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INTRACELLULAR SIGNALS ACTIVATED BY THE SRC ONCOPROTEIN

by

MARCELLO CURTO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment
of the requirements for the degree of Doctor of Philosophy
The City University of New York

1997

UMI Number: 9720083

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/15/97
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ABSTRACT

INTRACELLULAR SIGNALS ACTIVATED BY THE SRC ONCOPROTEIN

by

Marcello Curto

Advisor: Professor David A. Foster

How the oncogenic tyrosine kinase v-Src causes the uncontrolled cell division typical of neoplastic cells, is still unclear. The espression of immediate early genes (IEGs) is induced by several mitogenic stimuli as well as by v-Src, suggesting that increased expression of these mitogen-responive genes might contribute to v-Src-induced cellular transformation. Multiple intracellular signals mediate v-Src-dependent induction of IEGs, but the molecular mechanisms governing their activation are currently unknown. Here we have investigated the possibility that specific regions of the Src protein, are required to control different sets of intracellular signals leading to transcriptional activation of IEGs. To accomplish this, several Src genes, mutated at regions involved in the regulation of intracelluar signals, were tested for the ability to induce transcriptional activation of different promoter elements that regulate IEG expression. During the course of these experiments, we found that while transiently transfected Src-genes could induce strong activation of all the promoter element tested, these were poorly or not activated in cells chronically transformed by v-Src, suggesting that an increased expression of IEGs might not be necessary for determining a transformed phenotype. Therefore, we sought to identify other intracellular signals that may account for the induction of a proliferative phenotype by v-Src. In a different set of experiments, we found that two distinct receptors for the mitogens FGF and PDGF, are constitutively phosphorylated on tyrosine, and complexed with signal transducing molecules such as Grb2, Sos and PI3K. Since similar molecular events are induced upon stimulation with physiological mitogens, these experiments suggest that a v-Src-dependent intracellular activation of various growth factor receptors may result in the generation of unregulated mitogenic signals, ultimately leading to cellular transformation.

AKNOWLEDGMENTS

My deepest thanks must go to my mentor, Prof. David Foster, for his perceptive guidance, patience and understanding. He taught me an invaluable lesson of scientific pragmatism.

Thanks to all present and former members of the laboratory, Paul Frankel, Frank Santaniello, Dr. Qun Zang, Jing-Qing Luo, Dr. Ruchika Gupta, Dr. Janguo Song, Dr. You-Wei Jang, Dr. Hong Jang, Anne Saunders, Armand Hornia, Zhimin Lu, Nancy Barile-Butler, Thuy Nguyen and Angela Shen for making the laboratory such a special place to work.

Particular thanks to Albert Carrero, for his friendship, as well as the hundreds of transfections he helped me to perform. Many thanks to Sergei Bychenok, for being a good friend and intellectual "sparring partner".

I am profoundly indebted to my family, friends and mentors in Italy, for their constant support and encouragement. A very special thanks to Malcom, Mary, Anjali and Sheila Menzies, who provided me with such a cozy and caring family throughout these years.

I dedicate this work to my companion Sheila, whose love, grace and uniqueness made my New York years possible and unforgettable.

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LIST OF ABBREVIATIONS

ATF	Activating transcription factor
ATP	Adenosine-5'-triphosphate
CPM	Counts per minute
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CSF-1	Colony stimulating factor-1
DAG	Diacylglycerol
DMEM	Dulbecco Eagle modified medium
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis tetracetic acid
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptors
GTP	Guanosine-5'-triphosphate
HGF	Hepatocyte growth factor
HSV-TK	Herpes simplex virus - Thymidine Kinase
IEG	Immediate early genes
IP_3	Inositol 1,4,5 triphosphate
kDa	KiloDaltons
Mab	Monoclonal antibody
MBD	Modular binding domain
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI3K	Phosphatidyl inositol 3-Phosphate
PIP_2	Phosphatidyl inositol 4,5 diphosphate
PKA	Protein kinase cyclic AMP-dependent

PKC	Protein kinase Ca^{++}-dependent
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulphonyl fluoride
RPTK	Receptor protein tyrosine kinase
SDS	Sodium dodecyl sulphate
SH	Src Homology
SRE	Serum response element
SRF	Serum response factor
TCF	Ternary complex factor
TFs	Transcription factors
TGFα	Transforming growth factor α
TPA	12-O-Tetradecanoylphorbol 13-Acetate
TRE	TPA-responsive element

CHAPTER I

Introduction

The Src oncoprotein

The ability of Rous sarcoma virus (RSV) to cause tumors in chickens is entirely dependent upon the viral gene *v-src*, which arose from transduction and modification of the homologous cellular gene *c-src* (fig. 1). Both genes encode a 60 kDa protein tyrosine kinase, and while the viral v-Src readily induces neoplastic transformation, cellular c-Src can partially transform only when overexpressed (Johnson et al., 1985). Unlike the proto-oncogenic c-Src, v-Src has a constitutively high tyrosine kinase activity that is solely responsible for the initiation and maintenance of the many phenotypic changes occurring in transformed cells (Jove & Hanafusa, 1987). Although v-Src was the first oncogene to be identified (Stehelin et al., 1976) and its product, the first protein tyrosine kinase to be described (Hunter & Sefton, 1980), the mechanisms by which v-Src causes transformation, and the physiological role of its normal cellular counterpart, c-Src, are still poorly understood. Cells transformed by v-Src, show a profound subversion of several cellular functions involved in transduction of extra- and intracellular signals, phospholipid metabolism, cytoskeletal reorganization, regulation of cell cycle, and control of gene expression (Cantley et al., 1991; Bishop, 1991; Bortner et al., 1993; Shalloway & Shenoy, 1991). The fact that mutational activation of cellular c-Src can cause the same wide range of effects induced by v-Src (Kmiecik & Shalloway, 1987), suggested that c-Src might

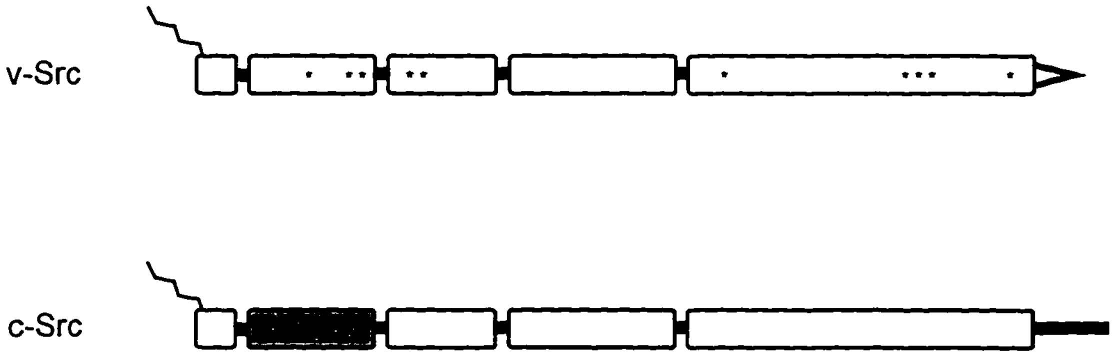


Fig.1 Viral v-Src differs from its cellular homologue c-Src. Retroviral transduction by Rous sarcoma virus (RSV), resulted in the substitution of 19 C-terminal amino acids in the avian c-Src (black bar) with 12 novel residues in v-Src (stippled arrowhead). Additional point mutations (*), have been acquired through multiple passages of the original RSV strain

have an essential role in controlling multiple critical functions within the cell, and that v-Src dependent transformation directly results from the excessive stimulation of the same functions controlled by c-Src.

The c-Src protein is ubiquitously expressed in animal cells with highest levels in embryonic and adult nervous tissue (Cotton & Brugge, 1983), in platelets (Golden et al., 1986), and adrenal medullary chromaffin cells (Parson & Creutz, 1986), suggesting a role in development, maintanance of a differentiated state and secretion. Disruption of the c-Src gene in knock-out experiments revealed that c-Src is not essential for cell growth and differentiation since *src*-null mice develop normally and have no obvious defects in neurons or platelets. These observations have been explained by the functional redundancy existing among the several members of its family (Lowell et al., 1994). Unexpectedly the only histopathological alteration responsible for the early death of the mice is an extensive osteopetrosis resulting from a deficiency in bone reabsorption (Soriano et al., 1991). The molecular basis of such an effect are currently unknown. Thus, clues concerning a Src-specific role that relates to its proto-oncogenic nature, have not been obtained genetically.

Structural determinants of Src functions

Although a clear picture of the molecular pathophysiology of Src is still elusive, our knowledge about its structural-functional features has rapidly improved in recent years. Src and the other eight members of the Src family of cytoplasmic tyrosine kinases share an amino acid sequence with identity ranging between 60 and 75% (Brickell, 1992). The higher degree of homology is found within five of the six conserved regions that define the overall molecular architecture of the family (fig. 2). Several functional properties of Src have been ascribed to these structurally separated domains which, in different variants, can also be found in most proteins involved in the transduction of cellular signals (Pawson, 1995; Cohen et al., 1995).

Localization signal: The first 15 N-terminal residues, or SH4 (Src homology domain) domain, contain a myristylation signal that directs the co-translational covalent attachment of a myristyl fatty acid to glycine 2. Although not sufficient by itself, this lipophylic group is necessary for localizing Src at the inner surface of plasma membranes and endosomes (Resh, 1993; Kaplan et al., 1992). Removal of Gly-2 abolishes v-Src myristylation and its ability to transform cells (Cross et al., 1984), although tyrosine kinase activity is not affected (Buss et al., 1986), indicating that proper membrane targeting is a critical requirement for v-Src oncogenic activity. At position 12, a serine residue is, together with serine

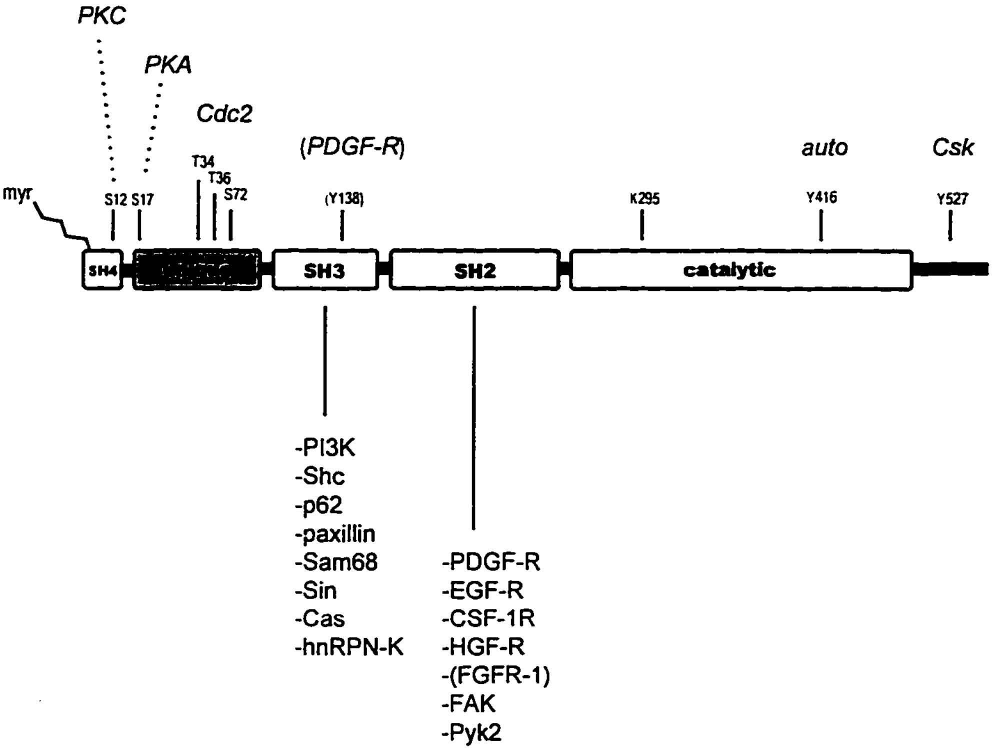


Fig. 2 Diagram depicting the general structure of the c-Src protein. Signaling molecules that either phosphorylate Src (top), or interact with the SH domains (bottom) are indicated (see text).

48, phosphorylated by PKC (Ca^{++}-dependent protein kinase). Mutation of these residues do not result in obvious alterations of the transforming ability of activated Src. The functional significance of serine phosphorylation at these sites, as well as the PKA (cAMP-dependent protein kinase) induced phosphorylation of the neighboring serine-17, is presently unknown (Yaciuk et al., 1989).

Unique domain: The following 50-70 amino acids, constitute a “unique domain” that is poorly conserved among the different members of the Src family. This domain has been shown to participate in protein-protein interactions in other members of the family, but not Src (Rudd et al., 1993). This region appears to be involved in regulating Src activity during mitosis since it contains three residues (Thr-34, Thr-46, and Ser-72) that are phosphorylated by the cell cycle specific kinase Cdc2 (Shenoy et al., 1989).

SH3 domain: C-terminal to this domain is the SH3 domain, a region of about 60 residues functioning as a modular binding domain (MBD). It is commonly found in several signaling molecules where it mediates associations with proline-rich helices on the interacting protein (Ren et al., 1993). Binding specificity and affinity are dependent upon the consensus sequences where the polyproline motifs reside (Feng et al., 1994). SH3-mediated interactions are known to direct subcellular localization of proteins, assembly of macromolecular complexes, substrate selection and regulation of enzymatic activities (Cohen et al., 1995; Feller et al., 1994). Mutations at the SH3 domain can prevent phosphorylation and binding of Src substrates, and alter the oncogenic potential of activated Src in a cell-specific manner (Hirai & Varmus, 1990a,b). Several signalling proteins involved in various cellular functions bind the SH3 domain of Src. These include: the p85 non-catalytic subunit of PI3K (phosphatidyl inositol 3-kinase) (Liu et al., 1993); the adaptor protein Shc (Weng et al., 1994); a 62 kDa protein that also binds to RasGAP (Ras-GTPase activating protein), PLCγ (phospholipase C gamma) and Grb2 (Weng et al., 1994; Richard et al., 1995); the cytoskeletal protein, paxillin (Weng et al., 1993); Sam68, a Src substrate that binds RNA and becomes tyrosine phosphorylated during mitosis (Taylor et al., 1995), and other less charactericized proteins such as Sin (Alexandropoulos & Baltimore, 1996), p130 Cas (Sakai et al,. 1994) and the heterogeneous ribonucleoprotein K (Weng et al., 1994). Recently, two groups reported that PDGF- and EGF-induced mitogenic signaling require an intact Src SH3 domain. and PDGF induces phosphorylation of the Tyr-138 (in murine Src), located on the binding surface of the SH3, that causes a down-regulation of the SH3 ability to bind its ligands (Broome & Hunter, 1996; Erpel et al., 1996). Recruitment and selection of Src substrates, often in cooperation with the adjacent SH2 domain, appears to be the major role of this domain. Although necessary for normal c-Src functions, the biological relevance of the SH3 domain with respect to Src-induced transformation, remains unclear, since SH3-deleted mutants of activated Src are still able to transform (Seidel-Dugan et al., 1992; this thesis).

SH2 domain: Next to the SH3 domain, is the SH2 domain which comprises about 100 residues. This was the first MBD to be identified for its ability to selectively bind phosphorylated tyrosines. Binding specificities are dictated by residues surrounding the phosphotyrosine on the cognate proteins, as well as within the SH2 domain itself (Songyang et al,. 1993). Like other MBDs, this domain is found in a variety of signal transducing proteins, mainly those capable of associating with receptor protein tyrosine kinases (RPTKs) (Pawson, 1995). Following stimulation by different growth factors, SH2-containing molecules translocate from cytosol to the submembrane region by recognizing and binding phosphorylated tyrosines on the cytoplasmic tails of activated RPTKs (Schlessinger & Ullrich, 1992; van der Geer et al., 1994). Src binding to activated receptors for PDGF (plateled-derived growth factor), EFG (epidermal-derived growth factor), CSF-1 (colony-stimulating factor-1) and HGF (hepatocyte growth factor) occurs via its SH2 domain (Mori et al., 1993; Luttrell et al., 1994; Courtneidge et sl., 1993; Ponzetto et al., 1994), which also mediates interactions with the tyrosine kinases FAK (focal adhesion kinase) and Pyk2 (Cobb et al., 1994; Dikic et al., 1996). The Src SH2 domain is responsible for negatively regulating Src kinase activity through an intramolecular association with its phosphorylated tyrosine at position 527. The SH3 domain too, seems to participate in this auto-inhibitory head-tail interaction that would fold the catalytic domain in a closed inactive conformation (Superti-Furga et al., 1993). SH2 and SH3 domains frequently cooperate also in binding and selecting substrates (Weng et al., 1994; Broome & Hunter, 1996). Tyrosine residues phosphorylated by Src, often lie within the consensus sequence recognized by its own SH2 domain (Songyang et al., 1995). This can potentially determine a local increase in activated Src-substrates that would contribute to Src activation by rising the concentration of phosphotyrosines competing for the binding of its Tyr-527 at the SH2. In addition, it has been proposed that a preferential binding to its own substrates might also result in the ability of Src to repeatedly phosphorylate multiple tyrosines on the same molecule, leading to the processive phosphorylation of the substrate (Mayer et al., 1995). Different mutations in the SH2 domain have been found to either increase or decrease the transforming potential of Src in a host-dependent manner and to cause variations in substrate preferences (Hirai & Varmus, 1990a,b; Seidel-Dugan, 1992), suggesting that SH2-mediated interactions are relevant for v-Src-dependent transformation.

Catalytic domain: The SH1, or catalytic domain is approximately 260 amino acids long and contains several highly conserved regions of similarity found in most tyrosine and serine/threonine kinases (Taylor et al., 1993). A universally conserved lysine residue at position 295 is necessary for ATP binding in the phosphotransfer reaction. Mutations at this site, abolish kinase activity, transformation and phosphorylation of Tyr 416 (Snyder et al., 1985). This residue is autophosphorylated *in trans* by Src itself and is hyper-phosphorylated in v-Src, but not in c-Src proteins. This modification appears to contribute to the high kinase activity of v-Src, perhaps by stabilizing the catalytic domain in its active conformation or facilitating the access of substrates to the active site (Cooper & Howell, 1993).

Auto-inhibitory region: The catalytic domain is followed by a short "tail" containing a conserved tyrosine residue at position 527 involved in the negative regulation of Src activity. In the currently accepted model, phosphorylated Tyr-527 interacts with the SH2 domain *in cis* to maintain Src in an inactive conformation (Cooper & Howell, 1993). This fits the observation that most transforming viral *src* genes have truncations or substitutions of the last 15-20 C-terminal amino acids, resulting in the absence of Tyr-527, and a protein with constitutively high kinase activity (Takeya & Hanafusa, 1983; Jove & Hanafusa, 1987). Consistently, mutation of Tyr-527 to phenylalanine is sufficient to convert proto-oncogenic c-Src into an oncogene (Kmiecik & Shalloway, 1987), and dephosphorylation of Tyr-527 is accompanied by a more than 10-fold increase in kinase activity (Courtneidge et al., 1985). Members of the related but distinct class of Csk (c-terminal src kinase) protein kinases are capable of phosphorylating Tyr-527 and repress Src kinase activity (Okada et al., 1993).

It is now apparent that Src, as the majority of signal transducing proteins, utilizes a combination of site-specific modifications, and MBD-directed interactions at the SH domains, to govern the pattern of its activation states, localization, selection of substrates and formation of multimeric signaling complexes (Pawson, 1995; Cohen et al., 1995). But, we must also agree with Brickell (1992) that "*now there is an almost embarassing wealth of possible interactions and the important task in the coming years will be to discern which of these are biologically relevant*".

Integration of Src in multiple cellular functions

Increased proliferative rate, decreased cell adhesion, cytoskeletal rearrangements and changes in the pattern of gene expression are major cellular phenotypic responses observed after mitogenic stimulation, as well as in oncogenically transformed cells. Most of the functional compartments that control these biological events have been implicated as potential critical targets for the transforming effects of oncogenic Src, either because c-Src is involved in the physiologically-induced responses, or because these can be altered in v-Src expressing cells (fig. 3)

1. Transmembrane receptors and their effectors.

Src has been shown to bind several receptor protein tyrosine kinases (RPTKs) following their ligand-dependent activation. In the case of PDGF-R and CSF-1R this also results in increased Src kinase activity (Mori et al., 1993; Courtneidge et al., 1993). The activation of Src appears to be essential for PDGF-, EGF-, and CSF-1-induced mitogenesis (Roche et al., 1995), as well as for differentiative signaling by NGF and FGF receptors in PC12 cells (Kremer et al., 1991). However, how the recruitment and activation of Src translates into a RPTK-initiated signal is still an open question.

Upon ligand binding, RPTKs dimerizes and trans-phosphorylate various tyrosine residues on their juxtamembrane cytoplasmic tails. The phosphotyrosines

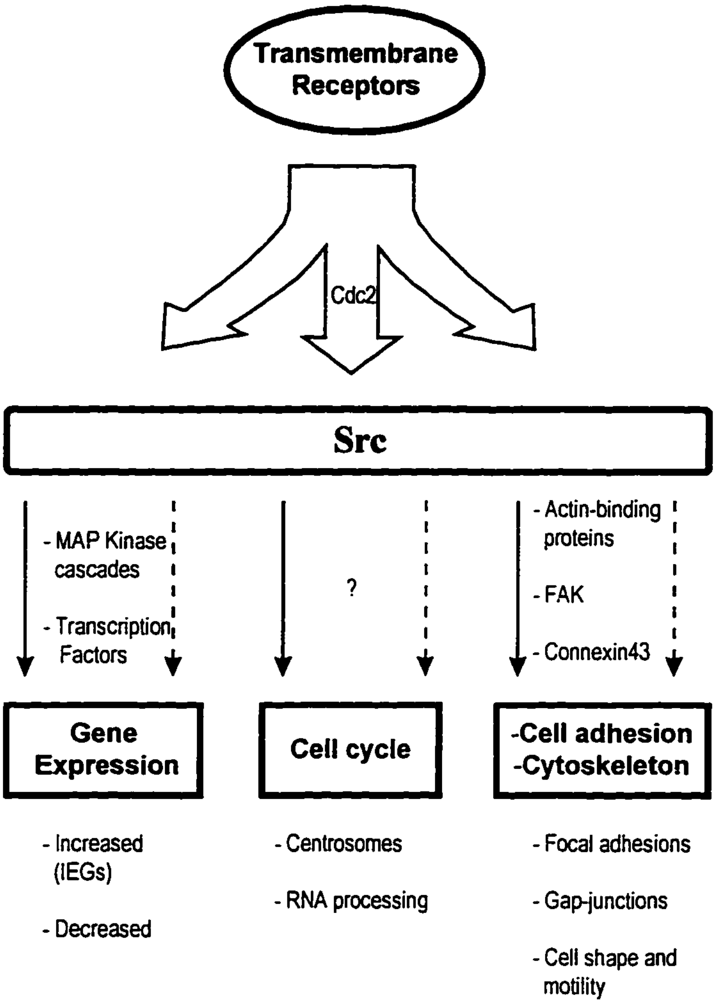


Fig. 3 Integration of Src in multiple cellular functions. Src is functionally interconnected with different cellular compartments that control the induction of a mitotic phenotype in response to physiological stimuli (continuous lines), or in the presence of a transforming Src oncoprotein (broken lines) (see text).

are subsequently recognized and bound by specific SH2 domains present on several signaling enzymes such as PI3K, PLCγ, RasGAP, and Src-type tyrosine kinases. Interacting molecules also include Shc, Grb2 and Nck adaptor proteins which, albeit lacking catalytic activities, contain SH2 or/and SH3 domains enabling them to act as "molecular bridges" that couple activated RPTKs to other signaling molecules (Schlessinger & Ullrich, 1992; van der Geer et al., 1995). Recruited SH2-containing molecules undergo further modifications and/or associations resulting in the initiation of different intracellular signals. For instance, recruitment of PLCγ brings the enzyme in proximity of its preferential substrate PIP_2 (phosphatidyl inositol 4,5 diphosphate), which is hydrolysed to DAG (diacylglycerol) and IP_3 (inositol 1,4,5 triphosphate). DAG is the physiological activator of PKC, whereas IP_3 causes Ca^{++} release from intracellular compartments. Similarly, recruitment of PI3K results in the generation of PIP_3 (3'phosphoinositides) from membrane phospholipids. Although the role of PIP_3 in cellular signaling remains unclear, PI3K is involved in the activation of the small GTPase Ras, and other members of its family (Rodriguez-Viciana et al., 1994; Reif et al., 1996). Ras is a membrane-bound protein that becomes activated when its bound nucleotide is exchanged from GDP to GTP. Sos is the guanine nucleotide exchange factor that catalyzes this conversion. In resting cells cytoplasmic Sos is complexed with the Grb2 adaptor protein. Upon RPTK activation, Grb2 is recruited to the receptor and the associated Sos is then capable of causing the activation of inactive membrane bound Ras. Grb2 binding to the receptor can occur either directly or through the other adaptor protein Shc. The prominent effect of GTP-bound Ras is to trigger the sequential activation of an enzymatic cascade involving multiple serine/threonine kinases such as Raf, Mek and MAP kinase, that leads to the phosphorylation/activation of nuclear transcription factors activating expression of genes involved in proliferative and differentiative responses (Egan et al., 1993; Davis, 1993) (fig. 4).

Why is Src activated and recruited by RPTKs? One suggested possibility is that by bringing Src-bound molecules in the proximity of the receptor, Src could increase the range of RPTK substrates and effectors (Broome & Hunter., 1996). Alternatively, Src might phosphorylate other effectors that are not substrates for the activated RPTKs, or further modify molecules already bound to the receptor (Roche et al., 1995). The transforming effect of v-Src might therefore result from the unregulated activation of the same effectors normally utilized in RPTKs induced signaling. The fact that, PLCγ (Nakanishi et al., 1993), RasGAP (Ellis et al., 1990; Brott et al., 1991), PI3K (Liu et al., 1993) and Shc (McGlade et al., 1992; Weng et al., 1994) are tyrosine phosphorylated in v-Src transformed cells, is consistent with this hypothesis, and it might also explain why Ras is a major mediator of v-Src transformation (Smith et al., 1986). The ability to "hijack" mitogenic signals normally utilized by transmembrane receptors might be a more

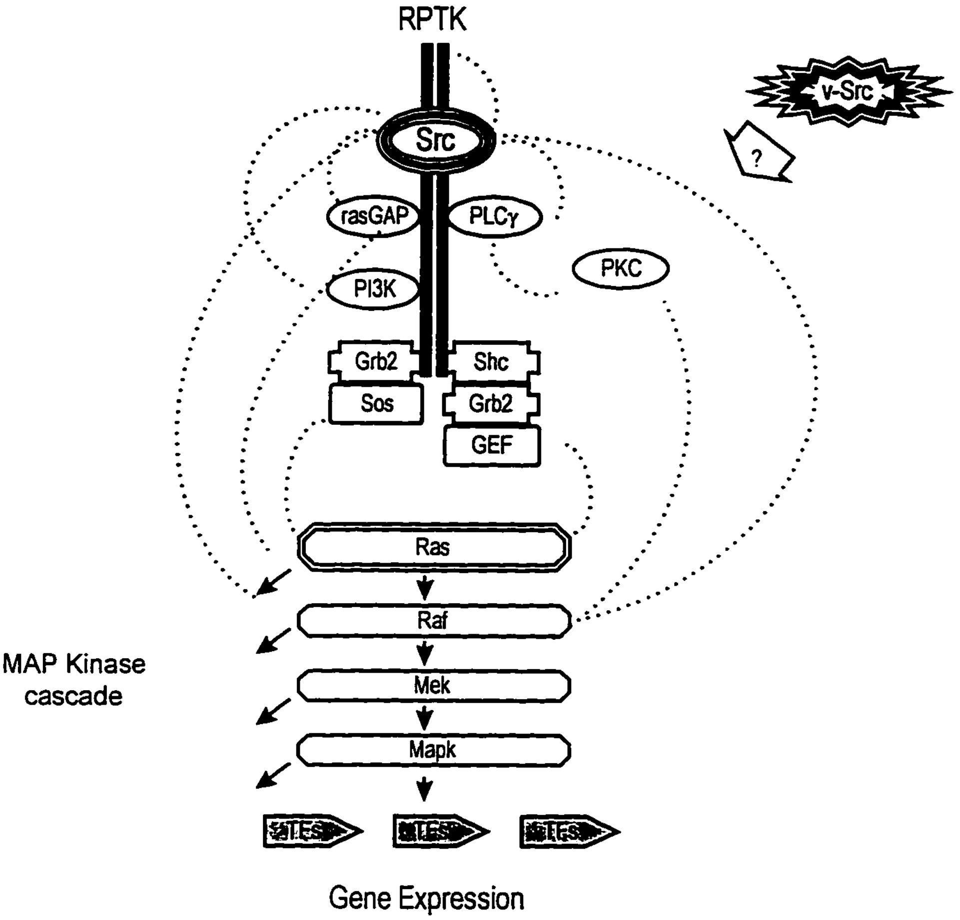


Fig. 4 Src is a potential regulator of signals initiated by transmembrane receptor protein tyrosine kinases (RPTKs). The Ras-MAP Kinase cascade is a signaling pathway central to cellular growth and differentiation. Multiple effectors orchestrate its activation following their recruitment to ligand-activated RPTKs. These effectors are also potential targets of Src, which might modulate their activation together with the RPTKs. A constitutively activated v-Src might then cause the unregulated activation of the same signal transduction machinery, resulting in cellular transformation (see text)

general aspect of v-Src activity. Indeed, mitogenic stimulation of seven-transmembrane (heptahelix) receptors that signal through heterotrimeric G-proteins (G-protein coupled receptors, GPCRs), has been recently shown to cause activation of Src and several other effectors such as PI3K, PLCγ, PKC and the Ras-MAP kinase pathway (Liu et al., 1996b; Luttrell et al., 1996; Chen et al., 1996).

2. Cell adhesion and cytoskeletal rearrangements.

Several cytoskeleton-associated proteins are highly tyrosine phosphorylated in v-Src transformed cells, suggesting that the major morphological alterations seen in these cells, might directly result from such modification (Jove & Hanafusa, 1987). These include the actin-binding proteins paxillin, talin, cortactin and vinculin, as well as integrin subunits and the focal adhesion kinase FAK. They are primarily localized in submembrane structures called focal adhesions, through which the intracellular actin cytoskeleton becomes anchored to the extracellular matrix via transmembrane integrin receptor (Jockusch et al., 1995; Schwartz et al., 1995). In normal cells, integrin binding to the substrate causes autophosphorylation of FAK, its binding to Src, PI3K and the Grb2/Sos complex (Schlaepfer et al., 1994; Chen & Guan, 1994). While FAK and Src might phosphorylate the cytoskeleton-associated proteins necessary to assemble the focal adhesion plaques, Grb2/Sos and PI3K could serve to coordinate cellular adherence with mitogenic signaling, since fibroblasts replication requires anchorage to a solid substratum (Stocker et al., 1968). Focal adhesions are reduced in number and structurally disorganized in v-Src transformed cells (Burridge et al., 1988), likely resulting in the typical low adherence and round cellular morphology. Interestingly, Grb2 and Sos are constitutively bound to tyrosine phosphorylated FAK in v-Src transformed cells (Schlaepfer et al., 1994). It has been proposed that the abnormally high levels of tyrosine phosphorylation of FAK and other focal adhesion proteins could allow transmission of mitogenic signals in the absence of a firm cell adhesion, thus resulting in the characteristic phenomenon of anchorage-independent growth of transformed cells (Calalb et al., 1995).

Similar events might also occur in regions of cell-cell contacts. Indeed, connexin43, the main component of fibroblast gap-junctions, is tyrosine phosphorylated in v-Src-transformed cells, and this correlates with a downregulation in the number of gap-junctional communications between cells (Azarnia et al., 1988). This could potentially cause the loss of contact inhibition of cell growth, also observed in oncogenically transformed cells.

3. Cell cycle regulation

A role for Src as regulator of cell cycle-dependent events, is suggested by the fact that during M-phase its activity is increased up to 40-fold (Shenoy et al., 1992). This activation is associated with the mitosis-specific phosphorylation of Src at Thr-32, Thr-46 and Ser-72 by the cyclin-dependent kinase Cdc2 (Shenoy et al., 1989). This modification is exclusively detectable at the onset and during mitosis, concomitantly with the increase in Src activity. It has been proposed that it might derepress inactive c-Src by destabilizing the intramolecular interaction between the SH2 and Tyr-527, and/or favouring dephosphorylation of Tyr-527 (Shenoy et al., 1992; Stover et al., 1994). Accordingly, activated form of Src lacking Tyr-527 are not further activated during mitosis (Bagrodia et al., 1991). The existence of a signaling mechanism from Cdc2 to Src supports the hypothesis that Src could act as an effector of Cdc2 in regulating mitotic-specific events. Among the potential targets are the cytoskeletal components controlling cell-substrate and cell-cell adhesion structures that are disassembled during mitosis (Shalloway & Shenoy, 1991; Jockusch et al., 1995). In M-phase, Src is concentrated in perinuclear endosomal membranes surrounding the centrosomes, where it could play a role in regulating mitotic-spindle formation (David-Pfeuty & Nouvian-Dooghe, 1990). The RNA-binding protein Sam68 is tyrosine phosphorylated and associated with Src only in mitosis, suggesting that Src might participate in regulating RNA processing during the cell cycle (Taylor & Shalloway, 1994). Finally, mitotically-activated Src could also promote activation of proliferation-specific genes, since v-Src is known to increase the expression of several genes that are normally induced by mitogenic stimuli (see below). Thus, another possible mechanism through which v-Src would cause transformation, is the unregulated activation of the same mitotic targets controlled by normal Src.

4. Gene expression

The induction and maintenance of any cellular phenotype is also determined by the underlying pattern of expressed genes. Relative to their normal counterparts, v-Src transformed cells display both, increased and decreased expression of several genes involved in disparate cellular functions. About 1000 new transcripts have been detected in v-Src transformed chicken embryo fibroblasts (Groudine & Weintraub, 1980). Although many of the affected genes might have a marginal contribution, others are likely to play a major role in establishing the transformed phenotype. For instance, down-regulation of extracellular matrix proteins such as fibronectin and collagen, coupled with the expression of the secreted proteases stromelesin, cathepsin-L and collagenase, might effectively promote invasiveness and metastatic potential (Bortner et al., 1993). Augmented levels of transcripts for the TGFα, TGFβ and PDGF-A growth factors (Ciardiello et al., 1990), might establish autocrine and paracrine loops resulting in proliferation under low serum conditions.

The enhanced growth rate of neoplastic cells, might also result from either down-regulation of growth-inhibitory genes, as well as elevation of growth-promoting genes (Lewin, 1991; Lin et al., 1995; Bortner et al., 1993). Among the latter, a particular group of genes called “primary response” or “immediate early” genes (IEGs), has been intensely investigated because in response to several mitogenic stimuli they are rapidly induced without requiring *de novo* protein synthesis. This mode of activation suggested that IEGs might be necessary to drive quiescent cells through the cell cycle following proliferative signals (Hershman, 1991). v-Src and other oncogenes can increase the expression of several IEGs, suggesting that their abnormal activation could be responsible for the cell cycle deregulation typical of transformed cells. The major mechanism for IEGs induction is the transcriptional activation of their promoters (Hershman, 1991). The signals through which v-Src causes their activation are not fully understood, however, it is evident that distinct transcriptional promoter elements such as SRE (serum response element) or TRE (TPA responsive element) direct v-Src dependent induction of IEGs. Although several signal transducing molecules such as PKC, Ras and Raf, have been shown to mediate activation of these response elements (see below), the molecular mechanisms by which v-Src causes their activation are currently unknown.

In this thesis, we have investigated the possibility that specific MBDs and/or phosphorylated sites on Src, are required to control different sets of intracellular signals leading to transcriptional activation of separate IEG-promoter elements, such as SRE, TRE and CRE (cyclic AMP responsive element). To accomplish this, several Src genes, mutated at regions involved in the regulation of its signaling properties, were tested for the ability to induce transcriptional activation of the above promoter elements. During the course of these experiments, we found that while transiently transfected Src-genes could induce strong activation of all promoter elements, these were poorly or not activated in cells chronically transformed by v-Src, suggesting that an increased expression of IEGs might not be necessary for maintaining a transformed phenotype.

Therefore, we sought to identify other intracellular signals that may account for the induction of a proliferative phenotype by v-Src. In a different set of experiments, we found that two distinct receptors for the mitogens FGF and PDGF, are constitutively phosphorylated on tyrosine, and complexed with signal transducing molecules such as Grb2, Sos and PI3K. Since similar molecular events are induced upon stimulation with physiological mitogens, these experiments suggest that a v-Src-dependent intracellular activation of various growth factor receptors may result in the generation of unregulated mitogenic signals, ultimately leading to cellular transformation.

CHAPTER II

Transcriptional activation of immediate early gene-promoter elements by different mutated forms of Src

INTRODUCTION

Understanding the mechanisms by which the oncoprotein v-Src causes transformation is complicated by the plethora of intracellular signals activated by this oncogene. Among the many cellular functions affected by v-Src, the enhanced expression of IEGs is likely to play a major role in determining the transformed phenotype since their expression is almost invariably induced by most mitogenic stimuli (Hershman, 1991). Studies from our laboratory and others, have demonstrated that v-Src can induce expression of the Egr-1 (Qureshi et al., 1991a), Collagenase (Shonthal et al., 1988) and TIS10 (Qureshi et al., 1991b) primary response genes. Three distinct transcriptional control elements found within the promoters of these genes mediate v-Src dependent transactivation: SRE (Qureshi et al., 1991a), TRE (Shontal et al., 1988) and CRE (Xie et al., 1994) respectively. In transiently transfected murine fibroblasts, each of the above elements can drive the transcription of a reporter gene cotransfected with a v-Src expression vector. Some of the signaling molecules through which v-Src induces transcriptional activation of these response elements have been identified (fig. 5). When fibroblasts are depleted of PKC by prolongued TPA (12-O-tetradecanoyl phorbol-13-acetate) treatment, TRE- and CRE-dependent transcription is inhibited, but transactivation of the SRE is unaffected (Qureshi et al., 1991; Xie et al., 1994). Co-transfection of a dominant-interfering mutant of the serine/threonine kinase Raf reduces the activation of the SRE, but not TRE, by v-Src (Qureshi et al., 1991c) . In the same system, a dominant-interfering mutant form of the small GTPase Ras blocks v-Src mediated transactivation of all three promoter elements (Qureshi et al., 1992; Xie et al., 1994). Since different transcription factors mediate the transcriptional output from these promoter sequences, their activation by v-Src occurs via three distinguishable signaling mechanisms that differentially utilize PKC, Ras and Raf (fig. 5).

How can such a diversified set of signals be generated in response to Src activation? The structural features shared by Src and most signaling proteins (Pawson, 1995; Cohen et al., 1995) suggest that post-translational modifications of specific sites within the protein, as well as protein-protein interactions occurring at its modular binding domains (see fig. 2), might allow Src to control the activation of distinct cellular signals. For instance, the association of Src with Shc (Weng et al., 1994), RasGAP (Brott et al, 1991) PI3K (Liu et al., 1993) and Raf could potentially result in the activation of a Ras-Raf dependent pathway. Similarly, the association with PLCγ (Nakanishi et al., 1993), could account for the production of DAG, which in turn could activate PKC and downstream signals. It is not known whether any or all of these associations are required to initiate v-Src-dependent transcriptional activating signals. This hypothesis can be tested by examining whether mutations at the putative source of a signal (i.e. a specific site on Src), result in the selective alteration of a given Src-inducible response

v-Src

PKC	-	+	+
Ras	+	+	+
Raf	+	-	nd
Gene	*egr-1*	*collagenase*	*Tis10*
Response Element	**SRE**	**TRE**	**CRE**
Transcription Factors	TCF/SRF	Fos/Jun	Jun/ATF

Fig. 5 Activation of three different transcriptional control elements by v-Src occurs via multiple signaling mechanisms that are dependent (+), or independent (-) from PKC, Ras and Raf. nd = not determined.

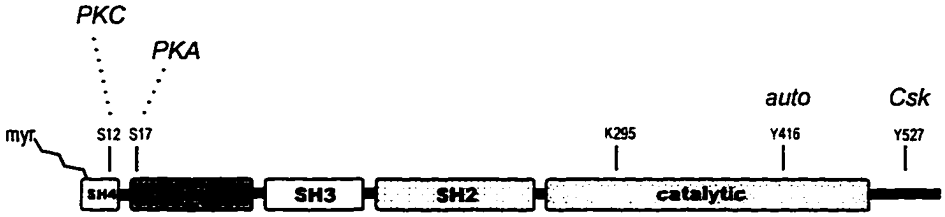
(i.e. activation of a specific promoter element). Detection of a preferential site-dependence for an intracellular signal, would enable us to further confirm or exclude its involvement in v-Src transformation, as well as the role played by the IEGs correspondingly affected. To test this hypothesis, we examined a set of Src mutants for their ability to induce activation of a luciferase reporter gene under control of the above three v-Src responsive transcriptional regulatory elements.

RESULTS

1. Transcriptional activation of IEG-promoter elements by different mutated forms of Src.

To verify whether specific regions within the Src protein are responsible for the transcriptional activation of Src-induced gene expression, we examined the ability of distinct Src-mutants to activate v-Src-responsive promoter elements in transient transfection assays. A series of expression vectors encoding for Src proteins mutated at sites implicated in the transduction of intracellular signals, was obtained fron Dr. D. Shalloway (Cornell University), and additional constructs were generated in our laboratory. The type(s) and the effects of the mutations are summarized in fig. 6.

Fig. 6 Schematic representation of the different Src-mutants used in this study. Each diagram corresponds to two Src proteins carrying the same mutation in either the wild type c-Src or its activated form c-Src527. Activated mutants are in parenthesis and followed by an asterisk indicating the Tyr-to-Phe mutation at position 527. The effect(s) of each mutation are also indicated.

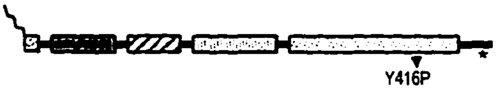


PROPERTIES ALTERED BY
THE MUTATION

c-Src
*(c-Src527)**

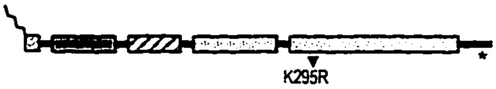
c-Src: none
(c-Src527): AutoinhibitoryTyr-527 removed.
Constitutively active Src kinase.

*(c-416/527)**



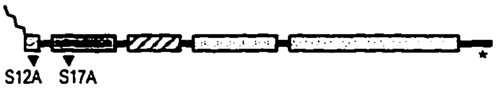
Autophosphorylation site removed. Full activation of kinase activity is inhibited.

c-Src295
*(c-295/527)**



ATP-binding site removed. Kinase activity is abolished.

c-Src12/17
*(AAF527)**

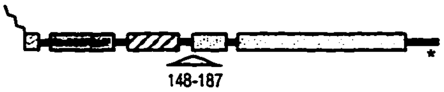


PKC (S12) and PKA (S17) phosphorylation sites removed. Regulation of Src by PKC and PKA is abolished.

dlA
*(dlA527)**

SH3 domain deleted (A-box). Protein-protein interactions with polyproline-containing ligands are abolished.

dlB
*(dlB527)**

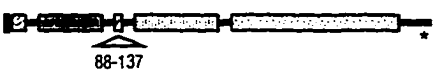


SH2 domain deleted (B-box). Protein-protein interactions with phosphotyrosine-containing ligands are abolished.

LN
*(LN527)**

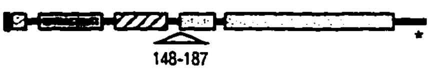
LN= N-terminal addition of 4 aa (MAAA) disrupts the myristylation site. Localization to membranes is abolished.

LNdlA
*(LNdlA527)**



Non-myristylated, SH3-deletion mutant. Membrane localization and SH3-mediated interactions are abolished.

LNdlB
*(LNdlB527)**



Non-myristylated, SH2-deletion mutant. Membrane localization and SH2-mediated interactions are abolished.

The transforming potential of v-Src results from the constitutive activation of its tyrosine kinase activity. Therefore the effect of each mutation has to be assessed in the context of an activated Src protein, relative to a non-activated Src, in order to detect changes in signaling properties. To accomplish this, all mutations were introduced into two genetic backgrounds: the wild type *c-src* (as a control), and the activated form *c-src527*, which carries a tyrosine-to-phenylalanine substitution at position 527. This dominant gain-of-function mutation abolishes the inhibitory tyrosine phosphorylation site in the C-terminal tail of Src and produces a constitutively active Src kinase that mimics the oncogenic properties of v-Src (Kmiecik et al., 1987). Using liposome-mediated transfection, each Src-encoding construct was transiently co-transfected into NIH 3T3 cells along with a luciferase reporter plasmid driven by either the SRE, TRE or CRE v-Src-responsive promoter elements previously described (fig. 7), and the relative induction of luciferase activity was measured in the cell extract.

First we tested a set of Src mutants with point-mutations at sites potentially involved in the regulation of Src kinase activity. As expected, increased activation of all reporters was induced by the two activated v-Src and c-Src527, while the wt c-Src caused only a slight increase above the levels recorded for the empty vector pEVX (fig. 8a). Autophosphorylation of tyrosine 416 is required for a full activation of Src kinase, and its mutation to phenylalanine (c-416/527) causes a decrease in kinase and transforming activities of activated c-Src527 (Kmiecik &

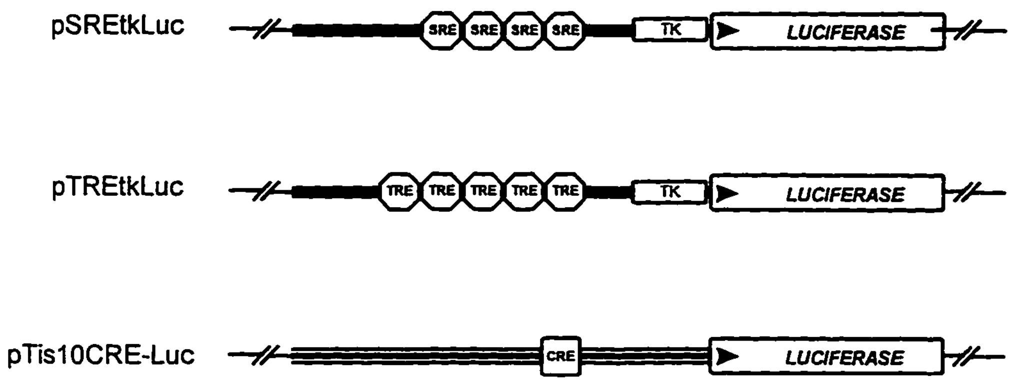
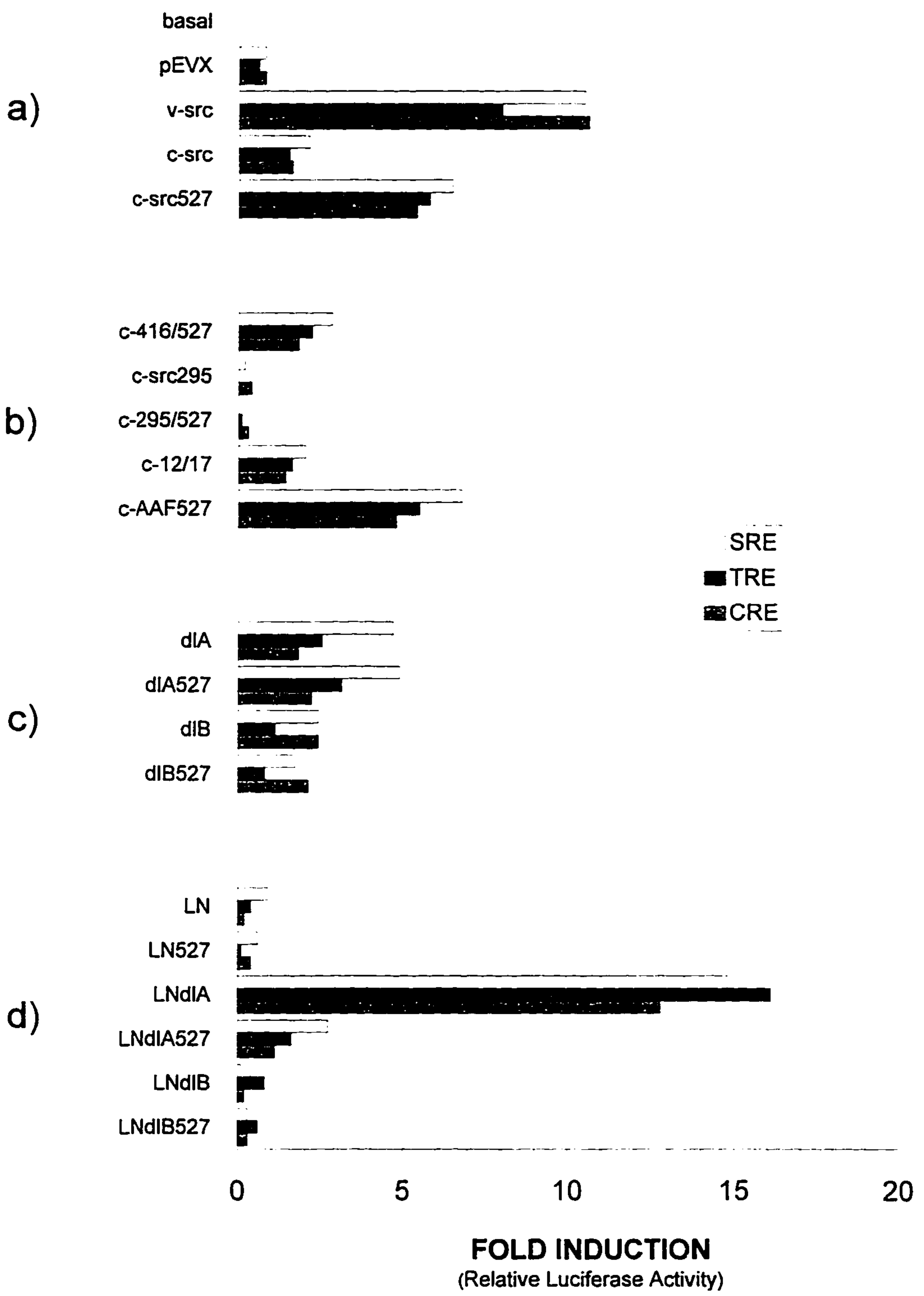


Fig. 7 Diagrammatic representation of the luciferase reporter plasmids used to determine the transcriptional activating potential of the Src-mutans. Promoter sequences containing SRE, TRE and CRE v-Src-responsive elements, were cloned upstream of the luciferase reporter gene in the pXP2 vector (see Methods and Materials).

Fig. 8 Determination of the transcriptional activating ability of different Src-mutants. Each luciferase reporter plasmid (see fig. 7) was transiently co-transfected into NIH 3T3 cells, along with a separate Src-mutant-encoding vector. After ~48 hours the luciferase activity was assayed as described in Methods and Materials. Bars represent the average of 3 to 5 independent transfections, each performed in triplicate samples. Values are normalized to the total protein concentration in the lysate, and are expressed as fold induction of luciferase activity over the basal zero-value of a reporter-only transfected sample.


a)
basal
pEVX
v-src
c-src
c-src527
b)
c-416/527
c-src295
c-295/527
c-12/17
c-AAF527
SRE
■TRE
▨CRE
c)
dlA
dlA527
dlB
dlB527
d)
LN
LN527
LNdlA
LNdlA527
LNdlB
LNdlB527
0
5
10
15
20
FOLD INDUCTION
(Relative Luciferase Activity)

Shalloway, 1987). Consistent with this, all reporters were poorly activated by the c-416/527 mutant, when compared to the control c-Src (fig. 8b). The catalytic activity of Src requires the ATP-binding site lysine-295 and its mutation to arginine results in a catalytically inactive Src (Snyder et al., 1985). As expected, neither of the two kinase-defective Src-mutants, c-Src295 and c-295/527, caused reporter activation (fig. 8b). This observation also allowed us to rule out the possibility that Src could initiate intracellular signals by simply engaging in protein-protein interactions, as in the case of adaptor proteins Shc and Crk (Mayer and Baltimore, 1993). In addition, together with the impaired activity observed for the c-416/527 mutant, these results confirmed that tyrosine kinase activity is required for Src-induced transactivation.

Two serine residues at position 12 and 17 on Src, are the major phosphorylation sites for PKC and PKA respectively (Yaciuk et al., 1989). To determine whether PKC or PKA affect Src-dependent transactivating signals, we used Src mutants in which both serines were substituted with alanines. As shown in fig. 8b both, non-activated c-Src12/17 and the activated AAF527, had the same transactivating potency of their respective non-mutated parental c-Src and c-Src527, indicating that transcriptional activation of all three responsive elements is not affected by PKC- and PKA-induced phosphorylation of Src ar these sites.

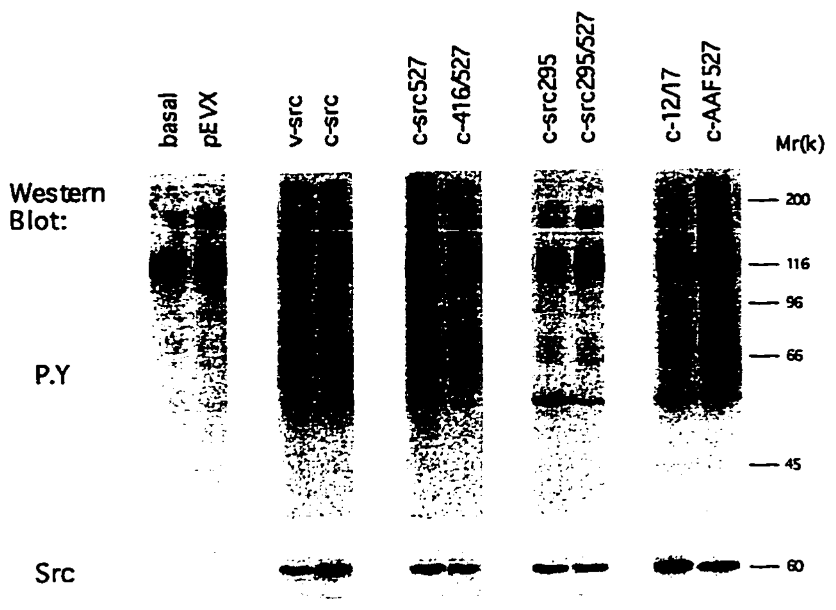
Most of the reported interactions between Src and other signalling proteins occur at its SH3 and SH2 domains (see fig. 2), suggesting that some of the bound molecules might act as signaling effectors of Src. To verify the role played by these MBDs in the activation of Src-induced transcriptional signals, we used a second set of mutants in which either the SH3 or the SH2 domain were disrupted by deletions (Seidel-Dugan et al., 1992; Bagrodia et al., 1992). As shown in fig. 8c, the SH3-deleted/activated Src mutant dlA527, retained a moderate SRE-activating effect, while signaling to the TRE and CRE appeared to be preferentially reduced. Interestingly, the same transactivating properties were displayed by the corresponding non-activated dlA. This observation is consistent with the fact that in addition to interacting with putative Src effectors, the SH3 also participates in the downregulation of Src activity (Superti-Furga et al., 1993). Therefore, disruption of the SH3 would result in a lesser inhibition of the non-activated dlA, but it would also impaire the full activating potential of the activated dlA527, since critical interactions might be lost. Disruption of the SH2 domain in the activated Src (dlB527) resulted in a lack of reporter transactivation indicating that SH2-mediated interactions are required for the initiation of transcriptional activating signals by Src (fig, 8c).

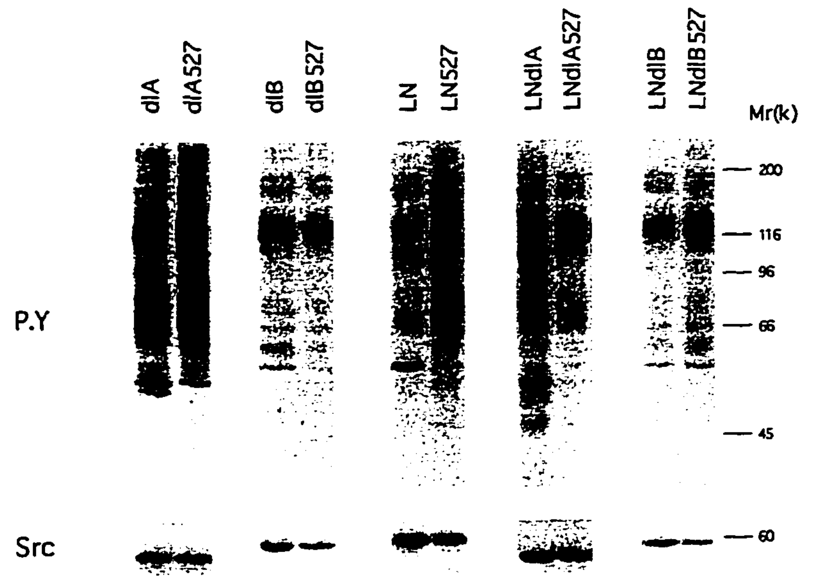
The accessibility to specific substrates and signaling effectors depends upon the subcellular localization of a signaling molecule (Faux, 1996). Since several Src-interacting and Src-activated molecules are not associated with membranes, we reasoned that a delocalization of Src from its normal submembrane compartment could allow certain interactions to occur, hence causing activation of specific signals. This hypothesis was tested by using a set of non-myristylated Src mutants, since the subcellular distribution of Src is mainly dictated by the insertion of its myristylated moiety into the lipid bilayer of endosomal and plasma membranes (Kaplan et al., 1992), and its removal causes Src to localize in the cytosol (David-Pfeuty & Nouvian-Doogue, 1990; David-Pfeuty et al., 1993). The LN mutation abrogates the N-terminal myristylation site on Src (Schuh & Brugge, 1988; Bagrodia et al., 1993), and was introduced into a third set of mutants encoding for wild type, SH3- and SH2-deleted Src. As shown in fig. 8d, relative to the c-Src control, all the constructs had a reduced transactivating ability with the only exception being the unmyristylated, non-activated, SH3-deletion mutant, LNdlA. Indeed, a remarkably high transcriptional activation of all three reporters was induced by this mutant, that even exceeded the one observed for v-Src (see fig. 8a). This finding was particularly surprising since the activated form of this mutant (LNdlA527), could not induce transcriptional activation. This implies that the signaling ability of LNdlA is dependent upon the C-terminal tyrosine 527, in contrast with the reported role of this residue in causing inhibition of Src activation. At the moment we are unable to explain such a discrepancy in the behavior of these two mutants. However, this suggests that a mechanism involving the interaction of phosphorylated Tyr-527 with another protein, might exists and cause the high transcriptional activation we observed. The non-myristylated SH2-deleted mutants LNdlB and LNdlB527 were both unable to induce transactivation (fig. 8d), consistent with the requirement for an intact SH2 domain for Src-dependent transcriptional signaling, and as already suggested by the effects of their corresponding myristylated forms (fig. 8c).

The differences in the transactivating potencies of the Src mutants, rather than being Src-specific effects could result from different expression levels among the mutants. This possibility was ruled out since most mutants were expressed at comparable levels in the transient transfection assay (fig. 9). Instead, some of the low transactivating forms were expressed above the average level.

We also tested each construct for its ability to induce tyrosine phosphorylation of cellular proteins in the transient transfection assay. As shown in fig. 9, the increase in protein tyrosine phosphorylation correlated with the transactivating effect of each mutant, with the relative exception of LN527 and LNdlA. Although unable to cause transcriptional activation, LN527 increased the phosphotyrosine content of a specific set of proteins, consistent with a similar effect previously reported in chicken embryo fibroblasts (Reynolds et al., 1989). On the other hand, despite having the highest transactivating potency measured among all constructs, LNdlA induced a significantly lower level of tyrosine phosphorylated proteins than those induced by the other activating mutants.

Fig. 9 Immunodetection of tyrosine phosphorylated proteins and Src-mutated proteins in NIH 3T3 cells transiently transfected with the indicated Src-mutant gene. Proteins (15μg/lane) from total cell lysates, were separated by SDS-PAGE on a 7.5% polyacrilamide gel, transferred to nitrocellulose, and immunostained with a monoclonal antibody (Mab) that specifically recognizes Src proteins encoded by the avian gene, or with a Mab against phosphotyrosine (P.Y).


basal
pEVX
v-src
c-src
c-src527
c-416/527
c-src295
c-src295/527
c-12/17
c-AAF527
Mr(k)
Western Blot:
P.Y
Src
200
116
96
66
45
60

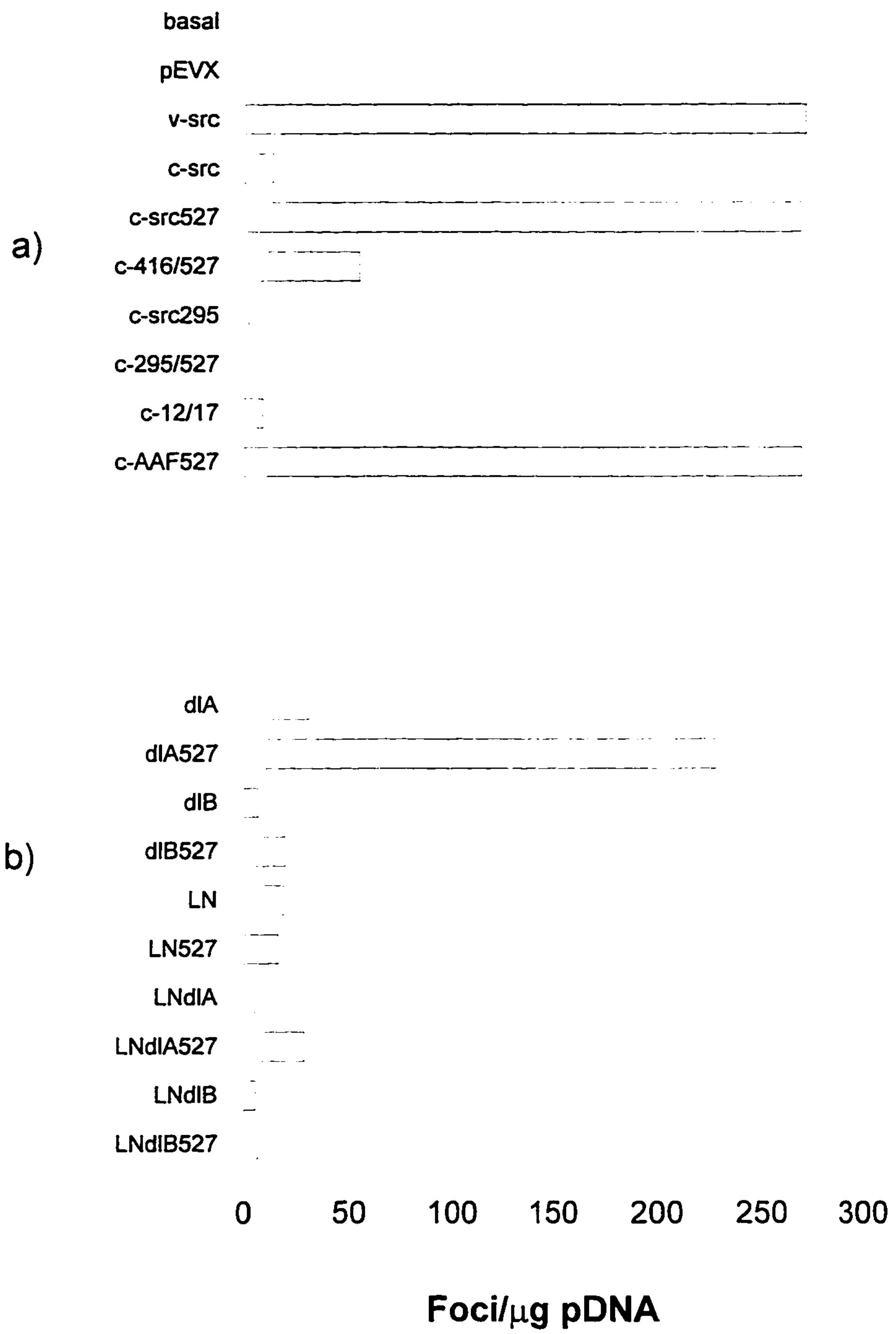

dlA
dlA527
dlB
dlB527
LN
LN527
LNdlA
LNdlA527
LNdlB
LNdlB527
Mr(k)
P.Y
Src
200
116
96
66
45
60

2. Transcriptional activation of IEG-promoter elements by Src is not correlated with its transforming effect.

Instead of further investigating the molecular mechanisms underlying the effects of the dlA, dlA527 and LNdlA mutants, the results described above caused us to ask the question whether the transactivating properties of LNdlA and other Src mutants were biologically significant in respect to the capacity of Src to cause transformation. To verify this, each Src mutant was tested for its ability to induce formation of transformed foci in monolayer cultures of transfected NIH 3T3 cells. fig 10a shows that the transforming efficiency of the first set of point-mutants was strictly correlated with their ability to induce both transcriptional activation and tyrosine phosphorylation of cellular proteins. Within the group of SH mutants (fig. 10b), only the activated, SH3-deleted Src (dlA527) was able to transform, while the strong transactivating LNdlA mutant was unable to cause transformation.

In agreement with previous reports (Hirai & Varmus, 1990a,b; Cross et al., 1984), these data indicated that, transformation of NIH 3T3 cells by Src requires both, an intact SH2 domain and membrane localization. But, in contrast to what reported by Erpel et al. (1995; 1996), in our experiments, transformation was not dependent upon the SH3 domain. In addition, despite its ability to cause a strong transcriptional activation, the unmyristylated, SH3-deleted mutant LNdlA did not induce formation of transformed foci. This indicated that, at least for the three

Fig. 10 Transforming efficiency of different *src*-mutant genes transfected into NIH 3T3 cells. The number of transformed foci in monolayer cell cultures was determined 12-14 days after transfection. Transfections were performed in duplicate plates as described in Methods and Materials. Values are geometric averages from four separate experiments, and are expressed as number of transformed foci/dish/μg of plasmid DNA.


a)
basal
pEVX
v-src
c-src
c-src527
c-416/527
c-src295
c-295/527
c-12/17
c-AAF527
b)
dlA
dlA527
dlB
dlB527
LN
LN527
LNdlA
LNdlA527
LNdlB
LNdlB527
0
50
100
150
200
250
300
Foci/μg pDNA

response elements considered here, the ability to increase transcriptional activation of IEGs is not sufficient to determine a transformed phenotype.

3. Transcriptional activation of IEG-promoter elements is minimal or absent in cells chronically transformed by v-Src and v-Ras

One possible explanation for the lack of transforming activity by the LNdlA mutant in spite of its high transactivating potency is that the high levels of IEG expression induced in the short time of a transient transfection assay might, in the long run, be incompatible with cell viability and cause a lethal phenotype. This possibility was excluded since we were able to establish cell lines stably expressing the LNdlA mutant (fig. 11a). Cellular growth and morphology were indistinguishable from the parental NIH 3T3 cells and, according with the data obtained in the transient transfection assay, the intracellular content of tyrosine phosphorylated proteins was significantly elevated in respect to the parental cell line (fig. 11b).

Then, to further substantiate the dissociation between transformation and trascriptional activation of IEG-promoter elements suggested by the previous experiments, we compared the levels of transactivation attained by the same reporter constructs in **i**) non-transformed parental NIH 3T3 , **ii**) non-transformed LNdlA-expressing cells, and **iii**) v-Src transformed cells. As an additional positive control we used a cell line transformed by an oncogenic v-Ras protein that can

Fig. 11 The LNdlA Src-mutant is expressed, and causes increased tyrosine phosphorylation of proteins in stably transfected NIH 3T3 cells. A vector encoding the SH3-deleted, non-activated, non-myristylated LNdlA mutant of Src, was transfected in NIH 3T3 fibroblasts along with the pSV2neo plasmid containing the neomycin-resistance gene. Neomycin-resistant clones were selected and amplified. Proteins (15 µg/lane) from three LNdlA-expressing clonal cell lines (N-LNdlA) were separated by SDS-7.5%PAGE and analyzed by Western blotting using monoclonal antibodies against avian Src **(a)**, or phosphotyrosine **(b)**

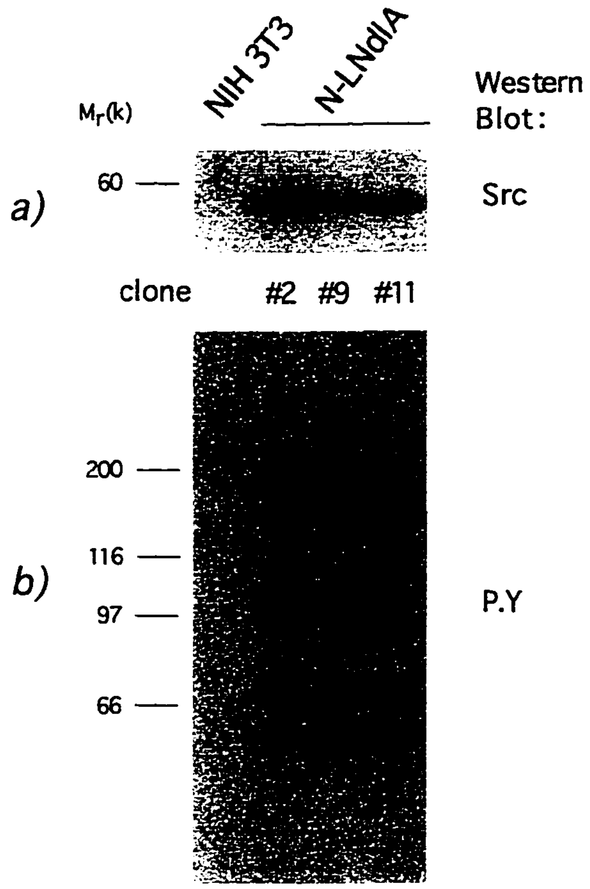
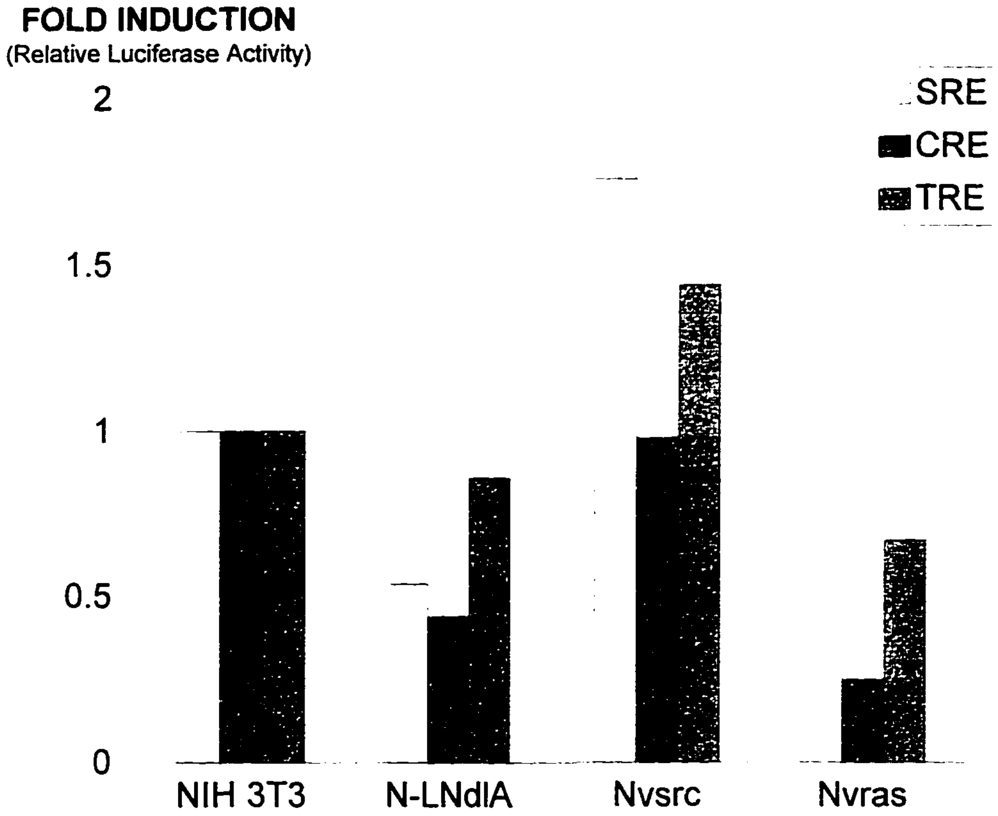

NIH 3T3
N-LNdlA
Western Blot:
M_r(k)
a)
60
Src
clone
#2 #9 #11
b)
200
116
97
66
P.Y

Fig. 12 Transcriptional activation of v-Src-responsive promoter elements in transformed *versus* non-transformed cell lines. NIH 3T3 cell lines stably expressing the LNdlA, v-Src and v-Ras proteins were generated as described in Methods and Materials. The SREtkLuc, TREtkLuc and Tis10CRE-Luc reporter vectors were transiently transfected in the indicated cell lines, along with a β-galactosidase expression plasmid to normalize for variations in transfection efficiencies among the different cell lines. Values are expressed as fold induction of luciferase activity/μg protein per β-galactosidase OD/μg protein, and are the averages of four independent experiments.


FOLD INDUCTION
(Relative Luciferase Activity)
2
1.5
1
0.5
0
SRE
CRE
TRE
NIH 3T3
N-LNdIA
Nvsrc
Nvras

induce SRE and TRE dependent transcription in transient transfection assays (Qureshi et al., 1992). Using the same liposome-mediated system, the three luciferase reporter vectors were transiently transfected in each of the above cell lines. As shown in fig. 12, a surprisingly low activation of all reporters was detected in all cell lines. SRE (0.78 fold) and TRE (0.45 fold) activation in v-Src transformed cells was almost negligible if compared to the levels (about 10 fold) measured following transient co-transfection of v-Src and the reporter, into NIH 3T3 cells (see Fig. 8). All cell lines showed no induction of the CRE element, and a moderate inhibition of all reporters was observed in the LNdlA-expressing cell line. v-Ras transformed cells exhibited a minimal SRE activation (0.4 fold), while both CRE and TRE activities were below the control values. These results indicated that activation of the SRE, TRE and CRE transcriptional control elements by transiently transfected v-Src does not reflect their state of activation in cells that are chronically transformed by the same oncogene. suggesting that increased levels of IEGs expression are dispensable in stably transformed cells.

DISCUSSION

Our initial aim was to discern Src-initiated intracellular signal(s) capable of preferentially activate a specific Src-responsive IEG promoter element. A preferential activation of the SRE element was observed by the two SH3-deleted Src mutants dlA and dlA527. However, this effect did not correlate with transformation since both had the same transactivating ability but only the activated one (dlA527) was efficiently transforming. Moreover, three activated Src genes (v-Src, c-Src527, AAF527) were efficient in causing both, transactivation and transformation, but the strongest transactivating construct LNdlA was unable to induce formation of transformed foci.

Regardless the molecular mechanisms responsible for the transcriptional activation of the SRE, TRE, and CRE elements by transiently transfected Src-mutants, and besides the discrepancy between transactivating and transforming ability, the major point that emerged from these studies was the substantial absence of reporter activation in v-Src-transformed cells. In these experiments, two separate phenotypes were examined in two distinct experimental conditions: a 48 hour-long transient transfection assay, versus a two-weeks focus forming assay. The simplest interpretation of the LNdlA-induced effects was that a short-timed excessive expression of IEGs would not be sustainable by the cell in the long run, and the consequent lethal phenotype would not allow formation of foci during the two weeks following the introduction of the transgene. However, this was not the case since it was possible to establish LNdlA-expressing cell lines that were non-tranformed and with an elevated content of tyrosine phosphorylated proteins. This, enabled us to further verify the separation of transcriptional activation from transformation in the context of a cellular phenotype attributable to a stably expressed Src-transgene. When compared to the parental NIH 3T3 cells, the intracellular signaling pathways regulating gene transcription from the tested SRE, TRE and CRE elements, were poorly or not activated in cells chronically transformed by v-Src and v-Ras, as well as in cells expressing the non-transforming Src mutant LNdlA. Since v-Src, LNdlA and v-Ras are all strong transcriptional inducers of the same response elments when transiently transfected, the validity of this assay should carefully be reconsidered before data from short-time expressed activating genes are extrapolated to a biological context derived from the continuous, long-lasting effects of the same gene.

Nearly all the studies on v-Src induced IEGs expression have employed short-term assays such as transient transfections or temperature activatable v-Src genes (Fujii et al., 1989; Spangler et al., 1989; Apel et al., 1992; Qureshi et al., 1991a, 1991b, 1991c, 1992; Xie et al., 1994; Yu et al., 1993; Sato et al., 1993). An often raised argument is that the effects observed in transient transfection assays could be secondary to the phenotypic changes occurring during the "long" time lag (usually ~48 hours) of the assay, rather than a direct effect of the transgene. To refute the argument, systems in which a stably transfected gene is conditionally activated for shorter times (i.e. temperature-sensitive mutants) have been employed. Ironically, the problem seems to consist in the short, rather than the long timing of the assay.

It would then appear that in the process of cellular transformation, oncogenes like v-Src or v-Ras might initially cause an elevated expression of IEGs which is not sustained once an overtly transformed phenotype is established. Several reports are in agreement with this possibility. Progression from G0 to G1-S and mitosis with no appreciable increase in IEGs such as c-Fos, c-Myc and c-Jun was reported in Rat-1 cells expressing a temperature activatabl v-Src (Welham et al., 1990). Yu et al. (1993) found that in four different v-Src transformed cell lines, as well as in cells trasformed by v-Ras, v-Raf and v-Sis, the serum-inducibility of c-Jun, c-Fos, Egr-1 and NGF1-B IEGs was attenuated, and none of them was expressed at detectable levels in unstimulated basal conditions. Transiently transfected activated Ras was shown to induce expression of the *egr-1* gene, whereas no expression was detectable in the same cells constitutively expressing activated Ras (Huang et al, 1994). Even more intriguing was the ability of overexpressed Egr-1 to inhibit cell growth in different neoplastic human cell lines (Huang et al, 1995). Perhaps with a similar mechanism, overexpression of the SRE-activating trancription factor SRF, causes a reversion of the Ras-transformed phenotype (Kim et al., 1994). It should be made clear that these, and our data, do not indicate that transcriptional-activating signals are silenced in v-Src transformed cells. Indeed, the transcription factors involved in IEGs transactivation might still be activated and bound to their promoter elements since enhanced AP1/TRE binding activity can be detected in v-Src transformed cells (Welham et al., 1990).

However, the transcriptional output might be inhibited by unknown feedback mechanisms, restoring normal levels of IEG expression.

That activation of IEGs expression might not be instrumental in causing transformation is also suggested by observations concerning nuclear oncogenes. Several transcription factors required to activate the promoters of IEGs, are themselves the product of IEGs such as c-Jun, c-Fos, c-Myc and c-Rel. They also constitute a major group of proto-oncogenes thought to cause tranformation via unregulated activation of proliferation-controlling genes such as IEGs. However this appears not to be the case. Indeed, transformation by v-Jun and v-Fos is not associated with increased AP1/TRE activation (Hawker et al., 1993), v-Jun inhibits, rather than stimulate transcription (Gao et al., 1996; Havarstein et al., 1992), and putative transformation-specific promoter sequences for v-Jun are still to be found (Shuur et al., 1995). In apparent contradiction, expression of JunB is costitutively elevated in v-Src tranformed cell lines (Apel et al., 1992), but JunB is not an activator of transcription from AP1/TRE sites, and antagonizes the transactivating function of c-Jun (Chiu et al., 1989). Conversion of c-Rel into oncogenic v-Rel results from the deletion of its C-terminal transactivation domain, and v-Rel transforms whether localized in the nucleus or cytoplasm (Gilmore & Temin, 1988). Perhaps, the strongest support to the argument that mitogenesis might not depend upon increased expression of IEGs comes from the finding that in the rat liver, a massive proliferative response to certain mitogens occurs with no increased expression of several IEGs (Columbano & Shinozuka, 1996).

Thus, activation of IEGs is unlikely to be a relevant determinant of the transformed phenotype. On the contrary, inhibition of IEGs expression, rather than their activation, could contribute to transformation. Trans-repression of growth inhibitory genes, rather than trans-activation of growth-promoting genes has been indeed proposed to play a major role in oncogenesis (Lewin, 1991; Havarstein et al., 1992; Lin et al., 1995). It is important to emphasize that our experiments do not exclude the possibility that an increased transcription of IEGs might play a critical role in the initiation of cellular transformation (Yu et al., 1993). However, our findings suggest that an increased transcriptional activation of IEGs is not likely required for the maintenance of the transformed phenotype induced by v-Src, as well as other oncogenes such as v-Ras.

Although contrasting, the data obtained from transient *versus* stable expression of v-Src nevertheless could reflect a unified process of adaptation where suppression of v-Src cytotoxic effects might occur via accumulation of resistant cells (Brugge et al., 1987; Wu et al., 1995), and/or increased endogenous tyrosine phosphatase activity (Nelson and Branton, 1984; Woodford-Thomas et al., 1992). Through a gradual homeostatic process the network of intracellular signals would thus re-regulate to accommodate for an increased activation of signaling effectors and the high levels of phosphotyrosine caused by v-Src. At the distal end of the signalling pathways, compensatory feedbacks, such as inhibitory phosphorylations of transcription factors (Hunter & Karin, 1992), might then restore expression of IEGs to levels compatible with cell viability.

This interpretation is consistent with the low IEG expression observed in v-Src transformed cells despite the constitutive activation of the intracellular signals physiologically involved in their activation. MAP kinase (Gupta et al., 1992; Schlaepfer et al, 1994), PI3K (Fukui & Hanafusa., 1991), PKC (Zang et al., 1995), PLCγ (Nakanishi et al., 1993) and Raf (Morrison et al., 1988) are all phosphorylated and/or activated in v-Src transformed cells, and Ras (Smith et al., 1986) and Rac (Minden et al., 1995) mediate transformation by v-Src. If the increased expression of IEGs is not required for v-Src to cause transformation, can we explain the transforming ability of v-Src solely on the basis of the activation of the above signaling molecules?

The typical transformed morphology of cells expressing v-Src (as well as other oncogenes), results mainly from rearrangements in the actin cytoskeleton. Similar, but ordered changes are induced by growth factors and other mitogenic stimuli, and are mediated by the Ras-superfamily small GTPases Rho, Rac and Cdc42 (fig. 13). Rho induces formation of acting stress fibers and focal adhesion complexes, Rac promotes ruffling and lamellipodia formation at the plasma membrane, and Cdc42 stimulates formation of filopodia (Nobes, 1995). These

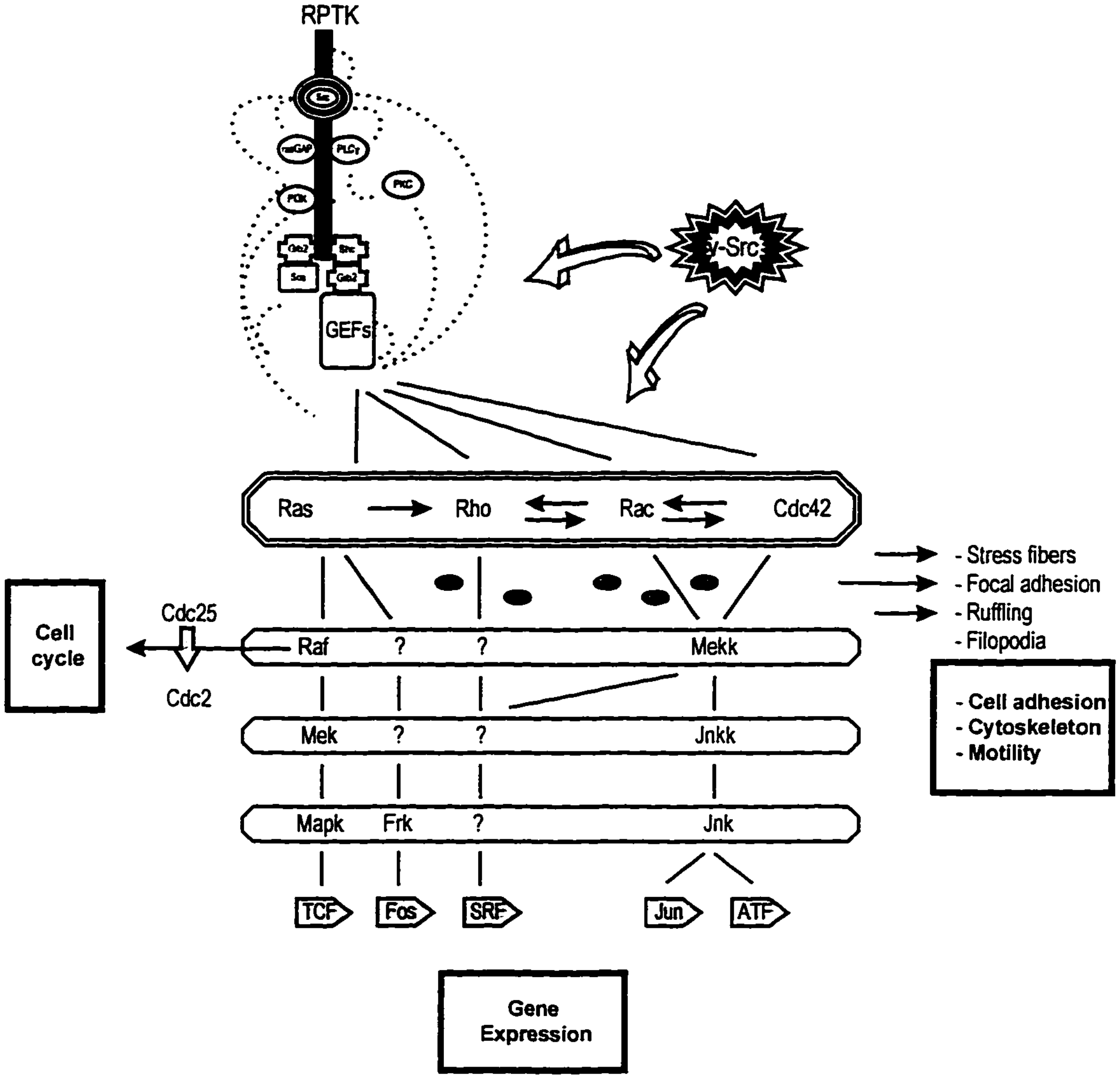


Fig.13 Diagram showing how different cellular functions responsible for the mitotic phenotype can be controlled via intracellular signals mediated by small GTPases of the Ras-superfamily. Their activation proceeds in a highly regulated fashion in response to RPTKs activation, while v-Src could cause their abnormal activation through as yet unidentified mechanisms (see text).

structures are abnormally formed in v-Src transformed cells (Burridge et al., 1988; Howell & Cooper, 1994; Kaplan et al., 1994).

Like Ras, Rho, Rac and Cdc42 also activate MAP-kinase-type cascades leading to activating phosphorylations of different transcription factors. Ras induces sequential activation of Raf-Mek-MAP kinase resulting in the activation of the SRE-binding TCFs (Treisman, 1994). And Ras-dependent activation of Frk causes activation of c-Fos (Deng & Karin, 1994). Rac and Cdc42 stimulate a distinct cascade containing the ser/thr kinases Mekk-Jnkk-JNK which activates Jun and ATF (Gupta et al., 1995; Minden et al., 1995), whereas Rho causes SRF activation via a similar, but still incompletely characterized cascade (Hill et al., 1995).

Like Ras, Rho-family GTPases also stimulate DNA synthesis and are required for G1-S progression in response to serum (Olson et al., 1995). Consistent with our data, activation of gene expression by these GTPases does not appear to be necessary for transformation. In fact, Rac and Cdc42 mutants unable to cause activation of the JNK/MAP kinase cascade can still mediate ruffling and transformation (Joneson et al., 1996; Lamarche et al., 1996) indicating that morphological and growth-promoting effects are not dependent upon Jun-directed transcription. These data are also in agreement with the elegant experiments of Beug et al. (1978), showing that enucleated cells expressing a temperature sensitive v-Src are still capable to attain a transformed morphology upon temperature shift.

What then links small GTPases to cellular proliferation? The link might be a surprisingly direct one. Indeed, at membrane ruffles both Ras and Raf colocalize with Cdc25A, a dual-specificity phosphatase that promotes G1-S transition through dephosphorylation/activation of Cdc2, which is the indispensable cyclin-dependent kinase that drives entry into the cell cycle (Nurbury & Nurse, 1992). Raf associates with, and phosphorylates Cdc25A causing its activation (Galaktionov et al., 1995). A sustained activation of Cdc25A would therefore be sufficient to explain the transforming activity of v-Src, v-Ras and v-Raf, although would exclude a role for the other GTPases Rho, Rac and Cdc42 which also appear to be required for proliferation and transformation. Since Ras can activate Rho and Rac (Khosravi-Far et al., 1995), and Rac activates Cdc42 (Nobes, 1995), activation of all the GTPases could be promoted only by Ras. An attractive speculation is that these GTPases might control other Raf-type kinases to regulate different Cdc25 phosphatases. In fact, mammals have three Cdc25 forms (A, B and C) that function at different times in the cell cycle. Raf associates mainly with Cdc25A, only weakly with Cdc25B and not with Cdc25C which is required for G2-M transition (Galaktionov and Beach, 1991). The several dephosphorylation-activatable cyclin-dependent kinases (CDKs) that regulate progression through the cell cycle might also constitute additional regulatory targets (Morgan, 1995).

In conclusion, our experiments did not support the original hypothesis that different transcriptional activating signals are induced by Src via specific regions within the Src protein. Rather, they pointed to the fact that activation of IEGs appears not to be necessary for maintaining the transformed phenotype induced by v-Src. If Ras, the other small GTPases, and the effectors involved in their regulation, can reasonably be assumed to act as key mediators in the induction of the v-Src-transformed phenotype, then the major question that arises is how v-Src causes their activation. Experiments addressing this issue will be described in the following chapter.

CHAPTER III

Receptor protein tyrosine kinases are tyrosine phosphorylated and bound to signaling molecules in v-Src transformed cells

INTRODUCTION

Although many proteins have been identified as putative v-Src substrates (Jove & Hanafusa 1987), none has yet been established as an obligatory substrate necessary for v-Src-induced transformation. Kinase activity, membrane binding and the SH2 domain of activated Src, are all absolutely required for transformation, implying that SH2-mediated interactions that regulate tyrosine phosphorylation of critical substrates by v-Src must occur within the submembrane compartment in order to cause transformation.

Among the possible candidates, PI3K and PLCγ localize at the membrane, are tyrosine phosphorylated, and activated in response to several mitogenic growth factors as well as in v-Src-transformed cells (van der Geer & Hunter, 1994; Fukui & Hanafusa, 1991; Wasilienko et al., 1991; Nakanishi et al., 1993). However, PI3K binds to v-Src via its SH3 domain (Liu et al., 1993), which is not required for transformation, and PLCγ is not a v-Src substrate (Wasilienko et al., 1991). Thus their activation is unlikely a direct effect of v-Src. Ras is membrane-bound and necessary for v-Src induced transformation (Smith et al., 1986), but is not a Src substrate. Its effector Raf, is tyrosine phosphorylated and activated in v-Src transformed cells, but its activation depends upon Ras (Williams et al., 1992; Jelinek et al., 1996). PKC α and δ are activated and membrane-bound in v-Src transformed cells, but their activation depends upon the diacylglycerol produced through PLC or PLD (phospholipase D) enzymes (Zang et al., 1995). Thus, a direct link between v-Src and the activation of these signal transducing molecules cannot be clearly established.

Receptor protein tyrosine kinases (RPTKs) satisfy all the conditions for a putative v-Src effector involved in the regulation of proliferative signals. Are membrane localized, contain phosphorylatable tyrosines, can associate with Src and directly promote mitogenesis upon growth factor stimulation (van der Geer, 1994). Ligand-activated tyrosine kinase receptors as PDGF-R (Mori et al., 1993), CSF-1R (Courtneidge et al., 1993), HGF-R (Ponzetto et al., 1994) and perhaps FGFR-1 (Zhan et al., 1994), bind Src via its SH2 domain. And Src activity is required for PDGF , CSF-1 and EGF induced DNA synthesis (Roche et al., 1995). Mitogenic responsiveness to EGF is enhanced in c-Src overexpressing cells (Luttrell et al., 1988), and c-Src associates with overxpressed EGF receptor (Luttrell et al., 1994). Given the close structural and functional interactions with Src, RPTKs are likely to be affected by alterations of c-Src function(s), as those observed in oncogenic v-Src.

Weber and coworkers, reported that IGF-1R (insulin-like growth factor-1 receptor) and EGF-R become tyrosine phosphorylated in v-Src-transformed cells (Kozma et al., 1990; Wasilienko et al., 1991). In principle, tyrosine phosphorylation of growth factor receptors could account completely for the transforming activity of v-Src, since virtually every phenotypic change induced by v-Src can also be induced by growth factors, including activation of PI3K, PKC, PLCγ or Ras which are activated in v-Src transformed cells (Aaronson, 1991; Jove & Hanafusa, 1987; Cross & Dexter, 1991; Cantley et al., 1991). Nevertheless, these findings have received little consideration, presumably because the hallmark of receptor activation - its interaction with signaling effectors - was not investigated. A possible correlation between tyrosine phosphorylation of RPTKs and v-Src transformation was also suggested by our previous observation that only the transforming Src-mutants examined in the transient tranfection assays (see fig. 9) were able to increase the level of phosphorylated tyrosines in proteins above ~100-120 kDa, a molecular weight range in which most transmembrane receptors are comprised.

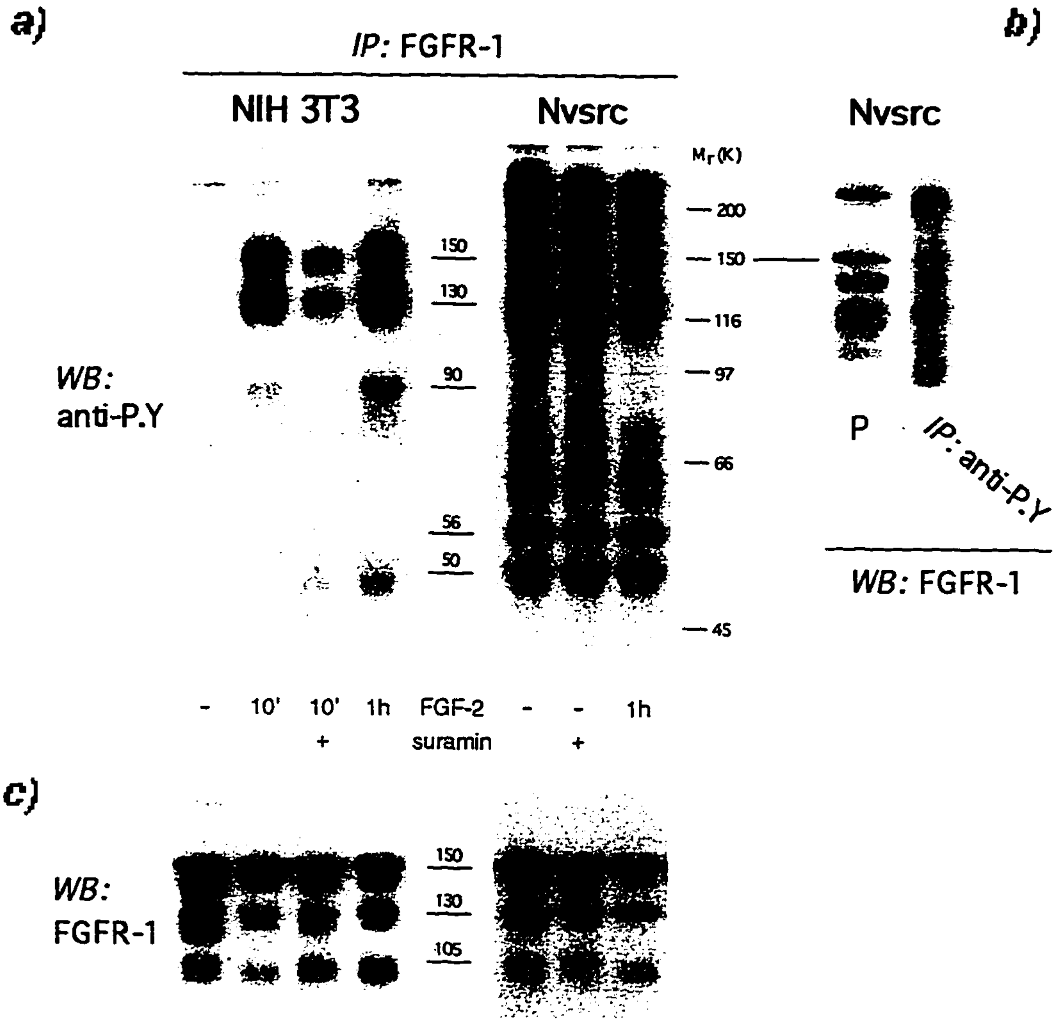
Therefore, we wished to determine whether other RPTKs were tyrosine phosphorylated in v-Src-transformed cells, and involved in the same type of molecular interactions that occur upon ligand-dependent activation. Since both FGF and PDGF are potent mitogens for fibroblasts (Burgess and Maciag, 1989; Ross et al., 1986), and Src is known to participate in signaling through their receptors, we investigated the state of the endogenous FGFR-1 and PDGF-Rβ in v-Src transformed fibroblasts.

RESULTS

1. FGFR-1 is tyrosine-phosphorylated and complexed with several tyrosine-phosphorylated proteins in v-Src-transformed NIH 3T3 cells.

We first examined tyrosine-phosphorylated proteins in FGFR-1 immunoprecipitates from NIH 3T3 and v-Src-transformed NIH 3T3 cells. Anti-FGFR-1 immunoprecipitates from FGF-treated and untreated NIH 3T3 cells were subjected to Western blot analysis using anti-phosphotyrosine antibody. In response to basic FGF (FGF-2) the 130 and 150 kDa forms of FGFR-1 (Safran et al., 1990) were most prominently tyrosine-phosphorylated (fig. 14a). In v-Src-transformed NIH 3T3 cells, FGFR-1 precipitated several tyrosine-phosphorylated proteins including proteins that appeared to be the 130 and 150 kDa FGFR-1 isoforms (fig. 14a). Consistent with this, the 130 and 150 kDa FGFR-1 isoforms could be detected in anti-phosphotyrosine immunoprecipitates from v-Src-transformed cells (fig. 14b). To exclude the possibility of an autocrine effect, the cells were treated with suramin, a compound that antagonizes the interaction between FGF and FGFR-1 (Moscatelli & Quarto, 1989). While suramin was effective, as expected, in inhibiting FGF-induced activation of the receptor in NIH 3T3 cells, a 24 hours incubation with suramin had no significant effect upon the tyrosine phosphorylation of proteins in FGFR-1 immunoprecipitates from v-Src transformed cells (fig. 14a). Conditioned medium from cultures of v-Src

Fig. 14 FGFR-1 is tyrosine-phosphorylated and complexed with several tyrosine-phosphorylated proteins in v-Src-transformed NIH3T3 cells. (**a**). The FGFR-1 was immunoprecipitated from 0.8 mg particulate fraction (P) protein from parental or v-Src transformed NIH 3T3 cells (Nvsrc) as described in Methods and Materials. FGF-2 (30 ng/ml) was added for the indicated time intervals at 30 ng/ml. Suramin (0.2 mM) was added together with FGF in NIH 3T3 cells, or for the 24 h preceding lysis in the Nvsrc cells. Immunocomplexes were analysed by Western blot analysis using an anti-phosphotyrosine antibody (PY20). (**b**). PY20 antibody was used to immunoprecipitate tyrosine-phosphorylated protein from lysates of Nvsrc particulate fractions. Immunocomplexes (IP) and 30 μg of protein from the particulate fraction (P) were subjected to Western blot analysis using anti-FGFR-1. (**c**). The level of immunoprecipitated FGFR-1 present in (a) were assessed by stripping the Western blot filter and reprobing with anti-FGFR-1 antibody.


a)
IP: FGFR-1
NIH 3T3
Nvsrc
Mr(K)
200
150
116
97
66
45
150
130
90
56
50
WB:
anti-P.Y
- 10' 10' 1h FGF-2 - - 1h
+ suramin +
b)
Nvsrc
P
IP: anti-P.Y
WB: FGFR-1
c)
WB:
FGFR-1
150
130
105

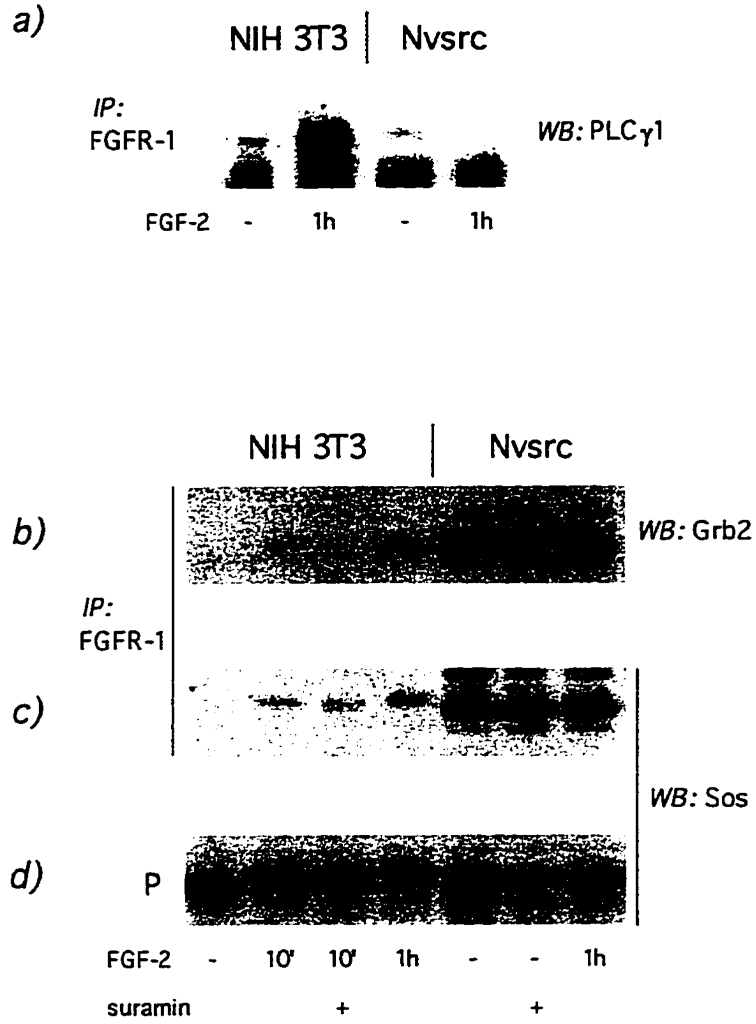
transformed cells could not induce tyrosine phosphorylation of proteins in FGFR-1 immunoprecipitates from parental NIH 3T3 (not shown). Together with the fact that none of the additional phosphotyrosine bands were induced by FGF, these results indicated that FGFR-1 tyrosine phosphorylation in v-src transformed cells was unlikely due to an autocrine mechanism.

There were significant differences in the tyrosine-phosphorylated proteins associated with the receptor in response to FGF and v-Src. The 130 kDa isoform appeared to be preferentially phosphorylated relative to the 150 kDa isoform in v-Src-transformed cells (fig. 14a). This difference was not due to variations in the amount of these two FGFR-1 isoforms since FGFR-1 levels were not significantly different under any of the conditions tested (fig. 14c). A previously reported 90 kDa tyrosine-phosphorylated protein that co-precipitates with FGFR-1 (Klint et al., 1995; Wang et al., 1996) was detected in FGF-treated NIH 3T3 cells, but not in v-Src-transformed cells (fig. 14a). And, several additional tyrosine-phosphorylated proteins were detected in the FGFR-1 immunoprecipitates from v-Src-transformed cells that were not observed in FGF-treated NIH 3T3 cells (fig.14a). These data demonstrate a ligand-independent stimulation of FGFR-1 tyrosine phosphorylation in v-Src-transformed cells that differs from the FGFR-1 tyrosine phosphorylation induced by FGF. Interestingly, FGF was unable to increase tyrosine phosphorylation levels in FGFR-1 immunoprecipitates from v-Src-transformed cells; in contrast, some bands seem to disappear with FGF treatment (fig. 14a), suggesting that responsiveness to FGF has also been altered or lost in v-Src-transformed cells.

2. Grb2 and Sos, but not PLCγ, co-immunoprecipitate with FGFR-1 from v-Src-transformed cells.

Tyrosine phosphorylation of FGFR-1 in v-Src-transformed cells suggested that signaling complexes could be assembling via phosphotyrosine - SH2 interactions (Mayer & Baltimore, 1993). The observation in fig. 14a that multiple tyrosine-phosphorylated proteins are detected in anti-FGFR-1 immunoprecipitates supported this hypothesis. We therefore examined for the presence of SH2-containing signaling molecules in the FGFR-1 immunoprecipitates from FGF-stimulated and v-Src-transformed NIH 3T3 cells. We looked first for the presence of PLCγ, the major signaling molecule reported to associate with FGFR-1 in response to FGF (Mohammadi et al., 1996; Burgess et al., 1990). While an increase in PLCγ levels in FGFR-1 immunoprecipitates from FGF-stimulated NIH 3T3 cells was easily detectable, we could not detect increased PLCγ in FGFR-1 immunoprecipitates from v-Src-transformed cells (fig. 15a). We also looked for phosphatidylinositol-3-kinase, Ras-GAP, Src and Grb2 in FGFR-1 immunoprecipitates from FGF-stimulated and v-Src-transformed cells. Of these SH2-containing proteins, only Grb2 was detectable in the FGFR-1 immunoprecipitates (not shown). Consistent with previous reports (Mohammadi

Fig. 15 Grb2 and Sos, but not PLCγ, co-immunoprecipitate with FGFR-1 from v-Src-transformed cells. FGFR-1 was immunoprecipitated from FGF-treated NIH 3T3 and v-Src-transformed NIH 3T3 cells as in Fig. 14a and subjected to Western blot analysis with anti-PLCγ (**a**) Grb2 (**b**) or anti-Sos (**c** and **d**) antibody. Suramin (0.2 mM) was added as shown. In (**d**), 40 μg of protein from the particulate fraction (P) was subjected to Western blot analysis using anti-Sos antibody.


a)
NIH 3T3
Nvsrc
IP:
FGFR-1
WB: PLCγ1
FGF-2 - 1h - 1h
NIH 3T3
Nvsrc
b)
WB: Grb2
IP:
FGFR-1
c)
WB: Sos
d)
P
FGF-2 - 10' 10' 1h - - 1h
suramin + +

et al., 1996), Grb2 associated very weakly with FGFR-1 in response to FGF (fig. 15b), presumably via an interaction mediated by the p90 phosphoprotein (Klint et al., 1995);, however, in v-Src-transformed cells, there was a very strong association between Grb2 and FGFR-1 that was more than 20 fold greater than that observed in response to FGF in the parental NIH 3T3 cells. The association was unaffected by either FGF or suramin (fig. 15b).

Grb2 has previously been demonstrated to provide a link between tyrosine-phosphorylated molecules and the Ras GDP/GTP exchange factor Sos (Egan et al., 1993). We therefore looked for the presence of Sos in FGFR-1 immunoprecipitates from FGF-treated and v-Src-transformed NIH 3T3 cells. In both v-Src-transformed and FGF-treated NIH 3T3 cells, Sos co-precipitated with the FGFR-1 and, as observed for Grb2, there was approximately 20 times more Sos associated with FGFR-1 in v-Src-transformed cells (fig. 15c). As with Grb2, the association was not affected by either suramin or FGF. A reduction in the electrophoretic mobility of Sos was observed upon FGF stimulation (fig. 15c and 15d). A MAP Kinase-dependent phosphorylation on Ser and Thr is known to cause a similar mobility shift of Sos that correlates with the attenuation of the Ras activation response (Buday et al., 1995; Cherniack et al., 1994). Interestingly, Sos had an intermediate mobility in v-Src transformed cells relative to that displayed in FGF-treated and untreated NIH 3T3 cells, suggesting a partially phosphorylated state. It is conceivable that an incompletely phosphorylated Sos is unable to undergo a downregulatory dissociation from FGFR-1 and therefore accumulates on the receptor. This would also explain the strong association seen between FGFR-1 and Sos.

3. Association between FGFR-1 and Sos is induced by activation of a temperature sensitive v-Src.

The observations presented in figs. 14 and 15 were further confirmed in a different cell line expressing a temperature-activatable v-Src gene (Maroney et al., 1989). Serum-starved UP1 cells mantained at the non permissive temperature (40°C), were shifted to the permissive temperature (35°C) for various time intervals. Increased tyrosine phosphorylation was detected in the FGFR-1 immunoprecipitates within 10 min after shifting to the permissive temperature for v-Src (fig. 16a). The association between FGFR-1 and Sos became evident within 30 minutes (fig. 16b). These v-Src-induced responses were not affected if the temperature shift occurred in the presence of suramin (fig. 16b). These data indicate that tyrosine phosphorylation of FGFR-1 and its association with Sos do not depend upon a specific cell type or a specific v-Src gene, and are a direct effect of the v-Src kinase activity rather than the result of an autocrine stimulation or phenotypic selection secondary to transformation.

Despite the high content in tyrosine phosphorylated proteins, UP1 cells are strongly contact-inhibited with a flat, non-transformed morphology at the

Fig. 16 Tyrosine-phosphorylated proteins and Sos associate with the FGFR-1 upon activation of a temperature-sensitive v-Src. UP1 cells, which express a temperature activatable Src gene (Maroney et al., 1989) were maintained at the non-permissive temperature for v-Src (40°C) and then shifted to the permissive temperature (35°C) for 10 and 30 min as shown. FGFR-1 immunoprecipitates were then subjected to Western blot analysis with anti-phosphotyrosine (**a**) or anti Sos (**b**) antibody.

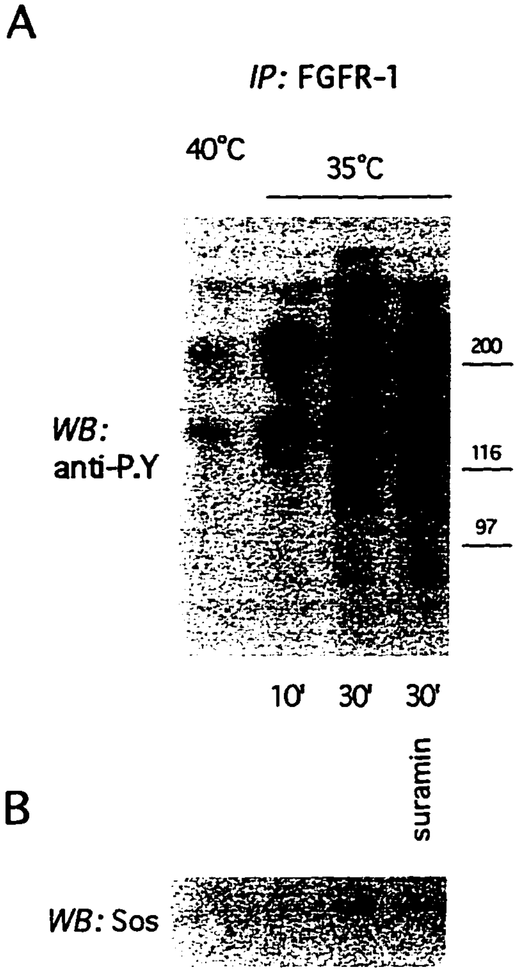
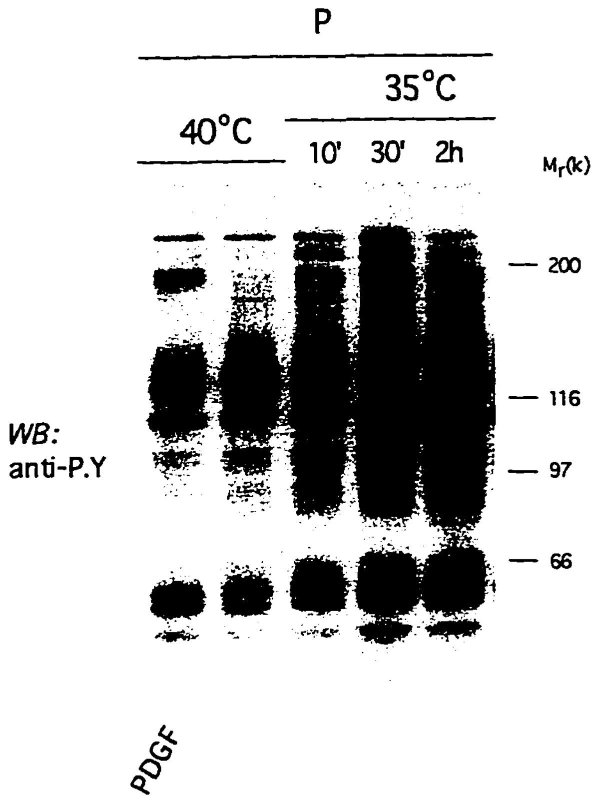

A
IP: FGFR-1
40°C
35°C
WB:
anti-P.Y
200
116
97
10'
30'
30'
suramin
B
WB: Sos

Fig. 17 High molecular weight tyrosine-phosphorylated proteins are induced upon activation of a temperature sensitive v-Src. UP1 cells maintained as in Fig. 16, were shifted to the permissive temperature for 10 min, 30 min and 2 hours, or treated with PDGF-BB (25 ng/ml) for 30 min. 40 µg/lane of protein from the particulate fraction (P) were subjected to Western blot analysis using anti-phosphotyrosine antibody.

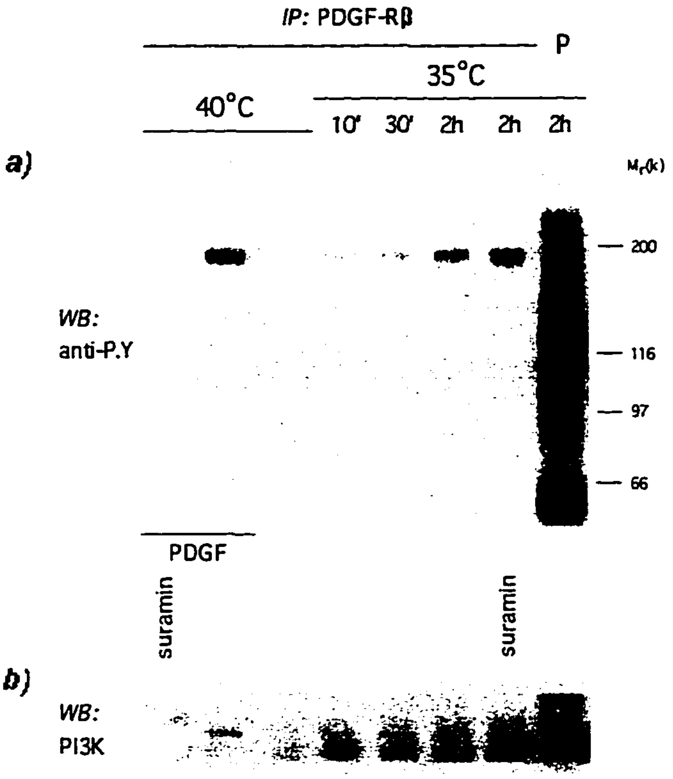

P
40°C
35°C
10'
30'
2h
Mr(k)
200
116
97
66
WB:
anti-P.Y
PDGF

non-permissive temperature (Maroney et al., 1989), consistent with the observation that the majority of the protein tyrosine phosphorylations induced by v-Src are neither necessary nor sufficient for transformation (Kozma et al., 1990; Kozma & Weber, 1990). Activation of v-Src kinase upon temperature shift, particularly increases tyrosine phosphorylation of high molecular weight proteins above 100 kDa~ in the particulate (P) fraction (fig. 17), and is followed by the appearance of a transformed phenotype (Maroney et al., 1989). In agreement with our previous transient transfection experiments, increased tyrosine phosphorylation in the same molecular weight range was only induced by the transforming Src mutants (see fig. 9). Since most RPTKs have similar molecular weights, this observations strenghthen the possibility that enhanced tyrosine phosphorylation of membrane-associated RPTKs might be a direct effect of v-Src and significantly contribute to v-Src-induced transformation.

4. PDGF-Rβ is tyrosine phosphorylated and associates with PI3K in v-Src expressing cells.

The PDGF-R is another potential target for v-Src since PDGF-induced mitogenic signaling requires Src (Roche et al., 1995), and Src binds to activated PDGF-R (Kypta et al., 1990). We therefore examined whether tyrosine phosphorylation of the PDGF-R (β isoform), could be detected following activation of the temperature-sensitive v-Src in UP1 cells. Serum-starved cells maintained at the non-permissive temperature, were stimulated with PDGF (BB homodimer) and anti-PDGF-R immunoprecipitates from the particulate fraction were subjected to Western blot analysis with an anti-phosphotyrosine antibody. A single 180 kDa phosphotyrosine band, corresponding to the mature form of the PDGF-Rβ (Keating & Williams, 1987) was induced by PDGF (fig. 18a, 19b). As expected, this effect was blocked by suramin, a polyanionic compound which also inhibits binding of PDGF to its receptor and dissociates receptor-bound PDGF (Williams et al., 1984). Temperature dependent activation of v-Src kinase resulted in increased tyrosine phosphorylation of the PDGF-R within 10 minutes, with a marked elevation at 2 hours (fig. 18a). In contrast to what was observed for the FGFR-1, no other phosphotyrosine bands co-precipitated with the PDGF-R, although several signaling molecules are known to associate with activated PDGF-R (Claesson-Welsh, 1994). One possible explanation is that the majority of the studies reporting associations between PDGF-R and its effectors, employed PDGF-R-overexpressing cells (Escobedo et al., 1988; Escobedo et al., 1991; Fantl et al., 1992; Valius & Kazlauskas., 1993; Kazlauskas et al., 1993). Nevertheless, we were able to detect the p85 non-catalytic subunit of PI3K in PDGF-R immunoprecipitates from PDGF- and v-Src-stimulated cells (fig. 18b). The time course of the association paralleled the increase in tyrosine phosphorylation of the PDGF-R and, as indicated by the two bands present in the control lane (P), specifically involved the PI3K form with decreased electrophoretic mobility

Fig. 18 PDGF-Rβ is tyrosine phosphorylated and associated with PI3K upon activation of a temperature sensitive v-Src. Up1 cells were maintained and treated as in Fig. 16. In addition, suramin (0.2 mM) was added together with PDGF, or 30 min before temperature shift. PDGF-Rβ immunoprecipitates from 0.8 mg particulate fraction (P) protein, and a control lane with 30 μg of P, were then analyzed by Western blotting using anti-phosphotyrosine (**a**), or anti-p85-PI3K antibody (**b**).


IP: PDGF-Rβ
P
35°C
40°C
10'
30'
2h
2h
2h
a)
Mr(k)
200
116
97
66
WB:
anti-P.Y
PDGF
suramin
suramin
b)
WB:
PI3K

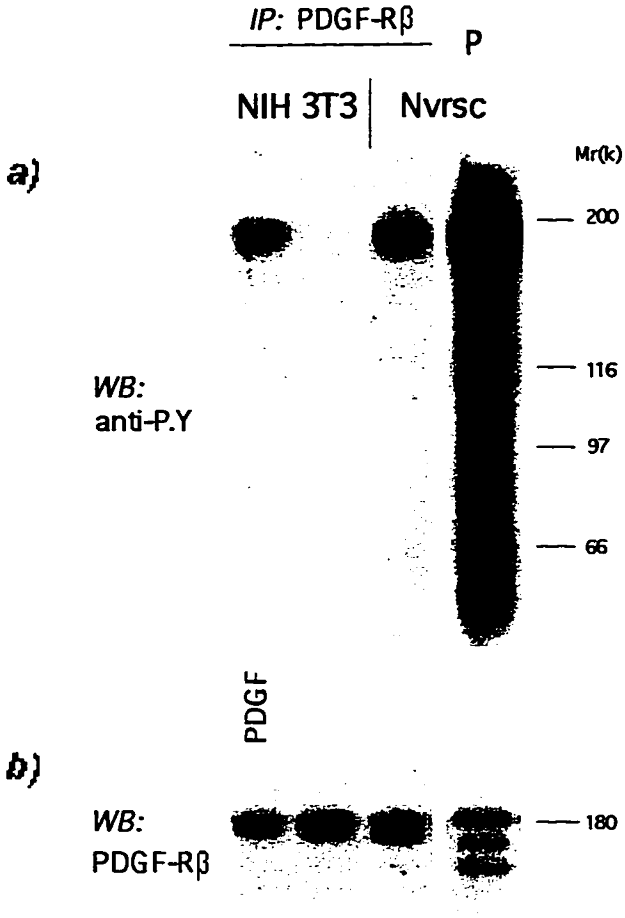
(fig. 18b), which likely represents the phosphorylated/active form of p85-PI3K (Domin et al., 1996). Addition of suramin had no effect upon either phosphorylation of the PDGF-R receptor or its binding to PI3K (fig. 18c), suggesting that v-Src-induced tyrosine phosphorylation of the PDGF-R, like the FGFR-1, occurs through an intracellular, non-autocrine mechanism. As expected, the PDGF-R was also constitutively tyrosine phosphorylated in 3T3 fibroblasts chronically transformed by v-Src (fig. 19).

These results demonstrate that the PDGF-R is another RPTK which becomes tyrosine phosphorylated in v-Src transformed cells, and suggest that this modification might cause the receptor to acquire the same signaling competence induced by the physiological ligand, since co-precipitation of PI3K and PDGF-R occurred in response to both PDGF and v-Src-induced stimulation.

DISCUSSION

In these experiments, we have shown that in v-Src transformed cells, both FGFR-1 and PDGF-Rβ are tyrosine phosphorylated in a ligand-independent manner, and associated with signaling effector molecules. Several tyrosine phosphorylated proteins co-precipitated with FGFR-1 in v-Src transformed cells as opposed to the FGF-treated parental cells (fig 14a).

Fig. 19 The PDGF-Rβ is constitutively phosphorylated in v-Src transformed NIH 3T3 cells. The PDGF-Rβ was immunoprecipitated from PDGF-treated (25 μg/ml) or untreated parental NIH 3T3, and v-Src-transformed cells (Nvsrc) as in Fig. 18, and subjected to Western blot analysis with anti-phosphotyrosine (**a**) or anti-PDGF-Rβ antibodies (**b**). Control lane: 40 μg of total protein from the particulate fraction (P).


IP: PDGF-Rβ
P
NIH 3T3
Nvrsc
a)
Mr(k)
200
116
97
66
WB:
anti-P.Y
PDGF
b)
WB:
PDGF-Rβ
180

In addition to receptor-bound proteins, these phosphotyrosine bands might represent other forms of the FGFR-1 itself. Indeed, the *fgfr-1* gene encodes for multiple alternative splice variants that include secreted and intracellular isoforms which are likely to heterodimerize in a combinatorial mode (Jaye et al., 1992; Shi et al., 1993). Moreover, FGFs and FGF receptors can function and localize in different intracellular compartments (Jaye et al., 1992; Maher, 1996; Jouanneau et 4l.,1991), allowing the possibility for a v-Src-induced intracrine activation of FGF-receptors. Although this eventuality cannot be ruled out from our experiments, the pool of intracellular FGF receptors, on the other hand, constitutes an additional potential target upon which v-Src might exert its effects.

In v-Src transformed cells, FGFR-1 tightly associated with the adaptor protein Grb2 and the Ras guanine nucleotide exchange factor Sos. Previous efforts to detect an interaction between FGFR-1 and SH2-containing proteins showed only an association with PLCγ (Mohammadi et al., 1996), which is not required for the mitogenic effects of FGF (Peters et al., 1992; Mohammadi et al., 1992). In v-Src-transformed cells, the tyrosine-phosphorylated FGFR-1 was not associated with either PLCγ or the p90 protein previously demonstrated to associate with FGFR-1 in response to FGF (Klint et al., 1995; Mohammadi et al., 1996). There was however, a very strong association of FGFR-1 with Grb2 and Sos. While we did detect an FGF-induced association between FGFR-1 and Grb2/Sos in NIH 3T3 cells that could be mediated by p90 (Mohammadi et al., 1996), the association was very weak relative to that observed in v-Src-transformed cells. The presence of Grb2/Sos and the lack of PLCγ and p90 in FGFR-1 immunoprecipitates from v-Src transformed cells suggests an alternative recruitment mechanism perhaps via phosphorylation of additional tyrosines on FGFR-1 by v-Src. This possibility is also suggested by the finding that v-Src-induced phosphorylation of the EGF receptor involves tyrosine residues that are not phosphorylated in response to EGF (Wasilienko et al., 1990), and that *in vitro* phosphorylation of EGFR by Src creates two novel high affinity binding sites for the SH2 domains of Src and PI3K (Stover et al., 1995). An altered phosphorylation pattern on FGFR-1 could also account for the apparent lack of FGF-responsiveness in v-Src-transformed cells. Possibly, the binding of several molecules at the cytoplasmic tail of FGFR-1 could oppose a steric hindrance precluding the access to the autophosphorylation sites normally induced by FGF. Alternatively, the bound molecules could act as a constraint for efficient dimerization of the extracellular FGF-binding domains. Regardless the mechanisms, normal FGF signaling seems to be abolished in v-Src transformed cells since increased tyrosine phosphorylation of the p130 and p150 FGFR-1 isoforms, the appearance of the p90 band, and the up-shift in the electrophoretic mobility of Sos, were not inducible by FGF.

Activation of Ras by Sos has been shown to occur by recruitment of Sos to tyrosine-phosphorylated receptors (Egan et al., 1993; Lowenstein et al., 1992), therefore it is likely that the strong association of Sos with FGFR-1 in v-Src-transformed cells causes substantial Ras activation. Ras is required for both multiple intracellular signals activated by v-Src (Qureshi et al., 1992) as well as v-Src-induced transformation (Smith et al., 1986). In this regard, it may be important that Grb2 and Sos have previously been demonstrated to associate with the v-Src-substrate FAK in v-Src-transformed cells (Schlaepfer et al., 1994). FAK mediates signals initiated by integrins, which may be important for cytoskeletal rearrangements and anchorage-independent growth (Richardson & Parson, 1995). However, since FAK cannot be oncogenically activated (Hildebrand et al., 1993), the Grb2/Sos complexes associated with FAK might be required to control a subcellular pool of Ras proteins that only mediates cytoskeletal rearrangements. Given the mitogenic function of the FGFR-1, its binding with Grb2/Sos appears to be the closest link demonstrated so far, between a v-Src-induced protein tyrosine phosphorylation and the activation of a Ras-dependent proliferative signal. Thus, association of Grb2/Sos with both FAK and FGFR-1 in v-Src-transformed cells may reflect the activation of multiple intracellular signals mediated by Ras leading to both anchorage independence and mitogenesis in v-Src-transformed cells.

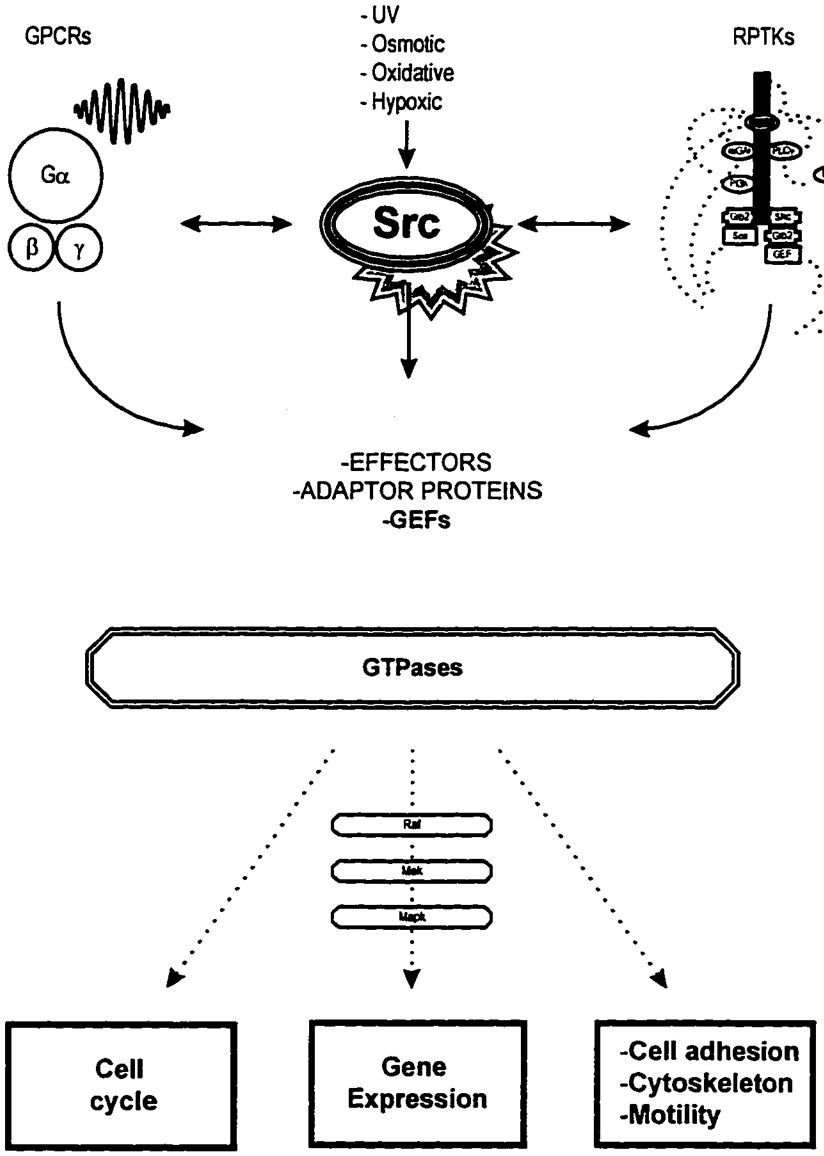
The PDGF-R was also tyrosine phosphorylated in v-Src transformed cells but, unlike the FGFR-1, no other tyrosine phosphorylated proteins were visible in PDGF-R immunoprecipitates. We did not detected Grb2, Sos or Src, but upon activation of v-Src kinase, the PDGF-R was able to co-precipitate the non-catalytic p85 subunit of PI3K in its apparently activated form (Domin et al., 1996). As expected, the same response was induced after PDGF stimulation, suggesting that v-Src can cause tyrosine phosphorylation of RPTKs also at the same autophosphorylation sites induced by the physiological ligand, in agreement to what recently reported for the IGF-1R (Peterson et al., 1996). If this is the case, v-Src-induced association of PI3K with the PDGF-R might contribute to the same PI3K-dependent effects elicited by PDGF, such as DNA synthesis, transcriptional activation of gene expression, cell motility and membrane ruffling (Claesson-Welsh, 1994). These cellular responses are also mediated by different monomeric GTPases (see fig. 13), and PI3K seems to play a major role in controlling their activation. Indeed, activated GTP-bound Ras associates with, and regulates the activity of the p110 non-catalytic subunit of PI3K (Rodriguez-Viciana et al., 1994), and the other three GTPases, Rho, Rac and Cdc42, all bind PI3K (Talias et al., 1995; Craig et al., 1996; Parson et al., 1996). All these data strongly suggest that the ligand-independent association of PI3K to the PDGF-R might significantly contribute to the transformed phenotype induced by v-Src.

In our experiments, we were unable to detect v-Src in FGFR-1 or PDGF-Rβ immunoprecipitates, consistent with the fact that no association between v-Src and RPTKs has been reported so far. This appear to be in contrast with the absolute requirement for the phosphotyrosine-binding SH2 domain of Src, and the proposed role of RPTKs' tyrosine phosphorylation in v-Src transformation. Binding of c-Src to ligand-activated PDGF-R and CSF-1R occurs transiently and with a low stoichiometry (Kypta et al., 1990; Courtneidge et al., 1993) indicating that such interaction is not a strong one; and SH2 domains appear to bind their cognate phosphopeptides with weaker affinities than generally presumed (Ladbury et al., 1995). Src and other non-receptor tyrosine kinases preferentially phosphorylate tyrosine-containing sequences recognized by their own SH2 domain (Songyang et al., 1995). In the tyrosine-rich submembrane compartment, a constitutively active v-Src would therefore generate a high local concentration of phosphotyrosines competing for binding at its SH2 domain. Since the dissociation rate of a specific SH2-phosphotyrosine interaction would consequently increase, the absence of v-Src from FGFR-1 and PDGF-R immunoprecipitates is not unexpected. Moreover, the v-Src-induced phosphorylation of RPTKs is likely to enhance their kinase activity (Peterson et al., 1994), causing a further increase in the number of tyrosine phosphorylated proteins potentially competing at the v-Src SH2 domain. In addition, this would also imply that RPTKs, and not only v-Src, contribute to the high level of tyrosine phosphorylated proteins seen in v-Src transformed cells.

Besides RPTKs, also G-protein coupled receptors (GPCRs) can be affected by Src. Different Gα subunits of heterotrimeric G-protein have been shown to be tyrosine phosphorylated in a v-Src dependent manner. (Hausdorff et al., 1992; Liu et al., 1996b). Although the significance of this modification is still unclear, a role for Src in GPCR-mediated signaling has been recently established. Mitogenic stimulation of heptahelix GPCRs for endothelin-1 (Liu et al., 1996b), angiotensin II (Schieffer et al., 1996), thrombin (Chen et al., 1994) and lysophosphatidic acid (LPA) (Luttrell et al., 1996; Dicik et al., 1996) cause activation of Src and several other effectors involved in RPTK signaling such as PI3K, PKC, PLCγ and the Ras-MAP kinase pathway (van Biesen et al., 1995). In response to endothelin-1, LPA and thrombin, also the EGF-R becomes tyrosine-phosphorylated and its activity is necessary for GPCR-induced DNA synthesis (Daub et al., 1996). Similarly, activation of the IGF1-R can be induced by thrombin (Rao et al., 1995). These findings indicate the existence of intracellular mechanisms that physiologically activate RPTKs in the absence of their own ligand. The system appear to work by-directionally since insulin and IGF-1 receptors can cause tyrosine phosphorylation of the β-adrenergic GPC Receptor (Karoor et al., 1996; Baltensperger et al., 1996). The molecular determinants of this receptor-trans-activating mechanisms are presently unknown, but Src appear a reasonable candidate for mediating a cross-talk among these signaling pathways. This possibility is further supported by the observation that following ultraviolet radiation (UV), the rapid activation of both Src and the EGF-R is required to trigger a cellular stress-response mediated by a (JNK)/MAP kinase signaling pathway (Devary et al., 1992; Sachsenmaier et al., 1994). Similar responses are induced by hyperosmotic, oxidative, hypoxic and perhaps mechanical stresses (King et al., 1989; Huang et al., 1996; Mukhopadhyay et al., 1995; Liu et al., 1996a), that might involve ligand-independent activation of multiple membrane receptors (Rosette & Karin, 1996).

Altogether, these findings can support a model (fig. 20) in which one physiological function of Src would be to promote tyrosine phosphorylation of RPTKs (and perhaps GPCRs) not only in the presence of their ligand, but also in response to stimuli originated elsewhere in the cell. Following the recruitment of SH2-containing proteins to the activated RPTK, Src could further phosphorylate other signaling substrates and modulate their activities. In cooperation with the other recruited effectors, different GEFs (guanine nucleotides exchange factors) such as Sos or Dbl, would then promote activation of Ras-type small GTPases which would ultimately determine the type of phenotypic response. In the presence of a constitutively activated v-Src, numerous RPTKs might become activated, perhaps with an abnormal set of recruited molecules, leading to the persistent activation of several signaling pathways. Their inability to undergo downregulation, would then result in the transformed phenotype.

Fig. 20 Model for Src contribution to the activation of receptor-mediated intracellular signals involved in mitogenic and adaptive responses. Different sets of transmembrane receptors become activated following ligand-dependent and independent stimuli. Ligands cause the activation of the bound receptor, as well as a Src-mediated ligand-independent cross-activation of other receptors. In the absence of ligand(s), various physical and chemical stimuli can also activate Src which, in turn, activates membrane receptors. In both cases, the output is a concerted stimulation of multiple signaling effectors and guanine nucleotide exchange factors (GEFs), that cooperate in the activation of distinct monomeric GTPases. Acting as a signal relay, GTPases activate different subcellular compartments through which mitotic and adaptive responses are mediated. In the presence of oncogenic v-Src, the constitutive, unregulatable activation of several receptors would result in the continuous mis-activation of downstream effectors and a consequent transformed phenotype.


GPCRs
Gα
β
γ
- UV
- Osmotic
- Oxidative
- Hypoxic
Src
RPTKs
PLCγ
PI3K
PKC
Grb2
Src
Sos
Grb2
GEF
-EFFECTORS
-ADAPTOR PROTEINS
-GEFs
GTPases
Raf
Mek
Mapk
Cell cycle
Gene Expression
-Cell adhesion
-Cytoskeleton
-Motility

CHAPTER IV

Methods and Materials

Plasmids

All Src-expression plasmid were variants of the pc-Src (or pM5HHB5) vector encoding wild type chicken c-Src (Kmiecik & Shalloway, 1987) (see fig. 6). Constructs encoding for the following Src proteins were obtained from Dr. D. Shalloway (Cornell University) : wild type **c-Src**; **c-Src527**, in which Tyr 527 is mutated to Phe, and **c-416/527** that carries the additional substitution of Tyr 416 to Phe (Kmiecik & Shalloway, 1987). **c-Src295** has Lys 295 mutated into Arg (Shenoy et al., 1989); **c-Src12/17** contains a double mutation that converts Ser 12 and Ser 17 into Ala, and **AAF527** has the additional Tyr-527 to Phe mutation (Yaciuk et al., 1989); **LN,** where the LN mutation that add four amino acids (MAAA) at the c-Src amino-terminus (Schuh & Brugge, 1988), was inserted into pc-Src (Bagrodia et al., 1993); **dlA** contains a deletion of aa 88 to 137 within the SH3 domain of c-Src (Seidel-Dugan et al., 1992); **dlB** carries a deletion of aa 147 to 187 within the SH2 domain of Src (Bagrodia et al., 1994); **LNdlA** and **LNdlB** carry the LN mutation in dlA and dlB respectively. Plasmids encoding for **dlA527**, **dlB527**, **LN527**, **LNdlA527** and **LNdlB527**, were constructed from the corresponding vectors described above, that carry the un-mutated Tyr 527. By cleaving them at the unique sites *SalI* (upstream of c-Src coding region) and *MluI* (codon 259), the mutation-containing fragment was inserted into the c-Src527-encoding plasmid cut at the same sites. The plasmid encoding for **c-Src295/527** was constructed by cleaving the c-Src295-encoding vector at the two *SphI* sites (upstream of the Src coding region, and at codon 518) and inserting the resulting fragment into the *Sph*I-cut vector for c-Src527. **pMv-Src** encodes the transforming Schmidt-Ruppin A *v-src* gene of Rous sarcoma virus (Johnson et al., 1985). All Src constructs were in the **pEVX** expression vector (Johnson et al., 1985; Kriegler et al., 1984). Plasmid pv-HaRas expressing an activated **HaRas** protein (Feig at al., 1986) was a gift from Dr. G.M. Cooper (Dana-Farber Cancer Institute, Boston). **pSV2neo** expresses the neomycin (G418)-resistance gene (Southern & Berg, 1982).

pTis10CRE-Luc (or Tis10s-Luc; a gift from Dr. H.Hershman, UCLA) (see fig. 7), contains the -371 to +70 promoter region of the TIS10/Pgs2 gene (Fletcher et al., 1992), cloned upstream of the luciferase gene in the pXP2 luciferase expression vector (Nordeen, 1988). v-Src-induced transactivation of the Tis10 promoter occurs via the single CRE (cyclic AMP responsive element) centered at position -60 (Xie et al., 1994). **pSREtkLuc** was contsructed by inserting the *Hind*III-*Bgl*II region of pSRETKCAT (Alexandropoulos et al., 1993), which contains a cluster of four v-Src responsive SREs (serum responsive elements) in the -425 to -250 region of the *egr-1* promoter linked to a downstream thymidine-kinase promoter from Herpes Simplex virus (HSV-TK), into the *Hind*III-*Bam*HI fragment of pXP2. **pTREtkLuc** was constructed by inserting the HindIII-BglII fragment of pTRETKCAT (Alexandropoulos et al., 1993), which contains a cassette of five TREs (TPA responsive elements) from the collagenase promoter

(Angel et al., 1987) upstream of the HSV-TK promoter, into the *Hind*III-*Bam*HI fragment of pXP2. **pCMVβgal** plasmid contains a β-galactosidase gene under control of the Cytomegalovirus promoter (gift of Dr S. Qureshi, Rockefeller U.). All Plasmid DNA were purified using the Wizard Maxiprep DNA purification system (Promega).

Cells and Cell Culture Conditions

All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% calf serum (Hyclone), 100 U/ml penicillin G, 100 ug/ml streptomycin, in a humidified, 10% CO2 atmosphere. **NIH 3T3** murine fibroblasts were obtained from the American Type Culture Collection. v-Src-transformed (**Nvsrc**), v-HaRas-transformed (**Nvras**), and LNdlA-expressing cells (**N-LNdlA**) were generated from NIH 3T3 cells as described below. **UP1** cells express the LA90 temperature sensitive strain of Rous sarcoma virus. They are non-transformed at the non-permissive 40°C temperature and transformed at 35°C (Maroney et al., 1992). Master cultures of all cell lines were grown at 37°C. Prior to addition of human recombinant FGF-2 (30 ng/ml, Life Technology), human recombinant PDGF-BB (25 ng/ml, Life Technology), suramin (0.2 mM, Calbiochem) or temperature shift, subconfluent cell cultures were placed in medium containing 1% serum for 24 h, at 37°C (all NIH 3T3-derived cells), or 40°C (UP1 cells).

Transfections

Transient transfections: for transient reporter-assays, $4x10^4$ NIH 3T3 cells were plated in triplicate 15-mm culture wells, 24 hours prior transfection. Each luciferase reporter plasmid (0.2 μg), was transfected along with a sparate Src-expression vector (0.2 μg), using the Lipofectamine (Life Technologies) method according to the manufacturer‘s protocol, keeping a Lipofectamine to DNA ratio of 10:1. After two hours incubation with the liposome:DNA complexes, cells were placed into medium containing 1% serum. After 48 hours, cells were harvested in Reporter lysis buffer (Promega), and extracts were used for determination of total protein content (BIO-RAD protein assay kit) and luciferase activity (see below). To determine the level of expression of transiently transfected Src-mutants, as well as their ability to induce tyrosine phosphorylation of cellular proteins, $10x10^4$ NIH 3T3 cells in 20-mm wells, were transfected with 1 μg of each Src-mutant encoding plasmid, using the Lipofectamine reagent. After two days, cells were harvested in Triton X 100 lysis buffer (see below) and the extracts subjected to Western blot analysis with anti-Src and anti-Phosphotyrosine antibodies (see below). Transient transfections of NIH 3T3, Nvsrc, Nvras, and N-LNdlA cell lines, were performed as above using 0.3 μg of luciferase reporter plasmid plus 0.1 μg of the pCMVβgal vector to normalize for transfection efficiency.

Establishment of cell lines: Nvsrc and Nvras transformed cell lines were generated from NIH 3T3 fibroblasts by transfecting 0.4 μg of the pMv-Src and pv-HaRas plasmids, respectively, using the same Lipofectamine-based protocol described above. After one day, cells were trypsinized, replated in two 100-mm dishes and grown for 12-14 days in complete medium until the appearance of transformed foci. Randomly selected foci were picked and expanded for further analysis. The N-LNdlA cell lines stably expressing the LNdlA mutant of Src, were obtained by co-transfecting LNdlA-encoding plasmid together with the pSV2neo encoding a neomicyn-resistance gene (Southern and Berg, 1982), at a 10:1 ratio (p LNdlA:pSV2neo). The next day, cultures were split in two 100-mm dishes and selected for 10 days in 400 µg/ml of G418 (Geneticin; Life Technologies). G418-resistant colonies were picked with cloning cylinders and expanded for further analysis.

Reporter assays

Cells were lysed in 120 µl of Reporter lysis buffer (Promega) 48 hours after transfection. Following a 30 min. incubation at room temperature, extracts were clarified by centrifugation at 10,000 x g for 5 min. The supernatant was collected and the total protein concentration was determined in a 30 µl aliquote with the Bio-Rad protein-assay system (BIO-RAD). Using a Luciferase assay kit (Promega), 6 µl of supernatant were mixed to 30 µl of luciferase reaction buffer and the luciferase activity was measured in a liquid scintillation counter (Beckman) as average single photon counts per min. (CPM) emitted within 1 min from the reaction start. Values were expressed as average CPM/μg total protein of triplicate samples.

β-galactosidase activity was measured in 70 μl of the above supernatants incubated for 30 min. with 70 μl of 2 x assay buffer [100 mM β-mercaptoethanol; 2 mM $MgCl_2$; 200 mM Na-phosphate buffer (150 mM Na_2HPO_4; 100 mM NaH_2PO_4); 1.33 mg/ml o-nitrophenyl-β-galactopyranoside]. Reactions were stopped with 500 μl of 1M Na_2CO_3 before measuring the optical density at 420 nm.

Focus formation assay

$4x10^4$ NIH 3T3 cells were plated in duplicate 15-mm wells and transfected with 0.4 μg of each Src-encoding vector using Lipofectamine reagent as described above. The next day, cells were trypsinized, replated into 60-mm dishes and grown in complete medium that was changed every 3-4 days. After 12-14 days, dishes were washed two times in PBS [pH 7.4], incubated at room temperature for 5 min. in 3 ml of staining solution (1% w/v Gentian violet in 70% ethanol), rinsed and dryed. The average number of foci per duplicate dishes was normalized to 1 μg of transfected plasmid DNA.

Antibodies

Anti-FGFR1/flg is a rabbit polyclonal antibody (pAb) directed against a carboxy terminal epitope (aa. 808-822) of the fibroblast growth factor receptor-1/*flg* (Santa Cruz Biotechnology), and was used for immunoprecipitation and western blotting. Anti-PDGF-Rβ is a rabbit pAb that recognizes residues 425 to 446 on the murine platelet derived growth factor receptor-β (Calbiochem), and was used for immunoprecipitation and western blotting. Anti-PLCγ1 rabbit pAb (Santa Cruz Biotechnology) recognizes a carboxy terminal epitope (aa. 1249-1262) of phospholipase Cγ-1, and was used for western blotting. Anti-Sos1 (Transduction Laboratories) is a mouse monoclonal antibody (Mab) against aa 1-109 of *Son of Sevenless* (Sos), the guanine nucleotide releasing factor for p21 Ras and was used for western blotting. Anti-Grb2 Mab (Transduction Laboratories) raised against a full-length growth factor receptor binding protein 2 (Grb2) was used for immunoblotting. Anti-PI3K p85 (Transduction Laboratories) Mab directed against aa. 563-724 of the 85 kDa non-catalytic regulatory subunit of phosphatidyl inositol 3-kinase, was used for immunoblotting. Anti-phosphotyrosine Mab PY20 (Transduction Laboratories) was used for immunoprecipitation and western blotting.

Cell lysis and cellular fractionation

Confluent monolayers in 150-mm culture dishes, were washed three times with ice-cold phosphate buffered saline (PBS, pH 7.4) (136 mM NaCl, 2.6 mM KCl, 4.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and scraped in 1 ml hypotonic buffer (20 mM Hepes [pH 7.4], 5mM $MgCl_2$, 1mM EDTA, 1mM EGTA,1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 5mM iodoacetic acid, 10 μg/ml each of aprotinin, pepstatin, leupeptin). Pooled samples from 3 to 6 dishes were incubated 20 min on ice, cells disrupted with 80 strokes in a Dounce glass homogenizer. The homogenate was then centrifuged at 100.000 x g for 1 h at 4°C. The particulate fraction containing nuclei and membranes, was resuspended in 1 ml Triton X 100 lysis buffer (1% Triton X-100, 50 mM Tris-Hcl [pH 7.4], 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 5 mM iodoacetic acid, 10 μg/ml each of aprotinin, pepstatin, leupeptin), homogenized in a 2 ml teflon-glass Potter homogenizer, and incubated 30 min. on ice before a second centrifugation at 100.000 x g for 1 h at 4°C. The supernatant was collected as the particulate fraction (P). Protein content in the fractions was measured using the Biorad Dc protein assay (Biorad).

Immunoprecipitation

Proteins from the particulate fraction were normalized at 1 mg/ml concentration. 0.8 mg of protein was precleared by incubating 2 h at 4°C with 35 µl protein-A agarose beads (Santa Cruz Biotechnology), with gentle rocking. Beads were removed after centrifugation at 10,000 x g for 10 min. and supernatants incubated with 3.5 µg of either anti-FGFR-1, anti-PDGF-Rβ, or 10 µg PY20 antibodies, overnight at 4°C. Antigen-antibody complexes were recovered by centrifugation after a 2 h additional incubation with 40 µl protein-A agarose beads, whashed four times in Triton lysis buffer, resuspended in 25 µl SDS-PAGE loading buffer, boiled 5 min. and stored at -70°C until used.

Western blot analysis

Immunoprecipitated samples, equal amount of proteins of total cell extracts from transient transfections (15 µg), or particulate fractions (30-40 µg), were separated through 7.5% SDS-polyacrilamide gel electrophoresis (Laemmli, 1970) and electrotransferred onto nitrocellulose filter membranes (MSI) at 250 mA overnight or 750 mA for 3 h. Membranes were incubated overnight at 4°C, or 1 h at room temperature, in blocking buffer [5% non-fat dry milk in TBST (20mM Tris-HCl [pH 7.4], 137 mM NaCl, 0.1% Tween-20), washed and incubated overnight at 4°C, or 1 h at room temperature, with 1 µg/ml of appropriate primary antibody diluted in blocking buffer. Depending on the origin of the primary antibody, filters were incubated 1 h at room temperature with either anti-mouse or anti-rabbit horse radish peroxidase conjugated IgG (Santa Cruz Biotechnology). Chemioluminescence-based detection of the antigen was performed using the Super Signal system (Pierce). When necessary, antibodies were removed from filters by 30 min incubation at 52°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris HCl [pH 6.7]), and the membranes reblotted as above. Relative levels of proteins were determined by densitometric analysis using a laser scanning densitometer (Molecular dynamics).

CHAPTER V

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