v-src-Dependent Downregulation of the Ste20-like Kinase SLK by Casein Kinase II

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Summary
We have previously shown that the Ste20-like kinase SLK is a microtubule-associated protein inducing actin stress fiber disassembly. Here, we show that v-src expression can downregulate SLK activity. This downregulation is independent of focal adhesion kinase (FAK) but requires v-src kinase activity and membrane translocation. SLK downregulation by v-src is indirect and is accompanied by SLK hyperphosphorylation on serine residues. Deletion analysis revealed that casein kinase II (CK2) sites at position 347/348 are critical for v-src-dependent modulation of SLK activity. Further studies show that CK II can directly phosphorylate SLK at these positions and that inhibition of CK II in v-src-transformed cells results in normal kinase activity. Finally, CK II and SLK can be co-localized in fibroblasts spreading on fibronectin-coated substrates, suggesting a mechanism whereby SLK may be regulated at sites of actin remodeling, such as membrane lamellipodia and ruffles, through CK II.

Introduction
Cell growth and differentiation are tightly regulated mechanisms involving a large number of signaling cascades. Dysregulation and accumulation of genetic aberrations in these signaling cascades are key components in the transformation of a normal cell to a cancer cell. Furthermore, a direct correlation has been found between the metastatic potential of cancers and the nature of the observed genetic mutations (1-5). Indeed, cellular transformation by the src oncogene, a non-receptor tyrosine kinase, results in loss of adherence, invasiveness and metastasis through increased phosphorylation of adhesion proteins and cytoskeletal disorganization.

c-Src and its viral counterpart v-src are the most studied members of src family kinases. Several studies have illustrated potential src-mediated mechanisms regulating cell survival and apoptosis (6). In addition, studies have demonstrated altered c-src kinase activity, and in some cases protein levels, in human cancers such as breast, colon and pancreatic cancers (3). V-src transformed cells have been widely used to elicit the oncogenic effect of a constitutively active c-src. Features characterizing these cancer cell line models include increased cell detachment and migration.

Casein kinase II (CK2) is a serine/threonine kinase tetramer complex composed of two catalytic subunits, α and/or α′ and/or α′′ and two regulatory β subunits. CK2 minimal amino acid consensus phosphorylation sequence is Ser-X-X-Acidic, where the acidic residue can be glutamic acid, aspartic acid, phospho-Ser or phospho-Tyr (7). CK2 is referred to as “a house keeping enzyme” given its increasing number of substrates (over 300). A role for CK2 has been shown in a wide range of cellular functions and properties such as cell proliferation, survival, differentiation, transformation and tumorigenesis (8-12). Recently, the the CK2α subunit has been shown to be phosphorylated by the src family kinases,
c-fgr and c-lyn, resulting in increased catalytic activity (13).

Previous studies in our laboratory have shown that the Ste20-like kinase SLK is redistributed to membrane ruffles and lamellipodia along with the microtubule network and adhesion components during cell spreading on fibronectin. In addition, SLK can induce actin stress fiber disassembly which can be inhibited by inactive Rac1 (14), suggesting that SLK plays a role in cytoskeletal reorganization.

To gain further insights into the role of SLK in this process, we investigated whether regulators of early adhesion signaling events impact on SLK activity. Our results show that SLK kinase activity is reduced in cells expressing an oncogenic form of c-src and that this regulation requires src kinase activity and translocation to the cell periphery. Furthermore, expression of v-src in FAK-null cells also resulted in SLK downregulation, suggesting that v-src-mediated SLK regulation is independent of FAK. Phosphoamino acid analysis of SLK in v-src expressing cells shows that SLK is hyperphosphorylated on serine residues and that v-src-dependent SLK regulation is mediated, in part, by direct phosphorylation by CK2 in the SLK kinase domain. Our results suggest that CK2 may be regulating SLK at sites of cytoskeletal remodeling.

Experimental Procedures

Cell Lines and Immunostaining

HEK293, COS1 and 49F cells were purchased from the American Tissue Type Collection. The mesodermal FAK-/- cells (p53-/-, FAK-/-) and wildtype counterpart were kindly provided by D. Ilic. The SYF cells, deficient for src, yes and fyn; and SYF cells stably expressing c-src were a generous gift from P. Soriano. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2 in Dulbecco’s modified Eagle’s medium (DME medium, Bio-Whitaker) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin.

V-Src transformed 49F cells were generated by puromycin (2 µg/ml, Sigma) selection of pBabePuro3-transfected cells. Cells stably expressing fpgv-1 or tsLA29 v-src were obtained by G418 (0.4mg/ml, Life Technologies) selection following transfection of TsLA29 v-src or empty vector. Prior to SLK kinase assays, cells stably expressing fpgv-1 or tsLA29 v-src (maintained at 40°C) were shifted to 35°C for 24-48 h. Other transfections were carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instruction.

For immunostaining the coverslips were fixed in 4% PFA for 10 min at room temperature, washed in PBS, and blocked with 1% BSA or 200 µg/ml ChromaPure Goat IgG (Jackson ImmunoResearch Laboratories). SLK was detected using an anti-SLK rabbit polyclonal antibody (15) in conjunction with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. CK2 and c-src proteins were detected with an anti-CK2 goat polyclonal (C-18, Santa Cruz) and an anti-c-src monoclonal (Sigma) in conjunction with tetramethyl rhodamine isothiocyanate (TRITC)-labeled secondary antibodies. TRITC-conjugated phalloidin was used to detect actin stress fibers. Samples were visualized on a Zeiss Axioskop100 epifluorescence microscope equipped with appropriate filters and photographed with a digital camera (Sony Corporation HBO50) using the Northern Eclipse software package.

Plasmids

The pBabePuro3 v-src encoding the avian v-src was kindly provided by M. McMahon. HA-tagged pcDNA3 expression vectors bearing full length FAK, FAK kinase dead (FAK-K454R), the FAK Y397F mutant FRNK and the FRNK S1084A mutant were kindly provided by D. Schlaepfer. The fpgv-1 control vector and the TsLA29 v-Src encoding the temperature sensitive v-src were generously provided by M.C. Frame.

The HA-v-src kinase inactive point mutant was generated using PCR-based mutagenesis converting lysine 298 to a methionine generating v-srcK298M. Similarly, a HA-v-src myristylation site mutant (HA-v-srcG2A) construction was also generated. The v-src cDNA and mutant PCR products were subcloned into an HA-tagged pcDNA3 expression vector (Invitrogen) using standard cloning procedures (16). All PCR-generated
mutants were subjected to sequencing analysis at the Ottawa Health Research Institute. Sequencing data was analysed using EditSeq and MegAlign (DNASTAR computer software).

Inactive SLK kinase domain (Myc-SLK\(^{1-373}\)K63R) truncations were generated using a PCR-based approach. Myc-SLK\(^{1-192}\)K63R, containing amino acid residues 1 to 192, was PCR amplified using Myc-SLK\(^{1-373}\)K63R as DNA template and the T7 primer with SLK192 (5' CCG CTC GAG AGC CAT CCA ATA TGG TGT 3'). Myc-SLK\(^{1-325}\)K63R, containing amino acid residues 1 to 325, was generated with Myc-SLK\(^{1-373}\)K63R as template with T7 and SLK325 (5' CCG CTC GAG CTA GAG ATT CTC TGC TTC 3') primers. Serine residues 340, 347, 348, 362 ans 364 were mutated to alanines by PCR using primers bearing the appropriate mutations. Subcloning of the PCR products into Myc-SLK pcDNA3 expression vector was performed using standard procedures.

**Fibronectin Replating Assays**

Subconfluent cultures were serum-starved in 0.25% FBS-DME medium for 24 h and harvested by trypsin-EDTA treatment as described previously (17). Cells were held in suspension (0.1% BSA in DMEM) for 1 h at 37°C and then plated onto FN (10 \(\mu\)g/ml, Sigma) or Poly-L-Lysine (10 \(\mu\)g/ml, Sigma) pre-coated coverslips.

**Immunoprecipitations, Kinase Assays and Western Blots**

Cells were rinsed in PBS and protein extracts were made in modified RIPA buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, and protease inhibitors (Sigma inhibitor cocktails). Samples (40 \(\mu\)g) were fractionated on 8% SDS-PAGE and transferred to PVDF membranes for Western blotting. Reactive proteins were detected by enhanced chemiluminescence (Perkin Elmer) using HRP-labeled secondary antibodies and visualized by autoradiography.

For SLK kinase assays, 400 \(\mu\)g of total cell lysate was immunoprecipitated using 2 \(\mu\)g of anti-SLK or 9E10 antibodies and 20 \(\mu\)l of protein A-Sepharose (Amersham Biosciences) for 4h at 4°C. Immunoprecipitates were washed three times with NETN and once with SLK kinase buffer (20 mM Tris-HCl (pH 7.5), 15 mM MgCl\(_2\), 10 mM NaF, 10 mM \(\beta\)-glycerophosphate, and 1 mM orthovanadate). Kinase reactions (20 \(\mu\)l in kinase buffer) were initiated by the addition of 5 \(\mu\)Ci of [\(\gamma\)-\(32\)P] ATP. After a 30 min incubation at 30°C, reactions were terminated by the addition of 4X SDS sample buffer and 20 \(\mu\)l aliquots were fractionated by 8% SDS-PAGE. Gels were transferred to PVDF membranes and exposed to X-ray film. PVDF membranes were then probed for SLK to evaluate the efficiency of the immunoprecipitation.

To assay CK2 activity, 400 \(\mu\)g of total cell lysate was immunoprecipitated using 2 \(\mu\)g of anti-CK2\(\alpha\) goat polyclonal antibodies (C-18, Santa Cruz Biotechnology), washed three times with NETN and once with modified CK2 kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MnCl\(_2\), 10 mM NaF, 10 mM \(\beta\)-glycerophosphate and 1 mM orthovanadate) (13). Reactions (20 \(\mu\)l in kinase buffer) were initiated by the addition of 3 \(\mu\)g of dephosphorylated casein (C4032, Sigma) and 5 \(\mu\)Ci of [\(\gamma\)-\(32\)P] ATP. Reactions (30 min/37°C) were terminated by the addition of 4X SDS sample buffer and 20 \(\mu\)l aliquots were fractionated by a gradient gel 8-12% SDS-PAGE. Gels were transferred to PVDF membranes, Ponceau stained then exposed to X-ray film. 40 \(\mu\)g of total cell lysates was subjected to Western blot with anti-CK2\(\alpha\) goat polyclonal antibodies (C-18, Santa Cruz Biotechnology) to detect endogenous CK2\(\alpha\) proteins.

**Recombinant CK2 kinase assays**

To assay SLK phosphorylation by CK2, recombinant SLK mutants were used in kinase assays with purified CK2. SLK GST fusions on glutathione agarose were washed with NETN and CK2 kinase buffer. Reactions were initiated by the addition of 2 \(\mu\)l of recombinant CK2 from rat liver (C3460, Sigma) and ATP as above. After a 30 min incubation at 37°C, beads were washed seven times with NETN to remove any traces of rCK2 and once with PBS.
For tryptic digests, beads were incubated in 50 mM NH₄HCO₃ with 10 μl of trypsin from bovine pancreas (T8802, Sigma) overnight at 37°C followed by the addition of another 10 μl of trypsin for 4 h at 37°C the next day. The reaction was terminated by adding 4X SDS sample buffer, boiled for 3 min and fractionated on a 20% Tricine-SDS-PAGE. Expression of the undigested GST fusions was confirmed by independent SDS-PAGE and coomassie staining of the purified undigested proteins.

**Phosphoamino Acids Analysis**

On the day of the assay, exponentially growing cells were washed twice and incubated in phosphate-free DME medium (Gibco-BRL) at 37°C in a humidified atmosphere containing 5% CO₂ for 2 h and [³²P] orthophosphate (250 μCi/ml) was then added for an additional 4 h. After the labeling period, cells were washed three times with TBS (pre-incubated at 4°C) and lysed using modified RIPA buffer with protease inhibitors as above. Equal amounts of cell lysates were used to immunoprecipitate endogenous SLK using 2 μg of anti-SLK antibodies overnight at 4°C. SLK protein was eluted off the beads with 50 μl of re-distilled 5.7 M HCl and hydrolysed at 110°C for 1 h. Dried samples were then dissolved in 2 μl of pH1.9 buffer (formic acid/acetic acid) containing, 1 mg/ml of xylene cyanol, phospho-serine (PS), phospho-threonine (PT) and phospho-tyrosine (PY) standards (Sigma). Samples were subjected to thin layer chromatography in pH1.9 buffer followed by electrophoresis in pH3.5 buffer (pyridine/acetic acid). Amino acids standards were visualized with 0.2% ninhydrin followed by exposure to x-ray film.

**Results**

**V-src Downregulates SLK Kinase Activity**

Fibronectin stimulation of fibroblasts has been shown to trigger the activation of FAK, the recruitment of c-src and the formation of adhesion signaling complexes mediating actin rearrangements (18,19). Furthermore, FAK and c-src appear to be critical for the turnover of these complexes, allowing cell migration. We have previously shown that SLK can induce actin disassembly and that it is redistributed to membrane structures reminiscent of ruffles and lamellipodia during cell spreading on FN (14). Therefore, to gain further insights into the role of SLK in cytoskeletal remodeling, we have investigated SLK activity in FAK- and SYF-deficient cell lines or v-src-transformed cells.

We performed SLK in vitro kinase assays on cell lysates from FAK-WT, FAK-/-, SYF-deficient, SYF+c-src and 49F fibroblasts stably expressing the v-src oncogene. *In vitro* kinase assays showed no change in SLK kinase activity in SYF-deficient, SYF+c-src, FAK-null or FAK-WT cells (Figure 1A). However, a 2 to 3-fold decrease in SLK kinase activity was consistently observed in v-src transformed cells (Figure 1), suggesting that activated c-src or its downstream effectors negatively regulates SLK. In addition, 49F cells stably expressing c-srcY527F, a constitutively active mutant of c-src, also showed a downregulation of SLK kinase activity (not shown).

To further understand the mechanism by which v-src downregulates SLK activity, we generated 49F cells stably expressing TsLA29v-src, a mutant v-src that is temperature-sensitive for translocation to adhesion sites (20). Shifting the cultures to the permissive temperature (35°C) results in the translocation of v-src to the cell periphery. Incubation of the cells at the permissive temperature resulted in a 2-3 fold decrease in SLK kinase activity concomitant with the characteristic v-src-induced morphological changes (see Figure 1F-H). Supporting a role for c-src in SLK regulation, immunofluorescence staining of exponentially growing 49F cells revealed that c-src and SLK proteins could be co-localized at membrane ruffles and lamellipodia (Figure 1C and D). Furthermore, shifting the TsLA29v-src cells to the permissive temperature also resulted in SLK v-src colocalization (Figure 1E and F). Interestingly, immunoprecipitation of SLK followed by immunoblotting for phosphotyrosine residues revealed that SLK is not tyrosine phosphorylated under these conditions (not shown). Taken together, these results suggest that v-src-mediated SLK downregulation is indirect and that this requires v-src translocation to the membrane.
v-Src Kinase Activity And Membrane Translocation Are Required For SLK Downregulation

Previous studies have shown that c-src kinase activity is required for Rac1- and cdc42-induced adhesion remodeling and directed cell migration whereas the src SH3 and SH2 domains are sufficient for its translocation to the cell periphery in a RhoA-dependent manner (20,21). Therefore, we tested the role of v-src kinase activity and myristylation site on SLK downregulation. A kinase inactive (v-srcK298M) and myristylation defective (v-srcG2A) mutant was engineered and co-transfected with Myc-SLK in HEK293 cells. SLK immunoprecipitation and in vitro kinase assays showed that both v-src point mutants could no longer downregulate SLK kinase activity compared to controls (Figure 2A). Together, these data suggest that both v-src kinase activity and membrane anchoring are required to negatively regulate SLK kinase activity.

v-Src-mediated downregulation of SLK Kinase Activity is Independent of FAK

Integrin signaling following FN stimulation proceeds through the recruitment and autophosphorylation of FAK on Tyr-397, causing its transient association with activated c-src and further phosphorylation (4). Similarly, in v-src transformed cells, FAK is hyperphosphorylated, in addition to other adhesion complex proteins (22-24). Therefore, we investigated whether SLK could also be downregulated by FAK. To test this, HEK293 cells were co-transfected with Myc-SLK and either wild-type FAK, alone or in combination with the dominant negative FRNK molecule, or the non-FAK binding mutant FRNK S1084A (25,26). In vitro kinase assays showed that FAK overexpression had no effect on SLK kinase activity (Figure 2B). Similarly, overexpression of FRNK, had no significant effect. Supporting these observations, SLK kinase activity was still downregulated in FAK-WT or FAK-/− cells stably expressing the oncoprotein v-src (Figure 2C). Together, these results suggest that v-src mediated SLK downregulation does not proceed through FAK and its downstream signaling pathways, but rather by activating an independent signaling system.

SLK is hyperphosphorylated in v-src-transformed cells

To investigate whether potential post-translational modifications on SLK, other than tyrosine phosphorylation, were responsible for the negative downregulation, we conducted phosphoamino acid analysis. Endogenous SLK protein was immunoprecipitated from 32P-labeled control or v-src-transformed cells, subjected to acid hydrolysis and resolved by two dimensional thin layer chromatography (TLC). Phosphoamino acid analysis revealed that SLK is hyperphosphorylated solely on serine residues in v-src- or c-srcY527F-transformed 49F cells in comparison to 49F cells (Figure 3A), supporting the notion that the v-src- or c-srcY527F-mediated effect is indirect. Treatment of 49F cells with phosphatase inhibitors followed by SLK immunoprecipitation showed no changes in its kinase activity (not shown), suggesting that v-src mediated SLK downregulation proceeds through the modulation of a serine/threonine kinase. To identify potential kinases involved in the downregulation of SLK activity, we have focused on amino acids 1-373, encompassing all of the kinase subdomains for the identification of target serine residues. To that end, Myc-tagged SLK kinase inactive constructs (Myc-SLK1-373K63R , Myc-SLK1-192K63R and Myc-SLK1-325K63R ) each designed to contain a small number of serine residues and encompassing the kinase domain were generated. Co-transfection of these constructs with or without v-src in HEK293 cells revealed that only the Myc-SLK1-373K63R truncation was hyperphosphorylated, suggesting a potential phosphorylation occurring within the last 9 serine residues of the kinase domain (Figure 3B). The peptide sequence containing these last serine residues was analysed for potential protein phosphorylation sites using ScanProsite from ExPASy. Primary sequence analysis showed the presence of putative PKA, PKC and CK2 phosphorylation sites (Figure 3C). To test the role of PKA and PKC on SLK kinase activity, HEK293 cells were transfected with HA-SLK and treated with either dibuturyl-cAMP (dbcAMP) or 12-O-tetradecanoylphorbol-13-acetate (TPA).
Following SLK immunoprecipitation and kinase assays, no changes in SLK kinase activity was observed (not shown). Supporting this, Myc-tagged SLK point mutants S340A and S364A, sites of potential PKA and PKC phosphorylation respectively, showed no changes in kinase activity at the basal level or when co-expressed with HA-v-src (not shown), suggesting that the v-src effect is not mediated by PKA or PKC.

**CK2 phosphorylates SLK and regulates its kinase activity**

To investigate the potential role of CK2 on SLK kinase activity, Myc-SLK point mutants for serine residues 347/348 and 362 were generated (Myc-SLK SS347/348AA and Myc-SLK S362A). HEK293 cells transfected with these constructs revealed that Myc-SLK SS347/348AA displayed a 2- to 3-fold increase in basal kinase activity when compared to Myc-SLK (Figure 4A). In addition, co-transfection of Myc-SLK SS347/348AA with HA-v-src did not result in the downregulation of SLK kinase activity (Figure 4A). Examination of the CK2α protein content in HEK293 cells revealed that it is highly expressed (Figure 4A), suggesting that CK2 may potentially regulate the catalytic activity of the transfected Myc-SLK (Figure 4A). Therefore, we next addressed whether SLK is directly phosphorylated by CK2 on serine 347/348 or 362. The kinase domain mutants were GST-tagged and subjected to an in vitro CK2 kinase assay in the presence or absence of recombinant rat liver CK2 (Sigma). Following the kinase assay, samples were either digested with thrombin, to remove the GST peptide, or with trypsin to reveal the 1.5 kDa peptide of interest containing serines 347/348 or 362 (Figure 5A). Both thrombin or trypsin digests showed that the SLK mutant SS347/348AA displayed a marked decrease in its phosphorylation level relative to GST- SLK\(^{1-371}\)K63R or GST-SLK\(^{1-372}\)K63R S362A. These results suggest that CK2 can phosphorylate SLK directly on serine residues 347/348 and that this results in SLK downregulation.

**Inhibition of CK2 rescues SLK kinase activity in v-src-transformed cells**

To assess the role of CK2 on SLK kinase activity in v-src-transformed cells, we used 4,5,6,7-Tetrabromo-2-azabenimidazole (TBB; Calbiochem), a specific CK2 inhibitor on v-src expressing cells (27). Control and v-src transformed 49F cells were first treated overnight with 50 mM TBB. SLK and CK2 were then immunoprecipitated independently from the same cell lysate and subjected to in vitro kinase assays. Our results show that after TBB treatment, SLK kinase activity in v-src-transformed cells is similar to that of wildtype 49F cells (Figure 6A). Interestingly, we observed a dramatic increase in CK2 kinase activity in v-src-transformed cells, suggesting that v-src transformation results in CK2 activation (Figure 6B). As previously reported, TBB treatment resulted in a decrease in CK2 kinase activity in both cell lines. Supporting a role for CK2 in the regulation of SLK activity, SLK and CK2α could be colocalized at the cell periphery in 49F cells following a 20 min replating assay on FN matrix (Figure 6 C).

**Discussion**

We have previously described that overexpression of SLK in various cell lines induces the rapid disassembly of actin stress fibers and cell death (28). Here we show that v-src expression leads to downregulation of SLK kinase activity, a process that requires v-src translocation to the membrane, kinase activity and membrane anchoring. Surprisingly, this downregulation does not proceed through FAK or by direct tyrosine phosphorylation of SLK, but rather through activation of CK2 which in turn downregulates SLK. We have observed a marked increase in SLK phospho-serine content in v-src and c-srcY527F transformed 49F cells in comparison to parental cells. Further mapping identified two serine residues located at position 347/348 (SS347/348) that are phosphorylated by CK2. Although we have focused on the catalytic region of SLK as the target of v-src- mediated downregulation, truncation analysis of the full length SLK revealed that SLK is also serine phosphorylated on its M-NAP and ATH domains (15,28), however at much lower levels (not shown). Therefore, serine phosphorylation of those domains cannot be excluded as other important regulatory sites. Nonetheless, serine 347/348 residues are the main CK2 targets within the kinase domain. Previous studies have
shown that the src family kinases, Lyn and c-Fgr, are capable of phosphorylating CK2 α subunit leading to an increase in its kinase activity in vitro (13). Interestingly, we have observed a marked increase in CK2 activity in v-src transformed cells that could not be attributed to differences in protein expression levels. Therefore, one possibility is that v-src may directly modulate CK2 activity. Supporting this, we have demonstrated that the inhibition of CK2 activity in v-src transformed cells restores the catalytic activity of SLK.

We have previously demonstrated that SLK is redistributed with vinculin to structures reminiscent of membrane lamellipodia and ruffles during cell spreading on FN (14). Similarly, CK2 can be co-localized with SLK in these structures (Figure 6). In addition, our previous results show that SLK-mediated actin stress fiber disassembly can be inhibited by the co-expression of a dominant negative Rac1 (RacN17). Interestingly, Timpton et al. showed that c-src kinase activity is required at peripheral adhesion sites for Rac1- and cdc42-induced adhesion remodeling and directed cell migration (21). Therefore, one possibility is that the regulation of cytoskeletal dynamics by SLK may be controlled by c-src. Alternatively, the observed decrease in SLK kinase activity in v-src-expressing cells may be specific to v-src-transformation.

A role for CK2 in cell survival has been demonstrated (8). In addition, SLK overexpression has been shown to induce an apoptotic response (15,28,29). Therefore, another possibility is that SLK activity is downregulated in v-src-transformed cells as part of an anti-apoptotic pathway. The signaling mechanisms that ensue downstream of SLK and how they regulate cytoskeletal remodeling and cell death will allow these hypotheses to be tested.

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Figure Legends

Figure 1. Reduced SLK kinase activity in v-src-transformed 49F cells. (A) In vitro kinase assays were performed on 49F, 49F+v-src, NIH3T3, FAK-Wt, FAK/-, SYF and SYF+c-src cells. Endogenous SLK was immunoprecipitated from equal amounts of cell lysates and subjected to in vitro kinase assays. The immunoprecipitates were blotted and probed for SLK to evaluate the efficiency of the immunoprecipitation. The extent of SLK autophosphorylation was used as a measure of kinase activity. Downregulation of SLK activity was observed in v-src transformed cells. (B) Cells stably expressing TsLA29 v-src were maintained at the restrictive temperature (40°C) and then shifted to the permissive temperature (35°C) for 16-18h. Endogenous SLK was then immunoprecipitated and subjected to kinase assays. Translocation of the Ts v-src to adhesion sites by incubation of the cultures at the permissive temperature (35°C) induced the characteristic v-src-transformed cell morphology (see panel H) and SLK activity downregulation. The membranes were probed with an anti-SLK antibody to evaluate the efficiency of the immunoprecipitation. Double immunostaining for endogenous SLK (C and D) and c-src (F and G) shows colocalization at both the restrictive and permissive temperature (arrowheads). The characteristic v-src-transformed cell morphology was clearly observed by phalloidin staining (E and H) following the shift to 35°C. The cells were photographed at 1000x.

Figure 2. v-src kinase activity and membrane localization are required for SLK regulation. (A) HEK293 cells were co-transfected with HA-v-src or mutants in the presence or absence of Myc-SLK. Myc-SLK was immunoprecipitated from cell lysates and subjected to in vitro kinase assay. The SLK IP was monitored by probing the kinase assays with anti-SLK antibodies. Expression of all mutants and SLK was confirmed by Western blotting of the total cell lysates for Myc and HA. (B) v-src-mediated SLK downregulation is independent of FAK. HEK293 cells were co-transfected with Myc-SLK and FAK constructs and subjected to SLK kinase assays. Overexpression of FAK or FRNK had no effect on SLK kinase activity. SLK IP were monitored by repробing the kinase assay for SLK protein. Expression of the various form of FAK was verified by Western blotting (bottom panel). (C) SLK was immunoprecipitated from FAK+/- or -/- MEFs stably expressing v-src and subjected to in vitro kinase assays and the IP was assessed as in (A) and FAK expression was also evaluated by Western blot analysis.

Figure 3. v-src-dependent phosphorylation of the SLK kinase domain. (A) Phosphoamino acid analysis of endogenous SLK protein from v-src and c-srcY527F-transformed cells. Cells were labeled with 32P-orthophosphate, lysed and subjected to SLK immunoprecipitation. The phosphorylated SLK was then hydrolyzed and spotted onto thin layer chromatography plates and exposed to X-ray film. The position of the phosphorylated standards is circled. (B) Myc-tagged SLK kinase inactive truncations, SLK1-192K63R, SLK1-325K63R and SLK1-373K63R were co-transfected with v-src and the cells were labeled with 32P-orthophosphate. SLK1-373K63R truncations were immunoprecipitated, transferred to polyvinylidene difluoride (PVDF) membranes and exposed to X-ray film. Control transfections consisted of the empty expression vector (pCanHA2) and Myc-SLK constructs. The PVDF membranes were probed with an anti-myc antibody to evaluate the efficiency of the immunoprecipitation (not shown). (C) The peptide sequence comprised between SLK residue 332 to 370 illustrating the potential serines hyperphosphorylated in v-src transformed cells as well as kinase target sites.

Figure 4. Regulation of SLK by CK2. Potential CK2 phosphorylation site mutants (S → A) were generated at residues 347/348 (SLK-AA) and 362 (SLK-A). Myc-SLK mutants were transfected into HEK293 cells in the absence or presence of HA-v-src and 9E10 immunoprecipitates were subjected to in vitro kinase assays. Samples were transferred to PVDF membranes, and exposed to X-ray film. The membranes were then probed with an anti-SLK antibody to evaluate the efficiency of the immunoprecipitations. Endogenous levels of CK2α expressed in HEK293 cells are also
shown (panel A). (B) Equal amounts of cell lysates probed with 12CA5 shows the efficiency of the HA-v-src transfection.

Figure 5. CK2 phosphorylates SLK at serine 347/348 in vitro. (A) GST-tagged kinase inactive SLK (SLK\(^{1-373}K63R\) ) harboring mutations at serine residues 347/348 and 362 were generated (SLK-AA and SLK-A) and subjected to in vitro kinase assays in the presence or absence of rat liver recombinant CK2 (rCK2). Samples were digested with thrombin (A), or trypsin (B), and resolved by SDS-PAGE and exposed to X-ray film. The Coomassie stained gel in (A) shows the GST fusions following thrombin treatment. The stained gel in (B) shows the GST fusions prior to trypsin digestion.

Figure 6. Inhibition of CK2 restores SLK kinase activity in v-src transformed 49F cells. 49F and v-src-transformed 49F cells were incubated with or without 50mM TBB and SLK (A), as well as CK2\(\alpha\) (B) were immunoprecipitated independently from the same cell lysate and subjected to in vitro kinase assays. SLK kinase activity was assayed based on its level of autophosphorylation (A) whereas, dephosphorylated casein was used as a substrate for CK2 activity (B). Immunoprecipitates were probed with anti-SLK or with anti-CK2\(\alpha\) antibodies to evaluate the efficiency of the immunoprecipitation. (C) Co-localization of SLK and CK2\(\alpha\) during cell spreading on FN. 49F cells were fixed and stained at 20 min following replating of suspended cells onto FN-coated mattrices. Co-localization of SLK and CK2 is observed in ruffles (arrowheads).
Figure 1
Figure 2
Figure 3

A.

B.

C.

332-PIPANKRASSDLSIAASSSEEDKLSQNCIELSVSERTEQS-370

PKA | CK2 | CK2 | PKC
A.

**IP:** 9E10

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**Kinase Assay**

**IB:** SLK

**Total CK2α**

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B.

**Blot:** anti-HA

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Figure 4
A. CK2 Kinase Assay

- GST
- GST-SLK
- GST-SLK 1-373K63R
- SS347/348AA
- SS362A
- S362A
- rCK2

1-373K63R

Thrombin digest

Coomassie blue

B. CK2 Kinase Assay

- GST
- GST-SLK
- GST-SLK 1-373K63R
- SS347/348AA
- SS362A
- S362A
- rCK2

Tryptic digest

Partial

Peptide

Coomassie blue

Protein lysate

Figure 5
Figure 6

A.

IP: SLK
Kinase Assay

IB: SLK

IP: SLK

B.

IP: CK2α
Kinase Assay

Ponceau stain
of Casein

IB: CK2α

C.

CK2α

SLK

Merged
v-src-dependent downregulation of the ste20-like kinase SLK by casein kinase II
Ziad Y. Chaar, Paul O'Reilly, Irwin Gelman and Luc A. Sabourin

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