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Essential role of Src suppressed C kinase substrates in endothelial cell adhesion and spreading

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Abstract

Integrin-mediated substrate adhesion of endothelial cells leads to dynamic rearrangement of the actin cytoskeleton. Protein kinase C (PKC) stimulates reorganization of microfilaments and adhesion, but the mechanism by which this occurs is unknown. Src suppressed C kinase substrate (SSeCKS) is a PKC substrate that may play an important role in regulating actin cytoskeleton. We found that SSeCKS was localized to focal adhesion sites soon after cell adhesion and that SSeCKS translocated from the membrane to the cytosol during the process of cell spreading. Using small interfering RNAs specific to SSeCKS, we show that RPMVEC cells in which SSeCKS expression was inhibited reduce adhesion and spread on LN through blocking the formation of actin stress fibers and focal adhesions. These results demonstrated SSeCKS modulate endothelial cells adhesion and spreading by reorganization of the actin cytoskeleton.
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The tight control of cell adhesion and motility is crucial for a wide variety of physiological and pathological processes such as embryogenesis, inflammation, angiogenesis, wound healing, and tumor metastasis. Integrins are heterodimeric cell surface receptors that mediate cell–cell and cell-extracellular matrix (ECM) interactions and have been involved in the regulation of cell growth, migration, survival, and metastasis [1,2]. Integrin affinity and avidity are modulated by intracellular signaling cascades (''inside-out'' signaling), leading to changes in adhesion and motility. Conversely, binding of integrins to ECM proteins elicits signals that are transduced into the cell (''outside-in'' signaling) to regulate cell growth, migration, and survival. A number of intracellular signaling pathways have been involved in the regulation of integrin adhesive functions, including protein kinase C (PKC), phosphatidylinositol 3-kinase, and the small GTP-binding proteins of the Ras and Rho families [3–5].

Among the proteins implicated in inside-out signaling, protein kinase C (PKC) has been found in many instances to play a crucial role in modulating integrin-mediated cell adhesion, spreading, and migration [6–8]. Compounds that activate protein kinase C, such as phorbol 12-myristate 13-acetate (PMA), enhance the adhesion of cells to a matrix [9]. Pharmacological inhibitors of PKC prevent not only focal adhesion formation but also stress fiber formation in several cells plated on LN [10]. However, despite considerable evidence describing the importance of PKC in integrin-mediated adhesion, the mechanism by which PKC regulates these processes remains poorly understood.

A means of PKC regulation is through their association with targeting proteins, providing a tight control of PKC subcellular localization and substrate specificity [11,12]. One such protein, SSeCKS (Src suppressed C kinase

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substrate), specifically binds to PKC. SSeCKS is a 280 and 290 kDa protein that have predicted rod-like structures that localize to the cortical cytoskeleton and plasma membrane sites, most likely via myristylation [13]. Upon PKC activation, SSeCKS relocates from the cell cortex to perinuclear sites. Inducible overexpression of SSeCKS results in cell flattening, elaboration of the cortical cytoskeleton and an increase in integrin-independent FAK phosphorylation and growth arrest [14]. SSeCKS binds F-actin in vitro and in vivo whereas its tyrosine phosphorylation causes translocation from F-actin to cortical cytoskeletal sites [15]. These studies suggest a strong correlation among PKC activation, SSeCKS phosphorylation, and dynamic actin remodeling. Furthermore, SSeCKS binding to the plasma membrane isoform of β-1,4-galactosyltransferase I (GalT) cytoplasmic domain is partly responsible for the ability of GalT to associate with the cytoskeleton and induce signal transduction cascades [16]. Cell surface GalT functions as a cell adhesion molecule by binding to extracellular oligosaccharide ligands [17], however, the possible involvement of SSeCKS in this process has not been investigated.

There is no cellular change in which reorganization of the actin cytoskeleton is more profound than that which occurs during adhesion and spreading. Based on previous findings of the importance of SSeCKS in a PKC signaling pathway that mediates cytoskeleton reorganization, we hypothesized that it is through SSeCKS that integrin signaling, via PKC activation, regulates actin cytoskeletal organization during this dramatic cellular morphological change in rat pulmonary microvascular endothelial cell (RPMVEC). Here, we report that SSeCKS translocation from the membrane to the cytosol in response to adhesion to laminin. Knockdown of SSeCKS by siRNA inhibited of RPMVEC adhesion, spreading specifically on laminin and inhibited the formation of F-actin-containing stress fibers and focal adhesions. We propose, therefore, that SSeCKS participate in the transmembrane linking of intracellular cytoskeletal components to extracellular matrix components which occurs in focal adhesions.

Materials and methods

Generation of rat pulmonary microvascular endothelial cell. Isolation and culture of RPMVEC has been described previously [18]. RPMVEC were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, and penicillin/streptomycin. The endothelial phenotype of the preparation was confirmed by evaluating cellular uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) and the presence of platelet endothelial cell adhesion molecule (PECAM-1).

siRNAs and transfection. Double-stranded RNAs of 19 nucleotides were synthesized by Ambion Research. The targeting sequence of rat SSeCKS mRNA (5′-CCG AGT AGA GAA GAA TCT T-3′) corresponds to the region 2099–2118, relative to the first nucleotide of the start codon (GenBank Accession No. AY695057). Non-silencing control siRNA is an irrelevant siRNA with random nucleotides and no know specificity. Transfection of RPMVECs with duplex synthetic siRNA was performed using Oligofectamine reagents (Invitrogen) according to the manufacturer's instructions. Cells were assayed after 48 h of transfection. For mock transfection, all procedures listed above were performed in the absence of siRNA duplex.

RNA isolation and real-time quantitative PCR. Total cellular RNA was extracted from cells 48 h post-transfection using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The level of SSeCKS mRNA expression was quantified by real-time quantitative PCR using the TaqMan PCR MASTER MIX kit (Applied Biosystems, Foster City, CA). Amplification and detection were performed using the Rotogene3000 Sequence Detection System starting with 2 μL of cDNA. The primers and probes used were: SSeCKS forward primer 5′-AAGTGCTGGCTTCGGAGAAAG-3′; SSeCKS reverse primer 5′-TGACTTCAGGAAC TTCAAGGCTC-3′, probe: FAM′ AGCCTGTCCAGT CTCAGAGCCCTGTG′TAMRA. GAPDH was used as an internal control. For relative quantification, the copy ratios of SSeCKS/GAPDH were calculated and used as an indication of the relative expression levels.

Cellular adhesion and spreading assays. Cells used in adhesion assays were trypsinized and washed with PBS, and 3×10^4 cells were plated on 96-well polystyrene assay plates uncoated or coated with 20 μg/ml laminin. One hour after plating, the cells were washed with PBS and fixed with 4% formaldehyde for 30 min before being stained with 0.25% crystal violet in 40% methanol for 3 h. After removing the crystal violet solution, the plates were washed extensively, dried, and the stain released using 2% SDS in PBS. Stain intensity was quantified by spectrophotometry (570 nm) using a plate reader (Multiscan Plus, Labststem, Franklin, MA, USA). Each line had 10 wells assayed, and experiments were repeated three times ($n = 30$). To confirm that these data represented not only the amount of protein but also the number of cells, 3×10^5 cells were also plated on laminin-coated 35-mm petri dishes, and the number of attached cells was determined. One hour after plating, the cells were washed with PBS, fixed, and stained with crystal violet solution, and photographs were taken using a monochrome CCD camera. The number of cells in each of the 6 images was determined. Spread cells were defined as previously described (flattened morphology and phase-dark) [19]. One hundred cells were counted at each time point and condition.

Western blotting. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and extracted in lysis buffer for 45 min on ice. Equal amounts of protein were separated by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA) using a mini trans-blot (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then blocked with PBST (PBS with 0.05% Tween 20) containing 5% non-fat dry milk for 1 h and incubated with a sheep polyclonal antibody against SSeCKS (Sigma, Oakvile, ON) overnight at 4 °C. Membranes were then washed with PBST and incubated with horseradish peroxidase conjugated rabbit anti-sheep immunoglobulin G (IgG) for 1 h. The blots were developed using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, USA).

Immunofluorescence. RPMVEC cells were washed once with phosphatebuffered-saline (PBS) and fixed in 4% paraformaldehyde for 15 min. They were then permeabilized by incubation in PBS containing 0.1% Triton X-100 for 20 min on ice, blocked in PBS containing 0.1% Tween 20 (PBST) and 3% bovine serum albumin for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies. After being washed with PBST, the cells were incubated for 1 h with an Rhodamine conjugated secondary antibody (Jackson laboratory). To visualize actin microfilaments, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR).The stained cells were then examined by a Zeiss Confocal Laser Scanning Microscope.

Statistical analysis. All data are expressed as means ± SD and were analyzed statistically using the one-way ANOVA followed by the Newman–Keuls test with P values less than 0.05 considered statistically significant. All statistical analyses were performed using SPSS software.

Results

SSeCKS localization during RPMVEC spreading

To determine if SSeCKS localization did in fact correlate with different phases of integrin-mediated cell spreading, we plated RPMVEC on LN and analyzed the cultures for changes in SSeCKS localization as a function of time. After 30 min on LN, SSeCKS was recruited to cellular structures resembling focal adhesions. To confirm that SSeCKS is indeed at focal adhesion sites in endothelial cell upon spreading, we co-stained cells with antibodies to SSeCKS and to FAK and found that they localized to the same sites after the cells were plated on LN for 30 min (Fig. 1A). Fig. 1B shows the localization of SSeCKS in endothelial cell plated for various times on LN. Soon after adhesion (30 min), SSeCKS was found in punctate structures typical of focal adhesion throughout the cell. After 1 h on LN, SSeCKS was observed more diffusely in the cytosol in most of the cells, although localization at focal contacts was still evident and very predominant at 30 min (Fig. 1A). At 24 h on LN, SSeCKS staining was observed in perinuclear region and diffusely in the cytosol in most of the cells. To further determine whether SSeCKS is indeed redistribution after adhesion to LN, we observed SSeCKS translocation from the membrane to the cytosol in RPMVEC plated on LN at 1 h (Fig. 1C). Thus, the translocation of SSeCKS to the cytosol occurs early during the transition from cell adhesion to cell spreading.

Specific reduction of SSeCKS mRNA and protein in RPMVEC

To assess directly the importance of SSeCKS in RPMVEC adhesion and spreading, we transfected the specific SSeCKS siRNA or either with vector alone or non-specific siRNA (both controls showed similar results). We first confirmed the specificity of siRNA induced gene silencing in RPMVEC. Western blot analysis showed strong and specific down-regulation of SSeCKS protein levels in RPMVEC cells treated with SSeCKS siRNA, but not with control-irrelevant siRNA or in the absence of siRNA (Fig. 2A). As expected, SSeCKS mRNA expression was considerably knocked down in SSeCKS siRNA-transfected

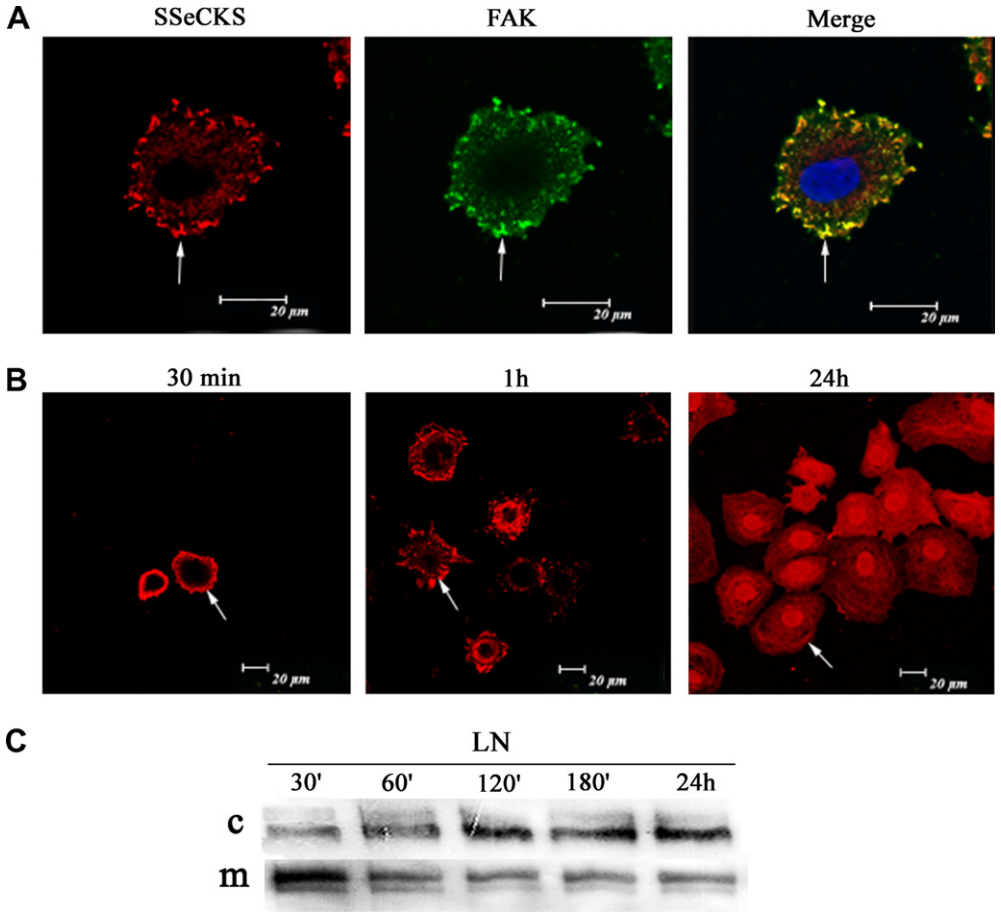

Fig. 1. SSeCKS localized and translocated in RPMVEC cells which plated on LN. (A) SSeCKS is localized to focal adhesion sites during the initial phases of RPMVEC cell attachment and spreading. The cells shown here were fixed 30 min after plating and show colocalization of SSeCKS and FAK, demonstrating the localization of SSeCKS at focal adhesion sites (arrow). (B) SSeCKS translocates from the membrane to the cytosol during RPMVEC cell spreading. SSeCKS localization to focal adhesion sites (arrows in each panel) is most prominent at early time points, decreasing in intensity as the cells spread. With time, SSeCKS becomes more diffusely distributed in the cytosol. (C) SSeCKS translocation is mediated during RPMVEC cell spreading.

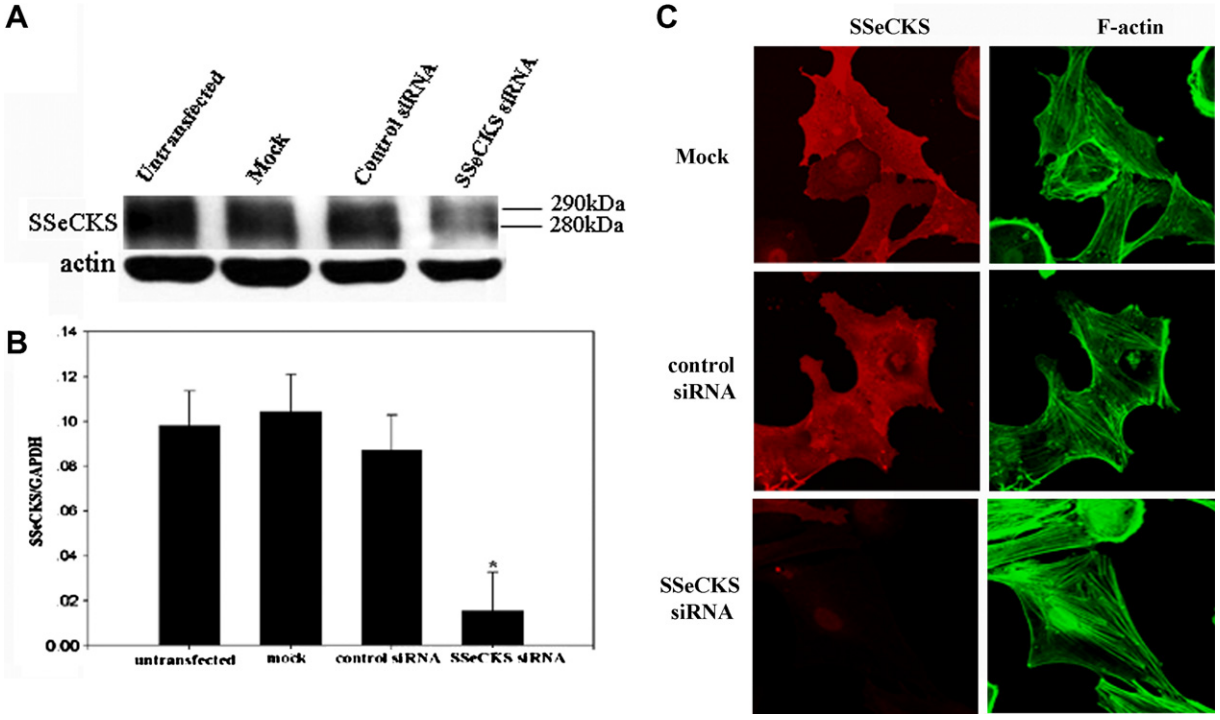

Fig. 2. SSeCKS expression is knocked down using SSeCKS siRNA. (A) Western blot analysis of corresponding protein expression. (B) Real time-PCR analysis of SSeCKS mRNA expression in mock- and siRNA-transfected RPMVECs. $^{*}P < 0.05$ vs untransfected group by one-way ANOVA. (C) Effects of SSeCKS siRNA on F-actin fibers. RPMVEC cells were both mock transfected and transfected with control siRNA and SSeCKS siRNA, and stained with both an anti-SSeCKS antiserum (left) and pholloidin (middle) at 2 days after the transfection. A merged image is shown in the right panel. Scale bar, 20 μm.

cells compared with mock-transfected or control siRNA-transfected cells (Fig. 2B). Although not 100% knocked down because of the limitation of transient transfection, the profound reduction of SSeCKS protein that did occur was indeed attributable to the effectiveness of SSeCKS siRNA and not to the overall inhibition of protein synthesis, because β-actin expression among samples remained unchanged (Fig. 2A). To additionally demonstrate the effectiveness of SSeCKS siRNA, transfected cells were observed for SSeCKS expression under confocal microscopy. Mock-transfected and control siRNA transfected RPMVECs exhibited typical SSeCKS staining at the cell edge and cytoplasm (Fig. 2C). However, in SSeCKS siRNA transfected cells, SSeCKS staining is barely visible, indicative of the extent of depletion of SSeCKS expression (Fig. 2C). Interestingly, in SSeCKS siRNA-transfected cells, we consistently observed an increased in F-actin structures in cytoplasm, suggesting that SSeCKS may be required for the organization of F-actin fibers in cultured endothelial cells. Together, these data confirm the generation of SSeCKS-depleted cells, which can now be used to study the effect of SSeCKS functional knockdown in laminin-induced events.

SSeCKS regulates cellular adhesion and spreading on different substrata

Previous studies identified a role for SSeCKS in the induction of filopodia and lamellipodia, structures involved in cell adhesion and spreading [20,21]. Therefore, we sought to analyse the role of SSeCKS in RPMVEC adhesion and spreading. Cells were plated on dishes for 1 h, fixed, and stained. By quantification using crystal violet, the siRNA control and no transfection control RPMVEC groups attached more than did SSeCKS siRNA transfected RPMVEC groups to coated or uncoated dishes (Fig. 3A). The number of cells attached to coated dishes was also determined by counting to confirm the data using crystal violet absorbance (Fig. 3B). From the images, SSeCKS siRNA transfected RPMVEC appeared only marginally attached by 1 h, whereas control RPMVEC cells had already started spreading by this time (Fig. 3C).

SSeCKS-knockdown cells show reduced focal adhesion and alteration of actin cytoskeletal organization

To test directly whether altered cell adhesion in the SSeCKS siRNA transfected RPMVEC was caused by impaired cytoskeleton organization, we analyzed focal adhesion and actin stress fiber formation during cell adhesion. The control and SSeCKS siRNA transfected cells showed very distinct focal adhesion contacts and stress fiber formation after 1 h on laminin (Fig. 4). By contrast, focal adhesion formation was markedly inhibited in SSeCKS siRNA transfected cells with focal adhesion kinase (FAK) more diffusely distributed along the leading edges of the cells (Fig. 4A). Furthermore, there was very limited stress fiber formation with actin staining seen more

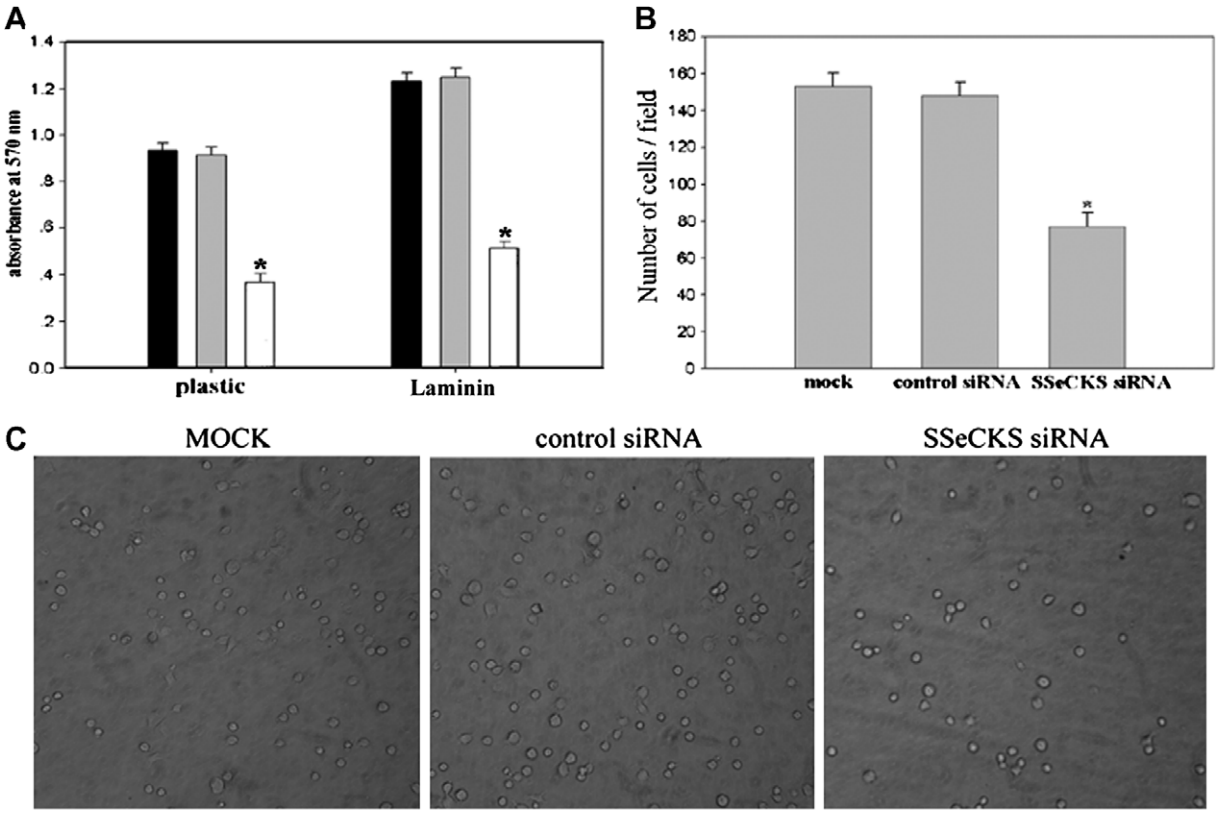

Fig. 3. Cell adhesion 1 h after plating on LN. (A) Cells attached to uncoated (plastic) or laminin-coated dishes (laminin) were quantified by staining with crystal violet. Black, gray, and white columns represent mock, control siRNA, and SSeCKS siRNA transfected cells, respectively. Error bars represent standard error of the mean (SEM). $^{*}P < 0.05$ vs mock by Student's *t*-test. (B) The average number of cells adhered to laminin-coated dishes per 100× field was determined by counting cells attached 1 h after plating. Error bars represent SEM. $^{*}P < 0.05$ vs mock Student's *t*-test. (C) Spreading of each cell 1 h after being plated on laminin-coated dishes. Note that attached SSeCKS siRNA transfected cells did not spread as well as control siRNA or mock cells.

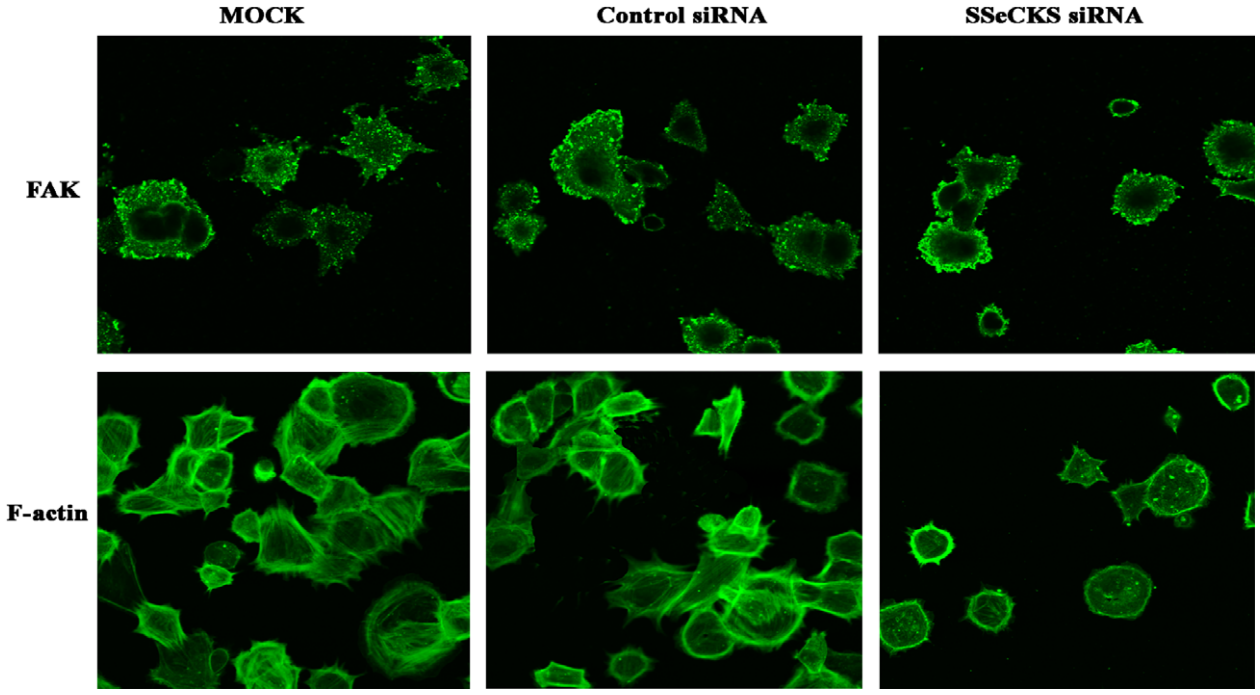

Fig. 4. Focal adhesion and actin stress fiber formation in SSeCKS knockdown cells. Focal adhesion sites and stress fiberts were labeled with an anti-FAK antibody and FITC-conjugated phalloidin, respectively, 1 h after cells were plated on laminin. Scale bar, 20 μm.

prominently in cortical regions compared to control cells (Fig. 4B). Taken together, these results demonstrate the negative role of the SSeCKS siRNA in cellular processes necessary for a complete cell adhesion, including those involved in focal adhesion and stress fiber formation.

Discussion

The results of the present study demonstrate that SSeCKS translocate between the membrane and cytosol during RPMVEC adhesion and spreading on the laminin. This process is initiated during the early phases of cell adhesion to laminin, an interaction that leads to an activation of specific PKC isoenzymes in a temporal fashion [22,23] and the subsequent displacement of SSeCKS from the membrane. Similarly, since PKC activation is thought to release SSeCKS from the cell surface, allowing it to translocate back to the perinuclear region [13].

Our results provide direct support for the necessity of SSeCKS translocation between membrane and cytosol to promote RPMVEC cell spreading, and indicate the clear temporal sequence that depends upon SSeCKS localization in a particular cellular compartment during the transition from initial adhesion through the process of cell spreading (Fig. 1A and B). The adhesion and spreading of all cells involves dramatic changes in the actin cytoskeletal network. The initial stages of cell adhesion require cytoskeletal reorganization to permit extensive cell shape changes. Such changes require that stress fibers are disassembled and filopodia and lamellipodia are extended at the leading edge of moving cells to make contact with the matrix [24]. At this stage, we observed that SSeCKS displayed punctuate structures throughout the cell edge. As cell spreading progresses, the cross-linking of actin, increases the viscosity and stiffness of the actin filament network. At this stage, we observed that SSeCKS distributed more diffusely in the cytosol in most of the cells.

The extracellular matrix (ECM) plays a critical role in modulating the morphology, growth, migration and differentiation of cells. The most thoroughly studied receptors are the integrins, which when bound by ligands, can induce an array of cytoplasmic signals. Another, although less well studied, receptor for the ECM is β-1,4-galactosyltransferase I (GalT) [25].

The major signaling pathways triggered by integrin activation that have been studied in the control of actin cytoskeleton organization are those mediated by PKCs and by the Rho family of monomeric GTPases [26]. The activation of PKC enhances focal adhesion formation and cell spreading in various cell types in response to cell binding to extracellular matrix proteins [22,23]. The possibility that PKC activation can directly regulate cytoskeletal organization is supported by the identification of several actin binding and modulating proteins, such as SSeCKS, as PKC substrates [14]. The facts that SSeCKS is a major PKC substrate in the cell and has a direct role in regulating actin polymerization suggest that SSeCKS might be the PKC-sensitive intermediate between integrin activation and the cytoskeletal reorganization that accompanies cell spreading.

Furthermore, anther mechanism by which SSeCKS has been suggested to regulate the organization of the actin cytoskeleton is by affecting the tyrosine phosphorylation/activation of FAK. The activation of FAK by Src-family kinases plays a central role in LPA-, neuropeptide-, and integrin-mediated signal transduction and cytoskeletal reorganization [27]. Earlier reports have demonstrated that aggregation of cell surface GalT with synthetic multivalent substrates or with anti-GalT antibodies induces a transient tyrosine phosphorylation of FAK and a loss of stress fibers [28]. Similarly, increasing the level of SSeCKS elevates the level of integrin-independent FAK tyrosine phosphorylation causing cells to flatten with a subsequent reduction of stress fibers [14]. In this respect, it is interesting to note that SSeCKS knocked-down RPMVEC cells show an abundance of actin stress fiber staining compared with control cells. The identification of the SSeCKS scaffolding protein as a cytoplasmic partner for the GalT cytoplasmic domain suggests that it is partly responsible for the ability of GalT to associate with the cytoskeleton and induce signal transduction cascades. These observations suggest that SSeCKS binding to the cytoplasmic domain of GalT is coupled, directly or indirectly, with the reorganization of the actin cytoskeleton that are induced by GalT adhesion to laminin.

Acknowledgments

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