Research Article

Casein Kinase Iγ2 Impairs Fibroblasts Actin Stress Fibers Formation and Delays Cell Cycle Progression in G1

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Actin cytoskeleton remodeling is under the regulation of multiple proteins with various activities. Here, we demonstrate that the γ2 isoform of Casein Kinase I (CKIγ2) is part of a novel molecular path regulating the formation of actin stress fibers. We show that overexpression of CKIγ2 in fibroblasts alters cell morphology by impairing actin stress fibers formation. We demonstrate that this is concomitant with increased phosphorylation of the CDK inhibitor p27Kip and lower levels of activated RhoA, and is dependent on CKIγ2 catalytic activity. Moreover, we report that roscovitine, a potent inhibitor of cyclin-dependent kinases, including Cdk5, decreases p27Kip protein levels and restores actin stress fibers formation in CKIγ2 overexpressing cells, suggesting the existence of a CKIγ2-Cdk5-p27Kip-RhoA pathway in regulating actin remodeling. On the other hand, we also show that in a manner independent of its catalytic activity, CKIγ2 delays cell cycle progression through G1. Collectively our findings reveal that CKIγ2 is a novel player in the control of actin cytoskeleton dynamics and cell proliferation.

1. Introduction

The Rho family of GTPases comprising RhoA, Rac1, and Cdc42 regulates the organization of the cytoskeleton in eukaryotic cells [1]. These proteins cycle between an active GTP-bound and inactive GDP-bound state through the action of GTPase exchange factors (GEFs) and GTPase activating proteins (GAPs) [2]. Once activated, RhoA regulates actin stress fibers formation [3], while Rac1 triggers the assembly of actin in lamellipodia and membrane ruffles [4] and Cdc42 induces filopodial extensions [5] at the leading edge of the cell. Over the years, Rho GTPases were found to be crucial regulators of actin remodeling involved in a great deal of normal cellular functions, including cell migration and adhesion, cell cycle progression, and membrane trafficking [6]. In addition, Rho GTPases contribute to pathological conditions, particularly to cancer initiation and metastasis by controlling cell proliferation, migration, and adhesion during oncogenic transformation [7–9].

Accumulating evidence suggests that Rho GTPases are regulated at least in part by the cyclin-dependent kinase inhibitors (CDKIs) p21Waf/Cip, p27Kip1, and p57Kip2 through different mechanisms. As example, p27Kip1, which depends on its abundance and nuclear localization to inhibit the cyclin-dependent kinases (CDKs), inhibits RhoA activation in a cell-cycle independent manner, thereby modulates actin dynamics [10]. In fact, p27Kip1 phosphorylation at Ser10 increases its stability and cytoplasmic localization [11, 12], where it binds to and inhibits RhoA by interfering with the interaction between RhoA and its activating GEFs [10]. Among protein kinases that regulate p27Kip1, cyclin-dependent kinase 5 (Cdk5), also known as a regulator of actin dynamics, was found to stabilize p27Kip1 through phosphorylation of p27Kip1 at Ser10 in cortical neurons [13]. However, whether Cdk5 possesses similar activity in nonneuronal cells remains to be determined.

Casein kinase I (CKI) encompass a large family of Ser/Thr protein kinases encoded by separate genes and several splice variants. The 7 mammalian CKI isoforms identified so far, namely, α, β, γ1–3, δ, and ε, share high degree of identity within their kinase domain, but differ significantly in the length and amino acid composition of their N- and C-termini [14]. Overall, CKIs are conserved throughout evolution and involved in diverse cellular functions [15]. CKIα, δ, and ε involved in vesicular trafficking [16–18] are also implicated in canonical Wnt signaling, but
with distinct role [19]. CKIδ transduces specific centrosome functions [20], but, like CKIε, it also contributes to the regulation of the circadian rhythm [21, 22], apoptosis [23], and neuronal neurite outgrowth [24]. Interestingly, among the CKI family, the closely related CKIγ proteins (CKIγ1, 2, and 3) are unique in carrying C-terminal lipid modification motif that is believed to anchor them at the plasma membrane [25, 26]. In agreement with CKIγ plasma membrane localization, expression of the Xenopus tropicalis CKIγ in vertebrates and Drosophila cells has been implicated in transducing early signaling events of LRP6, a cell surface membrane receptor involved in Wnt signaling [25]. However, very little is known regarding the function of individual mammalian CKIγ isoforms. Previously, we reported that the Src homology (SH) domain-containing adaptor protein Nck directly interacts with CKIγ2 through two of its SH3 domains [27], while we determined that a proline rich motif (P143DVPSQPR352) unique to the C-terminal noncatalytic tail of CKIγ2 is mediating binding of Nck (unpublished data). Given that Nck transduces signals from membrane receptor protein tyrosine kinases to effectors regulating crucial biological cellular responses such as actin cytoskeletal reorganization and cell proliferation, we further investigated CKIγ2 function in mammalian cells.

In this study, we provide evidence that the kinase activity is required for CKIγ2 to regulate actin cytoskeleton remodel ing through its ability to downregulate RhoA proteins and signaling via the activation of the Cdk5-p27kip1 pathway. In addition, our findings also reveal that in a manner independent of its catalytic activity, CKIγ2 also regulates cell proliferation.

2. Materials and Methods

2.1. CKIγ Constructs. The mouse CKIγ1, 2, and 3 full length cDNAs were subcloned downstream of a Kozak sequence and in frame with a HA epitope sequence into the mammalian expression vector pZeoSV2 (Invitrogen). A kinase deficient (KD) CKIγ2 full length cDNA was generated by introducing a point mutation (K53R) in the ATP-binding site. A cDNA (1–1020 nts) encompassing the kinase domain, but lacking the C-terminal extension of CKIγ2 (ΔC-term), was generated by PCR using appropriate specific primers and further subcloned into pZeoSV2 as reported above. All constructs were fully sequenced to confirm their identity and to ensure that no unwanted mutation had been introduced during their creation.

2.2. Stable Cell Lines of Fibroblast Overexpressing CKIγ2. Rat 2 fibroblasts were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Life Technologies, Inc) supplemented with 2 mM L-glutamine, 45 mM sodium bicarbonate, and 10% FBS at 37°C, in a humidified atmosphere of 95% air and 5% CO2. Using calcium phosphate precipitation, fibroblasts were transfected with indicated expression plasmids. Upon selection in medium containing high concentration of zeocin (500 μg/mL) or G418 (400 μg/mL) for cells transfected, respectively, with pZeoSV2 or pcDNA 3.1, individual clones were isolated, grown, and analyzed for expected proteins expression. Positive clones were propagated under the same conditions, except that 50 μg/mL zeocin or 40 μg/mL G418 was added to the culture medium. For fibroblasts transfected with the empty pZeoSV2 plasmid, instead of individual clones following zeocin selection procedure, a pool of resistant cells was propagated and used as control.

2.3. Cell Culture and Transient Transfection. Rat-2 and HaCaT cells were grown in DMEM and HepG2 cells in Minimum Essential Medium Alpha Medium (MEM) (Invitrogen) supplemented with anti biotic/antimycotic (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) at 37°C in 5% CO2/95% O2. For CKIγ1, 2, and 3 transient expression into Rat-2 cells, cells plated at 80% confluence in 60 mm dishes were transiently transfected with indicated expression plasmids using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instructions.

2.4. SiRNA Transfection. Human CKIγ2 siRNAs targeting two independent coding regions (R1 and R2) were purchased from Integrated DNA technologies (IDT) R1(5′-GCACUGAGUAUCCGUUC-3′) and R2(5′-GCGCUACGUAGCAUACAC-3′). Scrambled siRNA obtained also from IDT was used as control. HepG2 and HaCaT cells were transiently transfected with indicated siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 300 μmol of siRNA was added to 500 μL of Opti-MEM I Medium without serum (Invitrogen) in 6-well plates and mixed gently. 5 μL of Lipofectamine RNAiMAX reagent was added to each well containing diluted siRNAs, mixed gently, and incubated at room temperature for 20 min. In the meantime, cells were harvested, counted, and diluted at 200 000 cells/mL in MEM media without antibiotics. Then, 2.5 mL of cells suspension (i.e., 500 000 cells/well) were added to each well and mixed gently, making the final siRNA concentration at 100 nM. The cells were further incubated at 37°C for 48–72 hours.

2.5. Antibodies, Immunoprecipitation, and Western Blots. To immunoprecipitate HA-tagged CKIγ2, we used the commercial HA F-7 antibody (Santa Cruz). For western blot analysis, the following antibodies were used: HA Y-11 (Santa Cruz); p53 FL-393 (Santa Cruz), Nck 1794 (in house [27]), p21Cip1 C-10 (Santa Cruz); p27kip1 C-19 (Santa Cruz) and RhoA F- 1 (Santa Cruz). To detect CKIγ2, we generated a rabbit polyclonal antibody using a KHL-coupled CKIγ2 peptide encompassing aa 331–354 as antigen. In general, cells were lysed in lysis buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride) supplemented with 2 μg/mL leupeptin and aprotinin as well as with 1 mM phenyl-methylsulfonyl fluoride (PMSF) and 200 μM activated sodium orthovanadate. Clarified cell lysates were normalized to equal protein concentrations with the lysis buffer and protein immunoprecipitations performed.
using appropriate antibodies. Immune complexes were subsequently collected with Protein A-Agarose (SantaCruz), and, after several washes with the lysis buffer, proteins were eluted in Laemmli buffer [28], boiled, and subjected to SDS-PAGE. Western Blots were performed as previously described [29] using chemiluminescence (ECL Plus, GE Healthcare, UK). When mentioned, equal amounts of total cell proteins were subjected to SDS-PAGE and subjected to Western Blot analysis following the same protocol.

2.6. In Vitro Kinase Assays. Immunoprecipitated proteins immobilized on Protein A beads or recombinant GST fusion proteins were washed five times with lysis buffer and three times with the kinase buffer before being divided into two aliquots, which were, respectively, subjected to in vitro kinase assay and immunoblot. For CKI activity, the kinase buffer was composed of 20 mM Hepes, pH 7.5, 1 mM dithiothreitol (DTT), 5 mM MgCl2, 10 mM β-glycerophosphate, and 5 μg of α-casein as exogenous substrate. For all assays, following a preincubation at 30 °C for 5 min, the reactions were initiated by adding [γ-32P]-ATP (50 μM, 5–10 μCi) (DuPont, NEN) and further incubated for 20 min at 30 °C. The reactions were stopped by adding Laemmli buffer, boiled, subjected to SDS-PAGE and immunoblot. Phosphorylation of exogenous substrates was analyzed by densitometry (Imaging Densitometer, Model GS-800, BioRad). To assess whether Ctky2 phosphorylates RhoA in vitro, 200 ng of purified recombinant GSTCKI2 full length (FL) or truncated of its C-terminal (∆C-term) were incubated with 1 μg of purified recombinant RhoA as reported above.

2.7. Cell Proliferation. Proliferation of stable fibroblast cell lines was evaluated by counting the number of cells, different times after plating. Cells were seeded at 5 × 10^3 cells/60 mm plate, in triplicate for each time points and cell lines. On days 3, 5, and 7 after plating, the cells were trypsinized and counted using a hemocytometer.

2.8. 3H-Thymidine Incorporation. Cells were plated at 2 × 10^4 cells/well in 24 wells plates and grown for 24 hours in DMEM containing 10% FBS. The next day, the cells were starved for 36 hours in DMEM supplemented with 0.1% BSA. At the end of the starvation period, the medium was replaced by fresh starving medium with or without FBS at 2.5% or PDGF at 25 ng/mL and the cells incubated for an additional 24 hours. During the last 8 hours of stimulation, 0.5 μCi of 3H-Thymidine was added. Thymidine incorporation was stopped by replacing the medium by cold TCA (10%) and further incubation at 4 °C. Precipitated material was then solubilized in 0.3 N NaOH and incorporated 3H-Thymidine counted by liquid scintillation using a LKB 1219 Rack Beta Liquid scintillation Counter.

2.9. DNA Laddering. Following washes with PBS, serum growing cells in culture dishes were directly lyzed in 0.5 mL of DNAzol genomic isolation reagent (Molecular Research Center, Inc., Cincinnati, OH). The resulting lysates were subjected to repeated pipetting and DNA precipitation performed by adding 0.25 mL of 100% ethanol. Samples were mixed by inverting the tubes 5–8 times and kept at room temperature for 3 min. Precipitated DNA was then spooled using a pipette tip, washed twice in 70% ethanol, and dissolved in water. Samples of total DNA were separated on 1.8% agarose gel and stained with ethidium bromide. As positive control, primary rat thymocytes maintained in culture in DMEM supplemented with 10% FBS were treated with 10 μg/mL of anisomycin for 24 hours. Thymocytes were collected by centrifugation, washed with PBS and genomic DNA prepared as described above.

2.10. Cell Cycle Analysis. For flow cytometry analysis (FACS), 1 × 10^6 of serum growing cells were collected, fixed in 70% ethanol following incubation for 15 min on ice and storage for at least 1 hour at −20 °C. Fixed cells were washed in cold PBS, and stained with propidium iodide (PI, Sigma) using a solution containing 50 μg/mL of PI and 10 μg/mL of RNase in PBS at 37 °C for 30 min. Quantification of cell populations in different phases of the cell cycle was determined using the Cell Quest software (Becton Dickinson, CA).

2.11. Cell Morphology and Actin Staining. Cells plated on coverslips were rinsed with PBS before being fixed for 10 min at room temperature in 4% formaldehyde/PBS. Following fixation, coverslips were rinsed with PBS and the cells permeabilized in 0.2% Triton X-100/PBS for 5 min at room temperature. For filamentous actin staining, cells were incubated with rhodamine-conjugated phalloidin (0.1 μg/mL; Sigma, Oakville, ON, Canada) or phalloidin-coupled to Alexa Fluor 488Fluor for 30–60 min at room temperature. For HA-staining, we used the commercially available anti-HA 12CA5 (Roche Applied Science). Coverslips were washed with PBS and water prior to being mounted with Mowiol and examined on a Zeiss Axiosvert 200 microscope at 40X or 63X using Zeiss oil immersion. Fluorescence images were subsequently captured using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.). Images were transferred to Adobe Photoshop and assembled with PowerPoint.

2.12. Rho Activation Assays. Essentially, levels of activated RhoA (RhoA-GTP) were assessed using the Rho activation kit purchased from Millipore (cat. no. 17–294). Briefly, serum growing fibroblasts (R2Zeao and Z23), about 70% confluent, were transiently transfected with a vector-encoding Myc-tagged RhoA (100 ng) using Lipofectamine Plus (Invitrogen). Cells lysates prepared 16 hours after transfection were mixed with 60 μg of recombinant GST-Rhotekin Rho binding domain previously isolated on beads. Following 45 min at 4 °C, beads were washed three times, boiled in Laemmli sample buffer, and bound proteins separated on a 12% SDS-polyacrylamide gel. Levels of Myc-tagged RhoA proteins bound to the fusion protein or present in the whole cell lysates were evaluated by western blotting with a rabbit polyclonal anti-Rho antibody (RhoA, B, and C) provided with the kit and ECL Plus detection as reported above.
2.13. Cells Stimulation. Cells (6 × 10^4) were plated on coverslips 24 hours prior to be serum starved for 24 hours in DMEM/0.1% BSA and subsequently treated with 50 ng/mL of lysophosphatidic acid (LPA, Sigma) for 30 min at 37°C or overnight. For roscovitine experiments, we treated the cells overnight with 25 μM roscovitine (Sigma). Control cells were exposed to equivalent volume of vehicle. Cells were then washed, stained for filamentous actin using phalloidin and mounted for immunofluorescence microscopy or processed for western blot analysis as previously described.

3. Results

3.1. CKIγ2 Overexpression in Fibroblasts Alters Cell Morphology and Inhibits Actin Stress Fibers Formation in a Kinase-Dependent Manner. To investigate the role of CKIγ2 in mammalian cells, we generated fibroblasts that stably overexpress CKIγ2 by transfecting a plasmid encoding N-terminal HA-tagged wild-type CKIγ2 [29]. Fibroblasts transfected with an empty plasmid are considered as control. We selected a pool of empty plasmid transfected cells (R2Zeo) as control and three independent clones expressing different levels of the 50–55 kDa HACKIγ2 protein (A20 < Z6 < Z23) to further study (Figure 1(a)). We demonstrated the activity of HA-CKIγ2 by performing in vitro kinase assays on HA immunoprecipitates (IP) using α-casein as exogenous substrate (Figure 1(b)). Visual examination of these cells foremost revealed that fibroblasts overexpressing higher levels of CKIγ2 (Z6 and Z23) presented marked change of morphology when compared with fibroblasts overexpressing lower levels of CKIγ2 (A20) or mock-transfected fibroblasts (R2Zeo) (Figure 1(c)). We observed
that cells harboring higher levels of CKI2 (Z6 and Z23) lost their fibroblastic elongated shape to acquire a more rounded morphology. Actin staining with phalloidin demonstrated that the rounded shrunken morphology of these cells (Z6 and Z23) is associated with a drastic decrease in actin stress fibers (Figure 1(d)).

To assess whether loss of actin stress fibers in fibroblasts overexpressing CKI2 affects cell motility, we compared the migratory activity of fibroblasts overexpressing CKI2 (Z23) with control fibroblasts (R2Zeo) using in vitro wound healing assays. To ensure that cells in the wounded area result from cell motility, rather than proliferation, fibroblasts were deprived from serum for 24 hours prior to performing the wound. As shown in Figure 2, fibroblasts that overexpress CKI2 did not migrate and fill the wounded area at a rate comparable to control fibroblasts. Altogether, these observations indicate that overexpression of CKI2 in fibroblasts induces dissolution of actin stress fibers and impairs cell motility in vitro.

We next investigate whether the kinase activity is required for CKI2 to inhibit the formation of actin stress fibers.

For this, we generated two independent clones of fibroblasts stably overexpressing a kinase deficient form of CKI2 (KD1, KD30) at levels almost comparable to wild-type CKI2 levels detected in the Z23 cell line (Figure 3(a), upper panel). As expected, CKI2 KD (K75R) is devoid of catalytic activity as shown by the absence of α-casein phosphorylation in HA-immunoprecipitated CKI2 KD in in vitro kinase assays (Figure 3(a), lower panel). However, we observed similar to control cells (R2Zeo) morphology and levels of actin stress fibers organization in fibroblasts overexpressing kinase deficient CKI2 (KD) (Figure 3(b)). This demonstrates that the kinase activity of CKI2 is required for the inhibition of actin stress fibers formation.

To demonstrate that the regulation of actin stress fiber formation by CKI2 occurs not only in overexpressing conditions, we assessed actin stress fibers in HaCaT human keratinocytes transiently transfected with two siRNAs (R1, R2) derived from short hairpin-type RNA constructs targeting independent coding regions of hCKI2 that have been reported to effectively downregulate CKI2 in these cells [30]. As shown in Figure 4, HaCaT cells treated with CKI2 siRNAs substantially present increased formation of stress fibers, supporting a physiological role for CKI2 in regulating actin cytoskeleton reorganization.

3.2. CKI2 Overexpression in Fibroblasts Decreases RhoA Protein and RhoA-GTP Levels. Formation of actin stress fibers is under the control of the small GTPases Rho [3]; therefore, we first compared the levels of RhoA protein in fibroblasts overexpressing CKI2 with control fibroblasts (Figure 5(a)). Interestingly, we found that overexpression of CKI2 results in decreased levels of the RhoA proteins, suggesting that dissolution of actin stress fibers in CKI2 overexpressing fibroblasts might be due to low levels of RhoA proteins that yield to nonefficient RhoA signaling activity. To further investigate this point, we expressed Myc-RhoA in fibroblasts overexpressing or not CKI2 and determined the levels of active Myc-RhoA-GTP by measuring the amount of Myc-RhoA proteins bound to a GST fusion protein encoding the Rho-binding domain of Rhotekin. Consistent with decreased actin stress fibers and lower RhoA protein levels in fibroblasts overexpressing CKI2, we found lower levels of activated RhoA (Myc-RhoA-GTP) as well as total Myc-RhoA in cells overexpressing higher levels of CKI2 (Figure 5(b)). To further support that increased expression of CKI2 downregulates RhoA protein levels, we transiently transfected Rat-2 fibroblast with increasing amounts of plasmid encoding HA-CKI2 and determined the expression levels of HA-CKI2 and RhoA in total cell lysates by western blotting. In agreement with decreased levels of RhoA protein in fibroblasts overexpressing high levels of CKI2 (Z23), transient expression of high levels of CKI2 leads to lower levels of RhoA protein (Figure 5(c)). Altogether, these data suggest that CKI2 contributes to lowering the expression or enhancing the degradation of RhoA and this could result in attenuated RhoA signaling.

To determine whether fibroblasts overexpressing CKI2 can still be challenged by external stimuli to build up actin
Figure 3: The catalytic activity of CKIγ is required to induce change in cell morphology and actin stress fibers in fibroblasts. (a) Isolated clones of fibroblasts stably transfected with a plasmid encoding HA-tagged wild-type CKIγ (Z23), HA-tagged kinase deficient CKIγ (KD1 and KD30), or pool of stable fibroblasts transfected with an empty plasmid (R2Zeo) were analyzed for HA-CKIγ expression by HA Western Blot (WB) on HA immunoprecipitates (IP) and CKIγ activity as determined on HA immunoprecipitates from equal amounts of protein normalized cell lysates and *in vitro* kinase assays using α-casein as substrate. KD1 and KD30: clones that overexpress kinase deficient CKIγ at the same levels as cells stably overexpressing wild-type CKIγ (Z23). (b) Morphology of serum growing cells was visualized by phase contrast microscopy (40X) (upper panels) and actin organization by actin staining with rhodamine-conjugated phalloidin (63X) (lower panels).

Figure 4: Increased actin stress fibers in HaCaT cells transiently transfected with hCKIγ siRNAs. HaCaT cells transiently transfected with siRNA control or siRNAs targeting two independent coding regions of hCKIγ (R1, R2) were subjected to actin staining using phalloidin-coupled to AlexaFluor 488. Pictures were taken at 63X.
stress fibers, we treated these cells with the serum-borne phospholipid lysophosphatidic acid (LPA), a G-protein-coupled receptor agonist which regulates the assembly of actin stress fibers through the activation of RhoA [31]. Actin staining of fibroblasts expressing high levels of HA-CKIγ2 in response to LPA stimulation at 50 ng/mL for 10–30 min revealed that, in all conditions, LPA treatment results in formation of actin stress fibers (Figure 6(a)). Finally, actin stress fibers could be rescued by expressing a constitutively active RhoA (RhoAL63) in fibroblasts overexpressing CKIγ2. Altogether, these data suggest that signaling downstream of RhoA is intact in fibroblasts overexpressing CKIγ2 and it also could be efficiently challenged to lead to the formation of actin stress fibers (Figure 6(b)). Overall, our observations provide strong evidence supporting that CKIγ2-mediated inhibition of RhoA-dependent formation of actin stress fibers is reversible and could result from impaired expression and activation of the GTPases Rho.

3.3. RhoA Is Not Phosphorylated by CKIγ2 In Vitro. As serine phosphorylation of Rho proteins negatively regulates their activity, we determined whether CKIγ2 could directly phosphorylate RhoA in vitro. For this, we incubated GST fusion protein encoding CKIγ2 full length (FL) or truncated with its noncatalytic C-terminal domain deleted (ΔC-term), with recombinant RhoA in presence of γ-32P ATP and assessed 32P labeling of RhoA upon SDS-PAGE and autoradiography. As shown in Figure 7, CKIγ2 full length and CKIγ2 deleted of its C-terminal domain autophosphorylate in vitro, suggesting that these are active protein kinases. In contrast, RhoA was not phosphorylated by either GST-CKIγ2 constructs, suggesting that in vivo CKIγ2 does not induce actin stress fibers disassembly by directly phosphorylating and inhibiting RhoA.

3.4. CKIγ2 Overexpression in Fibroblasts Inhibits Cell Proliferation and Delays Cell Cycle Progression in G1. In addition to the effect of overexpressing CKIγ2 on cell morphology, we found that fibroblasts overexpressing CKIγ2 proliferate at a significant slower rate compared with control fibroblasts (Figure 8(a)). In addition, decreased proliferation appears to correlate with the extent of CKIγ2 overexpression. Diminished proliferation in cells overexpressing CKIγ2 was further confirmed by decreased incorporation of 3H-thymidine into DNA in response to PDGF, a potent mitogenic factor for fibroblast [32], or serum over a 24-hour period of stimulation (Figure 8(b)). For an unknown reason, incorporation of 3H-thymidine in response to PDGF or serum stimulation
in fibroblasts overexpressing higher levels of CKIγ2 (Z6 and Z23) is often decreased compared with their respective unstimulated basal levels (Z6: Bas 5,300 ± 196, PDGF 3,205 ± 103, FBS 4,098 ± 110; Z23: Bas 8,071 ± 192, PDGF 3,672 ± 212, FBS 6,853 ± 327 cpm). Therefore, to exclude cell death as an important factor contributing to decreased proliferation, all cell lines were subjected to DNA laddering assay (Figure 8(c)) and DAPI staining (data not shown). As a positive control for DNA laddering, we used primary cultured rat thymocytes treated for 24 hours with anisomycin (10 ug/mL). Using both approaches, we established that apoptosis is not responsible for the apparent decrease in proliferation of cells overexpressing CKIγ2. In agreement, significant increase in doubling time calculated from growth curves for all aforementioned cell lines overexpressing CKIγ2 compared with control fibroblasts suggests that overexpression of CKIγ2 increases cell cycle duration (Table 1). To test this hypothesis, we performed FACS analysis to determine the distribution of actively serum growing asynchronized cells stably overexpressing CKIγ2 throughout the different phases of the cell cycle. As reported in Table 2, 50% of control fibroblasts mock-transfected were detected in G1 and
the remaining cell population was evenly distributed into S and G2 phases (approximately 23%, resp.). In contrast, fibroblasts overexpressing CKI2 presented a significant larger population of cells in G1 (63–70%) and a reduced percentage of cells in S and G2 phases (12–17%). Collectively, these results indicate that CKI2 inhibits cell proliferation by modulating cell cycle progression through G1.

3.5. Overexpression of CKI2 in Fibroblasts Increases Expression of the CDK Inhibitors p21Cip1 and p27Kip1 and the Tumor Suppressor p53. Consistent with a larger population of cells in G1 and reduced thymidine incorporation into DNA during the S phase of the cell cycle, earlier G1 phase cell cycle events could account for the antiproliferative effect of CKI2. To address this, we then compared the expression of the CDKIs p21Cip1 and p27Kip1 and the tumor suppressor p53 in fibroblasts overexpressing CKI2 with control fibroblasts. Our investigation revealed that inhibition of cell proliferation and delay in cell cycle progression in fibroblasts overexpressing CKI2 correlate with increased expression of p21Cip1, p27Kip1, and p53 (Figure 9(a)). Surprisingly, the effects of CKI2 on cell cycle regulators are independent of its catalytic activity as shown in fibroblasts overexpressing CKI2 kinase dead (KD1 and 30) (Figure 3) that still shows increased expression of p21Cip1, p27Kip1 and p53 proteins. This is in contrast with the effects of CKI2 on actin reorganization that require the catalytic activity of CKI2 (Figure 9(b)). Interestingly, increased expression of p21Cip1 and p27Kip1 appear, to be CKI2 dosage independent compared to increased expression of p53 which correlates with the levels of CKI2 overexpressed (Figures 9(a) and 9(b)). Overall, these findings demonstrate that CKI2 impairs cell proliferation by delaying cells in the G1 phase of the cell cycle. Likewise, the fact that fibroblasts overexpressing CKI2 are still evenly distributed in S and G2 phases of the cell cycle suggests that these steps proceed normally and that the effects of CKI2 on cell proliferation are restricted to the G1 phase of the cell cycle.

To further demonstrate a role for CKI2 on expression levels of CDK inhibitors, we compared p27Kip1 protein expression levels between HepG2 cells transfected with CKI2 specific siRNAs and scramble siRNA (Figure 10). Using this approach, we found that efficient downregulation of CKI2 in HepG2 cells leads to decreased expression of p27Kip1 proteins.

CKI2 is closely related to CKI1 and 3, and, like CKI2, CKI1 and 3 are believed to also be membrane associated due to a putative palmitoylation site present in their C-terminus [25]. In attempt to determine to what extent the effects of CKI2 on p27Kip1 and actin stress fiber are isoform specific, we failed to establish stable fibroblast cell lines overexpressing CKI1 or 3, most likely due to toxicity as reported by others [33]. This was also the case for transient overexpression of CKI1 in fibroblasts, while transient overexpression of CKI2 or γ3 was possible. Therefore, we carried out transient transfection of fibroblasts with an empty plasmid as control, or plasmid encoding either HA-tagged CKI2 or γ3 and monitored p27Kip1 levels and actin organization using these cells (Figure 11). As reported above, expression of HA-tagged CKI2 or γ3 was detected using total cell lysates in Western Blot with anti-HA antibody (Figure 11(a)). Interestingly, as observed in stable cell lines overexpressing CKI2, transient

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**Figure 7:** RhoA is not phosphorylated by CKI2 in vitro. Recombinant GST-CKI2 full length (FL) or C-terminal truncated (Δ C-term) (200 ng) purified on beads was incubated with recombinant RhoA (1 μg) in vitro kinase assays. Incorporation of 32P was revealed by autoradiography of the kinase reactions resolved on SDS-PAGE. Respective proteins are indicated, and equivalent amount of RhoA used in the assays was revealed by Coomassie blue staining.

**Table 1:** Calculated doubling time from cell proliferation assays.

| Clones               | Doubling time (hours) |
|----------------------|-----------------------|
| Control R2Zeo        | 17.3 (0.3)            |
| HA-CKI-γ2-Wild Type  |                      |
| A20                  | 18.8 (0.4)*           |
| Z6                   | 23.1 (0.5)*           |
| Z23                  | 27.1 (0.3)*           |

Values are the means (SEM) of assays. *P at least ≤ 0.005 compared to control.

**Table 2:** Cell distribution in different phases of cell cycle determined by FACS analysis.

| Clones       | % fluorescence at cell cycle phase |
|--------------|-----------------------------------|
|              | G1      | S        | G2       |
| Control R2Zeo| 50.3 (0.2) | 23.6 (0.2) | 23.8 (0.1) |
| HA-CKI-γ2-Wild Type |     |         |          |
| Z6           | 67.6 (0.4)* | 12.3 (0.4)* | 15.3 (0.6)* |
| Z23          | 70.2 (0.3)* | 17.0 (0.2)* | 12.1 (0.4)* |

Cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to flow cytometry analysis. Values are mean (SEM) of 4 assays. *P at least ≤ 0.00001 compared to control.
Figure 8: CKIγ2 overexpression in fibroblasts reduces cell proliferation. (a) Cell proliferation was examined by seeding each cell line in triplicate at $3 \times 10^5$ cells/60 mm plate on day 0. On days 3, 5, and 7, cells were trypsinized and counted. Results are expressed as the average ± SEM of three independent experiments and for Z6 and Z23, the symbols include the SEM. *Significantly different at least at $P < 0.01$ as determined by Student’s $t$-test when compared with R2Zeo cells. (b) Incorporation of $\text{H}^3$-thymidine in response to PDGF (25 ng/mL) or serum (2.5% FBS) stimulation was performed in indicated cell lines. Results are expressed as the fold of thymidine incorporation in presence of PDGF or FBS over basal unstimulated condition and are the mean ± SEM of three independent experiments performed in quadruplicate. *Significantly different at least at $P < 0.05$ as determined by Student’s $t$-test compared with respective basal condition. (c) Indicated serum growing cells were submitted to DNA laddering assays according to standard procedures described in Materials and Methods. As positive control for apoptosis, primary rat thymocytes in culture were exposed 24 hours to anisomycin (10 μg/mL).
overexpression of CKIγ2 increases p27Kip1 protein levels. However, this is also observed in fibroblasts overexpressing CKIγ3 (Figure 11(a)). More importantly, transient overexpression of either CKIγ2 or CKIγ3 negatively impacts actin stress fibers formation (Figure 11(b)). These results suggest that, like CKI2, CKIγ3 could also regulate the expression of CDK inhibitors and actin cytoskeleton reorganization, at least when overexpressed.

3.6. Inhibition of Roscovitine-Sensitive Cyclin-Dependent Kinases Reduces the Level of p27Kip1 and Rescues Actin Stress Fibers Formation in Fibroblasts Overexpressing CKIγ2.

Since phosphorylation of p27Kip1 at Ser10 increases its stability and cytoplasmic accumulation [11, 12] where it can bind and inhibit RhoA [10], we first determined whether p27Kip1 phosphorylation at Ser10 was increased in fibroblasts overexpressing CKIγ2. Indeed, we found that the level of p27Kip1 phosphorylated at Ser10 was higher in CKIγ2 overexpressing than in control fibroblasts (Figure 12(a)). In addition, we found that roscovitine, a potent inhibitor of cyclin-dependent kinases with good selectivity toward Cdk1, Cck2, Cdk5, Cdk7, and Cdk9 [34], not only strongly reduced the levels of p27Kip1 proteins (Figure 12(b)), but also rescued actin stress fibers formation in fibroblasts overexpressing CKIγ2 (Figure 12(c)). Interestingly, we observed that Cdk5, a roscovitine-sensitive cyclin-dependent kinase that is phosphorylated and activated by CKI [35–37] and known to affect actin dynamics by interacting and phosphorylating p27Kip1 at Ser10 [13], is equally expressed in fibroblasts independently of CKIγ2 expression levels (Figure 12(a)). Collectively our findings indicate an important role for CKIγ2 in modulating actin dynamics through a Cdks- p27Kip1 pathway, potentially implicating Cdk5.

4. Discussion

In this study, we provide evidence that the isoform γ2 of CKI prevents the formation of actin stress fibers and delays cell cycle progression in G1. We showed that CKIγ2 induces phosphorylation and accumulation of p27Kip1 and decreases expression levels of RhoA, which could result in inadequate levels of activated RhoA to sustain actin stress fibers formation in fibroblasts expressing higher levels of CKIγ2. Moreover, we demonstrate that the effects of CKIγ2 on p27Kip1 and actin stress fibers are dependent on a subset of Cdks. The findings that CKI regulates Cdk5 activity [35–37] and that Cdk5 is expressed in fibroblasts suggest that the effects of CKIγ2 on actin dynamics in fibroblasts overexpressing CKIγ2 potentially implicate activation of Cdk5. Several studies indicated that Cdk5 affects actin remodeling in neuronal cells [13, 38–41]. In addition, recent evidence point to a critical role of Cdk5 in the regulation of p27Kip1 stability and cytoplasmic retention by directly phosphorylating p27Kip1 on Ser10 [13]. Interestingly, a role...
Figure 11: Overexpression of either CKIγ2 or CKIγ3 in fibroblasts increases expression of p27kip1 and inhibits formation of actin stress fibers. (a) Fibroblasts transiently transfected with plasmids encoding either HA-CKIγ2 or HA-CKIγ3 were analyzed for HA-tagged proteins and p27kip1 protein expression levels by Western Blot. The arrow represents HA-CKIγ2 or HA-CKIγ3, while nonspecific bands are indicated by asterisks. Actin was used as loading control. (b) Similar fibroblasts were stained for HA or actin organization using phalloidin coupled to AlexaFluor 488. Arrows indicate HA-positive cells, while arrow heads point nontransfected cells. Pictures were taken at 63X.

for p27kip1 in the regulation of RhoA activation [10] has been reported. Indeed, p27kip1 directly interacts with RhoA, inhibiting RhoA activation by interfering with RhoGEFs. Therefore, these findings are consistent with our model suggesting that CKIγ2 regulates actin remodeling through a Cdk5-p27kip1-RhoA pathway (Figure 13).

The yeast homologs of the mammalian CKIγ isoforms (Yck1/2, Cki1+/2+) [26] have been implicated in various biological functions. In S. cerevisiae, independent loss of function of the YCK1 and YCK2 genes did not alter growth, but simultaneous loss of function of both genes resulted in lethality [42]. This established the YCK genes as an essential genes pair. In contrast, in S. pombe, gene disruption experiments showed that neither cki1+ nor cki2+ is essential for cell viability [43]. However, overexpression of cki2+, but not cki1+, resulted in growth inhibition accompanied by aberrant morphology. This suggests that, despite overall similarity in structure, high homology in amino acids
sequence and probable overlap in substrate specificity, close related isoforms might have non overlapping functions and play distinct role in cells.

In this study, we showed that CKIγ2 stably overexpressed in fibroblast, alters cell morphology and formation of actin stress fibers concomitant with lower levels of activated RhoA, a small GTPase that regulates actin stress fibers formation in response to growth factors [3]. Interestingly, actin stress fibers were restored by directly activating RhoA signaling following LPA treatment or expression of a constitutively active RhoA, suggesting that CKIγ2 regulates upstream events leading to RhoA expression and activation. Meanwhile, we also found that CKIγ2 increases expression of the tumor suppressor p53 and the CDK inhibitors p21Cip1 and p27Kip1 and negatively regulates cell proliferation by delaying cell progression through G1. To explain poor proliferation of CKIγ2 overexpressing fibroblasts, we propose that level of RhoA activity in these cells is too low to efficiently counteract the induction of the CDK inhibitors and promote adequate timing of expression of the cyclin D1, both processes normally under the control of RhoA [44–46]. Interestingly, Cdk5 activation in neuronal cells occurs only in postmitotic neurons [47], suggesting that, in fibroblasts overexpressing CKIγ2, modulation of the cell cycle resulting in decreased mitotic activity may precede and be required for the activation of Cdk5 by CKIγ2. Although additional experiments are required to investigate this point, here we propose a model in which CKIγ2 induces the activation of

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**Figure 12:** Roscovitine reverses the effects of CKIγ2 overexpression on p27Kip1 and actin stress fibers. (a) From indicated cell lines, equivalent amount of total cell lysate proteins was subjected to Western Blot analysis using specific antibodies against total and phospho-Ser10 p27Kip1, Cdk5, and HA. β-Actin was used as loading control. (b) Equivalent amount of total cell lysate proteins from indicated cells treated or not with roscovitine that were subjected to Western Blot analysis using specific antibodies against p27Kip1 or β-actin was used as loading control. (c) Cells overexpressing CKIγ2 were incubated with roscovitine (25 μM, 16 hrs) before to be stained with phalloidin to visualize actin organization. Images were taken at 63X.
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