# The SH4-Unique-SH3-SH2 domains dictate specificity in signaling that differentiate c-Yes from c-Src

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## Summary

**c-Src and c-Yes are highly homologous members of the Src family of non-receptor tyrosine kinases. The overall sequence similarity between c-Src and c-Yes allows them to perform many overlapping functions. However, the phenotypes of the c-*src* and c-*yes* knockout mice, and cells derived from them, are quite different, indicating functional specificity between the two proteins. Specifically, c-*src*$^{-/-}$ cells are deficient in several processes that require dynamic regulation of the actin cytoskeleton. In order to begin to understand why c-Yes is unable to compensate for c-Src signaling, we used a series of Src/Yes chimeras in which the non-catalytic functional domains of Src$^{527F}$ were replaced by those of c-Yes. Using chicken embryo fibroblasts as a model system, our results indicate that the c-Yes N-terminal SH4-Unique domains are sufficient to inhibit the ability of Src$^{527F}$ to alter cell morphology, induce actin filament rearrangements or stimulate motility or invasive potential. The data also indicate that the SH4-Unique-SH3-SH2 domains of c-Yes work cooperatively and prevent activation of signaling proteins associated with Src$^{527F}$ transformation, including activation of phosphatidylinositol 3-kinase, phosphorylation of c-Raf and Akt and downregulation of RhoA-GTP. These data indicate that c-Yes may not modulate signals associated with c-Src-induced changes in actin filament integrity and may explain why c-Yes fails to compensate for c-Src signaling in *src*$^{-/-}$ cells.**

Key words: Yes, Src, Actin filaments, SH4 domain, Unique domain

## Introduction

The Src family of non-receptor tyrosine kinases is composed of nine members: c-Src, c-Yes, Fyn, Lyn, Lck, Hck, Blk, Fgr, and Yrk. These proteins participate in a variety of cellular signal transduction pathways, governing such diverse processes as cell division, differentiation, survival, motility, and vesicular trafficking (Thomas and Brugge, 1997). Members of the Src family are defined by their amino acid homology and functional domain architecture. All Src family members are comprised of an N-terminal membrane-localization domain (Src Homology 4 or SH4 domain), a poorly conserved Unique domain, an SH3 domain, an SH2 domain, a tyrosine kinase or SH1 domain, and a short carboxy-terminal regulatory sequence (Thomas and Brugge, 1997). c-Src and c-Yes are two of the most ubiquitously expressed and highly homologous members of the Src family (Sudol and Hanafusa, 1986; Zhao et al., 1990; Sugawara et al., 1991), sharing over 80% homology outside of their Unique domains (Kitamura et al., 1982).

Given the high homology between these two kinases and their widely overlapping tissue distributions, it is of little surprise that they are capable of performing redundant functions. c-Src and c-Yes are both activated downstream of a multitude of cell surface receptors, including receptor tyrosine kinases, G-protein-coupled receptors, and cytokine receptors (Kypta et al., 1990; Landgren et al., 1995; Fuhrer and Yang, 1996a). Additionally, both kinases are activated during the cell cycle transition from $G_2$ to M phase. Roche et al., provided strong evidence for functional overlap between c-Src, c-Yes, and Fyn, in demonstrating that inhibition of all three Src family kinases blocked cell cycle progression at the $G_2$/M transition (Roche et al., 1995). Inhibition of c-Src alone did not block cell cycle progression unless c-Src was the only Src family member expressed (Roche et al., 1995). Further evidence for functional redundancy between c-Src and c-Yes has been derived from the c-*src* and c-*yes* knockout mice. While the loss of neither gene individually is embryonic lethal, mice lacking both genes fail to survive after birth (Stein et al., 1994).

Despite the evidence for functional overlap, several studies have also indicated specificity between c-Src and c-Yes (reviewed by Summy et al., 2003). The two kinases differ in their sub-cellular localization (Sargiacomo et al., 1993), intermolecular binding partners (Fuhrer and Yang, 1996b), activation in response to cellular stimulation (Mukhopadhyay et al., 1995), and ability to mediate downstream signaling (Schieffer et al., 1996). The inactivation of the c-*src* and c-*yes* genes has provided the most compelling evidence for functional specificity. With the exception of reduced transcytosis of the polyimmunoglobulin (pIg) receptor, the c-*yes*$^{-/-}$ mice display no overt phenotype (Luton et al., 1999). However, mice lacking the c-*src* gene develop osteopetrosis due to a perturbation of osteoclast function that prevents bone resorption (Soriano et al., 1991). The defective osteoclasts are additionally unable to form membrane ruffles and actin ring structures (Boyce et al., 1992). Osteoclasts, however, are not the only cells that are affected by the loss of the c-*src* gene. Additional defects in c-*src*$^{-/-}$ cells include inefficient motility (Hall et al., 1996), decreased rates of fibroblast spreading (Kaplan et al., 1995), and neurite extension (Ignelzi, Jr et al., 1994). It is of interest to note that all of these processes are dependent on the dynamic regulation of the actin cytoskeleton. As cytoskeletal rearrangements are a hallmark of cell transformation, it is likely that c-Src and c-Yes may play divergent roles in the onset or progression of the transformed phenotype. In support of this notion, it has been observed previously that induction of mammary tumors by middle T antigen is impaired in c-*src*$^{-/-}$ cells, whereas tumor formation occurs normally in the absence of a functional c-*yes* gene (Guy et al., 1994). We have hypothesized that functional domain differences prevent c-Yes from compensating for c-Src in signaling pathways that regulate actin cytoskeletal dynamics (Summy et al., 2003).

Given the significant amino acid sequence homology between c-Src and c-Yes, it is likely that functional domain specificity may result from subtle differences in amino acid composition, and thus each functional domain may contribute to signaling specificity. Previous studies have indicated minor differences in the ligand specificity of the c-Src and c-Yes SH3 domains in vitro (Rickles et al., 1995). Data obtained in our laboratory indicate that the c-Yes SH3 domain is incapable of efficiently binding several c-Src SH3 domain binding partners, including the actin filament associated protein AFAP-110 (Summy et al., 2000). In contrast to the SH3 domain, little data exists to suggest specificity between c-Src and c-Yes, or other Src family members, at the level of the SH2 domain. However, we recently demonstrated co-immunoprecipitation of an 87 kDa tyrosine-phosphorylated protein (pp87) with Src$^{527F}$/c-Yes chimeras containing the c-Yes SH2 domain, indicating that specificity may also be derived from SH2 domain differences (Summy et al., 2000).

Several recent studies have pointed to the role of the Src family N-terminus in dictating signaling specificity between Src family kinases. All Src family members with the exception of c-Src and Blk contain one or more cysteine residues downstream of the myristoylated glycine residue at amino acid position two (Resh, 1994). These cysteine residues are sites of palmitoylation and incorporation of one or more palmitate residues facilitates localization to detergent-resistant membrane fractions, also known as lipid rafts (Resh, 1994; Robbins et al., 1995). Localization to lipid rafts is important for Src family kinase participation in Fcε receptor and T-cell receptor signaling (Kabouridis et al., 1997). Hoey et al. recently demonstrated that replacement of the Src$^{527F}$ N-terminus (including the SH4 and Unique domains) with that of c-Yes prevented upregulation of heme oxygenase 1 (HO-1) message and protein (Hoey et al., 2000). Thus it is evident that multiple functional domains may contribute to specificity in signaling, and hence function, between c-Src and c-Yes. In the present study, we have sought to gain a better understanding of signaling specificity between c-Yes and c-Src. In order to accomplish this, we have replaced the non-catalytic functional domains of Src$^{527F}$ with those of c-Yes and assessed the ability of the resulting chimeras to induce differential cellular signals, morphological and cytoskeletal changes associated with overexpression of constitutively active c-Src in chicken embryo fibroblast cells (CEF).

## Materials and Methods

### Plasmids

cDNA constructs encoding LA29 temperature-sensitive v-Src, c-Src, Src$^{527F}$, Y3$^{527F}$, Y2$^{527F}$, Y32$^{527F}$, Y4U32$^{527F}$, Y4U$^{527F}$, Y4$^{527F}$ and YU$^{527F}$ were generated within the Rous Sarcoma Virus (RSV) as described previously (Felice et al., 1990; Summy et al., 2000; Hoey et al., 2000).

### Cells

Chicken embryo fibroblasts (CEFs) were prepared as described previously from day 10 eggs (Spafas) (Flynn et al., 1993). Cells were passed 1:4 every 48 hours in Falcon 100 mm tissue culture dishes or Falcon 250 ml vented-cap tissue culture flasks. CEFs were transfected at one half confluence with 15 μg of plasmid DNA using the Clontech Calphos kit, as per the protocol. Confluent cells were viewed through a Nikon Phase Contrast II microscope at 40× total magnification (Plan 2 filter) and photographed with a Nikon N 2000 camera using Kodak TMax 400 black and white film.

### Antibodies

The rabbit polyclonal anti-Src antibody was raised against an epitope in the Src C-terminus as described previously (Summy et al., 2000). This antibody was used at a 1:1000 dilution for western blot analysis. The anti-phosphotyrosine and anti-pp85 cortactin antibodies were obtained from BD Transduction and were used at a 1:1000 dilution for western blot analysis. The rabbit anti-phospho-Y$^{416}$ was obtained from Upstate Biotechnology and used at a dilution of 1:1000. The anti-Shc antibody is a rabbit polyclonal antibody commercially available from BD Transduction; it was used at a 1:1000 dilution for western blot analysis and a 1:200 dilution for immunoprecipitation. The anti-Grb2 antibody was obtained from BD Transduction and used at a 1:5000 dilution for western blot analysis. The anti-phospho-c-Raf antibody was obtained from Biosource International; it recognizes c-Raf phosphorylated on tyrosines 340 and 341, and was used at a 1:1000 dilution for western blot analysis. The rabbit anti-c-Raf-1 antibody was obtained from BD Transduction; it was used at a 1:1000 dilution for western blot analysis. The monoclonal anti-phospho-MAP kinase antibody was obtained from Upstate Biotechnology. It is immunoreactive against Erk 1 and Erk 2 phosphorylated at the pT-E-pY motif and was used at a dilution of 1:1000 for western blot analysis. The monoclonal anti-MAP kinase antibody was obtained from Upstate Biotechnology. This antibody recognizes Erk 1 and 2 and was used at a 1:1000 dilution for western blot analysis. The rabbit anti-phospho-Akt antibody was obtained from Cell Signaling. This antibody recognizes Akt-1 phosphorylated at serine 473 and was used at a 1:1000 dilution for western blot analysis. The monoclonal anti-Akt antibody was obtained from BD Transduction and used at a 1:500 dilution for western blot analysis. The rabbit anti-rat PI3K p85 antibody was obtained from Upstate Biotechnology and used at a dilution of 1:1000 for western blot analysis and 1:50 for immunoprecipitation. Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Life Science and used at a dilution of 1:1000.

### Immunofluorescence

Immunofluorescence was carried out as described previously (Qian et al., 1998). Briefly, CEF were split onto coverslips and fixed in 3.7% formaldehyde at 50% confluence. The cells were washed three times in PBS, permeablized in 0.4% Triton X-100, washed, and stained with rhodamine-conjugated phalloidin (2 μg/μl in 5% BSA/PBS). Cells were stained for 20 minutes, washed three times in PBS, and mounted on coverslips using Flouromount G (Southern Biotechnology Associates). Cells were visualized using a Zeiss LSM 510 confocal microscope (63× objective).

### Western blot analysis

Cells were lysed at confluence in RIPA buffer as described previously (Hoey et al., 2000). Cell lysates were quantitated for total protein content using the Pierce BCA assay as per the protocol. Thirty or fifty μg of cell lysates were boiled in Laemmli's sample buffer (LSB) and resolved by 8% SDS-PAGE. The proteins were transferred to PVDF membrane and washed as described previously (Hoey et al., 2000). The membranes were blocked overnight in 1% BSA formulated in Tris buffered saline with 1% Tween 20 (TBS-T) at 4°C or 5% nonfat milk in TBS-T for 30 minutes at room temperature. The membranes were probed with primary antibody (diluted in TBS-T) for 1 hour at room temperature or overnight in 5% nonfat milk/TBS-T at 4°C. Secondary antibodies were applied for 45 minutes in TBS-T. Bound antibodies were visualized by incubation with ECL reagents (Amersham Pharmacia), followed by X-ray film (Kodak) exposure (Hoey et al., 2000).

### Immunoprecipitation

Five hundred μg of RIPA lysates were incubated with antibody for 1.5 hours at 4°C with rotating. Twenty μl of Protein A/G agarose (Santa Cruz) was added for an additional 1.5 hours at 4°C with rotation. The beads were centrifuged for 30 seconds at room temperature, followed by two washes in RIPA and two washes in TBS. Bound proteins were eluted by boiling in LSB and resolved by SDS-PAGE. Western blot analysis was carried out as described above.

### Cell fractionation

Cells were separated into Triton X-100 soluble (C) and insoluble (R) fractions as described previously (Hamaguchi and Hanafusa, 1987). The insoluble (R) fraction contains the cytoskeletal associated cellular proteins. Briefly, cells were washed twice with cold Tris-buffered saline. Cells were then incubated with 1 ml of cold CSK buffer (10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 1% Triton X-100) on ice with gentle rocking for 1, 4 or 10 minutes. The soluble material was removed, and the remaining material was solubilized in 1 ml of RIPA buffer as described previously. Lysates were clarified by 5 minutes centrifugation at 13,100 ***g*** and 4°C in a Hermle Z 360 K bench-top centrifuge, and supernatants were quantitated for total protein content. Samples were prepared for western blot analysis as described above. Triton-soluble fractions were equilibrated to RIPA by addition of 1% sodium deoxycholate and 10 mM Tris-HCl, pH 8.0, prior to protein quantitation.

### Phosphatidylinositol 3-kinase and RhoA-GTP binding assays

The phosphatidylinositol 3-kinase (PI3K) assay was performed as described elsewhere (Jiang et al., 1998). Briefly, CEF were scraped from tissue culture flasks in cold PBS and pelleted at 1000 rpm and 4°C for 5 minutes in a Sorvall RT6000B bench-top centrifuge. Cells were then lysed and total protein concentration was determined. Four hundred μg of lysate were pre-cleared with 20 μl of Protein A/G agarose for 1 hour at 4°C. Lysates were immunoprecipitated with 2 μl of anti-PI3K antibody for 1 hour at 4°C. Twenty-five μl of protein A/G agarose beads were added and incubated with rotation for an additional 1 hour at 4°C. The immunoprecipitations were washed with TNE buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA), centrifuged, and the pellet was then resuspended in 50 μl of PI3K assay buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.2 mg/ml phosphoinositol, 60 μM ATP, 2 μCi [$\gamma$-$^{32}$P] ATP). The reactions were incubated at room temperature for 15 minutes. Following the reaction, the products were extracted by addition of 80 μl 1 M HCl and 160 μl chloroform/methanol. The upper phase was removed, and the lower phase, containing the lipid products, was dried. The pellets were then resuspended in chloroform and spotted on thin layer chromatography (TLC) plates. Once the buffer front had migrated to the top of the plate, the plates were removed, dried, and exposed via phosphorimaging.

Rho activation assays were conducted as described (Edlund et al., 2002). Briefly, the cells were washed with 1× PBS supplemented with 1 mM $MgCl_2$. After the washing, the cells were lysed immediately with lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin and 1 mM PMSF). The lysates were centrifuged at 18,400 ***g*** for 15 minutes. The supernatants were added to GST-rhotekin in GST beads to pull-down Rho proteins, followed by incubation at 4°C for 20 minutes. After the incubation, the beads were washed twice with cold wash buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin, and 0.1 mM PMSF). The Rho protein was eluted with sample buffer and subjected to 15% SDS-PAGE. The western blot analysis was performed using anti-Rho polyclonal antibody. Both ECL cell attachment matrix and anti-Rho antibody are from Upstate Bio. GST-rhotekin is a kind gift from Pontus Aspenström (Ludwig Institute for Cancer Research, Uppsala, Sweden).

### Transwell migration assay

Transwell migration assays were conducted using modifications of the method described by the manufacturer (BD Biosciences). Briefly, the cells were serum-starved overnight. The transwells were coated with E-C-L cell attachment matrix (Upstate Biotechnology) at 20 μg/ml and incubated for one hour at 37°C. The top chambers of the transwell were loaded with $4\times10^5$ cells/ml in 0.5% serum DMEM media and the bottom chambers were filled with 5% FCS DMEM media. The 5% FCS served as an attractant for the cells. The transwells were incubated in 0.5% $CO_2$ at 37°C for 16-18 hours. After the incubation, the cells that had migrated were fixed with 10% formalin, stained with Harris Modified Fisher Hematoxylin (Fisher Co.), and mounted on slides. Cells were also preincubated for 2 hours with 20 μM LY294002 prior to the migration assay, to determine the effects of inhibiting PI3K upon migration. The images were taken using an Olympus inverted microscope, and represent the typical fields per each sample. Cell numbers were counted, or estimated, depending upon the degree of cell clumping, within 10× fields of magnification in order to determine the numbers of cells that migrate. Treatment with the PI3K inhibitor, LY294002, was performed using a 20 μM concentration for 22 hours prior to analysis of cells for motility or invasion. After 22 hours of treatment, cells are routinely analyzed for survival after removal of the drug. Cell survival was noted with little evidence of cell death. Cell migration assays were conducted and quantified as described (Huack et al., 2002). Briefly, the cells were serum-starved overnight, the transwells were coated with ECL cell attachment matrix (Upstate Biotechnology) at 20 μg/ml. The top chamber of transwell was loaded with 0.2 ml of $4\times10^5$ cells/ml in serum-free media and the bottom chamber with 0.6 ml of DMEM medium containing 0.5% FCS. The cells were incubated in the transwells at 37°C in 5% $CO_2$ for 14 hours. Migrated cells were fixed, stained with 0.1% crystal violet. Washing five times with 1× PBS removes extraneous, unbound crystal violet, leaving bound crystal violet associated with only migrated cells. Following washing of the migrated cells, elution of bound crystal violet with 10% acetic acid enables quantification of the relative levels of cell-associated dye by spectrophotometry. The microplate reader was used to measure the OD of the eluted solutions to determine the migration values. The mean values were obtained from three individual experiments and were subjected to a *t*-test ($P<0.01$, $n=3$).

### Invasion assay

Invasion assays were performed according to manufacturer's protocol (BD Biosciences). The cells were serum-starved overnight. Cells (0.5 ml of $1.0\times10^5$ cells/ml) were loaded on pre-coated matrigel 24-well invasion chamber (BD Biosciences). 0.5 ml of 5% FCS DMEM media was added to the wells of the BD Falcon TC Companion plate to serve as the chemoattractant for the cells. The matrigel invasion chambers were incubated in 0.5% $CO_2$ at 37°C for 22 hours. Cells were also preincubated for 2 hours with 20 μM LY294002 prior to the migration assay, to determine the effects of inhibiting PI3K upon migration. After the incubation, the invading cells were fixed with 10% formalin, stained with Harris Modified Fisher Hematoxylin (Fisher Co.), and mounted onto slides. The invading cells were counted and analyzed according to manufacturer's instruction.

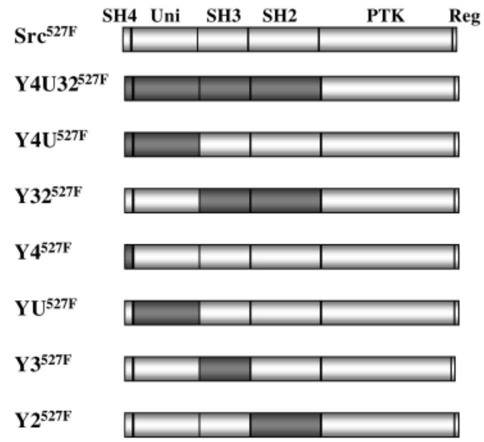


**Fig. 1.** Src$^{527F}$/c-Yes chimeric constructs. Src$^{527F}$/c-Yes chimeras are depicted subdivided into the following functional domains: SH4 domain; Unique domain; SH3 domain; SH2 domain; protein tyrosine kinase (PTK) domain; and regulatory sequence (Reg). Gray boxes represent domains from c-Yes; white boxes represent domains from Src$^{527F}$. The generation of the Y3$^{527F}$, Y2$^{527F}$, Y32$^{527F}$, Y4U32$^{527F}$, Y4U$^{527F}$, Y4$^{527F}$ and YU$^{527F}$ chimeric constructs has been described previously (Summy et al., 2000). All proteins were expressed in CEF via the Rous Sarcoma Virus (RSV) vector.

## Results

### Expression of Src$^{527F}$/c-Yes chimeric constructs in CEF

Chicken embryo fibroblasts were transfected at 50% confluence with RSV constructs expressing Src$^{527F}$, or Src$^{527F}$/c-Yes chimeric constructs, where Src$^{527F}$ contained the c-Yes SH4-Unique-SH3-SH2 domains (Y4U32$^{527F}$), the SH4-Unique domains (Y4U$^{527F}$), the c-Yes SH3 and SH2 domains (Y32$^{527F}$), SH4 domain (Y4$^{527F}$), Unique domain (YU$^{527F}$), SH3 domain (Y3$^{527F}$) or the c-Yes SH2 domain (Y2$^{527F}$) (Fig. 1). The temperature-sensitive v-Src variant, LA29 was used as an additional positive control, and is well known to affect upregulation of tyrosine phosphorylation and changes in the actin cytoskeleton at permissive temperature, 35°C (van der Valk et al., 1987; Felice et al., 1990). After 12 days, cells were lysed in RIPA, 30 μg of cell lysates were resolved by SDS-PAGE, and expression of tyrosine phosphorylated proteins verified by western blot analysis using an anti-phosphotyrosine antibody. As seen in Fig. 2A, all constructs generated an overall increase in cellular tyrosine phosphorylation. A light exposure is shown in order to analyze the cells for changes in the profile and content of phosphotyrosine-containing proteins that might correlate with the functional domains present in each chimera (Fig. 2A). Although most protein bands revealed equivalent steady-state levels of phosphotyrosine content when comparing all constructs (e.g. at 75 kDa), there was some evidence for reduced tyrosine phosphorylation in the Y4U32$^{527F}$-expressing cells. These include a reduction in phosphorylation of 190 kDa, 180 kDa and 65 kDa proteins, while increased phosphorylation was seen in a 47 kDa protein. To ensure equal protein loading, cell lysates were probed with anti-pp85 cortactin, which revealed equivalent levels of pp85 cortactin among each sample (Fig. 2B). Identical results were obtained by western blot analysis of cell lysates for pp130cas or pp125FAK (data not shown). Analysis of Src construct expression levels indicate some minor differences in expression levels (Fig. 2C). Western blot analysis of Src levels indicated that Src$^{527F}$, Y3$^{527F}$ and Y4U32$^{527F}$ were expressed at comparable levels. Y2$^{527F}$ and Y32$^{527F}$ were expressed at relatively higher levels than Src$^{527F}$, while Y4U$^{527F}$ was expressed at relatively lower levels. The temperature-sensitive vSrc variant, LA29, is always expressed at very low levels (J.M.S. and D.C.F., unpublished) and was used as a control to correlate expression with changes in cellular phosphotyrosine content. Western blot analysis with anti-phosphoY416 revealed that each of the chimeric constructs, in addition to Src$^{527F}$, demonstrated increased phosphorylation at Tyr416, a signature for activation (Fig. 2C). Src$^{527F}$, Y3$^{527F}$ and Y2$^{527F}$ display similar levels of autophosphorylation, while Y32$^{527F}$, Y4U$^{527F}$ and Y4U32$^{527F}$ appear to have higher steady state levels of autophosphorylation. Thus, each of these constructs can be expressed in CEF cells and are activated based on increased autophosphorylation and increased cellular tyrosine phosphorylation. These data also indicate that although there may be minor differences in expression levels, each of the chimeric constructs direct upregulation of cellular tyrosine phosphorylation levels to equivalent levels.

### The Y4U32$^{527F}$ and Y4U$^{527F}$ chimeras do not induce morphological changes or rearrangement of cytoskeletal actin

We hypothesized that differences in one or more of the functional domains were responsible for the inability of c-Yes to compensate for c-Src in regulation of cellular pathways controlling actin cytoskeletal dynamics. We chose chicken embryo fibroblast cells (CEFs) as a model system for these studies, as CEFs have a well-defined system of actin filament stress fibers that undergo a characteristic rearrangement upon overexpression of constitutively active Src (Reynolds et al., 1989). Transfected cells were evaluated for the Src-transformed phenotype, the hallmarks of which include a rounded morphology, the lack of a well-organized monolayer, and an absence of contact-dependent inhibition of cell growth. As seen in Fig. 3A, the Y4U32$^{527F}$ and Y4U$^{527F}$ chimeras

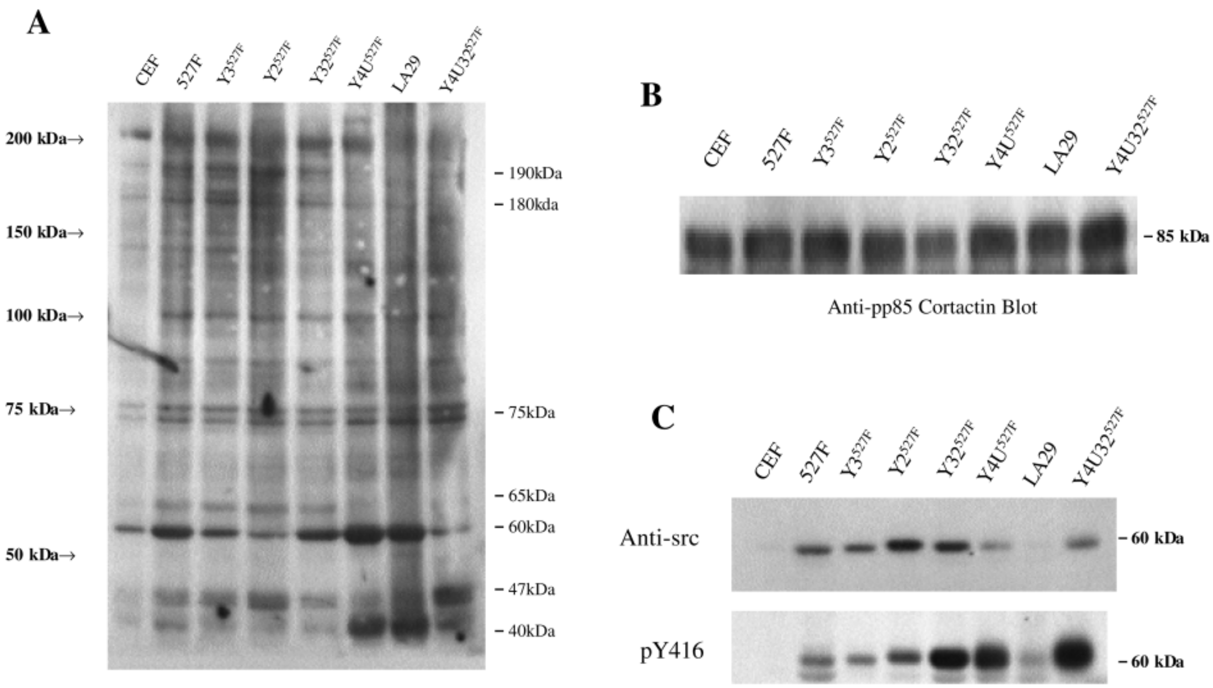


**Fig. 2.** Effects of Src$^{527F}$/c-Yes chimeras on cellular phosphotyrosine. cell morphology, and the actin cytoskeleton. (A) 30 μg of day 12 RIPA lysates from mock-transfected CEF or cells expressing Src$^{527F}$, LA29, or the chimeric constructs were separated by 8% SDS-PAGE. Lysates were transferred to PVDF membrane, and probed with a rabbit anti-phosphotyrosine antibody Molecular weight markers are shown on the left side of the figure. Protein bands of note are highlighted by their $M_r$, on the right side of the figure. (B) 50 μg of cell lysate (as used in Fig. 2A) was resolved by 8% SDS-PAGE, followed by western transfer and probed with the anti-pp85 cortactin antibody. (C) By using the same lysates as in Fig. 2A, 30 μg of lysate was resolved by 8% SDS-PAGE and western blot analysis performed with rabbit anti-src antibodies to quantify the steady state levels of Src and the chimeric constructs (top panel), or with anti-phosphoY416 to detect the activation state of the Src or chimeric constructs.

failed to induce changes in cell morphology, as the morphology of these cells was difficult to distinguish from that of mock-transfected cells. Y32$^{527F}$, however, induced morphological changes similar to those induced by Src$^{527F}$ (Fig. 3A), as did Y3$^{527F}$ and Y2$^{527F}$ (data not shown). As the inability to induce radical changes in cell morphology were associated primarily with the c-Yes N-terminal SH4 and Unique domains, Y4$^{527F}$ and YU$^{527F}$ chimeras were generated in an attempt to determine whether these affects could be attributed primarily to either the c-Yes SH4 or Unique domains individually. Upon expression of these constructs in cultured fibroblasts, it was observed that Y4$^{527F}$ and YU$^{527F}$ displayed more evidence for disorganization in monolayer than CEFs. Expression levels of the Y4$^{527F}$ and YU$^{527F}$ chimeras were equivalent to those of Src$^{527F}$ (data not shown). These data indicate that the c-Yes SH4-Unique-SH3-SH2 domain, collectively, do not permit Y4U32$^{527F}$ to induce changes in cell morphology or to affect the organization of the monolayer. Further, the SH4-Unique domains alone appear to play a major role in preventing these changes, and these domains appear to all work cooperatively.

The change in cell morphology induced by constitutively active Src$^{527F}$ occurs concomitantly with rearrangements of the actin cytoskeletal structure. Src$^{527F}$-transformed cells display a loss of actin stress fibers and focal adhesions, with the actin repositioning into rosettes, lamellipodia and filopodia. In order to determine the effects of the c-Yes functional domains on the ability of Src$^{527F}$ to exert its influence on the actin-based cytoskeleton, cells were fixed on coverslips, stained with rhodamine-conjugated phalloidin, and visualized via confocal laser microscopy. As can be seen in Fig. 3B, cells expressing Src$^{527F}$ were morphologically distinct, and actin staining was detected in punctate rosette structures along the cell periphery or in actin-based motility structures such as lamellipodia or filopodia. Similar actin staining was also detected in cells expressing Y32$^{527F}$, Y4$^{527F}$ and YU$^{527F}$. However, Y4U$^{527F}$ and Y4U32$^{527F}$ appeared to have contiguous, well-formed actin filaments (Fig. 3B). These data indicate that the cYes SH4-Unique-SH3-SH2 domains do not support changes in the actin based cytoskeleton associated with the activated tyrosine kinase and that the presence of the c-Yes SH4-Unique domains is largely responsible for preventing these changes in actin filament organization, which correlates well with changes in cell morphology.

## Src$^{527F}$/c-Yes N-terminal chimeras are associated with the Triton X-100 insoluble cytoskeletal fraction

To investigate the mechanisms associated with the inability of Y4U32$^{527F}$ and Y4U$^{527F}$ to alter actin filament integrity, we next examined the subcellular distribution of these chimeric proteins. One of the hallmarks of transformation-competent variants of Src is that they are associated with cellular membranes via their SH4 domains. Fractionation of cells into membrane and cytosolic preparations revealed that Src$^{527F}$ and

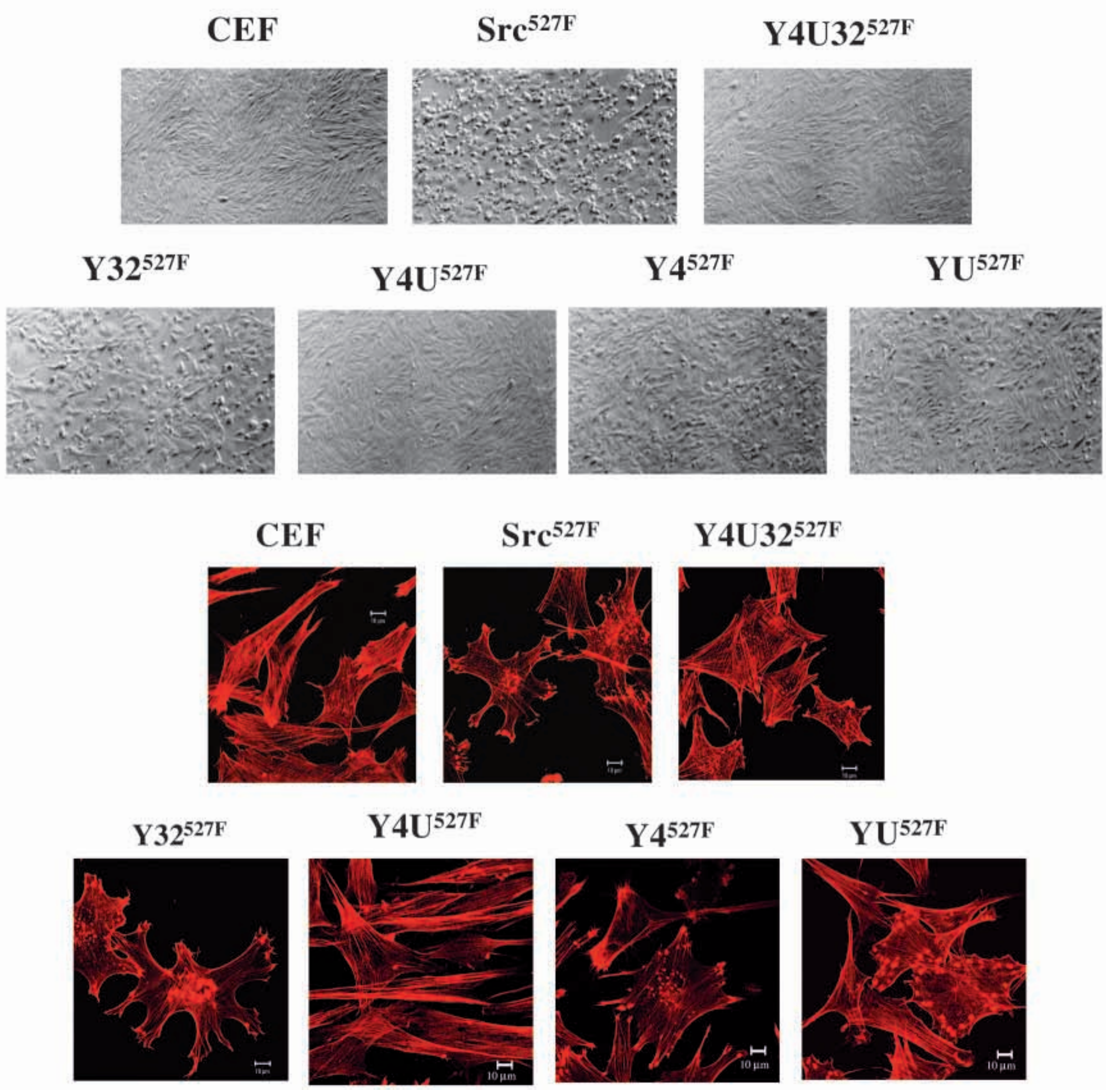


**Fig. 3.** Expression of Src$^{527F}$/c-Yes N-terminal chimeras in CEF and their effects on cell morphology and the actin cytoskeleton. (A) The SH4-Unique domains of c-Yes prevent Src$^{527F}$ from affecting cell morphology. Confluent CEF that were uninfected, or expressing Y4U32$^{527F}$, Y32$^{527F}$ Y4U$^{527F}$, Y4$^{527F}$ or YU$^{527F}$ were photographed at day 12 post-transfection in 100 mm tissue culture plates (Falcon). Cells were photographed at 40× total magnification using a Plan 2 filter. (B) The SH4-Unique domains of c-Yes prevent Src$^{527F}$ from affecting actin filament integrity. CEF expressing Y4U32$^{527F}$, Y32$^{527F}$ Y4U$^{527F}$, Y4$^{527F}$ or YU$^{527F}$ were fixed at 50% confluence on coverslips in 3.7% formaldehyde, permeablized in 0.4% Triton X-100, and stained with rhodamine-phalloidin (2 µg/ml). Cells were visualized via Zeiss LSM 510 confocal microscopy (63× objective). Bars, 10 µm.

all Src$^{527F}$/c-Yes chimeras were associated predominantly with the membrane fraction (data not shown). Thus the inability of Y4U32$^{527F}$ to induce cytoskeletal rearrangements was not due to an inability to localize with cellular membranes. Another feature of transformation-competent variants of Src is that they associate predominantly with the Triton X-100 insoluble cytoskeletal fraction, whereas endogenous c-Src is primarily Triton-soluble (Hamaguchi and Hanafusa, 1987). Thus, in order to determine whether Src$^{527F}$/c-Yes N-terminal chimeras were able to associate with the Triton-insoluble fraction, mock-transfected CEF or cells expressing c-Src, Src$^{527F}$, Y4U32$^{527F}$, or Y4U$^{527F}$ were separated into Triton-soluble and Triton-insoluble fractions. Both endogenous c-Src from mock-transfected cells and overexpressed c-Src displayed a shift in solubility from the Triton insoluble fraction to the Triton soluble fraction after four minutes of incubation in CSK buffer, whereas Src$^{527F}$ and Y4U32$^{527F}$ remained predominantly associated with the Triton-insoluble fraction, even after 10 minutes of incubation (Fig. 4). Similar results were obtained with Y4U$^{527F}$ (data not shown). These results indicate that the inability of Y4U32$^{527F}$ to effect actin redistribution does not correlate with an inability of the protein to partition into the Triton-insoluble cytoskeletal fraction.

## The c-Yes N-terminal region does not prevent activation of the MAP kinase pathway

The inability of Src$^{527F}$/c-Yes N-terminal chimeras to induce rearrangement of the actin cytoskeleton may be attributed to failure to activate one or more downstream pathways that are

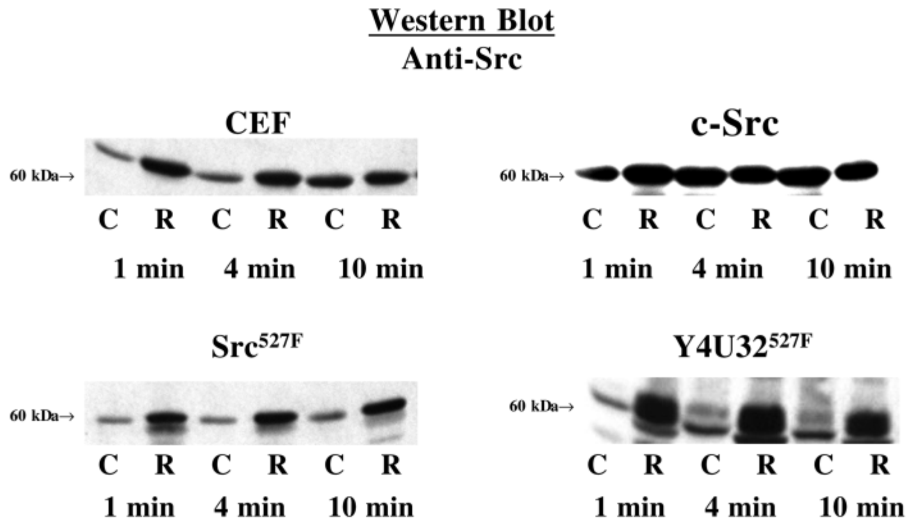


**Fig. 4.** Y4U32$^{527F}$ is associated with the Triton X-100 insoluble cytoskeletal fraction. Mock-transfected CEF or cells expressing c-Src, Src$^{527F}$ or Y4U32$^{527F}$ were grown to confluence in 100 mm dishes. Cells were incubated in 1 ml of CSK buffer for 1, 4 or 10 minutes. The Triton-soluble fractions were collected, and the Triton-insoluble material at each time-point was solublized in RIPA buffer. 50 μg of cell lysates from each fraction were separated by 8% SDS-PAGE, transferred to PVDF membrane, blocked with 5% nonfat milk/TBS-T, and probed with rabbit anti-Src. Results are shown for mock-transfected CEF, c-Src, Src$^{527F}$ and Y4U32$^{527F}$. C, CSK buffer; R, RIPA buffer. Cytoskeletal-associated proteins are defined in the R fraction.

normally upregulated in response to oncogenic Src$^{527F}$. Thus, we next investigated the activation status of signaling proteins that function downstream of Src$^{527F}$. The proteins chosen in this study can broadly be classified into two distinct yet overlapping categories: those that are involved in the mitogenic response to Src$^{527F}$ activation and those that are associated with the effects of Src on the actin-based cytoskeleton.

The MAP kinase pathway is one of the most well-characterized pathways activated in response to Src and is involved in the induction of a mitogenic response (Gupta et al., 1992). One of the first steps in the activation of the MAP kinase pathway downstream of Src$^{527F}$ is the formation of stable complex between Shc and Grb2 (Rozakis-Adcock et al., 1992). Thus in order to assess the effects of the c-Yes N-terminus on the ability of Src to induce activation of the MAP kinase pathway, we first assayed complex formation between Shc and Grb2. CEF lysates were immunoprecipitated with a rabbit anti-Shc antibody and the immunoprecipitates were resolved by SDS-PAGE. Western blot analysis was performed using an anti-Grb2 monoclonal antibody. Blots were stripped and re-probed with the anti-Shc antibody to demonstrate that equal amounts of Shc were immunoprecipitated (Fig. 5A, bottom panel). Minimal complex formation was detected between Shc and Grb2 in lysates from mock-transfected cells. Robust complex formation was detected between Shc and Grb2 in Src$^{527F}$-transformed cells (Fig. 5A, top panel). Cells expressing Y4U32$^{527F}$ or Y4U$^{527F}$ displayed increased Shc/Grb2 complex formation over mock-transfected CEF (approximately 2.5-fold); however, levels were not quite as high as in Src$^{527F}$-expressing cells. Similar results were obtained when

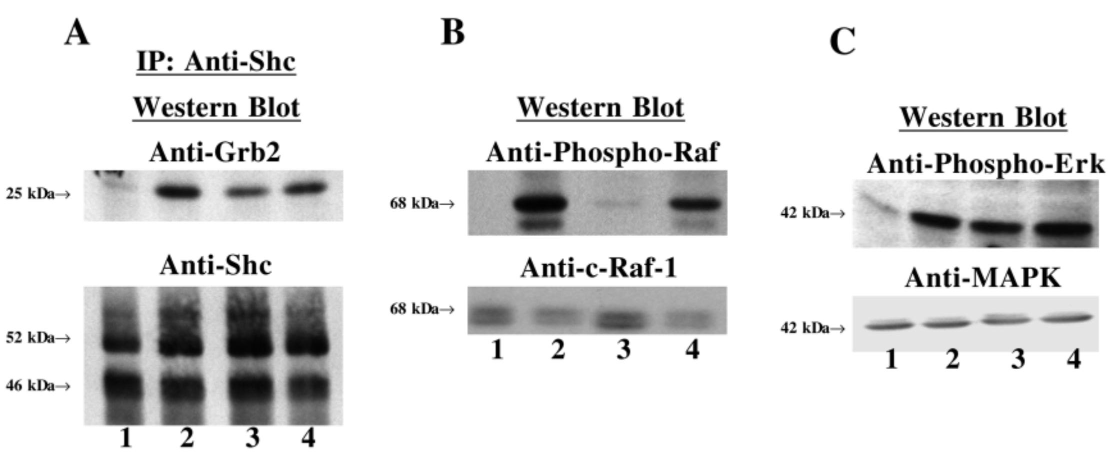


**Fig. 5.** Activation of the MAP kinase pathway by Src$^{527F}$ and Src$^{527F}$/c-Yes chimeras. (A) 500 μg of RIPA lysates from mock-transfected CEF or cells expressing Src$^{527F}$, Y4U32$^{527F}$ or Y4U$^{527F}$ were immunoprecipitated with an anti-Shc antibody and separated by 10% SDS PAGE. Immunoprecipitated proteins were transferred to PVDF membrane, blocked in 5% nonfat milk/TBS-T and probed with an anti-Grb2 antibody (top panel). The blots were stripped and re-probed with the anti-Shc antibody, immunoreactive against the 46 and 52 kDa isoforms of Shc (bottom panel). (B) 50 μg of RIPA lysates from mock-transfected CEF or cells expressing Src$^{527F}$, Y4U32$^{527F}$, or Y4U$^{527F}$ were resolved by 8% SDS-PAGE, transferred to PVDF membrane, blocked in 5% nonfat milk/TBS-T and probed with an anti-phospho-Raf antibody (top panel). Western blot analysis was also performed with an anti-c-Raf antibody to determine relative protein levels (bottom panel). (C) 50 μg of RIPA lysates from mock-transfected CEF or cells expressing Src$^{527F}$, Y4U32$^{527F}$ or Y4U$^{527F}$ were processed for western blot analysis as described above. Membranes were probed with an anti-phospho-Erk antibody (top panel) or an anti-p42/44 MAPK antibody to determine relative protein levels (bottom panel). (A-C) Lane 1, CEF; lane 2, Src$^{527F}$; lane 3, Y4U32$^{527F}$; lane 4, Y4U$^{527F}$.

immunoprecipitating with the anti-Grb2 antibody and probing with the anti-Shc antibody (data not shown). These data indicate that the c-Yes N-terminus alone is not sufficient to abrogate Src-induced Grb2/Shc complex formation.

We next looked further downstream at c-Raf activation. c-Raf signaling was assessed by western blot analysis using an antibody against the phosphorylated form of c-Raf, which recognizes a phosphorylation site ($Y^{340/341}$) that appears to be regulated in a Src-dependent manner (Diaz et al., 1997). CEF lysates from mock-transfected cells, or cells expressing $Src^{527F}$, $Y4U32^{527F}$ or $Y4U^{527F}$ were separated by SDS-PAGE, and western blots were probed with the anti-phospho-Raf antibody. As seen in Fig. 5B, no phosphorylation of $Y^{340/341}$ on c-Raf was detected in mock-transfected cells; however, lysates from cells expressing $Src^{527F}$ were strongly immunoreactive with the anti-phospho-Raf antibody (Fig. 5B, top panel). $Y4U32^{527F}$ lysates were weakly immunoreactive with the anti-phospho-Raf antibody in comparison to $Src^{527F}$; however, $Y4U^{527F}$-induced c-Raf phosphorylation more closely approximated levels induced by $Src^{527F}$ (Fig. 5B, top panel). Blots were additionally probed with an anti-c-Raf antibody in order to determine relative levels of c-Raf present in the cell lysates (Fig. 5B, bottom panel). The markedly lower levels of phospho-c-Raf present in $Y4U32^{527F}$ lysates were somewhat surprising and indicate that the SH3-SH2 domains, or a combination of the SH4-Unique-SH3-SH2 domains of cYes do not support activation of c-Raf.

We next assessed the effects of the c-Yes N-terminus on the ability of Src to induce MAP kinase (MAPK) activation. In order to evaluate MAPK activation, western blot analysis was performed on lysates from mock-transfected CEF or cells expressing $Src^{527F}$, $Y4U32^{527F}$ or $Y4U^{527F}$ using an anti-phospho-Erk antibody. As seen in Fig. 5C, $Src^{527F}$, $Y4U32^{527F}$ and $Y4U^{527F}$ each induced significant Erk activation above levels detected in mock-transfected cells (Fig. 5C, top panel). Western blots were additionally probed with an anti-MAPK antibody, specific for Erk 1/2, to ensure that equal levels of protein were present in the lysates (Fig. 5C, bottom panel). These results indicate that the presence of the c-Yes N-terminus is insufficient to abrogate Erk activation downstream of $Src^{527F}$. These data indicate that the SH3-SH2 domains of c-Yes can enable formation of the Shc/Grb2 complex and activation of Erk1/2. However, specificity in signaling by the SH3-SH2 domains is evident in that $Y4U32^{527F}$ was unable to direct increased phosphorylation of c-Raf at $Tyr^{340/341}$. Thus the inability of chimeric proteins with the c-Yes N-terminus to induce rearrangement of the actin cytoskeleton and changes in cellular morphology do not correlate with an inability to activate the MAPK pathway. Further, these data indicate that either $Y4U32^{527F}$ can activate Map kinase signaling in a c-Raf-independent manner or that only low levels of c-Raf activation are required to achieve subsequent MAPK activation.

## Differential signaling to the PI3K/Akt and RhoA pathway

While many proteins are known to be important for Src-mediated actin filament rearrangement, the precise pathway that regulates this process has not been defined. Phosphatidylinositol 3-kinase (PI3K), a lipid and protein kinase named for its ability to phosphorylate the 3′ hydroxyl group of inositol phospholipids, may mediate some of the effects of Src on the actin cytoskeleton, as it functions downstream of Src and is known to be involved in actin filament rearrangements (Penuel and Martin, 1999). We first assessed the activation of PI3 kinase indirectly, using the activation state of Akt as an indicator of PI3 kinase activity. Akt is a Ser/Thr kinase that is activated downstream of PI3K and an important mediator of cellular survival signals (Krasilnikov, 2000). In order to evaluate Akt activation, western blot analysis was performed, using an antibody against the phosphorylated and active form of Akt, on lysates from cells that were mock-transfected or expressing $Src^{527F}$ and the chimeric constructs. As shown in Fig. 6A, no Akt phosphorylation was detected in lysates of mock-transfected CEF; however, significant anti-phospho-Akt immunoreactivity was detected in cell lysates expressing $Src^{527F}$ and all the chimeric constructs, with the exception of $Y4U32^{527F}$ (Fig. 6A, top panel). These results indicate that the presence of the c-Yes SH4-Unique-SH3-SH2 domains were sufficient to ablate the ability of $Src^{527F}$ to induce activation of Akt, presumably through PI3K. Interestingly, both $Y4U^{527F}$ and $Y32^{527F}$ induced activation of Akt phosphorylation, indicating that the combination of the c-Yes SH4-Unique-SH3-SH2 domains may coordinately play a role in directing activation of Akt. In order to assess directly the ability of these proteins to induce PI3K activity, cell lysates were immunoprecipitated with an antibody against the 85 kDa subunit of PI3K, and the immunoprecipitated proteins were subjected to a PI3K assay. The radio-labeled kinase assay products were then spotted on thin layer chromatography plates for separation. The results were visualized using phosphorimager analysis. Little PI3K activity was detected in lysates from mock-transfected cells, while PI3K activity was readily detected in $Src^{527F}$-transfected cells (Fig. 6B, top panel). However, $Y4U32^{527F}$ did not induce PI3K activity above background levels (Fig. 6B, top panel). Western blot analysis of PI3K immunoprecipitates using an anti-PI3K p85 antibody revealed that equivalent amounts of PI3K were present (Fig. 6B, bottom panel). These results confirm that $Y4U32^{527F}$ is unable to induce activation of PI3K and indicate that the c-Yes SH4-Unique-SH3-SH2 domains may function interdependently to prevent PI3K activation, while the same domains from c-Src enable PI3K activation.

$Y4U^{527F}$ is able to stimulate phosphorylation of Akt, as well as PI3K activity (data not shown), but is unable to induce changes in actin filament integrity. These data indicate that activation of the PI3K pathway is not linked with the failure of $Y4U^{527F}$ and $Y4U32^{527F}$ to alter actin filament integrity. The actin cytoskeleton is dynamically regulated and re-organized in transformed cells and during cell motility. The family of small GTPases of the Rho family, in particular Rac1, RhoA and Cdc42, regulate the organization of the actin cytoskeleton and provides the force for cell motility (Wittmann and Waterman-Storer, 2001; Ridley, 2001). RhoA activation is associated with the formation of stress filaments, and its activity has been reported to be downregulated in Src-transformed cells, which may be important for changes in actin filament integrity (Fincham et al., 1999). CEF cells expressing the chimeric constructs were assessed for endogenous RhoA-GTP activity by affinity absorption from cell lysates with the GST-Crib fusion protein generated from rhotekin, which has higher affinity for RhoA-GTP over RhoA-GDP. Western blot analysis indicates that GST-Crib was able to affinity absorb

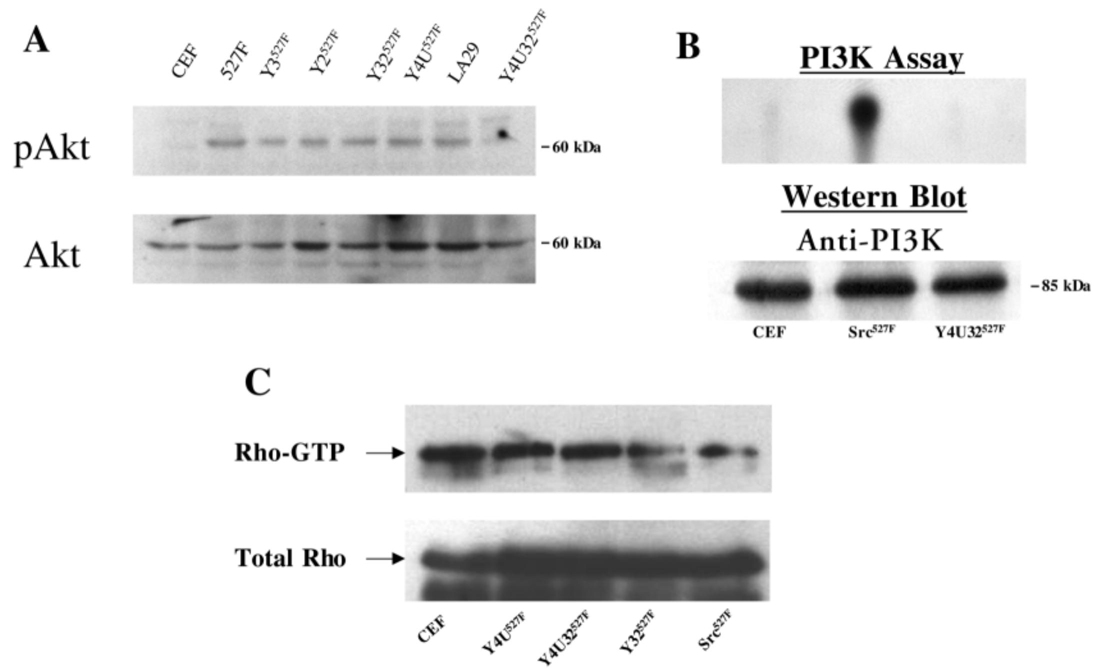


**Fig. 6.** Differential signaling to the PI3K/Akt and RhoA pathway. (A) Akt phosphorylation. 50 μg of RIPA lysates from mock-transfected CEF or cells expressing Src$^{527F}$ or the chimeric constructs were resolved by 8% SDS-PAGE, transferred to PVDF membrane, blocked with 5% nonfat milk/TBS-T, and probed with an anti-phospho-Akt antibody (top panel) or an anti-Akt antibody to determine relative protein levels (bottom panel). (B) PI3K assay. 400 μg of lysates from mock-transfected CEF or cells expressing Src$^{527F}$ or Y4U32$^{527F}$ were immunoprecipitated with an anti-PI3K antibody and subjected to PI3K assay as described in Materials and Methods. Kinase assay products were resolved by thin layer chromatography and visualized by Phosphorimager analysis (top panel). Anti-PI3K immunoprecipitates were also resolved by 8% SDS-PAGE, transferred to PVDF membrane, blocked in 5% nonfat milk/TBS-T, and probed with an anti-PI3K p85 antibody to verify that there were equal amounts of PI3K in the lysates (bottom panel). (C) RhoA-GTP assay. CEF cells expressing the chimeric constructs or Src$^{527F}$ were lysed and 500 μg of cell lysate processed for affinity absorption with GST-Crib, expressing the Crib domain of rhotekin.

higher levels of RhoA from CEF cells, as well as Y4U32$^{527F}$ and Y4U$^{527F}$ cells, relative to the Src$^{527F}$-transformed cells or the Y32$^{527F}$-transformed cells (Fig. 6C). These data indicate that the presence of the SH4-Unique domains of c-Yes is associated with a failure to downregulate RhoA activity, which can be linked with a failure to induce changes in actin filament integrity.

### The SH4-Unique domains of c-Yes do not support increased motility or invasive potential

As the SH4-Unique domains of c-Yes do not permit significant changes in actin filaments, it was predicted that chimeric constructs that contain the c-Yes SH4-Unique domains may be less motile or invasive, compared with Src$^{527F}$. To test this, CEF cells were infected with retrovirus encoding Src$^{527F}$, Y32$^{527F}$, Y4U$^{527F}$ and Y4U32$^{527F}$, the cells achieved confluent expression of these constructs within 12 days and were subjected to a transwell migration assay. Fig. 7A qualitatively demonstrates that upon loading of equal cell numbers, Src$^{527F}$- and Y32$^{527F}$-expressing cells migrated across the transwells more efficiently than normal CEF cells. Y4U$^{527F}$- and Y4U32$^{527F}$-expressing cells did not exhibit increased migration potential relative to CEF. To quantify these changes in migration, the migrated cells were processed for analysis by spectrophotometry to measure migration relative to each group of CEF cells expressing the chimeras (Fig. 7B, see Materials and Methods for details). The assay indicates that Y32$^{527F}$- and Src$^{527F}$-expressing cells were more efficient in migrating across the transwells than the CEF cells. In addition, the Y4U$^{527F}$- and Y4U32$^{527F}$-expressing cells were no more efficient in migration than CEF cells. These data indicate that, consistent with an inability to affect changes in actin filament integrity, Y4U$^{527F}$ and Y4U32$^{527F}$ were unable to increase cell migration efficiency.

An invasion assay was also performed using the Matrigel 24-well invasion chamber (Fig. 7C). Under conditions of equal cell numbers, CEF cells expressing Src$^{527F}$ or Y32$^{527F}$ were three-times more invasive than untransfected CEF cells, whereas Y4U$^{527F}$ or Y4U32$^{527F}$ demonstrated invasive potential equivalent to untransfected CEF cells. These data indicate that the SH4-Unique domains do not support increased motility or invasion, which correlates well with an inability to support changes in cell morphology or dynamic changes in actin filament integrity.

## Discussion

c-Yes and c-Src are two of the most highly homologous members of the Src family of non-receptor tyrosine kinases, yet despite their significant similarity, specificity in signaling exists between the two proteins. The c-*src* and c-*yes* gene knockout mice and cells derived from them have proved useful in uncovering cellular processes and pathways in which these proteins function distinctly from one another. As mentioned above, several studies carried out in cells derived from the c-*src*$^{-/-}$ mice have indicated deficiencies in cellular processes that are dependent on dynamic regulation of the actin cytoskeleton. c-Yes, despite normal expression in these cells, is unable to compensate for the loss of c-Src in these processes.

The experiments described in this report were undertaken in an effort to gain an understanding of the roles of the c-Yes

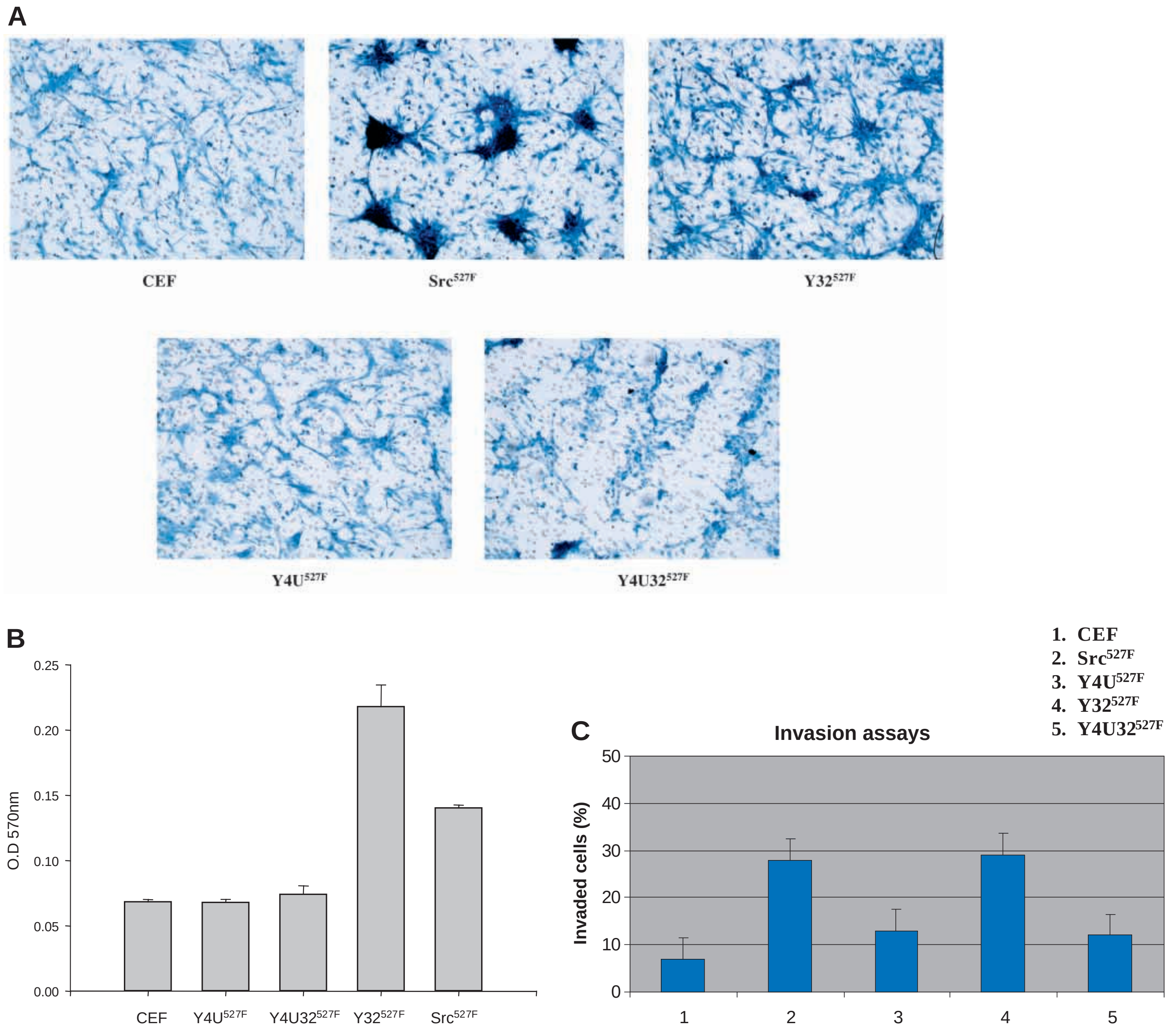


**Fig. 7.** Y4U32$^{527F}$ is unable to induce motility or invasion of CEF cells. (A) Equal numbers of mock transfected CEF cells, or CEF cells expressing Src$^{527F}$, Y32$^{527F}$, Y4U$^{527F}$, Y4U32$^{527F}$ subjected to (A) a transwell migration assay where cells migrated across the transwell and were visualized by staining. (B) The transwell migration assay was repeated and the total number of cells that migrated was isolated and quantified spectrophotometrically, to determine the relative numbers of cells that migrated. The experiment was done in triplicate and error bars indicate standard deviation. (C) A Matrigel invasion assay was also performed under the same conditions with the same cells, where the number of cells capable of invading through the Matrigel were counted. The experiment was carried out twice.

functional domains in the apparent inability of c-Yes to compensate for c-Src signals, especially those known to regulate actin cytoskeletal rearrangements. In these experiments, the cytoskeletal and morphological changes that occur concomitantly with Src$^{527F}$-induced transformation of primary and mortal CEF cells were used as a model system. We discovered that replacement of the Src SH4, Unique, SH3 and SH2 domains with the corresponding c-Yes domains resulted in a loss of actin repositioning from stress fibers to punctate rosettes and actin-based membranous motility structures such as lamellipodia and filopodia. Replacement of the c-Src N-terminus with that of c-Yes eliminated the ability of Src$^{527F}$ to induce the phenotypic changes that are typically observed upon overexpression of the protein. The inability to induce morphological and cytoskeletal changes was associated primarily with the c-Yes N-terminus (SH4 and Unique domains). Differences in signaling were also associated with a combination of the SH4-Unique-SH3-SH2 domains of c-Yes. These data indicate that the ability to induce morphological changes may be largely due to the SH4-Unique domains and that signaling specificity may be interdependent upon each of these functional domains.

Having identified the c-Yes N-terminus as the region responsible for the failure of these chimeric proteins to induce morphological and cytoskeletal changes, the obvious question is why does this occur? One possible mechanism that may prevent Y4U$^{527F}$ or Y4U32$^{527F}$ from altering actin filament integrity is altered subcellular localization. Although we found no evidence for gross changes in cellular localization to membranes or the cytoskeleton, it is possible that changes in subcellular regions may not be detected by these methods, but may be highly relevant to stimulating specific signaling cascades. One obvious difference between the SH4 domains of c-Yes and c-Src is palmitoylation of Cys$^{3}$ in c-Yes, which could direct it to lipid rafts. Raft localization may not be the full explanation, however, as the palmitoylation site is sufficient for localization of Src family kinases to lipid rafts (Shenoy-Scaria et al., 1994), yet Y4$^{527F}$ was predicted to be palmitoylated and yet was able to induce actin filament rearrangement and morphological changes. It is possible that the Unique domain also contributes to membrane compartmentalization, as occurs with Lck (Bijlmakers et al., 1997), or targets the kinase to proteins with which it would not normally interact. Conversely, the c-Yes Unique domain may prevent the interaction of Src$^{527F}$ with substrates or binding partners that are necessary for repositioning of cellular actin and induction of morphological changes. However, the ability of the YU$^{527F}$ chimera to induce actin filament rearrangement and morphological changes suggests that this is also not the complete story and that both the SH4 and Unique domains may work cooperatively to contribute to the inability of Src$^{527F}$/c-Yes N-terminal chimeras to induce morphological changes and actin filament rearrangement. The SH4 and Unique domains may act synergistically, both through sequestration to lipid raft fractions and through altered protein/protein and/or protein/substrate interactions. The function of the c-Yes Unique domain has not been previously explored.

If altered sub-cellular localization is responsible for the inability of Y4U32$^{527F}$ and Y4U$^{527F}$ to induce actin filament rearrangements and morphological changes consistent with cell transformation, it must result in failure to activate the appropriate signaling pathways necessary to effect these transformation-associated changes. Unfortunately, determining which signaling pathway or pathways that are normally activated by transforming Src variants but not activated by Y4U32$^{527F}$ and Y4U$^{527F}$ is a particularly vexing issue, as there are many proteins that are involved in Src-mediated actin filament rearrangement, and they do not necessarily function in a linear pathway. In fact, it is likely that the effectors of Src transformation act through multiple pathways, both parallel and overlapping. One pathway that is activated downstream of Src and is essential for cell transformation by oncogenic Src is the MAPK pathway (Cowley et al., 1994). MAPKs are activated downstream of Src through a pathway that can be initiated through Shc/Grb2 interaction (Rozakis-Adcock et al., 1992). Shc/Grb2 complex formation allows the Grb2/SOS complex to activate Ras, which is followed by activation of Raf, MEK1/2, and finally the MAP kinases themselves (Klein and Schneider, 1997). In these studies, the ability of Src$^{527F}$ to induce Shc/Grb2 complex formation was not ablated by the presence of the c-Yes N-terminus. Although Y4U32$^{527F}$ did not induce the robust activation of the MAPK pathway that Src$^{527F}$ did, differences mediated by Src$^{527F}$ and Y4U$^{527F}$ were less pronounced. The reduced levels of MAPK pathway activation induced by Y4U32$^{527F}$ may be due in part to the presence of the c-Yes SH3 and SH2 domains, which, as noted above, have been previously demonstrated to differ in their ligand-binding, and hence signaling, capacities (Sparks et al., 1996). Interestingly, Y4U32$^{527F}$ failed to induce phosphorylation of c-Raf on Tyr$^{340/341}$, unlike Y4U$^{527F}$. This phosphorylation of c-Raf is associated with c-Src activity. Thus the reduced c-Raf phosphorylation associated with Y4U32$^{527F}$ may be due to either differences in SH3-SH2 mediated signaling, or to interdependent signaling by the combination of the c-Yes SH4-Unique-SH3-SH2 domains. The inability of c-Yes to phosphorylate and possibly activate c-Raf may have functional meaning. c-Yes will associate with adherens junctions (Tsukita et al., 1991). Nusrat et al. demonstrated that the tight-junction-associated protein, occludins, uniquely associate with c-Yes and not c-Src (Nusrat et al., 2000). Occludins are transmembrane proteins that regulate extracellular interactions in tight junctions. Activation of Raf-1 is associated with downregulation of occludin expression (Li and Mrsny, 2000). These data are consistent with a role for activated c-Yes as a binding signaling partner for occludins, while activation of c-Src might be predicted to direct phosphorylation and activation of Raf-1 and downregulation of occludins. Thus, it is possible that activated c-Yes may play a role in participating in the maintenance of tight junction interactions, whereas activation of c-Src is known to cause their dissociation. Thus, it may not be functionally advantageous for c-Yes to activate c-Raf, if c-Yes plays a role in regulating occludin function.

While activation of the MAP kinase pathway is important for the mitogenic response to Src, the role that MAP kinase activation plays in rearrangement of the actin cytoskeleton downstream of Src is unclear. Fincham and colleagues demonstrated mitogeneis-independent inactivation of Rho, a key modulator of the actin cytoskeleton, downstream of Src, indicating that separate pathways may be involved in the induction of cytoskeletal and mitogenic responses to Src activation (Fincham et al., 1999). Mek has also been shown to play a role in regulating actin filament dynamics in some cells (Pawlak and Helfman, 2002); however, in our CEF system, Mek inhibitors were unable to block changes in actin filament integrity (data not shown). Although Y4U32$^{527F}$ was able to induce activation of Erk1/2, it was unable to induce phosphorylation of c-Raf. Interestingly, it has been demonstrated that c-Raf-knockout cells (raf$^{-/-}$) or raf$^{-/-}$ cells that are engineered to express c-Raf$^{Y340/341F}$ (c-raf-FF) are able to grow, and Erk activation in these cells is normal (Huser et al., 2001). These data indicate that Erk1/2 activation may proceed in a Raf-independent manner.

PI3K is another important downstream mediator of Src, which has been implicated in directing actin cytoskeleton rearrangements. It has been reported previously that PI3K may function in parallel with the MAP kinase pathway (Penuel and Martin, 1999). The 85 kDa subunit of PI3K is both a substrate and SH3 domain binding partner of Src, and the ability of Src to associate with PI3K correlates with its ability to induce cell transformation (Hamaguchi et al., 1993). Fincham et al. recently demonstrated that binding of the v-Src SH3 domain to PI3K is important in targeting Src to focal adhesion structures (Fincham et al., 2000). The exact mechanism by which PI3K exerts its influence on the actin cytoskeleton remains unclear; however, it may be involved in the regulation of Rho family members, such as Rac-1, Cdc42 and RhoA (Reif et al., 1996). One of the major effector proteins downstream of PI3K is Akt (Krasilnikov, 2000). Akt activation contributes to cell survival and may also play a role in cell transformation (Krasilnikov, 2000). Our data indicate that Y4U32$^{527F}$ is unable to efficiently induce activation of Akt, as demonstrated by western blot analysis with the anti-phospho-Akt antibody. However, Y4U$^{527F}$ and Y32$^{527F}$ were able to induce phosphorylation of Akt, indicating that interdependent signaling between each of these functional domains may divert c-Yes from being able to activate Akt. This conclusion correlates with the inability of Y4U32$^{527F}$ to induce activation of PI3K. PI3K has been implicated in modulating changes in actin filament integrity, cell motility and invasion and, consistent with these observations, CEF cells expressing chimeric constructs of Src$^{527F}$ that contained the c-Yes SH4-Unique-SH3-SH2 domains were less motile and invasive compared with Src$^{527F}$. Interestingly, Y4U$^{527F}$-expressing cells were also less motile and invasive, which correlates well with the morphology of cells expressing this construct, although this chimera was able to direct phosphorylation of Akt, indicating that PI3K activation may not be sufficient to induce changes in cell morphology in CEF cells. The reason for this may be attributed to an inability of Y4U$^{527F}$ and Y5U32$^{527F}$ to downregulate RhoA. Our data indicate that Src$^{527F}$/c-Yes N-terminal chimeras fail to inactivate RhoA, which may explain why the Y4U32$^{527F}$ and Y4U$^{527F}$ chimeras failed to affect changes in actin filament integrity.

The investigation of one question invariably leads to the uncovering of another: in this case, why is Y4U32$^{527F}$ unable to induce activation of PI3K? Traditionally, Src has been hypothesized to induce PI3K activation through direct binding and/or tyrosine phosphorylation (Pleiman et al., 1994). Binding of Src to PI3K occurs through an interaction between the Src SH3 domain and the 85 kDa subunit of PI3K (Pleiman et al., 1994). Mutants of v-Src that fail to bind PI3K also fail to induce cell transformation (Catling et al., 1994). While there is no previous evidence of direct interaction between c-Yes and PI3K, there is little reason to believe that the c-Yes SH3 domain would be unable to bind to p85, as the SH3 domain residues that have been reported to be essential for p85 binding in other Src family members are conserved in c-Yes (Mak et al., 1996), and v-Yes is able to induce elevated PI3K activity (Fukui et al., 1991). Indeed, Y32$^{527F}$ appears to co-immunoprecipitate with PI3K as efficiently as Src$^{527F}$ (data not shown). It is possible that sequestration to the lipid raft fraction may be in part responsible for the inability of Src$^{527F}$/c-Yes N-terminal chimeras to activate the PI3K pathway and thus induce morphological and cytoskeletal changes. This explanation would be in agreement with the ability of v-Yes to induce PI3K activation and cell transformation, as v-Yes is an N-terminal fusion of the Yes protein with the retroviral Gag protein and is not palmitoylated, relying instead on the Gag protein for membrane targeting (Ghysdael et al., 1981). It should also be noted that the inability of Y4U32$^{527F}$ to induce PI3K activation in CEF may be unrelated to direct interaction between the chimeric proteins and PI3K. It has been demonstrated that in Shp-2 knockout cells, the PI3K/Akt pathway was inefficiently activated by v-Src, and this correlated with reduced complex formation between c-Cbl and p85 (Hakak et al., 2000). The reason behind the reduced activation of the PI3K/Akt pathway by Y4U32$^{527F}$ is currently under investigation.

PI3K/Akt activation may not be sufficient to induce disruption of the actin stress fiber network, as Y4U$^{527F}$ was able to activate Akt phosphorylation (a signature for PI3K activation) but did not exert a significant effect on actin filament organization. We speculate that the SH4-Unique-SH3-SH2 domains may work cooperatively to position c-Yes to activate specific signals that are not associated with actin filament changes. We speculate that the SH4-Unique domains may function as one functional domain and position c-Yes in specific subcellular regions that prevent it from affecting significant changes in actin filament organization and/or activation of specific signals associated with actin filament rearrangements. Together, the SH4-Unique-SH3-SH2 domains of c-Yes may target this tyrosine kinase for distinct functions, which may explain why c-Yes is unable to compenstate for cSrc in affecting dynamic changes in actin filament organization in *src*$^{-/-}$ cells.

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