A Novel src- and ras-suppressed Protein Kinase C Substrate Associated with Cytoskeletal Architecture*

Xueying Lin, Eugene Tombler, Peter J. Nelson, Michael Ross, and Irwin H. Gelman‡

From the Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

We previously identified a novel src- and ras-suppressed gene, 322, encoding a mitogen regulatory function (Lin, X., Nelson, P. J., Frankfort, B., Tombler, E., Johnson, R., and Gelman, I. H. (1995) Mol. Cell. Biol. 15, 2754–2762). Here, we characterize the 322 gene product as an in vivo and in vitro substrate of protein kinase C (PKC). Hence, we named this product SSeCKS (pronounced essex) for Src Suppressed C Kinase Substrate. Rabbit polyclonal sera raised against glutathione S-transferase (GST)-SSeCKS recognized a myristylated 280–290-kDa doublet in Rat-6 fibroblasts. SSeCKS levels in src- and ras-transformed Rat-6 cells were 15- and 8-fold less, respectively, than those in untransformed cells. Short-term addition of phorbol ester resulted in a 5-fold increase in SSeCKS phosphorylation which was inhibited by bis-indolylmaleimide. In vitro phosphorylation of GST-SSeCKS by purified rabbit brain PKC-α was enhanced by phosphatidylinerine and blocked by excess PKC pseudosubstrate inhibitor peptide. GST-SSeCKS bound purified PKC-α or PKC from Rat-6 lysates in a phosphatidylinerine-dependent manner. Four SSeCKS domains containing Lys/Arg-rich motifs similar to the PKC phosphorylation site in MARCKS were phosphorylated in vitro by PKC. Immunofluorescence analysis showed SSeCKS present throughout the cytoplasm with enrichment in podosomes and at the cell edge. Short-term addition of phorbol esters caused the movement of SSeCKS from plasma membrane sites to the perinuclear coincident with a loss of actin stress fibers. These data suggest a role for SSeCKS in the control of cellular cytoskeletal architecture.

The ability of the oncopeptide product encoded by Rous sarcoma virus, p60src, to induce morphological transformation and tumorigenesis is dependent on its intrinsic protein tyrosine kinase activity and ability to recognize cellular substrates. The transforming activity of p60src is controlled by at least four modular domains including a Src-homology 1 (SH1) encoding the kinase domain, an SH2 and an SH3 domain which binds phosphotyrosine residues or polypeptide tracts, respectively, on specific signal proteins, and an SH4 motif which directs N-terminal myristylation and thus, association with the plasma membrane (2–4). The src oncogene is known to alter cellular signal pathways controlling mitogenesis, cell cycle, and morphology. p60src is thought to initiate mitogenic signal cascades by directly phosphorylating signaling proteins at plasma membrane sites, resulting in the association with and activation of messenger molecules such as GAP, phospholipase-C, p21ras, the c-RAF serine/threonine kinase, MAP kinase kinase, and MAP kinase. Indeed, MAP kinase is activated early after v-src expression (5). This signal ultimately results in the expression and/or modification of "immediate-early" transcription factors such as those comprising the AP-1 complex, NGFI-A, KC, and PC4, followed by induction of DNA replication and cell proliferation (6, 7). Recent evidence indicates that v-src can directly activate STAT-3, presumably at plasma membrane sites, resulting in the translocation of STAT-3 to the nucleus where it induces the transcription of mitogen-response genes (8).

In contrast, more recent evidence suggests that src controls cytoskeletal architecture via PKC and GTPase proteins such as Rho and Rac. These pathways may require nuclear events as well as direct effects by p60src in the cytoplasm. For example, p60src affects cell architecture and cell-to-cell interactions by either down-regulating the transcription of several cytoskeletal and extracellular matrix components such as tropomyosin, fibronectin, and type I collagen (9, 10), or by directly inducing the phosphorylation of extracellular matrix ligand proteins such as N-cadherin (11) or cytoskeletal proteins such as tropomyosin and talin (12). p60src also phosphorylates and activates the focal adhesion kinase (p125FAK), which controls the formation of actin stress fibers and adhesion plaques (12). The association of these fibers with integrins is further modulated by the tyrosine phosphorylation of tethering proteins such as tensin and vinculin (12, 13). Because integrins bind directly to extracellular matrix proteins, these events effectively regulate controls on contact inhibition and interactions with neighboring cells. Most importantly, several studies demonstrate an in vivo co-localization of p60src with p125FAK, PKCα, vinculin, and actin fiber ends in focal adhesions (12–15).

We previously characterized a novel gene, 322 (1), isolated in a screen for candidate tumor suppressor or regulatory genes (16). 322 transcription is sustained in confluent, non-dividing fibroblasts, but is suppressed in response to serum growth factors or activation of a Tα-v-src allele. Furthermore, the constitutive overexpression of 322 via retroviral vectors or by stable transfection is toxic, resulting in the deletion of variants deleted of the transduced 322 cDNA. These data suggested that 322 may encode a regulator of mitogenesis.

In this paper, we have characterized the protein product of the 322 gene, determined its cellular localization in rat fibro-

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‡ To whom correspondence should be addressed: Box 1124, Dept. of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Tel.: 212-241-3749; Fax: 212-534-1884; E-mail: igelman@msmtplink.msm.edu.

1 The abbreviations used are: GAP, GTPase-activating proteins; MAP, mitogen-activated protein; FAK, focal adhesion kinase; PKC, protein kinase C; kb, kilobase(s); GST, glutathione S-transferase; a.a., amino acid; PS, phosphatidylinerine; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; ORF, open reading frame; MARCKS, myristylated alanine-rich C kinase substrate.
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blasts, and defined it as a PKC substrate in *vitro* and *in vivo*. Based on these characteristics, we have named the 322 product SSeCKS for Src Suppressed C Kinase Substrate. Our data suggest a role for SSeCKS in the formation of the actin-based cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—A full-length 322 cDNA was constructed by splicing a 1.2-kb XhoI/BstEII fragment from a 5′-rapid amplification of cDNA end clone, p35x2 (kindly provided by Sue Jaken, W. Alton Jones Cell Science Center, Lake Placid, NY), into a BstEII-partial/XhoI fragment of the 13.2 322 cDNA (1). The resulting “SSeCKS” cDNA was sequenced using Sequenase 2.0 kits (U. S. Biochemical Corp.) and the data were entered into the GenBank in the SWISSPROT database (accession number U23140). GST fusion constructs were produced using pGEX-5x-1 (Phar- macia) and His-tag constructs were produced using pET28 (Novagen). Retroviral constructs of the SSeCKS cDNA were produced in pBABE- hygro and packaged in Ω cells as described previously (17). The SSeCKS cDNA was also spliced into pCEV27 (18) containing the Moloney leukemia virus-long terminal repeat promoter, and stably trans- fected into Rat-1 cells (19) followed by infection in 400 μg/ml Geneticin (Life Sciences) as described previously (17).

**Expression of GST- and His-tag Fusion Proteins**—A fragment of the SSeCKS ORF (a.a. residues 389 to 894) was amplified by polymerase chain reaction from the 13.2 cDNA using primers 322-13-13 (cDNA coordinates 1167 to 1184) and 322-11b (cDNA coordinates 2725 to 2739) and inserted in-frame into EcoRI/SalI-cut pGEX-5x-1. The GST fusion constructs were produced using pGEX-5x-1 (Phar- macia) and His-tag constructs were produced using pET28 (Novagen). Retroviral constructs of the SSeCKS cDNA were produced in pBABE- hygro and packaged in Ω cells as described previously (17). The SSeCKS cDNA was also spliced into pCEV27 (18) containing the Moloney leukemia virus-long terminal repeat promoter, and stably trans- fected into Rat-1 cells (19) followed by infection in 400 μg/ml Geneticin (Life Sciences) as described previously (17).

**In Vitro PKC Phosphorylation Assay**—PKC assays were variations of previously described assays (21). Briefly, 40 μl reactions contained 10 μl of 0.3 mg/ml target polypeptide, 10 μl of 1 μCi/μl [γ-32P]ATP (DuPont NEN), 10 μl of rabbit brain PKC enzyme (10–25 ng), and 10 μl of 4 × buffer (20 mM Tris-HCl, pH 7.5, 1 mM CaCl2, 5 mM MgCl2, 0.05% Triton X-100, and freshly added 0.31 mg/ml leupeptin, antipain, pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.06 mg/ml 1,2-dioleoyl-rac-glycerol, and 0.4 mM ATP) were incubated for 30 min at 37°C. Target proteins included various GST- SSeCKS products, PKC substrate peptide [Ser25]PKC[19–36] (Life Technologies) was used at 0.15 μg/ml of protein and was phosphorylated using the same conditions as described above.

In Vivo Phosphorylation Analysis—10° Rat-6 cells were incubated overnight in Dulbecco's modified Eagle's medium (Bio-Whittaker) sup- plemented with 5% calf serum. PKC substrate peptide Ac-Myelin Basic Protein[4–14] (the latter two from Life Technologies). A specific inhibitor (peptide-substrate) peptide PKC[19–36] (Life Technologies) was used at 0.15 μg/ml of protein and was phosphorylated using the same conditions as described above.

**Production of Immune Sera**—After roughly 10 ml of preimmune sera was obtained, two New Zealand giant rabbits were immunized with 150 μg each of GST-1322 protein emulsified in an equal volume of complete Freund's adjuvant. The specificity of the sera was determined by probing preimmune sera against GST-1322, and BL21 lysate alone, followed by incubation with alkaline phosphatase-labeled sheep anti-rabbit Ig (Boehringer Mannheim), washing in Western blot buffer (below), and developing with 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium (Promega). Both rabbits gave high titers (>5000) of anti-SSeCKS antibodies. Immunofluorescence-purified an- ti-SSeCKS antibodies were isolated as follows. Glutathione-Sepharose columns were saturated with either GST or GST-SSeCKS, and then treated with 25.5 mM dimethyl pimelimitide cross-linker (Pierce). 10 μl of Rb anti-SSeCKS sera was passed repeatedly over the GST column (the bound antibodies were eluted with glutathione after each round) until all the anti-GST reactivity (as determined by slot blot Western analysis) was removed. The resulting sera was passed over the GST-1332 column, and the bound antibodies were eluted with Trit- glycine buffer, pH 2.8, as described previously (20). This fraction was shown by slot blot Western analysis to retain GST-1332 and Hisα1322 binding at dilutions of 1:1000, and no cross-reactivity to GST protein alone at dilutions of 1:100.

In Vivo Transsection/Translation of SSeCKS—Plasmid DNAs (1 μg) containing either the full-length SSeCKS cDNA or the 5.4-kb 13.2 cDNA (1) cloned into pBluescript SK II were linearized at the 3′-ends of the cDNA inserts (Smal) and incubated at 30°C for 90 min in a 50 μl of coupled transcription/translation reaction (TNT, Promega) containing 50 μCi of translation-grade [35S]methionine (DuPont NEN), according to the manufacturer's specification. 5 μl of the resulting protein products were electrophoresed on a 6% SDS-polyacrylamide stacking gel. The gels were fixed in methanol/acetic acid (80:20, v/v) for 30 min, incubated in Amplify (Amersham), and fluorographed with Kodak X-AR film at ~70°C.

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6 M. A. Mansfield (Millipore Corp.), personal communication.
Inc.) were co-incubated with 135 μl of glutathione-Sepharose pre-bound to 50 μg of GST-13.22 for 4 h at 4 °C (rotating) in RIPA buffer containing 150 mM NaCl, 5 mM MgCl$_2$, and 0.2 mM CaCl$_2$. PS was added in some cases at 0.37 mg/ml. The pellets were washed three times and then analyzed by SDS-PAGE and immunoblotting as described above using mouse monoclonal anti-PKC type III (Upstate Biologicals, Inc.).

**Subcellular Fractionation of Plasma Membrane and Cytosol Components**—10$^6$ Rat-6 or Rat-6/PKCα over-expressor cells were washed three times in ice-cold Tris-Glu buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM sodium phosphate, 0.1% glucose). The cells were scraped into Tris-Glu, and pelleted by centrifugation at 1,500 × g for 5 min. The cells were swollen on ice for 10 min in 20 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Trasylol, and 1 mM phenylmethylsulfonyl fluoride. The cells were Dounce homogenized (40 strokes with pestle B), and then NaCl was added to a final concentration of 100 mM. The nuclei and cell debris were pelleted at 1,500 × g for 10 min (4 °C) yielding initial pellet (P1) and supernatant (S1) fractions. The S1 fraction was loaded into polycarbonate tubes and centrifuged in a SW41 rotor (Beckman) for 30 min at 100,000 × g, yielding a secondary pellet (P100), containing plasma membranes, and supernatant (S100). Aliquots of these fractions were analyzed by SDS-PAGE and immunoblotting as above.

**Immunofluorescence Analysis**—Rat-6 cells were seeded onto sterile 22-mm coverslips at a density of roughly 70% and then incubated overnight or until the cells were confluent for at least 2 days. The coverslips were fixed as described previously (23). After washing in phosphate-buffered saline, the cells were incubated for 1 h with immunofluorescence-purified rabbit polyclonal anti-SSeCKS (above; 1:50 dilution) and rhodamine-labeled phalloidin (1:400; Sigma). Secondary antibodies—specifically recognizes a 280/290-kDa doublet in Rat-6 cells (Beckman). The coverslips were mounted in Aqua-Mount (Lerner Laboratories, Pittsburgh, PA) containing 20 mM p-phenylenediamine (Kodak) as an anti-bleaching agent.

**RESULTS**

**SSeCKS Is Identical to a Previously Described ">200-kDa" PKC Substrate**—Recent work from the Jaken laboratory identified several novel substrates of PKC using overlay assays (24). Preliminary sequencing of the >200-kDa gene showed >99% homology to our 322 gene sequence, which encodes SSeCKS (GenBank accession U23146).3 Most importantly, rabbit sera raised against GST/SSeCKS (GST/13.2.2., see Fig. 2) specifically recognizes a 280/290-kDa doublet (resolved better in Fig. 1) from a rabbit immunized with GST-13.2.2 protein (5 μg/lane) and lysate from Rat-6 cells (150 μg/lane). A 280/290-kDa doublet (resolved better in Fig. 1) and a minor 240-kDa SSeCKS species were recognized. Antibody binding was completely blocked by the addition of GST/13.2.2 (20 μg) but not by GST alone (20 μg), B, in vitro transcription and translation of SSeCKS. One μg of plasmid DNA encoding the full-length SSeCKS cDNA or a N terminally truncated SSeCKS cDNA (clone 13.2.2) were incubated in a coupled T7 transcription/translation reaction (TNT, Promega) containing [35S]methionine as described under “Experimental Procedures.” One-tenth of the labeled products were analyzed by SDS-PAGE followed by fluorography. Protein size markers are shown at the left. Note that a shortened version of SSeCKS, synthesized from an internal ATG start site in clone 13.2.2, is not produced in the context of the upstream ATG start site in the full-length SSeCKS cDNA. C, in vivo myristylation of SSeCKS. Lysates from 5 × 10$^6$ Rat-6 cells incubated overnight in media containing 100 mCi/ml [3H]myristate were analyzed by immunoprecipitation using preimmune or anti-SSeCKS sera, SDS-PAGE, and fluorography (5-month exposure).

3 S. Jaken, personal communication.
acidic residues as well as an inherent rod-like structure. The smaller polypeptides purified on glutathione columns are considered C-terminal breakdown products inasmuch as Western blotting using anti-GST sera identified the same band pattern as Coomassie Blue staining (data not shown).

Figs. 1, 3, 9, and 11 show that immune rabbit serum recognizes a 280/290-kDa doublet and a minor 240-kDa SSeCKS form (in Figs. 1 and 3, the 280/290-kDa doublet is unresolved). The 280/290-kDa doublet form is consistent with the in vitro product generated using the TNT system (Fig. 1B) and the bacterially expressed GST-SSeCKS products (Figs. 4 and 6), further suggesting that post-translational modifications are only minor contributors to the molecular masses of the mature SSeCKS products in Rat-6 cells. It is unclear whether the 240-kDa species represents an in vivo utilization of the internal ATG start site described above or a specific proteolytic cleavage product. Interestingly, the 240-kDa species is not readily labeled in vivo with [32P]orthophosphate (Fig. 3, top panel), although predicted casein kinase II and PKC sites are found throughout the SSeCKS ORF. Our previous data (1) showed a single mRNA hybridizing to 322 cDNA, and thus, the multiple forms of SSeCKS are most likely due to protein modifications rather than to multiple SSeCKS allelic products.

SSeCKS contains a predicted N-terminal myristylation signal, MGAGSSTEQR, which conserves the Gly-2 and Ser-6 motifs encoded by retroviral GAGs and the HIV nef product (Ref. 4). Fig. 1C shows that the 280/290-kDa SSeCKS form could be labeled in vivo with [3H]myristate. We assume this post-translational modification facilitates the association of SSeCKS with plasma membrane fractions shown in Fig. 11.

We found it difficult to metabolically label SSeCKS in either subconfluent or confluent cultures using either [35S]methionine/cysteine or [3H]leucine, although p60c-src was easily labeled in the same lysates (data not shown). This could not be due to a dearth of Met, Cys, or Leu residues in SSeCKS (20, 15, and 86, respectively). In contrast, SSeCKS could be immunoblotted easily under the same conditions, suggesting that its relative rate of de novo synthesis is low. We showed previously that SSeCKS is not glycosylated in an in vitro mammalian translation system (1). The addition of tunicamycin to Rat-6 cells did not alter the electrophoretic mobility of SSeCKS as determined by [35S]methionine/cysteine labeling or Western blotting (data not shown), indicating that SSeCKS is not significantly glycosylated in vivo.

SSeCKS as a PKC Substrate—Activation of PKC by the short-term addition of nanomolar concentrations of phorbol esters is known to result in the rapid phosphorylation of PKC substrates such as MARCKS (28). Fig. 3 (top) indicates that the relative phosphorylation level of the 280/290-kDa SSeCKS species in vivo rapidly increases 5–6-fold in response to PMA, and

4 M. Resh (Sloan Kettering Institute), personal communication.
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Fig. 3. In vivo phosphorylation of SSeCKS by PKC. Confluent Rat-6 cells grown overnight in Dulbecco’s modified Eagle’s medium lacking calf serum were starved of phosphate for 2 h and then labeled for 4 h with $^{32}$P orthophosphate. At the end of the labeling period, some cells were treated with 200 nM PMA and the PKC-specific inhibitor, bis-indolylmaleimide. SSeCKS protein was immunoprecipitated from equal aliquots (400 μg) of lysates from untreated or treated cells, and Western blotted onto a polyvinylidene difluoride membrane. The lower panel represents immunoblotting using rabbit anti-SSeCKS serum (showing equal amounts of SSeCKS protein loaded) whereas the upper panel represents autoradiography of the blotted protein (showing an increase in $^{32}$P labeling of SSeCKS following PMA treatment). The 280/290-kDa doublet (unresolved in this gel) is indicated by an arrow, and the minor 240-kDa form of SSeCKS can be detected in the upper panel. A better resolution of these SSeCKS species is shown in Fig. 11.

Fig. 4. In vitro phosphorylation of SSeCKS by PKC. GST and GST/322 fusion protein (see Fig. 2) were expressed and purified from bacteria as described under “Experimental Procedures” (panel A). Coomassie-stained SDS-polyacrylamide gel. Five μg of the GST samples were added to PKC assays containing [γ-$^{32}$P]ATP (“Experimental Procedures”) in the presence or absence of the PKC peptide inhibitor (19–36). The products were then bound to glutathione-Sepharose beads, precipitated, and washed, and analyzed by SDS-PAGE and autoradiography (panel B). Protein size markers are indicated on the appropriate sides. Radioactive labeling was detected in GST-322 (160 kDa and breakdown products) only.

We then determined that purified rabbit brain PKC containing α, β, and γ isoforms could phosphorylate GST-322 protein in vitro (Fig. 4, panel B). This phosphorylation is inhibited by the addition of excess PKC pseudosubstrate peptide (a.a. 19–36) indicating that PKC and not a contaminating kinase is responsible for the phosphorylation. The PKC-specific phosphorylation of GST-322 paralleled that of myelin basic protein in its dependence on PS (Fig. 5) and Ca$^{2+}$ (data not shown). PI could supplant PS in this assay although the relative level of GST-322 phosphorylation was roughly 2-fold less than that of myelin basic protein using PI (Fig. 5). As previously reported using myelin basic protein, PC did not stimulate PKC activity of GST-322 (29). Although several C-terminal breakdown products smaller than 70 kDa are present in the preparation of GST-322, the 70 kDa are phospho-

Fig. 5. Phospholipid preference for the in vitro phosphorylation of SSeCKS by PKC. Myelin basic protein (panel A), GST-322 and GST proteins (panel B) were phosphorylated in vitro as in Fig. 4, in the presence or absence of various lipids including PS, phosphatidylcholine (PC), or phosphatidylinositol (PI). In some cases, excess PKC peptide inhibitor (19–36) was added as in Fig. 4. The extent of labeling in the peptide substrates was determined by spotting the reaction products on phosphocellulose discs (Whatman), precipitating peptides with washes of 0% trichloroacetic acid, followed by scintillation counting.

SSeCKS Binding to PKC—The ability of PKC to phosphorylate GST-322 indicates some level of interaction between these proteins, yet the conditions required for binding are unclear. Thus, we characterized the ability of PKC to bind GST-1322 in vitro. Our results (Fig. 6) indicate that SSeCKS binds both purified PKC and PKC in Rat-6 lysates in a PS-dependent manner. Thus, SSeCKS and PKC most likely interact via a PS bridge, although it cannot be ruled out that there is a lower affinity protein-protein interaction with domains in SSeCKS not encoded by GST-1322. Pre-phosphorylation of GST-1322 by
PKC decreases this binding at least 10-fold (data not shown), suggesting that phosphorylated SSeCKS has decreased binding affinity for PS.

Identification of in Vivo PKC Phosphorylation Sites on SSeCKS—The consensus motifs for PKC phosphorylation have been identified as (S/T)(X/K/R) or (K/R)(X/S/T), with a greater preference for serine over threonine (31). However, our observations of previously characterized in vivo PKC phosphorylation sites indicate that they typically contain a high concentration of basic residues and at least 2 or 3 of the overlapping phosphorylation motifs described above. Analysis of the SSeCKS sequence yielded 4 such putative phosphorylation sites shown in Table I. These sites share some linear sequence homology and predicted secondary structural similarity with the PKC phosphorylation site in MARCKS. A minimal MARCKS 23-peptide containing this site (32) also binds calmodulin and F-actin (Table I).

In order to determine whether the SSeCKS sequences described in Table I could be phosphorylated by PKC in vitro, polymerase chain reaction products containing these individual sites or several sites in tandem were generated, fused in-frame to GST-expressing vectors (Fig. 2), and checked by sequencing. Fig. 7 indicates that sites 1–4 could be phosphorylated efficiently by purified rabbit brain PKC and that this phosphorylation was blocked by excess pseudosubstrate peptide inhibitor. It is yet unclear whether these sites are utilized during in vivo phosphorylation.

SSeCKS Is Resistant to Heat Denaturation—Besides having predicted rod-like structures, many PKC substrates share peculiar characteristics such as resistance to heat denaturation (31). Fig. 8 shows that the 280/290-kDa form of SSeCKS remained soluble after 5 min of boiling in RIPA buffer containing 0.05% SDS. Additionally, boiled SSeCKS retained roughly 50% of its immunoreactivity with rabbit immune serum. These data strengthen the notion that SSeCKS assumes a rod-like structure in vivo. In contrast, GST fusions of SSeCKS are heat-labile PKC substrates, a characteristic most likely conferred by the GST moiety (not shown).

SSeCKS in src- and ras-transformed Cells—The SSeCKS encoding gene, 322, was originally isolated based on its being transcriptionally suppressed in src-transformed NIH3T3 cells (16). We showed subsequently that 322 is down-regulated at least 10-fold at the steady-state RNA level in src- and ras-transformed Rat-6 fibroblasts but not in cells transformed by activated raf-1 (1). Fig. 9 shows that the relative level of SSeCKS in src- and ras-transformed Rat-6 fibroblasts is 15- and 8-fold lower, respectively, than in untransformed cells. An additional 305-kDa SSeCKS form found in the ras- and src-transformed cells might represent a modified form of the 280/290-kDa SSeCKS doublet. These data suggest that the relative abundance of SSeCKS in transformed cells is controlled at the transcriptional level.

Cell Localization of SSeCKS—We determined where SSeCKS is found in subconfluent and confluent Rat-6 cells. Immunofluorescence analysis using immunofluorinity-purified α-SSeCKS antibody indicates that SSeCKS localizes to the cytoplasm but is enriched at the cell edge, in structures resembling podosomes, and in the perinucleus (Fig. 10). An apparent intranuclear staining of SSeCKS (Fig. 10J) most likely represents deposits of SSeCKS in or on the nuclear cage based on confocal microscopy (data not shown). The association of SSeCKS with cortical actin-like structures (Fig. 10, A, C, and E) and cellular components such as podosomes (Fig. 10I) further suggests a role for SSeCKS in the control of actin-based cytoskeletal architecture.

Previous data indicated that short-term treatment (≤10 min) of quiescent fibroblasts with PMA led to a rapid detachment of MARCKS from plasma membrane sites into a soluble cytoplasmic compartment, followed by its re-association with membrane structures and progressive movement toward the perinucleus (30). This effect was coincident with a ruffling of rat actin fibers at the plasma membrane. We determined the effect of PMA on SSeCKS localization. Fig. 10 (A–F) shows that after 10 min PMA treatment, membrane ruffling of actin was apparent in rat fibroblasts, but SSeCKS was still uniformly associated with cortical actin-like structures throughout the cytoplasm. With longer PMA treatment (60 min), SSeCKS localized predominantly to the perinucleus. This delayed movement of SSeCKS toward the perinucleus, when compared with MARCKS, suggests that it is a consequence of exocytosis, which is known to be induced by short-term PMA treatment.

Table I

| Sequence | CaM binding | Actin binding | PKC phosphorylation |
|----------|-------------|---------------|---------------------|
| MARCKS (bovine/chicken) | 28435 | + | + |
| MARCKS (mouse) | KRFPSKKSFKLGSFSPKKSKEA | + | + |
| MacMARCKS/F52 | KRFSSKSFKLGSFSPK | + | + |
| Myosin light chain kinasea | KRSKKKAPAWSARFVKK | ? | ? |
| SSeCKS-1 (rat) | ETTSKAFYTVSFSK | ? | ? |
| SSeCKS-2 (rat) | KFFSSQGLKSLGKKGGG | ? | ? |
| SSeCKS-3 (rat) | EGIPEWFSK | ? | ? |
| SSeCKS-4 (rat) | EGWSTWEEKRLVTPKK | ? | ? |

a Ref. 42
purification of the GST-SSeCKS fusion proteins. Five-
(1–4) in SSeCKS, were subjected to an
individual or combinations of the predicted PKC phosphorylation sites

...press only 5–10-fold SSeCKS over background whereas control
cells routinely express 100-fold luciferase reporter over back-
ground, suggesting that there is selective pressure against expressing high levels of SSeCKS.

All three SSeCKS forms (240 and the 280/290 kDa doublet) contain multiple potential serine or threonine phosphorylation sites, yet only the doublet species is phosphorylated significantly in vivo. Thus, it is unlikely that the 240-kDa SSeCKS species is a breakdown product or proteolytically-matured form of the protein. As our data indicate that PKC can phosphorylate SSeCKS in vitro and in vivo, it is possible that the 240-kDa form may localize to cellular compartments unavailable to PKCs or other serine/threonine kinases. Indeed, this species is relatively enriched in soluble components (S100) of rat fibroblasts. Our ability to show a relationship between the three SSeCKS protein species using trypic maps has been confounded by a difficulty in metabolically labeling these proteins in vivo.

SSeCKS has a predicted rod-like structure, with a set of turns in the molecule’s center flanked by N- and C-terminal tubular domains (Fig. 2). This conformation, as well as the

...bacterially sated from uninduced (lane a) or induced (lane b) bacteria, or 5 μg of GST-SSeCKS fusion protein eluted from glutathione-Sepharose columns (lane c), were analyzed by SDS-PAGE, and then stained with Coomassie Blue. Arrows indicate the size of the unfragmented protein product.

...fivefold luciferase reporter over background whereas control cells routinely express >100-fold luciferase reporter over background...
concentration of acidic residues in the N-terminal third of the protein, are most likely responsible for the retarded mobility of SSeCKS in SDS-PAGE. Indeed, bacterially-expressed GST- or His-tag fusions of SSeCKS show similarly retarded mobilities as bacterially-expressed GST- or His-tag fusions of SSeCKS show similarly retarded mobilities in SDS-PAGE. Indeed, bacterially-expressed GST- or His-tag fusions of SSeCKS show similarly retarded mobilities as bacterially-expressed GST- or His-tag fusions of SSeCKS show similarly retarded mobilities.

SSeCKS can be phosphorylated by PKC in vivo in response to short-term treatment by phorbol esters (≥2 min). This phosphorylation is abrogated in the presence of the PKC-specific inhibitor, bis-indolylmaleimide. It is unclear which isoform of PKC phosphorylates SSeCKS in vivo. The majority of PKC in Rat-6 cells is ε and δ, although α is fairly abundant. Additionally, the specificity of the PKC inhibitor we use (bis-indolylmaleimide) has been established for the so-called Group A PKC isoforms (α, β, βII, and γ) but has not been tested rigorously for other isoform groups. The ability of the PKC fraction used for our in vitro assays (enriched for α, β, and γ) to phosphorylate specific SSeCKS polypeptides is likewise not direct proof that these isoforms are responsible for the in vivo phosphorylation activity.

The SSeCKS coding sequence contains 4 domains of overlapping PKC phosphorylation motifs (ST/X/K/R) or (K/R)XX/S/T representing potential phosphorylation sites. Each of these sites (SSeCKS 1–4) can be phosphorylated in vitro by purified rabbit brain PKC in a PS- and Ca2+-dependent manner. The in vitro phosphorylation of SSeCKS could also be supported by PI but not by PC, confirming previous data on the phospholipid cofactor requirements of PKCa (29). Moreover, the binding of SSeCKS to PKCa in vitro is PS-dependent which agrees with the PS-dependent binding of PKC by the >200-kDa protein (24). We are currently determining whether SSeCKS 1–4 are phosphorylated in vivo by activated PKC, and whether the phosphorylation of one or more dominates over others.

3. Results

The first two PKC phosphorylation sites in SSeCKS (SSeCKS-1 and -2, Table I) contain significant similarities with a 23-mer MARCKS peptide encoding a minimal PKC phosphorylation site as well as binding ability to calmodulin and F-actin (32). These SSeCKS sites also are enriched for basic residues, as has been previously reported for other PKC sites (31). In contrast to SSeCKS-1 and -2, whose sequences are not similar to the putative serine-phosphorylation sites in SSeCKS 1–4 (Table I), with the requirement that potential phosphoserine residues be retained. No significant similarities to SSeCKS-1 were found. However, the SSeCKS-2 putative PKC site showed 50% identity to a sequence in the retinoic acid receptor-α (SWISSPROT: Rra1_Mouse) and the SSeCKS-3/4 consensus peptide showed 46.2% identity to the protein kinase A anchor protein, AKAP-79 (SWISSPROT: AK79_Human). It is unknown whether these other proteins are phosphorylated by PKC at these sites. However, these similarities to SSeCKS strengthen the notion of a function for SSeCKS at the plasma membrane.

We searched the SWISSPROT databank for similarities to the putative serine-phosphorylation sites in SSeCKS 1–4 (Table I), with the requirement that potential phosphoserine residues be retained. No significant similarities to SSeCKS-1 were found. However, the SSeCKS-2 putative PKC site showed 50% identity to a sequence in the retinoic acid receptor-α (SWISSPROT: Rra1_Mouse) and the SSeCKS-3/4 consensus peptide showed 46.2% identity to the protein kinase A anchor protein, AKAP-79 (SWISSPROT: AK79_Human). It is unknown whether these other proteins are phosphorylated by PKC at these sites. However, these similarities to SSeCKS strengthen the notion of a function for SSeCKS at the plasma membrane.

Analysis of the in vitro SSeCKS phosphorylation sites using the PAM250 and Dhalve program (94) predicts amphipathic helical structures for SSeCKS-1, -2, and -4 but less so for SSeCKS-3. It is difficult to predict whether there is any interplay between these phosphorylation sites as they are separated by between 60 and 100 residues on a proposed rod-shaped
Novel PKC Substrate, SSeCKS

molecule. In the case of MARCKS, McLaughlin and Aderem (35) postulate that MARCKS probably associates with plasma membranes via its N-terminal myristyl group and its concentration of positively charged amino acid residues in the PKC phosphorylation site. PKC phosphorylates three serines in this site that align along one axis of a short amphipathic helix. They further postulate that the resulting confluence of electrostatic phosphoserine charges causes MARCKS to detach from plasma membranes. Indeed, SSeCKS is enriched at the cell edge and in podosomes (Fig. 10), as was previously demonstrated for MARCKS (28). However, following the activation of PKC, SSeCKS did not detach appreciably from membrane sites or from subcellular fractions enriched for plasma membranes (Fig. 10, A–F, and Fig. 11). This suggests that the phosphoserine charges in SSeCKS are insufficient to counteract its affinity for membranes. It cannot be ruled out that only a minor component of SSeCKS is membrane-associated in the cell and that phosphorylation by PKC induces this component to move to a soluble cytoplasmic compartment.

Although SSeCKS and MARCKS share little sequence similarity past their PKC phosphorylation sites, they share several biochemical and structural characteristics common to other PKC substrates implicated in the regulation of cytoskeletal architecture such as Igloo, GAP-43, and neurogranin. These include: (i) a predicted elongated or rod structure, (ii) enrichment for alanine, serine, lysine, and glutamic acid residues, (iii) binding to plasma membranes (GAP-43, for example, is palmitoylated), (iv) association with focal contact sites or cellular processes, (v) predicted or proven phospholipid binding activity, and (vi) predicted or proven calmodulin and F-actin binding domains (29, 36, 37). Additionally, the over-expression of SSeCKS or MARCKS is growth inhibitory (Ref. 1; this study). This correlates with the increase in SSeCKS and MARCKS expression as cells enter G0 (1, 38). These data suggest that SSeCKS and MARCKS share some overlapping functions and regulatory motifs. However, unlike MARCKS, which is expressed throughout mammalian tissues, and SSeCKS, which is primarily expressed in the brain, genitouriary tract, intestines, and kidney (1), GAP-43, Igloo, and neurogranin are brain-specific (29, 36, 37). Additionally, GAP-43, Igloo, and neurogranin, but not MARCKS and SSeCKS, encode PKC phosphorylation sites with the so-called “IQ” motif, KIQSFRGHR (39).

SSeCKS localizes to focal contact sites (Fig. 10, H and I) known to be enriched for PKCα, p125Fak, and actin-binding proteins. These structures mediate the interaction of cytoplasmic actin fibers with extracellular matrices via integrins (12, 15). Indeed, SSeCKS seems to associate with a cortical actin-like cytoskeletal matrix in confluent Rat-6 cultures (Fig. 10, C, and E). Our preliminary data show binding of SSeCKS to F-actin in vitro. Thus, SSeCKS may be involved in the regulation of actin-based cytoskeletal architecture.

Recent data indicate that actin fiber formation is controlled by rac- and rho-mediated pathways distinct from the rafl MAP kinase-mediated pathways controlling proliferation (40). SSeCKS transcription is suppressed in src- and ras- but not raf-transformed cells (1). Thus, the raf-independent control of SSeCKS expression parallels the rac- and rho-dependent control of actin-based cytoskeletal architecture. Since actin-based structures regulate cell morphology, motility, metastasis, and cell-to-cell interactions, we are interested in elucidating the role of SSeCKS in controlling these processes in both transformed and transformed cells.

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