierre J. Courtoy^{1,*}

¹CELL Unit, Christian de Duve Institute of Cellular Pathology, Université catholique de Louvain, UCL 75.41, Avenue Hippocrate, 75, 1200 Bruxelles, Belgium

²Laboratory of Toxicology, Université Libre de Bruxelles, Boulevard du Triomphe, 1050 Bruxelles, Belgium

*Author for correspondence (e-mail: courtoy@cell.ucl.ac.be)

Accepted 14 June 2004

Journal of Cell Science 117, 4849-4861 Published by The Company of Biologists 2004
doi:10.1242/jcs.01359

Summary

In Rat-1 fibroblasts, v-Src causes a profound remodelling of cortical actin cytoskeleton. This transformation includes membrane ruffling, a hallmark of the leading edge in migrating cells, and results from activation of phosphoinositide 3-kinase (PI 3-kinase), phospholipase C (PLC) and phospholipase D (PLD). We therefore re-examined whether motility is constitutively triggered by v-Src and studied whether this response is controlled by the same signalling pathway. The study was performed using Rat-1/tsLA29 and MDCK/tsLA31 cells, each harbouring a different thermosensitive v-Src kinase, active at 34°C but inactivated at 40°C. In both cell lines, overnight v-Src activation induced transformation and accelerated spontaneous motility by approximately twofold, as evidenced by wound-healing assay and by single-cell track, time-lapse recording in Dunn chambers. Inhibitors of PI 3-kinase, PLC and PLD selectively abrogated acceleration of motility by v-Src. Since mechanisms that co-ordinate spontaneous, as distinct from oriented, cell migration are

separable, we further analysed in Dunn chambers chemotactic response of Rat-1/tsLA29 cells to PDGF and of MDCK/tsLA31 cells to EGF. In both cases, v-Src decreased the steady-state level of growth factor receptors at the cell surface twofold, and abrogated movement directionality at comparable level of occupancy as in non-transformed cells. The burst of pinocytosis in response to growth factors was also abolished by v-Src. Altogether, these results indicate that v-Src triggers motility in a PI 3-kinase-, PLC- and PLD-dependent manner, but abrogates directionality by suppressing polarised signalling downstream of growth factor receptors.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/117/20/4849/DC1>

Key words: v-Src, Cell migration, PI 3-kinase, PLC, PLD, Chemotaxis

Introduction

Cell motility plays a key role in embryogenesis, wound healing, immune surveillance, inflammation, atherosclerosis, as well as primary invasion of cancer cells and metastasis (for a review, see Lauffenburger and Horowitz, 1996). Our laboratory has demonstrated that the two paradigmatic oncogenes, v-Src and K-Ras, trigger macropinocytosis, a form of endocytosis directly driven by actin (Veithen et al., 1996; Amyere et al., 2000). In the course of such studies, we noticed that cell motility was accelerated as well. Since endocytosis and motility may be linked (Bretscher and Aguado-Velasco, 1998; de Curtis, 2001), we decided to investigate the molecular mechanisms underlying accelerated cell motility triggered by v-Src, using two different cell lines bearing two different v-Src mutants with thermosensitive kinase activity. Propagation of these cells at the non-permissive temperature indeed minimises the risk of phenotypic drift due to permanent transformation.

The Src family comprises nine related non-receptor tyrosine kinases that relay growth factor-induced gene expression,

proliferation, differentiation, adhesion and motility (for a review, see Thomas and Brugge, 1997). In particular, the ability of c-Src to control cell adhesion and spontaneous motility has been demonstrated in several experimental systems. Fibroblasts with c-Src knockout exhibit impaired motility and spreading on plastic, which can be restored by transfection with an expression vector for normal, but not kinase-defective c-Src (Kaplan et al., 1995). Similarly, in cells with triple knockout of the ubiquitous Src family kinases, Src, Yes and Fyn, which display reduced integrin-dependent cell migration, c-Src transfection is sufficient to restore motility (Klinghoffer et al., 1999).

Reactivation of various thermosensitive mutants of v-Src in fibroblasts induces extensive reorganisation of the actin cytoskeleton leading to stress fibre breakdown and membrane ruffling (Boschek et al., 1981), as well as the loss of focal adhesions (Fincham et al., 1995), all characteristic features of migrating cells. In Madin-Darby canine-kidney cells (MDCK), activation of the tsLA31 thermosensitive v-Src mutant further

results in the loss of differentiated epithelial status [also known as 'epithelio-mesenchymal transformation' (reviewed by Hay, 1995; Thiery, 2002)] and induces an invasive phenotype (Behrens et al., 1993). However, the effect of v-Src on spontaneous motility seems to be cell-type specific and conflicting results have been reported (see Discussion). The first goal of this work was thus to clarify the role of v-Src in spontaneous motility of fibroblasts and epithelial cells.

In Rat-1 fibroblasts, both v-Src and K-Ras have been found to cause a profound remodelling of cortical actin that drives membrane ruffling, a hallmark of the leading edge of migrating cells. Ruffle closure generates macropinosomes (Veithen et al., 1996), a process that depends in these cells on the permanent activation of phosphoinositide 3-kinase (PI 3-kinase) as well as phospholipase C (PLC) and phospholipase D (PLD), both downstream of PI 3-kinase (Amyere et al., 2000) (M.A., M.M., A.P., P. Van Der Smissen and P.J.C., unpublished data). Since macropinocytosis and cellular movement commonly depend on actin cytoskeleton reorganisation, both ultimately may share the same regulators and effectors. We therefore next examined whether motility triggered by v-Src is controlled by the same early signalling pathway as v-Src-induced macropinocytosis, namely via PI 3-kinase, PLC and PLD.

A growing body of evidence implicates increased expression and/or activity of Src in a variety of human carcinomas, including of colon (Malek et al., 2002), ovary (Wiener et al., 2003), breast (Verbeek et al., 1996) and pancreas origin (Lutz et al., 1998). Furthermore, Src kinase activity often correlates with the stage of cancer progression (Frame, 2002). However, although the vast majority of cancers are of epithelial origin, most experimental studies to dissect the role of Src in cancer have been carried out in fibroblasts (for a review, see Frame, 2002). Since properties of fibroblasts and epithelial cells differ, we felt it was important to clarify the effects of v-Src activity on motility in both cell types. Moreover, while leukocytes, lymphocytes, fibroblasts and neuronal cells migrate as individuals, epithelial and endothelial cells often move as sheets or loosely associated groups of cells. In particular, during angiogenesis and wound healing as well as tumour invasion, cells may migrate as clusters or strands that maintain cell-cell adhesions and communication while moving (Friedl and Bröcker, 2000). As a third goal, we compared motility and its regulation in Rat-1 fibroblasts and MDCK epithelial cells, upon activation of thermosensitive v-Src kinase. In addition, to explore distinct motility properties exhibited by fibroblasts and epithelial cells, as well as differences in the behaviour of scattered and confluent cells, we applied two complementary assays. Migration of confluent populations of cells was tested by a wound healing assay, best suited for testing a wide range of pharmacological inhibitors. In parallel, motility of individual cells or small epithelial cell clusters was examined by direct viewing in Dunn chambers, which have the added advantage that a chemotactic gradient can be generated.

Indeed, while motile cells spontaneously adopt a polarised morphology with a leading and a trailing edge, their migration can be oriented by a chemotactic gradient, that stabilises and coordinates directionality (Rorth, 2002). The role of Src in growth factor-induced chemotaxis is not clear. Cells with combined deletion of Src, Yes and Fyn display reduced migration in wound healing assays but retain the oriented motility triggered by chemotactic response to PDGF, indicating

that none of these ubiquitous Src kinases is required (Klinghoffer et al., 1999). However, transfection of NIH 3T3 cells with v-Src abolished their chemotactic response towards PDGF (Kundra et al., 1994a). The last goal of this study was to clarify the effect of Src on chemotactic response to growth factors and the possible underlying mechanism. With this aim, we analysed directionality of Rat-1/tsLA29 fibroblasts towards a PDGF gradient and that of MDCK/tsLA31 cells towards an EGF gradient.

Integrating these various approaches, we report that, in both fibroblasts and epithelial-derived cell lines, v-Src triggers spontaneous motility in a PI 3-kinase-, PLC- and PLD-dependent manner, but abrogates directionality of movement by suppressing response to growth factors.

Materials and Methods

Cell culture

Rat-1 cells infected with the tsLA29 subclone of Rous sarcoma virus (Rat-1/tsLA29), therefore expressing a thermosensitive v-Src kinase, were kindly provided by Dr G. Rousseau (UCL-ICP, Brussels, Belgium). MDCK cells (type I) infected with a murine leukemia construct, in which the v-Src gene of tsLA31 subclone of Rous sarcoma virus was inserted in place of pol-env genes (Wyke and Linial, 1973), were a kind gift from Dr J. Behrens (University of Essen Medical School, Germany) and Dr M. Mareel (Ghent University, Belgium). In both cell lines, v-Src tyrosine kinase is inactive when cells are cultured at 40°C, but is expected to regain its activity upon transfer to 34°C. All cell lines were propagated in Dulbecco's modified Eagle's Medium (Life Technologies, Grand Island, NY, USA) supplemented with 20 mM glucose, 4 mM glutamine, 10 mM NaHCO₃, 10 mM Hepes, 10 µg/ml streptomycin, 66 µg/ml penicillin and 10% (v/v) foetal calf serum (FCS, Life Technologies, Paisley, UK) under 5% CO₂ at 37°C (Rat-1/tsLA29) or 40°C (MDCK/tsLA31). Cells were seeded at ~50,000 cells/cm² on 9.6 cm² Petri dishes (Sarstedt, Newton, NC, USA) for wound healing assays and at ~12,000 cells/cm² on 3.24 cm² glass coverslips for assays in Dunn chambers, then transferred at either 34°C or 40°C overnight.

Western blotting

Confluent cells, cultured overnight at 40°C or 34°C, were preincubated for 2 hours with mitomycin C as above, then incubated for 6 hours with 10 nM wortmannin, 10 µM LY294002, 10 µM NCDC or 0.05% 1- or 2-butanol, washed three times with PBS-Ca²⁺ and solubilized in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM NaF, 1 mM Na₃VO₄, 0.25% (w/v) sodium deoxycholate and Complete[®] protease inhibitor mixture (Roche, Mannheim, Germany). Suspensions were sonicated for 15 seconds and protein content was measured by the bicinchoninic acid procedure (Smith et al., 1985), with BSA as a standard. Proteins (15 µg) were analysed by SDS-PAGE (9% acrylamide) using the Mini Gel Protean system 3 (Bio-Rad), then transferred onto polyvinylidene difluoride membrane (Boehringer, Ingelheim, Germany) for 90 minutes at 90 V in 25 mM Tris-HCl, 192 mM glycine and 20% methanol, pH 8.3, using the Mini Trans-Blot system (Bio-Rad). Membranes were incubated for 1 hour at 37°C with anti-avian Src mouse monoclonal IgG_{2b} (clone EC10) or a phosphotyrosine peptide-specific rabbit antiserum raised against human pY⁴¹⁸-Src peptide, corresponding to pY⁴¹⁶ in avian Src (BioSource Europe, Nivelles, Belgium). Blots were revealed with horseradish peroxidase-labelled sheep anti-mouse IgG antibodies or donkey anti-rabbit IgG antibodies (1:100,000, Amersham) followed by enhanced chemiluminescence (NEN, PerkinElmer Life Sciences, Boston, MA, USA) and scanned with AGFA Snapscan 600 scanner.

Fluorescence microscopy

Cells were plated at $\sim 12,000$ cells/cm² on 0.8 cm² glass coverslips and cultured overnight at 40°C or 34°C. The following procedure was performed at room temperature. After a brief rinse with PBS-Ca²⁺ (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 3.6 mM CaCl₂ and 3 mM MgCl₂, pH 7.3), cells were fixed with 4% (w/v) formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 minutes, then permeabilised with 0.05% (w/v) saponin in PBS for 15 minutes. Non-specific binding sites were quenched by a subsequent 30-minute incubation with Q-PBS [PBS supplemented with 0.01% (w/v) saponin, 5% (w/v) BSA, 0.1% (w/v) lysine and 0.02% (v/v) azide, pH 7.4]. Cells were then incubated for 1 hour with 2.5 µg/ml of mouse monoclonal IgG_{2b} antibody specific to avian Src (clone EC10, Upstate Biotechnology, NY, USA) together with 0.25 µg/ml of anti-paxillin mouse monoclonal IgG₁ antibody (clone 165, BD Transduction Laboratories, San Diego, CA, USA) in Q-PBS. After extensive washing (6 × 5 minutes) with Q-PBS, samples were incubated with secondary goat anti-mouse IgG₂ antibodies conjugated to Alexa 488, together with goat anti-mouse IgG₁ antibodies coupled to Alexa 647 and 130 nM Alexa 568-phalloidin (all from Molecular Probes, Eugene, OR, USA) for 30 minutes in the dark. The effect of v-Src on focal adhesion was alternatively examined by immunolabelling of α v integrin (mouse monoclonal IgG₁, Transduction Laboratories) and focal adhesion kinase (rabbit polyclonal antibodies, Upstate Biotechnology). As the control, cells were incubated with conjugated secondary antibodies alone or with irrelevant mouse IgG followed by secondary antibodies. After extensive washing (6 × 5 minutes) with Q-PBS, samples were post-fixed with 4% formaldehyde for 5 minutes, mounted in Moviol (Calbiochem, La Jolla, CA, USA) with 2.5% DABCO (Sigma, St Louis, MO, USA) and analysed with an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to a MRC 1024 equipment (Bio-Rad, Richmond, CA, USA).

Single cell migration assays in Dunn chambers

Single-cell migration and chemotaxis were assessed by time-lapse cinematography in direct-viewing Dunn chambers (Zicha et al., 1991; Weber Scientific International Ltd, Teddington, UK), as described previously (Allen et al., 1998). This device is derived from a Helber bacteria counting chamber, by grinding a circular well in the central platform to leave an annular bridge (approximately 1 mm wide) between inner and outer wells. Chemoattractants added to the outer well of the chamber diffuse across the bridge towards the inner well and rapidly form a stable gradient (illustrated at Fig. 5A). Glass coverslips with cells that had been incubated overnight at 40°C or 34°C were placed on Dunn chambers and transferred to a microscope stage in humidified incubators that maintained temperatures in the wells at 40°C or 34°C \pm 0.1°C, under 5% CO₂. Phase-contrast images of cells were recorded using an inverted Olympus microscope coupled to a CCD video camera, at 2-minute intervals for 7 hours. Directionality was determined in medium without FCS, supplemented with 0.1% BSA, by comparing cell migration in the absence or presence of a chemoattractant gradient, that was generated by placing in the outer well of the Dunn chamber 1.33 nM rat PDGF-BB (for Rat-1/tsLA29 fibroblasts) or 1.66 nM human EGF (for MDCK/tsLA31 cells; both from Sigma). The actual slope of growth factor gradients was calculated according to the method of Zicha et al. (Zicha et al., 1991). Recording the interface between inner and outer wells started within 30 minutes after assembling the chamber, by which time a linear diffusion gradient of growth factor is established. This gradient slowly vanishes, with a half-life of \sim 30 hours (Zicha et al., 1991). Frames were compressed into a movie (see supplementary material) and trajectories of individual cells were reconstructed using mouse-based 'Retrac' software that also provides velocity statistics

(<http://mc11.mcri.ac.uk/retrac/>). For quantitation of growth factor-induced directionality, cells were divided into three groups, based on concentric annular zones of decreasing growth factor concentrations. A chemotactic response was demonstrated by a significant increase in the percentage of cells that migrated towards the outer well as source of chemoattractant.

Wound healing assays

Population-based movement was measured by a wound healing assay (André et al., 1999). In this assay, cells are grown to confluency on plastic Petri dishes, then induced to re-populate a 'wound' created by stripping a sharply defined channel using a razor-blade. To prevent growth during migration, cells were pretreated for 2 hours with 4.5 µM (Rat-1/tsLA29 fibroblasts) or 30 µM (MDCK/tsLA31 cells) mitomycin C (Sigma). These concentrations were optimised for each cell line, based on strong inhibition of [³H]thymidine (Amersham, Piscataway, NJ, USA) incorporation, lasting for at least 24 hours after drug removal. After band-stripping, cells were allowed to migrate into the wound for 6 hours in DMEM containing 10% FCS, supplemented or not with pharmacological inhibitors (see below), fixed with 3% formaldehyde in PBS for 20 minutes at 4°C, then stained with Crystal Violet. End-point result of the population movement was determined as number of cells that had colonised the wound margin in random microscopic fields (magnification, 320×; 10 fields per assay). In wound healing assays, PI 3-kinase was inhibited with the indicated concentrations of wortmannin (Sigma) or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, Calbiochem, La Jolla, CA, USA) and PLC with 2-nitro-4-carboxyphenyl N, N-diphenylcarbamate (NCDC, Sigma), all dissolved as 1,000-fold concentrated stock solutions in DMSO. At 0.1% (v/v), DMSO did not affect cell migration. PLD was inhibited with 1-butanol (Merck, Darmstadt, Germany), diluted in culture medium containing 10% heat-inactivated serum; 2-butanol was used as a negative control for non-specific effects.

Growth factor binding assays

Subconfluent MDCK/tsLA31 cells were grown overnight at 40°C or 34°C in 24-well plates (Greiner, Frickenhausen, Germany), then transferred to DMEM without FCS for 30 minutes, rapidly chilled in ice-cold PBS, and incubated with increasing concentrations of human ¹²⁵I-EGF (900 Ci/mmol; Amersham) in 150 µl binding buffer (DMEM containing 0.1% BSA) for 2 hours on ice under gentle rocking. After the incubation, cells were extensively washed (3 rinses with PBS-Ca²⁺, one 5-minute wash with DMEM-0.1% BSA, and 3 rinses with PBS-Ca²⁺), then lysed with 300 µl of 0.01% Triton X-100 in 0.3 M NaOH. Since PDGF exhibited a strong non-specific binding to plastic wells, Rat-1/tsLA29 fibroblasts were first scraped off with a rubber policeman, incubated in suspension in plastic tubes with increasing concentrations of ¹²⁵I-PDGF (2,000 Ci/mmol; Amersham) and washed as above by sedimentation/resuspension. Samples were counted in a gamma counter (1275 Minigamma Counter, Wallac, PerkinElmer, Belgium). Specific binding was calculated by subtracting non-specific binding (determined by addition of a 100-fold excess of cold growth factor to half of the wells; never exceeding 30% of total) and normalised to cell protein content (bicinchoninic acid assay).

Pinocytosis assay

After incubation in FCS-free medium for 30 minutes, cells were incubated for exactly 5 minutes in medium supplemented with 4 mg/ml horseradish peroxidase (HRP type 2, Roche) in the presence or absence of growth factors or phorbol esters (Sigma), added concomitantly. After extensively washing, HRP uptake was assayed and normalised as described previously (Cupers et al., 1994).

Statistical analyses

For wound healing and pinocytosis assays, values did not significantly deviate from a normal distribution and were thus appropriate for analysis by two-tailed Student's *t*-test. Values obtained from Dunn chamber assays showed non-Gaussian distribution and were therefore analysed by the non-parametric Mann-Whitney test. To evaluate chemotactic response, comparison between groups was performed by difference-in-probabilities tests. For each test, values were regarded as significant for $P < 0.05$, *, ** denotes $P < 0.01$; ***, $P < 0.001$.

Results

Comparison of the two cell lines for v-Src, actin cytoskeleton and focal adhesions

We first compared the expression level and activation state of v-Src in both Rat-1/tsLA29 and MDCK/tsLA31 cells incubated overnight at the non-permissive (40°C) or permissive temperature (34°C) for v-Src thermosensitive kinase, based on western blotting of total lysates using respectively an avian Src-specific monoclonal antibody (EC10: total v-Src protein) and phosphopeptide-specific rabbit antibodies to the peptide centred on tyrosine 418 in human (characteristic of fully activated Src kinase; position 418 in human Src corresponds to 416 in avian Src). These immunoblots confirmed that, at the permissive temperature, v-Src was indeed expressed in both cell lines at a comparable level (Fig. 1A). Whereas in MDCK/tsLA31 cells, v-Src abundance was indistinguishable at both temperatures, its content in Rat-1/tsLA29 fibroblasts was clearly decreased at 40°C. Such decrease was also detected by immunoprecipitation in chick embryo fibroblasts freshly infected with the tsLA29 Src virus (Stoker et al., 1986) and indicates that thermoinactivation of tsLA29 Src kinase decreases its half-life as a result of improper protein folding. Immunoblotting with Src P-Y⁴¹⁸ phosphopeptide-specific antibodies clearly showed the inactivation of v-Src at the non-permissive temperature in both cell lines (Fig. 1A).

In chicken and mice fibroblasts with a thermosensitive tsLA29 mutation, v-Src has been reported to partly relocate upon activation of the tyrosine kinase (temperature switch), from the perinuclear region to the plasma membrane including its residual adhesions (Stoker et al., 1986; Fincham et al., 1996). This translocation appeared to depend on the integrity of stress fibres. To verify these observations for the two cell lines we used, and to further test for a polarised relocation towards the leading edge, v-Src was visualised with a mouse monoclonal antibody specific to avian Src, after 20 hours of culture at either 40°C or 34°C. To simultaneously examine the effect of v-Src on actin cytoskeleton and focal adhesion organisation, cells were also labelled with Alexa 568-phalloidin and immunolabelled for paxillin, α v integrin or focal adhesion kinase.

As shown in Fig. 1B, a clear morphological transformation of both Rat-1 and MDCK cells was induced at 34°C. Upon v-Src activation, Rat-1/tsLA29 cell bodies were generally no longer spread but instead assumed a round shape with one or several thin cytoplasmic extensions of various lengths (lower left panel). Shifting of MDCK/tsLA31 epithelial cells from 40°C to 34°C caused the loss of cell-cell contacts so as to induce transformation from small epithelial clusters to fibroblast-like, spindle-shaped individual cells (Fig. 1B). The majority of cells had very motile protrusions that

rapidly changed position during migration (see Movie 1, supplementary material). In Rat-1/tsLA29 fibroblasts at 40°C F-actin was mostly associated with parallel stress fibres (Fig.

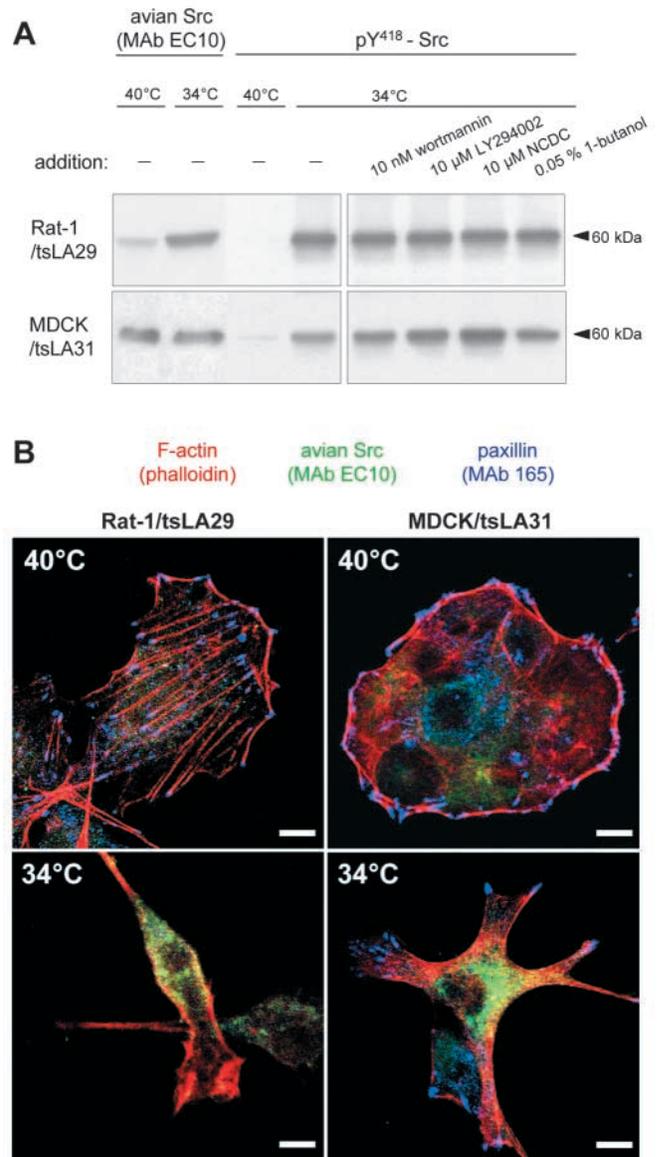


Fig. 1. Phenotypic comparison of the two cell lines studied. (A) Western blotting. Left: the effect of temperature shift on v-Src content (anti-avian Src antibody EC10) and activation (p-Y⁴¹⁸ phosphopeptide-specific antibodies). Right: evidence that pharmacological inhibitors used below do not interfere with v-Src expression and/or activation. (B) Immunolocalization of v-Src and paxillin in comparison with actin filaments. Rat-1/tsLA29 and MDCK/tsLA31 cells were cultured at low density on glass slides and incubated for 20 hours at non-permissive (40°C; upper row) or permissive temperature (34°C; lower row) for v-Src kinase activity, then fixed and permeabilised. v-Src was labelled by anti-avian Src-specific mouse monoclonal IgG_{2b} antibody (clone EC10), and revealed by Alexa 488-labelled goat anti-mouse IgG₂ antibodies (green). Paxillin, a focal adhesion marker, was detected by mouse monoclonal IgG₁ antibody (clone 165), and revealed by Alexa 647-labelled goat anti-mouse IgG₁ antibodies (blue). F-actin was decorated by Alexa 568-phalloidin (red). Cells were analysed by confocal microscopy. Scale bars: 10 μm.

1B, upper left) and in MDCK/tsLA31 cells with bundles along cell edges, most predominantly at the periphery of clusters (Fig. 1B, upper right). Upon transformation, both stress fibres and circumferential bundles disappeared, and F-actin was mostly localised to the cytocortex, in particular at ruffling membrane domains (Fig. 1B, lower panels).

Immunofluorescence intensity of avian v-Src protein greatly increased in both cell lines after transfer to 34°C (Fig. 1B). For tsLA29 v-Src expressed in Rat-1 fibroblasts, this increase was expected from western blotting analysis and was already observed in NIH 3T3, but not in Swiss 3T3 or chick embryo fibroblasts (Fincham et al., 1996). For MDCK/tsLA31 cells, however, this increase came as a surprise. It could indicate a concentration of the immunofluorescent signal at membranous cytoplasmic structures, probably endosomes, to which v-Src re-locates upon thermoactivation (Stoker et al., 1986; Kaplan et al., 1992). A second surprise was that v-Src localization always remained cytoplasmic; in other words, shift to the permissive temperature did not lead to a detectable redistribution to the plasma membrane, either in Rat-1/tsLA29 fibroblasts or in MDCK/tsLA31 cells, in contrast to a previous report (Fincham et al., 1996). One possible explanation could be that stress fibres, invoked for this redistribution to the plasma membrane, were no longer detected in our experiments.

The effect of v-Src on focal adhesion integrity in the two cell types was finally analysed by comparing paxillin, α v integrin and focal adhesion kinase immunolabelling. In non-transformed Rat-1/tsLA29 fibroblasts, paxillin was localised to well-organised focal adhesion plates (Fig. 1B, upper left) that completely disappeared upon v-Src thermoactivation (Fig. 1B, lower left). This was confirmed by the loss of punctate immunolabelling for α v integrin and focal adhesion kinase (data not shown). In non-transformed MDCK cells, paxillin was concentrated at foci around the edges of cell clusters (Fig. 1B, upper right). Surprisingly, in transformed, scattered MDCK/tsLA31 cells, focal adhesions bearing paxillin, α v integrin and focal adhesion kinase were still well visible, albeit smaller and more numerous than in the non-transformed state (Fig. 1B, lower right). This difference, combined with the higher motility in Src-transformed MDCK cells, supports the proposal that large focal adhesion plates favour anchorage, whereas small focal adhesions facilitate propulsive forces during migration (Beningo et al., 2001).

v-Src accelerates spontaneous motility of individual cells in Dunn chambers

Motility of Rat-1/tsLA29 and MDCK/tsLA31 cells was first tested at the single-cell level in direct-viewing Dunn chambers, by time-lapse video-microscopy recording, analysis of

compressed frames and reconstruction of individual trajectories. There was no detectable difference in the motility of parental Rat-1 and MDCK cell lines when compared at 40°C and 34°C (data not shown). However, striking differences were evidenced for both cell lines upon v-Src transformation. Whereas Rat-1/tsLA29 fibroblasts grown at 40°C were well spread and moved only sporadically (Fig. 2A; see also Movie 1, supplementary material), transfer to 34°C led to an approximate threefold average increase in spontaneous motility (Fig. 2B; see also Movie 2, supplementary material). A similar acceleration was noted using the phagokinetic track method introduced by Albrecht-Buehler (Albrecht-Buehler, 1977) (data not shown). At 40°C, sparsely plated single

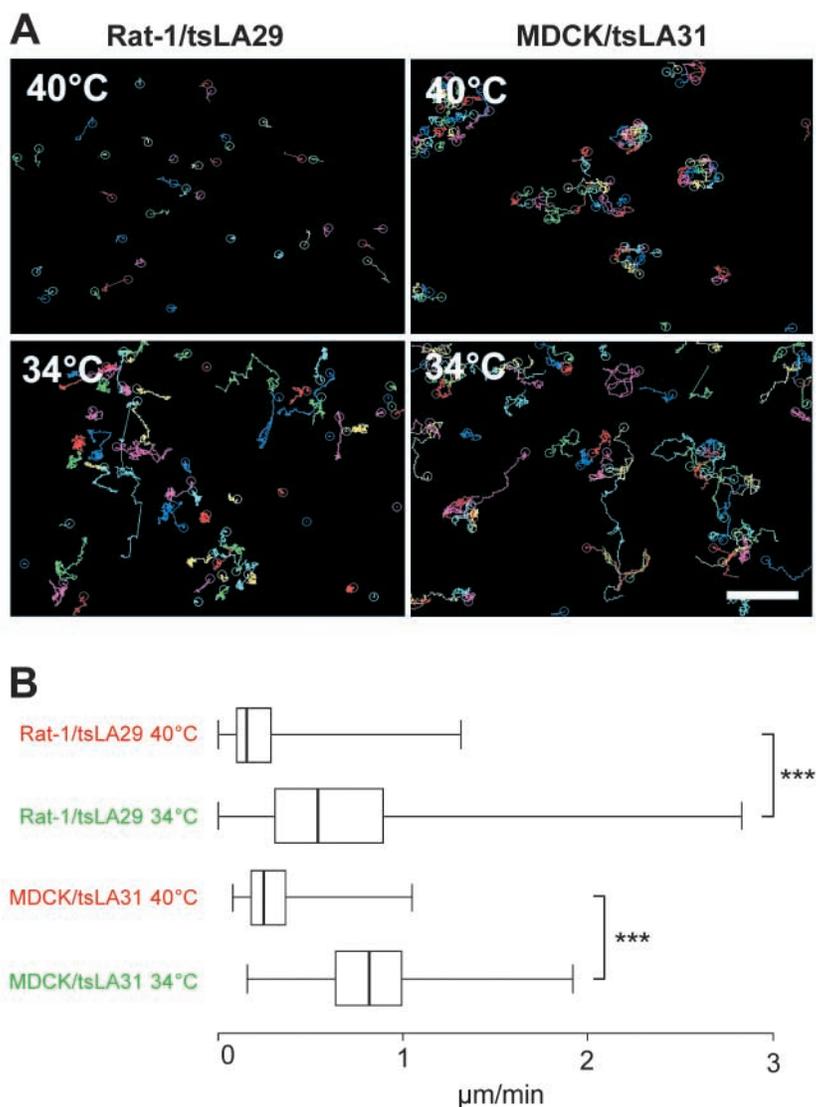


Fig. 2. v-Src increases spontaneous motility in Dunn chambers. To analyse individual movement, cells were plated at low density on glass slides. These were placed in Dunn chambers in the absence of added growth factor and phase-contrast images were recorded at 2-minute intervals. (A) Trajectories during 7 hours of migration; circles indicate initial positions. Scale bar: 200 μm (see also Movies 1-4, supplementary material). (B) Mean speed of migration: data were pooled from 3-4 experiments ($n=150$ to 180 cell trajectories) in total; box plots represent median (bold vertical line), interquartile range (boxes) and full values range (extremes); *** $P<0.001$ by Mann-Whitney test.

MDCK/tsLA31 epithelial cells spontaneously gathered into small clusters, where moving cells maintained contact with their neighbours to the point that movement of individuals did not result in a substantial displacement of the cluster (Fig. 2A; see also Movie 3, supplementary material). Upon shifting to the permissive temperature, MDCK/tsLA31 colonies dispersed into highly motile individual cells; their speed of migration was even higher than that of transformed Rat-1/tsLA29 fibroblasts (Fig. 2B; Movie 4, supplementary material).

v-Src accelerates wound healing

Since the mechanisms underlying migration of confluent populations differ from those of single-cell motility (Etienne-Manneville and Hall, 2002), we further tested the ability of Rat-1/tsLA29 and MDCK/tsLA31 cells to migrate into a wound introduced in a confluent cellular monolayer. Again, there was no detectable difference when this assay was performed at 40°C or 34°C in either parental Rat-1 fibroblasts or MDCK epithelial excluding an effect of temperature per se (data not shown). As evaluated by this assay, about twice as many Rat-1/tsLA29 fibroblasts migrated into the wound at the permissive temperature (34°C) than at the non-permissive temperature (Fig. 3, left). Consistent with the seminal observation that epithelio-mesenchymal transition correlates with a gain of invasiveness into collagen gels (Behrens et al., 1993), MDCK/tsLA31 cells also displayed accelerated migration into the wound at 34°C (Fig. 3, right). Once transformed, fibroblasts and epithelial cells migrated in a less coordinated manner, resulting in a much lower density in the re-colonised area, as compared with intact monolayer (Fig. 3A, lower row).

Role of PI 3-kinase and PLC in Src-dependent motility

Since PI 3-kinase and PLC act in sequence in v-Src-transformed fibroblasts to trigger actin cytoskeleton reorganisation and constitutive macropinocytosis (Amyere et al., 2000), we further tested if these early enzymatic relays would also signal v-Src-induced acceleration of spontaneous motility of Rat-1/tsLA29 and MDCK/tsLA31 cells. To this aim, the wound healing assay was selected, not only as a more convenient test of a range of concentrations of pharmacological inhibitors, but also to increase statistical power by being able to use parametrical tests.

In Rat-1/tsLA29 cells, low concentrations of the two unrelated PI 3-kinase inhibitors, wortmannin (<20 nM) and LY294002 (<1 µM), did not affect motility at the non-permissive temperature (40°C), but suppressed the acceleration of wound healing at the permissive temperature for v-Src (34°C) in a dose-dependent manner (Fig. 4A). Higher concentrations of wortmannin (30-100 nM) and LY294002 (10 µM) inhibited migration at both temperatures. Similarly, PLC inhibition by low concentrations of NCDC (<30 µM) did not affect motility at the non-permissive temperature, but slowed down wound healing at the permissive temperature, to, or below, the level of non-transformed cells (Fig. 4A). The response was even clearer in MDCK/tsLA31 cells, where a broader range of PI 3-kinase and PLC inhibitors had no effect on migration at the non-permissive temperature (40°C) but slowed down wound

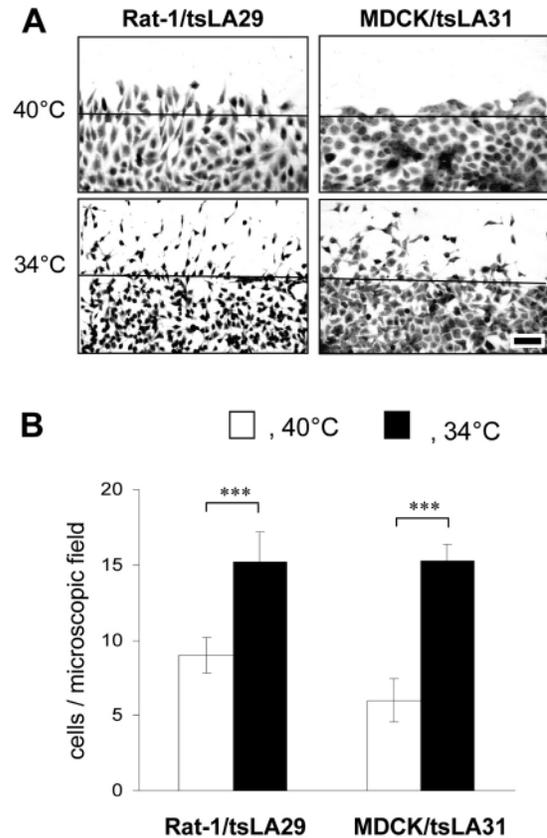


Fig. 3. v-Src accelerates wound healing. (A) Microscopy. Rat-1/tsLA29 fibroblasts (left) and MDCK/tsLA31 cells (right) were seeded on plastic dishes at high density and incubated overnight at 40°C or 34°C. To prevent growth, cells were treated with mitomycin C for 2 hours before band-stripping, then allowed to migrate into the wounded area for 6 hours in DMEM with 10% FCS, fixed and stained. The photographs present typical examples, with horizontal lines, clearly marked on plastic, indicating the initial edge of the wound (top half of each panel). Scale bar: 100 µm. (B) Quantitation. Spontaneous motility was estimated by counting cells that had colonised the margins of stripped areas after 6 hours. Values are means±s.e.m. of 40 random microscopic fields for each condition, pooled from four experiments (*** P <0.001 by Student's t -test).

healing at the permissive temperature (34°C) exactly to the level of non-transformed cells (Fig. 4B).

Role of phospholipase D (PLD) in Src-dependent motility

PLD activity is also stimulated by v-Src (Song et al., 1991) and its activation is necessary and sufficient to cause the reorganisation of the actin cytoskeleton that is typical of transformed cells (M.A., M.M., A.P., P. Van Der Smissen and P.J.C., unpublished data). We therefore investigated its potential role on v-Src-induced acceleration of motility. Inhibition by 1-butanol is a straightforward method to address the role of PLD. Indeed, PLD preferentially incorporates primary alcohols such as 1-butanol over water, so as to release phosphatidylalcohol instead of phosphatidic acid and to disrupt signalling downstream of PLD (Exton, 2002). By contrast,

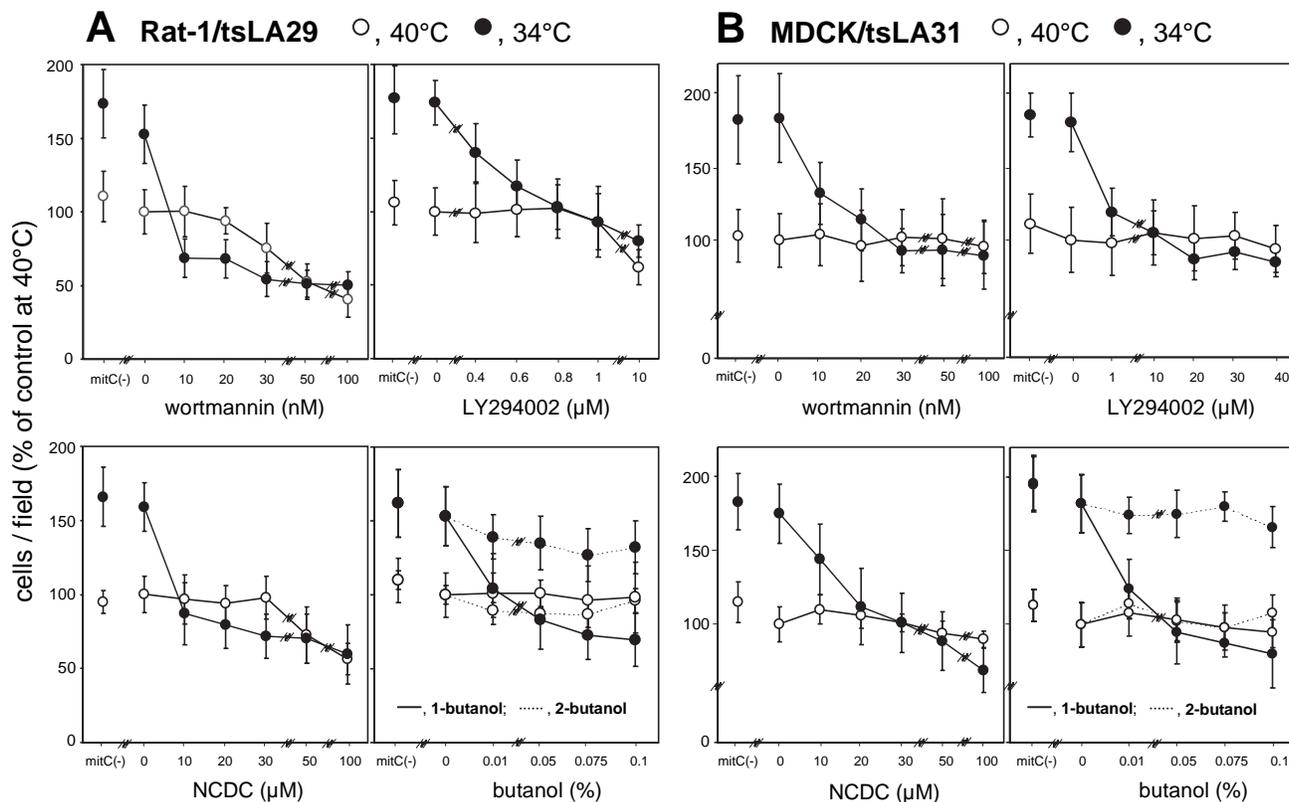


Fig. 4. Acceleration of motility by v-Src depends on PI 3-kinase, PLC and PLD. Rat-1/tsLA29 fibroblasts (A) and MDCK/tsLA31 cells (B) were pretreated with mitomycin C [except for 'mitC(-)' group], and tested for wound healing during 6 hours in DMEM with 10% heat-inactivated FCS supplemented by the indicated concentrations of wortmannin, LY294002 (notice the different concentrations used for the two cell lines), NCDC, 1-butanol or 2-butanol. Values are means \pm s.e.m. of 20-50 microscopic fields for each condition, pooled from 2-5 experiments and expressed as the percentage of migration at 40°C over the group treated with mitomycin C alone (condition '0').

secondary alcohols such as 2-butanol cannot substitute for water and therefore serve as control for non-specific effects.

To test for the role of PLD in v-Src-dependent motility, we accordingly compared the effect of 1- and 2-butanol in both Rat-1/tsLA29 and MDCK/tsLA31 cells by the wound healing assay (Fig. 4). In both cell lines, up to 0.1% (v/v) 1-butanol had no effect on migration at the non-permissive temperature (40°C), while a strong, dose-dependent inhibition was obvious at the permissive temperature (34°C). No appreciable effect of 2-butanol could be observed at any temperature. These results indicate that PLD is also involved in the acceleration of spontaneous motility by v-Src.

Pharmacological inhibitors do not affect Src activity

The early effects of v-Src on the cytoskeleton depend on de novo protein synthesis (Meijne et al., 1997). To verify that prolonged incubation (6 hours) with any of the pharmacological inhibitors used for wound healing assay did not, conversely, affect v-Src expression or activity, lysates of cells treated with mitomycin C, wortmannin, LY294002, NCDC, 1- or 2-butanol were analysed by western blotting with pY⁴¹⁸-phosphotyrosine-specific rabbit antibodies. No interference could be detected with v-Src expression/activity, either in Rat-1/tsLA29 or MDCK/tsLA31 cells (Fig. 1A, four right lanes).

v-Src transformation abolishes chemotactic response to PDGF and EGF

The different molecular mechanisms that coordinate spontaneous and oriented migration seem to be separable in the same cells (Kundra et al., 1994a; Klinghoffer et al., 1999). We therefore analysed, at the single cell level in Dunn chambers, PDGF-induced chemotaxis of Rat-1/tsLA29 fibroblasts and EGF-induced chemotaxis of MDCK/tsLA31 cells. Chemoattractant gradient was generated by placing PDGF or EGF in the outer well of the Dunn chamber. As a control, the formation of a chemical gradient was visualised by replacing growth factors by 5 μ g/ml of fluorescent dextran (Alexa 568-dextran, molecular mass 10 kDa). Thirty minutes after chamber assembly, there was a continuous gradient of fluorescence intensity across the bridge (Fig. 5A, left), which was measured using the Laserpix programme (Fig. 5A, right). The concentrations of PDGF and EGF across this bridge were calculated as described previously [(Zicha et al., 1991); the slope of PDGF is represented by the dotted line at Fig. 5A right, and was similar for EGF].

For quantification of chemotaxis, cells were divided into three groups, based on three concentric annular zones of decreasing growth factor concentrations (shown by dotted lines in Fig. 5A, left). In the outer zone of the gradient, the growth factor concentration was saturating for the two receptors analysed (~1.3-0.8 nM PDGF and ~1.6-0.9 nM EGF; see

Fig. 6). In the middle zone, concentration was subsaturating (~ 0.8 – 0.2 nM PDGF and ~ 0.9 – 0.3 nM EGF). In the central zone, values were below K_d values (<0.2 nM PDGF and <0.3 nM EGF). Directionality of trajectories in the chemoattractant gradients was quantified by calculating the fraction of cells that migrated (i) towards the source of chemoattractant (i.e. outer well); (ii) down the gradient of chemoattractant (i.e. towards the inner well); or (iii) parallel to the wells; in addition the fraction of non-motile cells was quantified. A chemotactic

response was assessed by a statistically significant increase in the percentage of cells that migrated towards the source of chemoattractant, as compared with migration in the absence of a gradient.

As shown in Fig. 7, both PDGF and EGF gradients triggered a chemotactic response at 40°C , even at concentrations below K_d . As expected, the fraction of cells that migrated towards the source of chemoattractant decreased with actual growth factor concentration within the gradient. By contrast, at the permissive temperature (34°C), movement no longer showed directionality even at saturating concentrations of growth factors. We also noted that the fraction of non-motile Rat-1/tsLA29 fibroblasts significantly decreased at 34°C compared with 40°C .

v-Src does not abolish growth factor binding but abrogates growth factor-stimulated endocytosis

The complete suppression by v-Src of chemotactic response could indicate that either v-Src suppresses the overall sensitivity to growth factors, e.g. by strongly down-regulating their receptors or downstream signal transduction pathways, or that the overall control by v-Src of the cytoskeleton or membrane trafficking overcomes polarised changes induced by growth factors. To test for a loss of plasma membrane receptors, cells were shifted to the permissive temperature overnight, then incubated at 4°C with increasing concentrations of appropriate ^{125}I -growth factors. Radioligand binding curves showed that surface pools of PDGF receptors on Rat-1/tsLA29 fibroblasts and of EGF receptors on MDCK/tsLA31 cells were both decreased by approximately twofold upon v-Src activation (Fig. 6, left). Scatchard plots revealed a single class of binding sites for both growth factor receptors, with K_d values insensitive to v-Src activation (calculated from non-linear fitting: for ^{125}I -PDGF, 0.17 ± 0.018 nM at 40°C vs 0.17 ± 0.023 nM at 34°C ; for ^{125}I -EGF, 0.22 ± 0.022 nM at 40°C vs 0.28 ± 0.022 nM at 34°C , see Fig. 6, right).

By definition, chemotactic response is achieved with only partial receptor occupancy. Hence, a two-fold difference in surface receptor pools did not seem to be sufficient to explain the loss of chemotaxis after v-Src transformation. This assumption is supported by the observation that the fraction of non-transformed cells exposed to actual growth factor concentrations below K_d and moving towards the growth factor source was still significantly higher than in transformed cells exposed to saturating concentrations of the growth factors (Fig. 7). In other words, the loss of directionality cannot be solely accounted for a defective binding of growth factors at the cell surface.

To test for a different downstream response to growth factor receptors, we measured the acute pinocytotic burst normally triggered by these growth factors, or to the phorbol ester, phorbolmyristyl acetate (PMA) (Swanson and Watts, 1995). In Rat-1/tsLA29 fibroblasts, transformation by v-Src accelerated fluid-phase endocytosis twofold (Veithen et al., 1996). In MDCK/tsLA31 cells, thermoactivation of v-Src does not consistently lead to an acceleration of fluid-phase endocytosis in their non-polarised state, but selectively accelerates apical endocytosis in their polarised state (Mettlen et al., in preparation). In both cell lines, saturating concentrations of

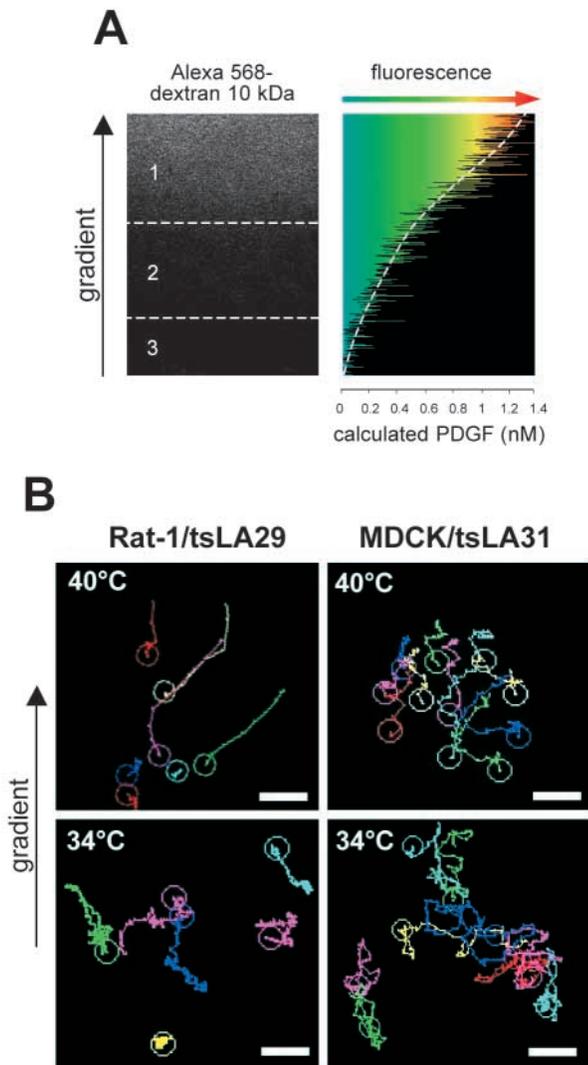


Fig. 5. Generation of, and response to, chemical gradients in Dunn chambers. (A) Alexa 568-dextran (10 kDa) was introduced in the outer well (next to the top of the figure) and the bridge was examined by confocal microscopy after 30 minutes (left; zones 1–3 selected for quantitation of chemotactic response are delimited by horizontal dotted lines). Fluorescence intensity was measured by the Laserpix programme (right). The actual slope of PDGF gradient was calculated according to the method of Zicha et al. (Zicha et al., 1991) and superimposed as a broken line (right). EGF gradient showed a similar slope. (B) Typical trajectories of Rat-1/tsLA29 cells and MDCK/tsLA31 cells during 7 hours of migration in Dunn chambers under chemoattractant gradient (PDGF or EGF, respectively). Increasing growth factor concentration is indicated by the vertical arrow at left. Scale bars: 50 μm .

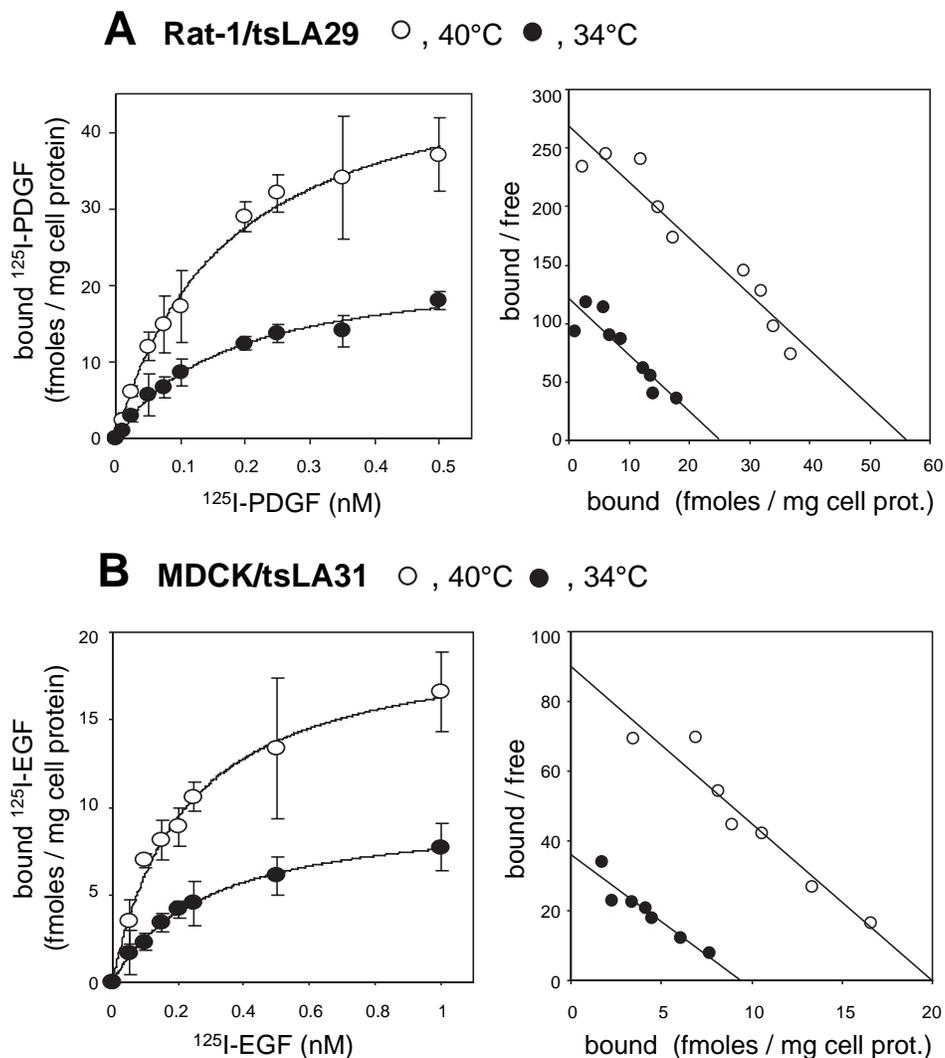


Fig. 6. Growth factor binding isotherms at 4°C for 2 hours. (A) Rat-1/tsLA29 fibroblasts were incubated in suspension with the indicated final ^{125}I -PDGF concentrations; (B) ^{125}I -EGF binding on MDCK/tsLA29 cells was performed in Petri dishes. Values are means \pm s.d. of specific binding in 6-9 dishes pooled from 3-4 experiments. Curves were drawn after non-linear fitting ($r^2 > 0.99$), based on the hyperbola $y = B_{\text{max}} * x / (K_d + x)$, where x is radioligand concentration, y is specific binding, B_{max} is a maximum specific binding and K_d is equilibrium dissociation constant. Graphs on the right result from Scatchard transformation.

growth factors or 1 μM PMA accelerated pinocytosis at 40°C; this response was also abrogated upon v-Src transformation (Fig. 8). Thus, the loss of responsiveness to growth factors in v-Src-transformed cells is not limited to chemotaxis, but appears to reflect overall refractoriness to growth-factor signalling (see Discussion).

Discussion

The role of Src family kinases in cell motility is poorly understood. Several lines of evidence indicate that c-Src may be necessary for spontaneous motility. Indeed, fibroblasts with c-Src knockout exhibit impaired motility and spreading on plastic, which can be restored by transfection with an expression vector for normal, but not kinase-defective c-Src (Kaplan et al., 1995). However, *c-Src*^{-/-} mice show normal migration of neurons in developing brains (Soriano et al., 1991). In cells with triple knockout of the ubiquitous Src family kinases, Src, Yes and Fyn, which display reduced integrin-dependent cell migration, c-Src is sufficient to restore motility (Klinghoffer et al., 1999). In addition, studies on the effect of v-Src on spontaneous motility yielded conflicting results. v-Src was reported to stimulate motility of murine and

rat fibroblastic cell lines in a hyaluronan-dependent manner (Hall et al., 1996; Sohara et al., 2001). By contrast, v-Src expression in 3T3 (Kundra et al., 1994a; Hauck et al., 2002) or chick embryo fibroblasts (Fincham and Frame, 1998) was reported to have no effect on spontaneous motility and even to decrease cell migration on fibronectin (Sankar et al., 1995; Hauck et al., 2002).

However, it should be noted that several studies addressing the effect of v-Src on cell motility have been performed by comparing non-transformed versus *stably* transformed cell lines, which could lead to uncontrolled genetic drift. To minimise this problem, we have used two cell lines expressing a thermosensitive v-Src kinase: Rat-1/tsLA29 fibroblasts and epithelial MDCK/tsLA31 cells, where Src kinase can be activated shortly before the motility assays. This paper demonstrates that in both cell lines, v-Src activation at 34°C not only induces morphological transformation and reorganisation of actin cytoskeleton, but also accelerates spontaneous motility, as evidenced by single-cell and population-based assays.

Cell migration involves multiple changes in the cytoskeleton, membrane trafficking, cell-substrate adhesion and extracellular matrix remodelling. Src directly

phosphorylates focal adhesion kinase (Fincham et al., 1995; Meijne et al., 1997) and several actin-regulatory proteins such as cortactin (Wu and Parsons, 1993) and gelsolin (De Corte et al., 1997). Alternatively, v-Src can indirectly control the cytoskeleton by activation of phosphatidylinositol 3-kinase type I (PI 3-kinase) (Haefner et al., 1995). We have previously reported that v-Src causes constitutive macropinocytosis in Rat-1 fibroblasts (Veithen et al., 1996) via the permanent activation of PI 3-kinase, PLC and PLD (Amyere et al., 2000) (Amyere et al., manuscript in preparation). We here demonstrate that v-Src-induced motility is controlled by the same early signalling cascade. However, effects on motility and endocytosis were not systematically linked. In Rat-1/tsLA29 fibroblasts, v-Src accelerates fluid-phase but not receptor-mediated endocytosis of transferrin (Veithen et al., 1996). By contrast, in the MDCK/tsLA31 clones studied, it does not detectably affect fluid-phase endocytosis, but accelerates receptor-mediated endocytosis of transferrin approximately twofold (our unpublished observations). Whereas the significance of these discrepancies remains to be clarified, they

could reflect differences between fibroblastic and epithelial cells in the contribution of endocytic trafficking to cell migration (Bretscher and Aguado-Velasco, 1998).

PI 3-kinase serves as an important knot in signalling pathways leading to cell migration and various PI 3-kinase isoforms can differentially regulate cell motility (Vanhaesebroeck et al., 1999). Class I PI 3-kinases activate Cdc42 and Rac and thereby regulate formation of filopodia and lamellipodia that are necessary for cell migration. The regulatory subunit (p85 α) of PI 3-kinase type I is essential for chemotaxis, linking PDGF receptor activation to Cdc42 stimulation (Jimenez et al., 2000). Local generation of 3-phosphoinositides by PI 3-kinase and their rapid degradation are key processes for spatial sensing, governing directional migration in fibroblasts (Haugh et al., 2000) and *Dictyostelium discoideum* (Funamoto et al., 2002), and also in generation of phagocytic cups (Marshall et al., 2001). Moreover, a growing body of evidence implicates PI 3-kinase in the increased motility and metastasis of cancer cells (for a review, see Roymans and Slegers, 2001). For example, the loss of

phosphatidylinositol-3 phosphatase, PTEN, correlates with the progression of tumours to a metastatic state (Li et al., 1997). Conversely, inhibition of PI 3-kinase by wortmannin inhibits v-Src-induced invasion of MDCK cells into type I collagen gel (Kotelevets et al., 1998). However, since motility and invasion can be differentially regulated (Banyard et al., 2000; Hsia et al., 2003), it is important to distinguish both

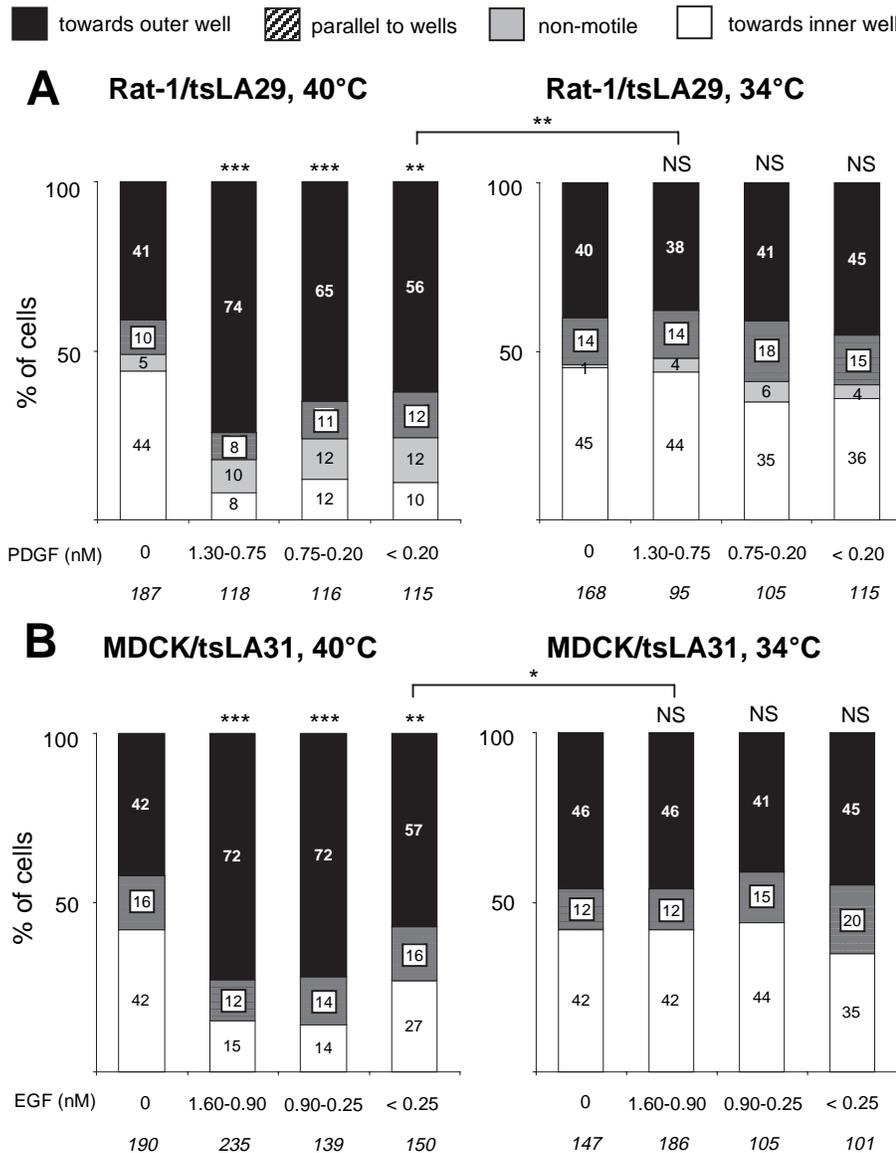


Fig. 7. Quantification of chemotaxis in Dunn chambers: v-Src abolishes this response. (A) Rat-1/tsLA29 fibroblasts; (B) MDCK/tsLA31 cells. Same procedure as for Fig. 2, except that 1.33 nM PDGF-BB or 1.66 nM EGF were introduced into the outer well of Dunn chambers, respectively. Recording of cell migration started after 30 minutes of chambers assembly, when a linear diffusion gradient of growth factors had been established. For quantification of directionality, orientation of each cell trajectory was determined in the three zones of decreasing growth factor concentrations defined at Fig. 5A (ranges are indicated below bar histograms and numbers of cells analysed in each group are specified in italics). The fraction of cells that had migrated towards the outer well (positive chemotactic response), the inner well, parallel to both wells of the chamber, or non-motile cells, was determined. Values are pooled from 4-8 experiments (** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by difference-in-probabilities test for the fraction of cells moving towards the growth factor source, as compared with no gradient; or depending on v-Src activation for a comparable level of growth factor/receptor complexes, as indicated by the horizontal bracket).

processes as each can contribute to tumour progression. This study focuses on regulation of v-Src-triggered motility and demonstrates that PI 3-kinase inhibition by wortmannin or LY294002 suppresses v-Src-induced acceleration of spontaneous motility in both Rat-1 and MDCK cells.

PI 3-kinase lipid products can directly activate phospholipase C- γ 1 (PLC- γ 1), which propagates signalling further downstream (Falasca et al., 1998). In addition, PLC- γ 1 may also associate with, and be phosphorylated by, Src (Nakanishi et al., 1993). Activated PLC generates diacylglycerol (DAG), a potent activator of PKC, and inositol 1,4,5-trisphosphate, which triggers release of calcium from internal stores and thereby affects actin polymerisation machinery. Pharmacological inhibition of PLC γ 1 reduces invasion of various tumour cell lines overexpressing EGF receptors (Turner et al., 1997; Price et al., 1999). In addition, EGF promotes MDA-MB-231 breast cancer cell migration via a PI 3-kinase- and PLC-dependent mechanism (Price et al., 1999; Piccolo et al., 2002), pointing to the involvement of PLC in EGF-induced motility. To the best of our knowledge, our

results provide the first evidence for a role of PLC in the control by Src of spontaneous motility as well.

The function of the ubiquitous enzyme, PLD, remains poorly defined. One of postulated roles for PLD is regulation of the actin cytoskeleton rearrangements. Its product, phosphatidic acid, may activate type I PI 4-phosphate 5-kinases which synthesise PtdIns(4,5) P_2 that, in turn, influences actin-associated cytoskeletal proteins (Sechi and Wehland, 2000). PLD activity is indeed required for actin stress fibres formation in fibroblasts (Kam and Exton, 2001) and epithelial cells (Porcelli et al., 2002). v-Src is known to stimulate phospholipase D in fibroblasts (Song et al., 1991) and this activity is increased in several human cancers (Noh et al., 2000; Zhao et al., 2000). Overexpression of PLD2 dramatically alters cortical actin dynamics in rat embryo fibroblasts (Colley et al., 1997), but not in the 3Y1 fibroblastic cell line (Shen et al., 2002). This report demonstrates that inhibition of PLD with 1-butanol decreases spontaneous motility in v-Src-transformed cells down to the level of control cells. Taken together, our studies show that v-Src-induced macropinocytosis and motility share the same early signalling cascade, namely via PI 3-kinase, PLC and PLD.

The role of Src in chemotaxis is also far from clear. Fibroblasts carrying a combined deletion of Src, Yes and Fyn, which display reduced migration in wound healing, migrate, as do wild-type cells, towards a PDGF gradient (Klinghoffer et al., 1999), suggesting that the Src family of non-receptor-tyrosine kinases are not required for chemotaxis. However, in cells transfected with PDGF α/β receptor chimeras, mutation of Tyr579 and/or Tyr581 at Src-association sites abolishes chemotaxis, although involvement of other signal transduction molecules docking to the same sites could not be excluded (DeMali et al., 1999). Moreover, transfection of NIH 3T3 fibroblasts with v-Src does not affect spontaneous motility but abolishes chemotaxis towards PDGF (Kundra et al., 1994a) or, conversely, increases serum-stimulated chemotaxis (Hauck et al., 2002). Although these various reports are not easy to integrate, they nevertheless imply that the molecular mechanisms that co-ordinate spontaneous versus oriented migration are separable in the same cells (Kundra et al., 1994a; Klinghoffer et al., 1999). Our results showing that v-Src, in spite of triggering spontaneous migration, abolishes chemotactic response to PDGF in Rat-1 cells and to EGF in MDCK cells, confirm this hypothesis. The loss of response to these two growth factors is not specific to the motile response, since a growth factor-stimulated pinocytic burst was also inhibited by v-Src, indicating an overall refractoriness to growth factor signalling in (some) transformed cells.

Oncogenes can strongly decrease surface expression or affinity of PDGF β and EGF receptors (Gray and Macara, 1988; Wasilenko et al., 1990; Vaziri and Faller, 1995). Our results show that the surface pool of these two growth factor receptors was decreased by about twofold upon v-Src transformation, without detectable change in affinity. This down-regulation is likely to result from accelerated internalisation, and/or rerouting from signalling endosomes to lysosomes for degradation (Sorkin and von Zastrow, 2002). However, we still demonstrate a significant chemotactic response of non-transformed cells at actual growth factor concentrations below K_d , contrasting with the loss of detectable chemotactic response in transformed cells exposed to saturating

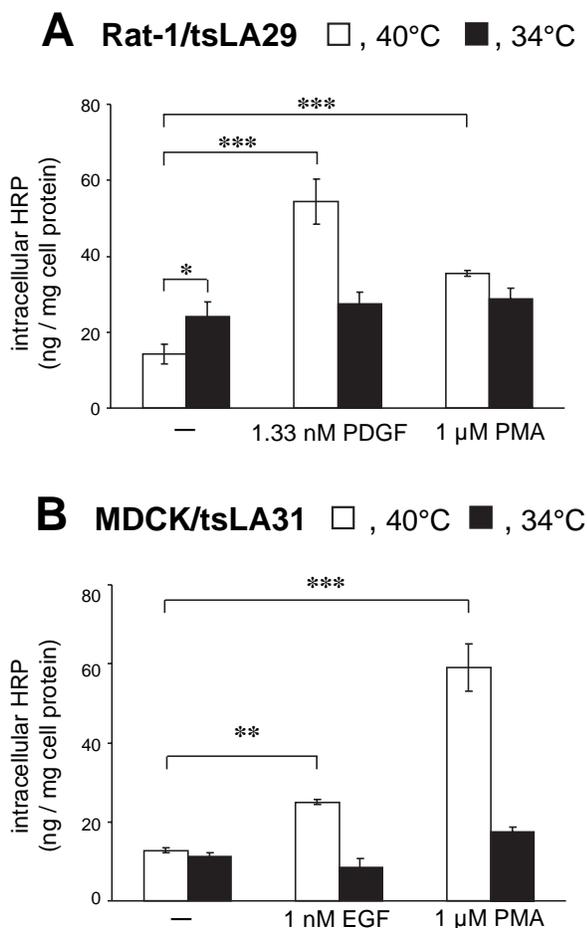


Fig. 8. (Macro)pinocytic burst in response to growth factors or phorbol ester. (A) Rat-1/tsLA29 fibroblasts; (B) MDCK/tsLA31 cells. Fluid-phase endocytosis was measured by a 5-minutes uptake of HRP in the presence or absence of the indicated concentrations of PDGF-BB, EGF or PMA added concomitantly. Values represent means \pm s.d. of three dishes from one representative experiment out of three (** P <0.01, *** P <0.001 by Student's t -test).

concentrations of growth factors. Thus, the complete loss of chemotactic and pinocytic responsiveness to PDGF and EGF cannot be solely accounted for by defective ligand binding to transformed cells and indicates suppression of polarised response downstream of growth factor receptors.

v-Src could possibly affect signalling downstream of growth factor receptors by (i) altering their phosphorylation state; (ii) over-recruiting downstream effectors all over the cell surface; or (iii) interfering with membrane trafficking, resulting in the loss of surface polarity. Not only does v-Src directly phosphorylate EGF receptor (Wright et al., 1996), but it also leads to constitutive activation of PI 3-kinase, PLC and PLD (Amyere et al., 2000) (Amyere et al., manuscript in preparation) and we here show that PI 3-kinase, PLC and PLD are necessary for the stimulation of spontaneous motility by v-Src. Since these signalling molecules also mediate PDGF and EGF signalling (Kundra et al., 1994b; Thorsen et al., 2003), it is conceivable that up-regulation of these early enzymatic relays by v-Src overcomes the effect of growth factors by abolishing the polarisation of lipidic second messengers generation that mediates the chemotactic response. Indeed, signalling downstream of growth factor receptors requires *silence* to be effective. Since Src and growth factor receptors share similar, if not identical, signal transduction pathways, constitutive activation of v-Src throughout the cell presumably activates these signals all around the pericellular membrane, creating *noise* which prevents polarised responses such as chemotaxis of micropinocytosis. Alternatively, v-Src could perturb trafficking of the recycling of growth factor receptors or anchoring molecules, so as to prevent their selective transcytotic transfer to the leading edge. Experiments designed to test these hypotheses are currently in progress.

We are grateful to Drs J. Behrens, M. Mareel and G.G. Rousseau for providing cell lines harbouring ts v-Src mutants and Dr V. Rigot for helpful advice on cell motility assays and immunolabelling of focal adhesion components. The secretarial assistance of Mr Y. Marchand is greatly appreciated. A.P. is a Research Fellow and I.C. is a Postdoctoral Researcher at the FNRS. This work was supported by the FNRS, the ARC and the IUAP, at which M.A. was a postdoctoral fellow.

References

- Albrecht-Buehler, G. (1977). The phagokinetic track of 3T3 cells. *Cell* **11**, 395-404.
- Allen, W. E., Zicha, D., Ridley, A. J. and Jones, G. E. (1998). A role for cdc42 in macrophage chemotaxis. *J. Cell Biol.* **141**, 1147-1157.
- Amyere, M., Payraastre, B., Krause, U., van Der Smissen, P., Veithen, A. and Courtoy, P. J. (2000). Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on sequential permanent activation of phosphoinositide 3-kinase and phospholipase C. *Mol. Biol. Cell* **11**, 3453-3467.
- André, F., Rigot, V., Remacle-Bonnet, M., Luis, J., Pommier, G. and Marvaldi, J. (1999). Protein kinases C- γ and - δ are involved in insulin-like growth factor I-induced migration of colonic epithelial cells. *Gastroenterology* **116**, 64-77.
- Banyard, J., Anand-Apte, B., Simons, M. and Zetter, B. R. (2000). Motility and invasion are differentially modulated by Rho family GTPases. *Oncogene* **19**, 580-591.
- Behrens, J., Vakaet, L., Friis, R., Winterhager, E., van Roy, F., Mareel, M. M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/ β -catenin complex in cells transformed with a temperature-sensitive v-Src gene. *J. Cell Biol.* **120**, 757-766.
- Beningo, K. A., Dembo, M., Kaverina, I., Small, J. V. and Wang, Y. L. (2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J. Cell Biol.* **153**, 881-887.
- Boschek, C. B., Jockusch, B. M., Friis, R. R., Back, R., Grundman, E. and Bauer, H. (1981). Early changes in the distribution and organization of microfilament proteins during cell transformation. *Cell* **24**, 175-184.
- Bretscher, M. S. and Aguado-Velasco, C. (1998). Membrane traffic during cell locomotion. *Curr. Opin. Cell Biol.* **10**, 537-541.
- Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altschuler, Y., Bar-Sagi, D., Morris, A. J. and Frohman, M. A. (1997). Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.* **7**, 191-201.
- Cupers, P., Veithen, A., Kiss, A., Baudhuin, P. and Courtoy, P. J. (1994). Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. *J. Cell Biol.* **127**, 725-735.
- De Corte, V., Gettemans, J. and Vandekerckhove, J. (1997). Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60^{c-src} catalyzed phosphorylation of gelsolin and related actin-binding proteins. *FEBS Lett.* **401**, 191-196.
- de Curtis, I. (2001). Cell migration: GAPs between membrane traffic and the cytoskeleton. *EMBO Rep.* **2**, 277-281.
- DeMali, K. A., Godwin, S. L., Soltoff, S. P. and Kazlauskas, A. (1999). Multiple roles for Src in a PDGF-stimulated cells. *Exp. Cell Res.* **253**, 271-279.
- Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.
- Exton, J. H. (2002). Regulation of phospholipase D. *FEBS Lett.* **531**, 58-61.
- Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A. and Schlessinger, J. (1998). Activation of phospholipase C γ by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J.* **17**, 414-422.
- Fincham, V. J. and Frame, M. C. (1998). The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J.* **17**, 81-92.
- Fincham, V. J., Wyke, J. A. and Frame, M. C. (1995). v-Src-induced degradation of focal adhesion kinase during morphological transformation of chicken-embryo fibroblasts. *Oncogene* **10**, 2247-2252.
- Fincham, V. J., Unlu, M., Brunton, V. G., Pitts, J. D., Wyke, J. A. and Frame, M. C. (1996). Translocation of Src kinase to the cell periphery is mediated by the actin cytoskeleton under the control of the Rho family of small G proteins. *J. Cell Biol.* **135**, 1551-1564.
- Frame, M. C. (2002). Src in cancer: deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta* **1602**, 114-130.
- Friedl, P. and Bröcker, E.-B. (2000). The biology of cell locomotion within three-dimensional matrix. *Cell. Mol. Life Sci.* **57**, 41-64.
- Funamoto, S., Meili, R., Lee, S., Parry, L. and Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* **109**, 611-623.
- Gray, G. M. and Macara, I. G. (1988). The pp60^{v-src} tyrosine kinase desensitizes epidermal growth factor binding to 3T3 fibroblasts by two distinct protein kinase C-independent mechanisms. *J. Biol. Chem.* **263**, 10714-10719.
- Haefner, B., Baxter, R., Fincham, V. J., Downes, C. P. and Frame, M. C. (1995). Cooperation of Src homology domain in the regulated binding of phosphatidylinositol-3-kinase - a role of the Src homology 2 domain. *J. Biol. Chem.* **270**, 7937-7943.
- Hall, C. L., Lange, L. A., Prober, D. A., Zhang, S. and Turley, E. A. (1996). pp60(c-src) is required for cell locomotion regulated by the hyaluronan receptor RHAMM. *Oncogene* **13**, 2213-2224.
- Hauck, C. R., Hsia, D. A., Puente, X. S., Cheresch, D. A. and Schlaepfer, D. D. (2002). FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. *EMBO J.* **21**, 6289-6302.
- Haugh, J. M., Codazzi, F., Teruel, M. and Meyer, T. (2000). Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J. Cell Biol.* **151**, 1269-1279.
- Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta. Anat.* **124**, 689-703.
- Hsia, D., Mitra, S. K., Hauck, C. R., Streblov, D. N., Nelson, J. A., Ilic, D., Huang, S., Li, E., Nemerow, G. R., Leng, J. et al. (2003). Differential regulation of cell motility and invasion by FAK. *J. Cell Biol.* **160**, 753-767.
- Jimenez, C., Portela, R. A., Mellado, M., Rodriguez-Frade, J. M., Collard, J., Serrano, A., Martinez, A. C., Avila, J. and Carrera, A. C. (2000). Role of PI3K regulatory subunit in the control of actin organization and cell migration. *J. Cell Biol.* **151**, 249-261.

- Kam, Y. and Exton, J. H.** (2001). Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell. Biol.* **21**, 4055-4066.
- Kaplan, K. B., Swedlow, J. R., Varmus, H. E. and Morgan, D. O.** (1992). Association of p60c-Src with endosomal membranes in mammalian fibroblasts. *J. Cell Biol.* **118**, 321-333.
- Kaplan, K. B., Swedlow, J. R., Morgan, D. O. and Varmus, H. E.** (1995). c-Src enhances the spreading of src^{-/-} fibroblasts on fibronectin by a kinase-independent mechanism. *Gene Dev.* **9**, 1505-1517.
- Klinghoffer, R. A., Sachsemaier, C., Cooper, J. A. and Soriano, P.** (1999). Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J.* **18**, 2459-2471.
- Kotelevets, L., Noe, V., Bruyneel, E., Myssiakine, E., Chastre, E., Mareel, M. and Gspach, C.** (1998). Inhibition by platelet-activating factor of Src- and hepatocyte growth factor-dependent invasiveness of intestinal and kidney epithelial cells. Phosphatidylinositol 3'-kinase is a critical mediator of tumor invasion. *J. Biol. Chem.* **273**, 14138-14145.
- Kundra, V., Soker, S. and Zetter, B. R.** (1994a). Excess early signalling activity inhibits cellular chemotaxis towards PDGF-BB. *Oncogene* **9**, 1429-1435.
- Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T. and Zetter, B. R.** (1994b). Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. *Nature* **367**, 474-476.
- Lauffenburger, D. A. and Horowitz, A. F.** (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.
- Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliareis, C., Rodgers, L., McCombie, R. et al.** (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947.
- Lutz, M. P., Esser, I. B., Flossmann-Kast, B. B., Vogelmann, R., Luhrs, H., Friess, H., Buchler, M. W. and Adler, G.** (1998). Overexpression and activation of the tyrosine kinase Src in human pancreatic carcinoma. *Biochem. Biophys. Res. Commun.* **243**, 503-508.
- Malek, R. L., Irby, R. B., Guo, Q. M., Lee, K., Wong, S., He, M., Tsai, J., Frank, B., Liu, E. T., Quackenbush, J. et al.** (2002). Identification of Src transformation fingerprint in human colon cancer. *Oncogene* **21**, 7256-7265.
- Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T. and Grinstein, S.** (2001). Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fcγ receptor-mediated phagocytosis. *J. Cell Biol.* **153**, 1369-1380.
- Meijne, A. M. L., Ruuls-Van Stalle, L., Feltkamp, C. A., McCarthy, J. B. and Ross, E.** (1997). v-Src-induced cell shape changes in rat fibroblasts require new gene transcription and precede loss of focal adhesions. *Exp. Cell Res.* **234**, 477-485.
- Nakanishi, O., Shibasaki, F., Hidaka, M., Homma, Y. and Takenawa, T.** (1993). Phospholipase C-gamma 1 associates with viral and cellular src kinases. *J. Biol. Chem.* **268**, 10754-10759.
- Noh, D. Y., Ahn, S. J., Lee, R. A., Park, I. A., Kim, J. H., Suh, P. G., Ryu, S. H., Lee, K. H. and Han, J. S.** (2000). Overexpression of phospholipase D1 in human breast cancer tissues. *Cancer Lett.* **161**, 207-214.
- Piccolo, E., Innominato, P. E., Mariggio, M. A., Maffucci, T., Iacobelli, S. and Falasca, M.** (2002). The mechanism involved in the regulation of phospholipase Cgamma1 activity in cell migration. *Oncogene* **21**, 6520-6529.
- Porcelli, A. M., Ghelli, A., Hrelia, S. and Rugolo, M.** (2002). Phospholipase D stimulation is required for sphingosine-1-phosphate activation of actin stress fibre assembly in human airway epithelial cells. *Cell Signal.* **14**, 75-81.
- Price, J. T., Tiganis, T., Agarwal, A., Djakiew, D. and Thompson, E. W.** (1999). Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism. *Cancer Res.* **59**, 5475-5478.
- Rorth, P.** (2002). Initiating and guiding migration: lessons from border cells. *Trends Cell Biol.* **12**, 325-331.
- Roymans, D. and Slegers, H.** (2001). Phosphatidylinositol 3-kinases in tumor progression. *Eur. J. Biochem.* **268**, 487-498.
- Sankar, S., Mahoofi-Brooks, N. and Madri, J. A.** (1995). Modulation of cell spreading and migration by pp125FAK phosphorylation. *Am. J. Pathol.* **147**, 601-608.
- Sechi, A. S. and Wehland, J.** (2000). The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. *J. Cell Sci.* **21**, 3685-3695.
- Shen, Y., Zheng, Y. and Foster, D. A.** (2002). Phospholipase D2 stimulates cell protrusion in v-Src-transformed cells. *Biochem. Biophys. Res. Commun.* **293**, 201-206.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C.** (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.
- Sohara, Y., Ishiguro, N., Machida, K., Kurata, H., Thant, A. A., Senga, T., Matsuda, S., Kimata, K., Iwata, H. and Hamaguchi, M.** (2001). Hyaluronan activates cell motility of v-Src-transformed cells via Ras-Mitogen-activated protein kinase and phosphoinositide 3-kinase-Akt in a tumour-specific manner. *Mol. Biol. Cell* **12**, 1859-1868.
- Song, J. G., Pfeffer, L. M. and Foster, D. A.** (1991). v-Src increases diacylglycerol level via type D phospholipase-mediated hydrolysis of phosphatidylcholine. *Mol. Cell. Biol.* **11**, 4903-4908.
- Soriano, P., Montgomery, C., Geske, R. and Bradley, A.** (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693-702.
- Sorkin, A. and von Zastrow, M.** (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* **3**, 600-614.
- Stoker, A. W., Kellie, S. and Wyke, J. A.** (1986). Intracellular localisation and processing of pp60^{v-src} proteins expressed by two distinct temperature-sensitive mutants of Rous sarcoma virus. *J. Virol.* **58**, 876-883.
- Swanson, J. A. and Watts, C.** (1995). Macropinocytosis. *Trends Cell Biol.* **5**, 424-428.
- Thiery, J. P.** (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442-454.
- Thomas, S. M. and Brugge, J. S.** (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13**, 513-609.
- Thorsen, V. A., Vorland, M., Bjorndal, B., Bruland, O., Holmsen, H. and Lillehaug, J. R.** (2003). Participation of phospholipase D and alpha/beta-protein kinase C in growth factor-induced signalling in C3H10T1/2 fibroblasts. *Biochim. Biophys. Acta* **1632**, 62-71.
- Turner, T., Epps-Fung, M. V., Kassisi, J. and Wells, A.** (1997). Molecular inhibition of phospholipase Cgamma signaling abrogates DU-145 prostate tumor cell invasion. *Clin. Cancer Res.* **3**, 2275-2282.
- Vanhaesebroeck, B., Jones, G. E., Allen, W. E., Zicha, D., Hooshmand-Rad, R., Sawyer, C., Wells, C., Waterfield, M. D. and Ridley, A. J.** (1999). Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat. Cell Biol.* **1**, 69-71.
- Vaziri, C. and Faller, D. V.** (1995). Repression of platelet-derived growth factor β-receptor expression by mitogenic growth factors and transforming oncogenes in murine 3T3 fibroblasts. *Mol. Cell. Biol.* **15**, 1244-1253.
- Veithen, A., Cupers, P., Baudhuin, P. and Courttoy, P. J.** (1996). v-Src induces constitutive macropinocytosis in rat fibroblasts. *J. Cell Sci.* **109**, 2005-2012.
- Verbeek, B. S., Vroom, T. M., Adriaansen-Slot, S. S., Ottenhoff-Kalff, A. E., Geertzema, J. G., Hennipman, A. and Rijksen, G.** (1996). c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. *J. Pathol.* **180**, 383-388.
- Wasilenko, W. J., Nori, M., Testerman, N. and Weber, M. J.** (1990). Inhibition of epidermal growth factor receptor synthesis caused by the src oncogene product, pp60^{v-src}. *Mol. Cell. Biol.* **10**, 1254-1258.
- Wiener, J. R., Windham, T. C., Estrella, V. C., Parikh, N. U., Thall, P. F., Deavers, M. T., Bast, R. C., Mills, G. B. and Gallick, G. E.** (2003). Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers. *Gynecol. Oncol.* **88**, 73-79.
- Wright, J. D., Reuter, C. W. and Weber, M. J.** (1996). Identification of sites on epidermal growth factor receptors which are phosphorylated by pp60^{src} in vitro. *Biochim. Biophys. Acta* **1312**, 85-93.
- Wu, H. and Parsons, J. T.** (1993). Cortactin, an 80/85-kilodalton pp60^{src} substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* **120**, 1417-1426.
- Wyke, J. A. and Linial, M.** (1973). Temperature-sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. *Virology* **53**, 152-161.
- Zhao, Y., Ehara, H., Akao, Y., Shamoto, M., Nakagawa, Y., Banno, Y., Deguchi, T., Ohishi, N., Yagi, K. and Nozawa, Y.** (2000). Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. *Biochem. Biophys. Res. Commun.* **11**, 140-143.
- Zicha, D., Dunn, G. A. and Brown, A. F.** (1991). A new direct-viewing chemotaxis chamber. *J. Cell Sci.* **99**, 769-775.