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Calcyclin (S100A6) Regulates Pulmonary Fibroblast Proliferation, Morphology, and Cytoskeletal Organization In Vitro

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Abstract Calcyclin (S100A6) is a member of the S100A family of calcium binding proteins. While the precise function of calcyclin is unknown, calcyclin expression is associated with cell proliferation and calcyclin is expressed in several types of cancer phenotypes. In the present study, the functional role of calcyclin was further elucidated in pulmonary fibroblasts. Antisense S100A6 RNA expression inhibited serum and mechanical strain-induced fibroblast proliferation. This attenuated proliferative response was accompanied by a flattened, spread cell morphology, and disruption of tropomyosin labeled microfilaments. Changes in cytoskeletal organization did not correspond with a decrease in tropomyosin levels. These observations suggest a role for calcyclin in modulating calcium dependent signaling events that regulate progression through the cell cycle. J. Cell. Biochem. 88: 848–854, 2003. © 2003 Wiley-Liss, Inc.

Key words: S100A6; tropomyosin; mechanical; strain; antisense; retrovirus

Calcyclin belongs to a family of S100A calcium binding proteins that bind molecules of calcium through conserved EF-hand domains. The genes encoding these small (10–15 kDa) proteins are tightly clustered on human chromosome 1 and range from S100A1 through S100A13 with calcyclin being identified as S100A6 [Schäfer et al., 1993]. Recently a wide variety of cellular functions has been attributed to the products of this gene family. In particular, calcyclin has been implicated in intracellular calcium homeostasis and signaling, ion transport [Courtois-Coutry et al., 2002], exocytosis of insulin from pancreatic cells [Okazaki et al., 1994], ubiquitinylated proteolytic degradation [Filipek et al., 2002], and proliferation [Calabretta et al., 1986]. Unlike calmodulin, which is ubiquitously expressed, calcyclin expression follows the cell type specific pattern of many S100A proteins and is expressed mainly in fibroblasts and epithelial cells with some expression in smooth muscle cells and neuronal cells [Kuznicki et al., 1992]. Furthermore, high levels of S100A6 are also found in tumor cells such as acute myeloid leukemia [Calabretta et al., 1986] and lung carcinoma cells [Takenaga et al., 1997]. However, calcyclin plays a role in nontransformed cells and is expressed at sites of ischemic kidney injury [Lewington et al., 1997] and in response to the hormone, arginine vasopressin is critical for glomerular transepithelial ion and water transport regulation. Recently, an increased level of calcyclin was measured in lungs ventilated at high inflation pressures [Breen et al., 1999], and calcyclin upregulation potentially reflects the proliferation of alveolar epithelial or mesenchymal cells stimulated by increased mechanical tension [Liu and Post, 2000].

As with many S100 proteins, calcium binding induces a conformational change that allows S100A6 to interact with specific intracellular target proteins [Sastry et al., 1998]. S100A6 does not posses enzymatic properties itself but likely modulates signaling pathways by altering the binding potential or availability of calcyclin specific target proteins. In vitro biochemical studies have identified several calcium-dependent S100A6 target proteins,

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which reside in the nuclear envelope, cytoplasm and plasma membrane [Stradal and Gimona, 1999]. For instance, calcyclin was originally identified as a target protein for annexin XI [Mizutani et al., 1992]. The nuclear localization signal of annexin XI allows the translocation of calcyclin from the cytoplasm to the nucleus. Calcyclin is also located in the cytoplasm and potentially binds to the actin-binding protein, tropomyosin [Golitsina et al., 1996]. Plasma membrane localized annexins II and VI are also calcyclin targets and have been shown to form cytoskeleton plasma membrane complexes in smooth muscle cells [Babiychuk et al., 2000]. At each of these subcellular locations, calcyclin could potentially modulate protein–protein interactions and regulate calcium dependent signaling. More recently calcyclin has been reported to associate with another S100 protein, S100B, suggesting the interactions between S100 proteins could influence protein–protein interactions and modify signal transduction pathways by controlling the availability of S100 or target proteins to associate with their known binding partners [Deloulme et al., 2000].

In the present study, the cellular function of calcyclin was further elucidated in an intact cell, the pulmonary fibroblast, through the expression of calcyclin antisense RNA. Inhibition of calcyclin resulted in an attenuation of serum growth factor or mechanical strain-induced pulmonary fibroblast proliferation. Decreased pulmonary fibroblasts proliferation was preceded by a flattened, spread cell morphology, and altered cytoskeletal organization.

MATERIALS AND METHODS

Cell Culture

Rat fetal lung fibroblasts were isolated from late-gestation pups (day 19) as previously described [Liu and Post, 2000]. Briefly, fetal lungs were dissected and minced in Hank's balanced salt solution without calcium or magnesium [HBBS (−)]. Lung tissue was then digested with a trypsin [0.125% (wt/vol)] and DNase (40 mg/ml) solution at 37°C for 20 min. The resulting homogenate was filtered through a 100 μ nylon cell filter and resuspended in fresh MEM-10% FBS. Cells were pelleted at low speed ($420 \times g$) and resuspended in MEM plus 0.1% collagenase for a second digestion step at 37°C for 15 min. Cells were again centrifuged at $420 \times g$, resuspended in fresh MEM-10% FBS and placed in sterile 10 cm culture dishes. After one hour, nonadherent epithelial cells were removed and fibroblasts were cultured overnight. Cells were routinely checked by immunohistochemical staining for vimentin, but not α smooth muscle actin or cytokeratin to confirm the isolation of a homogenous pulmonary fibroblasts population. Fibroblasts cultures were maintained in minimal essential medium (MEM) containing penicillin-streptomycin (50 U/ml) (GIBCO BRL, Grand Island, NY) and 10% charcoal-stripped fetal calf serum (Hyclone, UT) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Passages 2 through 5 were used in experiments.

Construction of an Antisense Retroviral Expression Vector

An approximately 500 bp BamHI/Pvu II DNA coding sequence from the mouse calcyclin (S100A6) cDNA [Guo et al., 1990] was subcloned into the retroviral plasmid vector pLNCX (Clontech Laboratories, Inc., Palo Alto, CA) in the sense (HindII/ClaI restriction sites) or antisense (HindIII/HpaI restriction sites) orientation. pLNCX, pLNCX/S100A6-sense and -antisense plasmids were amplified in Electro-Ten-Blue *E.coli* (Stratagene, La Jolla, CA). Correct sequence and orientation were confirmed by nucleotide sequence analysis (UCSD CFAR Molecular Biology Core Facility). Retrovirus was produced using a transient assay for the generation of high titer, helper-free retroviruses [Pear et al., 1993]. Briefly, 10 μg of pLNCX plasmid (without target gene), pLNCX/S100A6-antisense or -sense plasmids were transfected into Retro Pack PT67 cells (Clontech Laboratories, Inc.) using a standard calcium phosphate transfection method. PT67 cells were seeded at 1.5×10^6 cells/100 mm culture dish 24 h prior to transfection. Retroviral containing cell supernatant was collected 48 h later, filtered through a 0.45 μm cellulose acetate filter to remove PT67 cells and used immediately.

Cell Proliferation

Pulmonary rat fetal fibroblasts were seeded at a concentration of 1.5×10^4 cells/35 mm culture well. The next day cells were infected with pLNCX, pLNCX/S100A6-sense or -antisense retrovirus at approximately 10^6 pfu/ml in MEM plus 10% FBS and 8 μg/ml of polybrene

(Specialty Media-Division of Cell & Molecular Technologies Inc., Phillipsburg, NJ). Fibroblasts were infected again 12 and 24 h following the first retroviral infection. Approximate retroviral titers were determined by infection of NIH3T3 cells with serial dilutions of a reporter retrovirus, which expresses the enhanced green fluorescence protein, pLNCX/EGFP. pEGFP-C-N1 plasmid was purchased from Clontech. In addition, the infection of pulmonary fibroblast with equivalent amounts of pLNCX, pLNCX/S100A6-sense or -antisense retrovirus was confirmed by synthesizing incorporated viral DNA using pLNCX Seq/PCR primers by the polymerase chain reaction. Cell number was determined by hemocytometer counting in five to six wells at each time point.

In Vitro Application of Mechanical Strain

An electromagnet driven strain apparatus was used to cyclically stretch three-dimensional cultures of pulmonary fibroblasts grown in gelatin sponges according to the method of Skinner [1989] with some modifications. In this system one edge of a $2 \times 2 \times 0.25$ cm gelatin sponge (Gel Foam, Pharmacia & Upjohn Co., MI) is glued to the bottom of a 35 mm untreated suspension culture dish. To the opposite edge of the gelatin sponge, a 2×1 mm diameter plastic coated iron rod (weighing $0.178\ g \pm 0.007$) was attached. This allowed culture dishes to be stacked in front of a parallel pole electromagnet (AEC Magnetics, Cincinnati, OH) charged with an oscillator driven power supply. Fibroblasts were seeded into sponges at an initial density of 8×10^5 cells/sponge in MEM containing only 1% fetal bovine serum. Twenty-four hours after the initial seeding, fibroblasts/sponges were infected with pLNCX/S100A6-sense or -antisense retrovirus (approximately 10^6 pfu/ml). After an additional 24 h, fibroblast culture media was exchanged with 3 ml of media containing 1 μCi/ml of [^{3}H] thymidine in addition to retrovirus for each fibroblast/sponge containing culture dish. Fibroblast/sponges were cyclically stretched 5% of the original sponge length at a rate of 60 cycles/min (cpm) for 24 h. Resting, control cultures were placed in front of the magnetic field but the sponge was freely floating, unattached to the bottom of the culture dish. At the end of the strain period fibroblast/sponges were washed twice with phosphate-buffered saline (PBS) and precipitated with 10% trichloroactetic acid (TCA) at 4°C. Sponges were then rinsed three times with cold 5% TCA and directly counted with a scintillation detector. Cell proliferation was reflected as [^{3}H] thymidine incorporation per sponge.

Immunofluorescent Subcellular Localization of Tropomyosin and Calcyclin

Subcellular localization of calcyclin and tropomyosin were detected simultaneously according to the method of Takenaga et al. [1994]. Cells were grown on poly-D-lysine 8 well culture slides (BD Biosciences Labware, Bedford, MA). Fibroblasts were seeded at an initial density of 1.5×10^4 cells/2 cm^2 chamber and infected with pLNCX or pLNCX/S100A6-antisense retrovirus as described above. Following a 24 h infection period, cells were gently washed three times with phosphate-buffered saline (PBS) and fixed in a 1% paraformaldehyde/2.5% sucrose/PBS solution for 15 min at 4°C. Fibroblasts were permeabilized with 0.5% triton X-100/PBS for 4 min at room temperature, washed three times with PBS, blocked one hour with 3% BSA/PBS/0.1 M glycine and washed again with PBS before incubation of primary antibodies. Cells were simultaneously incubated with a mouse monoclonal tropomyosin antibody (TM311, Sigma, St. Louis, MO) and a rabbit calcyclin antibody (SWANT, Bellinzona, Switzerland) in a 1 mg/ml BSA/PBS solution. Primary antibody staining was detected using anti-rabbit FITC-conjugated and anti-mouse Texas Red secondary antibodies (Molecular Probes, Inc., Eugene, OR). Slides were mounted with Slow-Fade and viewed by fluorescent microscopy.

Western Analysis

Pulmonary fibroblasts were homogenized in lysis buffer (1% SDS, 62.5 mM Tris, pH 6.8, 1 μg/ml aprotinin, 100 μg/ml PMSF) and boiled for 5 min. Total protein levels in each samples were determined using the Bio-Rad DC-Protein assay and confirmed by staining with colloidal gold total protein stain (Bio-Rad Laboratories, Hercules, CA). 200 ng of protein was electrophoresed on a 10% SDS–PAGE gel under reducing conditions and probed with a monoclonal anti-tropomyosin antibody, TM311, which detects tropomyosin isoforms 1, 2, and 3. Primary antibodies were detected with HRP-conjugated secondary antibodies and ECL western blotting detection kit (Amersham Biosciences Corp., Piscataway, NJ).

RESULTS

Antisense Expressed Calcyclin RNA Inhibits Pulmonary Fibroblast Proliferation

Fetal rat pulmonary fibroblasts, cultured in medium that contains serum growth factors, were infected with pLNCX retrovirus, which either expressed calcyclin RNA in an antisense orientation or the same gene sequence expressed in a sense orientation. No difference in cell number was observed between cells infected with a sense calcyclin retrovirus or retroviral vector alone over a 4 day period (Fig. 1). Pulmonary fibroblasts infected with antisense calcyclin retrovirus revealed 30 and 32% decreases in cell number compared to retroviral vector or sense infected fibroblasts, 48 and 72 h post-infection, respectively. Ninety-six hours post-infection fibroblasts cultures approached confluence. No significant difference between antisense S100A6 retroviral infected cells and control groups was observed at this time. Similar results were observed with the human fetal fibroblast cell line, IMR-90 cells (data not shown).

Antisense Expressed Calcyclin RNA Inhibits Mechanical Strain-Induced Fibroblast Proliferation

Fetal pulmonary fibroblasts can also be stimulated by cyclic mechanical strain to increase their proliferation rate. In this experiment we observed a 50% increase in [^{3}H] thymidine incorporation, reflecting an increased cell number, in fibroblasts subjected to a 5% increase in mechanical strain applied cyclically over a 24 h period (Fig. 2). Fibroblasts infected with an antisense calcyclin retrovirus 24 h prior to the application of increased mechanical strain, demonstrated a only a 33% increase in [^{3}H] thymidine incorporation compared to unstrained, resting fibroblasts. This is equivalent to a 34% decrease of the [^{3}H] thymidine incorporation resulting from mechanical strain stimulation.

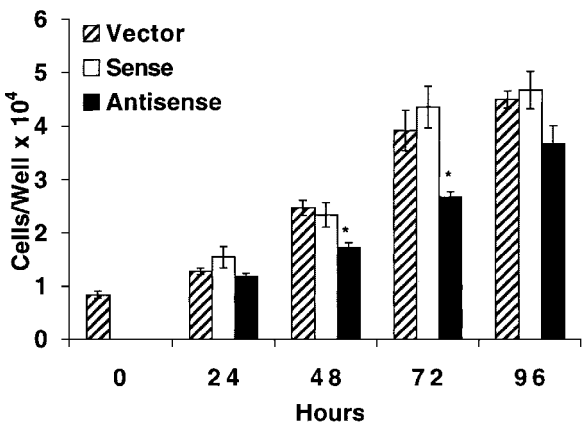

Fig. 1. Antisense S100A6 expression inhibits cell growth of fetal pulmonary fibroblasts. Fetal rat pulmonary fibroblasts were infected with pLNCX retrovirus, which expresses S100A6 in an antisense orientation (Antisense), sense orientation (Sense), or retroviral vector alone (Vector). Cell number was determined at 24 h intervals following retroviral infection. N = 6 ± SD. $P < 0.05$ was considered significant (*).

Altered Cytoskeletal Organization Independent of a Change in Tropomyosin Levels

Twenty-four hours post-infection with antisense calcyclin retrovirus, fibroblasts displayed a dramatic change in cellular morphology evident as a large and more flattened phenotype. Fibroblast morphology reverted to a normal appearance over the next 24 h. This change in cell morphology was accompanied by a reduction and disorganization of tropomyosin-associated cytoskeleton filament networks (Fig. 3) and a parallel reduction in F-actin

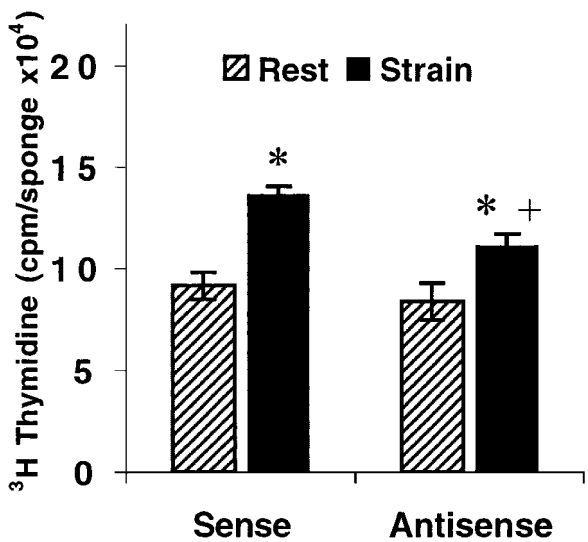

Fig. 2. Antisense S100A6 expression inhibits strain-induced fibroblast proliferation. Fetal rat fibroblasts were infected with pLNCX/S100A6-sense (Sense) or -antisense (Antisense) retrovirus 24 h prior to the application of increased mechanical strain. Infected fibroblasts were then subjected to increased cyclic mechanical strain (Strain) or allowed to remain unstrained (Rest) in the presence of 1 μCi/ml or [^{3}H] thymidine over the next 24 h period. Cell proliferation is reflected as TCA-precipitable counts per sponge. N = 3 ± SEM. * Indicates a difference between resting and mechanically strained fibroblasts cultures. + Indicates a difference between strained cultures with retroviral expressed Sense or Antisense S100A6 RNA.

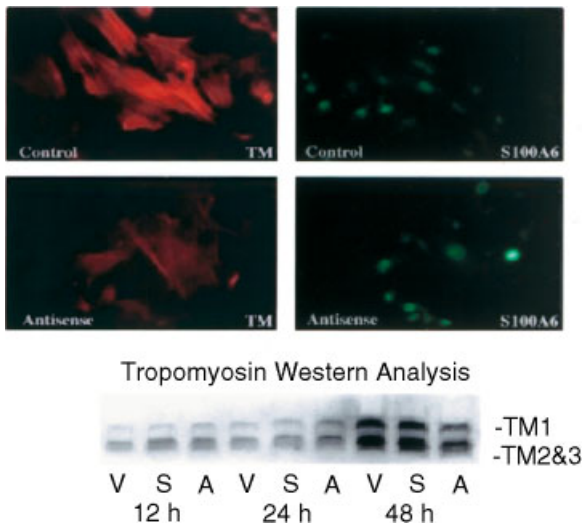

Fig. 3. Fibroblasts expressing S100A6 antisense RNA display altered cell shape and cytoskeletal organization. **A**. Pulmonary fibroblasts infected with antisense S100A6 retrovirus have a flatted, spread cell shape 24 h post retroviral infection. Immunofluorescent detection of tropomyosin reveals decreased levels of organized stress fibers. Anti-calcyclin antibodies demonstrate dispersed calcyclin nuclear localization. **B**. Western analysis reveals equivalent tropomyosin isoform 1, 2, and 3 levels 24 h post-retroviral infection.

stress fibers (data not shown). Calcyclin (S100A6) was localized in large spread nuclei of these triton-X permeabilized fibroblasts preparations. Tropomyosin immunoblot analysis did not demonstrate a difference in tropomyosin isoforms 1, 2 and 3 levels between vector, sense and antisense calcyclin (S100A6) RNA expressing fibroblasts (Fig. 3).

DISCUSSION

Calcyclin Attenuates Pulmonary Fibroblast Proliferation

The major outcome of this experiment is that decreasing calcyclin expression results in an attenuated fibroblast growth under normal physiologic intracellular calcium levels. However, lowering normal calcyclin levels did not completely inhibit fibroblast proliferation but reduced cell number approximately 30% when fibroblasts were grown under normal serum conditions or 34% when cell were stimulated by increased mechanical strain in low serum (1% serum). This incomplete inhibition of fibroblast proliferation suggest that calcyclin plays a role in modulating the intracellular signaling events necessary for cells to divide. Thus, calcyclin could potentially play a role in calcium homeostasis and signaling of intracellular calcium transients required at various control points of the cell cycle in mitogen stimulated cells [Wahl and Gruenstein, 1993]. Likewise, pulmonary fibroblasts respond to strain with an increase in intracellular calcium levels via gadolinium sensitive calcium channels and release of the mitogen, PDGF. A functional PDGF cis-acting DNA element has been identified in the calcyclin promoter [Ghezzo et al., 1988]. Thus, the ability to synthesize calcyclin through the serum mitogen, PDGF, and the potential to transduce changes in intracellular calcium level into cell signaling events could be a common calcyclin-mediated control points for both serum and strain induced fibroblast proliferation.

Calcyclin as a Regulator of Actin Binding Protein–Protein Interactions

While the functions ascribed to calcyclin and several other S100 proteins appear numerous, many of these cell processes (exocytosis, ion transport, migration, contractility, and proliferation) require the actin cytoskeleton. Previous studies have demonstrated through antisense RNA or chemical blockade that inhibition of S100A1, S100A4 [Takenaga et al., 1994], or S100A13 [Landriscina et al., 2000] decreases proliferation and disrupts cytoskeletal organization. For example, in nonmuscle cells it has been reported that PEL98 (S100A4), binds to the nonmuscle tropomyosin isoform 2, regulates tropomyosin–actin interactions and suppresses the metastatic potential of Lewis lung carcinoma cells [Takenaga et al., 1994]. In a similar manner, S100A1 expression in PC12 cells has been demonstrated by antisense inhibition experiments to be important in regulating tubulin levels, neurite organization and proliferate capacity [Zimmer et al., 1998]. S100A1 and S100A4 normally co-localize to actin stress fibers within the cell [Mandinova et al., 1998]. While, calcyclin is capable of binding to skeletal and smooth muscle TM and TM: actin thin filaments in vitro and decreased tropomyosin expression has been correlated with reorganization and the cytoskeleton and unregulated cell growth [Boyd et al., 1995], calcyclin has not been observed to co-localize with actin stress fibers in intact cells [Boyd et al., 1995, Hsieh et al., 2002]. However, calcyclin could potentially regulate actin bundling indirectly through its calcium–dependent

interaction with tropomyosin or other actin binding target proteins. This mode of action has been reported for an isoform of calcyclin, caldesmon, as well as S100A1 and SA100B. S100–caldesmon interactions indirectly effect cytoskeletal function by weakening actin–myosin interactions and relieving caldesmon-dependent inhibition of actin-activated myosin ATPase activity resulting in reduced stress fiber formation and contractility in SV80 fibroblasts [Mani and Kay, 1995, Helfman et al., 1999]. Amlexanox, an anti-inflammatory agent that binds S100A13, has also been reported to inhibit proliferation and src-dependent actin stress fibers assembly. This response is rapidly reversible upon withdrawal of amlexanox and occurs in the absence of a change in total F-actin levels. This described transient cell shape change is similar to the transient morphological changes observed in antisense calcyclin RNA expressing pulmonary fibroblasts which occur without a change in tropomyosin levels. Interestingly, amlexanox treatment has recently been reported to inhibit the calcium-initiated translocation of S100A6 via an actin filament based mechanism [Hsieh et al., 2002]. Alternatively, calcyclin may directly interact with annexins II or VI and strengthen calcium-dependent cytoskeleton-membrane complex formation at the plasma membrane [Babiychuk et al., 2000], analogus to the proposed role of S100A10–annexin II interactions during endo- and exocytosis [Harder and Gerke, 1993].

Potential Role of Nuclear Calcyclin for Cell Cycle Progression

The experiments reported in this study were evaluated at resting intracellular calcium levels and suggest that calcyclin is required but not sufficient to initiate cell division. One of the well known and first identified targets of calcyclin is nuclear annexin XI. Previous studies have suggested that annexin XI is distributed throughout the nucleoplasm during interphase of the cell cycle while it is localized to the mitotic apparatus during M phase in the embryonic fibroblast cell line, 3Y1 [Mizutani et al., 1992]. Furthermore, in cells transformed by v-src a subset of annexin XI molecules is phosphorylated on Ser and Thr residues allowing translocation from the nucleus to the cytoplasm [Mizutani et al., 1992]. Annexin XI-S100A6 interactions could potentially regulate nuclear calcium levels. Precedent for a nuclear S100 control point can be inferred from the role of calmodulin, which is dynamically regulated during the cell cycle. Calmodulin is thought to diffuse freely through nuclear pores and associate with binding proteins, which facilitate nuclear Ca^{2+}-calmodulin accumulation in response to elevated intracellular free Ca^{2+} [Liao et al., 1999]. Thus, nuclear Ca^{2+} levels in turn control the activation of calcium sensitive transcription factors (i.e., CREB, c-Jun) required for cell cycle progression [Cruzalegui et al., 1999]. Pulmonary fibroblasts expressing antisense calcyclin RNA exhibit large nuclei with diffuse calcyclin staining possible reflecting a block in annexin XI-calcyclin translocation to the cytoplasm or inhibited transition from G_0 through the G_1 pre-replicative phase.

Overall, these studies confirm the role of calcyclin in modulating pulmonary fibroblast proliferation in an intact cell. The changes in cytoskeletal structure and cell morphology that accompany this attenuated growth response suggest that the actin cytoskeletal is involved in this cellular process. Whether calcyclin signaling is involved in pre-replicative nuclear calcium signaling or calcium dependent protein–protein interactions with actin binding proteins or plasma membrane annexins will require further detailed investigation of the target protein interactions and function in an intact cellular environment.

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