The case for high-dose adjuvant chemotherapy in breast cancer: (II) clinical experience

Citation for published version:

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
European Journal of Surgical Oncology (EJSO)

Publisher Rights Statement:
available via europepmc open access link

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
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

V.J. Fincham, M. Unlu, V.G. Brunton, J.D. Pitts, J.A. Wyke, and M.C. Frame
The Beatson Institute for Cancer Research, CRC Beatson Laboratories, Glasgow, Scotland G61 1BD

Abstract. We have isolated Swiss 3T3 subclones that are resistant to the mitogenic and morphological transforming effects of v-Src as a consequence of aberrant translocation of the oncoprotein under low serum conditions. In chicken embryo and NIH 3T3 fibroblasts under similar conditions, v-Src rapidly translocates from the perinuclear region to the focal adhesions upon activation of the tyrosine kinase, resulting in downstream activation of activator protein-1 and mitogen-activated protein kinase, which are required for the mitogenic and transforming activity of the oncoprotein. Since serum deprivation induces cytoskeletal disorganization in Swiss 3T3, we examined whether regulators of the cytoskeleton play a role in the translocation of v-Src, and also c-Src, in response to biological stimuli. Actin stress fibers and translocation of active v-Src to focal adhesions in quiescent Swiss 3T3 cells were restored by microinjection of activated Rho A and by serum. Double labeling with anti-Src and phalloidin demonstrated that v-Src localized along the reformed actin filaments in a pattern that would be consistent with trafficking in complexes along the stress fibers to focal adhesions. Furthermore, treatment with the actin-disrupting drug cytochalasin D, but not the microtubule-disrupting drug nocodazole, prevented v-Src translocation. In addition to v-Src, we observed that PDGF-induced, Rac-mediated membrane ruffling was accompanied by translocation of c-Src from the cytoplasm to the plasma membrane, an effect that was also blocked by cytochalasin D. Thus, we conclude that translocation of Src from its site of synthesis to its site of action at the cell membrane requires an intact cytoskeletal network and that the small G proteins of the Rho family may specify the peripheral localization in focal adhesions or along the membrane, mediated by their effects on the cytoskeleton.

The product of the v-src gene is a protein tyrosine kinase that, in its active form, localizes to the plasma membrane where it initiates a cascade of signaling events leading to altered cell growth and behavior. We, and others, have extensively studied the G1/S to G2/M progression induced by v-Src in rat and chicken embryo cells withdrawn from the cell cycle by serum deprivation (Bell et al., 1975; Durkin and Whitfield, 1984; Welham et al., 1990; Catling et al., 1993; Wyke et al., 1993). Over the past few years, some of the intracellular signals that are crucial for the mitogenic activity of the v-Src oncoprotein have been identified. These include the signaling pathway that links p21 Ras to the mitogen-activated protein (MAP)1 kinases and their targets (Gupta et al., 1992; Howe et al., 1992; Wang and Erickson, 1992; Cowley et al., 1994; Wyke et al., 1995) and the transcription factor AP-1, which is activated by a variety of mechanisms in different cell types (Welham et al., 1990; Catling et al., 1993). In addition, we have demonstrated that activation of MAP kinase and activator protein-1 (AP-1) occur at times during G1 when v-Src activity is required and that both are necessary for mitogenesis (Frame et al., 1994; Wyke et al., 1995). We have also shown that the ability of v-Src to morphologically transform fibroblasts is not impaired by attenuation of either MAP kinase or AP-1 activation, indicating that the pathways that regulate mitogenesis are biochemically distinct from those that induce cell shape changes (Frame et al., 1994; Wyke et al., 1995). However, both the mitogenic and morphological effects of v-Src require its localization at the cell membrane (Catling et al., 1993).

Despite our increasing understanding of the role of the Ras/MAP kinase pathway and AP-1 in the mitogenic activity of v-Src, there is still no clear information on the upstream events that initiate this process. Our studies to date have used Rat-1 or chicken embryo fibroblasts expressing a temperature-sensitive mutant of v-Src (ts LA29), in

1. Abbreviations used in this paper: AP-1, activator protein-1; CEF, chicken embryo fibroblasts; MAP, mitogen-activated protein; MBP, myelin basic protein; PI, phosphatidylinositol.
which a single point mutation in the kinase domain gives rise to both temperature-dependent kinase activity and temperature-dependent localization to the cell periphery (Welham and Wyke, 1988). In this study, we describe Swiss 3T3 cell clones that are resistant to the mitogenic effects of ts LA29 v-Src upon shifting serum-deprived cells to the permissive temperature and describe characterization of the defect that is responsible for the lack of v-Src-induced MAP kinase and AP-1 activation and their biological consequences. For comparison, we used ts LA29 v-Src-expressing NIH 3T3 cells that undergo C_3 to S-phase transition in response to shift to permissive temperature. We demonstrate that cytoskeletal disruption, which occurs in Swiss 3T3 cells that have been deprived of serum (Ridley and Hall, 1992), is associated with their resistance to v-Src-induced mitogenesis as a consequence of impaired translocation of ts LA29 v-Src to the cell periphery. We further show that serum-induced reformation of the organized actin cytoskeletal network restores v-Src translocation upon shift to permissive temperature. These observations led us also to examine the subcellular localization of c-Src in response to a biological stimulus known to require c-Src activity and to conclude that the cytoskeleton also plays a crucial role in the redistribution of c-Src from the perinuclear cytoplasm to its membrane sites of action. We provide evidence that stimulus-specific cytoskeletal rearrangements, which are known to be mediated by members of the Rho family of small G proteins, may specify the localization of Src kinases at the plasma membrane.

Materials and Methods

Generation of Cell Lines

Swiss 3T3 and NIH 3T3 cell lines grown in DME supplemented with 10% FCS were transfected with 1 μg of the Moloney murine leukemia virus based vector pGV-1 (DeClue and Martin, 1989), into which the coding sequences for ts LA29 v-src had been cloned (provided to us by Andy Catling, The Beatson Institute). Transfections were by the DOTAP method (Boehringer Mannheim Corp., Indianapolis, IN) as per manufacturer's instructions, and neomycin-resistant colonies were selected in G418 (GIBCO BRL) at 500 μg/ml. Single colonies were isolated and those shown to express high levels of v-Src by immunoblotting (see Fig. 1) were chosen for study. C2F/S92 used for comparison in these localization studies were generated by transfection of the replication competent Rous-associated virus encoding ts LA29 v-src as described previously (Catling et al., 1994). Cytochalasin D (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and added to the culture medium to a final concentration of 0.1 μg/ml. Nocodazole (Sigma Chemical Co.) was dissolved in DMSO and used at 0.5 μg/ml.

Immunoblotting and Src Kinase Assays

v-Src was detected by preparing lysates in 0.1 M Tris, pH 8, 2% SDS, and 1 mM DTT. v-Src was detected by preparing lysates in 0.1 M Tris, pH 8, 2% SDS, and 1 mM DTT. Protein preparations were checked for activity by the method of Catling et al. (1994). GST-Val 14-Rho A protein was prepared, cleaved by thrombin (Sigma Chemical Co.), and collected from 50 μg lysate was reacted with mAb EC10 (Upstate Biotechnology, Inc.) and collected on rabbit anti-mouse-coated protein A-Sepharose. Immunoprecipitates were washed in NP-40 buffer and kinase assay buffer (100 mM Pipes-sodium, pH 6.8, 20 mM manganese chloride, 10 μM sodium orthovanadate) before resuspending in kinase assay buffer. An aliquot of v-Src-protein A-Sepharose (amount checked so that assay conditions were linear) was incubated with 5 μCi of [γ-32P]ATP (3,000 Ci/mM) (Amersham Corp.) and 1 μg denatured enolase in a final assay volume of 20 μl. The reaction was stopped by addition of 20 μl 0.1 M Tris, pH 8, 2% SDS, and 1 mM DTT, and the supernatant was separated by 10% SDS-PAGE. Lysate protein concentrations for immune-complex kinase assays and AP-1 DNA-binding assays were determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

AP-1 DNA-binding and MAP Kinase Assays

Cells from 100-mm dishes were lysed in 100 μl of buffer containing 20 mM Hepes, pH 7.9, 5 mM EDTA, 10 mM EGTA, 5 mM sodium fluoride, 0.1 μg/ml okadaic acid, 1 mM DTT, 0.4 M potassium chloride, 0.4% Triton X-100, 10% glycerol, and a mixture of protease inhibitors including pepstatin A, leupeptin, and aprotinin (all at 5 μg/ml), together with 1 mM benzamidine and 0.25 mM PMSF. Lysates were clarified by high-speed spin at 4°C. DNA-binding reactions were carried out in 20 mM Hepes buffer, pH 7.9, with 50 mM sodium chloride, 5 mM magnesium chloride, and 3 μg poly-dIdC. An oligonucleotide probe representing the collagenase AP-1 site (AGCTGTCGACCTAGGTGC) was end labeled using [γ-32P]ATP and T4 polynucleotide kinase (Northumberland Biologicals) and incubated with 15 μg protein for 30 min on ice. DNA–protein complexes were resolved by electrophoresis in 4% polyacrylamide gels.

The activity of p42 MAP kinase (ERK2) in 50 μg cell lysate was measured by immune-complex kinase assays using an antisense raised against the carboxy-terminal peptide of ERK2 as was used by Leevers and Marshall (1992), exactly as described in Wyke et al. (1995).

Immunofluorescence

For immunofluorescence confocal microscopy, cells were grown on coverslips, fixed at 4°C for 15 min with 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100 before incubation with 1:1,000 anti-Src mAb EC10, 1:100 anti-vinculin (Sigma Chemical Co.), 1:500 anti-tubulin (Sigma Chemical Co.), or 1 ng/ml TRITC-conjugated phalloidin (Sigma Chemical Co.) for 60 min at room temperature. Antibody detection was by reaction with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 45 min at room temperature. Antibody detection was by reaction with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 45 min at room temperature. Antibody detection was by reaction with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 45 min at room temperature. Antibody detection was by reaction with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) for 45 min at room temperature.

Microinjection of Rho A

pGEX-2T-rho A was kindly provided to us by Alan Hall (University College, London, UK). We derived Val 14-rho A by PCR-based mutagenesis. GST-Val 14-Rho A protein was prepared, cleaved by thrombin (Sigma Chemical Co.) and purified as described for Rac proteins (Ridley et al., 1992). Protein preparations were checked for activity by the method of Hall and Sefl (1986). Val 14-Rho A protein (1 μg/ml) in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM Mg Cl2, and 0.01 mM DTT was mixed with filtered Lucifer yellow CH dye (2%) and injected into about 40 quiescent Swiss 3T3-E92 cells per experiment using a microinjector system (model 2524, Eppendorf, Inc., Fremont, CA). After microinjection, cells were incubated in low serum medium at 4°C for several hours before switching to 35°C. Swiss 3T3-E92 cells are not coupled by GAP junctions, and Lucifer yellow provided a marker of injected cells. As controls, we examined
cells microinjected with dye alone and cells microinjected with Val-14-Rho A maintained at the restrictive temperature.

**Results**

**Swiss 3T3 Cells Are Resistant to v-Src-induced Mitogenesis**

Initial characterization of several independent clones of Swiss 3T3, which stably express high levels of ts LA29 v-Src, demonstrated that the oncoprotein was unable to induce a mitogenic response in Swiss 3T3 cells that had been made quiescent by serum deprivation. This was shown by the lack of stimulation of thymidine uptake after shift to the permissive temperature (35°C) (Fig. 1 a) and was in contrast to fibroblasts from other species, including rat and chicken (Welham et al., 1990; Catling et al., 1993). v-Src was also able to act as a mitogen in quiescent NIH 3T3 cells expressing similar amounts of ts LA29 v-Src (Fig. 1 b), showing that the lack of response in Swiss 3T3 was not a general property of mouse fibroblasts. Furthermore, addition of 10% serum induced reentry of quiescent Swiss 3T3 into cell cycle (Fig. 1 a), indicating that these cells were able to respond mitogenically to an appropriate stimulus.

**Figure 1.** Swiss 3T3 cells are resistant to the mitogenic effects of v-Src. (a) Swiss 3T3-f29 cells were serum deprived at the restrictive temperature of 40°C (control), and stimulated by addition of serum or shift to 35°C for up to 36 h. S-phase entry was monitored by thymidine uptake (cpm x 10^-4). (Inset) Level of ts LA29 v-Src expressed in the subclone of Swiss 3T3 used (Swiss f29) and is compared with parental Swiss 3T3 cells (control). Immunoblots were of lysates from equivalent cell numbers run on the same gel. (b) Mitogenesis of NIH 3T3-f29 cells as described for Swiss 3T3-f29 in a above.

**Figure 2.** AP-1 DNA binding and MAP kinase are not stimulated by v-Src in quiescent Swiss 3T3 cells. (a) Gel retardation analysis of AP-1 DNA-binding activity in lysates prepared from Swiss 3T3-f29 cells (upper) and NIH 3T3-f29 cells (lower) that had been made quiescent by serum deprivation at 40°C (0) or stimulated by serum addition or shift to 35°C for 0.25, 0.5, 1, 2, or 4 h. (b) MAP kinase activity immunoprecipitated from lysates of Swiss 3T3-f29 cells (left) and NIH 3T3-f29 cells (right) that had been made quiescent by serum deprivation at 40°C (0) or stimulated by serum addition or shift to 35°C for 1, 2, or 4 h. The substrate used was MBP. The lower panel shows the level of p42 MAP kinase determined by immunoblotting. MAP kinase activation, quantified by scanning laser densitometry of MBP phosphorylated in the in vitro MAP kinase assays, is presented as a bar chart.
Figure 3. Serum-deprived Swiss 3T3 cells are resistant to morphological transformation by v-Src. (a) Phase contrast images of Swiss 3T3-f29 cells that are growing (10% serum, upper) or serum deprived (lower) at 40 or 35°C. (b) Images of serum-deprived NIH 3T3-f29 cells at 40 or 35°C.
AP-1 and MAP Kinase Are Not Activated by v-Src in Quiescent Swiss 3T3 Cells

Since we had previously shown that AP-1 and MAP kinase activation are essential components of v-Src-induced mitogenesis, we examined whether these were activated in serum-deprived Swiss 3T3-ts and NIH 3T3-ts after addition of serum or shift to 35°C. Although serum induced AP-1 DNA-binding activity, as measured by gel retardation of an AP-1 consensus oligonucleotide, shift to the permissive temperature did not appreciably induce AP-1 DNA-binding activity, as measured by gel retardation of an AP-1 consensus oligonucleotide, shift to the permissive temperature did not appreciably induce AP-1 DNA binding (Fig. 2 a, top). In contrast, activation of ts LA29 v-Src by shift to 35°C in NIH 3T3 cells resulted in an obvious stimulation of AP-1 DNA binding (Fig. 2 a, lower). Similarly, measurement of MAP kinase activity, using an immune-complex kinase assay with antisera specific for the carboxy terminus of p42 MAP kinase and myelin basic protein (MBP) as substrate, demonstrated that shift to permissive temperature resulted in a classical early and transient activation in NIH 3T3-ts29 (Fig. 2 b, right) but induced no response in Swiss 3T3-ts29 (Fig. 2 b, left). These data show that AP-1 and MAP kinase are stimulated upon shift to 35°C in v-Src–responsive quiescent NIH 3T3 cells, but not in the v-Src–unresponsive Swiss 3T3 clones.

v-Src Is Not Translocated to the Cell Periphery in Quiescent Swiss 3T3 Cells

Since the defect in quiescent Swiss 3T3-ts29 that renders the cells nonpermissive for v-Src–induced mitogenesis was apparently upstream of activation of MAP kinase, one possibility was that v-Src was not activated or localized properly upon shift to 35°C. One prediction of the hypothesis that the defect is aberrant regulation of v-Src itself is that shift of serum-deprived Swiss 3T3-ts29 to 35°C would also not induce morphological transformation. Thus, we compared the cellular morphologies of Swiss 3T3-ts29 and NIH 3T3-ts29 that were either growing or serum deprived at the restrictive temperature (40°C) or after shift for 36 h to 35°C. As shown, serum-deprived Swiss 3T3-ts29 cells shifted to 35°C (Fig. 3 a, lower) do not assume the refractile rounded morphology characteristic of growing cells transformed at 35°C, although they appear granular (Fig. 3 a, upper). In contrast, serum-deprived NIH 3T3-ts29 become morphologically transformed at 35°C (Fig. 3 b) and appear similar to growing cells at this temperature (not shown). These observations confirm that the defect in v-Src activity in Swiss 3T3 cells is not confined to the mitogenic pathways, but that other biological consequences of v-Src activity are not induced when these cells are serum deprived. This is consistent with aberrant regulation of v-Src itself in serum-deprived Swiss 3T3 cells. Further characterization of Swiss 3T3-ts29 clones showed that ts LA29 v-Src was able to induce not only morphological cell rounding when the cells were growing but also an enhanced growth rate and growth in soft agar in a temperature-dependent manner (data not shown), indicating that v-Src was able to function as an efficient oncoprotein in Swiss 3T3 cells grown in the presence of serum.

As mentioned, ts LA29 v-Src is both catalytically activated and relocated from the perinuclear region to the cell membrane in response to shift to permissive temperature in rat cells (Welham and Wyke, 1988). Thus, we tested the reactivation kinetics of the v-Src tyrosine kinase and its subcellular localization. The v-Src kinase from quiescent Swiss 3T3-ts29 cells reactivated upon shift to 35°C with similar kinetics to v-Src from NIH 3T3-ts29 cells, as demonstrated by immune-complex kinase assays using exogenous enolase as substrate (Fig. 4 a). The level of v-Src protein was relatively invariant during temperature-shift of both cell lines, indicating that reactivation was due to increased specific kinase activity (Fig. 4 b). Furthermore, the reactivation kinetics were similar to those for ts LA29 v-Src from quiescent chicken embryo fibroblasts (CEF/R29), in which this v-Src mutant is an effective mitogen and transforming agent (Catling et al., 1993). In addition, both quiescent Swiss 3T3-ts29 and NIH 3T3-ts29 exhibited temperature-dependent tyrosine phosphorylation of cellular proteins upon activation of v-Src (not shown), demonstrating that activation of the tyrosine kinase occurs in vivo.

Examination of the subcellular distribution of v-Src showed that temperature-induced translocation to the cell periphery was impaired in serum-deprived Swiss 3T3-ts29 (Fig. 5 a). Immunofluorescence confocal microscopy using mAb EC10 demonstrated that in growing and serum-deprived Swiss 3T3-ts29 at 40°C, v-Src was localized around the nucleus (Fig. 5 a, 1 and 2). Control cells treated with conjugated secondary antibody alone displayed nuclear fluorescence that was not seen when primary antibody reactive with v-Src was also used (not shown). Upon shift to 35°C, a proportion of the v-Src localized to the cell periphery in growing cells, predominantly to discrete adhesions at early times (Fig. 5 a, 2, upper), and to the membrane/residual adhesions at later times in rounded transformed cells in which v-Src had induced focal adhesion degradation (Fig. 5 a, 2, lower). In contrast, v-Src remained mainly in the perinuclear region in cells, which had been deprived of serum, even after 4 h at the permissive temperature (Fig. 5 a, 4). At later times (up to 16 h) at 35°C, v-Src...
Figure 5. (a) Immunofluorescence confocal images of cells stained with anti-v-Src and a second antibody conjugated to FITC. 1 and 3 show growing and serum-deprived Swiss 3T3-f29 cells at 40°C, 2 shows growing Swiss 3T3-f29 cells that had been switched to 35°C for 2 h (upper) or 16 h (lower). The arrows point to v-Src present in focal adhesions at early times after transfer to 35°C or at the cell membrane and in residual adhesions at a later time. 4 shows serum-deprived Swiss 3T3-f29 cells that had been switched to 35°C for 2 h (upper) or 4 h (lower). (b) v-Src localization in quiescent CEF/R29 and NIH 3T3-f29 at restrictive temperature (41°C for CEF and 40°C for NIH 3T3) and after shift to 35°C for 30 min and 2 h, respectively. Bars, 20 μm.

remained perinuclear, although the serum-deprived Swiss 3T3-f29 cells became granular in appearance, indicating that the cells were unhealthy (Fig. 3 a). For comparison, the normal translocation of ts LA29 v-Src to focal adhesions induced in CEF/R29 after shift to 35°C for 30 min (Fig. 5 b, 2) and in quiescent NIH 3T3-f29 after shift to 35°C for 2 h (Fig. 5 b, 4) are shown. These observations demonstrate that, in contrast to v-Src-responsive cells, the v-Src in un-
responsive Swiss 3T3-f29 cells is not translocated to its site of action in focal adhesions at the cell periphery upon shift to the permissive temperature.

Translocation of v-Src Requires an Organized Actin Cytoskeleton

Since Swiss 3T3 are known to differ from other fibroblasts by disorganizing the cellular stress fibers and focal adhesions upon serum deprivation (Ridley and Hall, 1992), we examined the cytoskeleton of Swiss 3T3-f29 cells upon serum deprivation and after serum refeeding. As expected, phalloidin staining revealed that in serum-deprived Swiss 3T3-f29 cells, there were few stress fibers, with much of the actin having a punctate distribution (not shown); in contrast, stress fibers were detected in quiescent NIH 3T3-f29 (not shown). Upon refeeding Swiss 3T3-f29 cells with serum, new stress fibers formed rapidly, a process that is known to be dependent on the activity of the small G protein Rho (Ridley and Hall, 1992). Thus, the loss of organized actin stress fibers in quiescent Swiss 3T3 f-29 cells correlated with the lack of v-Src translocation to the cell periphery. We then tested whether serum-induced reformation of actin stress fibers restored the ability of ts LA29 v-Src to translocate to the membrane. Addition of serum for 1 h at 35°C resulted in the redistribution of some v-Src from around the nucleus outwards to the cell periphery (Fig. 6 a). The staining pattern indicated that v-Src was present in discrete structures or complexes that appeared to align with the newly formed actin stress fibers, shown in Fig. 6 b for the same cell. When the confocal images from staining with anti-v-Src (detected by FITC as green in Fig. 6 a) and phalloidin (detected by rhodamine as red in Fig. 6 b) were overlaid, the colocalization of the v-Src-containing complexes with the stress fibers became evident (detected as yellow) (Fig. 6 c). This pattern was consistent with the idea that v-Src-containing complexes may track along the newly formed actin cables to focal adhesions at the stress fiber termini. Overlaid images of a group of cells after shift to permissive temperature for a longer period in the pres-
Figure 6. Reassembly of the organized actin cytoskeleton is permissive for v-Src translocation. Swiss 3T3-f29 cells, which had been made quiescent by serum deprivation at 40°C, were refed with 10% serum or microinjected with Val 14-Rho A and shifted to 35°C before double staining with anti-v-Src (detected by FITC) and phalloidin (detected by rhodamine), or stained with antivinculin (detected by FITC), and visualized by confocal microscopy. (a) v-Src distribution after 1 h of serum at 35°C, (b) actin distribution in the same cell, (c) overlaid images showing distribution of both v-Src and actin stress fibers, and (d) overlaid images of a group of cells that had been refed with serum at 35°C for 4 h. (e) v-Src distribution after 2 h at 35°C in serum-deprived cells that had been microinjected with Val 14-
Figure 7. Cytochalasin D blocks v-Src translocation and v-Src-induced mitogenesis. (a) Swiss 3T3-f29 cells that had been made quiescent by serum deprivation at 40°C were refed with 10% serum and shifted to 35°C for 4 h in the presence of 0.1 µg/ml cytochalasin D before double staining with phalloidin (detected by rhodamine in 1) and anti-v-Src (detected by FITC in 2). (b) Quiescent NIH 3T3-f29 cells shifted for 4 h to 35°C in the presence of cytochalasin D were stained as for Swiss 3T3-f29 cells in a. Bars, 20 µm.

ence of serum (4 h), showed that much of the v-Src was now localized in adhesions at the ends of stress fibers (Fig. 6 d). The v-Src-containing complexes at these termini assumed a more elongated shape, reminiscent of mature focal adhesions in Swiss 3T3 cells (Barry and Critchley, 1994). Microinjection of an activated form of the small G protein Rho A (Val 14-Rho A), which is known to induce formation of actin stress fibers (Ridley and Hall, 1992), was able to substitute for serum in permitting translocation of v-Src upon shift to the permissive temperature (Fig. 6 e). Control cells that were microinjected with Lucifer yellow dye and shifted to the permissive temperature and microinjected cells maintained at the restrictive temperature did not show localization of v-Src in focal adhesions (not shown). Although it hasn’t been possible for double labeling of cells with vinculin and v-Src (since both antibodies were raised in mice), we observed that the staining pattern of the focal adhesion component vinculin was similar to v-Src. 4 h after refeeding quiescent Swiss 3T3-f29 cells with serum, vinculin was predominantly present in focal adhesions with some punctate staining also distributed throughout the cell (Fig. 6 f). The major difference between the vinculin and v-Src distribution under these conditions is the retention of a substantial amount of v-Src around the nucleus, presumably as a result of its vast overexpression. These observations suggest that v-Src may be transported along actin stress fibers in complexes that are destined to become focal adhesions at the stress fiber termini.

To determine if the serum-induced reformation of actin
The cytoskeleton was severely disrupted in cytochalasin D-treated quiescent Swiss 3T3-f29 cells that had been refed with serum and shifted to 35°C for 4 h, as shown by the lack of stress fibers (phalloidin staining in Fig. 7 a, 1). In addition, v-Src remained tightly located around the nucleus upon temperature shift (Fig. 7 a, 2), implying that the cytoskeleton plays an essential role in the transport of v-Src to the cell periphery. Similarly, translocation of v-Src is blocked in quiescent NIH 3T3-f29 cells upon shift to the permissive temperature (Fig. 7 b, 2). Consistent with the known requirement for v-Src activity at the cell periphery for mitogenic activity (Catling et al., 1993), ts LA29 v-Src was unable to activate G0/G1- to S-phase progression in cytochalasin D-treated NIH 3T3-f29 cells upon shift to 35°C (not shown). These data indicate that the actin cytoskeleton plays a crucial role in the translocation, and hence the biological activity, of v-Src.

**Translocation of v-src to Focal Adhesions Does Not Require Organized Microtubules**

The observations described above do not rule out the possibility that the activated v-Src-containing complexes are associated with structures that align in parallel with the Rho-induced actin stress fibers. This, together with the reported colocalization of c-Src with microtubules (Kaplan et al., 1992), prompted us to examine whether the microtubular network might be involved in the translocation of v-Src to the cell periphery. Anti-α-tubulin staining of Swiss 3T3-f29 cells displayed a highly cross-linked array of microtubules in the cytoplasm, which was distinct from stress fiber staining and which was unaffected by serum deprivation (Fig. 8 a, for comparison see Figs. 6 b and 8 b). Although the staining patterns of α-tubulin and phalloidin appeared distinct, the microtubular and actin networks must cross in the cytoplasm and local coalignment may occur. However, treatment of serum-deprived Swiss 3T3-f29 cells with 0.5 μg/ml of nocodazole, which led to severe disruption of the microtubular network (Fig. 8 b), did not block the translocation of v-Src to focal adhesions (Fig. 8, c and d), indicating that the peripheral recruitment of v-Src can occur independently of microtubules.

**Recruitment of c-Src to the Cell Periphery also Requires the Actin Cytoskeleton**

To determine whether the cytoskeleton was a general mediator of biologically stimulated intracellular transport of Src kinases, we tested whether PDGF, a potent fibroblast growth factor that is known to require the activity of c-Src or its close family members for its effects in NIH 3T3 cells (Twamley-Stein et al., 1993), was able to induce translocation of c-Src to the cell periphery in a cytoskeleton-dependent manner. We treated quiescent parental NIH 3T3 cells with 5 ng/ml PDGF and examined the subcellular distribution of c-Src using mAb N2-17. In unstimulated cells, c-Src was distributed throughout the cytoplasm (Fig. 9 a). The strong nuclear staining was also seen when the cells were incubated with FITC-conjugated second antibody alone (data not shown), indicating that it may be nonspecific. In PDGF-treated cells, some c-Src translocated to the cell periphery within 30 min (Fig. 9 b), where it remained for 60 min (Fig. 9 c). In some cells, a staining pattern was observed that was consistent with stress fiber alignment at early times after PDGF treatment (e.g. Fig. 9 b, left). Upon double labeling with phalloidin, it was evident that the peripheral regions to which c-Src was recruited coincided with regions of dense actin staining (not shown), a property that is associated with structures known as membrane ruffles, which are induced by PDGF (Mellström et al., 1988; Ridley and Hall, 1992). Consistent with a role for the actin cytoskeleton in the translocation of c-Src, cytochalasin D blocked PDGF-induced recruitment of c-Src to the putative membrane ruffles (Fig. 9 d).

**Discussion**

In order for v-Src to work as an effective oncoprotein, it must be translocated from its site of synthesis to its site of action at the cell periphery. Early efforts to locate v-Src within Rous sarcoma virus-infected cells produced apparently paradoxical views on its subcellular distribution. Biochemical fractionation, immunofluorescence, and electron microscopy suggested that the majority of v-Src in transformed cells was associated with the plasma membrane (Rohrschneider et al., 1979; Willingham et al., 1979; Courtneidge et al., 1980). However, there were also reports that v-Src was concentrated in the perinuclear region (Rohrschneider et al., 1979), associated with the cellular cytoskeleton (Burr et al., 1980), and located in residual focal adhesions (Rohrschneider, 1979; Nigg et al., 1982; Kreuger et al., 1984; Kellie et al., 1986). One explanation for these seemingly contradictory claims is provided by our studies using Swiss 3T3 cells that stably express high levels of v-Src protein, but that are resistant to its mitogenic effects as a result of aberrant membrane localization. Immunofluorescence, combined with the resolving power provided by the confocal microscope, has allowed us to propose that the actin cytoskeleton plays an important role in targeting v-Src, and its cellular homolog c-Src, to their sites of action at the cell periphery.

The ts LA29 mutant v-Src differs from the wild-type

---

**Figure 8.** v-Src transport to focal adhesions is independent of microtubules. (a) Swiss 3T3-f29 cells that had been made quiescent by serum deprivation at 40°C were stained with anti-α-tubulin (detected by FITC) or (b) refed with 10% serum for 1 h at 40°C in the presence of 0.5 μg/ml nocodazole and triple labeled with phalloidin (detected by rhodamine, left) and v-Src and tubulin (both detected by FITC, right). In c and d, cells refed with 10% serum and shifted to 35°C for 1 or 2 h, respectively, in the presence of nocodazole, were double stained with anti-v-Src and anti-α-tubulin (both detected by FITC). White arrows show v-Src at the cell periphery and broken white arrows show collapsed microtubular network. That the FITC staining at the cell periphery was due to v-Src was confirmed by single staining of a parallel coverslip. Bars, 20 μm.
Figure 9. PDGF-induced translocation of c-Src to the cell periphery is blocked by cytochalasin D. Parental NIH 3T3 cells were made quiescent by serum deprivation (a), treated for 30 or 60 min with 5 ng/ml PDGF (b and c, respectively), or treated with PDGF for 60 min in the presence of 0.1 μg/ml cytochalasin D (d), before staining with mAb N2-17 specific for c-Src (detected by FITC). Arrows point to regions of the cell membrane to which v-Src has been recruited. Bars, 20 μm.

protein by three amino acid substitutions, one of which is responsible for both temperature-sensitive tyrosine kinase activity and temperature-sensitive localization to the cell periphery (Welham and Wyke, 1988). Although the ts LA29 v-Src kinase was activated upon shift to permissive temperature in unresponsive serum-deprived Swiss 3T3-f29 cells, it remained perinuclear and, in this location, the kinase activity was not able to induce the downstream signaling consequences of v-Src as judged by activation of MAP kinase and the AP-1 transcription factor. This is consistent with the properties of a myristylation-defective version of ts LA29 v-Src, which is unable to stimulate AP-1 or mitogenesis in CEF (Catling et al., 1993), and demonstrates conclusively that translocation to the vicinity of its substrates at the cell periphery is an absolute requirement for these biological activities.

A clue to the defect in serum-deprived Swiss 3T3 cells that rendered them nonpermissive for activation-dependent v-Src translocation came from knowledge of the extreme phenotype that these cells display in disorganizing the actin cytoskeleton when they are made quiescent by serum-deprivation (Ridley and Hall, 1992; Barry and Critchley, 1994). Consistent with this, v-Src was translocated to the cell periphery in Swiss 3T3 when the cells were growing in the presence of serum, conditions that restored the actin cytoskeleton and were permissive for v-Src-induced growth in suspension, enhanced growth rate, and morphological transformation. In addition, v-Src
is an effective mitogen and transforming agent in other fibroblast cell lines that maintain some organized cytoskeleton when serum deprived.

Serum-induced reorganization of the actin cytoskeleton, which is known to be mediated by the small G protein Rho A in Swiss 3T3 cells (Ridley and Hall, 1992), led to the rapid redistribution of activated v-Src within the cell. At early times after activation, v-Src was present in oval shaped 'structures,' which appeared to align along the newly formed stress fibers. At later times, more v-Src was present in structures that had an elongated shape reminiscent of mature focal adhesions (Barry and Critchley, 1994) at the ends of stress fibers. This pattern of localization suggested that activated v-Src could be recruited into complexes that were destined to become focal adhesions and that these complexes were in turn recruited to the position of functional focal adhesions at the ends of stress fibers, possibly by tracking along the cytoskeleton. The blocking of serum-induced v-Src translocation in Swiss 3T3 cells, and in quiescent NIH 3T3 cells, by the actin cytoskeleton-disrupting drug cytochalasin D supports a role for the actin stress fibers in the translocation of v-Src to the cell periphery. Furthermore, the ability of microinjected active Rho A to permit v-Src translocation to focal adhesions indicates a role for the small G protein regulators of the cytoskeleton in actin-dependent protein trafficking.

Although little is known about the mechanics of focal adhesion assembly and the targeting of components to these structures, vinculin staining of Swiss 3T3 cells several hours after serum refeeding displayed a similar distribution in focal adhesions at the cell periphery with some staining in punctate smaller complexes through the cytoplasm. In addition, cytochalasin D treatment resulted in attenuation of vinculin transport to the focal adhesions as judged by the perinuclear localization of vinculin under these conditions (data not shown). Thus, it is possible that active v-Src-containing complexes that move out from the perinuclear region in response to serum may represent immature focal adhesion complexes. If true, this implies that the actin stress fibers have an important role in the assembly and trafficking of complexes containing focal adhesion components to their sites of action at the stress fiber termini.

In addition to v-Src, we also observed that the cytoskeleton was required for the stimulus-induced translocation of endogenous c-Src from its cytoplasmic location to the cell periphery. In particular, PDGF, a mitogen that requires Src family kinase binding to its activated receptor for activity in NIH 3T3 cells (Twamley-Stein et al., 1993), stimulated redistribution of c-Src to the plasma membrane, specifically to regions of dense actin staining around the cell periphery. Furthermore, this redistribution was blocked by cytochalasin D, demonstrating that c-Src translocation to the cell periphery is also dependent on the actin cytoskeleton. Since PDGF induces dense regions of actin known as membrane ruffles in Swiss 3T3 cells (Mellström et al., 1988; Ridley and Hall, 1992), a process that involves actin rearrangements mediated by the small G protein Rac (Ridley et al., 1992), it seems likely that c-Src is being directed to membrane ruffles in NIH 3T3 cells by treatment with PDGF. These data, together with the requirement for the serum-induced, Rho-mediated stress fiber assembly to translocate v-Src to focal adhesions, suggest that the exact peripheral location of Src kinases may be specified by rearrangements of the actin cytoskeleton under the influence of Rho and Rac.

Although little is known about the mechanism or dynamics of v-Src transport from its site of synthesis to its sites of action, molecules that act as binding partners for v-Src before its translocation are candidate mediators of this process. One such signaling intermediate that fulfills this criteria is the p85/p110 form of phosphatidylinositol 3-kinase (PI 3-kinase) that binds v-Src with kinetics that are indistinguishable from those of activation of the v-Src tyrosine kinase itself in CEF (Haefner et al., 1995). This, together with the known role of the yeast homologue of the p110 catalytic subunit of PI 3-kinase, VPS34, in vesicular protein trafficking (Schu et al., 1993) and the recently identified role of PI 3-kinase in the transport of GLUT 4 glucose transporter to the cell membrane (Cheatham et al., 1994; Clarke et al., 1994; Kamohara et al., 1995; Kotani et al., 1995), suggests that PI 3-kinase is a candidate regulator of v-Src translocation. In addition to PI 3-kinase, other possible regulators of this process include molecules that interact with both v-Src and focal adhesions, such as focal adhesion kinase or paxillin (for review see Schaller and Parsons, 1994), or v-Src and the cytoskeleton, such as cortactin (Wu and Parsons, 1993) or AFAP (for actin filament-associated pp60 [Src] substrate; Flynn et al., 1993). The mechanism of actin-dependent Src recruitment to the cell periphery and the potential role of molecules that interact with Src in this process require extensive further experimentation.

In conclusion, we have identified the actin cytoskeleton as an important component of the cellular machinery that translocates Src kinase from its site of synthesis to its sites of action. Furthermore, our observations, when considered with work from Alan Hall's group demonstrating that Rho and Rac are crucial regulators of cytoskeletal assembly (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995), implicate these small G proteins as potential determinants of Src localization mediated by their effects on actin organization. Thus, in addition to its more recognized roles in maintaining cell shape and attachment, in regulating motility, and in basic cell functions, such as cytokinesis, we have demonstrated that the actin cytoskeleton is probably also involved in intracellular transport processes, as has been suggested (Nobes and Hall, 1995). Examples of protein trafficking along actin tracks have been described (Fath and Burgess, 1994). More recently, cytoskeletal integrity has also been shown to be required throughout the growth factor–stimulated G1-phase of the cell cycle in normal cells and to mediate the anchorage-dependent expression of cyclin D1 (Böhmer et al., 1996). In the case of mitogenesis stimulated by the v-Src oncoprotein, however, we have shown that induction of G0/G1-to S-phase progression requires the cytoskeleton for localization at the cell periphery necessary for initiating the mitogenic cascade. Thereafter, v-Src–induced cell cycle progression proceeds independently of cytoskeletal integrity since v-Src activity induces rapid cytoskeletal disorganization (within 1–2 h in CEF) and subsequent transit of G1. Details of the mechanism by which v-Src and other oncoproteins that can induce anchorage-independent growth overcome the normal cytoskeletal-dependent checkpoint in G1 remain to be established.
We are grateful to Kate Nobes and Alan Hall (University College, Lon-
don, UK) for advice and for pGEX-2T- rho A, to Anne Wyke for MAP ki-
nase assay, to Burkhard Haefner for discussions on the role of PI 3-
kinase in v-Src transformation, to the Beatson Technology Service (Pe-
ter McHardy) for help with the confocal microscope and image process-
ning, and Dewi Owens for help with photography.

This work is supported by the Cancer Research Campaign (UK). V.
Brunton is supported by The Association for International Cancer Re-
search. M. unl is supported by Cumhuriyet University, Turkey.

Received for publication 21 May 1996 and in revised form 19 August 1996.

References

adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphory-
lation and the recruitment of both p125FAK and protein kinase C-4 to

Bell, J.G., J.A. Wyke, and I.A. MacPherson. 1975. Transformation by a temper-

Brunton is supported by The Association for International Cancer Re-
search (UK). V. Brunton is supported by The Association for International Cancer Re-
search. M. Ulus is supported by Cumhuriyet University, Turkey.

Received for publication 21 May 1996 and in revised form 19 August 1996.

References

adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphory-
lation and the recruitment of both p125FAK and protein kinase C-4 to

Bell, J.G., J.A. Wyke, and I.A. MacPherson. 1975. Transformation by a temper-

Brunton is supported by The Association for International Cancer Re-
search (UK). V. Brunton is supported by The Association for International Cancer Re-
search. M. Ulus is supported by Cumhuriyet University, Turkey.

Received for publication 21 May 1996 and in revised form 19 August 1996.

References

adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphory-
lation and the recruitment of both p125FAK and protein kinase C-4 to

Bell, J.G., J.A. Wyke, and I.A. MacPherson. 1975. Transformation by a temper-

Brunton is supported by The Association for International Cancer Re-
search (UK). V. Brunton is supported by The Association for International Cancer Re-
search. M. Ulus is supported by Cumhuriyet University, Turkey.

Received for publication 21 May 1996 and in revised form 19 August 1996.