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# Characterization of SSeCKS, A Src-Suppressed C Kinase Substrate, Involved in Tumor Suppression, Growth Arrest and Scaffolding

by

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

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# Abstract

## Characterization of SSeCKS, A Src-Suppressed C Kinase Substrate, Involved in Tumor Suppression, Growth Arrest and Scaffolding

by

Xueying Lin

**Advisor: Dr. Irwin H. Gelman**

Identification and characterization of novel genes involved in the regulation of mitogenesis and suppression of oncogene-induced tumor formation is of particular interest. Using a modified PCR-based subtractive hybridization technique developed previously, clone 322 was isolated whose transcripts were down-regulated in v-*src* transformed NIH3T3 cells. Later, the 322 product was identified as a PKC substrate/binding protein. Thus, the 322 gene product was named SSeCKS (Src Suppressed C Kinase Substrate).

SSeCKS transcript levels are suppressed in v-*src*, v-*ras*, but not in v-*raf* transformed rodent fibroblasts. The down-regulation of SSeCKS is dependent on Src kinase activity and is not a non-specific consequence of cell transformation. The finding that the down-regulation of SSeCKS correlates with anchorage-independent growth, and

that SSeCKS decreases v-*src*-induced colony formation in soft agar, suggests that SSeCKS may encode a potential tumor suppressor. To study the putative tumor suppressive function of SSeCKS, conditionally transformed NIH3T3 cell lines (expressing ts72v-*src*) with tetracycline-regulated SSeCKS expression were developed.

Forced expression of SSeCKS suppresses parameters of v-*src*-induced malignant transformation, such as increased refractility, increased saturation density, growth factor- and anchorage-independent growth. The tumor suppressive effects of SSeCKS are not mediated by inhibition of Src kinase activity and MAPK (ERK2 and JNK) activity, nor by inhibition of Src and ERK2 expression. Interestingly, ERK2 activity is enhanced in v-*src* transformed cells upon SSeCKS overexpression. Forced expression of SSeCKS also restores cytoskeletal architecture such as formation of stress fibers and focal adhesion plaques, which are disrupted in transformed cells. These data suggest that SSeCKS functions as a tumor suppressor, likely by its ability to control cytoskeletal architecture and regulate cell signaling.

Forced expression of SSeCKS in untransformed NIH3T3 cells induces G1 arrest, which correlates with suppression of cyclin D1. The decreased expression of cyclin D1 likely results from a suppression of serum-inducible ERK2 activity. Interestingly, ectopic expression of cyclin D1 fails to rescue SSeCKS-induced growth arrest in G1. SSeCKS sequence contains two potential cyclin-binding (CY) motifs which mediate association of cyclin D1 and SSeCKS *in vitro*. Overexpression of SSeCKS results in the sequestration of cyclin D1 in the cytoplasm, very likely via the CY motif-mediated binding. These data

strongly support the notion that SSeCKS controls G1-S progression by inhibiting cyclin D1 expression and/or sequestering cyclin D1 in the cytoplasm.

Along with the progressive understanding of SSeCKS function, other groups showed that SSeCKS is highly homologous to AKAP250 (gravin), a mammalian scaffolding protein. Scaffolding proteins interact with more than one enzyme simultaneously and direct their associated molecules to specific subcellular compartments. By doing so, scaffolding proteins coordinate different signals to tightly regulate physiological functions spatially and temporally. SSeCKS has been shown to function as an anchoring protein for PKA. SSeCKS is a PKC substrate/binding protein, a calmodulin-binding protein, and a cyclin D1-interacting protein *in vitro*. Taken together, it is proposed that SSeCKS controls transformation and cell cycle progression by acting as a scaffolding protein.

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Finally, I would like to express my deepest gratitude to my parents for their love and understanding. They always stand behind me when I am in need.

I would like to dedicate this thesis to my parents.

# Format of Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. Chapter III contains results published as Lin and Gelman, 1997. Chapter V contains results published as Lin et al., 1996. Chapter III, IV and V each has a brief introduction and discussion. A general introduction is placed at the beginning and a general discussion at the end of the thesis. "Materials and Methods" and "References" have been consolidated and placed in the respective sections. Figures and tables are placed at the end of each chapter.

# Table of Contents

# List of Tables and Figures

# Chapter I

# Introduction

## I. V-*src* Oncogene and Malignant Transformation

Oncogenes were originally isolated as retrovirus-encoded genes from tumors in birds and rodents. Later, they were demonstrated to be dominant mutants of their cellular counterparts (proto-oncogenes) (Cantley et al., 1991). The v-*src* oncogene, encoded by the Rous sarcoma virus, is derived from a normal cellular homolog which had been transduced by the virus (Brickell, 1992). $p60^{v\text{-}src}$ was the first protein tyrosine kinase discovered, and is a prototype of non-receptor tyrosine kinases (Hunter and Cooper, 1985). $p60^{v\text{-}src}$ expression leads to morphogenic and mitogenic transformation (Jove and Hanafusa, 1987).

The Src family is composed of nine members, including Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck and Blk. Whereas most members of this family exhibit tissue specific distribution such as expression in haematopoietic cells, Src, Fyn, Yes and perhaps Yrk are ubiquitously expressed (Courtneidge, 1994). Knock-out animal models demonstrate that there is considerable functional redundancy among the members of the Src family. While mice lacking most individual Src family members have only mild effects, mice lacking multiple family members usually show more severe phenotypes and embryonic lethality (Lowell and Soriano, 1996).

All Src family members share the same domain structure (Brown and Cooper, 1996). The SH1 domain (Src homology 1) is the catalytic domain, which catalyzes the phosphorylation of proteins on tyrosine residues. The SH2 motif (Src homology 2) encodes consensus sequences for binding to phosphotyrosine containing proteins. This motif is critical for translocation of Src itself to ligand-stimulated membrane receptors or phosphorylated signal transducers, and for recruitment of other signaling molecules. The SH3 motif (Src homology 3) also mediates signal transduction by binding to proline-rich region of proteins, and is presumably involved in the interaction of Src with cytoskeletal components. The SH4 motif (Src homology 4) encodes N-terminal myristylation consensus sequence, which is necessary for plasma membrane association (Brickell, 1992). A regulatory domain located in the carboxy-terminus contains the hallmark regulatory tyrosine residue (Tyr527 in Src), which is highly phosphorylated in nonactivated cells. The phosphorylated Tyr527 negatively regulates Src activity by facilitating intramolecular association with the SH2 domain (Cooper, 1994).

The v-*src* product exhibits structural similarity to the cellular c-*src* product except the c-terminal regulatory domain, resulting in constitutive tyrosine kinase activity. The oncogenic activity of Src can also be achieved through mutations (in the kinase domain, SH2 and SH3 domains). The amino acid substitutions are thought to affect either Src kinase activity (Hanafusa et al., 1984) or interactions with regulatory proteins or substrates (Brickell, 1992).

Recently, the X-ray crystal structures of Src family members confirm some previous hypotheses, while revealing an unexpected mechanism of regulation (Xu et al.,

1997; Sicheri et al., 1997; Williams et al., 1997). The enzymatic activity of Src is not only controlled by intramolecular association between the SH2 motif and c-terminal Tyr527, but also by intramolecular association between the SH3 motif and a SH2-SH1 linker. Both the linker and flanking sequence around Tyr527 have relatively low binding affinity to the SH3 and SH2 motifs, providing the possibility that these intramolecular interaction can be displaced by high affinity intermolecular association. Therefore, the potential mechanisms for Src activation may include: dephosphorylation of Tyr527, competition with the C-tail for the SH2 domain, and competition with the SH2-SH1 linker for the SH3 domain. This suggests that it is hard to distinguish between upstream activators and downstream effectors of Src (Shalloway and Taylor, 1997; Williams et al., 1998).

The v-*src* oncogene has potent transforming ability. Cells transformed by v-*src* exhibit a distinct rounded morphology, concomitant with a loss of stress fibers and a marked reduction in focal adhesion plaques (Felice et al., 1990). The v-Src protein has long been recognized to be enriched in focal adhesions and to phosphorylate FAK (focal adhesion kinase). FAK phosphorylation and degradation induced by Src is linked to the turnover of focal adhesions in Src transformed cells (Fincham et al., 1995; Hanks et al., 1992). Thus, FAK seems to play a crucial role in Src-induced morphological transformation.

The signaling pathways that mediate Src transformation are only partially understood. Recent evidence shows that transformation by Src results in increased Ras activity (Satoh et al., 1990), suggesting that a Ras-transduced signal cascade is

responsible for transformation by Src. This notion is supported by the observation that the MAPK (mitogen activated protein kinase) pathway is activated in Src transformed cells (Gupta et al., 1992). However, constitutive MAPK activity is not required for transformation (Stofega et al., 1997). Several studies indicate that oncogenic Ras requires Raf-MAPK pathway and other pathways regulated by Rho family members to complete mitogenic and morphogenic transformation (Prendergast et al., 1995; Khosravi-Far et al., 1995; White et al., 1995; Qiu et al., 1997). Rho family small GTPases play very important roles in cytoskeletal remodeling. In addition, they are also involved in transcriptional activation, growth control, and membrane trafficking. It is believed that Rho family members regulate signal transduction by linking extracellular stimuli to cytoskeletal re-organization (Van Aelst and D'Souza-Schorey, 1997). Therefore, it is possible that Src modulates morphological transformation by indirectly activating Rho family members. However, recent studies demonstrate that Src is able to regulate cytoskeletal architecture by interacting with p190-Rho GAP and p120-Cdc42 GAP, modulators of Rho family GTPases, suggesting a more direct effect (Barfod et al., 1993; Chang et al., 1995). The STAT pathway is also involved in Src transformation since Stat3 is constitutively activated to bind to DNA in v-Src-transformed cells (Cao et al., 1996).

Transformation by v-*src* is associated with an approximately 10-fold increase in tyrosine phosphorylation of cellular proteins (Jove and Hanafusa, 1987). The putative substrates of Src are proteins involved in i) cell shape such as tropomyosin, talin, vinculin, paxillin (Luna and Hitt, 1992), ii) cell-cell interaction such as N-cadherin (Hamaguchi et al., 1993), iii) signal transduction such as PI 3-K (phosphatidylinositiol 3-

kinase), $G_\alpha$ GAP (GTPase activating protein) and its associated proteins (Hausdorff et al., 1992; Liu et al., 1993). However, whether these proteins are directly phosphorylated by Src and whether their phosphorylation is required for transformation, is unclear. It is of interest that integrins and cytoskeletal proteins are substrates of Src. The increased tyrosine phosphorylation of these proteins may result in the disruption of focal contacts and interaction with ECM in cells transformed by v-*src* (Lo and Chen, 1994).

Transformation by v-*src* is accompanied by alteration in the transcription level of a number of genes. Among the up-regulated genes are c-*fos*, c-*jun*, c-*myc*, *rhoB*, urokinase-type plasminogen activator, 9E3/CEF-4, TGF-β1 (transforming growth factor β1), and Egr-1. The molecular mechanism of Src induced transcription appears to be due to the presence of AP-1 and SRE (serum responsive element) in the promoter regions of some of these genes. Additionally, several other promoter elements, TATAA, CCAAT, and PRDII/κB are also involved. On the other hand, certain genes are transcriptionally suppressed in v-*src* transformed cells, including MARCKS (myristylated alanine-rich C kinase substrate), SSeCKS (Src-suppressed C kinase substrate), *fibronectin*, *tropomyosin*, *NO3*, PKC (protein kinase C), type I collagen, MHC Class I, and *maspin*. The molecular mechanism of transcriptional down-regulation remains unclear (Frankfort and Gelman, 1995; Scholz et al., 1996).

The transcriptionally suppressed genes in transformed cells are of particular interest because they may encode potential tumor suppressors. Several lines of evidence suggest that the down-regulated genes function as tumor suppressors. For example, re-expression of *NO3* (Ozaki and Sakiyama, 1994), *tropomyosin* (Prasad et al., 1993,

Janssen and Mier, 1997) and *maspin* (Zou et al., 1994) in transformed cells reduces the morphological transformation and aberrant cell proliferation.

## II. Tumor suppressor genes

Tumor suppressor genes function in contrast to oncogenes. Classically, they are defined as genes whose loss of function leads to tumorigenicity (Knudson, 1993; Weinberg, 1991; Levine, 1993). The concept of tumor suppressor genes originated from two lines of evidence. First, hybrid cell lines derived from the fusion of a cancerous cell with a non-tumorigenic normal cell no longer produce tumors in animal models (Harris et al., 1969). Second, tumor cells frequently reveal a loss of heterozygosity (LOH) for certain chromosome markers (Cavenee et al., 1983). The definitive evidence for tumor suppressor genes is obtained first from the introduction of entire chromosomes harboring putative tumor suppressor genes into tumor cells (Weissman et al., 1987). This analysis is ultimately refined to individual genes (Huang et al., 1988; Finlay et al., 1989).

The tumor suppressor genes exert their inhibitory effect on tumorigenicity through multiple mechanisms. These include inhibiting angiogenesis (Rastinejad et al., 1989), increasing responsiveness to negative growth factor such as TGF-β (Gerwin et al., 1992), blocking cell cycle progression (Hinds et al., 1992), or inducing cell differentiation (Rosengard et al., 1989).

Tumor suppressor genes play very important roles in the development of human malignancies. First, loss of tumor suppressor genes are responsible for familial cancer syndromes. Concurrent with the functionally recessive nature of tumor suppressor genes,

germline mutations have been demonstrated to be transmitted from one generation to another. For example, mutations in the *BRCA1* gene are responsible for approximately 45% of inherited breast/ovarian cancers (Easton et al., 1993) and mutations in *RB* and *INK4A* (p16) are associated with familial retinoblastoma (Lee et al., 1987) and familial melanoma (Haber, 1997), respectively. Second, loss of tumor suppressor genes is implicated in sporadic cancers. In addition to germline mutations, tumor suppressor genes are also mutated somatically in sporadic forms of human cancers. For example, mutation in the *P53* gene is involved in more than 50% of nonfamilial cancers (Levine, 1993). *RB* and *INK4A* gene mutations are involved in several sporadic tumors such as osteosarcomas and bladder cancer (Brown, 1997; Haber, 1997).

However, one common question that arises from literature search is the lack of detectable mutations in many tumor suppressor genes. Therefore, attempts to search for alternate mechanisms for disrupting tumor suppressor function have been commenced. Taken together, the functional inactivation of tumor suppressor genes may be achieved by a variety of mechanisms (Brown, 1997). These mechanisms can be grouped into four classes: i) coding region mutation, including point mutation, deletion, or insertion; ii) other targets of mutation, including promoter mutation, splice site mutation such as *BRCA1* (Xu et al., 1996), and RNA editing such as *NF1* (Skuse et al., 1996); iii) modifier effects, including hypermethylation, dominant-negative effects of viral and cellular oncoproteins such as *mdm-2* (Oliner et al., 1993), and antisense regulation (overlapping transcripts) (Imai et al., 1996; Rao et al., 1996); iv) change in subcellular localization (Moll et al., 1995). The existence of multiple mechanisms of inactivating tumor

suppressor genes suggests that they are likely to play even greater roles than previously thought in the development of human cancers (Brown, 1997).

During the past several years, DNA hypermethylation has been under extensive investigation as a mechanism for transcriptional suppression (Baylin et al., 1998). The hypermethylation of multiple CpG islands in gene promoter regions typically occurs in tumor cells. Increased DNA-methytransferase activity in cancer cells is likely responsible for the hypermethylation (Belinsky et al., 1996; Lee et al., 1996). However, the underlying mechanism for DNA hypermethylation remains to be determined. The hypermethylation has been shown to serve as an alternative to mutations/deletions for functional inactivation of tumor suppressor genes such as *INK4A* (Merlo et al., 1995), *VHL* (Herman et al., 1994), and E-cadherin (Graff et al., 1995; Yoshiura et al., 1995).

A number of transcriptionally suppressed genes in transformed or tumor cells have been demonstrated to act as tumor suppressors (Prasad et al., 1993; Gluck et al., 1993; Zou et al., 1994). The transcriptionally suppressed tumor suppressor genes are of particular interest. Because the genes themselves are unmutated, their expression may be derepressed by drugs, providing a great opportunity for cancer therapy. In addition to therapeutic application, these genes are also good candidates for early diagnostic and prognostic markers (Lee et al., 1991).

Because retroviral oncogenes such as v-*src* induce tumorigenicity rapidly, it is very likely that they abolish functions of certain tumor suppressor genes by transcriptional down-regulation. This speculation is supported by the notion that v-*src* causes loss of normal p53 expression in the early step of immortilization of chicken

fibroblasts (Ulrich et al., 1992). Based on this hypothesis, our laboratory developed a modified PCR-based subtractive hybridization technique to identify down-regulated, potential tumor suppressor genes in v-*src*-transformed cells (Frankfort and Gelman, 1995). One such gene encodes SSeCKS, which exhibits tumor suppressive activity (Lin and Gelman, 1997).

SSeCKS is a cytoskeletal protein, and its tumor suppressive function is likely mediated by its ability to re-organize cytoskeletal architecture (Lin and Gelman, 1997). Several cytoskeletal proteins have been shown to function as tumor suppressors (Ben-Ze'ev, 1997). Among them, tropomyosin is the best documented. The expression of certain tropomyosin isoforms is diminished in transformed and tumor cells, and restored once the cells are reverted to normal (Cooper et al., 1985). When tropomyosin isoforms are ectopically expressed in transformed cells, oncogenicity is repressed (Prasad et al., 1993; Gimona et al., 1996; Janssen and Mier, 1997). When tropomyosin levels are suppressed by antisense RNA expression in normal cells, some parameters of transformation are induced (Boyd et al., 1995). Although the 3' untranslated region of tropomyosin 1 suppresses tumorigenicity (Rastinejad et al., 1993), the molecular basis of tropomyosin's tumor suppressive effect is not clarified. The fact that some down-regulated cytoskeletal proteins are tumor suppressors suggests that the dual role in cell structure and signaling are far more important than previously thought.

## III. Cell Cycle Regulation

The time period of a cell going through one division cycle is defined as one cell cycle. The length of a cell cycle depends on different cell types and cell lines, varying, typically, from 10 to 48 hrs. The cycle is operationally divided into four distinct phases: G1 (gap1), S Phase (DNA synthesis), G2 (gap2) and M Phase (mitosis) (Grana and Reddy, 1995). There is also a G0 (growth arrest) Phase, meaning quiescence. Cells enter G0 Phase under conditions of either low concentration of growth factors or contact inhibition (DelSal et al., 1996). In order to accurately transmit genetic information, the cell cycle progression must be tightly regulated.

The progression of the cell cycle in eukaryotes is mediated by the sequential assembly and activation of different kinase holoenzymes. Each holoenzyme is composed of a regulatory subunit, called cyclin and a catalytic subunit, named cyclin dependent kinase (CDK). Cyclin dependent kinase inhibitors (CKIs) are also associated with the complexes. Monomeric CDKs are activated upon association with cyclins, and further activated by phosphorylation (by CAK, the CDK activating kinase) and dephosphorylation (by the CDC25 phosphatase). The activated CDK can be inactivated by i) reducing cyclin levels, ii) Tyr phosphorylation (by the Weel kinase), iii) or interacting with CKIs (Hunter and Pines, 1994; Grana and Reddy, 1995; Morgan, 1995). Recently, the folding of CDKs has been suggested as an important step towards activation (Hunter and Poon, 1997).

The crystal structures of cyclin A, inactive free CDK2, partially active cyclin A-CDK2, fully active cyclin A-CDK2($PO_4$) and inactive cyclin A-CDK2(($PO_4$)-p27 have been determined (Brown et al., 1995; Jeffrey et al., 1995; Russo et al., 1996). The

structure of cyclin A is rigid, and there is no conformational change between the free and CDK2-bound forms. However, on association with cyclin A, CDK2 reorients its structure to expose the Thr160 hydroxyl group. Phosphorylation of Thr160 on CDK2 by CAK further stabilizes this conformation and results in a fully active cyclin A-CDK2 complex. The CDK inhibitor p27 binds to the cyclin A-CDK2($PO_4$) complex by interacting with both cyclin A and CDK2. p27 inhibits CDK2 kinase activity by causing its conformational change, but does not have any effect on the structure of cyclin A (Noble et al., 1997; Martin-Castellanos and Moreno, 1997).

p27 associates with cyclin A through an N-terminal sequence motif, SACRNLFG. A similar motif, SAKRRLFG, in p107 (a pRb family member) is necessary for binding cyclin A-CDK2. Another similar sequence, ACRRLFGP, in the N-terminus of p21 is required for interacting with cyclins independently of CDK2, and is important for optimal inhibition of CDK2 activity *in vitro* and G1 arrest *in vivo* (Chen et al., 1996). This conserved motif is named the cyclin-binding (CY) motif. The CY motif is also present on other CKIs such as p57, other substrates of CDKs such as p130 and E2F1, and on phosphatase CDC25A (Saha et al., 1997).

Cyclins were originally identified as proteins with cyclic accumulation and degradation (Evans et al., 1983). Later, they were discovered to be CDK partners and are essential for CDK activity (Pines, 1993). Cyclin levels are controlled by transcriptional activation and ubiquitin-mediated degradation. Cyclin D expression is rapidly induced during G1 as a delayed early response to mitogenic stimulation, and remains at relative high level throughout the cell cycle (Sherr, 1995). The expression of cyclin D1 is largely

dependent on the sustained activation of the ERK subfamily of MAP kinases (Lavoie et al., 1996a; 1996b). Although ERK activity can be stimulated by both growth factors and cell adhesion to the ECM (extracellular matrix), both stimuli are required for the expression of cyclin D (Schwartz et al., 1995; Bottazzi and Assoian, 1997). After the induction of cyclin D, cyclin E is expressed later in G1, with a maximal level at the G1-S transition. Cyclin E-associated kinase activity but not cyclin E expression is dependent on cell adhesion to ECM (Fang et al., 1996; Zhu et al., 1996). Cyclin A is expressed prior to the initiation of DNA synthesis. The expression of cyclin A is also dependent on the presence of growth factors and ECM (Schulze et al., 1995).

Accumulating evidence suggests that cyclins not only activate CDKs but also target CDKs to their substrates (Hubbard and Cohen, 1993). The latter function presumably ensures that distinct CDKs phosphorylate appropriate substrates during cell cycle progression. Nuclear translocation of cyclin B1 at the beginning of M phase enables CDC2 to phosphorylate compartmentalized substrates such as lamins, leading to nuclear envelope breakdown (Pines and Hunter, 1991; Li et al., 1997). Recent studies indicate that the cytoplasmic location of CDK4 is mediated by cyclin D1 and is linked to G1 arrest (Diehl and Sherr, 1997; Taules et al., 1998).

Cell cycle progression is controlled by the activity of cyclin-CDK complexes. Ectopic expression of either cyclin D or cyclin E accelerates the G1 phase, indicating that both cyclins are rate-limiting controllers of G1-S progression (Quelle et al., 1993; Resnitzky et al., 1994). However, the two cyclins have distinct functions. Cyclin D-CDK4/6 complexes are required for G1 progression (Baldin et al., 1993; Quelle et al.,

1993) whereas Cyclin E-CDK2 complexes are required for G1/S transition (Ohtsubo et al., 1995). S phase progression is dependent on the activity of cyclin A-CDK2. Cyclin A also interacts with CDC2, which is indispensable for G2/M transition (Pagano et al., 1992). Cyclin B-CDC2 holoenzyme regulates M phase (King et al., 1994).

Cyclin-CDK complexes function through phosphorylation of down-stream targets. A protein is generally considered as a putative substrate of cyclin-CDK complex if it fulfills the following criteria: i) it is phosphorylated on a CDK consensus site *in vitro* and *in vivo*; ii) its phosphorylation results in phenotypic changes; iii) it is co-localized with the holoenzyme at the same subcellular compartment at the time that phosphorylation takes place (Grana and Reddy, 1995). Among all the substrates, pRb is the best characterized. pRb phosphorylation begins in mid G1 by cyclinD-CDK4/6, and then by cyclinE-CDK2 (DelSal et al., 1996; Taya, 1997). The resulting hyperphosphorylated pRb loses its growth inhibitory function by releasing bound members of the E2F transcription factor family. E2F then transactivates genes required for S phase entry (Taya, 1997; Grana and Reddy, 1995). pRb phosphorylation is the most important function of cyclin D-CDK4/6 in cell cycle regulation (Lukas et al., 1995a; Lukas et al., 1995b). In contrast, cyclin E-CDK2 can phosphorylate other critical substrates in addition to pRb (Roussel et al., 1995; Ohtsubo et al., 1995).

While cyclins and CDKs usually provide positive signals for cell cycle progression, CKIs often confer negative signals. CKIs are supposed to satisfy the following criteria: First, a CKI must interact with a CDK or cyclin-CDK complex *in vivo*. Second, CKIs should inhibit CDK activity *in vitro*. Third, CKIs should not covalently

modify either cyclins or CDKs (Peter and Herskowitz, 1994). Two families of CKIs have been identified (Sherr and Roberts, 1995). The INK4 family is composed of p15, p16, p18, and p19, which inhibit exclusively CDK4/6 activity by binding in competition with cyclin D (Hannon and Beach, 1994; Sherr and Roberts, 1995). The Cip/Kip family includes p21, p27, and p57, which suppress a wide range of CDKs including CDK4/6 and CDK2 by preferentially forming cyclin-CDK-CKI ternary complexes (Toyoshima and Hunter, 1994; Poon et al., 1996). Some CKIs function in response to extracellular signals including mitogenic or antimitogenic signals, as well as ECM binding. For example, the levels of p27 are high in quiescent cells as a result of contact inhibition, growth factor deprivation or growth in suspension, and are low as cells progress into G1 phase following mitogenic signals (Bottazzi and Assoian, 1997). The expression of p27 is also increased in response to antiproliferative signals such as cAMP and immunosuppressant rapamycin (Grana and Reddy, 1995). Other CKIs are involved in the feedback loops of checkpoint control such as p21 (Sherr and Roberts, 1995).

Precise transmission of genetic information requires the existence of a number of surveillance systems. These surveillance systems are named checkpoints, which are able to interrupt cell cycle progression once errors are detected, and allow the cell cycle to resume after damages are repaired (Paulovich et al., 1997). They also ensure that each phase occurs only once for each cell cycle (Hartwell and Kastan, 1994). The checkpoints are separated into four classes: restriction point, DNA damage checkpoint, spindle checkpoint, and spindle pole body (centrosome equivalent) duplication checkpoint (Paulovich et al., 1997; Hunter and Pines, 1994).

The restriction point is at a point in late G1 when the cell commits to another round of DNA replication and division. After this point, growth factors are no longer required for the completion of the cell cycle until the next G1. The restriction point is regulated primarily by cyclin D-CDK4/6 complexes, suggesting that deregulation of cyclin D expression might make cells less dependent on growth factors (Hunter and Pines, 1994).

The DNA damage checkpoint functions at three stages of the cell cycle: G1/S transition, S phase progression, and G2/M transition (Elledge, 1996). G1/S transition is regulated by cyclin D-CDK4/6 and cyclin E-CDK2 complexes. This transition is also modulated by INK4 and Cip/Kip families. p53, induced in response to DNA damage, then activates p21 transcription. By binding to cyclin D-CDK4/6 and cyclin E-CDK2 complexes, p21 inhibits their kinase activities to cause G1 arrest (El-Deiry et al., 1994; Xiong et al., 1993). The arrest allows DNA repair proteins to correct DNA lesions and to restore the integrity of the genome. The S phase checkpoint ensures that there is only one S phase in each cell cycle, only one round of genome replication in each S phase, and that the length of the S phase is correctly gated. Cyclin A-CDK2 is required for ongoing DNA replication (Wuarin and Nurse, 1996). G2/M transition is signaled by the activation of cyclin B-CDC2. This kinase complex is activated at the end of G2 via dephosphorylation by CDC25. In response to DNA damage by radiation or alkylating reagents, the prevention of dephosphorylation results in the G2 arrest (Dunphy, 1994). G2/M CDKs also play an additional role in the onset of S phase. Inactivation of G2/M CDK at the end of mitosis enables the chromatin to be permissive for a new round DNA replication

(Wuarin and Nurse, 1996). Failure in controlling checkpoints allows cells to accumulate genetic changes which eventually contributes to the tumor phenotype.

Dysregulation of cell cycle gene expression is closely connected to oncogenesis. While cyclin D1 and CDC25 have been identified as oncogenes (DelSal et al., 1996; Galaktionov et al., 1995), p16 has been demonstrated to be a tumor suppressor gene (Kamb et al., 1994; Serrano et al., 1996). Most interestingly, at least one component of the p16-cyclin D/CDK4-pRb axis is inactivated (tumor suppressor gene) or overexpressed (cyclin D) in most tumor cells (Sherr, 1996). Thus, pRb is constitutively inactive, and causes uncontrolled cell cycle progression.

## IV. Targeting and Scaffolding Proteins

One-third of all proteins in a typical mammalian cell are thought to contain covalently bound phosphate. Genomic sequencing data has lead to estimates that about 2-3% of all eukaryotic genes may code for protein kinases. Approximately one-tenth of the anticipated 2000 protein kinases and 1000 phosphatases have already been identified (Hubbard and Cohen, 1993; Faux and Scott, 1996a). Moreover, a protein kinase or phosphatase can have multiple substrates, and one substrate can be the target of multiple kinases and phosphatases. Given this complexity, the regulation of kinase and phosphatase activity must be a sophisticated process. An important aspect of the regulation is how protein kinases and phosphatases are compartmentalized, that is, how their subcellular localization modulates their specificity and activity (Mochly-Rosen, 1995; Faux and Scott, 1996a; Pawson and Scott, 1997).

One decade ago, Philip and Patricia Cohen proposed that the regulation of protein phosphorylation occurs at several levels, including modification of catalytic activity and subcellular location (Cohen and Cohen, 1989). Later, the 'targeting hypothesis' was introduced (Hubbard and Cohen, 1993). The concept of this hypothesis is that phosphorylation events are regulated not only by the balance between protein kinases and phosphatases, but also by their localization in the cells. The subcellular location is accomplished by the presence of a targeting moiety. The targeting moiety could be a domain of a enzyme or a separate molecule, which directs the catalytic subunit to a restricted subcellular compartment through association with a 'targeting locus'. The targeting locus could be an organelle or a structural protein which is a component of membranes or cytoskeleton. Thus, the targeting moiety ensures the specificity and fidelity of protein phosphorylation by targeted kinases or phosphatases at the correct time in the mitogenic process (Hubbard and Cohen, 1993).

A variation of the targeting hypothesis is the formation of protein tyrosine kinase and phosphatase signaling complexes. In this scenario, the targeting of protein tyrosine kinases and phosphatases is mediated by adaptor proteins, which contain protein-binding modules such as SH2, PTB, PDZ, SH3, WW, and PH domains (Pawson, 1995; Bork et al., 1997; Pawson and Scott, 1997). Many modular proteins such as Grb2, p85, IRSs, Crk and Nck bear one SH2 domain and two SH3 domains, which connect upstream signaling enzymes to downstream targets including cytoskeletal components and low molecular weight G-proteins (Pawson, 1995). These adaptor proteins act as platforms for assembly

of molecules involved in intracellular signal cascades, which are initiated from receptor or non-receptor tyrosine kinases.

The targeting of Ser/Thr kinases or phosphatases is achieved by anchoring proteins, which restrict these kinases or phosphatases to certain subcellular structure and localize them close to their site of action (Faux and Scott, 1996b; Lester and Scott, 1997). In contrast to Tyr kinases and phosphatases, Ser/Thr kinases and phosphatases appear to constitutively co-localized with their substrates at appropriate subcellular compartment. Once stimulating signals are available, they become activated (Pawson and Scott, 1997). Ser/Thr phosphatases, protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and components of MAPK pathways have now been shown to be tethered to their substrates by associating with different types of targeting proteins (Mochly-Rosen, 1995; Lester and Scott, 1997; Pawson and Scott, 1997; Whitmarsh and Davis, 1998).

PKC consists of a family of Ser/Thr kinases implicated in regulating mitogenesis, differentiation, cell morphology, metastasis and vesicle translocation (Dekker and Parker, 1994). PKCs are composed of at least eleven different isoforms which can be classified into three groups based on the sequence homologies and requirements for the cofactor PS (phosphatidylserine), $Ca^{2+}$ and DAG (diacylglycerol): conventional PKCs ($\alpha$, $\beta$, $\gamma$) require all three cofactors; novel PKCs ($\delta$, $\varepsilon$, $\eta$, $\theta$) are dependent on PS and DAG; atypical PKCs ($\zeta$, $\lambda$) are only stimulated by phospholipid (Nishizuka, 1992; Newton, 1995). Because many cells express multiple isoforms of PKC, the subcellular localization of specific PKC isoforms may contribute to a certain degree of selectivity towards their physiological substrates (Lester and Scott, 1997).

The targeting of PKCs requires not only protein-lipid interactions, but also protein-protein interactions. Over the past several years, a number of PKC targeting proteins have been identified, and they are grouped into at least three classes (Lester and Scott, 1997). The first class of PKC targeting proteins, termed PKC substrate/binding proteins, were isolated by overlay assays (Liao et al., 1994). They associate with PKC in a PS-dependent manner, and phosphorylation negatively regulates their association with PKC. MARCKS and adducins belong to this class (Chapline et al., 1993; 1996). The second class of PKC targeting proteins, named RACKs (Receptors for Activated C-kinase), were also identified by overlay assay. RACKs are PKC binding proteins but not substrates. They interact with PKC in a PS-independent manner (Mochly-Rosen, 1995). The third class of PKC targeting proteins, designated PICKs (Proteins that Interact with C-Kinase), were cloned by two-hybrid screening. Phosphorylation of PICK-1, the only one characterized, does not affect its association with PKC (Staudinger et al., 1995). The fourth class of PKC targeting proteins are likely to exist, and presumably interact with PKC through PH (plextrin homology) domain (Dellambra et al., 1995). However, whether these proteins are PKC targeting proteins *in vivo* remains to be determined.

The targeting of PKA is mainly achieved by association of its RII regulatory subunit with AKAPs (A-kinase anchoring proteins) (Rubin, 1994). AKAPs were originally identified by overlay assay, and recently by screening cDNA expression libraries using recombinant RII probe (Carr et al., 1992; Nauert et al., 1997). AKAPs contain two distinct functional domains: a conserved amphipathic helix which is an RII binding site, and an unique targeting sequence which is responsible for localization to

intracellular sites. Therefore, AKAPs should be considered as targeting loci based on the targeting hypothesis. AKAPs direct PKA to specific subcellular compartments such as ER (AKAP100), plasma membrane (AKAP79), mitochondria (AKAP84), peroxisomes (AKAP220), and golgi (AKAP85) (Faux and Scott, 1996).

Recently, a new class of targeting proteins, named scaffolding proteins, have been identified. The scaffolding proteins simultaneously interact with multiple kinases or phosphatases and coordinate the location of multienzyme signaling complexes (Faux and Scott, 1996a). A "classic" scaffolding protein is expected to possess the following properties: i) association with several enzymes using distinct binding sites; ii) facilitating signaling of bound enzymes; iii) insulating bound enzymes from non-specific stimuli. The scaffolding proteins were originally thought to be specific for Ser/Thr protein kinases and phosphatases, but later were expanded to include Tyr kinases. Typical scaffolding proteins include STE5 in yeast, INAD in drosophila, caveolin, JIP-1 (JNK-interacting protein 1), AKAP79 and AKAP250 in mammalian (Lester and Scott, 1997; Whitmarsh and Davis, 1998; Okamoto et al., 1998).

In the budding yeast *S. cerevisae*, each component of the mating MAPK pathway associates simultaneously with a distinct region of scaffolding protein STE5. STE5 clusters components of this MAPK cascade to achieve tight regulation and to provide selectivity by preventing cross-talk between functionally unrelated MAPK modules in the same cell (Yashar et al., 1995). The presence of STE5 suggests that a mammalian homolog may exist. Recent studies indicate that JIP-1 selectively associates with multiple

components of JNK signaling cascade, and that JIP-1 functions similarly to STE5 (Dickens et al., 1997; Whitmarsh et al., 1998).

Caveolins are major components of caveolae, a vesicular invagination of the plasma membrane. Caveolins interact with a number of signaling molecules, such as G-protein α subunits, Ha-ras, Src family tyrosine kinases, endothelial nitric oxide synthase (eNOS), EGFR and PKCs, to generate preassembled signaling complexes (Okamoto et al., 1998).

Certain AKAPs are scaffolding proteins. For example, AKAP79 associates simultaneously with PKA, PKC and $Ca^{2+}$/*calmodulin*-dependent protein phosphatase 2B, calcineurin (Klauck et al., 1996; Coghlan et al., 1995). AKAP79 restricts the two multifunctional kinases and one broad-specificity phosphatase to postsynaptic densities, suggesting that reversible phosphorylation by kinases and phosphatases in synaptic signaling is coordinated by AKAP79 (Klauck et al., 1996). AKAP250 is also named Gravin. Distinct regions of AKAP250 interact with PKA and PKC, indicating a role of AKAP250 as a scaffolding protein (Nauert et al., 1997). Although evidence suggests that AKAP250 is involved in the cell adhesion to ECM, the physiological function of AKAP250 remains unclear. Sequence analysis indicates that the N-terminal half of Gravin is homologous to SSeCKS. It is proposed that either Gravin and SSeCKS represent human and murine homologs of the same protein, or they are distinct members of an emerging family of mammalian scaffolding proteins.

# Chapter II

# Materials and Methods

**Cells:** NIH3T3 fibroblasts (ATCC # CRL1658) and NIH/v-*src* (Gelman and Hanafusa, 1993) were grown in Dulbecco's modified Eagle's media (DMEM) containing 10% heat-inactivated calf serum (GIBCO), 100 units/ml of penicillin, 100 units/ml of streptomycin and 250 ng/ml of amphotericin B (Fungizone) (GIBCO). Rat-6 embryonic fibroblasts, Rat-6/*src*, Rat-6/*ras*, Rat-6/*myc*, Rat-6/*neu*, Rat-6/*raf*, Rat-6/*mos*, and Rat-6/*fos* (gifts of Robert Krauss, Mount Sinai School of Medicine), were grown as described above (Borner et al., 1992). 3Y1 (Fisher) rat fibroblasts and 3Y1/*ts*72*src* cells, containing a temperature sensitive allele of Rous sarcoma virus (Mayer et al., 1986), were kept at 35$^0$C (permissive temperature for ts *src* allele) at the same media. S2-6 cell line is a gift of David Schatz (Yale School of Medicine), and grown in histidine-deficient DME (Irvine Scientific) supplemented with 0.5 μM L-histidinol (Sigma), calf serum, penicillin/streptomycin/amphotericin B, and 0.5 μg/ml of tetracycline (tet) (Sigma). S2-6 is a derivative of NIH3T3 transfected with pTet-tTa, in which pUHD15-1 plasmid (Gossen and Bujard, 1992) was modified by addition of 7 copies of the tetracycline operator sequence (Shockett et al., 1995). $\Omega_e$ and BOSC23 (Pear et al., 1993), two packaging cell lines, were grown in the DMEM media, too.

## Generation of stable cell lines:

**1. Constitutive SSeCKS overexpressing cell lines:** Full length and an SSeCKS cDNA missing the N-terminal 1.1 kb were released from pBluescript II SK (Stratagene) by EcoRI digestion and then inserted into the replication-defective retroviral vector pBabe*hygro* (Morgenstern and Land, 1990). 10 μg of the resulting constructs or empty vector were transfected into 70-80% confluence $\Omega_e$ packaging cells (seeded the prior day) using the calcium phosphate precipitation technique, and transiently-expressed virus was harvested 24 h later. Target cells seeded at 50-70% confluence were infected for 2 hours with 1ml of filtered virus (0.22mM; Gelman Science) in the presence of 8 μg/ml of polybrene (Sigma). Two days later, these cells were split 1:10 and grown in media supplemented with 85 μg/ml of hygromycin (Sigma) for 2-6 weeks for the formation of colonies. Production of these cell lines was also carried out by direct transfection, in which the same constructs were introduced into 50-70% confluence target cells by the method described above. At 40-48 h post-transfection, the cultures were split and grown in hygromycin containing media.

**2. Tetracycline-regulated SSeCKS overexpressing cell lines:** Full length SSeCKS cDNA was spliced into pUHD10-3, a plasmid containing a tTA-dependent promoter in front of a multiple cloning site for a gene of interest (gift of Hermann Bujard, Gossen and Bujard, 1992), at the EcoRI site. 10 μg of the resulting pUHD10-3/SSeCKS or empty pUHD10-3 together with 1 μg of pBabe*puro* were co-transfected into S2-6 cells using calcium phosphate precipitation method. Selection of stable cell lines was performed as described above using media specific for S2-6 cells supplemented with 2 μg/ml of puromycin (Sigma). The S2-6/S24 cell line, one of the resulting clones, was used later for the production of temperature-sensitive *src*-expressing cell lines and cyclin D1 overexpressing

cell lines. S2-6/V3, a control clone, was also used later for generating cyclin D1 overexpressing cell lines.

**3. Temperature-sensitive *src*-expressing cell lines:** pLJ/ts72*src* and pLJ retroviral vectors, harboring a neomycin-resistance gene ($neo^R$), were independently introduced into $\Omega_e$ packaging cells using the calcium phosphate precipitation method. Supernatants were harvested after 24 h and used to infect S2-6/S24 cells in the presence of 8 μg/ml of polybrene (Sigma). Following G418 (400 μg/ml) (GIBCO) selection at 35°C (permissive temperature = PT), individual G418-resistant, morphologically transformed colonies were expanded and maintained in G418 media at 35°C. Clones were selected which were morphologically transformed at the PT but untransformed after 2-3 days growth at the non-permissive temperature (NPT = 39.5°C).

**4. Cyclin D1 overexpressing cell lines:** Viruses producing pMV7/cyclin D1 or pMV7 (gifts of Robert Krauss, Mount Sinai School of Medicine) were used independently to infect S2-6/S24 or S2-6/V3 cells. The infection procedure was carried out as mentioned above. Stable cell lines were selected by adding 400 μg/ml of G418 (GIBCO) into media.

**Northern blot analysis:** Total cytoplasmic RNA was isolated directly from cells on culture plates or from tissues by the RNA STAT-60 method (TEL-TEST, INC). 20-30 μg/lane of total RNA was electrophoresed through 1% agarose gels containing formaldehyde, transferred onto Immobilon N membrane (Millipore Corporation), and hybridized with $^{32}$P-SSeCKS cDNA in Rapid Hybridization Buffer (Amersham).

**Southern blot analysis:** Genomic DNA was isolated from cultured cells as described previously (Sambrook et al., 1989). 10 μg of DNA was cut to completion with EcoRI or HindIII, and then electrophoresed through 1% agarose gel, blotted onto Immobilon N membrane (Millipore Corporation), hybridized with $^{32}$P-SSeCKS cDNA in Rapid Hybridization Buffer (Amersham).

**Western blot analysis:** Cells grown in various conditions were washed three times with ice-cold PBS (phosphate buffered saline), scraped into micro centrifuge tubes, and lysed with RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 8% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 2 μg/ml of antipain, 2 μg/ml of pepstatin). Protein content was normalized using Bio-Rad Protein Assay Kits (Bio-Rad Laboratories). Equal amount of protein from different samples were separated by SDS-PAGE (5-10%) and then electrophoretically transferred to PolyScreen PVDF membrane (NEN) in transfer solution (25 mM Tris base, 192 mM glycine and 20% methanol). The filter was blocked with TBS-T (10 mM Tris-HCl pH7.4, 50 mM NaCl and 0.1% Tween-20) containing 5% non-fat dry milk for 2 h at room temperature, and then incubated with primary antibody for 2 h at room temperature. Following washing in TBS-T three times (15 min each time), the filter was subjected to binding of either horseradish peroxidase (HRP)-, or alkaline phosphatase-conjugated secondary antibody for 1 h at room temperature. After extensive washing, the

blot was visualized either by color development using Western Blue (Promega) as substrate of alkaline phosphatase, or by the enhanced chemiluminescence (ECL) method (Amersham). For detection of pRb phosphorylation, cells were lysed in NETN buffer (1% NP-40, 2 mM EDTA, 50 mM Tris-HCl pH8.0, 250 mM NaCl, 1 mM dithiothreitol, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 2 μg/ml of antipain, 2 μg/ml of pepstatin), and then the same protocol was used. When PY20 (anti-phosphotyrosine Ab) was utilized as a primary antibody, BSA instead of dry milk was used in the blocking solution.

**Antibodies:** Rabbit anti-SSeCKS polyclonal antibody was prepared in our laboratory (Lin et al, 1996). Anti-avian SRC monoclonal EC10 antibody was a gift of S. Parsons, and anti-SRC polyclonal TBR antibody was purchased from East-Acres Biologicals. Mouse monoclonal PY20 antibody is specific for phosphotyrosine (Transduction Labs). Polyclonal antibodies for p18 and p19 were gifts of Selina Chen-Kiang. Polyclonal antibodies for p21, p27, cyclin A, D1, E, CDK2, CDK4, CDK6 and ERK2 were purchased from Santa Cruz Biotechnology. Monoclonal antibodies for cyclin D1 and pRb were purchased from PharMingen. Anti-p16 polyclonal antibody was purchased from Clontech. Anti-PKC type III monoclonal antibody was purchased from Upstate Biotechnology Inc. Anti-vinculin monoclonal antibody was purchased from Sigma. Alkaline phosphatase-labeled sheep anti-rabbit IgG or goat anti-mouse IgG (Boehringer Mannheim) were used as secondary antibodies for color development. HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Chemicon) were used as secondary antibodies for ECL.

**Proliferation assay:** $10^4$ S24/ts*src* or S24/pLJ cells were seeded into 24-well plates, and the next day the cells were trypsinized and counted to establish a baseline plating efficiency. The remaining cells were grown in media containing either high (10%) or low (0.5%) heat-inactivated calf serum (GIBCO) in the presence or absence of tetracycline at the PT or NPT. Duplicate wells were trypsinized and counted every two days using a hemacytometer (Fisher). S24, V4, S24/D1 and S24/V cells were treated similarly except they were incubated in media supplemented with 10% calf serum at $37^0$C.

**Transformation assays:** Cellular transformation was evaluated by visual examination of cell morphology, contact-independent growth, and anchorage-independent growth. Contact-independent growth was monitored by foci formation as follows: 1 μg of pMv-*src* plasmid DNA and 10 μg of SSeCKS/pBabe*hygro* or 10 μg of pBabe*hygro* were co-transfected into NIH3T3 cells using the calcium phosphate precipitation method. Foci were counted following fixation with methanol and staining with 0.4% crystal violet 18 days after transfection. Aliquots of the transfected cells were grown in the presence of 85 μg/ml of hygromycin for two weeks, and then the numbers of hygromycin-resistant colonies were scored to serve as a control. Anchorage-independent growth was examined by colony formation in soft agar as follows: two days after co-transfection, 2-20 x $10^6$ cells were mixed with top agar (0.4%) components supplemented with 85 μg/ml of hygromycin and cultured for 3 weeks. The colony number was determined by counting. To compare the anchorage-independent growth of S24/ts*src* to S24/pLJ, $10^5$ cells were mixed with top agar

in the presence or absence of tetracycline and then grown for 3 weeks at the PT or the NPT.

***In Vivo* Phosphorylation analysis:** $10^6$ Rat-6 cells were incubated overnight in DMEM supplemented with 0.5% calf serum and then twice for 1 h in DMEM without sodium phosphate (Life Technologies). Labeling was for 2 h in modified essential medium without phosphate supplemented with 150 μCi of [$^{32}$P]orthophosphate (DuPont NEN). In some cases, phorbol 12-myristate 13-acetate (PMA, 200 nM) was added for various durations at the end of this labeling period. The PKC-specific inhibitor, *bis*-indolylmaleimide (Boehringer Mannheim; 10 μM), was added at the beginning of the labeling period and again when PMA was added. After washing the cells three times with ice-cold phosphate-buffered saline, the cells were lysed in RIPA buffer, and analyzed by SDS-PAGE.

## *In Vitro* Kinase assays:

**1. SRC kinase assay:** Cells grown under various conditions were lysed with RIPA buffer. Lysates containing 200 μg of protein were incubated with rabbit anti-SRC polyclonal antibody pre-bound to Affi-Prep protein A beads (Bio-Rad Laboratories). The immunocomplex was washed twice with RIPA buffer containing 300 mM NaCl, twice with 10 mM NaCl, and twice with kinase buffer (50mM Tris pH8.0/10mM $MnCl_2$). SRC kinase activity was performed in the kinase buffer containing 10uCi of $\gamma$-$^{32}$P[ATP] for 10 min at room temperature as previously described (Gelman et al., 1993). The reaction mixtures

were washed three times with RIPA buffer and electrophoresed through a 7.5% SDS-PAGE. Then the gel was fixed with 10% methanol and 7% acetic acid, and dried. The kinase activity was detected by autoradiography.

**2. ERK2 Kinase assay:** Cells were serum-starved overnight and then stimulated with 10% calf serum-containing media for various periods. Following lysis in RIPA buffer, the lysates were immunoprecipitated with rabbit anti-ERK2 antibody prebound to Affi-Prep protein A beads. The immunocomplex was washed twice with RIPA buffer and twice with kinase buffer (10mM Hepes pH7.5, 10 mM magnesium acetate). Approximate 20 μl of the bead-antibody-antigen complex was resuspended in 20 μl of myelin basic protein (MBP, 2 mg/ml) and 20 μl 3X hot mix (30 mM Hepes pH7.5, 30 mM MgAc, 150 μM cold ATP, 10 μCi $\gamma$-$^{32}$P[ATP]), and incubated for 30 min at 30°C. The reaction was stopped by adding 60 μl of 2X protein loading dye. This mixture was boiled and electrophoresed through a 15% SDS-PAGE, followed by autoradiography.

**3. JNK kinase assay:** pGEX-5x-1/JUN expression vector (gift of Lu-Hai Wang, Mount Sinai School of Medicine) and empty vector were introduced independently into BL21(DE3)pLysS bacteria to induce fusion protein expression. 50 μg of GST-JUN-, or GST-glutathione-sepharose beads were incubated with cell lysates in RIPA buffer. The beads were then washed twice with RIPA buffer, followed by two washes with kinase buffer (50 mM Tris pH8.0, 5 mM $MnCl_2$, 5 mM $MgCl_2$). The kinase activity was assayed in kinase buffer supplemented with 10 μCi of $\gamma$-$^{32}$P[ATP] for 10 min at room temperature. After three washes with RIPA buffer, the reaction mixtures were boiled and electrophoresed through a 10% SDS-PAGE, followed by autoradiography.

**4. CDK2 kinase assay:** Cells were lysed in RIPA buffer, and the lysates were incubated with anti-cyclin E antibodies prebound to Affi-Prep protein A beads. The immunocomplex was washed three times with RIPA buffer and two times with Histone H1 assay buffer (50 mM Hepes pH7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, 1mM DTT), and resuspended in a small volume of the assay buffer supplemented with 20 μM cold ATP and 4 μg Histone H1 (GIBCO). The kinase assay was initiated by adding 10 μCi of $\gamma$-$^{32}$P[ATP]. After 10 min incubation at 30°C, the supernatants were collected and electrophoresed through a 10% SDS-PAGE, followed by autoradiography.

**Cell cycle analysis:** Cell cycle progression was monitored by quantifying the percentage of cells in different phases of cell cycle by flow cytometry as described previously (Zhu et al., 1993; Kaplan et al., 1998). Synchronized cells were harvested by trypsinization, washed in PBS, and fixed in ice-cold 70% ethanol ($10^6$ cells/1 ml ethanol) for at least 2 h at -20$^0$C. Before flow cytometry analysis, the cells were pelleted, washed in PBS, and stained with 20 μg/ml of propidium iodide (Sigma) containing 1 μg/ml of RNase A for at least 2 h at room temperature. Analysis was performed on a FACScan machine (Becton Dickenson). Percentages of cells in specific cell cycle phases were determined by the intensity of propidium iodide staining using the CellFIT analysis software.

**Construction and expression of GST fusion proteins:** pGEX-5x-1 vector (Pharmacia) was used to generate GST fusion constructs. All SSeCKS cDNA fragments

were produced by PCR amplification from 1322/pBluescript (1322: SSeCKS cDNA deleted of 1.2kb N-terminus), SSeCKS/pBluescript or p53ext2 (pBluescript SK II carrying an N-terminal 1.2Kb portion of SSeCKS cDNA, a gift of Susan Jaken), and cloned in-frame into pGEX-5x-1. Each of the sense primers was derived from coding sequence of SSeCKS cDNA with additional nucleotides in the 5'-end to produce an EcoRI site. All of the resulting constructs were sequenced with Sequenase 2.0 kits (US Biochemicals) to assure that there were no mutations. BL21 (DE3) pLysS bacteria (Novagen) were transformed with these constructs, grown in LB/Amp medium containing 20mM glucose at $37^0$C. Upon reaching an $OD_{600}$ of 0.6, the bacteria were centrifuged, resuspended in the same medium plus 1mM IPTG (Jersey Lab Supply), and grown for another 2 h at $30^0$C to induce protein expression. The pelleted bacteria were lysed with lysis buffer (50 mM Tris-HCl, PH8.0, 1 mM EDTA, 100 mM NaCl, 1 mM NaF, 1 mM $Na_3VO_4$, 2 μg/ml each of aprotinin, leupeptin, antipain, pepstatin A and 1 mM PMSF) containing 270 μg/ml lysozyme (Sigma) and 4 mg/ml deoxycholic acid as described (Sambrook et al, 1989). When the lysates became viscous, endonuclease (benzonase, Sigma) and $MgSO_4$ were added to a final concentration of 300U and 5mM respectively. When the lysates lost viscosity, debris was removed by centrifugation at 16,000 rpm for 15 min at $4^0$C. The supernatants were filtered, applied to a glutathione-Sepharose column (Pharmacia) according to the manufacturer's description, and rotated overnight at $4^0$C. The column was washed with lysis buffer for three times, and the bound GST fusion proteins were eluted with 50 mM Tris-HCl, pH8.0 containing 15 mM reduced glutathione (Sigma) for three times. The concentrations of the fusion proteins were detected by Bio-Rad Protein Assay. The expression of fusion proteins

were visualized following electrophoresis through SDS-PAGE and coomassie blue staining.

***In vitro* PKC phosphorylation assay and mapping of phosphorylation sites:** PKC assays were performed as previously described (Graff et al., 1989). Briefly, 1-10 μg of GST fusion proteins (or GST alone as control) were mixed with 0.2 μl of purified rabbit brain PKC (gift of Alan Aderem, Rockfellor University) in reaction buffer (20 mM Tris-HCl pH7.5, 5 mM $Mg(OAc)_2$, 0.01% leupeptin, and 0.5 mM $CaCl_2$) containing 20 μM of cold ATP and 0.31 mg/ml of PS (L-α-phosphatidyl-L-serine) (Sigma). The reaction was initiated by the addition of 10 μCi of $\gamma$-$^{32}$P[ATP]. After incubation for 30 min at 37$^0$C, the phosphorylated products were subjected to electrophoresis through SDS-PAGE and autoradiography. PKC[19-36] (Life Technologies), a PKC-specific inhibitor peptide (pseudosubstrate), was included (7.5 mM) in some reactions.

To identify specific phosphoamino acids, *in vitro* PKC phosphorylation was performed as mentioned above except that 50 μg of substrates, 1mM final concentration of ATP, and 1 μl of purified PKC were used in a 120 min reaction. DTT was then added to a final concentration of 2mM, followed by the addition of iodoacetamide (Sigma) (final concentration of 5mM). The mixture was incubated at room temperature for 15 min, and then DTT was added again (final concentration of 2mM). The phosphorylated GST fusion proteins were electrophoresed and transferred to PVDF membrane. Membrane pieces containing $^{32}$P-labeled fusion proteins were digested with 5 μg of *Staphylococcus aureus* V8 protease (Boehringer Mannheim) in 0.1 mM ammonium bicarbonate containing 1%

hydrogenated Triton X-100 (Cal Biochem) overnight at 37$^0$C. The released peptides were resolved by HPLC, and radioactive fractions were subject to microsequencing (performed by Ronald Kohanski, Mount Sinai School of Medicine).

## *In vitro* pull-down assay:

**1. PKC pull-down assay:** 50 μg of GST-1322 which was pre-bound to glutathione-sepharose beads was mixed with 1 mg of cell lysates from Rat-6 or Rat-6/PKCα overexpressor cells (gift of I. B. Weinstein, Columbia University), or 20 ng of purified rabbit brain PKC (a mixture of α, β, and γ isotypes) (Upstate Biologicals, Inc.), and rotated for 4 h at 4$^0$C in RIPA buffer containing 5 mM $MgCl_2$ and 0.2 mM $CaCl_2$. 0.31mg/ml of PS was included in some reactions to serve as a control. Following washing, electrophoresis, and transferring, immunoblotting was performed using monoclonal anti-PKC-type III antibody (Upstate Biologicals, Inc.).

**2. Cyclin D1 pull-down assay:** Cyclin D1 pull-down assays were carried out similarly as described previously (Chen et al., 1996). Briefly, cells were lysed in binding buffer (20 mM Tris-HCl pH7.4, 1 mM EDTA, 25 mM NaCl, 10% glycerol, 0.01% Nonide P-40, 1 mM DTT, 1 mM $Na_3VO_4$, 2 μg/ml each of aprotinin, leupeptin, antipain, pepstatin A and 1 mM PMSF). 500 μg of the lysates were incubated with 15 μg of wild type or mutant GST-SSeCKS2 or GST prebound to glutathione-sepharose beads for 3 h at 4$^0$C on a rotating wheel. After the beads were washed four times in the binding buffer, bound proteins were eluted by boiling in protein loading dye, separated by a 10% SDS-PAGE, transferred to PVDF membrane, detected by immunoblotting using anti-cyclin D1 antibody.

**Construction of CY motif mutants:** Mutations of two potential CY motifs were generated using a Transformer™ Site-Directed Mutagenesis Kit (Clontech). SSeCKS2 (a.a. residues 389-552) was released from pGEX 5x-1 by restriction digestion and cloned into pBluescript plasmid, and an unique restriction site *Sca* I at the pBluescript was chosen to serve as a selection marker (*Sca* I to *Stu* I, or vice-a-versa). "Trans" and "switch" selection primers are: 5'GTGACTGGTGAGGCCTCAACCAAGTC (*Sca* I to *Stu* I), and 5'GTGACT GGTGAGTACTCAACCAAGTC (*Stu* I to *Sca* I), respectively. Trans mutagenic primers are as follows: 5'GGAAGTCCCTTGTCGAGCCTCTTCAGTAGC (first KK to SS), 5'GCTC AGGCTTAAGCTCGCTGTCTGGG (second KK to SS), 5'CCCTTGAAGAAAAGC TTCAGTAGC (first L to S), 5'GGCTTAAAGAAGTCGTCTGGGAAG (second L to S). Switch mutagenic primers are: 5'CCCTTGTCGAGCAGCTTCAGTAGC (first L to S) and 5'GGCTTAAGCTCGTCGTCTGGGAAG (second L to S). After denaturation, the target plasmid pBluescript/SSeCKS2 was annealed with primers, followed by synthesis of mutant DNA strand. Primary selection was carried out by restriction digestion. The mutant plasmid was amplified, and was then subject to second restriction enzyme digestion and final characterization by sequencing. The resulting mutant SSeCKS2 was spliced back to pGEX 5x-1 for fusion protein expression.

**Cross-linking of GST-SSeCKS to calmodulin:** 10 μg each of the GST-SSeCKS fusion proteins containing a single calmodulin binding domain and consensus PKC

phosphorylation motif was incubated with 0.25 μCi of $^{125}$I-calmodulin (Du Pont) in 35 mM Hepes-NaOH pH7.0 containing either 5 mM $CaCl_2$ or 5 mM EGTA for 1 h at room temperature (Graff et al., 1989). The resulting mixture was incubated for another 20 min at room temperature following the addition of cross-linking reagent dimethyl pimelimidate (Pierce) (final concentration of 4 mM). In some cases, GST-SSeCKS was first phosphorylated by PKC as mentioned above except substituting 100 μM of cold ATP in stead of radiolabeled ATP. GST was included in some reactions to serve as a control. The final reaction mixture was electrophoresed on SDS-PAGE, followed by autoradiography.

**Immunofluorescence analysis:** Cells were seeded onto sterilized 22-mm$^2$ coverslips, washed in room temperature Hank's, and fixed in either 60% acetone/3.7% formaldehyde for 20 min at -20$^0$C as previously described (Gelman and Silverstein, 1986) or 2% formaldehyde/0.1% glutaraldehyde/0.05% Triton X-100 for 20 min at room temperature. After three washes in Hank's, the coverslips were incubated with primary antibodies including anti-SSeCKS, -cyclin D1, and -vinculin. Secondary antibodies were FITC-labeled anti-rabbit IgG (Boerhinger Mannheim) and TRITC-labeled anti-mouse IgG (Chemicon). TRITC-labeled phalloidin (Sigma) was used to visualize F-actin. In some experiments, cells were treated with phorbol ester (200 nM PMA) and/or 10 μM PKC-specific inhibitor, *bis*-indolylmaleimide (Boerhinger Mannheim) before fixing. Following washing, the coverslips were mounted to slides using Prolong Antifade Kit (Molecular Probes). Slides were visualized on a Zeiss Planopar fluorescent microscope or a Leica CLSM laser confocal microscope.

# Chapter III

# Re-expression of the Major Protein Kinase C Substrate, SSeCKS, Suppresses v-*src* Induced Morphological Transformation and Tumorigenesis

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## ABSTRACT

In an attempt to isolate novel regulatory /tumor suppressor genes, we identified cDNAs whose abundance is low in NIH3T3 cells and further decreased following the expression of the activated oncogene, v-*src*. One such gene, SSeCKS (pronounced *essex*), is down-regulated in *src*- and *ras*-, but not in *raf*-transformed rodent fibroblasts. Using a panel of *ras*-transformed or revertant Rat-6 cells which exhibit selective parameters of transformation, we show that down-regulation of SSeCKS correlates with anchorage-independent growth. Co-transfection of NIH3T3 fibroblasts with an SSeCKS expression plasmid decreased 6- to 9-fold the ability of a v-*src* expressor plasmid to induce colonies in soft agar. To investigate whether SSeCKS possesses tumor suppressive function, we developed conditionally-transformed cell lines (expressing ts72*src*) with tetracycline-regulated SSeCKS expression. SSeCKS suppressed the ability of v-*src* to induce increased cellular refractility, focus formation, soft-agar colony formation, growth in low serum (0.5%), and increased saturation density, but did not inhibit cell proliferation in high serum

(10%) at the permissive (35°C) temperature for *src* kinase activity. However, at the non-permissive (39.5°C) temperature, SSeCKS induced growth arrest. SSeCKS expression did not affect i) the protein level, *in vivo* or *in vitro* kinase activity of ts72*src*, ii) the activity of c-Jun N-terminal kinase (JNK), iii) the level of MAP kinase (ERK2) protein. However, ERK2 activity was induced 5- to 10-fold by SSeCKS in the presence of active *src*. SSeCKS reversed v-*src*'s ability to decrease the formation of vinculin-associated adhesion plaques and actin-based stress fibers. These data suggest a tumor suppressive role for SSeCKS via the control of cytoskeletal architecture and cell signaling.

## INTRODUCTION

Tumor suppressor genes function as anti-oncogenes. Functional inactivation of tumor suppressor genes is an essential step in the oncogenicity of several types of human cancers. The well-characterized tumor suppressor gene families such as p53 and Rb lose their functions as a result of mutation or deletion of the wild type allele (Knudson, 1993; Levine, 1993). They have been referred to as class I tumor suppressor genes (Lee et al., 1991). In addition to mutation/deletion, the function of tumor suppressor genes can be eliminated by other means (Brown, 1997). For example, Rb can be alternatively inactivated through disruption of gene transcription such as CpG island hypermethylation (Sakai et al., 1991; Greger et al., 1994). Recently, a number of transcriptionally down-regulated genes in transformed or tumor cells, such as non-muscle α-actinin (Gluck et al., 1993), tropomyosin (Prasad et al.,1993; Gimona et al., 1996), vinculin (Fernandez et al., 1992) and maspin (Zou et al., 1994), have been shown by gene transfer techniques to encode potential tumor

suppressors. They are referred to as class II tumor suppressor genes (Lee et al., 1991). Isolation and characterization of the class II tumor suppressor genes is of particular interest since these genes themselves are intact and therefore their expression could be restored by drug treatment, providing the possibility for novel therapeutic modalities for cancer.

The cloning and identification of the class I tumor suppressor genes was accomplished by cumbersome pedigree and cytogenetic analyses (Sager, 1989). Class II tumor suppressor genes such as *NO3* (Ozaki and Sakiyama, 1994), maspin (Zou et al., 1994) and elafin (Zhang et al., 1995) were isolated using screens for differential expression in oncogene-transformed or metastasis-specific tumors. These findings suggest that additional suppressor genes could be identified in gene populations whose expression is down-regulated in response to oncogenic stimuli such as activation of oncogenes. As described previously, a novel PCR-based subtractive hybridization method was used in our laboratory to isolate genes expressed at low basal levels in NIH3T3 cells and at suppressed levels in v-*src*-transformed NIH3T3 (Frankfort and Gelman, 1995). We envisioned that such identified genes might represent potential tumor suppressors or mitogenic regulators.

Several criteria are used to define these down-regulated genes as tumor suppressors. First, their expression is down-regulated in tumor cell lines and primary tumors. Second, ectopic re-expression of their products in transformed or tumor cell lines should suppress their tumorigenic phenotype such as loss of anchorage-independent growth. Third, forced expression in tumor cell lines would diminish their ability to form tumors *in vivo* (Brown, 1997). A central enigma in this field is whether re-expression of class II genes represents *bona fide* tumor suppression or non-specific toxicity. The application of tetracycline-

regulated gene expression system in controlling potential tumor suppressor production eliminates the toxicity problem since the levels of these putative suppressors can be regulated to approximate that in non-transformed, normal conditions by using different levels of tetracycline (Gossen and Bujard, 1992; Shockett et al., 1995; Paulus et al., 1996; Hofmann et al., 1996).

Many class II tumor suppressors are cytoskeletal and adhesion-plaque proteins (Ben-Ze'ev, 1997). The critical role of the cytoskeleton in transformation has been demonstrated for many years. In malignant transformation, cytoskeletal protein expression is altered, and cytoskeletal organization is disrupted (Hunter, 1997). A rapid disruption of actin stress fibers is one of the early events in oncogene-induced transformation (Tikoo et al., 1994). The changes in the levels of cytoskeletal and plaque proteins are suggested to induce alteration of focal adhesion structures, and thus may have far-reaching effects on the oncogenic growth of cells. However, it is likely that focal adhesion structures in tumor cells are turned over more rapidly than in normal cells, rather than totally abrogated. Several lines of evidence indicate that the ability of the focal adhesion kinase (FAK) to regulate adhesion plaque turnover (Ilic et al., 1995; Zachary and Rozengurt, 1992) is increased by v-*src*-induced tyrosine phosphorylation (Zachary and Rozengurt, 1992; Chrzanowska-Wodnicka and Burridge, 1994). The overexpression of cytoskeletal proteins, then, may drive the balance towards less turnover.

Clone *322* was isolated from the previous subtractive screening (Frankfort and Gelman, 1995). The steady-state level of *322* transcripts in NIH/v-*src* cells is >15-fold less than in NIH3T3 controls. Moreover, the *322* gene is identical to a PKC substrate/binding

protein, ">200 kDa", identified by overlay assay (Chapline et al., 1996). Based on these characteristics, we named the *322* gene product SSeCKS for Src-Suppressed C Kinase Substrate (Lin et al., 1996). Immunofluorescence studies reveal that SSeCKS is mainly localized in the cytoplasm, and associated with a cortical actin network (Gelman et al., 1998; Lin et al., 1996).

In this study, we show that the transcript level of SSeCKS is suppressed in *src*- and *ras*- induced transformation, and that the down-regulation is not a non-specific consequence of transformation. SSeCKS possesses tumor suppressive activity as demonstrated by inhibition of *src*-induced parameters of morphological transformation. The tumor suppressive function of SSeCKS is not due to non-specific toxicity since its re-expression to a "normal" physiological level is able to repress *src*-mediated anchorage-independent growth. Our data also suggest that SSeCKS' tumor suppressive effect is manifested through the re-organization of cytoskeletal architecture.

## RESULTS

**The level of SSeCKS transcripts is suppressed in v-*src*- and v-*ras*-, but not v-*raf*-transformed cells.**

SSeCKS, isolated in an attempt to identify v-*src*-suppressed novel tumor suppressors or mitogenic regulators (Frankfort and Gelman, 1995), hybridized to a 6.1-kb message. The level of SSeCKS transcript is decreased at least 15-fold in NIH/v-*src* cells when compared with that in NIH3T3 cells (Fig. 3-1A). This phenomenon is not cell type specific since the down-regulation is also observed in *src*-transformed Rat-6 and 3Y1 cells

(Fig. 3-1C and 3-1D). In addition, the basal steady-state transcript level of SSeCKS is >10-fold less than that of the abundantly expressed type I collagen (data not shown), which is also down-regulated by v-*src*. To test whether the suppressed expression of SSeCKS results from deletions in the gene locus, southern blot analysis was performed. Genomic DNAs from NIH3T3 and NIH/v-*src* cells appeared to have exactly same pattern following EcoRI and HindIII digestion, indicating that no gross alteration occurred to the SSeCKS allele (Fig. 3-1B).

We next asked whether the down-regulation of SSeCKS is a generic phenomenon in all transformed cells. Northern blot analysis was carried out to detect relative levels of SSeCKS transcripts in various oncogene-transformed cells. SSeCKS transcript levels are suppressed at least 15-fold in cells transformed by v-*src* and v-*ras*, and approximately 3-4 fold in *myc*-transformed cells. In contrast, cells transformed with v-*raf*, v-*mos* or activated *neu* showed no down-regulation of SSeCKS transcripts (Fig. 3-1C). This indicates that SSeCKS is specifically involved in v-*src* and v-*ras* mediated transformation, and that SSeCKS transcription is regulated by a *src*- and *ras*-dependent, *raf*-independent signaling cascade.

To determine whether the decreased level of SSeCKS transcripts is directly controlled by Src kinase activity, we assayed the steady-state level of SSeCKS RNA following shift of confluent rat 3Y1/*ts*72*src* cells to the permissive temperature (35°C) for the activation of Src kinase activity. SSeCKS transcript levels rapidly increased in the first 2 h after shift, and then rapidly decreased to suppressed levels after another 4-6 h (Fig. 3-1D). At this time point, morphological transformation has not yet occurred (usually 24 h after

shifting). Since down-regulation of SSeCKS transcripts occurred well before the onset of morphological transformation, it indicates that SSeCKS transcription is regulated by Src kinase activity and is not a nonspecific consequence of morphological transformation. The initial increase in SSeCKS transcripts suggests that SSeCKS transcription is regulated by mitogenic signals.

**Suppression of SSeCKS transcript level correlates with anchorage-independent growth.**

Despite the evidence of down-regulation of SSeCKS in v-*src*- and v-*ras*-transformed cells, it is unclear which parameter of *in vitro* transformation correlates with the down-regulation. To answer this question, a northern blot was probed for steady-state SSeCKS RNA expression in a panel of *ras*-transformed and revertant Rat-6 cells (Feinleib and Krauss, 1996). When compared to control PKC-β cells, the levels of SSeCKS RNA were not decreased in ER1-2 cells, flat untransformed derivatives of PKC-β cells, and ER1-2/*ras* cells, morphologically transformed derivatives of ER1-2 which fail to form colonies in soft agar. In contrast, SSeCKS transcripts were suppressed in two anchorage-independent cells: PKC-β/*ras* and ER1-2T which are flat derivatives of ER1-2 which produce colonies in soft agar (but not as many as produced by PKC-β/*ras*). In highly anchorage-independent PKC-β/*ras* cells, SSeCKS RNA was down-regulated to a greater extent (Fig. 3-2B). The data indicate a strong correlation between SSeCKS down-regulation and anchorage-independent growth. This correlation suggests that SSeCKS may act as a tumor suppressor since the ability of cells to grow in an anchorage-independent manner is generally related to their tumorigenicity *in vivo*.

**SSeCKS inhibits *src*-induced anchorage-independent growth in a co-transfection assay.**

To investigate the effect of SSeCKS expression on v-*src* transforming ability, co-transfection was performed in NIH3T3 using pMv-*src* with pBabe*hygro*/SSeCKS or pBabe*hygro* DNAs. In agreement with others (Johnson et al., 1985), transfection of pMv-*src*, a v-*src* expression plasmid, induced efficient colony formation in soft agar (Fig. 3-3A). The expression of SSeCKS suppressed the ability of v-*src*-induced soft agar colony formation 6- to 9-fold (Fig. 3-3A). Additionally, SSeCKS decreased *src*-induced focus-formation approximately 2-fold (Fig. 3-3B). The differences in colony-forming efficiency was not due to differences in delivery of DNA since the numbers of hygromycin-resistant colonies were comparable in pMv-*src*/pBabe*hygro*-SSeCKS- and pMv-*src*/pBabe*hygro*-transfected cells (data not shown). The ability of SSeCKS to inhibit v-*src*-induced anchorage-independent growth supports the notion that SSeCKS may encode a potential tumor suppressor gene.

**Generation of conditional SSeCKS and v-*src* overexpressing cell lines.**

To study the putative tumor suppressive function of SSeCKS, a great effort was made to establish stable SSeCKS overexpressing cell lines. However, we failed on multiple occasions to produce constitutive SSeCKS overexpression by either retrovirus infection or transfection. All attempts resulted in selection of variants with deletions of the transduced SSeCKS cDNA as demonstrated by southern blot analysis (data not shown). This indicates that the constitutive overexpression of SSeCKS is toxic.

To overcome SSeCKS-induced toxicity, a modified tetracycline-controlled

expression system (Shockett et al., 1995) was employed to generate stable cell lines with conditional overexpression of SSeCKS. One such cell line, S2-6/S24, was selected for further manipulation. We introduced a temperature sensitive v-*src* allele into the S2-6/S24 cells by retrovirus infection. The resulting S24/ts72*src* and control S24/pLJ (vector alone) cells were then examined for SSeCKS and Src expression by western blot analysis. Exogenous Src was detected in S24/ts72*src* but not in S24/pLJ cells using an avian *src*-specific antibody (Fig. 3-4B). SSeCKS expression was increased more than 25-fold (280/290 kDa doublet) in both S24/ts72*src* and S24/pLJ cell lines after removal of tetracycline (Fig. 3-4A). Pulse-chase analysis showed that SSeCKS protein levels began to rise 8 h after tetracycline withdrawal and reached a maximum 72-96 h later (Gelman et al., 1998). The individual S24/ts72*src* and control S24/pLJ cell lines were used to investigate whether SSeCKS overexpression can revert characteristics of cellular transformation such as increased refractility, growth factor-independence, contact-independence and anchorage-independence.

**SSeCKS overexpression suppresses v-*src*-induced transformed phenotypes.**

After characterizing these stable cell lines, we first addressed whether SSeCKS overexpression reverted *src*-induced transformed morphology by microscopic examination. In the absence of ectopic SSeCKS expression (+tet), S24/ts72*src* cells were transformed at the PT (35°C) as shown by increased refractility, smaller size and multilayer growth (piling up after forming confluent monolayer). Following SSeCKS overexpression (-tet), however, the cells displayed obvious morphological changes at the PT (35°C) as exhibited by decreased refractility, bigger size and most importantly, monolayer growth. Moreover, the

altered morphology was similar to that at the NPT (39.5°C) without SSeCKS overexpression. At the NPT, SSeCKS overexpression resulted in a much flatter and bigger morphology in S24/ts72*src* cells (Fig. 3-5). These data indicate that SSeCKS overexpression reverts transformed morphology.

We then examined whether SSeCKS overexpression could inhibit *src*-induced oncogenic growth by performing cell proliferation assays at the PT (35°C). In the presence of 10% serum, S24/ts72*src* cells proliferated rapidly and reached very high saturation densities (Fig. 3-6A). However, under conditions of SSeCKS overexpression, they proliferated initially, and then stopped proliferation once they reached confluence, which was consistent with a monolayer growth phenotype (Fig. 3-6A). Their saturation densities were similar to those of S24/pLJ cells in the absence of SSeCKS overexpression (Fig. 3-6A). SSeCKS overexpression in S24/pLJ cells resulted in growth arrest, suggesting that SSeCKS might have a negative mitogenic regulatory role (Fig. 3-6A). In the presence of 0.5% serum, S24/ts72*src* cells continued to proliferate but were growth arrested when SSeCKS was overexpressed (Fig. 3-6B). These data indicate that SSeCKS overexpression restores contact-inhibition and growth factor-dependence in v-*src*-transformed cells.

Anchorage-independent growth is the strongest indicator of the transformed phenotype in rodent fibroblasts. To assess the effect of SSeCKS overexpression on this phenotype, colony formation in soft agar was carried out. At the PT, efficient colony formation was observed in S24/ts72*src* cells in the absence of SSeCKS overexpression (Fig. 3-7A). However, in the presence of SSeCKS overexpression, these cells exhibited a reduction of both colony number and size (Fig. 3-7B and 3-7A), and there was an

approximately 25-35 fold decrease in colony numbers (Fig. 3-7B). This indicates that SSeCKS overexpression suppresses *src*-induced anchorage-independent growth.

The effect of SSeCKS overexpression on suppressing transformed phenotypes was manifest in several independently isolated S24/ts72*src* cell lines (S24/ts72*src* 1-4), and therefore was not clone specific. These data demonstrate a tumor suppressive activity for SSeCKS independent of growth inhibition since SSeCKS overexpression at the PT does not result in growth arrest. The data also indicate that v-*src* induces mitogenic signals not inhibited by SSeCKS during the process of tumor suppression.

**Re-expression of SSeCKS to a physiological level (the level produced by parental NIH3T3 cells) suppresses v-*src*-induced anchorage-independent growth.**

The apparent inhibition of v-*src*-mediated transformation was detected under the condition of a very high level of SSeCKS overexpression in our S24/ts72*src* cells at the PT (>25-fold over background after removal of tetracycline). This raised a possibility that the tumor suppressive activity of SSeCKS was due to a non-specific toxic effect. To test this possibility, we performed soft agar colony formation assay in the presence of "normal" levels of SSeCKS expression (as in the parental NIH3T3 cells). We took advantage of tetracycline-controlled expression system in which protein expression level can be regulated by varying the concentration of tetracycline. At 0.02 μg/ml tetracycline, the expression level of SSeCKS was comparable to that of parental NIH3T3 cells (Fig. 3-8A). This level of SSeCKS expression still efficiently repressed S24/ts72*src* cells to form colonies at the PT (Fig. 3-8C), and the reduction on colony number was approximately 15-20 fold (Fig. 3-8B). Thus, the tumor suppressive effect is not due to overexpression or non-specific toxicity.

**The tumor suppressive function of SSeCKS is not mediated by repressing the expression and activity of Src and MAP kinase.**

Down-regulation of SSeCKS expression was a direct effect of Src kinase activity as described above. SSeCKS overexpression did not alter the levels of transduced ts72*src* protein (Fig. 3-4B). We then asked whether the tumor suppressive effect of SSeCKS was due to its ability to inhibit Src kinase activity. An *in vitro* Src kinase assay was performed using immunoglobulin heavy chain as substrate. In agreement with previous reports (Maroney et al., 1992), the kinase activity of Src isolated at the PT was 50-fold greater than that isolated at the NPT (Fig. 3-9A). SSeCKS overexpression marginally increased Src kinase activity at the PT but had no effect at the NPT (Fig. 3-9A). The *in vivo* kinase activity of Src was assessed by tyrosine phosphorylation of cellular proteins. SSeCKS overexpression did not grossly decrease the level or number of the *src*-induced phosphotyrosine substrates in several S24/ts72*src* clones grown at the PT (Fig. 3-9B). Thus, the tumor suppressive effect of SSeCKS is not manifested through the inhibition of v-*src* expression or kinase activity.

Mitogenic signals in fibroblasts are controlled by MAPK pathways (Derijard et al., 1994; Minden et al., 1994). Among the three well studied MAPK pathways including ERK, JNK/SAPK and p38, the ERK pathway is implicated in oncogenesis (Hunter, 1997; Cowley et al., 1994). Although c-Jun phosphorylation by activated JNK/SAPK is required for Ras transformation, the involvement of JNK/SAPK pathway in fibroblast oncogenesis remains inconclusive (Johnson et al., 1996). To examine whether the tumor suppressive effect of SSeCKS was due to inhibition of MAPK pathways, we measured the expression levels of

ERK2 by western blot analysis, and the kinase activities of ERK2 and JNK/SAPK by *in vitro* kinase assay. MBP (myelin basic protein) and GST-JUN were used as *in vitro* substrates for ERK2 and JNK, respectively. ERK2 expression was not altered upon SSeCKS overexpression (Fig. 3-10A). However, the kinase activity of ERK2 was unexpectedly increased 5- to 10-fold in all four S24/ts72*src* cell lines in response to SSeCKS induction at the PT only (Fig. 3-10B). In agreement with a previous report that ERK activation by mitogenic signals is repressed in v-*src*-transformed cells (Stofega et al., 1997), ERK2 was not constitutively activated in S24/ts72*src* cell lines kept at the PT (Fig. 3-10B). Lastly, JNK activation by v-*src* (3-10 fold increase) was unaffected by SSeCKS overexpression (Fig. 3-10C). These data indicate that the tumor suppressive effect of SSeCKS is not manifested through the inhibition of ERK2 expression and activity, and of JNK activity. Additionally, the lack of growth arrest following SSeCKS overexpression at the PT may be explained by the enhanced ERK2 activity.

**SSeCKS overexpression induces cytoskeletal re-organization.**

Transformation is generally associated with an alteration in cell shape and cytoskeletal organization (Cooper et al., 1987; Chan et al., 1989). To investigate the effect of SSeCKS overexpression on cytoskeletal architecture, we performed immunofluorescence analysis. In agreement with others (Jove and Hanafusa, 1987; Lo and Chen, 1994), S24/ts72*src* cells at the PT appeared to lack actin-based stress fibers, vinculin-associated adhesion plaques, and cytokinetic structures such as lamellipodia (Fig. 3-11A, 3-11E, 3-11I and 3-11M). In contrast, SSeCKS overexpression at the PT resulted in a more normal array of cytoskeletal and cytokinetic structures as manifested by restoration of stress fibers and

adhesion plaques as well as increased production of lamellipodia (Fig. 3-11B, 3-11F, 3-11J and 3-11N). The restored stress fibers were thinner than those in normal cells (Fig. 3-11N and 3-11O). S24/ts72*src* cells grown at the NPT with SSeCKS overexpression were devoid of stress fibers and adhesion plaque structures (Fig. 3-11H and 3-11P). Because SSeCKS overexpression in S2-6/S24 cells did not change the levels of total cellular actin and vinculin (data not shown), the alteration in cell shape and cytoskeletal architecture is more likely due to a re-organization of cytoskeletal components rather than changes in *de nova* synthesis. These data suggest that the tumor suppressive function of SSeCKS may be mediated by its ability to organize cytoskeletal architecture.

## DISCUSSION

**Suppression of SSeCKS transcript level is observed in v-*src*- and v-*ras*-transformed cells, and is not a non-specific consequence of transformation.**

Malignant transformation is accompanied by changes in gene expression. Isolation and characterization of these transformation-associated genes is an essential step to elucidate the molecular mechanism of transformation. Those down-regulated genes are particularly important since they may include potential tumor suppressor genes.

SSeCKS was isolated by a novel subtractive hybridization method to identify candidate tumor suppressor and/or regulatory genes whose expression were down-regulated in v-*src*-transformed NIH3T3 cells. The hypothesis behind the cloning strategy is that oncogenic *src* induces morphological transformation rapidly (approximately 24 h), therefore, the suppressed rather than mutated or deleted genes are more likely to represent

tumor suppressor genes.

We demonstrate here that down-regulation of SSeCKS expression is directly regulated by v-*src*, and is most likely not the consequence of morphological transformation. SSeCKS transcription is suppressed by *src*-, *ras*-, but not *raf*-induced transformation. Thus, the transcription of SSeCKS is regulated by specific signal pathways rather than generic pathways common to all transformed or tumors cells. Although it has been established for many years that c-*raf*-1 transduces many signals from *src* or *ras* (Williams and Roberts, 1994), recent studies have uncovered other *ras*-dependent pathways (White et al., 1995; Marshall, 1996; Van Aelst and D'Souza-Schorey, 1997). For example, Rho family members including Rho, Cdc42 and Rac1 are downstream molecules of Ras, and are essential for Ras transformation (Khosravi-Far et al., 1995; Qiu et al., 1995a; 1995b; Qiu et al., 1997). It is plausible that SSeCKS transcription may be controlled through such "alternate" pathways.

**SSeCKS possesses tumor suppressive activity.**

Consistent with cloning strategy, our data indicate that SSeCKS functions as a tumor suppressor. SSeCKS overexpression suppresses many parameters of v-*src*-induced *in vitro* transformation such as increased refractility, contact-independence, growth factor-independence, and anchorage-independence. It is unlikely that SSeCKS overexpression is simply toxic since S24/ts72*src* cells proliferate at the PT. SSeCKS re-expression in a rat prostate tumor cell line (MLL) harboring an activated *ras* is able to inhibit transformed phenotypes *in vitro* and tumor formation *in vivo* (nude mice) (Xia and Gelman, manuscript in preparation). In addition, SSeCKS down-regulation is detected in certain breast and

prostate tumor cell lines and primary tumors (Gelman and Wang, unpublished observation). Taken together, there is strong evidence to define SSeCKS as a novel class II tumor suppressor.

Among the parameters of *in vitro* transformation, anchorage-independent growth is closely correlated with tumor formation *in vivo*. The tumor suppressive effect of SSeCKS correlates best with anchorage-independent growth. First, down-regulation of SSeCKS is observed in anchorage-independent cells, but not in cells with rounded transformed morphology. Normal levels of SSeCKS are re-established in untransformed, flat revertants of Rat-6/*ras* cells. Second, SSeCKS overexpression inhibits growth in soft agar at the PT without inducing growth arrest of S24/ts72*src* cells. Therefore, the repression of colony formation in *src*-transformed cells is due to SSeCKS-induced tumor suppression rather than growth suppression. Third, the ability of SSeCKS to inhibit *src*-induced soft agar colony formation is not due to the non-specific effect of overexpression since parental levels of SSeCKS induced by the tetracycline system exhibit similar inhibitory activity.

It is of interest that parental levels of SSeCKS re-expression are as potent as high levels of overexpression (>25 fold above normal) in reversing *src*-mediated transformation. A similar phenomenon is observed following restoration of tropomyosin 1 synthesis (Prasad et al., 1993). In that case, anchorage independence and tumorigenicity are suppressed by even modest levels of ectopic tropomyosin 1. However, restoration of cell morphology and contact inhibition requires higher level expression (Prasad et al., 1993). Several lines of evidence suggest that transformed cells retain nucleation complexes for structural integrity, yet exhibit altered cell morphologies due to an increased turnover of these

complexes (Wang and Ingber, 1994). It is conceivable that slight changes in the levels of cytoskeletal proteins such as SSeCKS are sufficient to re-establish normal controls on cytoskeletal architecture, most likely by changing the turnover rate of the structural complexes.

**The signaling pathways mediating SSeCKS' tumor suppressive function are not resolved.**

Several lines of evidence suggest that Ras-transduced signal cascades are responsible for transformation by v-*src* (Satoh et al., 1990; Gupta et al., 1992). Transformation by oncogenic *ras* requires activation of ERK pathway, one of the best characterized MAPK pathways (Cowley et al., 1994; Khosravi-Far et al., 1995). SSeCKS overexpression did not suppress ERK2 activity in the presence of activated Src, indicating that the tumor suppressive effect of SSeCKS is not mediated by inhibition of ERK2. The activation of JNK, another well studied MAPK, is also required for Ras transformation (Derijard et al., 1994; Johnson et al., 1996). However, unlike ERK, the role of JNK in oncogenesis remains to be established (Hunter, 1997). Recently, JNK activation by Ras was demonstrated to be via Rac or CDC42 (Coso et al., 1995; Macara et al., 1996; Qiu et al., 1997). Although Rac and CDC42 are necessary for Ras transformation, the pathways downstream which mediate transformation remain to be clarified (Qiu et al., 1997; Qiu et al., 1995a). The lack of change in JNK activity by SSeCKS overexpression indicates that the tumor suppressive effect of SSeCKS is not manifested through inhibition of JNK. Most likely, SSeCKS exerts its inhibitory effects on tumorigenicity either downstream of MAPK or through distinct Rho family member-dependent pathways.

V-*src* also activates STAT pathways (Yu et al., 1995; Cao et al., 1996). Whether SSeCKS' tumor inhibitory function is mediated by suppression of STAT signals remains to be determined.

Surprisingly, SSeCKS overexpression results in activation of ERK2 only in the presence of activated Src. Src has been shown to induce tyrosine phosphorylation of FAK, and subsequent activation of FAK. Whether the activated FAK in turn activates ERK is debatable (Hanks and Polte, 1997; Wary et al., 1998). We have noticed that SSeCKS overexpression provokes tyrosine phosphorylation of FAK in untransformed NIH3T3 independent of cell adhesion (Gelman et al., 1998), but does not activate ERK2 (next chapter). How ERK2 activity is enhanced by SSeCKS in the presence of active Src remains to be investigated.

**The role of SSeCKS in organizing cytoskeletal architecture is possibly related to its tumor suppressive effect.**

Early findings suggest that the expression of several cytoskeletal proteins are reduced in transformed and cancer cells, such as tropomyosin, gelsolin, α-actinin and vinculin (Ben Ze'ev, 1997; Hunter, 1997). These proteins have been shown to act as tumor suppressors (Ben Ze'ev, 1997), and belong to a tumor suppressor family of actin-cytoskeleton-associated proteins (ACAPs) (Tikoo et al., 1994). Restoration of the expression of these proteins represses phenotypes induced by transformation (Gluck et al., 1993; Frenandez et al., 1992; Prasad et al., 1993; Janssen and Mier, 1997). Suppression of the expression of these proteins with antisense constructs results in transformed phenotypes in untransformed fibroblasts (Fernandez et al., 1993; Gluck and Ben-Ze'ev, 1994; Boyd et

al., 1995). Thus, cytoskeletal proteins play an important role in the regulation of cellular transformation.

SSeCKS physically associates with the cytoskeletal matrix (Gelman et al., 1998). Overexpression of SSeCKS at the PT restores stress fiber formation and focal adhesion plaques, concomitant with restoration of growth properties characteristic of normal fibroblasts. This phenomenon is also observed in the overexpression of several other cytoskeletal proteins as mentioned above. Taken together, these data support the notion that a complex of changes in the expression of cytoskeletal proteins occurs during transformation, and thus, cytoskeletal integrity may be compromised. Then, re-expression of any one of the down-regulated proteins may be sufficient to reverse transformed phenotypes.

It has been shown that the re-establishment of cytoskeletal architecture can override oncogene-induced parameters of tumorigenesis and apoptosis (Frisch and Francis, 1994; Lo et al., 1994). In addition, the tensegrity model proposed by Ingber (Ingber et al., 1994) suggests that changes in structural integrity directly affect the shape of the nucleus, and consequently, nuclear events such as transcription. These data underscore the emerging importance of cytoskeletal architecture in controlling morphogenic and mitogenic signal pathways.

**Fig. 3-1. Suppression of SSeCKS transcripts.**

**A.** Northern blot showing SSeCKS RNA levels in NIH3T3 vs. NIH/v-*src* cells. 30 μg of total RNA from NIH3T3 or NIH/v-*src* cells was used for northern blot analysis. The amount of RNA loaded was normalized by densitometric analysis of the 28S and 18S rRNA bands. SSeCKS transcript level is suppressed in NIH/v-*src* cells. **B.** Southern blot showing that there is no gross deletion or translocation of the SSeCKS allele. pBluescript II KS or SSeCKS/pBluescript plasmid DNA were included as negative and positive hybridization controls, respectively. **C.** Northern blot showing SSeCKS transcripts in oncogene-transformed Rat-6 fibroblasts. 30 μg of total RNA from *mos*-, *src*-, *myc*-, *neu*-, *ras*-, or *raf*-transformed or untransformed Rat-6 cells was analyzed by northern blotting for steady-state levels of SSeCKS RNA. SSeCKS transcripts are down-regulated in *src*-, *ras*-, but not in *raf*- transformed cells. **D.** The relative levels of SSeCKS RNA after the activation of a ts-*src* allele. Confluent 3Y1/ts*src* cells or parental rat 3Y1 fibroblasts were grown at the non-permissive temperature (39.5°C) for 24 hours, then shifted to the permissive temperature (35°C) for v-*src* activity. Total RNA isolated at various times after shifting was analyzed for SSeCKS transcripts by northern blot analysis. SSeCKS transcripts are suppressed relatively soon after the activation of ts-*src*.

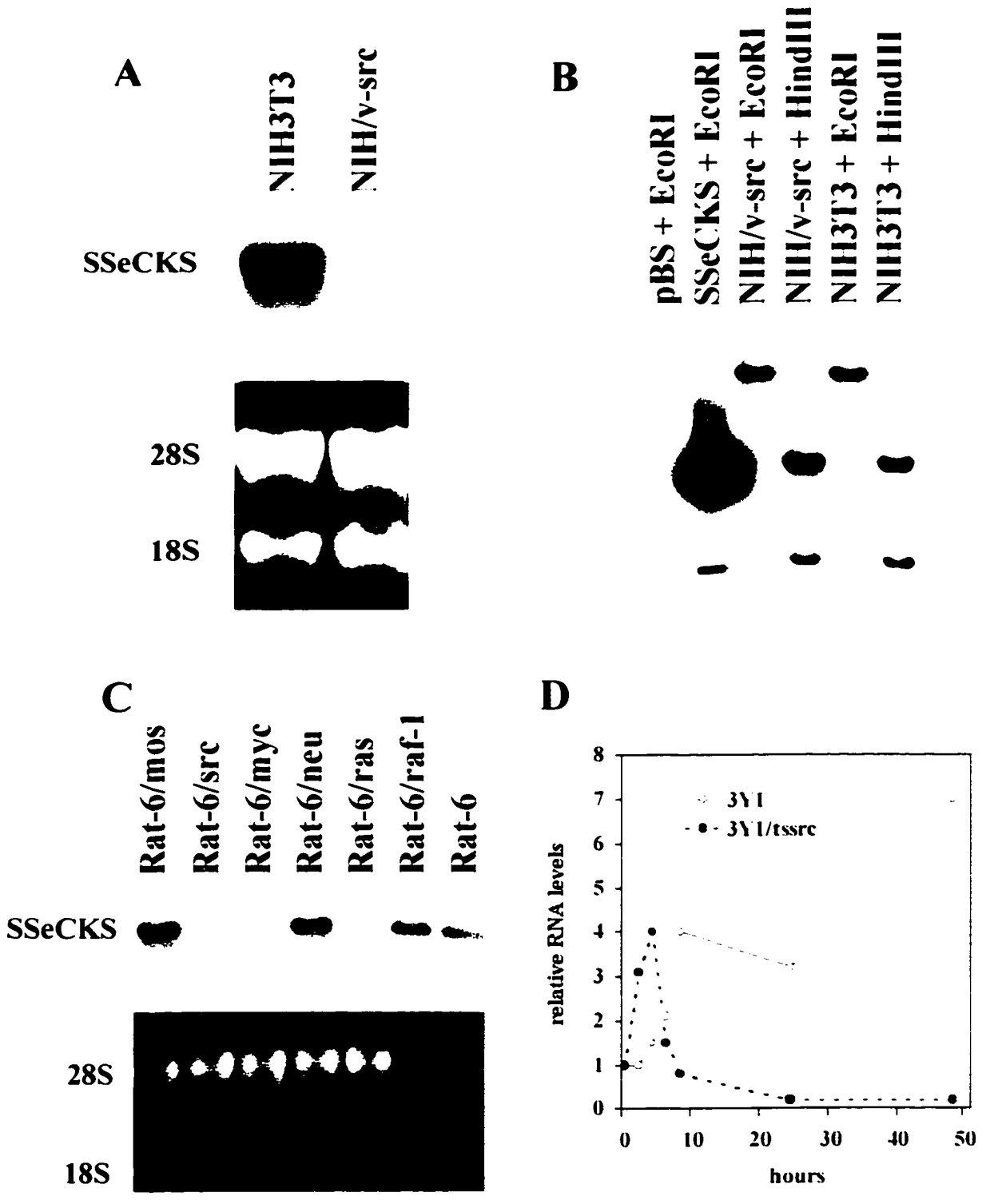



Fig. 3-1

**Fig. 3-2. The down-regulation of SSeCKS correlates with anchorage-independent growth.**

**A.** A panel of *ras*-transformed or revertant Rat-6/PKC-β cells described previously (Feinleib and Krauss, 1996). ER1-2 cells are flat untransformed derivatives of PKC-β, and ER1-2T are flat derivatives of ER1-2 that grow in soft agar (not as many colonies as produced by PKC-β/*ras* cells). ER1-2/*ras* are ER1-2 cells transduced with activated *ras*, which exhibit increased refractility but not growth in soft agar. **B.** A northern blot (gift of Feinleib and Krauss) containing total RNA from these cells was analyzed for SSeCKS RNA expression. SSeCKS RNA is down-regulated in two anchorage-independent cells (PKC-β/*ras* and ER1-2T).

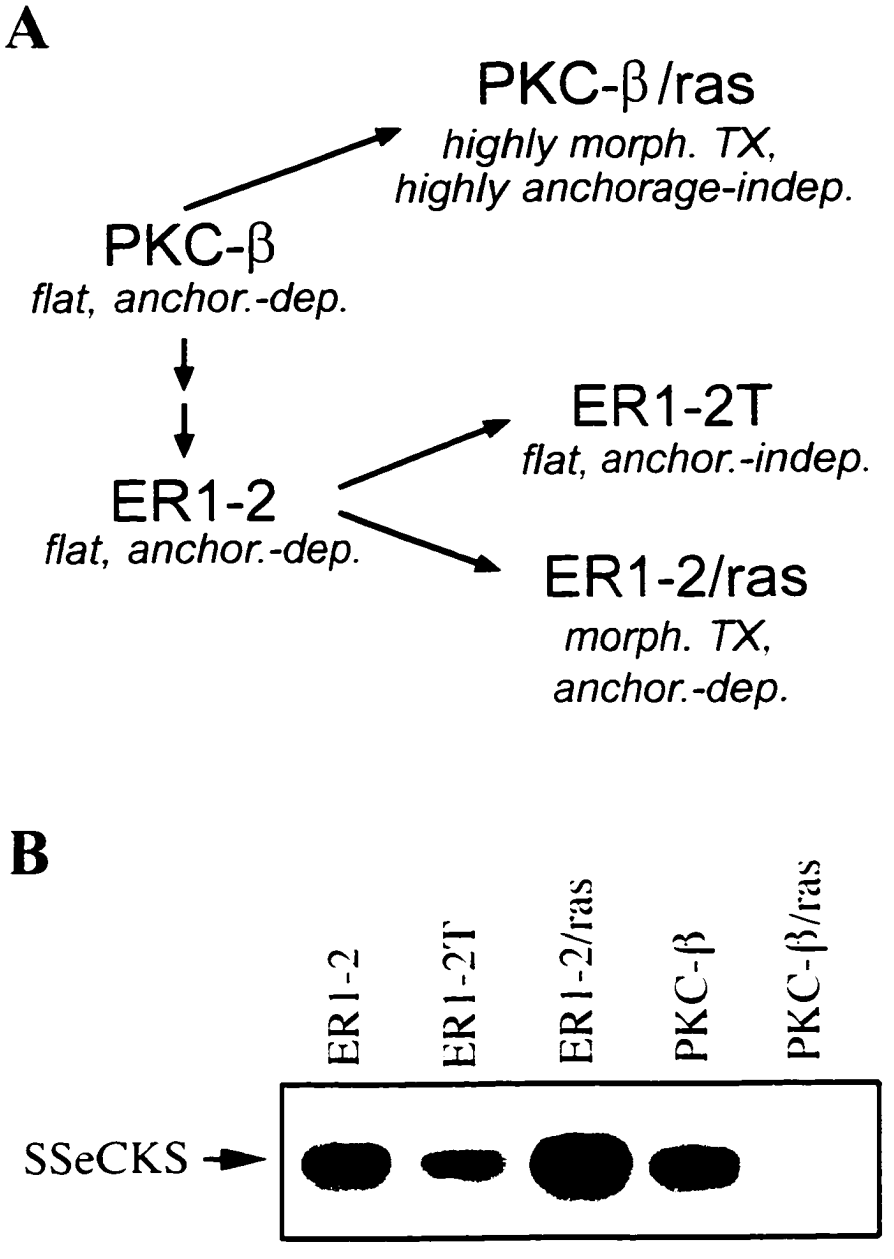



**Fig. 3-2**

**Fig. 3-3. V-*src*-induced transformation is suppressed by SSeCKS in a transient co-transfection assay.**

NIH3T3 cells were transfected with either pMv-*src* (1μg) plus pBabe*hygro* (10μg) or pMv-*src* (1μg) plus SSeCKS/pBabe*hygro* (10μg). **A.** Colony formation in soft agar assay showing that v-*src*-induced colony numbers are reduced by SSeCKS expression. $2x10^6$ and $20x10^6$ of the transfected cells were incubated in soft agar suspension supplemented with hygromycin for colony formation. **B.** Focus formation assay showing that v-*src*-induced foci are inhibited by SSeCKS expression. $0.25x10^6$ and $1x10^6$ of the transfected cells were plated onto dishes without selection for focus formation.

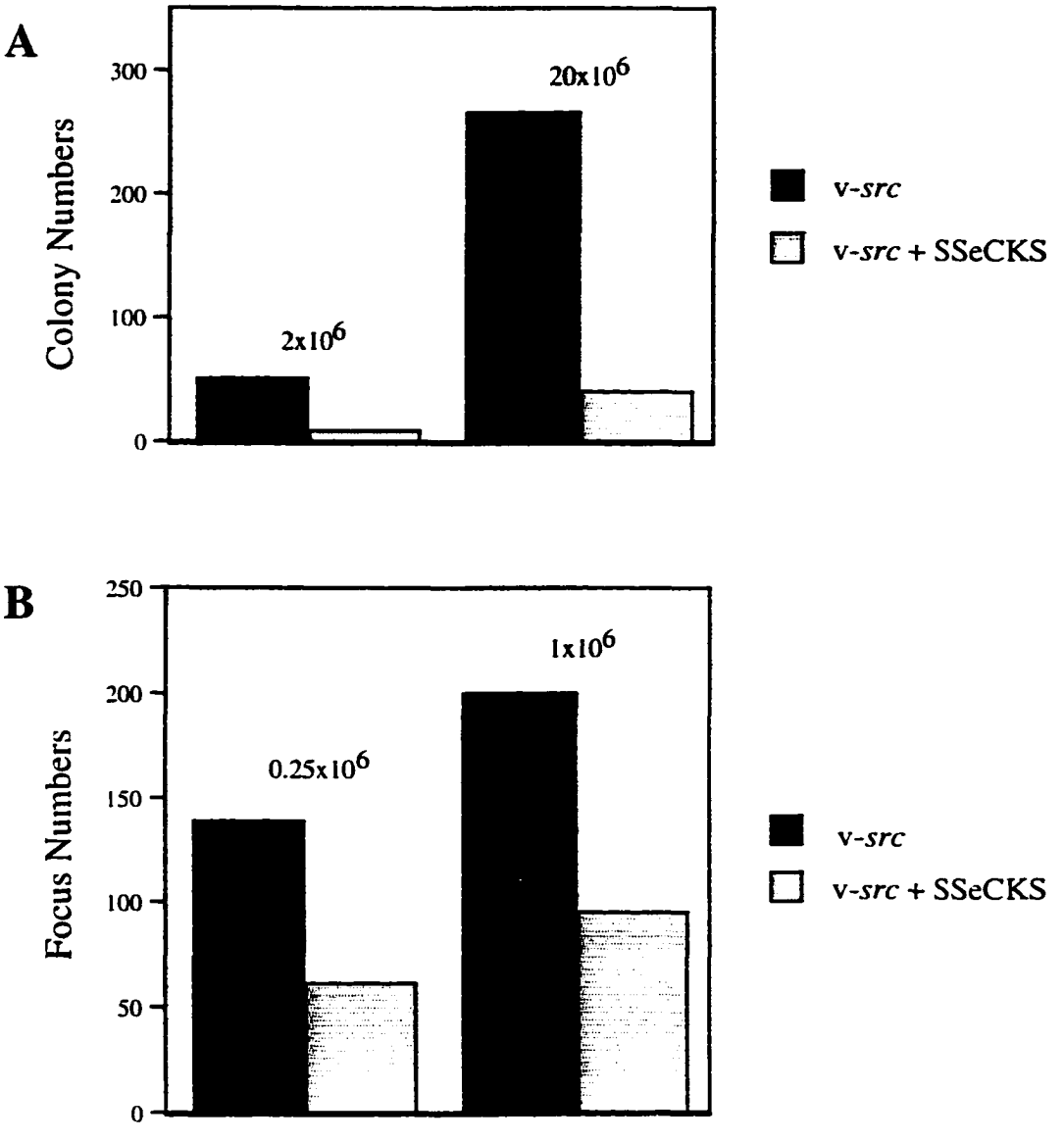



**Fig. 3-3**

**Fig. 3-4. Ectopic expression of SSeCKS and Src in NIH3T3 cells.**

Ectopic expression of SSeCKS was achieved by tetracycline-regulated system, in which the exogenous SSeCKS expression was induced after removal of tetracycline. Ectopic expression of Src was achieved by introducing a ts72*src* allele into an SSeCKS clone. The resulting S24/ts*src* clones and S24/pLJ control clones were grown at the permissive temperature (PT, $35^0$C) in the presence (+) or absence (-) of tetracycline, and lysed for western blot analysis. **A.** Western blot showing SSeCKS protein levels. There is a >25-fold increase in SSeCKS levels after the removal of tetracycline. **B.** Western blot showing ts*src* protein levels using monoclonal antibody EC10 which recognizes avian (exogenous) $p60^{src}$ only.

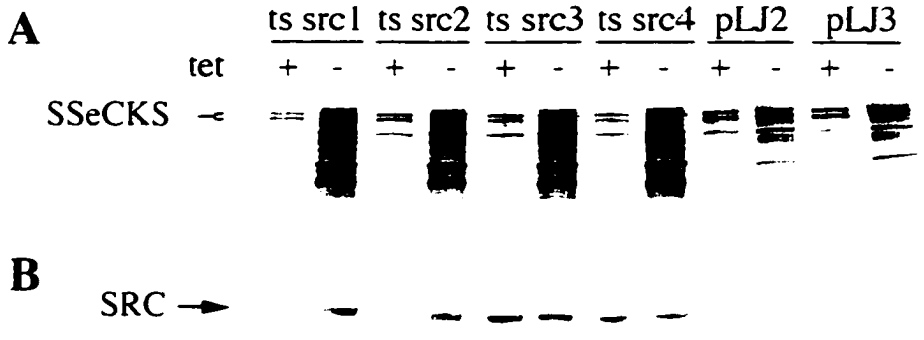



**Fig. 3-4**

**Fig. 3-5. SSeCKS expression reverts *src*-induced transformed morphology.**

S24/ts*src* cells were grown to confluence at either the PT (35°C) or NPT (39.5°C) for *src* kinase activity, in the presence (0.5 μg/ml) or absence of tetracycline. Morphological transformation is induced in the presence of tetracycline at the PT only. SSeCKS expression at the PT reverts *src*-induced increased refractility, multilayer growth, and smaller size.

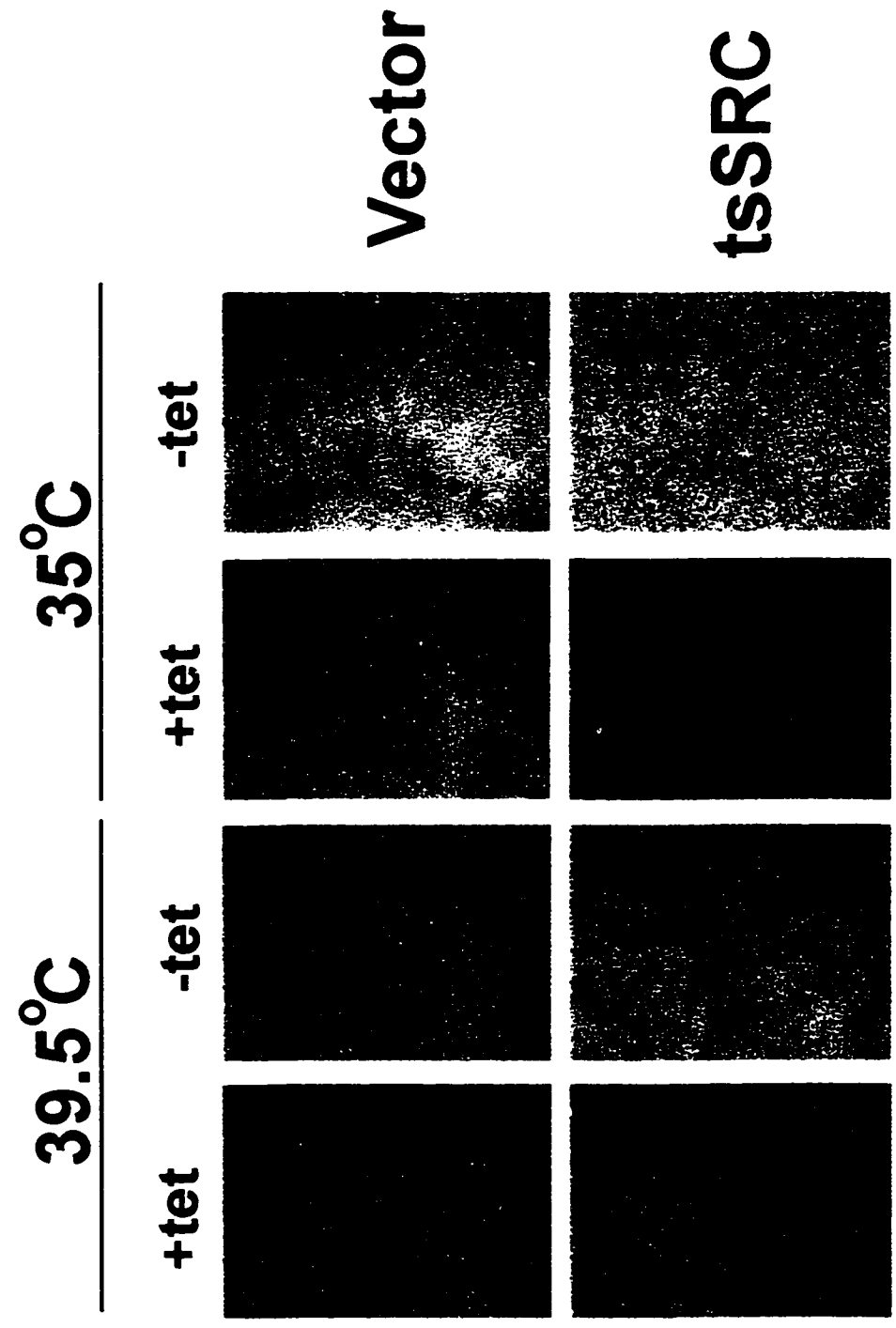



Fig. 3-5

**Fig. 3-6. Effect of SSeCKS expression on the proliferation rates of S24/ts*src* clones.**

**A.** Proliferation rate assay performed at the PT in DMEM supplemented with 10% calf serum. $10^4$ cells were seeded per well and allowed to proliferate in the presence (w) or absence (w/o) of tetracycline. Although the initial proliferation rates of the S24/ts*src* clones are similar in the presence (w) or absence (w/o) of tetracycline, those grown without tetracycline reach much lower saturation densities. **B.** Proliferation rate assay performed at the PT in DMEM supplemented with 0.5% calf serum. Growth of ts*src* cells are almost completely inhibited by SSeCKS expression.

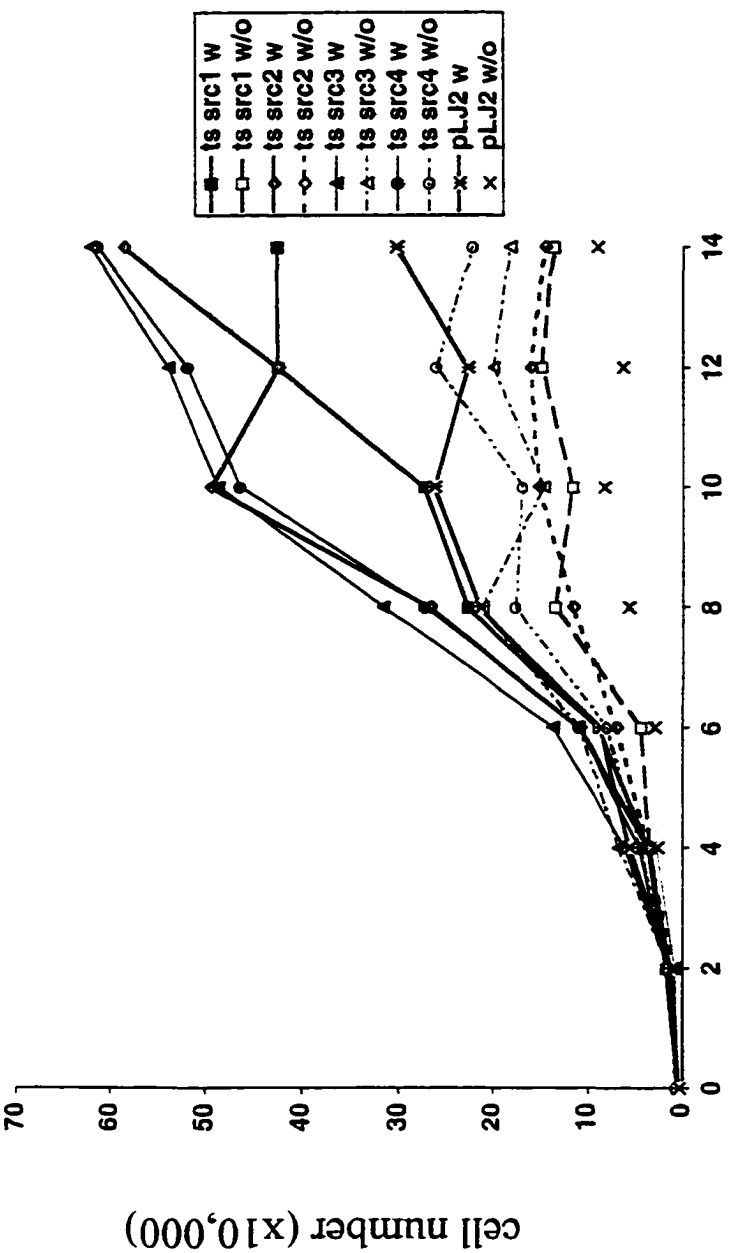



Fig. 3-6A

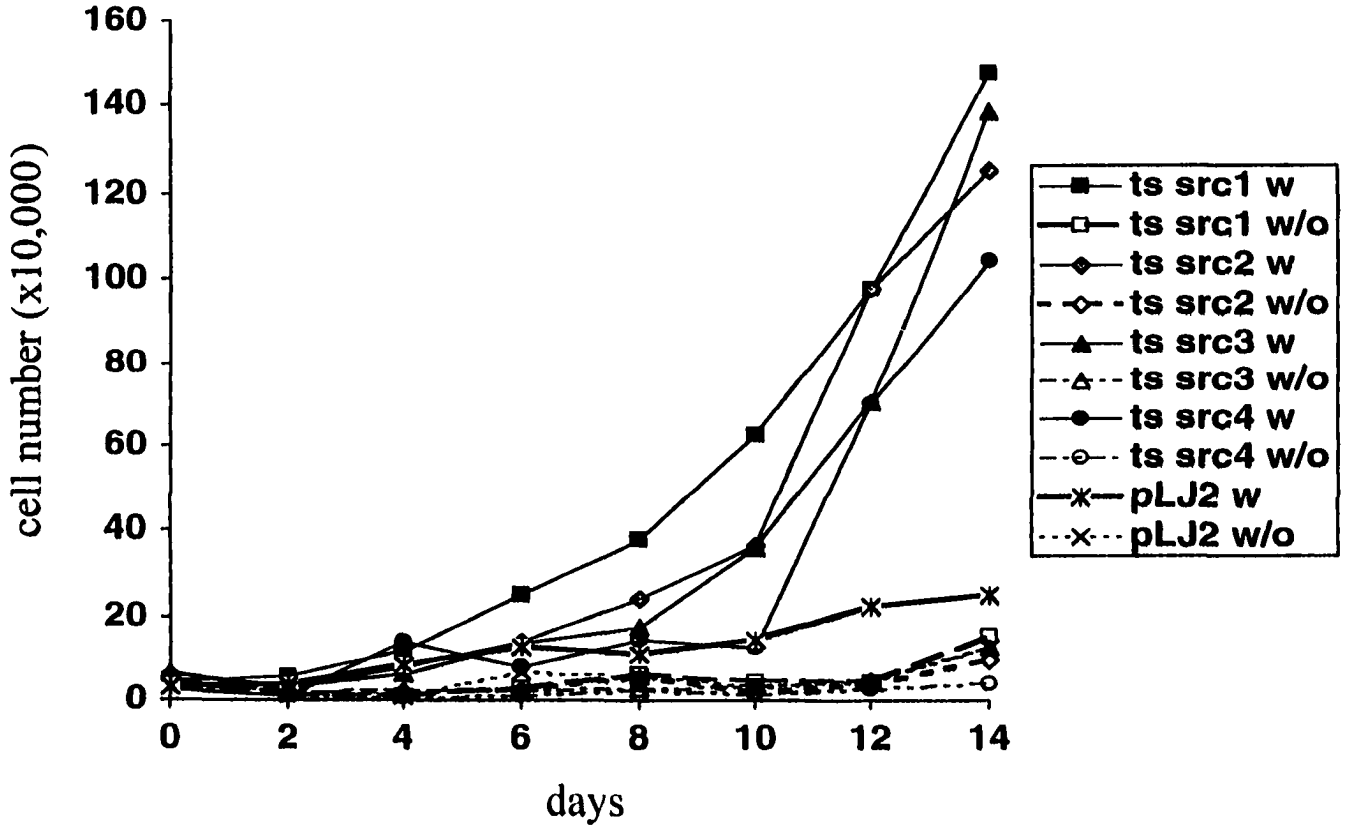
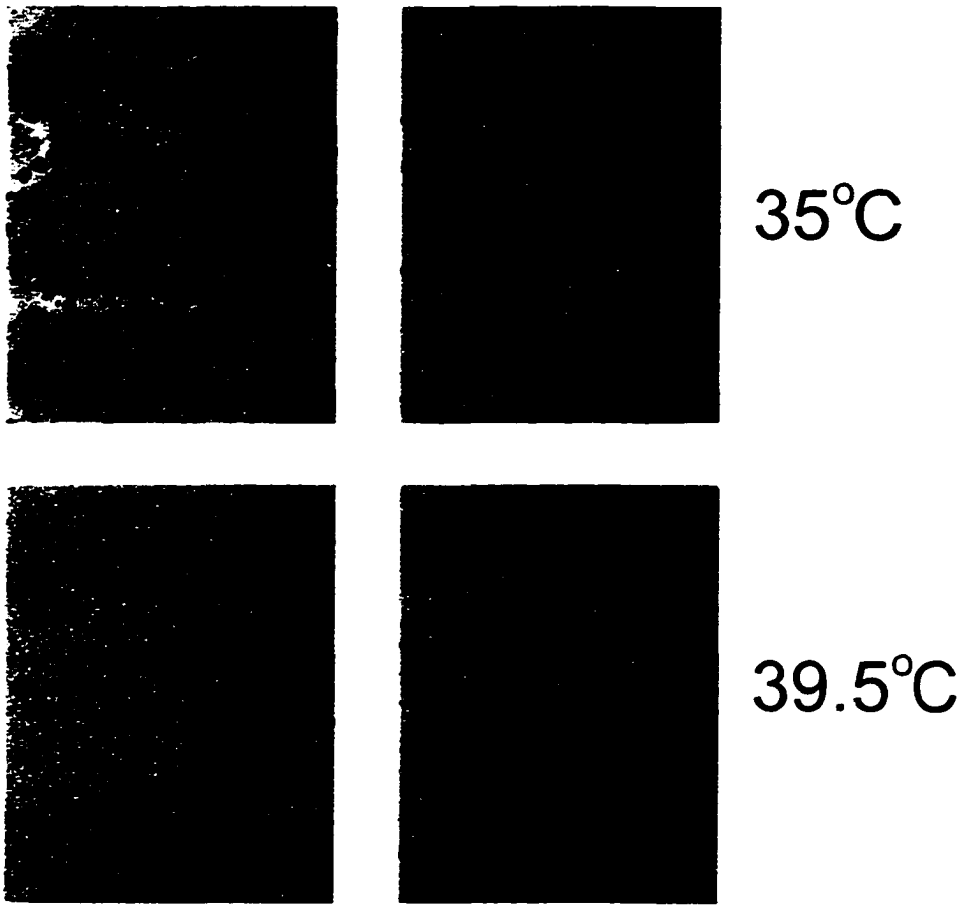



**Fig. 3-6B**

**Fig. 3-7. Overexpression of SSeCKS suppresses anchorage-independent growth of S24/ts*src* cells.**

$10^5$ S24/ts*src* cells were grown in soft agar suspension at either the PT or NPT in the presence or absence of tetracycline for 3 weeks. **A.** Colony size. SSeCKS expression results in uniformly smaller colonies at the PT. **B.** Colony numbers. SSeCKS expression results in a 25-35 fold reduction on colony numbers at the PT.

A




B

soft agar colony formation

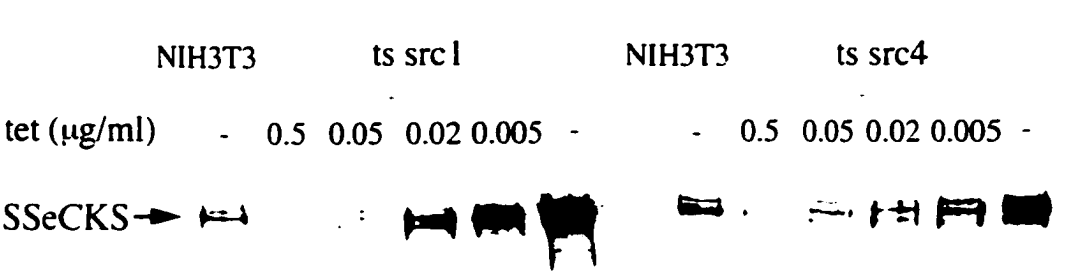
| | ts src1 | ts src2 | ts src3 | ts src4 | pLJ2 | pLJ3 |
|---|---|---|---|---|---|---|
| + tet | 2160 | 1640 | 2800 | 1080 | 0 | 0 |
| - tet | 60 | 60 | 110 | 35 | 0 | 0 |

**Fig. 3-7**

**Fig. 3-8. Re-expression of SSeCKS to "normal" levels suppresses anchorage-independent growth of S24/ts*src* cells.**

**A.** Western blot showing the effect of varying tetracycline concentration on the levels of SSeCKS in S24/ts*src* cells at the PT. The levels of endogenous SSeCKS are suppressed at the PT compared with parental NIH3T3 cells. The levels of SSeCKS in the presence of 0.02 μg/ml of tetracycline are comparable to that in parental NIH3T3 cells. **B.** Soft agar colony formation assay showing the effect of varying tetracycline concentration on colony numbers at the PT. 0.02 μg/ml of tetracycline gives a 15-20 fold decrease in colony numbers compared with 0.5 μg/ml of tetracycline. **C.** Colony size. The colonies formed in the presence of 0.02 μg/ml of tetracycline are typically smaller than those formed in 0.5 μg/ml of tetracycline.

**A**




**B** soft agar colony formation

| | 35°C | | | | | 39.5°C | |
|---|---|---|---|---|---|---|---|
| tet (μg/ml) | 0.5 | 0.05 | 0.02 | 0.005 | 0 | 0.5 | 0 |
| ts src1 | 2852 | 2464 | 174 | 51 | 22 | 0 | 0 |
| ts src4 | 1463 | 743 | 67 | 11 | 0 | 0 | 0 |

**C**

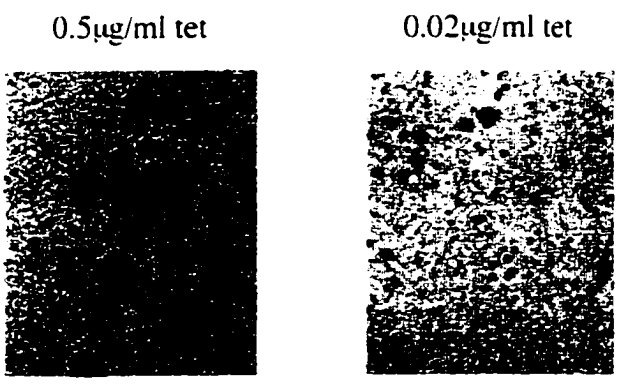



**Fig. 3-8**

**Fig. 3-9. Effect of SSeCKS expression on v-*src* activity in S24/ts*src* clones.**

**A.** *In vitro* kinase assay showing Src kinase activity. Src kinase activity from S24/ts*src* or S24/pLJ clones grown at the PT or NPT in the presence (+) or absence (-) of tetracycline was detected as transphosphorylation of immunoglobulin heavy chain. SSeCKS expression does not inhibit *in vitro* Src kinase activity at the PT. **B.** Anti-phosphotyrosine immunoblot showing *in vivo* Src kinase activity. Cell lysates of S24/ts*src* or S24/pLJ clones were analyzed for the levels of tyrosine-phosphorylated cellular proteins using monoclonal antibody PY20. SSeCKS expression does not grossly decrease the level or number of cellular phosphotyrosine proteins at the PT.

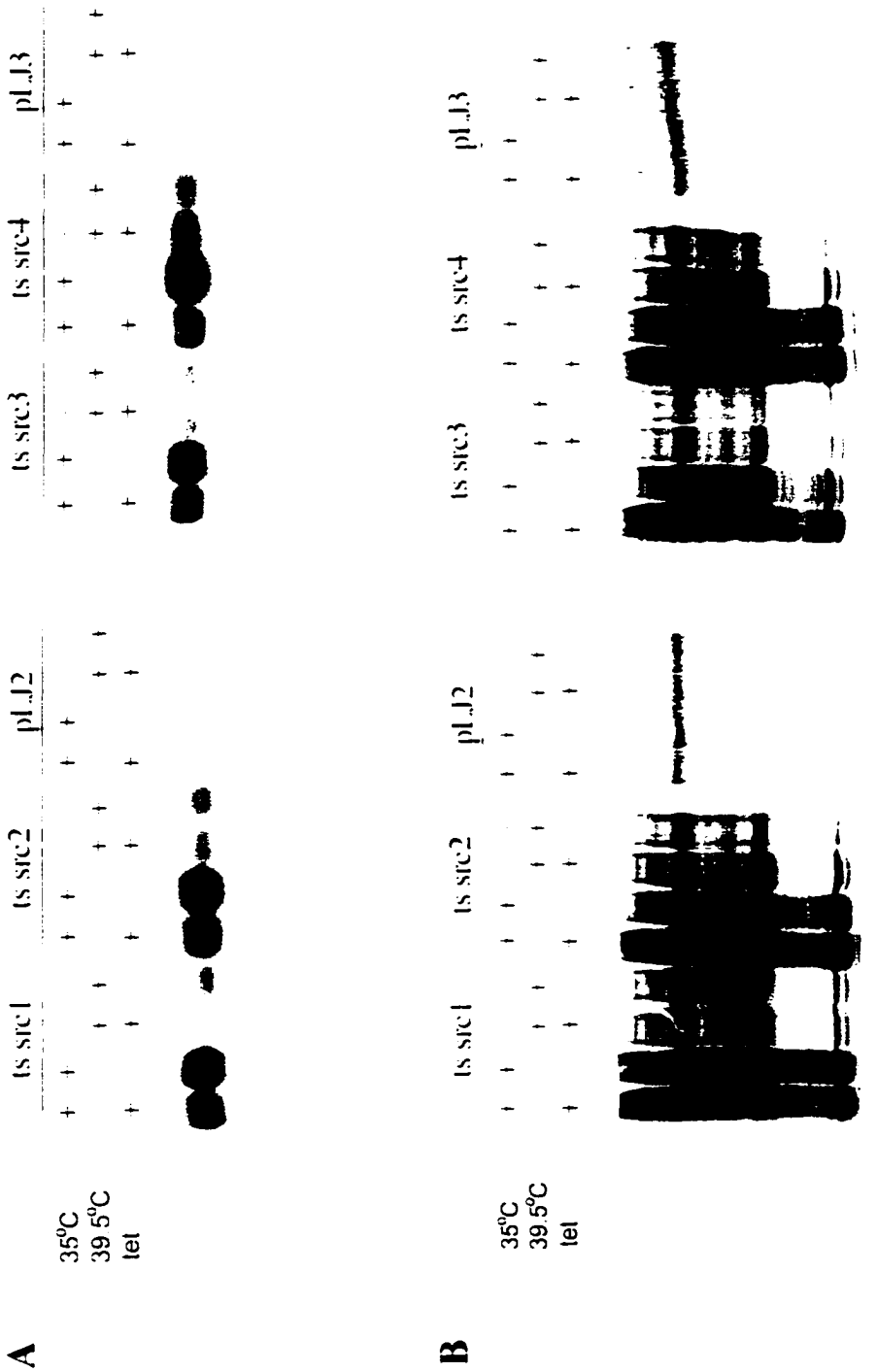



Fig. 3-9

**Fig. 3-10. Effect of SSeCKS expression on v-*src*-induced ERK2 and JNK activities.** Cell lysates were prepared from S24/ts*src* or S24/pLJ clones grown at the PT or NPT in the presence (+) or absence (-) of tetracycline, and analyzed for the expression of ERK2, and kinase activities of ERK2 and JNK. **A.** Western blot showing the levels of ERK2. SSeCKS expression has no effect on ERK2 expression at the PT or NPT. **B.** *In vitro* kinase assay showing ERK2 activity using myelin basic protein (MBP) as substrates. SSeCKS expression increases ERK2 activity only at the PT. **C.** *In vitro* kinase assay showing JNK activity using GST-JUN as substrates. SSeCKS expression has no effect on JNK activity at the PT or NPT.

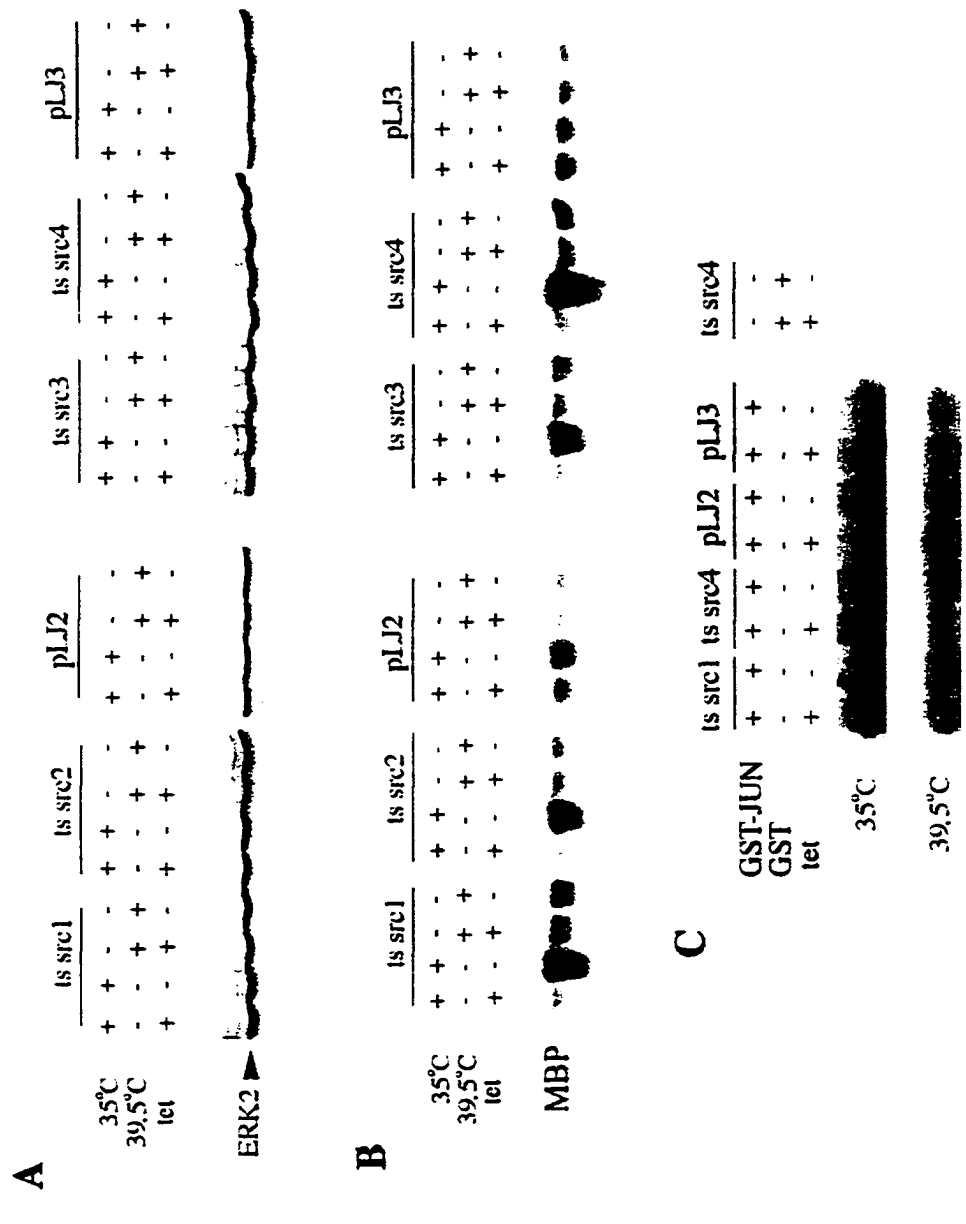



Fig. 3-10

**Fig. 3-11. SSeCKS expression restores v-*src*-induced loss of stress fibers and vinculin-associated adhesion plaques.**

S24/ts*src* cells were grown on coverslips at either the PT or NPT in the presence or absence of tetracycline, and then fixed and immunostained. A-D and I-L were immunostained for SSeCKS. E-H and M-P were immunostained with either mouse monoclonal anti-vinculin antibodies followed by rhodamine-labeled anti-mouse Ig or rhodamine-labeled phalloidin, respectively. Transformed cells have fewer but larger vinculin-associated adhesion plaques (E) compared to untransformed cells (G). Although SSeCKS expression causes an initial loss of typical adhesion plaques at the NPT (H), these structures are apparent at the cell's leading edge at the PT (F) as is seen in the untransformed cells in the presence of tetracycline (G). Similar effects on stress fiber formation are detected, with the caveat that SSeCKS induces smaller fibers at the PT (N) in comparison to untransformed cells with tetracycline (O).

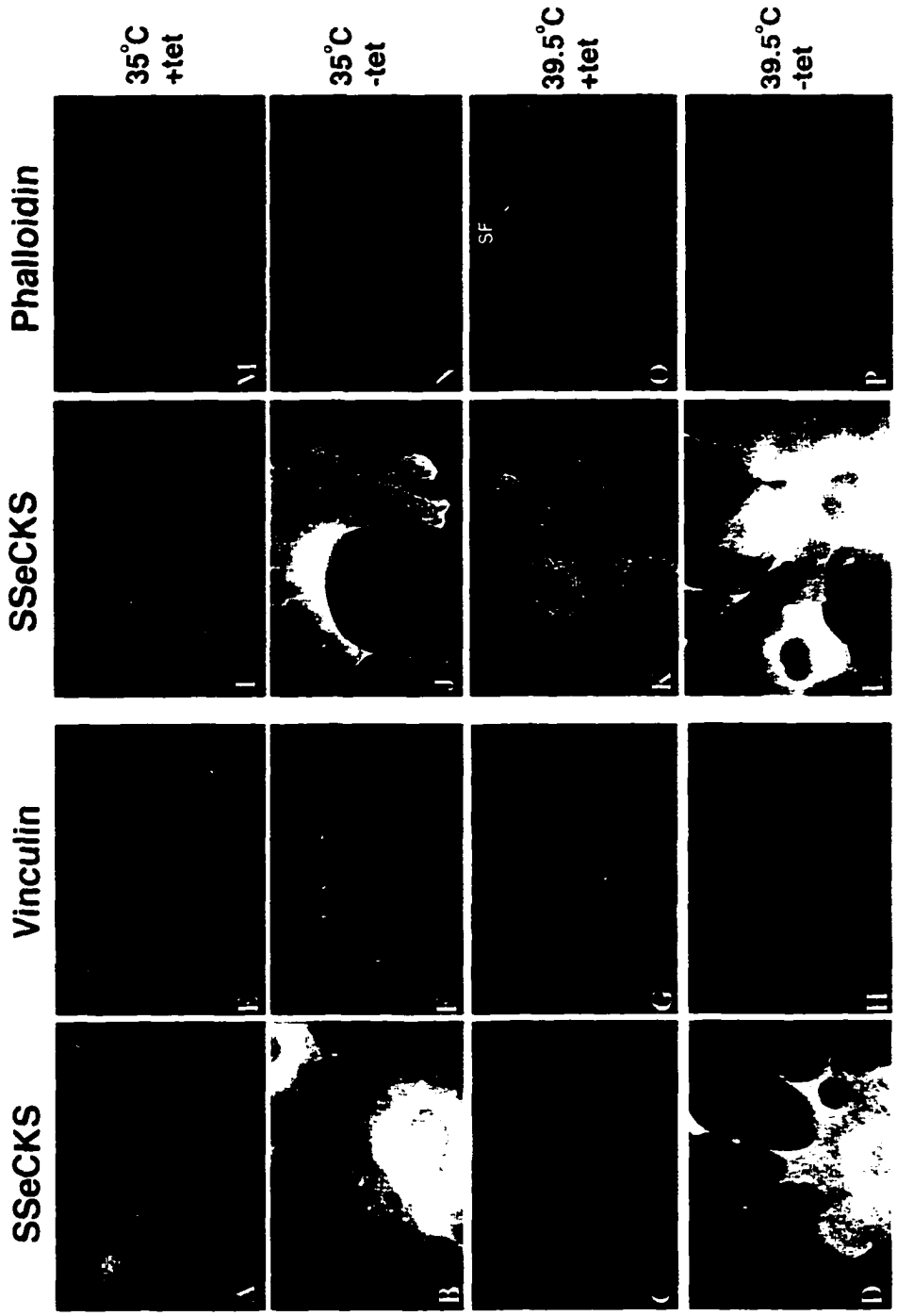



Fig. 3-11

# Chapter IV

# Regulation of G1-S Progression by the SSeCKS Tumor Suppressor: Control of Cyclin D1 Expression and Cellular Compartmentalization

Xueying Lin and Irwin H. Gelman (submitted for publication)

## ABSTRACT

SSeCKS is a major substrate of protein kinase C (PKC) associated with cytoskeletal architecture (Lin et al., 1996; Gelman et al., 1998). SSeCKS expression is down-regulated in *src*- and *ras*-transformed rodent fibroblasts, in human breast and prostate cancer cell lines, and in some prostate tumors (Lin et al., 1995; Gelman and Wang, unpublished data). Re-expression of SSeCKS suppresses oncogenic transformation in *src*-transformed NIH3T3 and rat prostate cancer cell lines, indicating that SSeCKS functions as a tumor suppressor (Lin and Gelman, 1997; Xia and Gelman, manuscript in preparation). Expression of SSeCKS is cell-cycle controlled: levels of underphosphorylated SSeCKS accumulate in contact-inhibited cells, but following addition of mitogens, SSeCKS levels decrease and phosphorylation of SSeCKS increases (Lin et al., 1995; Nelson and Gelman, 1997). Attempts to constitutively overexpress SSeCKS in untransformed rodent fibroblasts resulted in the selection of variants deleted of their transduced SSeCKS cDNA copies. Here, we show that the tetracycline-regulated expression of SSeCKS in NIH3T3 cells

induces growth arrest in G1, correlating with a reduction in cyclin D1 expression and pRb phosphorylation. Decreased cyclin D1 may be explained in part by a decrease in serum-inducible ERK2 activation following SSeCKS overexpression. The forced re-expression of cyclin D1 failed to rescue SSeCKS-induced G1 arrest possibly due to the sequestration of cyclin D1 in the cytoplasm. Bacterially-expressed protein encoding tandem cyclin-binding (CY) motifs in SSeCKS bound cellular cyclin D1, and mutation of either CY motif abrogated this binding. Our data indicate that SSeCKS controls G1-S progression by inhibiting cyclin D1 expression and/or sequestering cyclin D1 in the cytoplasm. These data suggest that SSeCKS plays a key role in controlling contact-inhibited growth, and that down-regulation of SSeCKS in tumor cells promotes contact-independent growth.

## INTRODUCTION

Cell cycle progression from the first gap (G1) to the DNA synthetic (S) phase is mainly controlled by the activity of cyclin D-CDK4/6 and cyclin E-CDK2 complexes (Sherr, 1993). Monomeric CDK catalytic subunits are inactive. The activation of CDKs is regulated at multiple levels: association with regulatory partner cyclins, posttranslational modification of CDKs, and interaction with CDK inhibitors (CKIs) (Morgan, 1995; Grana and Reddy, 1995; Pines, 1995). While cyclins and CDKs usually provide positive signals for CDK activation, the activity of CDKs is negatively regulated by association with CKIs. The members of INK4 family of CKI (p15, p16, p18, p19) specifically inhibit CDK4/6 activity by competing with cyclin D for association with CDK. In contrast, the members of Kip/Cip family of CKI (p21, p27, p57) inhibit the activity of both CDK4/6 and CDK2 by

forming a ternary complex (Sherr and Roberts, 1995; Grana and Reddy, 1995; Peter and Herskowitz, 1994).

The best studied downstream target of both cyclin D-CDK4/6 and cyclin E-CDK2 complexes is the tumor suppressor protein pRb. Hypophosphorylated pRb, which is the predominant form in G0 and G1, binds a family of transcription factors, E2Fs, thus preventing their role in transcribing proliferation factors (Weinberg, 1995). Phosphorylation of pRb by cyclin D-CDK4/6 occurs in mid-G1, and this initial phosphorylation makes pRb a competent substrate of cyclin E-CDK2 in late G1 (Delsal et al., 1996; Taya, 1997). The resulting hyperphosphorylated pRb has low affinity for E2Fs, which, in turn, are free to transcribe genes required for S phase entry. Several lines of evidence suggest that the primary role of cyclin D in cell cycle regulation is to phosphorylate pRb since microinjection of antibodies against cyclin D1 has no effect in $Rb^{-/-}$ cells (Lukas et al., 1995a; 1995b). Although cyclin E-CDK2 complexes can phosphorylate pRb, they also target other substrates involved in cell cycle regulation (Ohtsubo et al., 1995; Roussel et al., 1995) .

Current models suggest that cyclin D-CDK4/6 is responsible for G1 progression and cyclin E-CDK2 for G1-S transition. Cyclin D1 expression is induced during the delayed-early response to growth factors. Removal of growth factors during G1 phase results in rapid degradation of cyclin D1 and failure to progress to S phase, but has no effect once cells have passed a point in late G1 phase called the "R" or restriction point (Sherr, 1995). Ectopic expression of cyclin D1 accelerates G1 phase and reduces cell size and dependence on serum (Quelle et al., 1993). Fibroblasts microinjected in early G1 with

antibodies to cyclin D1 or with plasmids encoding antisence cyclin D1 are unable to enter S phase, whereas microinjection in late G1 has no effects, indicating a fundamental role for cyclin D1 in G1 progression (Baldin et al., 1993; Quelle et al., 1993).

The expression of cyclin D1 is mainly controlled at the transcriptional level and by ubiquintin-dependent proteolysis. Accumulation of cyclin D1 mRNA is dependent on mitogen- and anchorage-induced signals. Induction of cyclin D1 in early G1 requires the sustained activation of the ERK subfamily of MAP kinase (Lavoie et al., 1996; Bottazzi and Assoian, 1997). Degradation of cyclin D1 is dependent on phosphorylation-triggered, ubiquintin-mediated proteolysis (Diehl et al., 1997), which is also mitogen-dependent involving a Ras-PI3K-Akt-GSK3β-mediated pathway (Diehl et al., 1998). Cyclin D1 progressively translocates to the nucleus of human fibroblasts during G1 phase. Nuclear cyclin D1 levels decrease rapidly once cells enter S phase (Baldin et al., 1993). Recent evidence indicates that mutation of cyclin D1 residues required for correct protein folding (Diehl et al., 1998) causes retention of cyclin D1 in the cytoplasm and growth arrest in G1 (Diehl and Sherr, 1997).

One enigma in this field is how untransformed cells are growth arrested due to contact-inhibition even in the presence of growth factors and cell adhesion signals. This phenomenon implies the existence of at least one negative regulatory pathway that is dominant over the growth factor- and integrin-mediated effects on cyclin D expression. Contact-inhibition is defined in reality by a saturation density setpoint because untransformed cells do not exhibit growth arrest at the precise point of cell-cell contact. Mechanical forces defined by actin-based cytoskeletal architecture has been shown to

control mitogenic signaling during cell cycle progression (Huang et al., 1998). Thus, saturation density setpoints may be controlled by cytoskeletal proteins that influence cell shape in response to both growth factors and integrin-mediated adhesion.

SSeCKS transcripts are increased in contact-inhibited cultures but down-regulated in *src*- and *ras*-transformed fibroblasts (Lin et al., 1995). In untransformed rodent fibroblasts, SSeCKS RNA levels decrease rapidly following addition of serum or the activation of a temperature-sensitive v-*src*, whereas SSeCKS protein is rapidly phosphorylated in late G1 phase (Lin et al., 1995; Nelson and Gelman, 1997). Induction of SSeCKS phosphorylation by activated PKC causes a rapid translocation of SSeCKS from cytoskeletal and plasma membrane sites to the perinucleus (Lin et al., 1996; Gelman et al., 1998). These data suggest that SSeCKS function can be affected by either down-regulation or phosphorylation.

Multiple attempts to constitutively express SSeCKS showed that high levels inhibit cell proliferation, resulting in the selection of cell lines expressing deletion variants of SSeCKS (Lin et al., 1995). Recently, we demonstrated that high levels of SSeCKS expression can be achieved by tetracycline-regulated expression system (Lin and Gelman, 1997; Gelman et al., 1998). SSeCKS overexpression in NIH3T3 induces growth arrest marked by cell flattening and a transient loss of focal adhesion plaques and stress fibers (Gelman et al., 1998). SSeCKS overexpression suppresses morphological transformation and tumorigenicity in *src*-transformed rodent fibroblasts and in *ras*-activated rat prostate tumor cell lines, indicating a potential role as a tumor suppressor (Lin and Gelman, 1997; Xia and Gelman, manuscript in preparation)

In this report, we determine that SSeCKS-induced G1 arrest in NIH3T3 cells correlates with a lack of cyclin D1 induction, likely due to decreased levels of serum-inducible ERK2 activation. Unexpectedly, the forced expression of cyclin D1 failed to rescue growth arrest. SSeCKS expression resulted in the translocation of cyclin D1 from the nucleus to the cytoplasm, very likely mediated by direct binding to tandem cyclin binding (CY) motifs in SSeCKS. We conclude that SSeCKS controls cell cycle progression by inhibiting cyclin D1 expression and/or by sequestering cyclin D1 in the cytoplasm. This function of SSeCKS possibly influences the saturation density setpoint in untransformed cells.

## RESULTS

**Overexpression of SSeCKS results in growth arrest in G1 phase.**

Our previous attempts to produce stable cell lines with constitutive expression of SSeCKS resulted in the selection of variants deleted of their transduced SSeCKS cDNA copies (Lin et al., 1995). Using S2-6 cells (NIH3T3 with tetracycline-regulated expression of the tetracycline transactivator, tTA; gift of David Schatz, Yale University) we produced cell lines which express the full-length rat SSeCKS cDNA following the removal of tetracycline (Gelman et al., 1998). A number of resulting cell lines, such as S24 and S33, showed background levels of SSeCKS in the presence of tetracycline and more than 25-fold induction of the full length 280/290 kDa doublet forms of SSeCKS following tetracycline withdrawal (Fig. 4-1A). At least 40% of the cell lines selected showed a similar profile of inducibility of apparently full length protein (data not shown).

Consistent with our previous data (Gelman et al., 1998), SSeCKS expression induced morphological changes in cells. One day after removal of tetracycline, S24 cells became flat and enlarged. At day 4, the cells were extremely enlarged and flat (with almost indistinguishable cell edges) and apparently were growth arrested (Fig. 4-1B). No morphological changes were observed in V3 control cells (vector alone) in the absence of tetracycline.

We tested the effect of SSeCKS overexpression on proliferation rates in the presence of serum growth factors. In V4 control cells, removal of tetracycline decreased proliferation rates 20-30% (Fig. 4-1C), most likely due to squelching of endogenous transcription factors by the VP-16 moiety of the tTA as described by others (Gill and Ptashne, 1988). In contrast, cells expressing full length SSeCKS products such as S24 were growth arrested following tetracycline removal, showing only a 6-8 fold increase in cell numbers after 10 days of incubation (Fig. 4-1C). The finding of SSeCKS-induced growth arrest in a number of independently-derived clones indicates that this phenomenon is not due to the idiosyncrasies of a particular cell line.

The phenotype of SSeCKS-induced growth arrest is reminiscent of cellular senescence, which is characterized by a flat and enlarged morphology, and irreversible growth inhibition at subconfluent culture in the presence of serum (Stein and Dulic, 1995; Vojta and Barrett, 1995). To determine whether SSeCKS induces cellular senescence, tetracycline was added back to cell culture after cell flattening and growth arrest. Re-addition of tetracycline resulted in normal proliferation rates in two days (Fig. 4-1D), and

normal morphology in four days (data not shown). This suggests that cells did not senescence in respond to SSeCKS overexpression.

To determine where in the cell cycle SSeCKS arrests cell proliferation, cell cycle analysis was performed. S24 and control cells were put into G0 by serum starvation, then induced with serum in the presence or absence of tetracycline, followed by propidium iodide staining and FACS to analyze DNA content. Table 4-1 shows a 2-3 fold reduction in the percentage of cells in the S phase following expression of ectopic SSeCKS. Several independently derived tetracycline-regulated SSeCKS clones showed similar decreases in S phase concomitant with an increase in G1 phase (Table 4-1), indicating an overall G1 arrest. Interestingly, a small number of tetracycline-regulated SSeCKS clones, typified by S23 and S38, showed no G1 arrest (Table 4-1). Since these clones expressed apparently full length SSeCKS, we suspect that small deletions or mutations might occur in exogenous SSeCKS cDNA, affecting the ability of SSeCKS to induce G1 arrest.

**Overexpression of SSeCKS suppresses cyclin D1 expression, possibly by inhibiting ERK2 activation.**

G1-S progression in fibroblasts is mainly controlled by the activity of CDK4 and CDK2 (Sherr, 1995). Changes in the levels of G1 CDKs, cyclins, and CKIs as well as in the phosphorylation status of CDKs could result in G1 arrest. To investigate which cell cycle components are affected by SSeCKS overexpression, cell lysates from S24 and V3 cells were analyzed by western blotting. Among all the components examined, including cyclins such as cyclin D1, cyclin E and cyclin A, CDKs such as CDK2, CDK4 and CDK6, and CKIs such as p16, p18, p19, p21 and p27, only the expression of cyclin D1 was

dramatically reduced in S24 cells grown in the absence of tetracycline in comparison with V3 cells (Fig. 4-2A). The expression of p16 was undetectable in these NIH3T3-derived cell lines. p21 expression was decreased in both S24 and V3 cells after removal of tetracycline, probably due to the nonspecific effect of tTA. Similarly, the level of p19 was increased in both S24 and V3 cells in the absence of tetracycline.

Increase in the levels of hypophosphorylated pRb is a characteristic feature of G1 arrest in $Rb^{+/+}$ cells (Lukas et al., 1995a; 1995b). We then asked what was the phosphorylation status of pRb in the presence of SSeCKS overexpression. Western blotting revealed that majority of pRb was hypophosphorylated in S24 but not V3 cells after tetracycline withdrawal (Fig. 4-2B), which was consistent with SSeCKS-induced G1 arrest.

Because the expression of cyclin D1 is dependent on sustained ERK activation (Lavoie et al., 1996; Bottazzi and Assoian, 1997), we examined whether SSeCKS affects serum-inducible ERK activation. Lysates were prepared from serum-starved S24 cells grown in the presence or absence of tetracycline that were subsequently stimulated with serum for a various of time periods. SSeCKS overexpression had no effect on the levels of ERK2 protein as detected by western blotting analysis (Fig. 4-3A). However, serum-inducible ERK2 activation, as measured by the ability of ERK2 immunoprecipitates to phosphorylate myelin basic protein (MBP), was depressed by SSeCKS overexpression (Fig. 4-3B). The decreased activation of ERK2 is possibly responsible for cyclin D1 suppression.

**Overexpression of SSeCKS correlates with reduced proliferation rates, G1 arrest, suppression of cyclin D1 and cell flattening.**

We have shown previously that SSeCKS overexpression resulted in dramatic morphological changes including cell flattening, a transient loss of stress fibers and vinculin-associated adhesion plaques, and the formation of filopodia- and lamellipodia-like projections (Fig. 4-1B and Gelman et al., 1998). Table 4-2 shows a correlation between these effects and SSeCKS-induced G1 arrest in several independent tetracycline-regulated SSeCKS clones. In contrast, the putative variant clones such as S23 neither flattened nor arrested in G1. Additionally, G1 arrest always correlated with loss of cyclin D1 whereas the non-arresting clones expressed cyclin D1 in the absence of tetracycline. This indicates that these phenotypes are intrinsically connected with each other, and raises a possibility that reversion of one phenotype would lead to rescue of the others.

**Ectopic expression of cyclin D1 fails to rescue SSeCKS-induced growth arrest.**

Overexpression of cyclin D1 in rodent fibroblasts shortens the length of G1 phase and causes a smaller cell size (Quelle et al., 1993). If loss of cyclin D1 was sufficient to induce G1 arrest, we assumed that the forced expression of exogenous cyclin D1 should rescue the SSeCKS-induced arrest. To examine this possibility, human cyclin D1 was introduced into S24 cells by retrovirus infection. The resulting S24/D1 cells expressed high levels of cyclin D1, and cyclin D1 levels were unaffected by SSeCKS overexpression (Fig. 4-4A). Although these cells had a smaller size without SSeCKS induction, they still exhibited a flattened and enlarged morphology upon SSeCKS induction (data not shown; and Fig. 4-7A and 4-7B). Moreover, the forced expression of cyclin D1 failed to rescue SSeCKS-induced G1 arrest as shown by repressed proliferation in high serum (Fig. 4-4B) and by increased abundance of cells in G1 phase by FACS analysis (Fig. 4-4C). However,

S24/D1 cells reached higher saturation density than S24/V control cells (vector infected cells) in the absence of tetracycline (Fig. 4-4B). Most significantly, the majority of pRb remained hypophosphorylated in S24/D1 cells grown in the absence of tetracycline (Fig. 4-4D), however, there was no change in the steady-state levels of cyclins E and A, CDKs 2, 4, and 6, and CKIs p16, p18, p19, p21 and p27 (data not shown).

Because cyclin E-CDK2 is also responsible for pRb hyperphosphorylation and G1-S progression, we then asked whether SSeCKS overexpression affected the activity of cyclin E-CDK2. When measured by *in vitro* kinase assay using histone H1 as substrates, the cyclin E-dependent CDK2 activity was suppressed in both SSeCKS overexpressors (S24, S24/D1, and S24/V) and control cells (V3) grown in the absence of tetracycline (data not shown), which possibly results from the expression of VP-16 containing tTA. Thus, the reduction of cyclin E-CDK2 activity was not specifically induced by SSeCKS overexpression, and could not be responsible for pRb hypophosphorylation. The data imply that pRb hypophosphorylation is probably due to the inhibition of cyclin D-CDK4 activity by SSeCKS overexpression.

**SSeCKS binds cyclin D1 *in vitro*, mainly via tandem CY motifs.**

Work from Dutta's group identified a so-called cyclin-binding (CY) motif which facilitates the binding of cyclins to several cell cycle components such as p21 and CDC25 (Chen et al., 1996; Saha et al., 1997). SSeCKS encodes two closely spaced potential CY motifs, KKLFSxxxxKKLSG (two basic amino acids followed by two nonpolar ones, usually starting with L) (Fig. 4-5A). The existence of the potential CY motifs suggests that SSeCKS may binds cyclins. We therefore examined whether a GST fusion protein

containing the SSeCKS CY motifs could bind cyclin D1 in an *in vitro* pull down assay. GST-SSeCKS2 (a.a. residues 389 to 552 of SSeCKS) bound endogenous and exogenous cyclin D1 from lysates prepared from S24, S24/D1, S24/V and V3 cells grown in the presence or absence of tetracycline (Fig. 4-5B). GST alone failed to bind cyclin D1, indicating that the binding was directed by SSeCKS sequences (Fig. 4-5B). The levels of cyclin D1 bound by GST-SSeCKS2 corresponded to their expression levels. Higher amounts of cyclin D1 were bound in the S24/D1 cell lysates irrespective of tetracycline conditions, whereas in S24 cells, where SSeCKS overexpression suppresses cyclin D1 levels (Fig. 4-5C), less cyclin D1 was bound in the (-)tet condition compared to the (+)tet condition (Fig. 4-5B).

To strengthen the argument that the CY motifs mediate cyclin D1 binding, we subjected the conserved amino acid residues to *in vitro* mutagenesis, and then tested the binding efficiencies of these mutants. Specifically, the KKL residues were changed to serines, and these mutations were produced in either the upstream or downstream CY motifs or in both (Fig. 4-6A). Alteration of either the up- or down-stream KK residues reduced cyclin D1 binding by approximately 70%; KK mutations in both motifs reduced binding almost 90%. In contrast, the L to S mutations had little effect on cyclin D1 binding (Fig. 4-6B). As a control, Fig. 4-6C showed that none of the mutations affected the expression level or stability of the bacterially-expressed GST-SSeCKS fusion proteins. These data show a dependence on the charged residues in the CY motifs for cyclin D1 binding, and further indicate that CY motifs function both independently and in tandem.

These data also indicate that undefined flanking sequences of SSeCKS CY motifs mediate cyclin D1 binding to a much less extent than the CY sequences.

**SSeCKS overexpression redirects cyclin D1 to the cytoplasm.**

Cyclin D1-dependent kinase activity towards pRb is subject to not only cyclin D1 binding, but also subcellular localization of cyclin D1 (Diehl and Sherr, 1997; Taules et al., 1998). Because SSeCKS binds cyclin D1 *in vitro*, and majority of SSeCKS is localized in the cytoplasm as described previously (Fig. 4-1B; and Gelman et al., 1998), we considered the possibility that SSeCKS may sequester cyclin D1 in the cytoplasm via binding. To examine the subcellular location of cyclin D1, S24/D1 cells were immunostained with polyclonal antibody to cyclin D1. Cyclin D1 was located in the nucleus, but disappeared from the nucleus upon SSeCKS overexpression (Fig. 4-7A). To detect whether cyclin D1 was co-localized with SSeCKS, we performed double immunostaining analysis in S24/D1 cells using polyclonal antibody to SSeCKS and monoclonal antibody to cyclin D1. In the presence of SSeCKS induction, both SSeCKS and cyclin D1 were located in the cytoplasm, with brightest staining at the perinuclear region. When the two confocal images were superimposed, SSeCKS and cyclin D1 appeared to be co-localized in the cytoplasm to a considerable extent (Fig. 4-7B). Similar phenomenon was observed in the side-section of SSeCKS and cyclin D1 staining (Fig. 4-7C). The cytoplasmic staining of cyclin D1 and partial co-localization of SSeCKS and cyclin D1 imply that SSeCKS overexpression redirects cyclin D1 to the cytoplasm, very likely via CY motif-mediated interaction.

## DISCUSSION

**SSeCKS-induced growth arrest correlates with the down-regulation of cyclin D1.**

In this study, we present evidence that the inducible overexpression of SSeCKS in untransformed NIH3T3 (S24 cells) results in growth arrest in G1 accompanied by cell flattening and the loss of cyclin D1 expression. This result is predicted by our previous data showing that expression and phosphorylation of SSeCKS is cell cycle-regulated during G1-S, and that attempts to produce stable SSeCKS-expressing cell lines resulted in the selection of SSeCKS deletion mutants (Lin et al., 1995). SSeCKS-induced growth arrest cannot be viewed as simple toxicity since we showed elsewhere that *v-src* transformed NIH3T3 overexpressing SSeCKS (S24/ts72*src* cells) lose oncogenic growth characteristics but are not growth arrested (Lin and Gelman, 1997). Our data also indicate that SSeCKS suppresses expression of cyclin D1 possibly by preventing sustained activation of ERK2. However, unlike S24 cells, where the overexpression of SSeCKS inhibits serum-inducible ERK2 activation (Fig. 4-3B), the ability of S24/ts72*src* cells to proliferate is likely due to the superinduction of ERK2 activity when SSeCKS is overexpressed in the presence of active v-*src* (Lin and Gelman, 1997).

Sustained activation of the ERK subfamily of MAP kinase is required for cyclin D1 expression (Lavoie et al., 1996; Albanese et al., 1995). Although both growth factor stimulation and ECM attachment can activate ERK (Schwartz et al., 1995), cyclin D1 expression is dependent on the presence of both stimuli (Bohmer et al., 1996; Zhu et al., 1996). One possibility for this is that synergistic interaction between RTKs and integrins is required for eliciting sustained activation of ERK (Miyamoto et al., 1996; Zhu and Assoian, 1995). SSeCKS clearly affects ERK2 activation by serum growth factors.

SSeCKS has been shown to induce integrin-independent Tyr phosphorylation of FAK (Gelman et al., 1998). Because FAK activation has been tied to ERK activation (Schwartz et al., 1995; Zhu and Assoian, 1995), one might expect SSeCKS to induce rather than inhibit ERK2 activation. The fact that SSeCKS inhibits serum-inducible ERK2 activation suggests that SSeCKS probably up- and down-regulates growth factor- and integrin-mediated pathways for ERK2 activation, eventually resulting in the inhibition of ERK2 activity.

Cyclin E expression is controlled at the transcriptional and posttranslational levels. It has been shown that pRb mediates repression of cyclin E transcription since the levels of cyclin E are greatly enhanced in pRb knock-out mouse (Herrera et al., 1996). pRb is hypophosphorylated when SSeCKS is overexpressed, thus, one might expect reduced expression of cyclin E in the absence of tetracycline. However, the levels of cyclin E are not altered by SSeCKS overexpression. This might be explained by posttranslational control of cyclin E. Cyclin E protein has been shown to accumulate in physiologically G1 arrested cells such as senescent fibroblasts, most likely due to posttranslational mechanisms (Stein and Dulic, 1995). How posttranslational mechanisms regulate cyclin E expression in our system is not yet known .

There has been an enigma as to why untransformed cells fail to progress into S phase when they reach a specific saturation density even in the presence of growth factors and clustered integrins. One possibility could be due to the increased expression of p27 under contact inhibition (Hengst and Reed, 1996), which suppresses the activities of cyclin D-CDK4/6 and cyclin E-CDK2. Another possibility, we speculate, could be due to the

increased SSeCKS expression in contact-inhibited culture, which decreases cyclin D1 expression. This notion is supported by the fact that increased saturation densities are observed by decreasing SSeCKS expression (unpublished observation). It will be interesting to investigate whether saturation density is enhanced in SSeCKS knock-out cells.

**SSeCKS-induced G1 arrest is mediated by redirecting cyclin D1 to the cytoplasm.**

Cyclin-binding (CY) motif was originally identified as sequences in the N-terminus of p21 required for formation of complexes between p21 and cyclin-CDKs and for the inhibitory effects of p21 on CDK activity (Chen et al., 1996). The CY consensus motif is (K/R)/(K/R)/nonpolar/nonpolar, with the first non-polar residue usually leucine. Our results demonstrate that SSeCKS binds cyclin D1 *in vitro*, mainly mediated by tandem CY motifs between residues 503-507 and 512-515. Mutagenesis analysis revealed that mutation of the charged residues (KK) in either CY motif greatly reduced binding, and that mutation of KK in both motifs resulted in even greater loss of binding. This indicates that the two CY motifs are functional individually but that they also function cooperatively.

The binding of bacterially-expressed SSeCKS to cellular cyclin D1 suggests that SSeCKS and cyclin D1 may form a complex *in vivo*. However, we have not been able to detect association between SSeCKS and cyclin D1 inside cells by co-immunoprecipitation. Several possibilities may explain the failure: i) polyclonal antibodies against SSeCKS or cyclin D1 used for immunoprecipition may disrupt the complex, ii) the necessary inclusion of detergents in the lysis buffer may dissociate the interaction between SSeCKS and cyclin D1, iii) the association of the SSeCKS/cyclin complex with the cortical cytoskeleton may

prevent their accessibility to the soluble cell fraction after lysis. In defense of the latter notion, several groups including our own have been unable to demonstrate *in vivo* association between the SSeCKS/gravin protein and their *in vitro* binding partners such as PKC (Nauert et al., 1997).

Our immunostaining study clearly showed that cyclin D1 was translocated from the nucleus to the cytoplasm once SSeCKS was overexpressed. The co-localization of SSeCKS and cyclin D1 in the cytoplasm strengthens the notion that they associate with each other inside cells via CY motifs. However, we cannot rule out the possibility that SSeCKS forms complex with cyclin D1 via an intermediate. Whether SSeCKS redirects CDK4 localization is not defined yet. We assume that CDK4 also translocates to the cytoplasm together with cyclin D1 since the two proteins always co-localize (Diehl and Sherr, 1997; Taules et al., 1998). Nevertheless, nuclear CDK4 was not sufficiently activated to phosphorylate pRb and to stimulate cell cycle progression.

Recently, subcellular compartmentalization has been suggested as a key mechanism in regulating the activity and specificity of kinases (Hubbard and Cohen, 1993; Faux and Scott, 1996b). Cyclins have been considered as targeting subunits of CDKs, which direct CDKs to specific substrates by binding to the substrates (Hubbard and Cohen, 1993). Whereas cyclin D1 is progressively located in the nucleus during G1 phase and then rapidly diminished from the nucleus during S phase, the total level of cyclin D1 as determined by immunoblotting analysis declines only modestly during S phase, suggesting that the function of cyclin D1 may be regulated by celluler localization (Sherr, 1995). A recent study showed that a T156A substitution in cyclin D1 caused retention of kinase-

inactive cyclin D1-CDK4 complexes in the cytoplasm, and consequently G1 arrest (Diehl and Sherr, 1997). Additionally, inhibition of calmodulin activity caused G1 arrest marked by a translocation of cyclin D1-CDK4 complexes to the cytoplasm (Taules et al., 1998). This group suggested that cyclin D1 interacted with calmodulin in the cytoplasm via a calmodulin-binding protein, presumably Hsp90. Because SSeCKS is a calmodulin-binding protein (chapter V), it is possible that SSeCKS mediates association between cyclin D1 and calmodulin.

In this study, we present a framework of how SSeCKS, a cytoskeletal protein, controls cell cycle progression. SSeCKS overexpression-induced growth arrest in G1 could be due to its ability to suppress expression of cyclin D1 through inhibition of ERK2 activity. This may have relevance to normal cell cycle control. Under certain circumstances such as cyclin D1 overexpression, SSeCKS-induced growth arrest may also be due to the sequestration of cyclin D1 in the cytoplasm, which is a relatively novel mechanism for the inactivation of CDK4 catalytic activity. Growth arrest induced by SSeCKS under these two conditions strongly suggests that SSeCKS plays a very important role in cell cycle regulation.

**Fig. 4-1. SSeCKS overexpression results in growth arrest.**

**A.** Western blot showing expression of SSeCKS. Overexpression of SSeCKS was achieved by a tetracycline-regulated system, in which the expression of ectopic protein was induced after removal of tetracycline. Cell lysates of SSeCKS clones (S24 and S33) and control clones (V3 and V4) grown in the presence or absence of tetracycline were used for western blot analysis. In the absence of tetracycline, the ectopic expression of SSeCKS (280/290 kDa doublet) was turned on in S24 and S33 cells. **B.** Morphological changes after SSeCKS expression. S24 and V3 cells grown in the presence or absence of tetracycline were immunostained (a and b) for SSeCKS expression. c is Giemsa staining visualized by phase-contrast microscopy. SSeCKS overexpression results in cell flattening, decreased refractility, increased cell size, and production of projections. Removal of tetracycline does not affect the morphology of control V3 cells. **C.** Proliferation rate assay measuring cell numbers. The proliferation rate of S24 cells is dramatically reduced when SSeCKS is overexpressed. The approximately 30% reduction on proliferation rate of V4 cells is possibly due to the toxic effect of VP-16 encoded in the tetracycline transactivator. **D.** Proliferation rate assay showing that SSeCKS-induced growth arrest is reversible. Tetracycline was added back to cell cultures at day 6 after its removal. Two days later, S24 cells started to proliferate, indicating that SSeCKS overexpression does not induce cellular senescence.

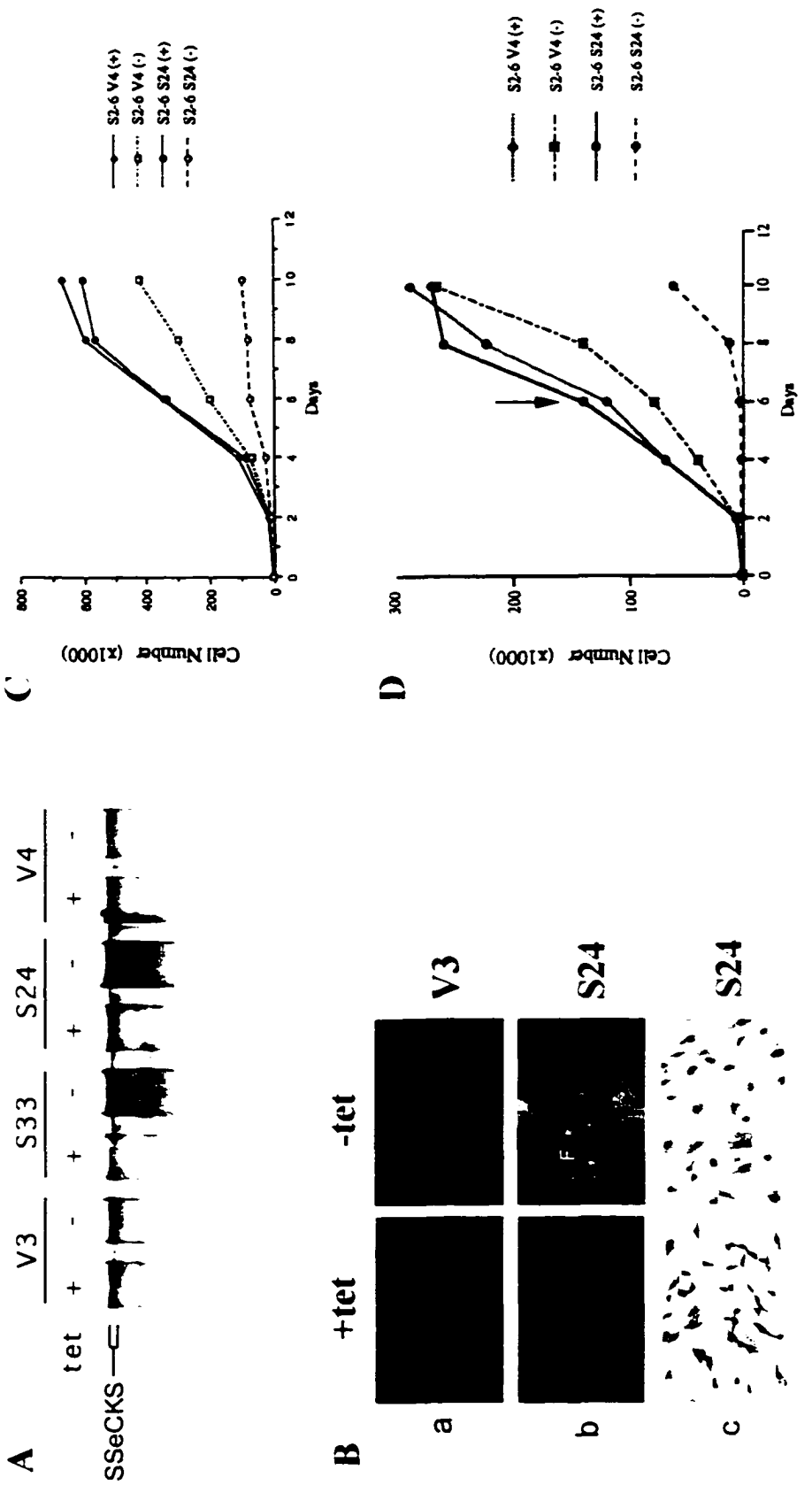



Fig. 4-1

**Table 4-1. SSeCKS overexpression results in G1 arrest.**

Cell cycle analysis showing the percentage of cells in each cell cycle stage by propidium iodide staining. SSeCKS clones (S24, S26, S33, S23, and S38) and control (V3 and V9) clones were synchronized by overnight serum starvation, and then stimulated with media containing 10% calf serum. The cells were fixed with 70% ethanol, labeled with propidium iodide, and analyzed for DNA content by FACS. S24, S26 and S33 clones are typical of the tetracycline-regulated SSeCKS overexpressing cell lines in that they express full-length SSeCKS protein and exhibit the G1 arrest phenomenon. S23 and S38 clones produce apparently full-length SSeCKS, but do not appear to be G1 arrested.

| | G0/G1 (%) | | S (%) | | G2/M (%) | |
|---|---|---|---|---|---|---|
| | +tet | -tet | +tet | -tet | +tet | -tet |
| V3 | 54.6 | 58.9 | 20.0 | 19.2 | 25.2 | 21.8 |
| V9 | 59.9 | 63.2 | 23.2 | 23.0 | 16.9 | 13.8 |
| S24 | 54.5 | 72.5 | 23.9 | 11.7 | 21.6 | 15.8 |
| S26 | 49.6 | 70.2 | 26.9 | 14.2 | 23.5 | 15.5 |
| S33 | 54.3 | 72.1 | 23.9 | 10.2 | 21.8 | 17.7 |
| S23 | 60.8 | 63.7 | 21.1 | 18.4 | 18.1 | 17.9 |
| S38 | 60.9 | 56.3 | 21.7 | 21.8 | 17.4 | 21.8 |

**Table 4-1**

**Fig. 4-2. SSeCKS overexpression results in suppression of cyclin D1 expression and pRb hypophosphorylation.**

S24 and V3 cells were grown in the presence or absence of tetracycline for 4 days before collecting cell lysates. **A.** Western blot showing the steady state levels of cyclins, CDKs, and CKIs. Among the examined cell cycle regulators, only the level of cyclin D1 is specifically reduced in response to SSeCKS overexpression. **B.** Western blot showing the phosphorylation status of pRb. The abundance of hypophosphorylated pRb (lower isoform) is greatly increased when SSeCKS expression is induced.

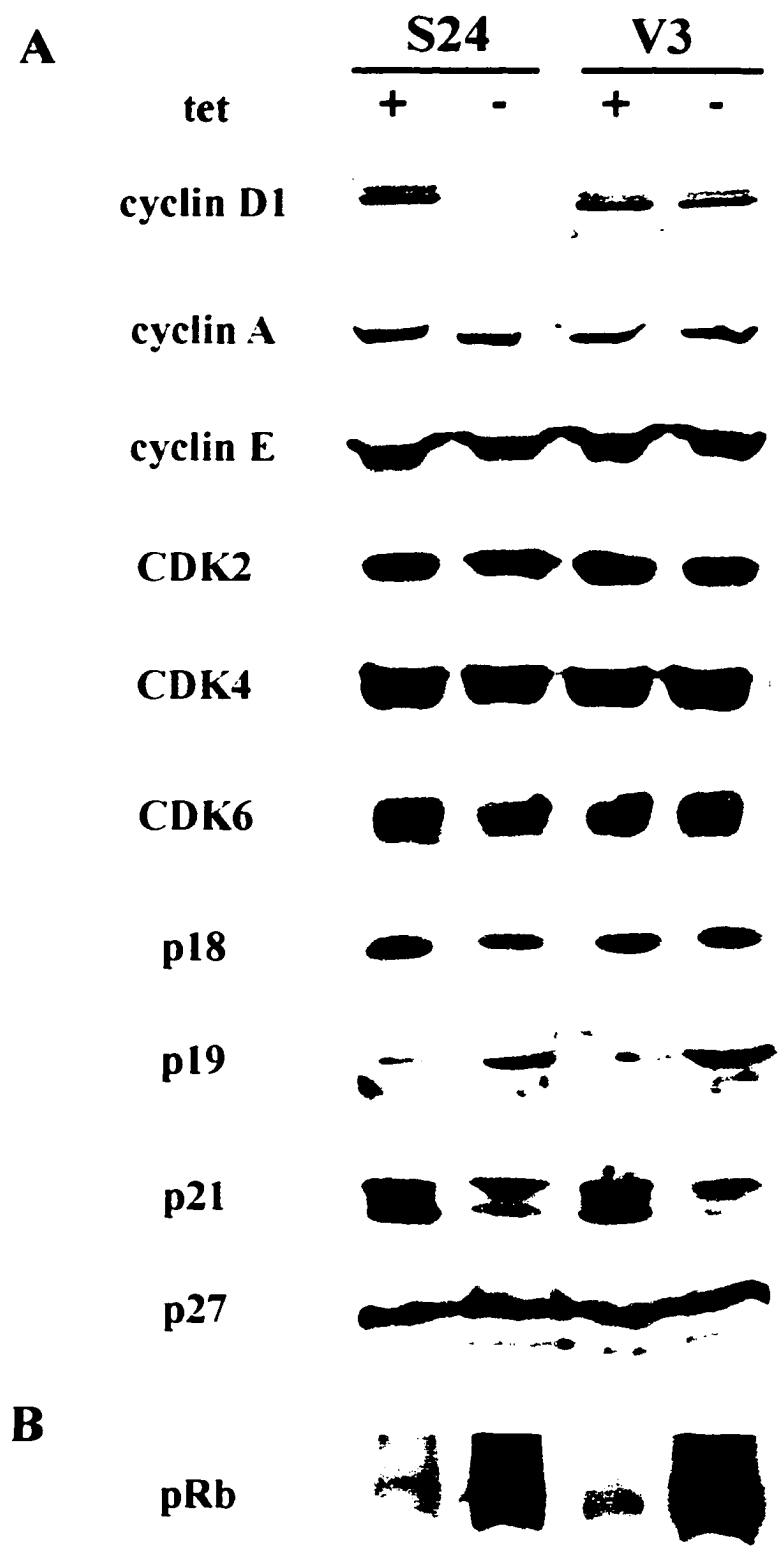



Fig. 4-2

**Fig. 4-3. SSeCKS overexpression inhibits serum-inducible ERK2 activity.**

S24 cells grown in the presence or absence of tetracycline were starved of serum overnight, and then stimulated with media supplemented with 10% calf serum for various times. **A.** ERK2 expression detected by western blot analysis. **B.** ERK2 kinase activity measured by *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. SSeCKS overexpression results in a decrease in serum-inducible ERK2 activity.

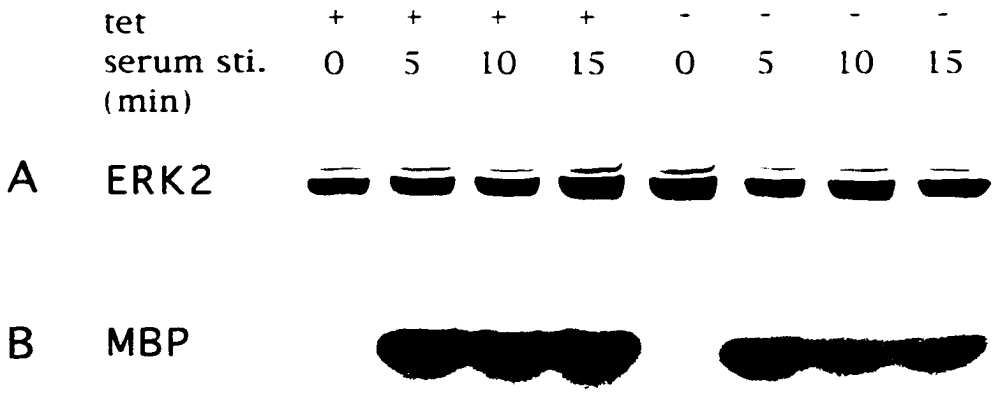



**Fig. 4-3**

**Table 4-2. SSeCKS overexpression correlates with reduced proliferation rates, G1 arrest, suppression of cyclin D1, and cell flattening.**

SSeCKS-induced phenotypes include cell flattening, dramatically reduced proliferation rates, G1 arrest, and suppression of cyclin D1. These phenotypic changes are all co-expressed. However, some of the SSeCKS clones such as S23 and S38 do not exhibit phenotypic alteration although they seem to express full-length SSeCKS.

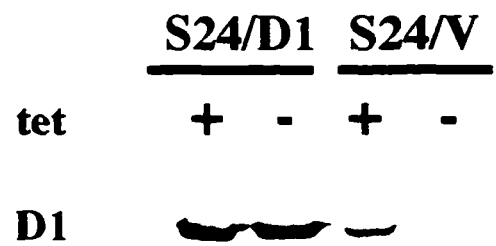
| | V3 | V9 | S24 | S26 | S33 | S23 | S38 |
|---|---|---|---|---|---|---|---|
| **flattening** | - | - | + | + | + | - | - |
| **growth arrest** | - | - | + | + | + | - | - |
| **G0/G1 arrest** | - | - | + | + | + | - | - |
| **suppression of cyclinD1** | - | - | + | + | + | - | - |

**Table 4-2**

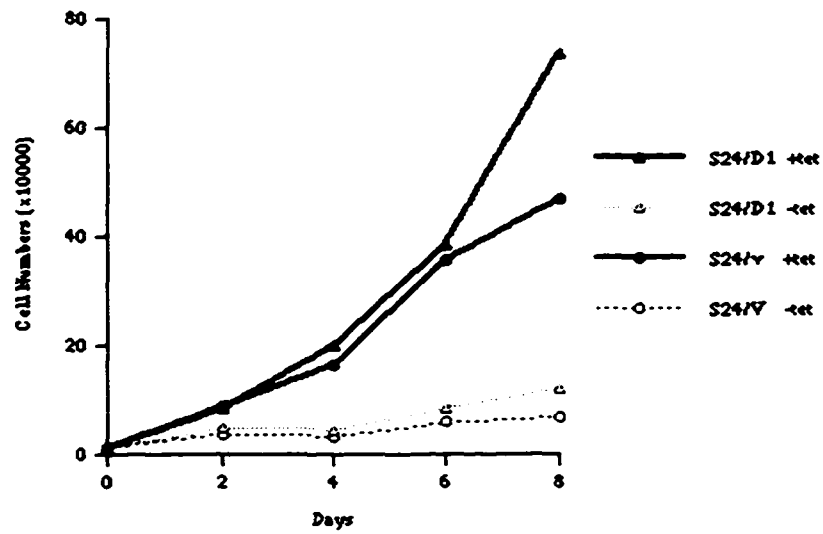
**Fig. 4-4. Ectopic expression of cyclin D1 fails to rescue SSeCKS-induced G1 arrest.**

**A.** Western blot showing expression of cyclin D1. Ectopic expression of cyclin D1 in S24 cells (S24/D1) was achieved by retroviral transduction, followed by G418 selection for stable clones. S24/V is vector-infected S24 cells. **B.** Proliferation rate assay measured by cell number. The proliferation rate of S24/D1 cells is inhibited by SSeCKS overexpression but to a little less extent when compared with S24/V cells. **C.** Cell cycle analysis showing the percentage of cells in each cell cycle stage by propidium iodide staining. SSeCKS overexpression results in increased abundance of S24/D1 cells in G1 phase. **D.** Western blot showing the phosphorylation status of pRb. The majority of pRb is hypophosphorylated in response to SSeCKS expression, even in S24/D1 cells.

A




B




C

| | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|
| S24/D1 (+tet) | 54.6 | 36.9 | 8.5 |
| S24/D1 (-tet) | 72.6 | 22.4 | 5.0 |

D


Fig. 4-4

**Fig. 4-5. Bacterially-expressed SSeCKS binds cellular cyclin D1 *in vitro*.**

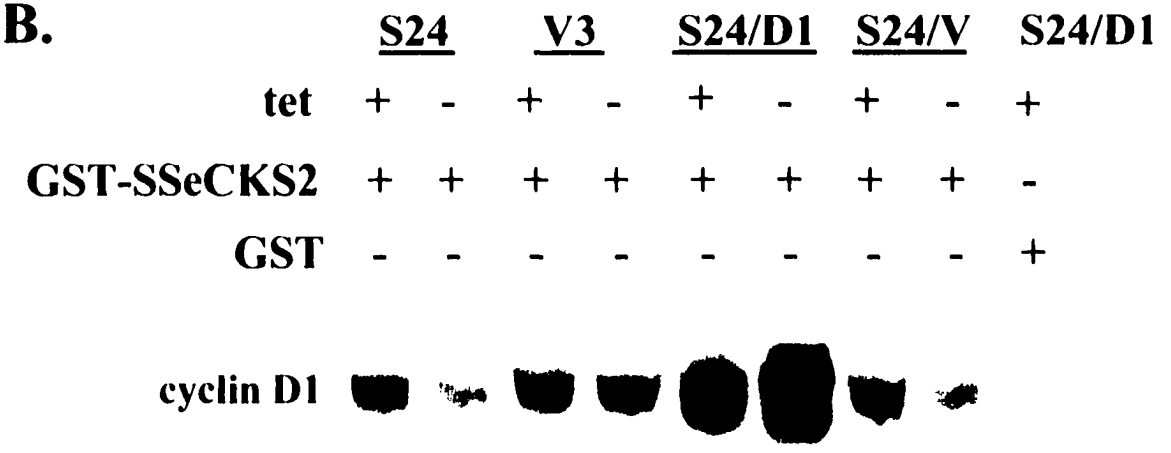
**A.** SSeCKS encodes two potential cyclin-binding (CY) motifs. **B.** *In vitro* pull down assay showing the binding of cyclin D1 to SSeCKS. Cells grown in the presence or absence of tetracycline were lysed in NP-40 containing lysis buffer. SSeCKS2, a fragment of SSeCKS bearing the potential CY motifs, was expressed as a GST fusion protein. GST-SSeCKS2 bound to glutathione-Sepharose beads was incubated with cell lysates, followed by western blot analysis using antibody against cyclin D1. GST-SSeCKS2, but not GST, bound cellular cyclin D1. **C.** Western blot showing cellular cyclin D1 levels in the cell lysates.

**A.** **Consensus Sequence of Cyclin-Binding (CY) Motif:**

**basic/basic/nonpolar/nonpolar**

**SSeCKS: SPLKKLFSSSGLKKLSGK**

**B.**




**C.**


**Fig. 4-5**

**Fig. 4-6. The binding of cyclin D1 to SSeCKS is mainly mediated by CY motifs.**

**A.** Sequence alignment showing mutations produced in the CY motifs. The consensus CY motifs were mutated by site-directed mutagenesis, with KK to SS and L to S. **B.** *In vitro* pull down assay showing the effect of CY mutations on the binding of cyclin D1 to SSeCKS. Wild type and mutant SSeCKS2 were expressed as GST fusion proteins, and then incubated with S24/D1 cell lysates, followed by western blot analysis using antibody against cyclin D1. The SSeCKS2 mutant with both CY motifs mutated (10) diminished but not completely abolished the binding of cyclin D1 to SSeCKS. **C.** Amounts of wild type and mutant GST-SSeCKS2 used in the pull down assay were shown by coomassie blue staining.

**A**

| | | | | | |
|---|---|---|---|---|---|
| wt | SPL | KKLFS | SSGL | KKLSG | K |
| 1 | SPL | **SS**LFS | SSGL | KKLSG | K |
| 2 | SPL | KKLFS | SSGL | **SS**LSG | K |
| 3 | SPL | **SS**LFS | SSGL | **SS**LSG | K |
| 4 | SPL | KK**S**FS | SSGL | KKLSG | K |
| 5 | SPL | KKLFS | SSGL | KK**S**SG | K |
| 6 | SPL | KK**S**FS | SSGL | KK**S**SG | K |
| 7 | SPL | **SSS**FS | SSGL | KKLSG | K |
| 8 | SPL | **SSS**FS | SSGL | **SS**LSG | K |
| 9 | SPL | **SSS**FS | SSGL | KK**S**SG | K |
| 10 | SPL | **SSS**FS | SSGL | **SSS**SG | K |
| 11 | SPL | KKLFS | SSGL | **SSS**SG | K |
| 12 | SPL | **SS**LFS | SSGL | **SSS**SG | K |
| 13 | SPL | KK**S**FS | SSGL | **SSS**SG | K |

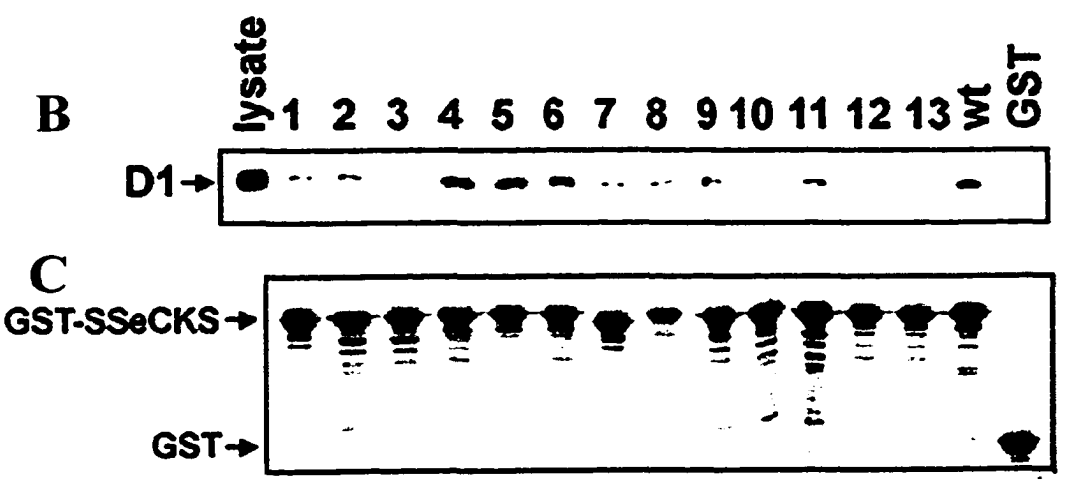



**Fig. 4-6**

**Fig. 4-7. SSeCKS overexpression redirects cyclin D1 to the cytoplasm.**

**A.** Cyclin D1 immunostaining. S24/D1 and V3/D1 (V3 cells overexpressing cyclin D1) cells grown in the presence or absence of tetracycline were fixed and immunostained for cyclin D1 expression using polyclonal antibody against cyclin D1. The nuclear staining of cyclin D1 is diminished in response to SSeCKS overexpression. **B.** Confocal images of SSeCKS and cyclin D1 double immunostaining. S24/D1 cells were immunostained for SSeCKS and cyclin D1 simultaneously using polyclonal antibody against SSeCKS and monoclonal antibody against cyclin D1. Cyclin D1 is located in the cytoplasm upon SSeCKS overexpression. SSeCKS and cyclin D1 exhibit a certain degree of co-localization. **C.** Confocal images of SSeCKS and cyclin D1 double immunostaining were viewed in the X-Z axis showing absence of nuclear cyclin D1.

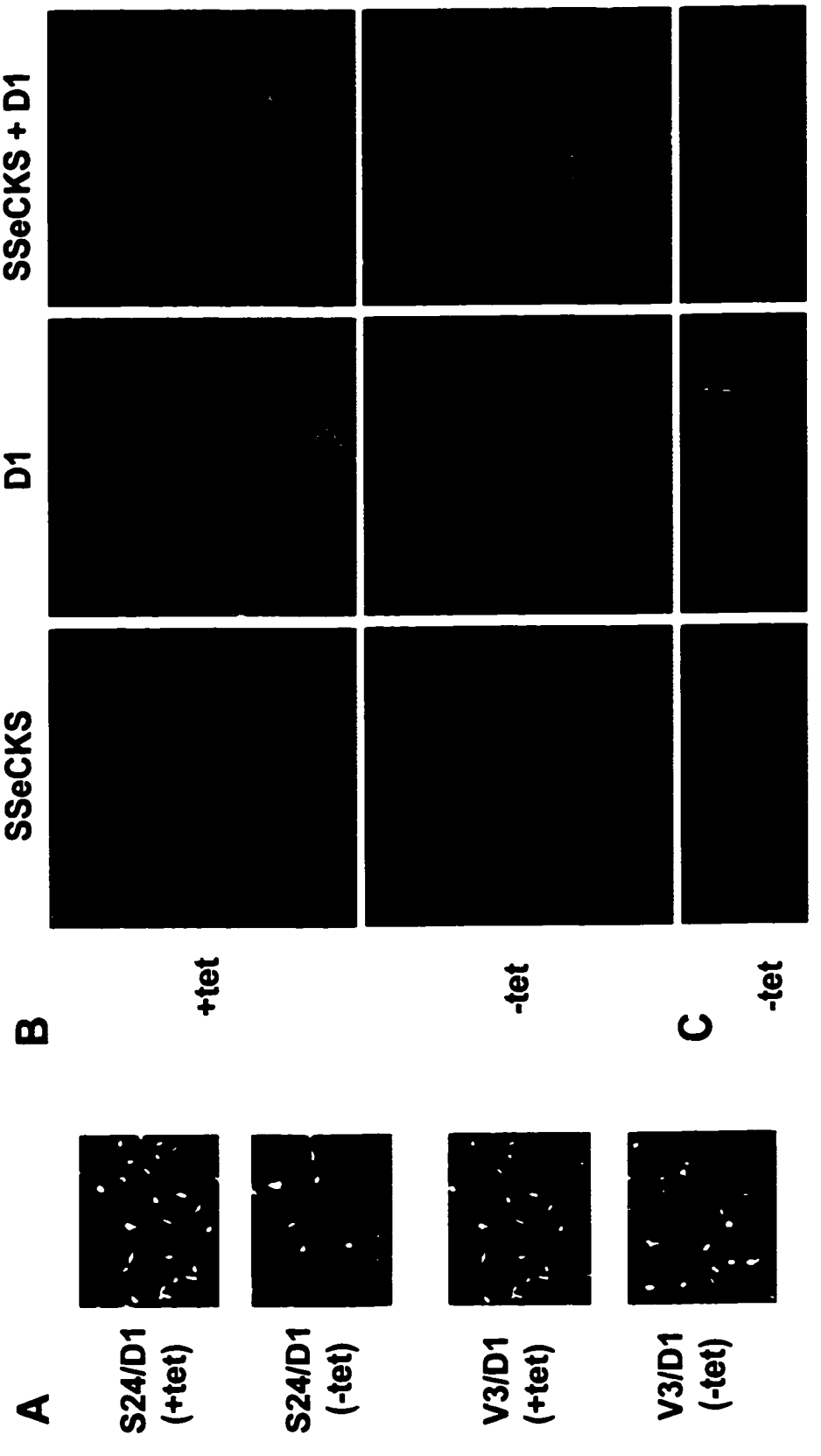



Fig. 4-7

# Chapter V

# SSeCKS, Coordinating Four Signaling Molecules, Is a Mammalian Scaffolding Protein

Xueying Lin, Eugene Tombler, and Irwin H. Gelman

## ABSTRACT

SSeCKS was previously described as a cytoskeleton-associated protein (Lin et al., 1996; Gelman et al., 1998). Overexpression of SSeCKS induces growth arrest in G1 by inhibiting cyclin D1 expression and/or sequestering cyclin D1 in the cytoplasm. The retention of cyclin D1 in the cytoplasm is most likely mediated by binding to CY motifs on SSeCKS (chapter IV). SSeCKS was cloned in a subtractive hybridization screen for transcriptionally suppressed genes in *src*-transformed cells (Frankfort and Gelman, 1995), and in an effort to identify PKC binding/substrate proteins by overlay assays (Chapline et al., 1996). SSeCKS is highly homologous to AKAP250, also named gravin, a scaffolding protein which binds to PKA and PKC (Nauert et al., 1997). SSeCKS is also demonstrated to be a PKA anchoring protein, which binds to RII regulatory subunits of PKA (personal communication, Susan Jaken). Here, we show that SSeCKS is a PKC-binding protein *in vitro*, requiring phospholipids as a bridge. SSeCKS is a PKC substrate *in vivo* and *in vitro*, with two *in vitro* phosphorylation sites mapped (Ser 515 and Ser 599). PKC activation causes rapid SSeCKS translocation from the cytoplasm to the perinuclear region. SSeCKS can interact with calmodulin *in vitro* in a $Ca^{2+}$- dependent manner.

Based on these biochemical data, we propose that SSeCKS regulates intracellular signal transduction by acting as a scaffolding protein for at least four signaling molecules: PKC, PKA, cyclin D1, and calmodulin. (The experiment involving *in vivo* phosphorylation of SSeCKS was performed by Eugene Tombler of our laboratory).

## INTRODUCTION

Intracellular signal transduction from the cell surface to specific subcellular compartments is a complicated and highly regulated process. The fundamental questions in this field include: how are different signaling pathways assembled, and where does the specificity come from? The assembly of protein kinases and phosphatases into architecturally defined complexes and the targeting of these complexes to appropriate subcellular compartments are critical events in conferring specificity and selectivity of signalings (Hubbard and Cohen, 1993).

The targeting of protein tyrosine kinases or phosphatases is usually mediated by adaptor proteins such as Grb2, p85, IRS-1 and Crk. These proteins often contain SH2 and SH3 domains which recognize, respectively, phosphotyrosyl residues and a proline-rich motif (P-X-X-P) (Pawson, 1995; Bork et al., 1997; Pawson and Scott, 1997). The targeting of serine/threonine kinases or phosphatases is mediated by targeting proteins or by covalent linkage to lipids which facilitate interaction with active sites on plasma membranes or the cytoskeleton (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). The targeting mechanisms for PKA and PKC are well documented. PKA targeting is achieved by AKAPs (A kinase anchoring proteins). One

class of PKC targeting proteins is PKC substrate/binding proteins, whose association with PKC is dependent on the presence of phospholipids and is negatively regulated by phosphorylation by PKC (Faux and Scott, 1996b; Lester and Scott, 1997).

Recently, a new family of targeting proteins has emerged, called scaffolding proteins (Faux and Scott, 1996b; Lester and Scott, 1997). In contrast to other targeting proteins which bind to only one kinase or phosphatase, scaffolding proteins act as platforms by binding more than one signaling molecule and bringing them to a specified subcellular compartment (Faux and Scott, 1996b; Lester and Scott, 1997). The best studied scaffolding proteins to date include Ste5 in yeast, JIP-1, AKAP 79 and AKAP 250 in mammalian cells.

AKAP 250, also called gravin, was originally isolated as a cytoplasmic autoantigen in myasthenia gravis patients (Gordon et al., 1992). Later, it was identified as an A-Kinase anchoring protein by an interaction cloning strategy using radiolabeled RII as a probe (Nauert et al., 1997). *In vitro* binding studies show that residues of 1526-1780 of gravin interact with regulatory subunit (RII) of PKA, and residues 265-556 associate with PKC. When human erythroleukemia cells are stimulated with the PKC activator, phorbol ester, gravin expression is induced and gravin co-localizes with an AKAP (Nauert et al., 1997). Moreover, gravin-PKA complexes can be isolated from these cells, suggesting that gravin binds PKA *in vivo*. Following PMA treatment, human erythroleukemia cells become more adherent and flatter. In these cells, gravin is located predominantly in the cytoplasm and filopodia, and co-stains with actin at the periphery of cells and filopodia, suggesting that gravin is involved in cell adhesion to ECM (Nauert et

al., 1997). Since distinct regions of gravin bind to PKA and PKC, gravin is proposed to be a mammalian scaffolding protein participating in adherence processes (Nauert et al., 1997).

Sequence analysis reveals that the N-terminal half of gravin is highly homologous to SSeCKS, with 69% identity at amino acid level (Nauert et al., 1997). SSeCKS was cloned independently by our laboratory and by Susan Jaken's laboratory (Frankfort and Gelman, 1995; Chapline et al., 1996). We isolated SSeCKS in an attempt to identify potential tumor suppressor genes which were transcriptionally suppressed in *src*-transformed cells using a modified subtractive hybridization technique (Frankfort and Gelman, 1995). Susan Jaken's laboratory cloned the same protein (clone 72) by overlay assays to identify PKC substrate/binding proteins. SSeCKS overexpression in rodent fibroblasts causes remarkable cell flattening and adhesion, with loss of stress fibers and formation of filopodia-like protrusions. SSeCKS is predominantly located in the cytoplasm in a cortical pattern associated with an actin-based cytoskeleton, which may explain the increased cell flattening and adhesion (Gelman et al., 1998). We previously described that SSeCKS overexpression induces G1 arrest by suppressing cyclin D1 expression and/or sequestering cyclin D1 in the cytoplasm. The latter is most likely due to direct binding to cyclin D1 via CY motifs in the SSeCKS sequence, suggesting that SSeCKS functions in determining the subcellular localization of cyclin D1 (Chapter IV). Recently, SSeCKS was demonstrated to be an AKAP, which binds regulatory subunit (RII) of PKA through an amphipathic helix at the C-terminus of SSeCKS (personal

communication, Susan Jaken). It is proposed that gravin and SSeCKS are members of an emerging family of scaffolding proteins (Nauert et al., 1997).

In this study, we show that SSeCKS is a PKC binding protein *in vitro*, and a PKC substrate *in vitro* and *in vivo*. SSeCKS also binds calmodulin in a $Ca^{2+}$-dependent manner. Taken together, our data strongly support the notion that SSeCKS acts as a scaffolding protein, which coordinates at least four signaling molecules (PKC, PKA, cyclin D1 and calmodulin) for optimal regulation and tight control of intracellular signaling events during G1-S progression.

## RESULTS

**SSeCKS coding sequence contains a number of protein interaction motifs.**

SSeCKS is a protein of 1597 amino acids which migrates as a 280/290 KDa doublet in SDS-PAGE. The apparent retardation of mobility in SDS-PAGE is assumed due to a rod-like shape and a high concentration of acidic residues (Lin et al., 1996). The SSeCKS coding sequence encodes several potential protein-protein or protein-lipid interaction domains (Fig. 5-1A).

The N-terminus of SSeCKS contains a conserved myristylation signal, MGAGSSTEQR, and SSeCKS is myristylated *in vivo* as demonstrated by [$^3$H]myristate labeling (Lin et al., 1996). This myristylation may facilitate the association of SSeCKS with plasma membranes.

The N-terminal region of SSeCKS is rich in polybasic amino acids believed to participate in the formation of a phospholipid bridge between PKC and its binding partners

(Chapline et al., 1993; 1996). There are four potential PKC phosphorylation sites in the N-terminal one-third of SSeCKS. The consensus motifs for PKC phosphorylation are (S/T)*X*(K/R) or (K/R)*XX*(S/T) in which serine is preferred (Pearson and Kemp, 1991). In actual PKC sites, several such motifs are clustered together (Lin et al., 1996).

These four potential PKC phosphorylation sites in SSeCKS share an certain degree of similarity with the PKC phosphorylation sites in MARCKS (Myristylated Alanine Rich C-Kinase Substrate), a major PKC substrate involved in coupling extracellular signals to actin cytoskeleton rearrangement (Hartwig et al., 1992). The PKC phosphorylation sites in MARCKS are also calmodulin-binding sites, suggesting that SSeCKS may bind calmodulin. Studies of more than 30 calmodulin-binding domains reveals that most of them are 16-35 amino acid stretches which show a segregation of basic and polar residues on one side and hydrophobic amino acids on the other in the α-helical wheel configuration (O'Neil and DeGrado, 1990; James et al., 1995). Additionally, calmodulin-binding domains are usually adjacent to PKC phosphorylation sites (James et al., 1995). The existence of the α-helical wheels in the SSeCKS structure (data not shown) further suggests that SSeCKS may bind calmodulin.

Coincident with the second potential PKC phosphorylation site are two potential cyclin-binding (CY) motifs, SPLKKLFSSSGLKKLSGK. In the previous chapter, I described that the CY motifs most likely mediate association between SSeCKS and cyclin D1, and subsequent sequestration of cyclin D1 to the cytoplasm. The C-terminal region of SSeCKS contains an amphipathic α-helical domain which is suggested to interact with regulatory subunits of type II PKA (RII) (personal communication, Susan Jaken)

The SSeCKS coding sequence also contains putative Zn finger motif and several nuclear localization signals (NLS). However, the role of these motifs is not clear since the majority of SSeCKS protein is located in the cytoplasm as shown by immunostaining (Gelman et al., 1998).

The existence of multiple protein-protein and protein-lipid interaction domains suggests that SSeCKS may associate with a number of proteins. To test this possibility, a set of GST-SSeCKS constructs encompassing these domains was generated using polymerase chain reaction (PCR) (Fig. 5-1B). The resulting GST-expressing vectors were checked by sequencing followed by protein expression in BL21(DE3)pLysS bacteria. These GST-fusion proteins were then used in *in vitro* binding and phosphorylation assays.

**SSeCKS binds PKC in a PS-dependent manner.**

The existence of a polybasic region in SSeCKS sequence suggests that SSeCKS may bind PKC. To examine the possibility, GST-1322, a large fragment of SSeCKS bearing the majority of the polybasic region, was expressed and immobilized to glutathione-Sepharose beads in order to perform *in vitro* pull down assay (Fig. 5-2A). GST-1322 binds both purified PKC and PKC from lysates of normal Rat-6 and PKCα-overexpressing Rat-6 cells. The binding was dependent on phosphotidylserine (PS) since the interaction was abolished without PS (Fig. 5-2B). Thus, SSeCKS and PKC most likely interact via a PS bridge. The interaction between SSeCKS and PKC was also detected by overlay assay in a PS-dependent manner by another student in our lab.

**SSeCKS is a PKC substrate *in vitro* and *in vivo*.**

The four potential PKC phosphorylation sites in SSeCKS contain a high concentration of basic residues and at least two or three overlapping PKC phosphorylation motifs, consistent with our observations of previously characterized *in vivo* PKC phosphorylation sites. To determine whether these sites could be phosphorylated by PKC, GST-fusion proteins containing these individual sites or several sites in tandem were expressed (Fig. 5-3A) and used as substrates for *in vitro* kinase assays. Each of these potential sites can be phosphorylated efficiently by purified rabbit brain PKC (Fig. 5-3B), which contains α, β, and γ isoforms. This phosphorylation was inhibited up to 80% by the addition of excess PKC pseudosubstrate inhibitor peptide (a.a. 19-36), indicating that the kinase is PKC rather than a contaminating kinase (Fig. 5-3B). GST alone was not phosphorylated by PKC.

To identify which serine or threonine residues were phosphorylated by PKC, GST-SSeCKS1, 2, 3, and 4, each of which bears individual PKC sites, were subject to endoprotease V8 digestion, HPLC fractionation, and microsequencing, following *in vitro* PKC phosphorylation. Two phosphoserines, Ser515 and Ser599, were identified as the phosphorylation sites in SSeCKS-2 and SSeCKS-3, respectively (Fig. 5-3C). Recently, Jaken's group confirmed Ser515 as an *in vivo* PKC phosphorylation site (Chapline et al., 1998). The phosphoserine/phosphothreonine residues in SSeCKS-1 and SSeCKS-4 have not been mapped yet.

We then investigated whether SSeCKS was phosphorylated by PKC *in vivo*. PKC activation by the short-term addition of phorbol esters results in the rapid phosphorylation of PKC substrates such as MARCKS (Aderem, 1992). In response to phorbol 12-myristate

13-acetate (PMA), the phosphorylation level of the 280/290 kDa SSeCKS doublet immunoprecipitated from Rat-6 cells rapidly increased within 2 min, and this phosphorylation was abolished by the addition of the PKC-specific inhibitor, *bis*-indolylmaleimide, GF-109203X (Fig. 5-4A). The PMA-induced phosphorylation lasted at least 10 min, and then decreased with treatment of longer than 60 min (data not shown), which may result from down-regulation of PKC. This data indicates that SSeCKS is likely an *in vivo* substrate of PKC. However, we cannot rule out that other kinases downstream of PKC are responsible for the rapid phosphorylation of SSeCKS.

To investigate the effect of phosphorylation on SSeCKS, we examined the location of SSeCKS following short-term PMA treatment. The tetracycline-regulated SSeCKS overexpressor (S24) and control (V3) cells were first withdrawn from tetracycline for 2 days to induce SSeCKS expression, and then treated with PMA for a various time. 5 min after PMA stimulation, both endogenously (V3) and exogenously (S24) expressed SSeCKS were translocated from the cytoplasm and plasma membrane sites to the perinuclear area. At 15 min, SSeCKS translocation was more obvious (Fig. 5-4B). At 60 min, some cells broke off from distal adhesion sites (Fig. 5-4Bg). These data suggest that phosphorylation of SSeCKS affects its biological function.

These data here indicate that SSeCKS is a PKC-binding protein *in vitro* and a PKC substrate *in vivo* and *in vitro*. Although *in vitro* binding suggests that SSeCKS and PKC form a complex *in vivo*, the definitive evidence for their association inside cells is not available yet..

**SSeCKS binds calmodulin in a $Ca^{2+}$ - dependent manner, and phosphorylation of SSeCKS decreases its calmodulin-binding activity.**

Calmodulin is a small acidic, $Ca^{2+}$-binding protein which participates in the regulation of many $Ca^{2+}$-dependent signaling cascades. Calmodulin binds to calmodulin-binding domains (amphiphilic helices) within a number of proteins. Because SSeCKS contains four potential calmodulin-binding domains. we asked whether SSeCKS binds calmodulin. GST-SSeCKS1, 2, 3, and 4 proteins, were expressed for *in vitro* calmodulin-binding assay using the cross-linking reagent dimethyl pimelimidate. This cross-linking technique had been previously used to demonstrate the binding between calmodulin and its partners (Vale, 1988; Graff et al., 1989). Each of the four SSeCKS fragments was capable of binding calmodulin in a $Ca^{2+}$- dependent manner since the binding was abrogated with the addition of the chelating molecule EGTA (Fig. 5-5). The resulting complexes showed retarded mobility in SDS-PAGE compared to the original GST-fusion proteins, suggesting that the GST-fusion proteins either bound more than one calmodulin (14kDa) or that SSeCKS may form multimers. This effect made it difficult to determine the binding stoichiometry. Because these GST-fusion proteins encoded PKC phosphorylation sites. we next examined the effect of phosphorylation by PKC on calmodulin binding. When these GST-fusion proteins were phosphorylated by purified rabbit brain PKC prior to incubation with $^{125}$I-CaM, the interaction was dramatically reduced, near 80%, except for SSeCKS-4 (minor reduction) (Fig. 5-5). These data indicate that SSeCKS binds calmodulin in a $Ca^{2+}$-dependent manner, suggesting that SSeCKS may be involved in controlling $Ca^{2+}$/calmodulin-dependent signaling pathways.

# DISCUSSION

**SSeCKS is a PKC substrate/binding protein.**

PKCs are a Ser/Thr protein kinase family consisting of at least 11 isoforms. The existence of multiple isoforms of PKC in the same cell has made it difficult to determine the role of each isoform in regards to cellular functions (Newton, 1995; Jaken, 1996). Recently, differences in PKC isoform subcellular localization has been suggested as a mechanism to regulate the isoform-selective phosphorylation of substrates (Jaken, 1996). In order to phosphorylate cytoskeletal and membrane proteins, soluble PKCs must be targeted first to appropriate cytoskeletal and membrane compartments, which requires specific interactions with their targeting proteins. One class of PKC targeting proteins is the PKC substrate/binding proteins. Members of this class identified to date are cytoskeleton-associated proteins that bind PKC in a PS-dependent manner. PS is presumably required to support a ternary complex of PKC and the substrate/binding protein. Another distinguishing feature of these proteins is that phosphorylation regulates their association with PKC. Consistent with this model, SSeCKS is a cytoskeletal-associated protein that interacts with PKC via a PS bridge and dissociates from PKC following phosphorylation (Chapline et al., 1996). This strongly suggests that SSeCKS is a PKC substrate/binding protein, and that may be involved in regulating PKC-mediated signal transduction by its targeting function.

**SSeCKS is a calmodulin-binding protein.**

At least six other calmodulin-binding proteins are PKC substrates, however, the effect of PKC phosphorylation on calmodulin-binding varies. For example, calcineurin phosphorylation by PKC does not affect its calmodulin-binding affinity (Tung, 1986). In

contrast, myosin light chain kinase shows a reduced calmodulin-binding affinity once phosphorylated by PKC (Nishikawa et al., 1985). PKC phosphorylation on GAP-43 decreases its binding affinity to calmodulin (Alexander et al., 1987; Graff et al., 1989). A unique feature of GAP-43 is that it binds calmodulin with higher affinity in the absence of $Ca^{2+}$. Phosphorylation of MARCKS by PKC suppresses the interaction between MARCKS and calmodulin (Graff et al., 1989). One possible physiological function of MARCKS is to sequester calmodulin during periods of PKC inactivation, perhaps in a specific subcellular compartment. Following PKC activation, MARCKS becomes phosphorylated and releases calmodulin. The increased free calmodulin levels and increased intracellular $Ca^{2+}$ concentrations resulting from PKC activation act together to activate $Ca^{2+}$/calmodulin-dependent signal transduction (Graff et al., 1989). Here, we show that SSeCKS binds calmodulin in a $Ca^{2+}$-dependent manner by the cross-linking technique, and that phosphorylation of SSeCKS by PKC decreases its calmodulin-binding affinity. SSeCKS and MARCKS share little sequence similarity except for their phosphorylation motifs, but they share several functional and structural features such as predicted rod-like shapes, binding of PKC via a PS bridge, growth inhibitory effects after overexpression, and phosphorylation regulated binding to calmodulin (Lin et al., 1996; Herget et al., 1993). Thus, it is possible that one function of SSeCKS is to bind and sequester calmodulin in a PKC inactive state in an appropriate subcellular location. However, whether SSeCKS and calmodulin form a complex in intact cells, and whether their association *in vivo* is antagonized by PKC phosphorylation needs to be investigated to support our hypothesis.

**SSeCKS is a scaffolding protein.**

Data presented in this chapter suggest that SSeCKS is a PKC- and calmodulin-binding protein, and participates in the targeting of PKC and calmodulin. In the previous chapter, SSeCKS is suggested to regulate the location of cyclin D1. Accumulating evidence indicates that cyclins direct CDKs to phosphorylate specific substrates at particular subcellular sites at the appropriate cell cycle stage (Hubbard and Cohen, 1993). SSeCKS may compete with other cyclin D1-binding proteins for the targeting of cyclin D1-CDK4 complex. Overexpression of SSeCKS switches the balance such that cyclin D1 is sequestered in the cytoplasm. In addition, SSeCKS is a PKA anchoring protein that binds RII subunits of PKA (Nauert et al., 1997). Taken together, at least four signaling molecules have been showed to bind to distinct regions of SSeCKS, and the binding is regulated by phosphorylation following activation. Thus, SSeCKS fulfills the characteristics of a scaffolding protein, most likely indicating that SSeCKS coordinates four signaling cascades through its scaffolding function.

**Fig. 5-1. Schematic representation of conserved sequence domains in SSeCKS and GST fusion constructs of SSeCKS domains.**

**A.** Schematic representation of conserved sequence domains in SSeCKS. **B.** GST fusion constructs of SSeCKS domains. SSeCKS-1, -2, -3, and -4 each contains individual PKC phosphorylation sites and individual calmodulin-binding domains. SSeCKS-2 also contains two potential cyclin-binding (CY) motifs.

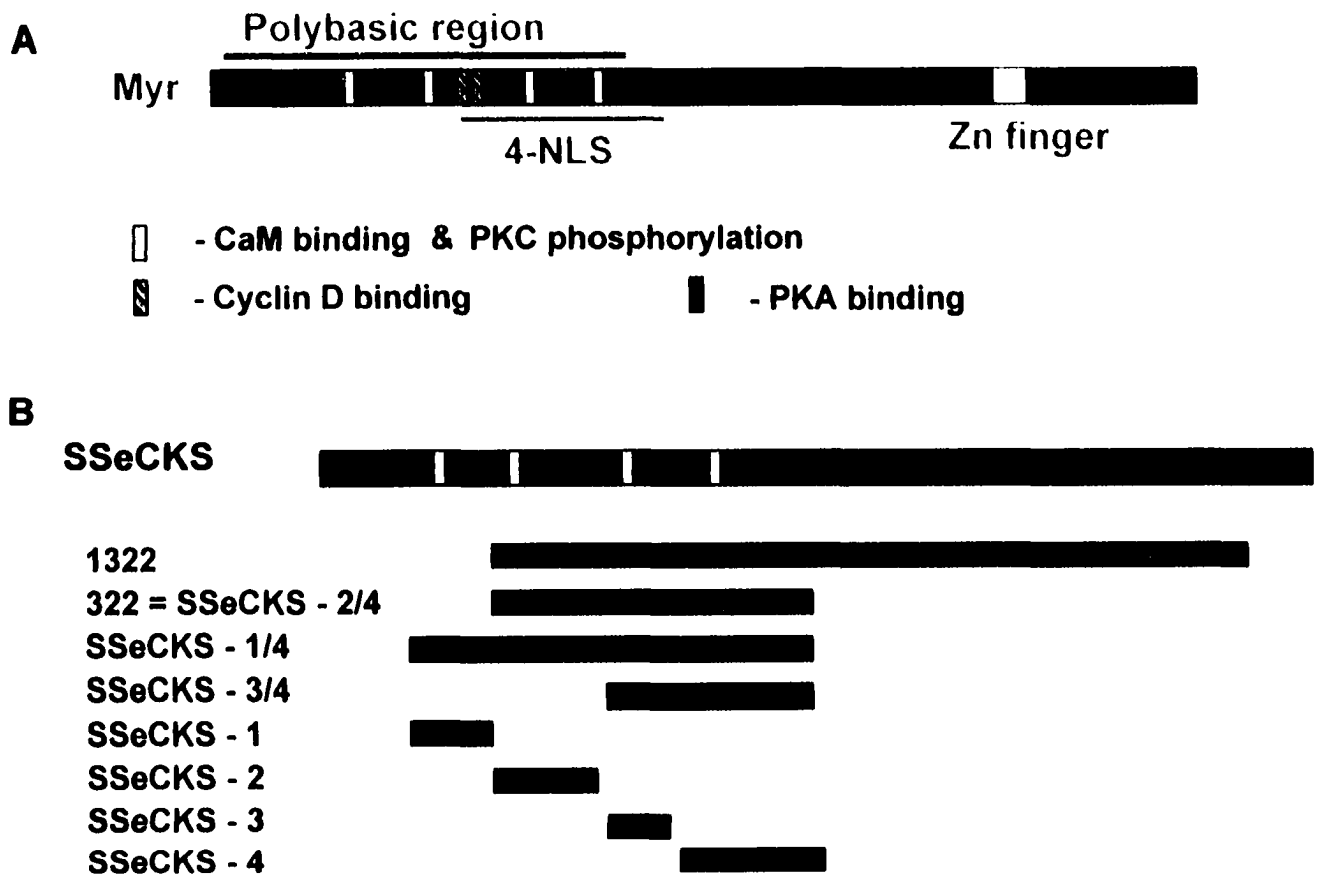



Fig. 5-1

**Fig. 5-2. SSeCKS binds PKC in a PS-dependent manner *in vitro*.**

**A.** Coomassie-stained SDS-PAGE gel showing the expression and purification of GST-1322 fusion protein. Last lane represents purified GST-1322. Like cellular SSeCKS, this fusion protein migrates slower than expected in SDS-PAGE. **B.** *In vitro* pull down assay showing that SSeCKS binds PKC. RIPA lysates (1 mg) from Rat-6 or Rat-6/PKC-α overexpressor cells, or purified rabbit brain PKC (20 ng, P-PKC-α) were incubated with 50 μg of GST-1322 (pre-bound to glutathione-Sepharose beads) in the presence or absence of phosphatidylserine (PS). The bound proteins were subjected to immunoblotting using antibody against PKC-type III. Bacterially-expressed SSeCKS binds PKC in a PS-dependent manner.

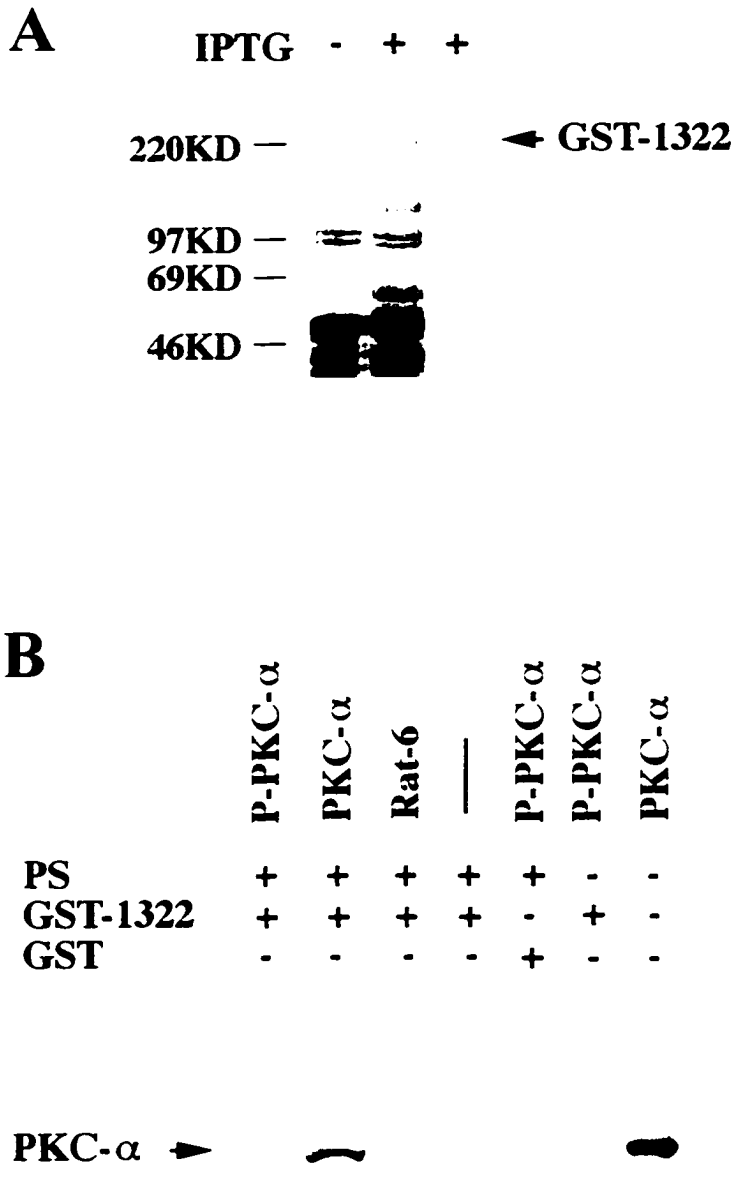



Fig. 5-2

**Fig. 5-3. SSeCKS is a PKC substrate *in vitro*.**

**A.** Expression and purification of GST-SSeCKS fusion proteins. Lysates from uninduced (a) or induced (b) bacteria, or glutathione-Sepharose purified GST-SSeCKS fusion proteins (c) were analyzed by SDS-PAGE, followed by coomassie blue staining. Arrowheads indicate the unfragmented products. **B.** 1 μg of various GST-SSeCKS fusion proteins were subjected to *in vitro* PKC phosphorylation reaction in the presence or absence of PKC[19-36], a PKC-specific inhibitor peptide. SSeCKS phosphorylation by purified rabbit brain PKC is inhibited by PKC[19-36]. **C.** Two *in vitro* PKC phosphorylation sites in SSeCKS were mapped: Ser515 in SSeCKS-2 and Ser599 in SSeCKS-3.

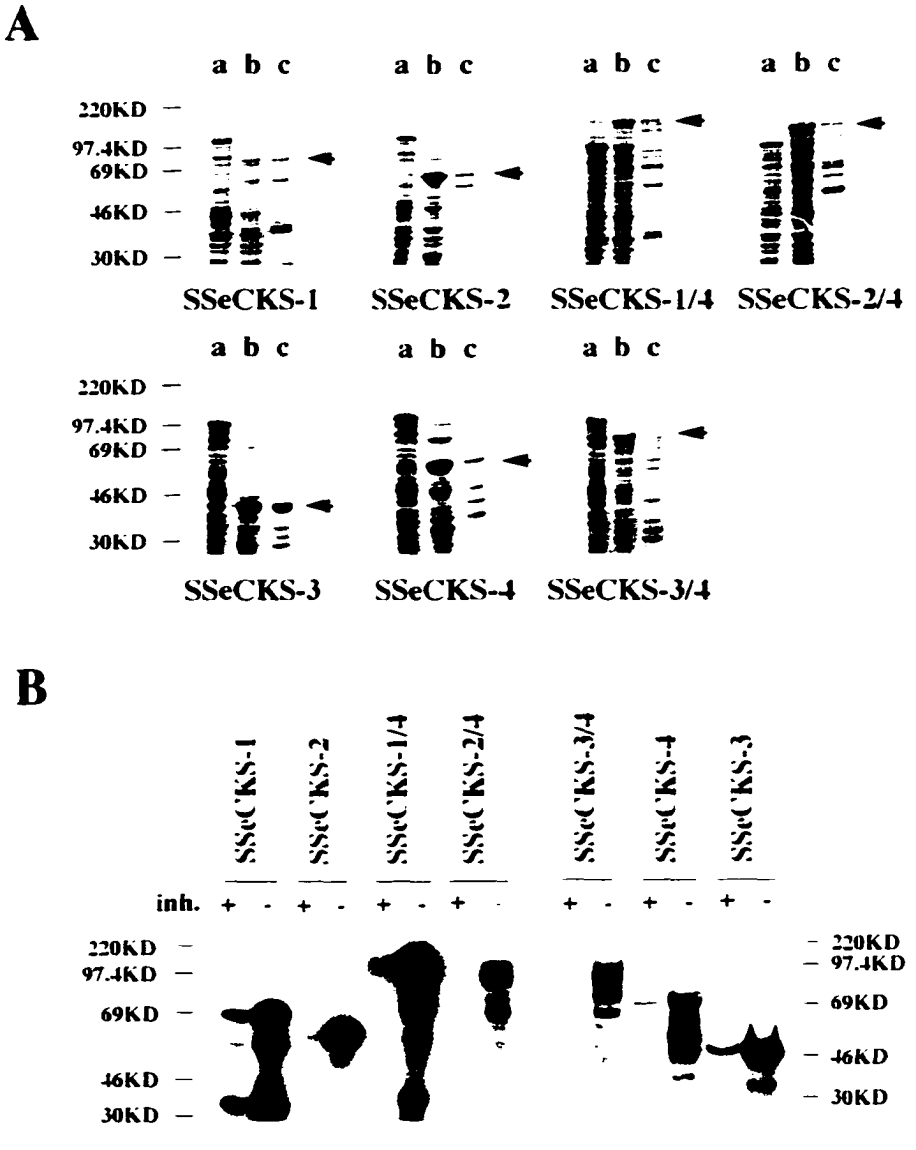



**Fig. 5-3**

**Fig. 5-4. *In vivo* phosphorylation of SSeCKS by PKC.**

**A.** Autoradiography showing SSeCKS phosphorylation. SSeCKS was immunoprecipitated from $^{32}$P-labeled Rat-6 cells, and western blotted onto PVDF membrane. SSeCKS phosphorylation is responsive to PMA stimulation. PKC-specific inhibitor, *bis*-indolylmaleimide, inhibits SSeCKS phosphorylation. Lower panel represents immunoblotting of SSeCKS. Equal amounts of SSeCKS precipitates were used for autoradiography. **B.** Immunostaining showing perinuclear translocation of SSeCKS after PKC activation. S24, an SSeCKS overexpressor cells (a, c, e, and g) or control V3 cells (b, d, and f) grown in the absence of tetracycline were stained for SSeCKS. Some of the cells were treated with 200 nM PMA for 5 min (c and d), 15 min (e and f), or 60 min (g). Activation of PKC results in the perinuclear translocation of both exogenous (S24 cells) and endogenous (V3 cells) SSeCKS.

A

| Time(min) | 0 | 2 | 10 | 10 |
|---|---|---|---|---|
| PMA | - | + | + | + |
| Inhibitor | - | - | - | + |

$^{32}$P-SSeCKS

SSeCKS

B

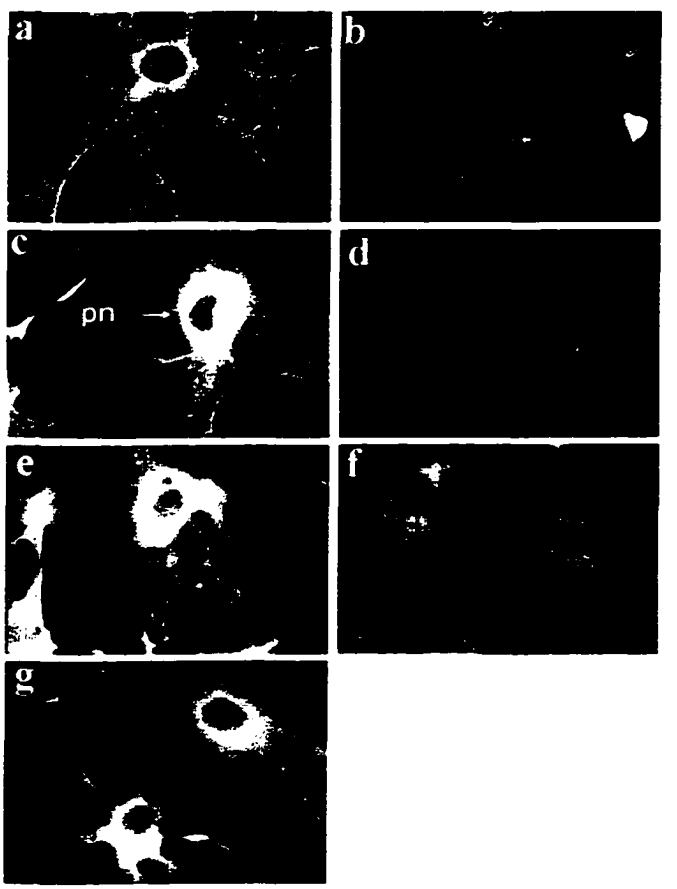
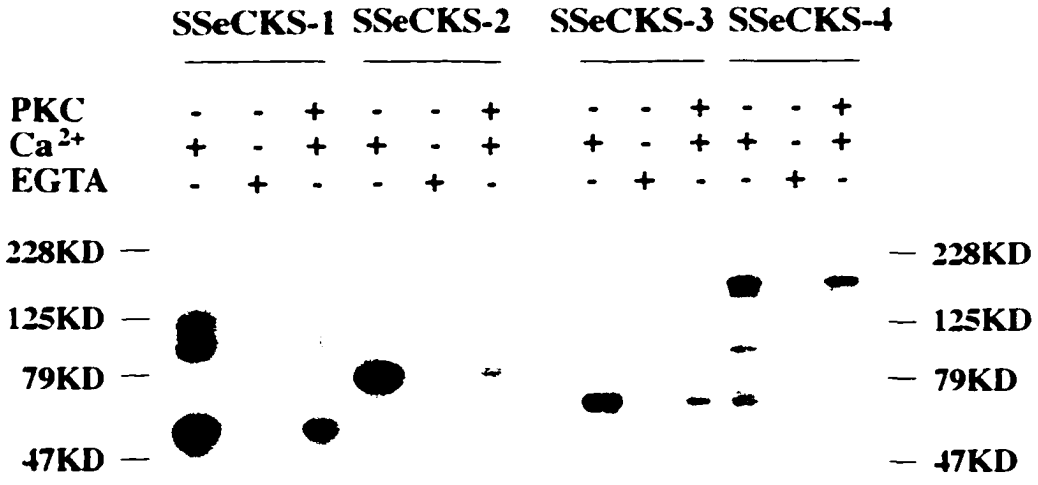



Fig. 5-4

**Fig. 5-5. SSeCKS binds calmodulin in a $Ca^{2+}$- dependent manner in *vitro*.**

GST-SSeCKS fusion proteins were incubated with $^{125}$I-calmodulin in the presence of $Ca^{2+}$ or EGTA. Following addition of the cross-linker dimethyl pimelimidate, the reaction mixtures were analyzed by SDS-PAGE. Pre-phosphorylated GST-SSeCKS fusion proteins by PKC were also included in cross-linking reactions. PKC phosphorylation inhibits SSeCKS' binding affinity to calmodulin.



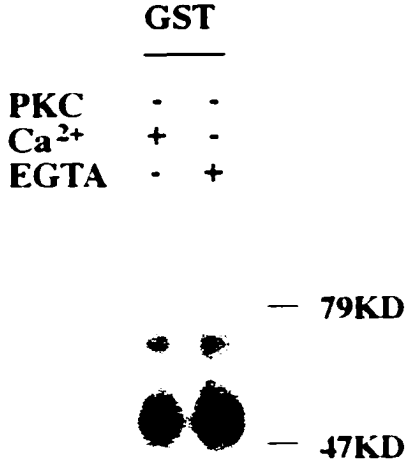




Fig. 5-5

# Chapter VI

## General Discussion and Future Perspectives

In this study, I investigated the roles of SSeCKS as a tumor suppressor, cell cycle regulator and scaffolding protein in rodent fibroblasts using a tetracycline-regulated mammalian expression system.

Normal cells grown on tissue culture dishes exhibit a number of properties such as contact-inhibition, anchorage-dependence, and growth factor-dependence. These characteristics are lost when cells become transformed by oncogenes. This phenomenon suggests that the oncogenes must overcome some cellular control functions to achieve transformation. In other words, these cellular control functions may be encoded by potential tumor suppressor genes. Thus, mutation or suppression of these genes would facilitate transformation and tumor formation. SSeCKS was identified by subtractive hybridization in an attempt to isolate down-regulated genes that might encode potential tumor suppressors. Suppression of SSeCKS transcripts in *src*- and *ras*-, but not in *raf*-transformed cells suggests that SSeCKS is specifically involved in *src*- and *ras*-mediated transformation. Interesting questions that remain are: how *src* or *ras* down-regulates SSeCKS transcripts (at the level of transcription initiation or by decreasing RNA stability), and how signals downstream of *ras* and *raf* are divorced in regards to SSeCKS regulation.

The cloning strategy used to isolate SSeCKS suggests that it might encode a tumor suppressor. Data presented here indicate that SSeCKS overexpression does suppress transformed phenotypes although the molecular mechanism has not been elucidated. In contrast to the classic definition in which a tumor suppressor gene is deleted or mutated in transformed cells or tumors, SSeCKS expression is suppressed in *src-* and *ras-* transformed fibroblasts, certain breast and prostate tumor cell lines, and certain advanced prostate tumors (Chapter IV; Gelman and Wang, unpublished data). However, the definitive evidence of SSeCKS as a tumor suppressor will result from extensive screening of primary human tumors in order to determine whether there is a good correlation between SSeCKS down-regulation and tumor development or progression, and from phenotypic analysis of knock-out mice.

In fact, the transcriptionally suppressed, so-called class II tumor suppressors, are attracting more and more attention recently. Because the genes themselves are intact, it is possible to restore their expression, which requires thorough understanding of the molecular mechanism of their down-regulation. The cloning of the promoter and enhancer region of SSeCKS will facilitate future attempts at forced up-regulation. Nevertheless, insight into the transcriptionally-suppressed class of tumor suppressor genes will provide a greater opportunity for cancer therapy than those mutated or deleted tumor suppressor genes.

Tumor suppressor genes function as anti-oncogenes. Oncogene and tumor suppressor gene products compete to control cellular processes such as cell cycle progression. Substantial experimental evidence indicates that cyclin D1 is an oncogene

that cooperates with Ha-*ras* in cellular transformation (Lovec et al., 1994), and promotes tumor formation in nude mice (Jiang et al., 1993). Cyclin D1 gene amplification is also detected in certain breast, esophageal, urinary bladder and squamous cell carcinomas (DelSal et al., 1996), and there is a correlation between cyclin D1 overexpression and invasive breast carcinoma (Weinstat-Saslow et al., 1995). The most important role for cyclin D1 in cell cycle regulation is to inactivate pRb. Moreover, at least one component of the p16-cyclinD/CDK4-pRb axis is inactivated or overexpressed in most human tumors. Thus, oncogenes and tumor suppressor genes both cooperate and compete to control physiological processes. When the balance is disrupted, abnormal phenomena will occur such as carcinogenesis or growth inhibition.

Overexpression of SSeCKS, a tumor suppressor, interferes with normal cell cycle progression and causes growth inhibition. Although the molecular mechanism of the tumor suppressive effect of SSeCKS remains to be further investigated, the molecular basis of SSeCKS overexpression-induced growth arrest in G1 is most likely due to its counteraction with oncogene cyclin D1. Data presented here indicate that growth inhibitory effect of SSeCKS results from its ability to suppress cyclin D1 expression and/or sequester cyclin D1 in the cytoplasm. Down-regulation of cyclin D1 possibly results from a suppression of serum-inducible ERK2 activation. One open question remaining is how ERK2 activity is inhibited upon SSeCKS overexpression. Unexpectedly, ectopic expression of cyclin D1 fails to rescue SSeCKS-induced growth arrest, and thus, our finding that cyclin D1 is sequestered in the cytoplasm is novel to the field of cell cycle regulation. Studies on CDK activation have been focused on cyclin

binding, CDK phosphorylation/dephosphorylation, and association with CKIs. Only recently, the subcellular localization of cyclins and CDKs has been demonstrated to be a new way to control cell cycle progression. For example, mutant cyclin D1 which stays in the cytoplasm and prevents CDK4 nuclear import can induce G1 arrest (Diehl and Sherr, 1997). Inhibition of calmodulin function decreases nuclear staining of cyclin D1 and CDK4 during G1 phase, and consequently decreases the abundance of hyperphosphorylated pRb (Taules et al., 1998). In the case of SSeCKS overexpression, we have not defined whether CDK4 is sequestered in the cytoplasm via association with cyclin D1. However, the available evidence indicates that cyclin D1 and CDK4 co-compartmentalize. Additionally, p21 binding to cyclin D1 via the CY motif only occurs when cyclin D1 is complexed with CDK4, suggesting that CY motif-mediated binding does not disrupt interaction between cyclin D1 and CDK4 (Chen et al., 1996). Therefore, CDK4 is possibly also sequestered in the cytoplasm.

Despite evidence of *in vitro* binding of cyclin D1 to SSeCKS, the retention of cyclin D1 in the cytoplasm, and partial co-localization of cyclin D1 and SSeCKS, whether SSeCKS directly sequesters cyclin D1 in the cytoplasm *in vivo* through CY-mediated binding is still an open issue. It will be interesting to investigate whether introduction of CY-containing peptides into cells will prevent the cytoplasmic retention of cyclin D1, and whether expression of CY-mutated, binding-defective SSeCKS in NIH 3T3 cells will lose growth inhibitory effects.

Recently, subcellular compartmentalization has been considered as a key step in the regulation of intracellular signal transduction. Scaffolding proteins, a new class of

targeting proteins, bind and coordinate several kinases and phosphatases at the same time to achieve an optimal regulation of physiological processes. The ability of SSeCKS to bind PKC, PKA, cyclin D1 and calmodulin through distinct but related regions suggests that SSeCKS fulfills the characteristics of a scaffolding protein. Thus, we consider SSeCKS as a scaffolding protein that binds inactive forms of these four molecules and directs them to specific subcellullar compartments. Following the mitogen-induced activation of kinases such as PKC, SSeCKS is phosphorylated and thus releases these molecules, which in turn regulate biological processes such as cell cycle progression. However, the *in vivo* association of SSeCKS with these molecules needs to be further investigated. The effects of SSeCKS phosphorylation by either PKC or PKA on the functions of SSeCKS remain to be established. Finally, involvement of SSeCKS-containing complexes in specific cellular processes also waits to be defined.

In summary, this study has characterized the role of SSeCKS in the suppression of cellular transformation and control of cell cycle progression. We speculate that the underlying mechanism of SSeCKS-induced phenotypes is due to its scaffolding activity.

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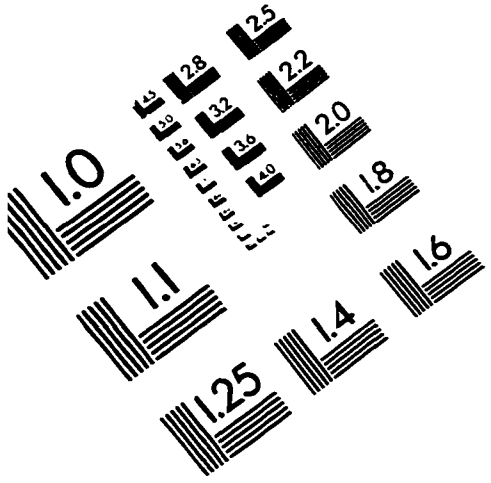
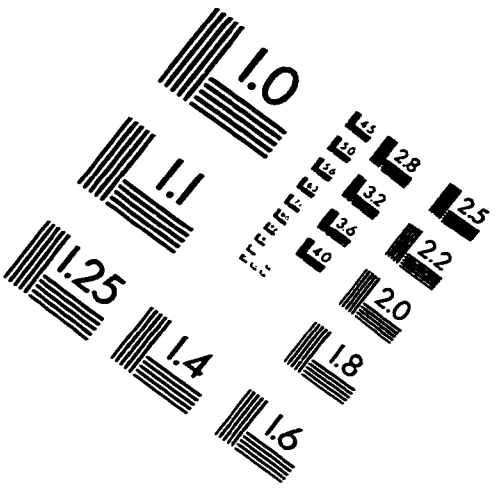
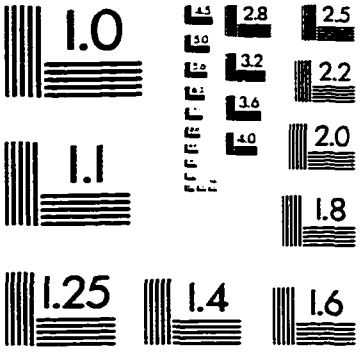
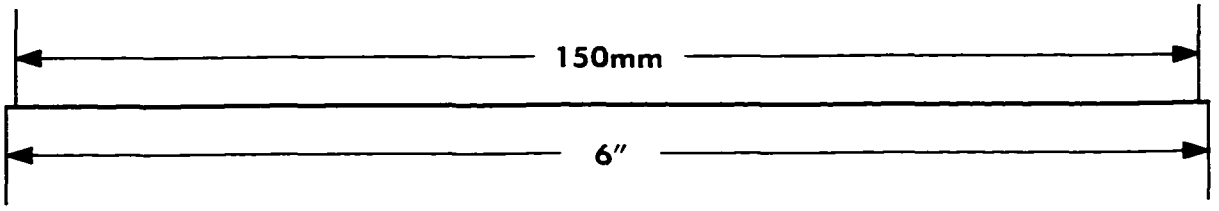
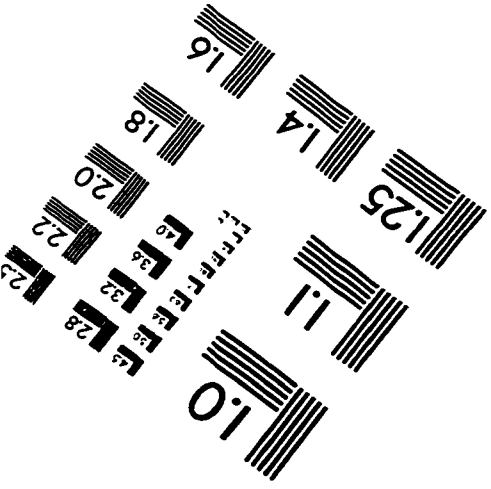
# IMAGE EVALUATION TEST TARGET (QA-3)

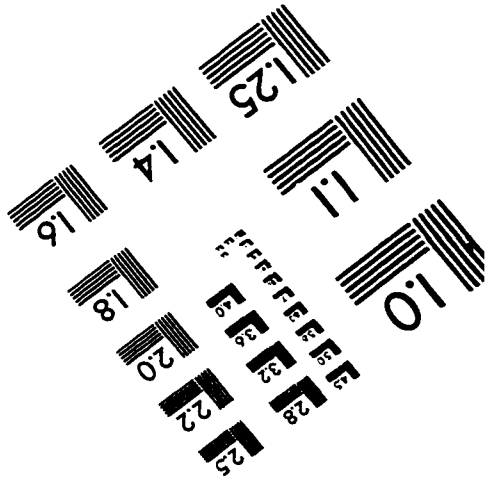
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