SSeCKS/Gravin/AKAP12 Metastasis Suppressor Inhibits Podosome Formation via RhoA- and Cdc42-Dependent Pathways

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Abstract
Podosomes are poorly understood actin-rich structures notably found in cancer cell lines or in v-Src-transformed cells that are thought to facilitate some of the invasive properties involved in tumor metastasis. The enrichment of the Tks5/Fish protein, a v-Src substrate, is required for formation of podosomes. We showed previously that the tetracycline-regulated reexpression of the Src-suppressed C kinase substrate (SSeCKS, also known as Gravin/AKAP12) inhibited variables of v-Src-induced oncogenic growth in NIH3T3, correlating with the induction of normal actin cytoskeletal structures and cell morphology but not with gross inhibition of Src phosphorylation activity in the cell. Here, we show that SSeCKS reexpression at physiologic levels suppresses podosome formation, correlating with decreases in Matrigel invasiveness, whereas there is no effect on total cellular tyrosine phosphorylation or on the phosphorylation of Tks5/Fish. Activated forms of RhoA and Cdc42 were capable of rescuing podosome formation in v-Src cells reexpressing SSeCKS, and this correlated with the ability of SSeCKS to inhibit RhoA and Cdc42 activity levels by >5-fold. Interestingly, although activated Rac I had little effect on podosome formation, it could partner with activated RhoA to reverse the cell flattening induced by SSeCKS.

These data suggest that v-Src-induced Tks5 tyrosine phosphorylation is insufficient for podosome formation in the absence of RhoA- and/or Cdc42-mediated cytoskeletal remodeling. Additionally, they strengthen the notion that SSeCKS suppresses Src-induced oncogenesis by reestablishing actin-based cytoskeletal architecture. (Mol Cancer Res 2006;4(3):151–8)

Introduction
Podosomes are defined as dynamic membrane protrusion sites enriched for filamentous actin (reviewed in ref. 1). These structures are found in monocyte-derived lineages, such as macrophages, dendritic cells, and osteoclasts, as well as in oncogene-transformed fibroblasts, carcinoma-derived epithelial cells, and smooth muscle cells. The enrichment of extracellular matrix–degrading metalloproteinase activity in podosomes and the correlation of podosome number with extracellular matrix—degrading activity in vitro have led to the hypothesis that podosomes control tissue invasiveness. Although they share many of the proteins enriched in focal adhesion and focal contact sites, podosomes are distinguished by their F-actin cores, more rapid formation, and the ability to assemble in the absence of de novo protein synthesis (reviewed in ref. 2). However, proteins unique to podosomes exist, such as cortactin (an actin-associated protein), dynamin (a GTPase), seprase and MT1-matrix metalloproteinase (proteases), and Tks5 (an adapter protein; ref. 3). Recent data indicate that podosome formation requires the presence of several specific proteins, such as members of the WASp family, RhoA and Tks5 (3-6).

Tks5, formerly known as Fish, was initially identified as a v-Src substrate in an in situ/kinase overlay assay (7). A recent study indicates that the RNA-mediated knockdown of Tks5 suppressed the formation of podosome structures in v-Src-transformed fibroblasts, correlating with a severe decrease in Matrigel invasion activity (3). This correlates with the finding that the fifth Tks5 SH3 domain binds ADAMS12 and ADAMS19, two members of a large metalloproteinase family (8).

The Src-suppressed C kinase substrate (SSeCKS), originally identified as a gene down-regulated in response to Src and Ras activation (9), is the rodent orthologue of human gravin, an autoantigen in some cases of myasthenia gravis (10). Besides binding protein kinase C (11), Gravin and SSeCKS are also known as AKAP12 because of their ability to scaffold protein kinase A through a COOH-terminal RII subunit binding motif (12). Gravin is a single-copy gene mapping to 6q24-25.2, a hotspot for deletion in advanced prostate, breast, and ovarian cancers (13, 14). The expression of SSeCKS/Gravin/AKAP12 is down-regulated by several oncogenes and strongly suppressed in various cancers, including prostate, ovary, and breast (14-17).

SSeCKS reexpression suppresses Src-induced oncogenesis by reducing anchorage-independent growth in soft agar and Matrigel invasiveness, and by restoring normal cell morphology and cytoskeletal architecture, without a significant effect on Src-induced tyrosine phosphorylation of cellular substrates (15). The tetracycline-regulated reexpression of SSeCKS in the rat prostate cancer cell line, MatLyLu (MLL), reduced anchorage-independent growth in cell
culture. In nude mice, SSeCKS reexpression severely decreased the formation of lung metastases without significantly affecting the growth of primary s.c. tumors (14). These findings led to the suggestion that SSeCKS functions as a potential metastasis suppressor, although its mechanism of action remains unclear (18).

Here, we address whether SSeCKS reexpression to physiologic levels in v-Src-transformed fibroblasts can affect

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**FIGURE 1.** SSeCKS reexpression inhibits Src-induced podosome formation. S24/ts72v-Src cells were grown on glass coverslips for 24 hours at the non-permissive temperature (39.5°C; A) or permissive temperature (35°C; B) for v-Src kinase activity and either with (+tet) or without (−tet) tetracycline. After fixation, the cells were incubated with Rb anti-Tks5 polyclonal antibody followed by FITC-labeled anti-Rb immunoglobulin antibody, rhodamine-phalloidin, and 4',6-diamidino-2-phenylindole (DAPI) as described in Materials and Methods. Arrows, podosomes, concentrations of F-actin, and Tks5 that sometimes have a circu-
lar, rosette shape. Bar, 30 μm.

**FIGURE 2.** Typical convergence of Tks5 and F-actin in S24/ts72v-Src cells grown at the permissive temperature. S24/ts72v-Src cells grown with tetracycline at the permissive temperature were fixed and stained for Tks5 and F-actin as described in Materials and Methods. These cells exhibit typical podosome structures as shown by the convergence of Tks5 and F-actin enrichment in rosette-like structures at the cell edge. The image series start at the bottom (left) to the top (right) of cells. Bar, 30 μm.
podosome formation and the Src-induced tyrosine phosphorylation of Tks5. Our data indicate that SSeCKS suppresses v-Src-induced podosome formation by inhibiting Rho family GTPase activation, whereas SSeCKS has no effect on Tks5 phosphorylation.

Results and Discussion

Mounting evidence connects podosome formation with increased tissue invasion by both normal and tumor cells (1, 2). Although the precise nature of podosome formation remains unclear, the requirement for proteins, such as Tks5, cortactin, and AFAP-110 (3, 19), plus the activation of specific signaling mediators, such as Src (20), suggests that podosome formation and function is highly regulated in a spatiotemporal manner.

Tks5 is phosphorylated directly by activated Src (7) and its short hairpin RNA–mediated knockdown suppresses podosome formation in Src/3T3 cells (3). Interestingly, Tks5 knockdown did not affect overall Src substrate tyrosine phosphorylation or secretion of matrix metalloproteinase-2 and -9, whereas it induced remodeling of a F-actin-based cellular cytoskeleton. Given these findings (7), it is conceivable that the tyrosine phosphorylation of Tks5 might be required for its podosome-forming function. However, it is also possible that podosome formation only requires remodeling of the actin-based cytoskeleton.

To address this issue, we employed NIH3T3 cells with tetracycline-regulated reexpression of the SSeCKS metastasis suppressor gene in the background of a temperature-sensitive v-Src, ts72v-Src. Our previous studies showed that reexpression of SSeCKS suppressed Src-mediated growth factor– and anchorage-independent proliferation without grossly affecting Src-induced tyrosine phosphorylation of total cellular substrates (15). However, SSeCKS reexpression was capable of restoring F-actin stress fibers and typical focal adhesion plaques, suggesting that SSeCKS suppressive effect related to the reestablishment of a “normal” cytoskeletal infrastructure.

At the nonpermissive temperature for Src kinase activity (39.5°C), S24/ts72v-Src cells show no podosome formation in the presence or absence of tetracycline based on Tks5 and phalloidin staining (Fig. 1A), although SSeCKS reexpression (-tetracycline) induces cell flattening and the formation of long, axonal-like extensions as we described previously (21). In contrast, cells grown in the presence of tetracycline at the permissive temperature for Src activity (35°C) showed abundant podosome formation (Fig. 1B, top, arrows) along with increased refractility (data not shown) and increased spindle-shaped cell morphology. The podosomes in our cells conform to standard definitions (see refs. 1, 2 for reviews); that is, concentrations of F-actin plus podosome-associated proteins, such as Tks5 (Fig. 2), paxillin, and vinculin (data not shown) at mostly peripheral sites. SSeCKS reexpression at the permissive temperature (-tetracycline) resulted in mild cell flattening, producing similar cell morphologies and sizes to that of untransformed NIH3T3, except for the retention of axonal-like extensions (Fig. 1B, bottom). Significantly, SSeCKS reexpression at the permissive temperature decreased the percentage of cells with podosomes roughly 4-fold (Figs. 1B, 3A). SSeCKS reexpression decreases total podosome formation.

**FIGURE 3.** SSeCKS reexpression decreases total podosome formation. S24/ts72v-Src cells grown at the permissive temperature in either the presence or the absence of tetracycline were fixed and stained for Tks5 and F-actin as in Fig. 2, and the percentage of cells exhibiting podosomes (A) or the frequency of podosomes per cell (B) was quantified. Columns, mean of six separate visual fields containing roughly 40 to 50 cells per field; bars, SE. *, P < 0.001.

SSeCKS/Gravin/AKAP12 Suppresses Podosome Formation

**FIGURE 4.** SSeCKS reexpression inhibits Matrigel invasiveness. S24/ts72v-Src cells (10⁴) were seeded onto Matrigel-coated Transwell chambers (24-well format) and incubated for 6 hours at the permissive temperature in either the presence or the absence of tetracycline, and Transwell filters were processed to stain and count the invasive cells as described previously (15). Columns, mean of invasive cells from triplicate Transwell assays; bars, SE. *, P < 0.001.
and 3A), although there was no significant change in the number of podosomes per cell (Fig. 3B). These data suggest that SSeCKS-induced cytoskeletal remodeling antagonizes podosome formation.

The presence of podosomes in Src-transformed fibroblasts and in human epithelial cancer cell lines correlates with extracellular matrix–degrading activity (1). Although this activity is considered a reflection of tumor cell invasiveness, there is no formal proof that individual podosomes are the direct facilitators of invasion. To test whether SSeCKS reexpression could suppress invasiveness, S24/ts72v-Src cells grown at the permissive temperature in the presence or absence of tetracycline were tested for the ability to invade through a layer of growth factor–reduced Matrigel in Boyden chamber assays. Figure 4 shows that SSeCKS reexpression reduced Matrigel invasiveness 4.5-fold, similar to the reduction in total podosome incidence in Fig. 3A. Cells grown at the nonpermissive temperature showed no significant invasiveness over parental NIH3T3 cells (data not shown). Our previous data indicated that SSeCKS reexpression in S24/ts72v-Src cells had no significant effect on proliferation in 10% serum or on motility in monolayer wound assays [i.e., comparing (+tetracycline) versus (-tetracycline) at the permissive temperature; ref. 15]. Taken together, this suggests that SSeCKS inhibits Matrigel invasiveness and podosome formation by an overlapping mechanism.

We next determined whether the SSeCKS-induced decrease in podosome formation and invasiveness correlated with changes in the abundance of either Tks5 protein or tyrosine phosphorylation level. Previous studies showed that rabbit polyclonal sera raised against a glutathione S-transferase (GST)-Tks5 fusion protein identify multiple isoforms between 125 and 140 kDa (7) but that only the upper three isoforms likely encoded Tks5, as they were lost after short hairpin RNA–mediated knockdown (3). Additionally, Seals et al. (3) showed that the slowest mobility isoforms (~140 kDa) likely result from Src-induced phosphorylations because these bands are present in immunoblots of Src/3T3 but not NIH3T3 lysates. Our results in Fig. 5A agree with this assessment in that there is an increase in the ~140-kDa band only in the cells grown at the permissive temperature irrespective of SSeCKS reexpression. The fact that ts72v-Src is only active at the permissive temperature was confirmed using a phospho-SrcY416–specific antibody (Fig. 5A).

SSeCKS reexpression at either physiologic or overexpressed levels had no effect on total cellular tyrosine phosphorylation or Tks5 tyrosine phosphorylation induced by v-Src. In the absence of tetracycline, where ectopic SSeCKS levels are >10-fold higher than those in parental NIH3T3 cells, or in 0.4 μg/mL tetracycline, which results in physiologic SSeCKS levels (Fig. 5B), SSeCKS fails to grossly change the pattern of cellular phosphotyrosine substrates detected in immunoblots (Fig. 5C). Similarly, there is no change in Tks5 tyrosine phosphorylation at 0.04 μg/mL tetracycline at the permissive temperature; paradoxically, there is a 2-fold increase in this phosphorylation in the absence of tetracycline, although these cell populations have significantly fewer podosomes (Fig. 3A). In sum, these data clearly indicate that physiologic levels of SSeCKS have no
effect on Tks5 abundance or Src-induced Tks5 tyrosine phosphorylation and, taken with the data in Fig. 3A, further indicate that Tks5 tyrosine phosphorylation is insufficient for podosome formation.

Although the tetracycline-regulated reexpression of SSeCKS induced cell flattening, we noted previously that, in any given culture lacking tetracycline, roughly 10% to 30% of the cells failed to flatten (21). Moreover, this was a stable phenomenon (as opposed to a contamination of non-tetracycline-regulated cells) because 20 cloned cells all showed between 70% and 90% cell flattening at a given time. We speculated that some of the tetracycline-regulated expression controls might be briefly attenuated by cell cycle effects in a nonsynchronized cell population because the “round” cells also showed little ectopic SSeCKS by immunofluorescence analysis. This would be consistent with our previous findings that most of SSeCKS expression is in the G1 phase (22) and that its overexpression in untransformed cells results in G1-phase arrest (23). Therefore, we addressed whether the ability of SSeCKS to suppress Src-induced podosome formation correlated with its ability to induce cell flattening. As shown in Fig. 6A, roughly 75% of the S24-ts72v-Src cells grown at the permissive temperature in the presence of 1.0 μg/mL tetracycline exhibited transformed morphologies, such as increased refractility (“round”). Growth in no tetracycline caused >90% of the cells to flatten, whereas at physiologic levels of SSeCKS reexpression (0.04 μg/mL tetracycline) there roughly was an even ratio of flat to round cells. Interestingly, there was a concomitant loss of round cells exhibiting podosome formation and an increase in the number of flat cells devoid of podosomes that correlated with increasing ectopic levels of SSeCKS (Fig. 6B). Moreover, SSeCKS did not change the percentage of flat or round cells exhibiting podosomes (Fig. 6B) or the podosome number per cell (Fig. 6C), only the total number of podosomes in the population (Fig. 6B). These data indicate a direct parallel between SSeCKS-induced cell flattening and suppression of podosome formation.

The suppression of Src-induced oncogenic transformation variables by SSeCKS reexpression correlated with the reestablishment of longitudinal F-actin stress fibers and vinculin-associated focal adhesion plaques (15). This contrasts to areas of membrane ruffling (ruff) in d. Bar, 30 μm.
suppression by SSeCKS. S24/ts72v-Src cells were transfected with constitutively activated clones plus pEGFP, and after 2 days of growth at the permissive temperature, podosome numbers were quantified in census of flat or round cells. At 0.04 μg/mL, individual constitutively activated mutants or paired combination had no significant effect on total podosome number in round cells, although the combination of all three constitutively activated mutants increased total podosome number by roughly 80% (Fig. 7B). Presumably, this is because the round cells are still “transformed.” In contrast, RhoA and/or Cdc42 increased total podosome formation >3-fold in the flat cells (Fig. 7A). Interestingly, the combination of Rac I and RhoA led to a decrease in total podosome number over controls (pEGFP alone) presumably because this combination induced increased cell rounding (Fig. 7C). Constitutively activated RhoA and/or Cdc42 also significantly increased the number of podosomes per cell in the flat cell population. Taken together, these data indicate that activated RhoA and/or Cdc42 are sufficient to rescue podosome formation downstream of the SSeCKS block, whereas a combination of activated Rac I and RhoA rescues SSeCKS-induced cell flattening irrespective of podosome formation.

Data in Fig. 7 strongly suggest that SSeCKS inhibits the activation of RhoA family GTPases by oncogenic Src. Thus, lysates from S24/ts72v-Src cells were precipitated with either GST-Rhotekin or GST-PAK beads (Fig. 8B), and these immunoprecipitates were probed with antibodies specific for RhoA, Rac I, and Cdc42. Indeed, SSeCKS reexpression in S24/ts72v-Src cells significantly inhibits activation levels of RhoA, Rac I, and Cdc42 2.5- to 5-fold without affecting the levels of each of these proteins (Fig. 8A).

To address whether the RhoA effects are manifested through activation of a downstream mediator, Rho kinase, S24/ts72v-Src cells grown at the permissive temperature in 1.0 μg/mL tetracycline were treated with a specific Rho kinase inhibitor, Y27632. Figure 8C shows that Y27632 inhibited total podosome formation 2.8-fold compared with vehicle-treated cells. The requirement for Rho kinase in podosome formation agrees with data from Sahai et al. (30) and Soh and Weinstein (31), which show a requirement for Rho kinase in RhoA-mediated oncogenic transformation and in tumor cell invasion. In contrast, Berdeaux et al. (6) show that Src-induced extracellular matrix–degrading activity associated with activated RhoA in podosomes is not blocked by Y27632.

Oncogenes, such as Src, manifest oncogenic transformation by subverting controls on signaling pathways governing cell proliferation, apoptosis, cytoskeletal architecture, motility, and adhesion to other cells and to extracellular matrix (32). Moreover, a critical role for Src activation in metastasis and tumor invasion has been established (33, 34). In this regard, Src-induced podosomes are thought to represent gross changes in the actin cytoskeletal infrastructure as well as in motility associated with tissue/tumor invasion. The ability of SSeCKS to suppress Src-mediated oncogenic growth seems related to its ability to induce cytoskeletal remodeling and not to a direct inhibition of Src kinase activity in cells. We speculate that the SSeCKS-induced cytoskeletal remodeling is dominant over the mechanisms underlying podosome formation possibly by disengaging the RhoA- and Cdc42-regulated filamentous actin cytoskeleton from sites of focal enrichment of podosome proteins, such as Tks5. We speculate further that the ability of SSeCKS to inhibit metastatic growth, but not generic cell

FIGURE 7. Effect of activated Rho family GTPases on total podosome number or podosome/cell frequency. S24/ts72v-Src cells were transfected with pEGFP plus constitutively activated forms of Rac I, RhoA, or Cdc42 as described in Materials and Methods and then incubated for 2 days in 0.04 μg/mL tetracycline or no tetracycline. The total podosome number (A and B) or podosome frequency per cell (D-G) was determined in flat versus round cells, and the total number of flat versus round green fluorescent protein (GFP)–expressing cells was counted (C). Columns, mean of three independent microscopic fields taken from each of duplicate transfections, containing 124 cells per field; bars, SE. *, P < 0.001.
motility, in prostate cancer cells with activated Src family kinases (14) relates to its suppression of Tks5-associated podosome formation. It will be interesting to determine if SSeCKS controls Src-mediated invasiveness through a direct interaction with Rho family GTPases or by preventing their translocation to membrane sites of activation.

Materials and Methods

Cell Lines and Reagents

S24/ts72v-Src cells are NIH3T3 cells expressing both a temperature-sensitive ts72v-Src allele and tetracycline-regulated reexpression of the rat SSeCKS cDNA (15). Cells were maintained in DMEM containing 10% heat-inactivated calf serum, tetracycline (0.7 μg/mL), hygromycin (1 μg/mL), and puromycin (1 μg/mL) at the permissive temperature for Src kinase activity (35°C). Cells were grown for at least 48 hours at the nonpermissive temperature (39°C) before seeding for experiments. Tks5 rabbit polyclonal antibody (7) was a gift of Sara Courtneidge (Van Andel Research Institute, Grand Rapids, MI). Constitutively active mutants of Rho family GTPases were gifts of Alan Hall (University of London, London, United Kingdom) and Andrei Bakin (Roswell Park Cancer Institute, Buffalo, NY). The Rho kinase inhibitor Y27632 was purchased from Calbiochem, San Diego, CA.

Immunofluorescence Analysis

Cells were plated onto sterilized 22-mm² glass coverslips the day before fixing. Fixation, staining, and microscopy were done as described previously (21) using a Nikon (Melville, NY) TE200-E fluorescent inverted microscope, SPOT INSIGHT-4 camera, and MetaVue imaging software package version 6.1 r6 (Universal Imaging Corp., Downingtown, PA). Cells were stained with Tks5 antibody (1:500), Rh-phalloidin (1:500; Sigma, St. Louis, MO), FITC-labeled goat anti-Rb immunoglobulin (1:500; Chemicon, Temecula, CA), and 4',6-diamidino-2-phenylindole (1:500; Sigma) in HBSS containing 3% bovine serum albumin.

Invasion Assay

Cell invasiveness was measured at 35°C for 6 hours as described previously (15) in 24-well plates containing Transwell chambers with 8-μm pores (Millipore, Billerica, MA) coated with growth factor–reduced Matrigel (BD Biosciences, San Jose, CA).

Protein Analysis

Radioimmunoprecipitation assay buffer lysates from S24/ts72v-Src cells grown at the nonpermissive or permissive temperature for 24 hours in the presence or absence of
tetracycline (0.7 μg/mL) were prepared as described (15) and then immunoblotted with RB polyclonal antibody anti-Tks5 (1:2,000), monoclonal antibody anti-actin (Sigma; 1:1,000), RB polyclonal antibody anti-SSeCKS (1:2,000), monoclonal antibody anti-phospho-tyrosine 4G10 (1:5,000; Upstate Biotechnology, Charlottesville, VA), or RB polyclonal antibody anti-phospho-Src(1416) (1:2,000; BioSource, Camarillo, CA). Secondary antibodies were either anti-Rb IgG or anti-mouse IgG labeled with horseradish peroxidase, and antibody binding was visualized following incubation with Lumi-Lite chemiluminescence reagent (Roche, Indianapolis, IN) using a ChemiGenius2 (Syngene, Frederick, MD) BioImager.

Pull-down Assays

Pull-down assays for activated forms of RhoA, Rac I, and Cdc42 were done as described previously (35) using GST-PAK (for Rac I and Cdc42) and GST-Rhotekin (for RhoA) beads. Lysates were prepared in pull-down buffer [50 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 2.5 mmol/L MgCl2, 10% glycerol, 1% NP40, 10 mmol/L NaF, 1 mmol/L Na3VO4, 1× protease inhibitor cocktail (Roche)] from S24/t527v-Src cells grown overnight at 35°C in the presence or absence of tetracycline. GST-PAK or GST-Rhotekin (5 μg) loaded onto glutathione-Sepharose beads was incubated for 1 hour at 4°C with 500 μg protein (±tetracycline), and the beads were washed twice with pull-down buffer, separated on 15% SDS-polyacrylamide gels, and immunoblotted blotted using antibodies for RhoA, Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA), or Rac I (Upstate Biotechnology).

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References

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