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THE ROLE OF SSeCKS/Gravin/AKAP12 SCAFFOLDING PROTEINS IN THE SPACIOTEMPORAL CONTROL OF SIGNALING PATHWAYS IN ONCOGENESIS AND DEVELOPMENT

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1. ABSTRACT

Scaffolding proteins are thought to facilitate the efficiency and specificity of enzyme/substrate reactions by coordinating their interaction along a cytoskeletal infrastructure in a spacial and temporal manner. Rodent SSeCKS, and its human orthologue, Gravin, seem to function as tumor suppressors and regulators of mitogenesis, inflammatory response, development and differentiation via novel scaffolding functions involving the selective binding of key G1→S phase signaling proteins such as protein kinase C (PKC), PKA, calmodulin, cyclins and ß-adrenergic receptors. The association of SSeCKS/Gravin with the actin-based cytoskeleton as well as to plasma membrane sites via N-terminal myristylation places these proteins at the junction of signaling and cytoskeletal pathways. The following review describes the regulatory and scaffolding functions of SSeCKS and Gravin and how mitogen-induced phosphorylation modulates their ability to regulate cell adhesion, signaling, mitogenesis and oncogenesis.

2. INTRODUCTION

Growth factors and hormones mediate diverse signals in many different cell types, yet they employ a similar set of secondary messengers such as Ca^{2+}, phospholipid, and cAMP. Most signals are mediated through the activation of protein kinases and/or phosphatases which modulate the phosphorylation state of target proteins. Protein phosphorylation also facilitates the recruitment of other signaling molecules via adapter domains such as SH2 or 14-3-3 that bind phosphotyrosine- and phosphoserine-containing peptides, respectively (57,71).

However, it is unclear how specific signal mediators activate the correct set of kinases and phosphatases both temporally and spatially in order to induce the appropriate intracellular cascades. In other words, how can different cells use the same second messengers to perform such diverse functions as proliferation, differentiation, and apoptosis? Recently, it has been suggested in both yeast and mammalian systems that the activity of signaling enzymes can be modulated by translocation from inactive to active sites in the cell (27,80). This compartmentalization is facilitated by so-called scaffolding proteins that sequester signaling proteins within the cell, and in some cases directly control their enzymatic activity during the cell cycle. In the review below, I will discuss the discovery of SSeCKS/Gravin proteins and the evidence suggesting how their scaffolding activities might control aspects of mitogenic and cytoskeletal signaling.

3. SSeCKS AS A TUMOR SUPPRESSOR

Several lines of evidence, described in detail below, strongly suggest that SSeCKS and Gravin are tumor

suppressors: i) expression of SSeCKS/Gravin is downregulated following oncogenic transformation by several oncogenes such as Src and Ras, ii) SSeCKS/Gravin RNA and protein levels are severely suppressed in many tumor types including prostate, ovarian and breast, iii) the gene encoding the human SSeCKS orthologue, Gravin, maps to a deletion hotspot in advanced prostate, ovarian and breast cancer, and iv) re-expression of SSeCKS or Gravin in cancer cells suppresses various parameters of oncogenic growth including anchorage-independence and metastasis.

SSeCKS was originally identified in a screen for genes severely downregulated by v-Src (28), but subsequently, we and others showed that it is also downregulated 4- to 20-fold by oncogenic forms of Ras, Myc, and Jun (63,15) and in SV40–transformed fibroblasts (11). Interestingly, transformation of Rat-6 fibroblasts by oncogenic forms of Raf, Mos or Neu does not lead to SSeCKS downregulation, indicating that the loss of SSeCKS expression is not a generic effect of transformation and suggesting that SSeCKS downregulation is controlled by specific signaling pathways. Fluorescence *in situ* hybridization (FISH) analysis using either full-length rat SSeCKS or human Gravin cDNAs identified only one signal (108), that being the map coordinates of the human Gravin gene. This site, 6q24-25.2, has been confirmed recently by the Sanger Sequencing Centre, UK, and by my lab using BAC clones and satellite polymorphism probes, and most important, analysis of the sequence data indicates no gene reiteration at this locus. This region is a deletion hotspot in advanced human prostate, breast and ovarian cancer suggesting that it encodes one or more tumor suppressor genes (49,79,110,20,4,95,103,21,19,104).

With the advent of high output gene expression screening analyses, there are an increasing number of oncogenic growth conditions correlating with downregulated expression of SSeCKS and Gravin. Multiple studies demonstrate 3- to 10-fold downregulation of SSeCKS or Gravin expression in various cancers including breast, prostate, lung, ovarian, and gliomas (1,81,101,24,106,86,30). Similarly, the loss of STAT3ß, whose activity is typically elevated in many types of cancer- especially those involving activation of Src (5,102,31,32), correlates with a 3.3-fold increase in SSeCKS RNA levels (109).

Another hint of SSeCKS function in oncogenesis comes from a study by Tchernitsa *et al.* (100) that shows downregulation of SSeCKS in immortalized cell lines permissive for H-*ras*-induced transformation compared to REF52 diploid embryo fibroblasts, which are refractile to transformation. Thus, they conclude that downregulation is a marker for susceptibility toward oncogenic transformation. This correlates with data from Kannan *et al.* (51) showing a 3.5-fold relative increase in SSeCKS expression in cells lacking p53 and thus, marked for immortalization. Finally, Coller *et al.* (16) demonstrate a roughly 4-fold decrease in Gravin RNA levels following the inducible expression of c-Myc. Downregulation of SSeCKS transcription is not a bystander effect in *src*- and *ras*-transformed cells, but rather contributes to the transformed state. For example, whereas SSeCKS transcription is downregulated in *ras*-transformed Rat-6 cells, the message levels in non-tumorigenic *ras*-revertants are similar to those in untransformed Rat-6 cells (60). However, in several retransformant clones derived from the flat revertants, SSeCKS downregulated correlated with anchorage-independent growth but not with morphological transformation.

SSeCKS' function as a potential tumor suppressor seems to be based on its ability to reorganize actin-based cytoskeletal architecture and control G1 phase signaling proteins via scaffolding activity. Using tetracycline-regulated SSeCKS expression in fibroblasts containing a temperature-sensitive v-*src* allele, it was demonstrated that SSeCKS re-expression could inhibit *src*-induced anchorage- and growth factor-independence and invasiveness through Matrigel, all without affecting the tyrosine kinase activity of v-Src or the level of total phosphotyrosine-containing proteins (60). Most important, re-expression of SSeCKS caused both stress fibers and vinculin-associated adhesion plaques to reorganize to similar structures found in control, untransformed cells. Thus, control of cytoskeletal pathways by SSeCKS is dominant over the oncogenic and/or proliferative pathways induced by v-*src*. Curiously, ERK2 was superinduced 10-fold over levels induced by v-*src* alone in the SSeCKS expressor cells. This indicates that activated ERK2 alone is insufficient to induce oncogenic transformation. In contrast, SSeCKS inhibits ERK2 activation in untransformed cells (62) Because SSeCKS causes growth arrest in untransformed cells but not when expressed with active *src*, it is likely that the superinduced ERK2 activity may be responsible for the proliferation of the SSeCKS/ts-v-*src* cells.

In agreement with the finding that Gravin maps to a deletion hotspot in advanced prostate and breast cancer, Gravin protein and RNA levels are severely decreased in 18 typically studied human prostate (108) and breast (I.H. Gelman, A. Bulua, A. Wang, unpublished data) cancer cell lines relative to normal epithelial cells. This indicates that loss of SSeCKS/Gravin, whether by chromosomal lesion or transcriptional downregulation, is typical in these cancer lines. A preliminary *in situ* analysis indicates that Gravin expression is deficient in 8/8 malignant prostate cancer lesions with Gleason scores >6, compared to normal prostatic epithelia (108).

The tetracycline-regulated re-expression of SSeCKS in MatLyLu (MLL), a metastatic rat prostate cancer cell line which expresses an activated H-*ras* allele (48,18), suppressed colony formation in soft agar, decreased refractility (induced cell flattening), and increased cell-cell interactions (108). Although SSeCKS/MLL cells induce primary tumors in nude mice that grow slower than controls, these tumors eventually lose their tet-regulated SSeCKS expression, indicating that sustained SSeCKS expression is antagonistic to *in vivo* tumor growth. Most interestingly, SSeCKS/MLL cells

induced 90% fewer lung metastases compared to controls. Taken with the data above on *src*-transformed fibroblasts, these findings indicate that SSeCKS can act as a tumor suppressor in various cell types.

4. SSeCKS AS A MITOGENIC REGULATOR

The SSeCKS coding gene was isolated first in a screen to identify genes that encode potential negative regulators of G1→S progression (28). SSeCKS transcript levels are severely reduced in *src*-, *ras*- and *jun*-transformed cells and following the addition of serum, and moderately reduced in *myc*-transformed cells (63,15). The finding that SSeCKS transcript levels are unaffected by *mos*-, *neu*- or *raf*-induced transformation (63) strongly suggests that SSeCKS expression is controlled by specific mitogenic and oncogenic signaling pathways.

Early attempts to constitutively express SSeCKS in fibroblasts or epithelial cells led to growth inhibition or deletion of the transduced SSeCKS cDNA (63,33). Using a tetracycline-controlled expression system, we demonstrated directly that SSeCKS promoted G1 phase arrest in untransformed fibroblasts (33,62) and prostate epithelial cells (108). Even marginal SSeCKS overexpression (2- to 4-fold over background) decreased cell proliferation and induced cell flattening and cytoskeletal reorganization (33,62). These characteristics will be described in more detail below.

The mechanism of SSeCKS-induced growth arrest in NIH3T3 cells was investigated recently (62). Among the cyclins, cyclin-dependent kinases and cyclin-kinase inhibitors involved in G1→S progression, only cyclin D RNA and protein were deficient in SSeCKS expressing cells. Loss of cyclin D expression correlated with an inhibition of serum-inducible ERK-2 activity by SSeCKS. Interestingly, the constitutive overexpression of cyclin D1 failed to rescue the SSeCKS-induced G1 arrest due to its sequestration in the cytoplasm. SSeCKS was shown to bind to cyclin D via two cyclin-binding domains (CY or RxL; ref. 13) in SSeCKS that overlap major PKC phosphorylation sites ($Ser^{507/515}$; below). Activation of PKC with phorbol esters or incubation with a cell-permeable peptide encoding the SSeCKS-CY domain induced cyclin D translocation to the nucleus, correlating with one extra round of cell replication. However, peptides encoding the CY domain containing phospho-$Ser^{507/515}$ failed to induced translocation of cyclin D, suggesting that SSeCKS scaffolding activity for cyclin D is antagonized after it is phosphorylated by PKC. SSeCKS also displays anti-apoptotic activity. Unlike control fibroblasts, SSeCKS-growth arrested cells do not undergo apoptosis when kept in suspension cultures for extended periods (anoikis), either in the absence or presence of 10% fetal calf serum (33). A similar effect was noted with tetracycline-regulated SSeCKS expression in MatLyLu rat prostate cancer cells (expressing activated *ras*): induction of ectopic SSeCKS expression prevented cell death following serum and integrin depravation (108). Although the exact mechanism controlling this effect is unclear, SSeCKS induces integrin-independent tyrosine phosphorylation of FAK in suspension cultures, a signal that is absent in the control cells undergoing (33). As activated FAK is known to activate PI_3 kinase-mediated anti-apoptotic pathways and cytoskeletal reorganization pathways (reviewed in ref. 90), these findings suggests a role for SSeCKS in FAK-mediated pathways.

5. CONTROL OF CYTOSKELETAL ARCHITECTURE BY SSeCKS

In actively dividing cells, SSeCKS associates with a cortical cytoskeleton and is enriched in podosomes, membrane ruffles and in lamellipodia (64,33). Overexpression of SSeCKS in untransformed NIH3T3 results in G1 growth arrest marked by severe cell flattening, elaboration of an SSeCKS-associated cytoskeleton, a transient loss of actin stress fibers and vinculin-associated adhesion plaques, and the production of filopodia- and lamellipodia-like projections (33,62). The loss of stress fibers is simultaneous with the onset of ectopic SSeCKS expression suggesting that SSeCKS directly effects the formation or maintenance of these fibers. After 2-3 days of SSeCKS overexpression, the stress fibers return, thinner and in more abundance. One interpretation is that the elaboration of an SSeCKS-based cytoskeleton forces the depolymerization of existing F-actin fibers but not the intrinsic mechanism for actin polymerization. Interestingly, SSeCKS-induced cell flattening is prevented by cytochalasin D or nocodazole, yet pre-flattened cells resulting from SSeCKS overexpression can be collapsed by cytochalasin D but not nocodazole (33), indicating that both microfilaments and microtubules are involved with SSeCKS-induced flattening whereas only microfilaments are involved in the maintenance of the SSeCKS-elaborated cytoskeleton.

When plated on fibronectin, mouse embryo fibroblasts show early coincident staining of SSeCKS with vinculin in developing focal contacts, followed by a diffusion of SSeCKS into larger podosome structures. Ablation of SSeCKS expression in glomerular mesangial cells (using retrovirus-transduced anti-sense cDNA) leads to thickening and polarization of F-actin fibers, an increase in the number of focal complexes per cell, and an increase of phosphotyrosine staining in focal complexes (75), all cytoskeletal hallmarks of early mitogenic activation. Ablation of SSeCKS converts the stellate morphology of human mesangial cells and rodent fibroblasts to a spindle morphology. Taken together, these data suggest that SSeCKS may play a role in the dynamic reorganization of the actin cytoskeleton during processes such as cell migration, process formation or cell division. Another finding- that SSeCKS rapidly enriches in the leading edges of fibroblasts moving into a monolayer wound- further strengthens this notion.

6. SSeCKS AND GRAVIN: ORTHOLOGUES

SSeCKS is closely related to human Gravin, an autoantigen in some cases of myasthenia gravis (35). Anti-Gravin sera recognizes a 250kDa isoform in human erythroleukemia cells and a 305kDa isoform in adherent

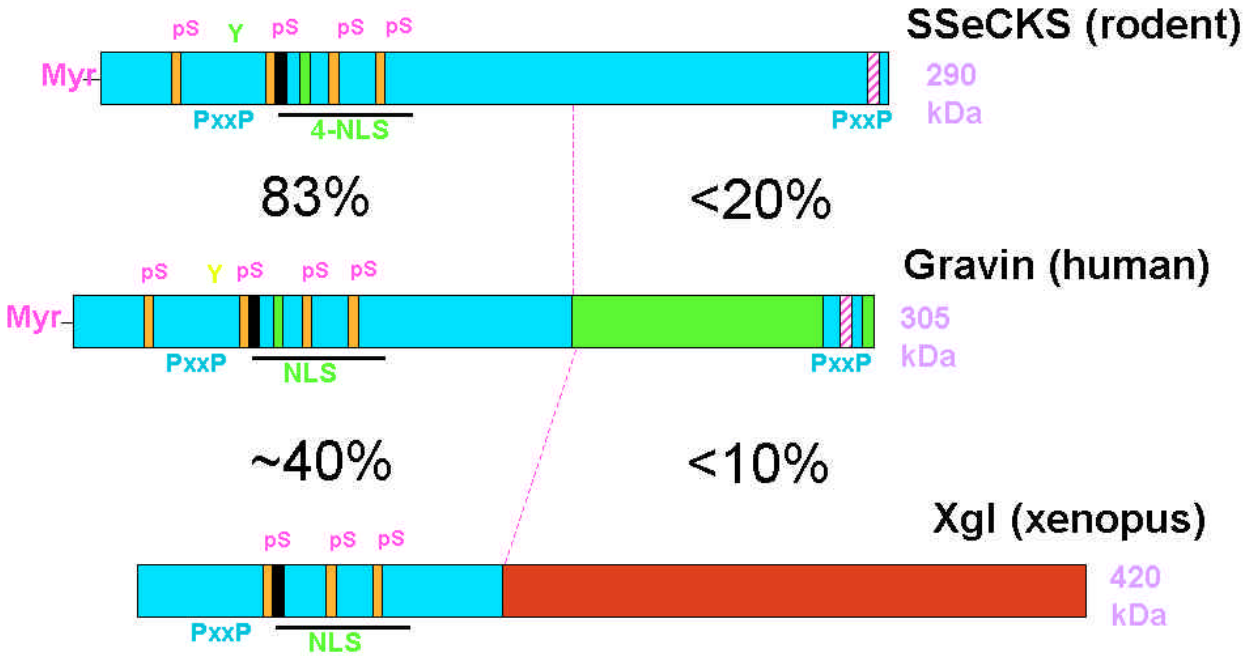

Figure 1A. Human, rodent and Xenopus orthologues of SSeCKS/Gravin/AKAP12. The myristylated isoform of rodent SSeCKS (see Figure2 for explanation of isoforms) contains several scaffolding domains and potential phosphorylation sites shared by human Gravin (middle) and xenopus Xenopus gravin-like (Xgl) (see Figure4 for domain structures). The percent of amino acid identity to rat SSeCKS is shown between the orthologues.

osteosarcoma cells. It is unclear what clinical effect Gravin autoantibodies manifest because although autoantibody to Gravin is expressed at higher levels in younger and nonthymomatous myasthenia gravis patients (87), it is also found in rare occasions in the serum of healthy patients (17). However, that some autoantibodies target the protein kinase A (PKA) anchoring domain (AKAP; described below) on Gravin (87) suggests that they might affect PKA signaling.

The cytoskeletal staining pattern of Gravin, its expression in cells with actin-based projections, and its absence in non-adherent cells led to the suggestion that it functions in cell adhesion or motility (35,38), although direct evidence for these functions is lacking. The original 1.8Kb terminal 3' Gravin cDNA sequence deposited in GenBank (accession #M96322) shares a high degree of similarity with the SSeCKS cDNA 3'-UTR, but there were only two similar 15 a.a. domains at the C-terminal coding region (Figure 1), leading to the original speculation that SSeCKS and Gravin proteins were not related. The enigma of the SSeCKS/Gravin relationship remained until Nauert *et al.*, in an overlay screen for AKAPs, identified Gravin as the previously described AKAP250 (72). Their full-length Gravin coding sequence (see UniGene Hs. 788) showed a high degree of identity between SSeCKS and Gravin in the first ~1000 a.a. (83%), followed by ~500 a.a. of relatively dissimilar sequence, and finally, short C-terminal homologies, one of which encodes the PKA binding site (Figs. 1 and 3). This contrasts with the recently published mouse SSeCKS sequence which has >90% similarity to rat SSeCKS over the whole coding sequence (GenBank accession #AB020886). Based on the PKA-binding activity of SSeCKS and Gravin (below), these genes have been re-designated "AKAP12" (UniGene). Mounting evidence indicates that SSeCKS and Gravin are orthologues. First, sera raised to the unique C-terminal domain of either rat SSeCKS or human Gravin fail to recognize any proteins from the other species (34). Second, Southern blotting with probes from these unique domains fails to hybridize stringently to DNA from the other species. As described above, FISH analysis of human chromosomes using either the human Gravin or rat SSeCKS cDNA identified the same region, 6q24-25.2, a known deletion hotspot in human prostate, ovarian and breast cancer.

Another confirmation that SSeCKS and Gravin are likely orthologues is based on the demonstration by Taketo *et al.* (98) that the "testes-specific gene," *Tsga12*, which is identical to mouse SSeCKS, maps to the centromeric end of chromosome 10, which is syntenic to human 6q24-27, the region to which Gravin maps (108).

7. SSeCKS/GRAVIN ORTHOLOGUES

It is somewhat surprising that GenBank lacks cDNAs from mammalian species other than rodents and humans that are orthologous to SSeCKS/Gravin. However, Klingbeil *et al.* (56) recently identified the so-called Xgravin-like (Xgl) gene that shares 39% identity with the N-terminal ~1000 a.a. of SSeCKS and Gravin yet lacking an obvious AKAP domain. Noteworthy is that Xgl is zygotically expressed in a dynamic fashion, with posterior mesoderm expression

Figure 1B. Human, rodent and Xenopus orthologues of SSeCKS/Gravin/AKAP12. Alignment of amino acid homologies between mouse SSeCKS, rat SSeCKS, human Gravin and xenopus Xgl. Conserved protein domains, such as a MARCKS-related domain, are identified beneath.

during gastrulation and a selective expression pattern during neurulation. Limited but significant cDNA homologies can be identified to short ESTs in Danio, C. elegans, and Oryzias, however whether these represent orthologues remains to be determined. In contrast, no significant homologies to Drosophila and yeast genomes exist, suggesting that the SSeCKS/Gravin orthologue family may be restricted to vertebrates.

8. TRANSCRIPTIONAL CONTROL OF SSeCKS

SSeCKS transcript levels dramatically increased in confluent cultures of untransformed fibroblasts, prostate epithelial cells, mesangial cells, and vascular smooth muscle cells irrespective of the effects of serum growth factors (63,108,75,14) SSeCKS transcript levels in subconfluent cultures are unaffected by either serum depravation or inhibition of DNA synthesis by either hydroxyurea or thymidine block (74). Thus, unlike the *sdr* gene, whose expression increases following serum starvation but not contact inhibition (40), or the GAS gene family, whose expression is induced by serum starvation or contact inhibition (42,70), SSeCKS belongs to a small group of genes including p27, PI3K, contactinhibin and neurofibromin (68,22,36,78) whose expression is induced by contact inhibition alone.

Independent of cell-cell contact, addition of serum factors to fibroblasts induces a transient 4- to 6-fold increase in SSeCKS transcription followed by a rapid decrease to suppressed levels (63,74). A similar biphasic control follows the activation of temperature-sensitive v-*src* in confluent cultures (63). Coats *et al.* (85) demonstrated a 6-fold induction of SSeCKS RNA levels by fetal calf serum in vascular smooth muscle cells, although they did not follow message levels after 3.5h.

In contrast, SSeCKS protein is long-lived (half-life >28h; (34)) although the coding sequence contains several PEST motifs linked to protein instability. Newly synthesized SSeCKS in confluent cultures is relatively underphosphorylated, whereas serum addition to subconfluent cultures results in a rapid serine and tyrosine phosphorylation concurrent with G1→S progression (74,107). S phase block (hydroxyurea) causes enrichment of phospho-SSeCKS whereas G2/M block (nocodazole) results in both protein decay and dephosphorylation (74). Release of G2/M block in confluent cultures causes a rapid increase in the levels of underphosphorylated SSeCKS protein. Several groups have shown that the underphosphorylated form has greatly increased *in vitro* binding affinity for phosphatidylserine (PS; ref. 72), calmodulin (CaM; ref. 61) and cyclin D (62) whereas prephosphorylation of SSeCKS by PKC decreases binding, presumably because of the proximity of the PKC phosphorylation sites to the binding sites. These scaffolding functions and how they relate to the role of SSeCKS and Gravin as negative mitogenic regulators, will be addressed below.

Interestingly, serum addition to confluent cultures fails to induce SSeCKS phosphorylation (74), suggesting that SSeCKS may play a role in contact inhibition by continually sequestering key signaling proteins. These data suggest that SSeCKS scaffolding activity may be highest in G0 or early G1, but that its phosphorylation by mitogen-induced kinases suppresses this activity during G1→S progression. Noting that i) SSeCKS and Gravin expression increase during contact inhibition, ii) loss of SSeCKS expression correlates with increased saturation density (75,62), and iii) expression of SSeCKS/Gravin is often lost in tumor cells, it is likely that SSeCKS and Gravin normally provide gating functions critical for the regulation of mitogen- and cytoskeletal signaling during contact inhibition.

SSeCKS and Gravin transcript levels are controlled by both changes in message stability and initiation rate. SSeCKS/Gravin RNA levels can be induced by phorbol ester (PMA) or cycloheximide (CY), however, a combination of PMA and CY leads to a superinduction (38,34), suggesting that both message stability and initiation mechanisms contribute to the steady-state control of message levels. Message instability, in part, may be facilitated by AU-rich motifs in both SSeCKS and Gravin 3'-UTRs that have been associated with decreased message half-life (93).

Several types of inflammatory or injury-inducing stimuli can induce SSeCKS/Gravin transcription in specific cell types. For example, angiotensin II induces SSeCKS transcription in vascular smooth muscle cells (14), lysophosphatidylcholine induces Gravin expression in vascular endothelial cells (88), and bacterial lipopolysaccharide induces SSeCKS expression in vascular endothelial cells in multiple tissues of treated mice (54). It may be that SSeCKS/Gravin fulfill common functions, such as cytoskeletal reorganization or signaling control, in all these cell types although it cannot be ruled out that these diverse stimuli induce SSeCKS expression through a common activated pathway. The notion that SSeCKS/Gravin expression correlates with cytoskeletal remodeling during differentiation is strengthened by the findings that retinoids induce SSeCKS expression in cardiac smooth muscle cells (12) and that thrombopoietin induces Gravin expression in megakaryoblasts (Isabelle Dusanter-Fourt, INSERM/France; personal communication). Indeed, our data with fibroblasts (33) and the data of Kitamura with endothelial cells (54) indicate that the ectopic expression of SSeCKS induces a transient loss of F-actin stress fibers, underlining a role for SSeCKS in the actin-based cytoskeletal architecture.

Coats *et al.* (14) identify a novel function for SSeCKS, namely that of a transactivator. Although angiotensin II (Ang II) is not thought to directly induce plasminogen activator inhibitor-1 (PAI-1) transcription, treatment of vascular smooth muscle cells with Ang II induced a similar increase in SSeCKS and mRNA levels (14). Interestingly, Coats *et al.* demonstrated that a fragment of SSeCKS (a.a. 626-1596) transactivated reporter expression from the PAI-1 promoter >10-fold, suggesting that SSeCKS is a downstream mediator of Ang II on PAI-1.

Figure 2. SSeCKS and Gravin protein isoforms. The major protein isoforms that are expressed in fibroblasts and epithelial cells for SSeCKS and Gravin are doublets of 290/280kDa and 305/287kDa, respectively. Minor isoforms, possibly representing proteolytic fragments of the major isoforms, are 240kDa (SSeCKS), 250kDa (Grain and 43kDa (SSeCKS and Gravin).

Typically, SSeCKS/Gravin expression is upregulated in microarray analyses that study paradigms of cell differentiation. For example, whereas induction of keratinocyte differentiation correlates with a 17-fold increase in Gravin expression, the expression of papillomavirus type 16 E6 and E7 oncoproteins in these cells correlates with a 5-fold decrease in Gravin levels (73). Likewise, SSeCKS expression rises 3-fold during osteoblast differentiation and maturation, correlating with decreased proliferation and increased cytoskeletal reorganization (83).

Selective control of SSeCKS/Gravin expression may contribute to subtle developmental differences in otherwise similar cell or tissue types. For example, although extraocular muscle is histologically similar to skeletal muscle from the jaw or legs, SSeCKS mRNA levels are 4- 6-fold higher in the former (82). Also, whereas SSeCKS expression is 5- to 12-fold higher in the developing embryonic rat kidney compared to the adult (96), renal ischemia-reperfusion in the adult mouse- a paradigm of acute renal failure marked by loss of epithelial cell polarity and cytoskeletal reorganization- correlates with a 14.5-fold increase in SSeCKS mRNA levels (111).

In agreement with data showing increased SSeCKS/Gravin expression following addition of inflammatory mediators (above), several studies demonstrate an increase in SSeCKS/Gravin expression using paradigms of heart failure or atherogenesis (reviewed in refs. 69,12). Many of these protocols are mediated by induction of TNF-a, and indeed, addition of TNF-a to human vascular endothelial cells induces a 4-fold increase in Gravin transcript levels (43). Lastly, whereas inflammatory cytokines such as IL-1ß + IFN-? induce SSeCKS expression in pancreatic insulin-producing ß-cells (7), treatment of lung airway epithelial cells with IL-13, which recapitulates the hyperresponsiveness and dedifferentiation in asthma, results in a 5-fold decrease in Gravin mRNA levels (58).

9. SSeCKS/GRAVIN PROTEIN PRODUCTS

Various cell and tissue types contain several SSeCKS isoforms and proteolytic products which may encode distinct functions. Most cells expressing SSeCKS/Gravin (fibroblasts, mesenchymal, smooth muscle, brain, some epithelial cells) contain either 280/290 (rodent) or 287/305kDa (human) major isoform doublets, and 240 (rodents) or 250kDa (human) minor isoforms (Figure 2), as detected by rabbit polyclonal sera raised against bacterially-expressed rat SSeCKS (64,34). Although not as yet formally proven, a stop codon in rodent SSeCKS ~100 a.a. upstream of the stop site in Gravin is most likely responsible for the roughly 10kDa difference between the human and rodent isoforms. Human and rodent cells also have a 43kDa proteolytically-cleaved product mapping to the N-terminal half of the larger

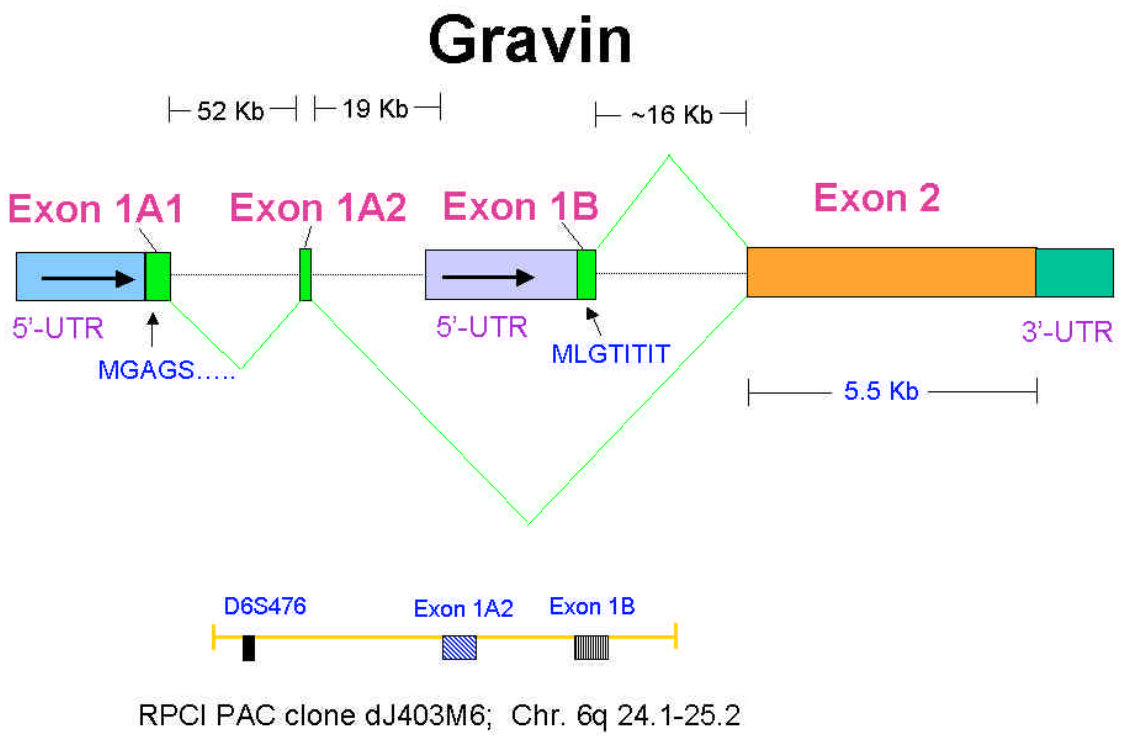

Figure 3. Promoter map for Gravin. The exon/intron map for the human Gravin gene on chromosome 6q24-25.2 is shown. Note the two independent promoters, each encoding an exon 1 (1A1/1A2 or 1B) with an ATG translational start site.

isoforms. In rat testes, for example, the 43kDa isoform is as abundant as the 280/290kDa forms, whereas in fibroblasts, the 43kDa isoform represents <10% of total SSeCKS protein (64,34).

Two sets of SSeCKS and Gravin protein products have been reported in Genbank which differ in their extreme N-terminal residues. One contains a 103 a.a. domain with a myristylation signal (MGAGS) at its N-terminus and the other is a 7 a.a. non-myristylated domain (MLGTITIT in SSeCKS, MLGTITVT in Gravin). Both are fused through alternative splicing to a common exon 2 sequence (Figure 3). No further introns are found in the remainder of the coding sequence. Each translational start site is flanked by its own 5'-UTR and promoter region. Moreover, the myristylated domain is derived from splicing of two exons, 1A1 (87Kb upstream of Exon 2) and 1A2 (35Kb upstream of exon 2). In contrast, the non-myristylated exon 1B domain is 16Kb upstream of exon 2. Polyclonal sera specific for the myristylated 103 a.a. domain (>70% similarity between human and rodent proteins) indicated that the larger SSeCKS and Gravin isoforms (290 and 305kDa, respectively) encode the myristylated products whereas the 280 and 287kDa isoforms encode the non-myristylated products (34). This correlated with our previous finding that only the 290kDa form is myristylated *in vivo* in rodent cells (64).

Isoforms of SSeCKS have been isolated from sperm and germ cell cDNA libraries that identify novel splicing variants. For example, Tsuchida and Nishimune (Osaka University, Japan) deposited an unpublished sequence for a mouse germ cell lineage protein, Gercelin, that is identical to SSeCKS except i) it starts at an internal ATG (a.a. 106 in SSeCKS), and ii) it contains 17 short deletions at the cDNA level suggestive of multiple, possibly testes-specific splicing events. The Gercelin transcript is identical to a testes-specific mRNA identified by Camus *et al.* (6) who knocked out expression of the upstream SSeCKS promoter (encoding the 290kDa myristylated isoform) using a gene trap vector insertion into mouse embryonic stem cells. The resulting mice, which were phenotypically normal, retained expression of the 280kDa isoform (J. Barra, personal communication) from the downstream promoter (exon 1B; see above), indicating that redundancy between the two major SSeCKS isoforms in regards to major organ and tissue development.

A continuing enigma is the presence of multiple $T_{antigen}$-like and bipartite nuclear localization signals (NLS) in SSeCKS and Gravin (see Figure 4). SSeCKS is found in the nucleus by either confocal microscopy (33) or cell fractionation, but this represents roughly 5% of the total cellular SSeCKS. Interestingly, the 43kDa isoform maps to the region containing most copies of the $T_{antigen}$-like NLS. A monoclonal Ab that recognizes a conformational epitope unique to the 43kDa isoform predominantly stains Rat-6 and NIH3T3 nuclei, with minor staining along F-actin fibers (Gelman, I.H.; unpublished data). This suggests that at least one of the SSeCKS isoforms has nuclear functions. Indeed, Coats *et al.* (53) demonstrate that the C-terminal half of SSeCKS can transactivate the plasminogen activator

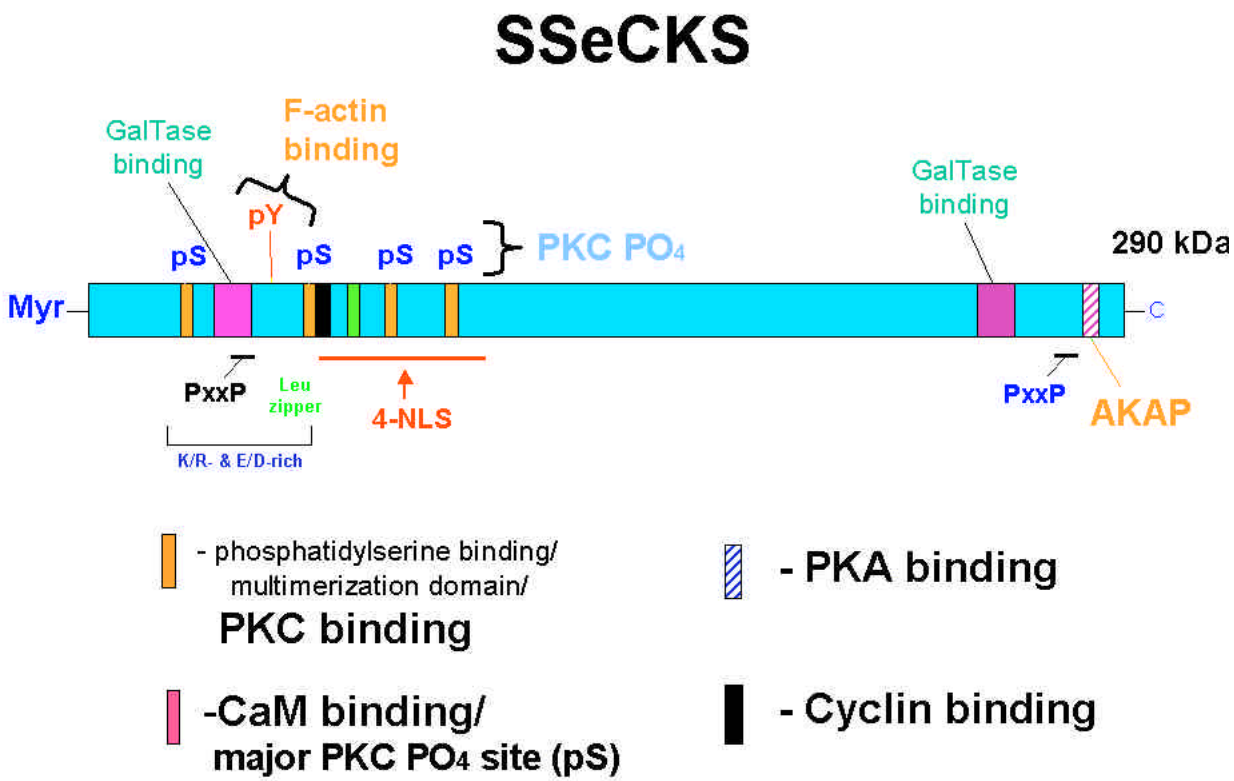

Figure 4. Scaffolding domains and phosphorylation sites on SSeCKS. SSeCKS contains various potential and demonstrated protein binding domains as well as PKC phosphorylation sites (pS) and a tyrosine phosphorylation site (pY). Potential SH3 binding sites on SSeCKS are shown as PxxP. SSeCKS also encodes at least 6 nuclear localization sites (NLS).

inhibitor-1 promoter, suggesting a role for SSeCKS in transcriptional control.

10. SSeCKS AS A PKC AND PTK SUBSTRATE

SSeCKS is a major *in vitro* and *in vivo* substrate of PKC (11,64). At least four *in vitro* PKC phosphorylation sites exist on SSeCKS, and at least two, Ser^{300} and Ser^{515}, are induced *in vivo* by the short-term (<5 minutes) activation of PKC with phorbol esters (64). These sites have been confirmed using polyclonal sera specific for these SSeCKS phosphopeptides (10). SSeCKS protein containing Ser^{300} and Ser^{515} is enriched in the lamellipodia forming at a monolayer wound front (10). Interestingly, PKC-induced phosphorylation of SSeCKS causes it to translocate from plasma membrane and cytoskeletal sites to the perinucleus in fibroblasts, mesangial and epithelial cells (64,10,75,108) suggesting that this event may play a role in the PKC-mediated reorganization of the actin cytoskeleton.

It is still unclear which PKC isoforms can phosphorylate SSeCKS *in vivo*. Purified PKCa phosphorylates SSeCKS *in vitro* and the level of *in vivo* SSeCKS phosphorylation is relatively increased in Rat-6 cells overexpressing PKCa compared to Rat-6 controls (64). Preliminary data indicate that partially-purified PKC a, ßI, ßII, ?, d, e, ?, and ? isozymes can phosphorylate GST-SSeCKS fusion proteins *in vitro*, although there is some isozyme preference for specific PKC phosphorylation sites (A. Bulua and I. H. Gelman, unpublished data). Given that SSeCKS expression is ubiquitous in mesenchymal and epithelial cells, SSeCKS phosphorylation is likely controlled by the set of PKC isozymes expressed and activated within a specific cell type.

SSeCKS is tyrosine phosphorylated *in vivo* in response to short-term treatment with either EGF, PDGF or serum, but not with insulin or IGF-1, and this phosphorylation antagonizes the binding of SSeCKS to F-actin *in vivo* (107). SSeCKS tyrosine phosphorylation was induced by EGF in cells deficient in Src-family, Abl, or JAK kinases, but not in FAK-deficient cells. Because purified FAK and Src failed to directly phosphorylate SSeCKS, it is likely that a novel FAK-induced or -activated tyrosine kinase is responsible for phosphorylating SSeCKS. Most interestingly, SSeCKS stains along F-actin fibers in FAK-/- fibroblasts whereas it is found at the cell edge and along a cortical cytoskeleton in most other fibroblasts. This correlated with a 10-fold increase in the amount of SSeCKS that co-immunoprecipitated with F-actin from FAK-/- compared to FAK+/+ cell lysates, as well as the finding that a bacterially-expressed, unphosphorylated SSeCKS fragment (a.a. 389-894) bound directly to F-actin *in vitro*. This binding activity may be facilitated by two motifs shared by SSeCKS and Gravin, ^{501}PLKK and ^{510}GLKK, that conform to the XLKK ABD-3 actin binding motif described previously (99). Taken together, these data strongly suggest that SSeCKS contains an F-actin binding domain whose activity is antagonized by mitogen-induced, FAK-mediated tyrosine phosphorylation.

11. PROTEIN MOTIFS AND BINDING PARTNERS

SSeCKS and Gravin encode multiple domains potentially involved in protein-protein, protein-lipid and protein-membrane interactions (Figures 1 and 3). The SSeCKS open reading frame lacks sequence motifs suggestive of any enzymatic activity, yet a growing corpus of data indicates that it can bind many key signaling mediators, and that some of these interactions are sensitive to SSeCKS phosphorylation

To date, SSeCKS domains have been identified that bind PKA (AKAP), cyclins, PKC (via phosphatidylserine bridge), ß$_2$-adrenergic receptor, calmodulin, ß-1,4 galactosyltransferase, and F-actin (Figure 4). Evidence will be presented below suggesting that the ability of SSeCKS to negatively control mitogenesis and cytoskeletal architecture relates to its selective scaffolding of signaling proteins during discrete periods in development and the cell cycle, and in discrete locations in the cell.

Rat SSeCKS was also identified (called "clone 72") in a screen for phosphatidylserine (PS)-dependent binding proteins of PKC (11). PS binding is likely facilitated by a domain shared by SSeCKS and its human orthologue, Gravin, ^{506}FSSSGLKKLSGKKQK^{520}, which is similar to the FxFxLKxxxKxR PS-binding motif previously identified in PKC (47). It is thought that polybasic domains facilitate the formation of phospholipid bridges between PKCs and their binding partners (50), and indeed, two polybasic regions shared by Xgl, SSeCKS and Gravin (a.a. 296-316 and 514-536 on rat SSeCKS) could inhibit PS-dependent binding of SSeCKS to PKC (72). Most significantly, PS plus the a.a. 256-556 polypeptide from SSeCKS could inhibit PKC activity *in vitro* in a saturable manner (55,72) suggesting that the scaffolding of PKC by SSeCKS *in vivo* attenuates its kinase activity. In addition to being a PKC binding partner, SSeCKS is a major PKC substrate (discussed above), and several major PKC phosphorylation sites on SSeCKS map close to the PS binding site. Interestingly, prephosphorylation of SSeCKS *in vitro* with PKC decreases PS-mediated PKC binding (64), although it has not been shown formally that this phosphorylation inhibits PS binding itself. This suggests that PKC binding by SSeCKS is modulated by the level of PKC phosphorylation.

SSeCKS binds calmodulin (CaM) in a Ca^{2+}-dependent manner (61), a function typical of other major PKC substrates such as MARCKS (37), adducins (66,89) or GAP-43/neuromodulin (65). SSeCKS and Gravin share seven potential CaM-binding sites conforming to the so-called 1-5-10 motif, (FILVW)x_3(FILV)x_3(FILVW), used by CaM kinase I and II, and by MARCKS to bind CaM (84). At least four SSeCKS domains have been demonstrated to bind CaM in a Ca^{2+}-dependent manner, and, as was reported previously with MARCKS, prephosphorylation of these domains with purified PKC severely decreases CaM binding (67). This antagonism reflects a general conflict in the use of common sites for CaM binding and PKC phosphorylation (9). Because CaM is a major calcium sensor involved in the regulation of ion channel function, cell-cycle progression and cytoskeletal organization, SSeCKS and Gravin may play regulatory roles by sequestering CaM in a phosphorylation-sensitive manner during discrete parts of the cell cycle.

Another major binding motif shared by SSeCKS and Gravin is a novel AKAP motif in the C-terminus, $LE^S/_TKS^N/_SK^I/_LV^Q/_L{}^N/_S{}^I/_V$IQ. Unlike the motif that facilitates binding to the R_I regulatory subunit isoform, this amphipathic helical domain in both Gravin and SSeCKS facilitates binding to the R_{II} isoform (72,91,76,25) and is similar to a peptide, Ht31 (IEEAASRIVDAVIE), shown previously to inhibit R_{II}/AKAP binding (8). However, it has yet to be determined whether the binding of PKA to SSeCKS/Gravin alters its kinase activity. Interestingly, Grove and Bruchey recently demonstrated that Gravin immunoprecipitates contain both PKA and PKCa, and that the associated PKA was enzymatically active. They also showed the converse, namely that Gravin could be eluted off of a cAMP-agarose column enriched for PKA (39). Taken together, these data indicate that SSeCKS, PKA and PKC can be found in a tripartite complex in cells. Interestingly, the PKA binding site on SSeCKS/Gravin is >1000 a.a. downstream of the PKC phosphorylation sites, leading to the speculation that PKA binding may be unaffected by PKC activation. If true, this might explain, in part, how PKA-mediated pathways are suppressed following PKC activation (44), and would put SSeCKS/Gravin at the center of crosstalk control of these two pathways.

SSeCKS binds the large isoform of ß1,4-galactosyltransferase (GalTase) *in vitro* and *in vivo* (77,105). This isoform functions as a transmembrane receptor for laminin (46,3), and most probably plays a role in adhesion mediated signaling. Two SSeCKS domains were found to independently bind the cytoplasmic domain of GalTase using a yeast 2-hybrid system, one at the N-terminus (a.a.- 51-330) and the other at the C-terminus (a.a.- 1271-1562). The N-terminal domain is homologous to Gravin, and thus, Gravin would presumably bind GalTase through this domain, however, the C-terminal domain shares little homology with Gravin. Interestingly, transfection of SSeCKS into mammary gland cells that express a dominant-negative, cytosolic GalTase mutant incapable of binding laminin, restored normative adhesion to laminin (105). This suggests that SSeCKS interacts with the cytoplasmic domain of GalTase to facilitate its plasma membrane association.

Gravin has been reported to bind the ß$_2$-adrenergic receptor in its unstimulated state, with increased binding after agonist-induced desensitization (59). The presence of Gravin seems to be required for the formation of a plasma membrane complex containing ß$_2$-adrenergic receptor, ß-arrestin, G-protein-linked receptor kinase-2 and clathrin inasmuch as anti-sense Gravin oligonucleotides disrupts this complex in unstimulated cells. The increased association of PKA and PKC with this complex after agonist stimulation may, in fact, be facilitated by Gravin's

binding domains. However, whereas Malbon's group showed interaction of Gravin with ß$_2$-adrenergic receptor using co-immunoprecipitation (co-IP) (92), Scott's group failed to find Gravin in ß$_2$-adrenergic receptor IPs analyzed by overlay with labeled PKA-R$_{II}$ subunit (29). In fact, the latter group found AKAP150, rather than AKAP250 (Gravin) in the ß$_2$-adrenergic receptor IP. It is possible that these disparate findings are due to different cell types (A431 vs. rabbit brain), lysis methods (SDS vs. CHAPS), or the methods used to isolate ß$_2$-adrenergic receptor complexes. Indeed, Fan *et al.* (26) map the Gravin binding domain to the cytoplasmic, intracellular tail of the ß$_2$-adrenergic receptor (a.a. 329-413), and show by confocal microscopy that Gravin remains associated even after agonist-induced receptor internalization. This underlines the notion that SSeCKS/Gravin manifest some of their scaffolding control functions by sequestration of associated signaling proteins to various cell compartments.

SSeCKS and Gravin encode common and unique protein motifs that may be involved in protein-protein interaction, but which have yet to be studied. For example, both SSeCKS and Gravin encode a potential leucine-zipper (a.a. 468-489 in SSeCKS) conforming to the motif, $Lx_6Lx_6Lx_6L$ (2), although Gravin has a Met and SSeCKS has an Ile substitution for the third Leu position. SSeCKS, but not Gravin, encodes a C_1H_3 motif that conforms to a Zn-binding domain. SSeCKS and Gravin encode several proline-rich domains that conform to the PxxP motifs recognized by proteins SH3 or WW domains (57,97). They also encode N-terminal R/K-rich and D/E-rich regions possibly involved in other protein-protein interactions. Both SSeCKS and Gravin encode a coiled-coils (residues 300-330 in SSeCKS) that would likely facilitate interaction with other proteins encoding similar domains. Lastly, both SSeCKS and Gravin encode potential calcineurin binding sites conforming to the motif, $WAS^{I}/_{F}{}^{K}/_{R}LVT^{K}/_{R}K^{K}/_{R}$ (55). This motif is also a competitive binding site on AKAP79 for phosphatidylinositol-4,5-*bis*-phosphate (23). Interestingly, in a study by Kashishian *et al.* (52), Gravin failed to inhibit calcineurin-dependent induction of NFAT activity (as measured by an NFAT promoter- luciferase reporter construct). However, the form of Gravin expressed in their system was 130kDa (full-length Gravin should be in the 300kDa range), suggesting some technical problems with expression and clouding the issue of whether Gravin may actually bind calcineurin or have effects on its signaling.

12. DEVELOPMENTAL, TISSUE AND SUB-CELLULAR EXPRESSION of SSeCKS/Gravin

SSeCKS is expressed in specific mesenchymal cells during early embryogenesis and in epithelial populations transitioning to mesenchyme. In late stage embryogenesis, most fibroblasts, connective tissue, and epithelial layers exhibit SSeCKS expression. Although Gravin expression was originally described as restricted to endothelial cells and especially absent in nonadherent cells (38), it is clearly expressed in WI-38 fibroblasts and epithelial cells (34), in the brain (29) and in epidermoid carcinoma cells (92) to name a few.

In the fetal mouse, early SSeCKS protein expression (E8.5 - 11) is focused in the loose mesenchyme, luminal surface of the neural tube, notochord, early heart and pericardium, urogenital ridge, and dorsal and ventral sections of limb buds. In later stages (E12-E14), SSeCKS is widely expressed in mesenchymal cells but is absent in the spinal ganglia. By E15, SSeCKS expression is ubiquitous, although the staining pattern varies from being striated within smooth muscle sarcomeres to filamentous in mesenchymal and select epithelial cells.

In the adult mouse, SSeCKS staining is relatively ubiquitous, with highest expression in the gonads, smooth and cardiac muscle, lung, brain and heart. High expression is also detected in fibroblasts and nerve fibers as well as in more specialized, process-forming cells such as glomerular mesangial cells and testicular Sertoli cells. SSeCKS expression in the rat testes correlates with the induction of puberty (25,98,34), and in mature mouse spermatozoa, SSeCKS is found in peripheral acrosome membranes and in a helix-like winding pattern within the midsection (34). Periodic enrichments of SSeCKS appearing as "pearls on a string" are found in sperm midsections and in developing axons, suggesting a role in architectural infrastructure. SSeCKS' staining pattern in developing axons is similar to that of Sec-8, a secretory protein involved in synaptogenesis (41,45), and thus, it is interesting to speculate that SSeCKS may help form synaptogenic complexes in developing neuronal cells. Siegel *et al.* (94) identified a staining pattern for SSeCKS in the rat brain in dorsal root ganglia and the dorsal horn of the spinal chord consistent with markers for small primary sensory neurons.

As with Gravin, high SSeCKS expression is absent in most epithelial cells, however, in contrast to Gravin (38), SSeCKS is expressed in Purkinje cells, cardiac muscle, macrophages and hepatic stellate cells, indicating overlapping yet distinct patterns of tissue expression in the SSeCKS/Gravin family. The data suggest roles for SSeCKS in the control of cytoskeletal and tissue architecture, formation of migratory processes and cell migration during embryogenesis. These patterns suggest multiple structural roles.

Depending on the cell type, SSeCKS and Gravin stain along a cortical cytoskeletal network and in the perinuclear area, with enrichments in some cells in podosome structures, focal adhesion complexes, and along the cell edge (64,33,75,39). Confocal microscopic analysis using immunoaffinity-purified polyclonal anti-SSeCKS antibody showed the majority of SSeCKS associating with a Triton-insoluble cytoskeleton and some found at the cell periphery (33). Similar results were described for Gravin using immuno-electron microscopy (39). However, one report indicates that Gravin is cytoskeletal (35) whereas another indicates that it is mostly cytosolic (72) although this may reflect differences in the fixation techniques used.

13. UNANSWERED QUESTIONS

Several major issues regarding SSeCKS remain unclear. First, the SSeCKS/Gravin proteins domains that

encode tumor suppressor activity are undefined, although there is strong evidence that the ability of SSeCKS to induce growth arrest in G1 is encoded by its cyclin D binding activity. It is possible that an SSeCKS knockout mouse might be prone to spontaneous or mutagen-induced tumor formation or hyperplasia, however, in the absence of other compensatory genes, SSeCKS deficiency may cause embryonic lethality, especially because there seem to be no other family members and because SSeCKS is so widely expressed throughout embryogenesis. Another enigma is whether SSeCKS plays a significant role in the nucleus, given its collection of nuclear localization motifs. It is unclear whether specific isoforms scaffold separate or even overlapping sets of signaling proteins. Clearly, the production of healthy mice deficient in the myristylated SSeCKS isoform (6) indicates that the 280kDa isoform as well as other smaller isoforms are sufficient for basic development. Lastly, it has yet to be demonstrated that SSeCKS/Gravin play central roles in regulating crosstalk between PKA and PKC signaling pathways.

14. PERSPECTIVES

The ability of SSeCKS/Gravin to scaffold key signaling and cytoskeletal proteins involved in G1®S progression in a cell cycle-dependent, phosphorylation-sensitive manner suggests that SSeCKS/Gravin are important regulators of mitogenesis. It is likely that SSeCKS/Gravin manifest their tumor suppressive activities via these binding functions, such that loss of SSeCKS/Gravin during oncogenesis leads to dysfunctional spaciotemporal signaling and cytoskeletal controls, and ultimately, to the loss of contact- and anchorage-dependent growth. SSeCKS/Gravin, therefore, represent significant targets for the future development of therapeutics for cancer and diseases associated with hyperplasia.

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