Newest findings on the oldest oncogene; how activated *src* does it

Margaret C. Frame

Cancer Research UK Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow, G61 1BD, UK
(e-mail: m.frame@beatson.gla.ac.uk)

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Summary

Oncogenic forms of the non-receptor tyrosine kinase Src alter cell structure, in particular the actin cytoskeleton and the adhesion networks that control cell migration, and also transmit signals that regulate proliferation and cell survival. Recent work indicates that they do so by influencing the RhoA-ROCK pathway that controls contractile actin filament assembly, the STAT family of transcription factors needed for transformation, and the Cbl ubiquitin ligase that controls Src protein levels. These studies also shed light on the role of focal adhesion kinase (FAK) downstream of v-Src and other signalling pathways in controlling migration, invasion and survival of transformed cells. Src directly phosphorylates integrins and can also modulate R-Ras activity. Moreover, it stimulates the E-cadherin regulator Hakai, interacts with and phosphorylates the novel podosome-linked adaptor protein Fish, and progressively phosphorylates the gap junction component connexion 43. A recurring theme is the identification of novel and important Src substrates that mediate key biological events associated with transformation.

Key words: v-Src, Protein tyrosine kinase, FAK, Focal adhesions, Integrins, Transformation

Introduction

The protein tyrosine kinase v-Src is the transforming product of Rous sarcoma virus, the first identified oncogenic retrovirus. Src, the cellular counterpart of v-Src, is a member of a multigene family of membrane-associated non-receptor tyrosine kinases that comprises nine members, some of which (Src, Fyn and Yes) are ubiquitous and some of which display more-restricted expression (for a general review on Src-family kinases, including their structures and modes of regulation, see Thomas and Brugge, 1997). v-Src differs from Src by substitution of sequences at the C-terminus, which results in loss of amino acids that normally bind to the SH domains and stabilize the 'closed' or inactive conformation of the molecule (reviewed by Frame, 2002). Other alterations, such as those in the SH3 domain, also contribute to v-Src activity (see below). v-Src and Src are known to modulate the actin cytoskeleton and cell adhesion structures from their peripheral sites of activity (reviewed by Frame, 2002). Specifically, Src-transformed cells display reduced numbers of bundled actin filaments and cellular contacts, particularly the integrin-associated focal adhesions of mesenchymal cells and cell-cell adhesions of epithelial cells, as well as inhibited gap junction communication between adjacent cells. v-Src and its tightly regulated endogenous counterpart in normal cells coordinate changes in cell structure with transmission of signals that influence cell growth. For example, v-Src-transformed fibroblasts also display constitutively altered cell-cycle control, with elevated expression of cyclin D, cyclin A and cyclin-dependent kinase 2 (CDK2), hyperphosphorylation of the retinoblastoma tumour suppressor protein (pRb) and stimulation of progression through G1-phase to S-phase (Riley et al., 2001). There is also abundant evidence that v-Src-transformed fibroblasts are finely balanced between proliferation and death, particularly when serum survival factors are limiting. Specifically, v-Src can both prime for apoptosis (by a mechanism that is independent of p53) and protect against it under low-serum conditions; survival signals are provided by the Ras and phosphoinositide 3-kinase (PI 3-K) pathways (Johnson et al., 2000; Webb et al., 2000). These findings are relevant to cancer, since it is reported that oncogenic mutations in Src arise in a subset of advanced human colon cancers tumours (Irby et al., 1999), although it is not yet clear that mutation is the most common mode of Src activation in tumours. Indeed, it remains likely that altered transcription, translation or protein stability might contribute to elevated Src activity in human cancers, although this is not well understood. The apparently opposing survival and apototic effects that v-Src can simultaneously induce in cells make it difficult to predict whether and, if so, how oncogenic Src influences survival signalling in cancer cells that have mutations in other proliferation and death pathways. In particular, whether its function in priming for cell death or in promoting survival can enhance or counteract the effects of DNA-damaging agents, particularly those currently used in the clinical treatment of cancer, is an intriguing question. Evidence is beginning to emerge that v-Src kinase activity can augment drug-induced apoptosis (Aouacheria et al., 2002; Boudny and Nakano, 2002).

Here, I discuss recent advances in our understanding of the mechanisms of transformation by v-Src or oncogenic forms of Src. I cover five particular areas: (1) v-Src-induced intracellular signals; (2) the roles of v-Src and focal adhesion kinase (FAK) in migration and invasion; (3) a novel Src effector at podosome structures in transformed cells; (4) epithelial cell junction regulation; and (5) Src survival signalling in colon cancer cells. The new findings reveal some previously unknown ways in

which Src probably contributes to cancer cell behaviour and tumour development. Because I focus here on recently published studies on oncogenic Src proteins, many more papers on the biological functions of endogenous, normal Src are not discussed. Of course, it is possible that some of the v-Src effector pathways referred to might also be engaged as a result of integrin clustering and/or growth factor receptor activation, in a Src-dependent manner.

v-Src-induced intracellular signals

The process by which v-Src disorganizes stress fibres

v-Src-induced loss of stress fibres and morphological transformation have previously been associated with activation of p190Rho-GAP (Fincham et al., 1999). However, new data suggest that activated Src does not always lead to decreases in Rho-GTP levels and, in some situations, actually increases the level of Rho-GTP. In such cases, v-Src may cause the dephosphorylation and activation of the actin-severing protein cofilin, thereby promoting actin filament disassembly (Pawlak and Helfman, 2002) (G. S. Martin, personal communication). These data indicate that v-Src can cause disassembly of actin filaments by more than a single mechanism and that a common effect of v-Src is to disrupt the Rho-ROCK-LIM kinase pathway that controls cofilin activity.

The STAT transcripton factors

Evidence gathered several years ago implicated a member of the signal transducers and activators of transcription (STAT) family – which is usually associated with cytokine signalling – in transformation by v-Src. Specifically, the levels of STAT3 are elevated in v-Src-transformed cells, and STAT3 expression potentiates anchorage-independent growth of NIH3T3 cells, whereas dominant-negative STAT3 inhibits v-Src-mediated transformation (Bromberg et al., 1998a). Interestingly, v-Src-induced activation of STAT3 requires both tyrosine and serine phosphorylation of the transcription factor. The serine phosphorylation involves Ras/Rac-dependent activation of p38 mitogen-activated protein (MAP) kinase and Jun N-terminal kinase (JNK) (Turkson et al., 1999). Other cooperating kinases, including Jak1 (a component of the STAT signalling pathway) and Etk, a member of the Btk family of tyrosine kinases, have also been implicated in the activation and function of STAT3 in Src-mediated transformation (Tsai et al., 2000; Zhang et al., 2000). Several studies have now led to the conclusion that STAT3 contributes to transcription of cell-cycle regulators, such as the D-type cyclins, cyclin E, $p21^{WAF1/CIP1}$ and Myc (Odajima et al., 2000; Sinibaldi et al., 2000). Indeed, v-Src-induced transformation requires STAT3-regulated Myc synthesis, as does the platelet-derived growth factor (PDGF)-induced mitogenesis that is known to require Src-family kinases (Barone and Courtneidge, 1995; Bowman et al., 2001; Twamley-Stein et al., 1993).

One interesting effect of v-Src-induced STAT3 activation is to stimulate expression of vascular endothelial growth factor (VEGF) through a STAT3-binding site in the promoter for VEGF (Niu et al., 2002). Because Src activity is required for hypoxia-induced VEGF production and consequent angiogenesis (Ellis et al., 1998; Mukhopadhyay et al., 1995; Shweiki et al., 1992), STAT3 may be a useful therapeutic target for suppression of angiogenesis in solid tumours. Src, of course, is also required downstream of VEGF, specifically to promote survival of endothelial cells and angiogenesis (Eliceiri et al., 1999).

STAT5b is also implicated in transformation by v-Src. In particular, STAT5b enhances v-Src-induced cell-cycle progression and cell survival, as well as promoting anchorage-independent growth and cell migration (Kazansky and Rosen, 2001). Recent studies have indicated that v-Src-induced tyrosine phosphorylation of STAT5b differs from that induced by other natural stimuli. For example, prolactin induces phosphorylation of Tyr699, whereas v-Src causes phosphorylation of STAT5b at additional tyrosine residues, including the Tyr679 residue that is associated with a conformational change in the protein. Furthermore, mutation of Tyr679 to Phe causes a qualitatively different subnuclear appearance of STAT5b when activated Src is present, although the significance of this is not known. However, expression of the Tyr679 to Phe mutant of STAT5b inhibits v-Src-induced, cyclin D1 promoter activity in transient transfection assays, which suggests that cyclin D transcription is under the control of tyrosine-phosphorylated STAT5b (Kabotyanski and Rosen, 2003). STAT5b is thus also an effector of v-Src that influences expression of important cell-cycle regulators.

c-Cbl, Hakai and Src ubiquitylation

Activated forms of Src are less stable than their wild-type or kinase-defective counterparts, largely because of poly-ubiquitylation and proteosome-dependent degradation (Hakak and Martin, 1999; Harris et al., 1999). Thus, when Src is active, ubiquitylation and degradation provide a means of attenuating signalling. A recent report has identified Cbl, particularly Cbl-c, as the E3 ligase responsible for ubiquitylation of Src and has shown that tyrosine phosphorylation of Cbl by Src leads to interdependent ubiquitylation and degradation of both proteins (Yokouchi et al., 2001; Kim et al., 2003). Cbl also reduces the levels of v-Src and inhibits v-Src-induced STAT3 activation (Yokouchi et al., 2001). v-Src can also regulate Hakai, a Cbl-like E3-ubiquitin ligase that binds to E-cadherin and causes disruption of epithelial cell-cell contacts (Fujita et al., 2002). Upon activation of Src in epithelial cells, E-cadherin and β-catenin become ubiquitylated by Hakai, ultimately leading to endocytosis of the E-cadherin complex (Fujita et al., 2002). These data indicate that activated Src can regulate ubiquitin-conjugating enzymes that, in turn, promote the degradation of Src itself or the functional suppression of Src effectors.

v-Src and FAK in adhesion regulation, migration and invasion

v-Src-induced proteolysis of FAK and transformation

In primary chicken embryo fibroblasts (CEFs), the temperature-sensitive (ts) LA29 v-Src protein induces an extreme phenotype by completely disrupting all focal adhesions over a 16-24 hour period, causing cell detachment (Fincham et al., 1995). In established fibroblasts, such as Rat-1 cells for example, or when a less transforming v-Src mutant is used, a more moderate phenotype is observed in which the ratio of adhesion turnover to adhesion assembly is increased without cell detachment. Studies in the ‘extreme

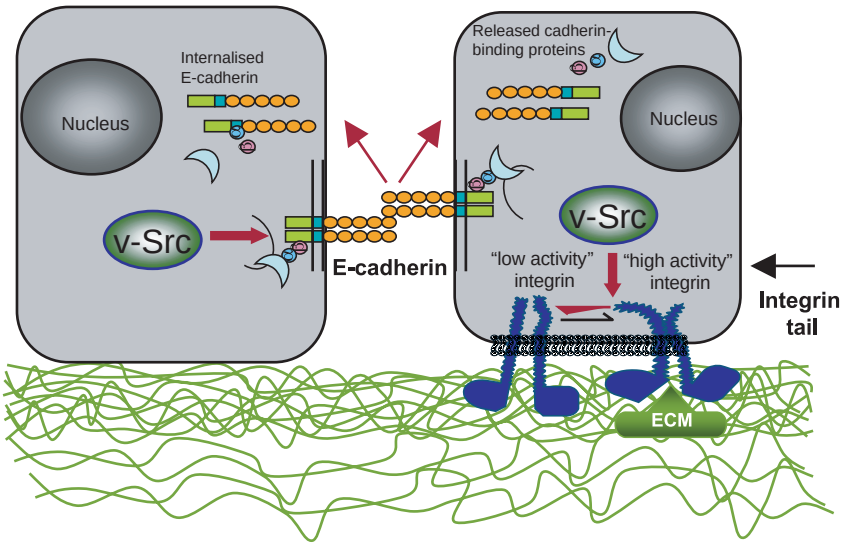

Fig. 1. v-Src, or activated Src527F, induces tyrosine phosphorylation of cell-cell adherens junction components (the cadherin/catenin system), or acts by phosphorylating protein regulators of adherens junctions, such as Hakai, to cause disruption, or weakening, of cell-cell adhesions. Internalised cadherin molecules and released binding proteins are indicated. In a somewhat analogous manner, oncogenic Src weakens the links between cell-matrix adhesions and the actin cytoskeleton in the cell interior, in some situations by causing phosphorylation of the integrin cytoplasmic tails, but also by phosphorylating R-Ras and causing a change in integrin activation status.

transformation' CEF system showed that strongly transforming v-Src mutants induce turnover of integrin-associated focal adhesions, and disruption of the associated actin filaments (Fincham et al., 2000; Fincham and Frame, 1998). This requires the catalytic activity of v-Src, and expression of a kinase-defective mutant inhibits dynamic turnover of the actin/adhesion network, leading to production of larger, more-static focal adhesions and impaired cell migration (Fincham and Frame, 1998). In these studies, a clear correlation emerged between v-Src-induced tyrosine phosphorylation of FAK and subsequent proteolysis of FAK by the enzyme calpain, this being particularly evident during the focal adhesion loss that accompanies transformation in CEFs (Carragher et al., 2001; Fincham and Frame, 1998).

More recently, we identified a v-Src-regulated complex comprising FAK, calpain and its upstream regulatory kinase, p42 ERK/MAP kinase, implicating the adaptor function of FAK in assembly of a protease complex that regulates focal adhesion turnover and migration of v-Src-transformed cells (Carragher et al., 2003). Furthermore, we also showed that v-Src increases synthesis of calpain 2, which localizes to focal adhesions (Beckerle et al., 1987), and degradation of its endogenous inhibitor calpastatin; inhibition of calpain suppresses v-Src-induced anchorage-independent growth (Carragher et al., 2002). These findings indicate that, at least in some instances, the calpain-calpastatin proteolytic system is an important regulator of the v-Src transformed phenotype.

v-Src affects integrin function

v-Src-induced structural changes include weakened cell adhesion and decreased deposition of extracellular matrix (ECM), both of which are associated with suppression of integrin function. Several lines of evidence now indicate that v-Src can directly impair integrin function through tyrosine phosphorylation (Fig. 1).

We have known for some time that v-Src induces tyrosine phosphorylation of β1A integrin subunits (Hirst et al., 1986; Johansson et al., 1994; Tapley et al., 1989). Recent work in GD25 mouse fibroblasts has established that v-Src induces phosphorylation of two particular tyrosine residues in the cytoplasmic domains of exogenously expressed β1A integrin (Tyr783 and Tyr795) (Sakai et al., 2001). Sakai et al. showed that mutation of these Tyr residues, particularly Tyr783, to Phe inhibits transformation of GD25 cells by impairing loss of adhesiveness and preserving focal contacts and actin stress fibres when v-Src is active (Sakai et al., 2001). Even in the absence of v-Src, cells expressing only the Tyr783/Phe795 β1A integrin double mutant have more focal contacts and assemble more ECM than cells expressing wild-type β1A integrin. This implies that phosphorylation of these tyrosines, perhaps by endogenous Src-family kinases, plays a role in the dynamic regulation of normal focal contacts and in matrix deposition (Sakai et al., 2001). However, phosphorylation of Tyr783 is not detected in v-Src-transformed cells by anti-phosphotyrosine antibodies, perhaps owing to its transience or the proximity of a coupled serine phosphorylation site (Ser785) that is linked to movement of β1A integrin in and out of focal contacts (Mulrooney et al., 2000; Sakai et al., 2001; Sakai et al., 1998). Tyrosine, and possibly also serine, phosphorylation of the β1A integrin subunit induced by v-Src thus might weaken links between the ECM and the cytoskeleton that are mediated by this integrin and thus contribute to reduced adhesiveness during cell transformation.

By contrast, experiments studying the effects of v-Src on the function of α5β1 integrin, the dominant fibronectin receptor in CEFs, have revealed that the ability of this integrin to assemble adhesive links with, and deposit, fibronectin is not strongly affected by v-Src in the short term (Datta et al., 2001). A longer-term reduction in α5β1-integrin-fibronectin links and cell adhesiveness is more likely to be due to increased production of proteases and/or hyaluronic acid, which either cause ligand removal or isolation of the integrin from surface-bound fibronectin ligand (Datta et al., 2001). However, v-Src suppresses the ability of cells to sense integrin-mediated adhesion and to induce 'outside-in' signalling, as judged by the lack of tyrosine phosphorylation of FAK on its Tyr397 autophosphorylation site and the adhesion insensitivity of proliferation (Datta et al., 2001). Taken together, these experiments indicate that, at least under the experimental conditions used in these studies, v-Src has profound effects on β1 integrin function and its ability to induce signals that elicit normal cellular responses.

v-Src-induced tyrosine phosphorylation of a second β integrin subunit, β3, at Tyr747 and Tyr759 reduces the strength

of αvβ3-mediated adhesion to fibronectin (Datta et al., 2002). Datta et al. used a 'spinning disk' assay to measure the relative strength and number of integrin-matrix bonds in live cells and to show that a Tyr747Phe mutation in β3 integrin restores normal adhesion to fibronectin, even in the presence of active v-Src. Interestingly, Tyr747 and Tyr759 of β3 integrin may be functionally equivalent to Tyr783 and Tyr795 of β1 integrin in providing Src-dependent negative regulation of adhesion strength, but there are also some clear differences in the detail of how tyrosine phosphorylation causes weakening of adhesion mediated by β1 and β3 integrins. In particular, phosphorylation of β3 integrin appears to have a direct effect on the integrin-fibronectin linkage, whereas the effects of β1 integrin tyrosine phosphorylation are consistent with a more indirect mode of action, which might exclude β1 integrin from focal adhesions and so prevent strong integrin-cytoskeleton and integrin-ligand interactions (discussed by Datta et al., 2002). In the case of both β1 and β3 integrin subunits, phosphorylation is probably transient and part of a regulated cycle of phosphorylation and dephosphorylation. Over-phosphorylation induced by v-Src might result in an imbalance that, in turn, leads to disassembly of focal adhesion complexes normally required for maintenance of the integrin-cytoskeleton and integrin-matrix links.

v-Src also has indirect activity on integrins. Zou et al. have revealed that v-Src, and a constitutively activated form of Src, can induce tyrosine phosphorylation of R-Ras (Zou et al., 2002), a Ras superfamily small GTPase member that controls integrin function (Zhang et al., 1996). Specifically, R-Ras is responsible for maintaining integrin activity in cells. Phosphorylation of a tyrosine residue in its effector domain, by an Eph receptor kinase, suppresses integrin activity and reduces cell-matrix adhesion (Zou et al., 1999). In the recent work, Zou et al. showed that R-Ras and v-Src form a complex and v-Src induces phosphorylation of R-Ras at a site (Tyr66) that mediates effects on integrin activity (Zou et al., 2002) (Fig. 1). In addition, co-expression of an R-Ras mutant that has a Tyr66Phe mutation partially inhibits the ability of v-Src to suppress cell adhesion, indicating that Src-dependent phosphorylation of Tyr66 of R-Ras is responsible, at least in part, for the v-Src-induced transformed phenotype (Zou et al., 2002).

v-Src and FAK stimulate invasion by promoting expression of MMPs

Recent evidence indicates that v-Src promotes invasion by forming v-Src-FAK signalling complexes. Hauck et al. showed that gain-of-function v-Src SH3 mutations [i.e. mutations that alter two amino acids in the RT loop region of the Src SH3 domain (from Arg97 and Thr98 in Src to Trp97 and Ile98 in v-Src)] specifically enhance formation of β1-integrin-enriched cell extensions termed invadopodia, and more-stable v-Src-FAK signalling complexes at these sites promote in vitro invasion (Hauck et al., 2002a). The same group also showed that expression of FRNK (FAK-related non-kinase), a putative endogenous inhibitor of FAK (Hauck et al., 2001; Richardson and Parsons, 1996; Schaller et al., 1993; Taylor et al., 2001; Zhao et al., 1998), specifically suppresses v-Src-induced cell invasion and experimental metastasis in vivo (Hauck et al., 2002). By contrast, FRNK does not interfere with other features of v-Src transformation, such as migration, anchorage-independent or low-serum growth, and tumour growth in nude mice (Hauck et al., 2002). FRNK expression is specifically associated with impaired phosphorylation of $p130^{CAS}$ (a Src-regulated protein that associates with the Crk adaptor protein) and FAK at its Src-dependent phospho-acceptor residues (Tyr861 and Tyr925), which suggests that the v-Src-FAK signalling complex is disrupted. Mutagenesis of FRNK further indicates that focal contact localization is important for its inhibitory effects (Hauck et al., 2002). These results are consistent with the observation that, although v-Src binds to FAK and induces its phosphorylation, FAK is not absolutely required for v-Src-induced changes in morphology and growth evident in transformed cells (Roy et al., 2002). Surprisingly, recent evidence suggests that lack of FAK expression might actually enhance the ability of v-Src to promote anchorage-independent growth, suggesting that, at least in some circumstances, FAK attenuates aspects of v-Src transformation (Moissoglu and Gelman, 2003). In this regard, multiple signalling pathways probably contribute to the effects of v-Src, and alternative routes to morphological transformation might be used in $FAK^{-/-}$ cells that have undergone adaptation to survive and proliferate in the absence of FAK.

One particularly interesting finding is that FRNK inhibits the production of matrix metalloproteinase 2 (MMP-2) which is normally stimulated by JNK-dependent signalling downstream of v-Src and FAK. Re-expression of MMP-2 can restore in vitro invasion (Hauck et al., 2002). A recent study also showed that v-Src-expressing $FAK^{-/-}$ cells are motile, but non-invasive (Hsia et al., 2003). FAK kinase activity and autophosphorylation are required for v-Src-induced invasion, which depends on a FAK-Src-containing signalling complex at lamellipodial focal contacts that includes $p130^{CAS}$ and DOCK180 (another Crk-binding adaptor protein that is involved in Rac activation by the Crk/$p130^{CAS}$ complex). In addition, cell invasion is associated with elevated Rac1 and JNK activities and increased expression of MMP-9 (Hsia et al., 2003) (Fig. 2). These findings from David Schlaepfer's lab support a role for oncogenic Src/FAK signalling in tumour cell invasion and metastasis that depends on the ability of FAK to integrate signals that coordinate migration and MMP-induced matrix re-modelling.

Numerous other studies support the conclusion that signalling downstream of v-Src induces metalloproteinase production. For example, v-Src transformation of cells is accompanied by a RalA-dependent increase in production of MMP2 and MMP9 (Aguirre-Ghiso et al., 1999), or a MEK-dependent increase in MMP1 production (Kurata et al., 2000). In MDCK cells v-Src induces production of active membrane type 1 MMP (an MMP that localizes to the surface of tumour cells, and probably also stromal cells, to cause the pericellular degradation of the ECM) (Cha et al., 2000; Kadono et al., 1998).

A Src effector found specifically at podosome structures in transformed cells

New information on podosomes (David-Pfeuty and Singer, 1980; Tarone et al., 1985), which are organized F-actin ring structures on the ventral surface of Src-tansformed cells, is beginning to throw light on potential mechanisms by which oncogenic Src promotes invasion. Podosomes are considered

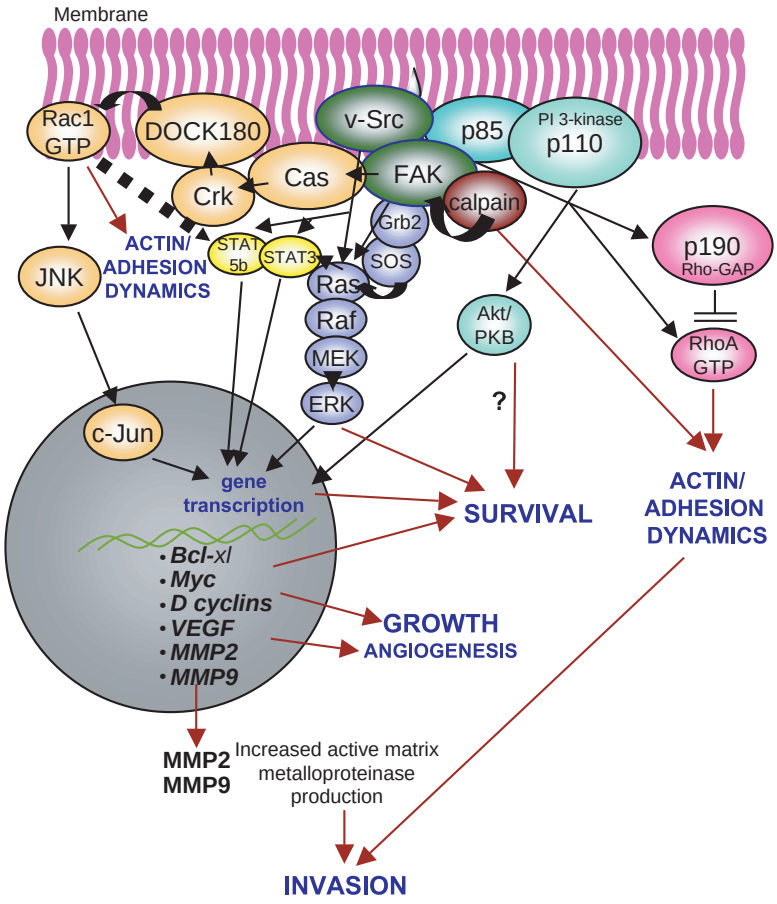

Fig. 2. Some of the major v-Src effector pathways (discussed in the text) that control and co-ordinate key cellular responses, including growth and survival, adhesion/actin dynamics, matrix-degrading enzyme production and invasion. This does not represent an exhaustive list of v-Src activities in transformed cells.

to be analogous to dynamic sites of matrix attachment found in normally invasive cells of the monocytic lineage, such as macrophages or osteoclasts. They contain proteins similar to those in focal adhesions, but are physically distinct because they do not have stress fibres tethered into them. In addition, podosomes contain several proteins that regulate actin organization, including cortactin, N-WASP, paxillin, p110^{AFAP} and p190$^{Rho\text{-}GAP}$. An inhibitory mutant of N-WASP impairs podosome formation as well as v-Src-induced matrix degradation, indicating a probable link between control of actin assembly and the formation of structures that mediate Src-induced matrix remodelling (Mizutani et al., 2002). Fish, first identified as a substrate of Src (Lock et al., 1998), is a novel component of podosomes (Abram et al., 2002). It contains an N-terminal Phox homology (PX) domain, which binds to 3-phosphorylated phosphatidylinositols (for review on PX domains, see Ellson et al., 2002), and five SH3 domains, as well as other motifs (Lock et al., 1998). The localization of Fish to podosomes is specified by the PX domain, which suggests that this domain interacts with particular lipids at podosomes (Abram et al., 2002). Intriguingly, a putative binding partner of the fifth Fish SH3 domain is ADAM19, a member of the ADAMs family of protease and disintegrin domain proteins thought to regulate integrin-mediated cell adhesion and release of surface-associated growth regulators. Their cytoplasmic domains also have protein interaction sites that might signal into the cell interior (reviewed by Black and White, 1998; Schlondorff and Blobel, 1999), iucluding SH3-binding PXXP motifs (Seals and Courtneidge, 2003). ADAM19, ADAM12 and ADAM15, but not ADAM9, can co-immunoprecipitate with Fish in cells, and ADAM12 also colocalizes with Fish at podosomes (Abram et al., 2003).

Precisely how Fish and associated ADAMs are coupled to lipid signalling pathways and are regulated by Src-dependent tyrosine phosphorylation remains to be worked out. Interestingly, podosome-like structures have been observed in cancer cells (Bowden et al., 1999; Seals and Courtneidge, 2003); by analogy with podosomes in osteoclasts, where the bone-remodelling active centre protrudes into the bone from an F-actin ring, podosomes of cancer cells may provide the actin structures from which extensions into underlying ECM originate, resulting in localized re-modelling activities associated with the invasive phenotype. Further studies on Fish regulation and function might reveal the processes used by invasive cancer cells to move through their immediate environment, and the relationship between these structures and the β1-integrin-containing invadopodia described in other studies (for example by Hauck et al., 2002).

Epithelial cell junction regulation

EMT and E-cadherin regulation

Oncogenic Src (and Ras) proteins induce a switch between epithelial- and mesenchymal-like phenotypes – the so-called epithelial-mesenchymal transition (EMT) – that is associated with loss of E-cadherin complexes at cell-cell adhesions and the acquisition of enhanced integrin-dependent cell-ECM contacts (reviewed by Thiery, 2002). In general, epithelial cancers that have undergone an EMT are regarded as potentially more migratory and consequently more invasive or metastatic. Two recent studies have now directly examined the effects of elevated intracellular Src activity on colon cancer cell behaviour. First, in SW480 and HCT 116 cells, activated Src induces a substantial decrease in calcium-dependent homotypic adhesion by a mechanism that requires Ras and FAK but not STAT3 (Irby and Yeatman, 2002). Second, expression of activated Src in KM12C colon carcinoma cells induces an EMT with assembly of integrin structures and de-regulation of E-cadherin (Avizienyte et al., 2002) (Figs 1, 3). Specifically, E-cadherin is unable to translocate to the cell membrane in response to calcium when oncogenic Src is expressed. Interestingly, Src must cooperate with integrin-dependent signals to suppress normal E-cadherin regulation and function (Avizienyte et al., 2002), and recent work has indicated that MEK/ERK-MAP kinase signalling to MLCK and myosin activity are crucial mediators of the Src-induced, integrin-dependent mesenchymal switch (E. Avizienyte and M.C.F., unpublished). A novel Src-binding protein, Hakai, acts

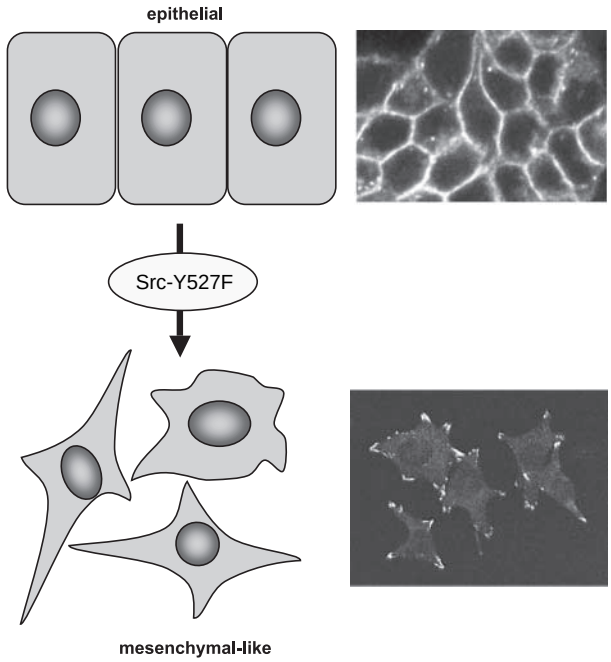

Fig. 3. Epithelial cells that are held together by intercellular contacts (top-left), and shown for cells from the KM12C colon cancer cell series (visualized by immunofluorescence) (top right), become dispersed as a consequence of expression of Src527F (bottom-left), shown for KM12C cells (bottom-right). As described in the text, cadherin-dependent cell-cell contacts are lost and assembly of integrin-associated adhesions are enhanced as a result of oncogenic Src, resulting in the 'adhesion switch' phenotype associated with acquisition of a more mesenchymal-like morphology. Cells in the top-right and bottom-right panels are stained with anti-E-cadherin and anti-vinculin, respectively. Images are courtesy of Egle Avizienyte.

as an E3 ligase to ubiquitylate the E-cadherin complex after activation of v-Src (Fujita et al., 2002) and stimulate its endocytosis, perturbing cell-cell contact. Hakai might therefore promote the EMT in epithelial cancer cells that have elevated Src (or other) tyrosine kinase activity (Fujita et al., 2002) (Fig. 1). Tsukamoto and Nigam have also reported that v-Src-induced scattering of MDCK cells is blocked by the proteosome inhibitors lactacystin and MG132, which specifically decrease E-cadherin turnover (Tsukamoto and Nigam, 1999). Since Src is often upregulated during the metastatic transition in colon cancers, for example in the Fidler model of colorectal metastasis (Jones et al., 2002; Mao et al., 1997), evaluating its mode of de-regulating the E-cadherin tumour suppressor protein might lead to new therapeutic targets. Interestingly, v-Src can also influence EMTs occurring normally during development, such as in developing glandular stomach (Shimizu et al., 2003). In particular, expression of v-Src, or activated MEK, can lead to loss of E-cadherin expression and acquisition of mesenchymal markers during EMT (Shimizu et al., 2003).

The Arf6 GTPase has emerged as a key regulator of MDCK cell adherens junction disassembly induced by v-Src, as well as other tyrosine kinases, such as the receptor for hepatocyte growth factor (HGF) (Palacios et al., 2001). Arf6-regulated membrane traffic is required for the cytoplasmic redistribution of E-cadherin during disassembly of cell-cell adhesions. Specifically, the GTPase cycle of Arf6 controls the translocation of E-cadherin between membrane and intracellular compartments, and is required for tyrosine-kinase-induced signals to drive E-cadherin out of adherens junctions (Palacios et al., 2001). As yet, how v-Src signals to Arf6, causing perturbation of E-cadherin at adherens junctions, is not understood.

The catenin $p120^{CTN}$ is a Src substrate located at cadherin-mediated cell-cell adhesions (Reynolds et al., 1989). It has been implicated in cell-cell adhesion assembly and strengthening mediated by its binding to the cadherin juxtamembrane domain that controls lateral diffusion and cadherin clustering (Thoreson et al., 2000; Yap et al., 1998). However, $p120^{CTN}$ has also been implicated in cell-cell adhesion disassembly (Aono et al., 1999; Ozawa and Kemler, 1998). These apparently contradictory findings can be reconciled if $p120^{CTN}$ acts as a switch that modulates assembly and disassembly of adherens junctions, and it has been proposed that serine/threonine phosphorylation of $p120^{CTN}$ might constitute the switch (reviewed by Anastasiadis and Reynolds, 2000). There is also evidence that tyrosine phosphorylation of $p120^{CTN}$ also modulates E-cadherin function at cell-cell contacts, at least in response to v-Src (Ozawa and Ohkubo, 2001). Specifically, in L-cells transfected with E-cadherin, tyrosine-phosphorylated $p120^{CTN}$ is found in anti-E-cadherin immunoprecipitates (Ozawa and Ohkubo, 2001). Ozawa and Ohkubo suggest that a conformational change caused by the binding of $p120^{CTN}$ to E-cadherin facilitates v-Src-induced phosphorylation of $p120^{CTN}$, which, in turn, suppresses cadherin function and promotes reduced cell-cell adhesion (Ozawa and Ohkubo, 2001). Recent data have clearly implicated $p120^{CTN}$ in maintaining cadherin stability in mammalian cells, at least in part by controlling cadherin trafficking (reviewed by Peifer and Yap, 2003). The particular role of Src-mediated phosphorylation of $p120^{CTN}$ in E-cadherin trafficking and stability at adherens junctions is not known, but $p120^{CTN}$ remains a strong candidate regulator as it is one of the major tyrosine-phosphorylated proteins in colon cancer cells in which elevated Src levels are responsible for de-regulation of E-cadherin trafficking (Avizienyte et al., 2002) (E. Avizienyte and M.C.F., unpublished)

The lipid phosphatase tumour suppressor protein PTEN, which antagonizes PI 3-K signalling, inhibits v-Src-induced cell-cell junction disassembly in MDCK cells (Kotelevets et al., 2001). Specifically, expression of PTEN, but not lipid-phosphatase-deficient mutants, reverts v-Src-induced biological effects. This suggests a crucial role for PI 3-K in v-Src-induced de-stabilization of E-cadherin-dependent adhesions. Interestingly, PTEN expression does not interfere with v-Src-induced tyrosine phosphorylation of E-cadherin, or associated catenins (Kotelevets et al., 2001). PTEN thus appears to stabilize adherens junctions and, consequently, restrains the invasive potential induced by active Src proteins (Kotelevets et al., 2001).

Gap junctions and connexin 43 phosphorylation

v-Src induces disruption of gap junctions, which form aqueous channels connecting the cytoplasms of adjacent cells to allow the flow of small (<1000 Da) molecules between connected

cells. Inhibition of gap junctions by v-Src is accompanied by tyrosine phosphorylation of the gap junction protein connexin 43 (Crow et al., 1990; Filson et al., 1990). This is probably the result of direct binding and phosphorylation of connexin 43 by v-Src (Loo et al., 1995; Loo et al., 1999). More-recent studies, using reconstitution of (or mutant) connexin 43 expression in knockout cells, have shown that disruption of gap junctional communication by v-Src is specifically inhibited by mutation of Tyr247 and Tyr265 in connexin 43 to phenylalanine (Lin et al., 2001a; Lin et al., 2001b). In addition, phosphorylation of connexin 43 Tyr265 appears to be required for efficient phosphorylation at Tyr247, clearly implicating progressive phosphorylation of connexin 43 in v-Src-induced disruption of gap junctions. However, the contribution of disrupting gap junctions to the transformed phenotype is not clear; a recent study has shown that maintenance of gap junctional communication in cells expressing v-Src does not affect growth properties associated with the transformed phenotype (Warn-Cramer et al., 2003). Nevertheless, loss of gap junctional communication is found in many cancer cells, indicating some likely contribution to the cancer phenotype.

Src survival signalling in colon cancer cells

There is considerable evidence that elevated expression and/or activity of Src is associated with the development of epithelial cancers (reviewed by Frame, 2002; Summy and Gallick, 2003), for example in colon and breast cancer, where many of the early observations were made. In addition to causing loss of intercellular adhesions (Avizienyte et al., 2002; Irby and Yeatman, 2002), Src contributes to the epithelial cancer phenotype in other ways. For example, Src regulates anoikis (detachment-induced cell death) in colon cancer cells, probably by activation of the PI 3-K/Akt pathway (Windham et al., 2002). Specifically, Src expression/activity levels correlate with resistance to anoikis. Moreover, expression of activated Src in SW480 cells (which have relatively low endogenous levels of Src) increases resistance to anoikis, whereas introduction of antisense Src causes HT29 cells (which have high endogenous levels of Src) to become more susceptible to anoikis (Windham et al., 2002). Activated Src kinase may thus contribute to the colon cancer phenotype by promoting survival when cells are dislodged from their normal environment. Recent studies have also demonstrated that v-Src can confer anoikis resistance in normal intestinal epithelial cells, probably by MEK/MAP-kinase-mediated induction of the anti-apoptotic Bcl2-family protein Bcl-x_L (Coll et al., 2002). Interestingly, STAT3 is also a transcriptional regulator of Bcl-x_L (Bromberg et al., 1998b), which suggests that v-Src-induced STAT3 activation could also be involved. These findings are consistent with previous work demonstrating that v-Src-transformed fibroblasts are primed to die by apoptosis when the Ras or PI 3-K pathways are inhibited and that this can be blocked by over-expression of Bcl-2 (Webb et al., 2000). Thus, Src activity in cancer cells might induce anoikis resistance by signalling pathways that converge on anti-apoptotic Bcl-2-family proteins and this, in turn, could promote progression of the disease.

Conclusion and perspectives

There is no doubt that studies on the v-Src oncoprotein continue to provide exciting novel mechanistic insights into cellular transformation. The evidence showing that Src is a crucial regulator of cell proliferation, and of the life and death choices that transformed cells make, continues to mount. Recent studies also provide more mechanistic information on how oncogenic Src causes reduced adhesiveness associated with the transformed phenotype. Specifically, v-Src affects integrin and cadherin function, or junctional communication between cells, by direct and indirect mechanisms. For example, v-Src subverts the normal physiological events that control the ability of integrins to bind to their extracellular ligands, or to the actin cytoskeleton in the cell interior, as well as influencing signalling pathways that regulate the activity state of integrins. New information is also emerging on how Src downmodulates cadherin-mediated cell-cell adhesions, and GAP junctional communication. Since cell migration is a function of adhesiveness, at least in part, and since Src is over-active in human epithelial cancers, its effects on cellular interactions are likely to be important in cancer development, particularly during the later stages as the cells acquire invasive and metastatic properties. The novel lamellipodial signalling pathway, involving FAK and $p130^{CAS}$/Dock180/Rac and JNK, also contributes to invasion-associated MMP production. Future studies will reveal how Src-induced signalling complexes, containing adaptor proteins such as Fish, are assembled at actin-based structures like podosomes or invadopodia that are associated with matrix re-modelling and invasion.

Many studies also now provide strong support for a key role for STATs in Src effects. Of particular interest is the role of STAT3 in Src-induced VEGF production that promotes angiogenesis.

In addition to providing insight into the mediators of Src transformation described in some detail above, more studies continue to identify new substrates or effector pathways by which v-Src might exert its oncogenic actions. For example, v-Src specifically relieves transcriptional repression by the putative tumour suppressor TEL-M1 (Lopez et al., 2003) and induces tyrosine phosphorylation of the low-density lipoprotein (LDL) receptor protein 1, enabling it to bind to the Shc adaptor protein (Barnes et al., 2003).

Finally, although Src already has a daunting track record in providing landmarks in cancer research, the application of new technologies and more-sophisticated molecular intervention to studies on Src will reveal yet more. A microarray study of Src-regulated changes in gene expression in a cell transformation model system provides such an example (Malek et al., 2002). Malek et al. compared gene expression in cells transfected with Src expression constructs and a panel of colon tumour samples and identified several interesting genes expressed in both highly transformed Src-expressors and human colon tumours; a subset of these are known to be associated with transformation (Malek et al., 2002). A Src transformation 'fingerprint' might thus help to predict genes that are aberrantly regulated during colon cancer development. Gene products identified include regulators of transcription, RNA processing, heat shock responses and chaperone functions, cell growth, lysosomal functions, biochemical signalling, protein trafficking, ubiqitin-dependent proteolysis and the cytoskeleton (Malek et al., 2002). Lasp-1 and APP1, which are postulated to play key roles in the dynamic regulation of actin-based structures and in adhesion and motility (Sabo et al.,

2001; Schreiber et al., 1998), are just two examples. It will be interesting to determine whether genes identified by these types of analyses are important, for example, in the altered actin and adhesion organization caused by Src activity in cancer cells. In addition to mechanistic insight, these types of assessments of genome-wide changes might reveal new surrogate markers of Src-dependent transformation that will aid evaluation of new anticancer drugs, particularly Src inhibitors.

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