Cardiovascular, Pulmonary and Renal Pathology

Cytoplasmic YY1 Is Associated with Increased Smooth Muscle-Specific Gene Expression

Implications for Neonatal Pulmonary Hypertension

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Immediately after birth the adluminal vascular SMCs of the pulmonary elastic arteries undergo transient actin cytoskeletal remodeling as well as cellular dedifferentiation and proliferation. Vascular smooth muscle phenotype is regulated by serum response factor, which is itself regulated in part by the negative regulator YY1. We therefore studied the subcellular localization of YY1 in arteries of normal newborn piglets and piglets affected by neonatal pulmonary hypertension. We found that YY1 localization changed during development and that expression of γ -smooth muscle actin correlated with expression of cytoplasmic rather than nuclear YY1. Analysis of the regulation of YY1 localization *in vitro* demonstrated that polymerized γ -actin sequestered EGFP-YY1 in the cytoplasm and that YY1 activation of *c-myc* promoter activity was inhibited by LIM kinase, which increases actin polymerization. Consistent with these data siRNA-mediated down-regulation of YY1 in C2C12 cells increased SM22- α expression and inhibited cell proliferation. Thus, actin polymerization controls subcellular YY1 localization, which contributes to vascular SMC proliferation and differentiation in normal pulmonary artery development. In the absence of actin depolymerization, YY1 does not relocate to the nucleus, and this lack of relocation may contribute to the pathobiology of pulmonary hypertension. (*Am J Pathol* 2005, 167:1497–1509)

During normal vascular development undifferentiated mesenchymal cells are recruited to an endothelial tube where they differentiate into vascular smooth muscle

cells (VSMCs) in response to various stimuli.¹ Once this process has been completed, the VSMCs in most vessels remain fully differentiated in the absence of pathological stimuli. This is not so, however, in the pulmonary circulation in which pulmonary arterial pressure and resistance fall dramatically after birth as blood flow increases. The pulmonary arterial VSMCs nearest the lumen change shape rapidly and undergo transient dedifferentiation with actin depolymerization and cytoskeletal remodeling, accompanied by a burst of cell replication.² These changes do not occur when there is maintenance of a high vascular resistance due to neonatal hypoxia.^{3–5}

There is an association between the level of expression of genes encoding cytoskeletal proteins and actin polymerization that is not limited to VSMCs. This association occurs in many cell types including other muscle cells and fibroblasts. Indeed, increased actin polymerization and the concomitant reduction of the monomeric, G-actin pool may be important contributors to muscle cell differentiation. Modulation of the activity of the transcription factor serum response factor (SRF) has been implicated in the modulation of gene expression by actin polymerization.^{6–8} Actin-dependent changes in SRF activity are regulated by one co-activator (Mal) and one inhibitory transcription factor (yin yang 1, YY1). Miralles and colleagues⁶ showed that Mal acted as a SRF co-activator and was localized primarily in the cytoplasm of cells in which actin was depolymerized, but in the presence of F-actin it translocated to the nucleus and increased SRF activity at its binding site (the CARG box).⁶ We previously showed that in VSMCs and C2C12 muscle cells the activation of the SM22 α promoter by increased actin poly-

Supported by the British Heart Foundation (grant PG/02/031/13573).

Accepted for publication August 16, 2005.

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merization required an intact SRF binding site and an intact inhibitory YY1 binding site.⁷ Increased actin polymerization resulted in reduced YY1 DNA binding activity and increased SRF DNA binding in the same cells. Taken together these data imply a dual control mechanism for the regulation of SRF activity at CArG boxes present in the promoters of genes encoding cytoskeletal proteins by actin polymerization.

YY1 is an important inhibitor of muscle cell differentiation and expression of muscle-specific genes, including smooth muscle-specific genes^{7,9–12} that acts primarily through the inhibition of SRF activity at CArG boxes. Furthermore, the proteolysis of YY1 appears to be important in muscle cell differentiation.¹³ YY1 has also been shown to be an important activator of a number of genes associated with the cell cycle, including histone genes¹⁴ and the *c-myc* gene.^{15,16} This dual role of YY1 as an inhibitor of muscle cell differentiation and activator of proliferation makes it a good candidate regulator of the changes in pulmonary VSMC phenotype observed during early postnatal life. We therefore determined the temporal and spatial expression of YY1 in the pulmonary arteries of normal and pulmonary hypertensive piglets by immunohistochemistry, and studied the localization of YY1 in cultured muscle cells. These experiments suggested that the localization of YY1 is regulated by the actin cytoskeleton. We therefore studied the effect of agents that alter actin polymerization on the localization of YY1 in muscle cells *in vitro*. We also determined the effect of YY1 and agents that modify the cytoskeleton on the *c-myc* promoter. These studies implicate YY1 in the remodeling of the pulmonary vasculature immediately after birth.

Materials and Methods

Cloning and Polymerase Chain Reaction (PCR)

To generate reporter vectors for the *Myc* promoter, a 540-bp fragment of the *c-myc* promoter (comprising bases 2081 to 2618 of HSMYCC) was amplified from human genomic DNA using the primers 5'-ATAAAGCTTAGCAAAAGAAATGGTATTCGCGCGTA-3' and 5'-ATATCTAGAAAAGCCCCCTATTCGCTCCGGATCTC-3'. This fragment was cloned into pGEM-T Easy (Promega, Madison, WI), sequenced, and then subcloned into pCAT-basic and pGL3-basic plasmids (Promega). The expression vectors pCYY1 and pLIMK have been described previously.⁷

The vector pEGFP-YY1 was generated by PCR amplification of EGFP from pCAGGS-EGFP using primers 5'-GGCAAAGAATTCGCCACCA-3' and 5'-GATATCC-TTGACAGCTCGTCATGCCGTGAGTG-3' and amplification of YY1 using primers 5'-GATATCGCCTCGGGCGA-CACCCTCTACATC-3' and 5'-TCTAGATCACTGGTTGT-TTTGGCTTTAGCGTGTG-3'. The EGFP cDNA fragment was cloned into pGEM-T Easy, digested with *EcoRI* and *EcoRV*, and cloned into *EcoRI/EcoRV*-digested pCDNA3 to generate pCEGFP. The YY1 fragment was cloned into pCEGFP using *EcoRV* and *XbaI* to generate pEGFP-YY1.

To make pIRES-YY1 the EGFP-YY1 sequence was removed from pEGFP-YY1 using *BamHI* and *XbaI* and was then cloned into *SmaI/XbaI*-digested pIRES-neo (Clontech, Palo Alto, CA). The cofilin and luciferase sequences were removed from pCDNA3-cofilin and pGL3 using *XbaI/EcoRI* and *NcoI/EcoRI*, respectively, and inserted into *BamHI*-digested pIRES-YY1 using blunt-end ligation. PCR was performed as described previously¹⁷ using Hi Fidelity *Taq* polymerase (Roche, Indianapolis, IN). All restriction enzymes were obtained from NEB (Beverly, MA).

Cell Culture, Luciferase, and CAT Assays

PAC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10% v/v) and were subcultured 1:6 by trypsinization at confluence. C2C12 and P19 cells were cultured as previously described.¹⁸ C2C12 cells were seeded into 24-well plates at a density of 1.8×10^4 cells/well. After 24 hours the cells were washed with serum-free DMEM and incubated with a mixture of 400 ng of DNA (prepared as indicated in the figure legends) complexed with 2 μ l of lipofectamine (Invitrogen, Carlsbad, CA) in OptiMEM (Invitrogen). Cells were incubated with the lipid/DNA mixture for 5 hours before the medium was replaced with DMEM supplemented with 10% fetal calf serum. Forty-eight hours after transfection, *Firefly* and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay (Promega, Madison, WI).

Twenty-four hours before transfection, P19 cells were seeded into six-well plates at a density of 2×10^5 cells/well. A 10- μ l aliquot of lipofectin (Invitrogen) in 100 μ l of OptiMEM (Invitrogen), prepared as described by the manufacturer, was mixed with a total of 2 μ g of plasmid (containing 1 μ g CAT vector, 0.5 μ g pCMV β gal, and 0.5 μ g comprised of 0.25 μ g of pCDNA3 plus 0.25 μ g of test vector or 0.25 μ g of each of two test vectors as detailed in the figure legend) in 100 μ l of OptiMEM and incubated at room temperature for 15 minutes. The cells were washed with serum-free α -MEM (Invitrogen) before the lipofectin/DNA mixture was added. After 18 hours the medium was changed to α -MEM supplemented with 10% (v/v) fetal calf serum. The cells were harvested 48 hours after transfection, lysed, and assayed for CAT activity and β -galactosidase activity as described previously.^{17,18}

YY1 Localization in Vitro and in Vivo

YY1 Localization in Vivo in Porcine Pulmonary Arteries

The pulmonary vasculature of the newborn piglet resembles that of the newborn child and therefore has been used extensively as an experimental model of normal development.^{19–23} In addition, exposing newborn piglets to chronic hypobaric hypoxia (50.8 kPa) results in maintenance of a high pulmonary vascular resistance and pulmonary hypertension.^{3,4,24} In the present study, nor-

mal Large White piglets were sacrificed within 5 minutes of birth and at 3, 6, or 14 days of age ($n = 5$ at each age). Other animals were exposed to chronic hypoxia from 3 days to 6 (6-day hypoxic) or 14 (14-day hypoxic) days of age ($n = 4$ for each time point) and sacrificed immediately after removal from the chamber. All piglets were sacrificed by an intraperitoneal injection of sodium pentobarbital (100 mg/kg). All animals received humane care in compliance with the British Home Office Regulations and the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Blocks of tissue were taken from the mid-lung region of all animals and processed for histology, 5- μ m sections were cut, and adjacent sections were stained with antibodies to YY1 (sc-281; Santa Cruz Biotechnology, Santa Cruz, CA) or γ -smooth muscle-specific actin (γ -SM actin) (Dr. J. Lessard, University of Cincinnati, Cincinnati, OH). After incubation with biotin-conjugated goat anti-mouse antibody, binding was visualized by diaminobenzidine (StrepABComplex; DakoCytomation, Ely, UK) before light counterstaining with Mayer's Hemalum. Nuclear staining with YY1 was assessed in duplicate sections without counterstaining. Control sections were incubated with 4% goat serum instead of primary antibody.

YY1 Localization in Cultured Cells

C2C12 or PAC-1 cells were seeded into four-well Lab-Tek chamber slides (Nunc, Naperville, IL) at a cell density of 1.5×10^4 cells/well 24 hours before transfection. Cells were transfected with 470 ng of DNA (pYY1-luc or pYY1-cof) and with 2 μ l of lipofectamine as described above and incubated overnight in DMEM supplemented with 10% fetal calf serum. The cells were treated with cytochalasin D (Calbiochem, Nottingham, UK) latrunculin B, or jasplakinolide (Molecular Probes, Eugene, OR) for 6 hours, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes, and stained with 0.5 μ g/ml of phalloidin TRITC (Sigma-Aldrich, St. Louis, MO) and 300 nmol/L DAPI (Molecular Probes). Images were generated using an Olympus TX 70 inverted microscope coupled to an Ultraview LCI confocal imaging system (Perkin Elmer).

Influence of Actin Mutants on YY1 Localization

C2C12 or PAC-1 cells were seeded into four-well Lab-Tek chamber slides (Nunc) at a cell density of 1.5×10^4 cells/well 24 hours before transfection. The cells were washed with serum-free DMEM and incubated with a mixture of 470 ng of DNA comprised of 350 ng EGFP-YY1-cofilin vector and 120 ng of pEF-FLAG actin expression plasmids (as indicated in the figure legends) complexed with 2 μ l of lipofectamine (Invitrogen) in OptiMEM (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after transfection the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilized for 10 minutes in 0.3% Triton X-100 in PBS.

Cells were incubated for 30 minutes in PBS containing 1% bovine serum albumin, 1 hour with anti-M2 flag antibody (Sigma-Aldrich), and 1 hour with anti-rabbit-Cy3 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) according to the manufacturer's instructions. Images were generated as described above. The actin and mutant actin expression plasmids were a gift from Prof. R. Treisman, Cancer Research UK London Research Institute, London, UK.

siRNA Transfection

The YY1-specific siRNA (siY1) used in this study has been described previously.²⁵ The control siRNA (siY2) was a double-stranded 20-mer designed in the YY1 non-coding sequence and corresponds to the nucleotide sequence: 5'-TTCCAAGTGTGCATATTGTA-3'. Both double-stranded RNAs were made by Dharmacon Research, Inc. (Lafayette, CO). The siRNA duplexes were transfected into C2C12 cells using lipofectamine as described below.

For Western blot and proliferation studies, 18,000 C2C12 cells were seeded into each well of a 24-well plate and cultured to 60% confluency. Cells were transfected as described above using 2 μ l of lipofectamine per well and 150 ng of siRNA. For RNA extraction, 5×10^5 cells were seeded into 100-mm Petri dishes and cultured to 60% confluency. Cells were transfected as described above using 32 μ l of lipofectamine per dish and 4.6 μ g of siRNA.

Western Blot Analysis

Cells were harvested 48 hours after transfection and lysed for 5 minutes at 4°C in sample buffer (2% sodium dodecyl sulfate, 2 mmol/L ethylenediamine tetraacetic acid, 20% glycerol, and 100 mmol/L Tris, pH 7.5). The protein concentration was determined²⁶ and dithiothreitol (25 mmol/L) was added. Protein samples (30 μ g) were denatured and solubilized by heating for 5 minutes at 95°C, electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes as described previously.⁷

Cell Proliferation Assay

After transfection cells were allowed to proliferate for 48 hours, and cell number was determined by a colorimetric assay using the CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega).

Reverse Transcription

Total RNA was extracted from C2C12 cells with the RNeasy kit (Qiagen, Valencia, CA). RNA concentration was quantified fluorimetrically using Ribogreen RNA-binding dye (Molecular Probes), according to the manufacturer's protocol. cDNA was synthesized from 250 ng of

RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

Quantitative Real-Time PCR Analysis

RNA from C2C12 cells was reverse-transcribed as described above. PCR was performed in a total volume of 25 μ l with 12.5 μ l of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), 2 μ l of cDNA, and 2.5 μ l of each primer (10 pmol/ μ l; forward: 5'-GCTGTGACCAAAACGATGGA-3'; reverse: 5'-GGCTGTCTGTGAAGTCCCTCTTA-3'). To assay 18S rRNA levels, real-time PCR was performed using a VIC-labeled TaqMan probe and TaqMan real-time PCR chemistry using an 18S rRNA detection kit (Applied Biosystems, Foster City, CA).

Reactions were performed in an ABI-Prism 7000 sequence detector (Applied Biosystems) in triplicate, initiated with hot-start by heating to 95°C for 10 minutes, and amplified using the following conditions: 95°C for 30 seconds and 60°C for 1 minute for 40 cycles. The final cycle was followed by an additional extension time of 10 minutes at 60°C. Analysis was performed after normalization of samples to their 18S rRNA expression levels as described previously.²⁷

Results

Expression and Localization of YY1 in Pulmonary Vascular Smooth Muscle *in Vivo*

In normal vessels γ -SM actin expression in the inner medial smooth muscle layers of the elastic arteries of 6-day-old piglets was reduced compared to the levels observed at birth (Figure 1). By day 14 both the distribution and staining intensity of γ -SM actin was similar to that seen at birth. In 6-day-old piglets exposed to chronic hypoxia from 3 days of age (3 days hypoxia) there was no reduction in γ -SM actin in inner medial layers, and staining was uniform across the media and of similar intensity to that seen at birth. In 14-day-old piglets exposed to chronic hypoxia from 3 days of age (11 days hypoxia), staining for γ -SM actin was uniform across the vessel wall and was consistently more intense than that seen in 14-day-old normal piglets. This increase in staining intensity is consistent with previous ultrastructural studies showing increased myofilament density in hypoxic animals.³

YY1 protein was expressed in the VSMCs of all pulmonary arteries of all normal and hypertensive piglets (Figure 1) but with spatial and temporal differences. In the elastic arteries of normal newborn, 6- and 14-day-old piglets YY1 was strongly and uniformly expressed in the nuclei of all of the VSMCs in all medial layers across the vessel wall (Figure 1), but expression in the VSMC cytoplasm varied with age. In newborns this staining was weak and uniformly expressed across all medial layers. At 6 days the intensity of cytoplasmic immunostaining for YY1 appeared less than at birth in the inner medial layers. By 14 days it had increased in all medial layers and was now particularly strong in the outer layers. In the elastic

arteries of 6-day-old hypoxic animals, YY1 was uniformly expressed in the cytoplasm of all medial layers. Similarly, a uniform cytoplasmic staining for YY1 was observed in the elastic arteries of 14-day-old hypoxic piglets, but in these arteries there was a striking absence of immunostaining in many of the nuclei in the outer medial layers. In the smaller muscular pulmonary arteries, YY1 protein was present in both the nuclei and cytoplasm of all VSMCs in both normal and hypertensive animals (Figure 1). In all animals the bronchial SMCs showed strong and uniform cytoplasmic and nuclear expression of YY1 (Figure 1).

Comparison of YY1 cytoplasmic staining with that of γ -SM actin, in adjacent sections, revealed close spatial similarities in the intensity of staining (Figure 1). Thus in newborn and 14-day-old piglets, both γ -SM actin and YY1 were uniformly expressed across the elastic media whereas at 6 days of staining of both was weaker in the inner media. In the hypoxic, hypertensive 6-day-old animals, there was no loss of either γ -SM actin or cytoplasmic YY1 staining in the inner medial layers. These experiments indicate an association between the expression of SM γ -actin and the presence of cytoplasmic YY1 in remodeling pulmonary VSM.

Localization of YY1 in Muscle Cells *in Vitro*

C2C12 cells provide a readily manipulable muscle cell background in which changes in actin polymerization alter the activity of the promoters of genes that encode contractile proteins.⁷ To determine whether YY1 localization in muscle cells *in vitro* was restricted to the nucleus, C2C12 cells were immunostained for YY1 protein. Fluorescent staining for YY1 was readily detectable in both the nucleus and cytoplasm (Figure 2A). These data indicate that unlike many transcription factors, YY1 does not exist in the nucleus by default, suggesting that YY1 localization is regulated. To study the factors regulating the localization of YY1 in muscle cells *in vitro* we transfected C2C12 cells with an expression vector for an EGFP-YY1 fusion protein (pEGFP-YY1). Transfection of these cells with pEGFP-YY1 showed that like the native YY1 protein, the EGFP-YY1 fusion protein was distributed throughout the cell in both the nucleus and cytoplasm (data not shown).

One possible mechanism regulating the localization of YY1 was thought to be via the actin cytoskeleton. Therefore, cells were co-transfected with pEGFP-YY1 and a cofilin expression vector. The primary function of cofilin is to facilitate actin depolymerization, making it an important regulator of the cytoskeleton, and to mediate nuclear localization of actin.²⁸ In these experiments all of the cytoplasmic EGFP-YY1 protein relocated to the nucleus in the majority of the EGFP-YY1-expressing cells (data not shown). However, it was not possible to know whether or not the cells in which EGFP-YY1 remained detectable in the cytoplasm had also been transfected with the cofilin expression vector. Therefore, we cloned the EGFP-YY1 fusion protein under the control of an IRES with either luciferase (pYY1-luc) or cofilin (pYY1-cof) on the same mRNA, transfected the C2C12 cells, and found that lucif-

Figure 1. Localization of YY1 in the vessels of newborn piglets. Immunohistochemical localization of YY1 and SM α -actin in lungs of normal piglets at birth; 3, 6, and 14 days after birth; and of piglets exposed to chronic hypoxia from 3 to 6 (6-day hypoxic) or 14 (14-day hypoxic) days of age. Lungs from at least three different animals were analyzed at each time point for the amount of nuclear and cytoplasmic YY1. At each age representative areas of the same artery from adjacent sections were stained for γ -SM actin plus counter stain or YY1 without counter stain. Scale bar, 50 μ m.

erase did not modify the localization of EGFP-YY1 compared to transfection with pEGFP-YY1 alone (Figure 2B). However, in the presence of cofilin, all of the cytoplasmic EGFP-YY1 became localized to the nucleus (Figure 2C). Consistent with these data, immunofluorescence staining of the endogenous YY1 in cells transfected with the cofilin

expression vector alone showed a significant proportion of cells with markedly increased nuclear YY1 and little or no detectable cytoplasmic YY1 (data not shown). Taken together these data indicate that cofilin can modify the localization of cytoplasmic YY1 and that the EGFP-YY1 fusion protein is a reliable marker of YY1 localization.

Figure 2. Localization of YY1 in C2C12 cells. **A:** C2C12 cells were seeded into four-chamber Lab-Tek slides and grown for 24 hours. The cells were fixed, and the localization of YY1 was determined by immunofluorescence as described in Materials and Methods. **B and C:** C2C12 cells were transiently transfected with expression plasmids encoding EGFP-YY1-luciferase (**B**) and EGFP-YY1-cofilin (**C**). Twenty-four hours after transfection, the cells were fixed in paraformaldehyde and stained as described in Materials and Methods. The first column shows YY1 localization (EGFP), the second column shows F-actin staining (phalloidin), the third column shows nuclear staining (DAPI), and the fourth column shows a merge of the first three columns. Original magnifications, $\times 60$.

To confirm these data in a pulmonary SMC line, we transfected PAC-1 cells with the YY1-EGFP constructs (Figure 3). In these experiments PAC-1 cells transfected with pYY1-luc showed fluorescence in both the cytoplasm and nucleus (Figure 3A) whereas fluorescence in PAC-1 cells transfected with pYY1-cof was restricted to the nucleus (Figure 3B). These data indicate that the changes observed in the C2C12 cells were also observed in the SMCs.

Effect of Drugs that Modify Actin Polymerization on EGFP-YY1 Localization

Actin polymerization can be modified by a number of chemical agents including cytochalasin D (which sequesters actin dimers²⁹), jasplakinolide (which stabilizes actin filaments³⁰), and latrunculin B (which sequesters actin monomers³¹). The effect of these agents on the localization of EGFP-YY1 was studied in C2C12 cells transfected with pYY1-luc, in which EGFP-YY1 was present in both the nucleus and the cytoplasm. None of the drugs appeared to have any effect on the localization of EGFP-YY1 (Figure 4A). However, it was possible that the apparent lack of effect was due to YY1 binding so strongly to the nuclear matrix that any change in free

nuclear YY1 would be difficult to detect. We therefore investigated the effect of these drugs on the location of YY1 in cells transfected with cofilin, in which all of the YY1 was nuclear, making a change in cytoplasmic YY1 more obvious. In cells transfected with pYY1-cof, latrunculin B had no effect on the localization of EGFP-YY1, but both cytochalasin D and jasplakinolide increased the proportion of cells with detectable EGFP fluorescence in the cytoplasm (Figure 4B). To quantify the observed response, we measured the average fluorescence of the cytoplasm compared to that of the nucleus for each cell. In untreated cells and in cells treated with latrunculin B, cytoplasmic EGFP fluorescence was less than 5% of the nuclear fluorescence, whereas in cells treated with jasplakinolide or cytochalasin D the cytoplasmic fluorescence had an intensity of $\sim 30\%$ of the nuclear fluorescence in the same cell (Figure 4C). These observations indicate a relationship between actin polymerization and YY1 localization and are consistent with our previous data indicating that jasplakinolide reduces the binding of YY1 to DNA.⁷ Similar analysis in PAC-1 cells showed that jasplakinolide and cytochalasin D also caused a relocation of YY1 (Figure 3, C and D) into the cytoplasm of SMCs.

Figure 3. Localization of YY1 in PAC-1 cells. PAC-1 cells were transiently transfected with expression plasmids encoding EGFP-YY1-luciferase (**A**) and EGFP-YY1-cofilin (**B–D**). Twenty-four hours after transfection, the cells were fixed in paraformaldehyde and stained as described in Materials and Methods. The first column shows YY1 localization (EGFP), the second column shows DAPI staining, and the third column shows a merge of the first two columns and the corresponding images for F-actin staining (phalloidin). Cells were treated for 6 hours without (**A** and **B**) or with 0.25 $\mu\text{mol/L}$ jasplakinolide (**C**) or 1 $\mu\text{mol/L}$ cytochalasin D (**D**). After incubation the cells were fixed with paraformaldehyde and stained as described in Materials and Methods. Original magnifications, $\times 60$.

Effect of Mutant Actin on EGFP-YY1 Localization

A number of mutant actins have been developed that either cannot polymerize into actin filaments (R62D and G13R) or constitutively polymerize into actin filaments (V159N and S14C).³² In cells transfected with pYY1-cof and the wild-type actin, EGFP-YY1 was localized in the nucleus whereas staining for the FLAG-tagged actin was detected in the cytoplasm. A similar pattern of localization was observed for cells transfected with pYY1-cof and the expression vectors for the R62D and G13R forms of actin (Figure 5). By contrast, in cells transfected with pYY1-cof and the expression vectors V159N and S14C, EGFP-YY1 was localized in both the nucleus and the cytoplasm. Quantitation of the EGFP-fluorescence showed that, in cells co-transfected with wild-type β -actin or the actin mutants R62D and G13R, cytoplasmic fluorescence was less than 10% of the nuclear fluorescence, but in cells co-transfected with V159N and S14C, the intensity of cytoplasmic EGFP fluorescence was $\sim 50\%$ of nuclear fluorescence (Figure 5). V159N but not G13R

also caused a relocalization of YY1 out of the nucleus in PAC-1 cells indicating that the phenomenon is not restricted to C2C12 myoblasts (Figure 5). These data are consistent with the changes observed in response to jasplakinolide and cytochalasin D and indicate that an increase in polymerized actin causes nuclear YY1 to translocate to the cytoplasm.

Modulation of YY1-Dependent Gene Expression by Actin Polymerization

Because increased actin polymerization causes YY1 to translocate to the cytoplasm we predicted that it would increase cytoskeletal gene expression and reduce the expression of cell cycle genes. LIM kinase inactivates cofilin and thereby promotes actin polymerization, and we have previously shown that it increases SM22 α promoter activity by reducing YY1-dependent inhibition of SRF activity.⁷ Here we investigated the effect of LIM kinase on YY1-dependent activation of the promoter of the cell cycle-associated gene *c-myc*, which

A. EGFP-YY1-luciferase

Figure 4. Effect of cytochalasin D, jasplakinolide, and latrunculin B on YY1 localization in C2C12. C2C12 cells were transiently transfected with expression plasmids encoding EGFP-YY1 and luciferase (**A**) or EGFP-YY1 and cofilin (**B**). Twenty-four hours after transfection, cells were treated for 6 hours without (**Aa, Ba**) or with 1 μ mol/L cytochalasin D (**Ab, Bb**), 0.25 μ mol/L jasplakinolide (**Ac, Bc**), or 0.5 μ mol/L latrunculin B (**Ad, Bd**). After incubation the cells were fixed with paraformaldehyde and stained as described in Materials and Methods. The first column shows YY1 localization (EGFP), the second column shows nuclear staining (DAPI), and the third column shows a merge of the first two columns and F-actin staining (phalloidin). **C:** The effect of cytochalasin D (cytD), jasplakinolide (jasp), and latrunculin B (latB) on EGFP-YY1 relocalization induced by cofilin was evaluated by determining the intensity of cytoplasmic staining as a percentage of nuclear staining. Five different fields of the preparation were selected at random. Statistical analysis was performed using analysis of variance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Original magnifications, $\times 60$.

B. EGFP-YY1-cofilin

C. Quantification

is known to be activated by YY1 through a YY1 binding site at -260.¹⁵ We generated reporter vectors containing a 540-bp fragment of the *c-myc* promoter. YY1 had

a weak activating effect on this promoter (129% of control) in C2C12 cells whereas LIM kinase inhibited promoter activity by 33%. This inhibitory effect of LIM

A. EGFP-YY1-cofilin in C2C12 cells

B. Quantification

C. EGFP-YY1-cofilin in PAC-1 cells

Figure 5. Effect of actin mutants on YY1 relocalization induced by cofilin in C2C12. **A:** C2C12 cells were transiently transfected with expression plasmid encoding EGFP-YY1-cofilin and expression plasmid pEF-FLAG actin [wild-type, nonpolymerizing actin (G13R and R62D), or actin that favor F-actin formation (S14C or V159N)]. Cells were fixed in paraformaldehyde and stained as described in Materials and Methods. The first column shows YY1 localization (EGFP), the second column shows β -actin staining (anti flag, red), and the third column shows a merge of the first two columns. **B:** The effect of expressing the actin mutants on EGFP-YY1 localization was evaluated by determining the intensity of cytoplasmic staining as a percentage of nuclear staining. Five different fields of the preparation were selected at random. Statistical analysis was performed using analysis of variance ($*P < 0.05$, $**P < 0.01$). **C:** PAC-1 cells were transiently transfected with expression plasmid encoding EGFP-YY1-cofilin and expression plasmids for wild-type actin or the mutant actins G13R and V159N. Cells were fixed, stained, and imaged as above. The first column shows YY1 localization (EGFP), the second column shows β -actin staining (anti flag, red), and the third column shows a merge of the first two columns. Original magnifications, $\times 60$.

A. C2C12 cell

B. P19 cell

Figure 6. Modification of Myc promoter activity by YY1 and LIM kinase. **A:** C2C12 cells were transfected with pMyLuc and pRL-TK in the presence of pCDNA3 (control), pYY1, and/or pLIMK. Cells were harvested 48 hours after transfection, and *Firefly* and *Renilla* luciferase activities were determined. Data are presented as fold activation relative to transfection with pCDNA3 alone. Each experiment was performed in triplicate and repeated three times. The data presented are the means of three independent experiments. **B:** P19 cells were transfected with pMyCAT and pCMV β -gal in the presence of pCDNA3 (control), pYY1, and/or pLIMK. Cells were harvested 48 hours after transfection, and CAT activity and β -galactosidase activity were determined. Data are presented as fold activation relative to transfection with pCDNA3 alone for the same cell type. Each experiment was performed in triplicate and repeated three times. The data presented are the mean of three independent experiments.

kinase was reversed by co-transfection with YY1 (Figure 6A). These data imply that the activity of YY1 is inhibited by transfection with LIM kinase and that this inhibition can be overcome by the addition of exogenous YY1.

Confirmatory studies were performed in cells that lack endogenous YY1 DNA binding activity (P19 cells⁷). In these experiments expression of exogenous YY1 increased the activity of the *c-myc* promoter approximately threefold. LIM kinase had no effect on the basal activity of the promoter but reduced the stimulation of the promoter by YY1 by 50% (Figure 6B). These data indicate that YY1-dependent activation of the *c-myc* promoter is modified by actin polymerization.

Knockdown of YY1 Increases SM22 α Expression and Inhibits Cell Proliferation

To determine whether YY1 modified endogenous contractile protein gene expression and proliferation as well

as reporter gene activity, we analyzed the effect of YY1 knockdown on SM22 α gene expression and cell proliferation in C2C12 cells. Cells were transfected with siRNA duplexes and harvested 48 hours later for protein extraction, RNA extraction, or determination of cell number. Western analysis showed that YY1 protein was barely detectable in cells treated with an siRNA (siY1) previously shown to knock down YY1 expression,²⁵ whereas protein was readily detectable in cells treated with an siRNA (siY2) to bases 1707 to 1726 of YY1 (accession number BC055899). We therefore used siY2 as a control oligonucleotide. Quantitative RT-PCR for SM22 α mRNA showed that knockdown of YY1 by siY1 markedly increased the expression of SM22 α . Furthermore the number of cells present 48 hours after transfection was reduced by transfection with siY1 compared to transfection with siY2 (Figure 7). These data indicated that reduced levels of YY1 promote cell differentiation and inhibit cell proliferation, consistent with the roles proposed for YY1 by the reporter assays.

Discussion

Localization of YY1 in Muscle Cells in Vitro and in Vivo

YY1 is an important regulator of smooth muscle-specific gene expression. In this study we have shown that as the porcine elastic pulmonary arteries remodeled after birth there was a temporal and spatial association between the protein expression of cytoplasmic YY1 and γ -SM actin in the VSMCs. The expression of both proteins decreased transiently after birth in the inner medial SMCs undergoing cytoskeletal remodeling. By contrast, expression of cytoplasmic YY1 increased noticeably in the medial SMCs of hypertensive elastic arteries. Exposure to hypoxia results in an increase in actin in hypertensive arteries⁵ and in the present study the increase in cytoplasmic YY1 in these arteries correlated with the increased γ -SM actin. Furthermore, the loss of nuclear YY1 staining in the arteries of chronically hypoxic piglets at 14 days is consistent with the increased myofilament density previously described in these animals.³ In SMCs that do not normally dedifferentiate immediately after birth, such as bronchial SMCs, YY1 is distributed throughout the cell cytoplasm at and after birth.

Our observations showing both a nuclear and cytoplasmic localization of YY1 are consistent with previous studies. YY1 was found in the nucleus and cytoplasm of the cells in the blastocyst of the preimplantation embryo³³ and in the cell cytoplasm of the developing *Xenopus* embryo, demonstrated by Western blotting of cytoplasmic and nuclear extracts plus immunocytochemistry, although confined to the nucleus in the adult *Xenopus* liver.³⁴ The presence of YY1 protein in both the nucleus and cytoplasm of pulmonary vascular SMCs *in vivo* in our study is consistent with our observations on the localization of YY1 in muscle cells *in vitro*. Others have also shown that YY1 can be present in both the nucleus and cytoplasm of cultured cells. Jurkat cells contain both

A. YY1 expression

B. Cell differentiation

C. Cell proliferation

Figure 7. Effect of reduced YY1 protein on differentiation and proliferation. C2C12 cells were transfected with siRNAs siY1 (YY1) and siY2 (control) as described in Materials and Methods. **A:** Cells were harvested 48 hours after transfection. Total protein extracts were prepared, and the protein content was determined by Bradford assay. Thirty μ g of each sample was run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Western analysis was performed as previously described.⁷ **B:** RNA was extracted from cells 48 hours after transfection. The amounts of SM22 α mRNA and rRNA in the samples were quantified by quantitative real-time PCR in triplicate. The experiment was repeated three times. The SM22 α mRNA level was normalized to the rRNA level as previously described.²⁷ **C:** Cell number was determined 48 hours after transfection with siRNA duplexes. Cell number was normalized to 100% in the presence of the siRNA siY2 (control) in each experiment. Each experiment was performed in triplicate and the data presented are the mean of the three independent experiments. Statistical significance was determined by analysis of variance (** $P < 0.01$).

cytoplasmic and nuclear forms of YY1, which differ in apparent molecular weight due to differential phosphorylation.³⁵ Conversely, Austen and colleagues³⁶ showed only nuclear localization of YY1 in RK-13 cells. These observations suggest that the localization of this protein may be cell-type-dependent. Other studies on the localization of YY1 in CHO cells have suggested that localization is dependent on the cell cycle, with YY1 accumulating in the nucleus during late G₁ and early S phase and then relocating to the cytoplasm late in S phase.³⁷ This observation may explain the absence of YY1 in the nuclei of the outer medial SMCs of the pulmonary hypertensive animals found in the present study.

Association between YY1 Localization and Actin Polymerization: Cause and Effect

Immediately after birth the VSMCs in the outer media of the elastic pulmonary arteries normally retain a well-differentiated phenotype whereas those of the inner media show a transient reduction in actin filaments and an increase in G-actin.² In this study the protein expression of both cytoplasmic YY1 and SM γ -actin was greater in the well-differentiated cells of the outer media. That these events were causally related was supported by our *in vitro* observation that increased actin polymerization in muscle cells led to increased cytoplasmic YY1. In cells transfected with cofilin, increasing the amount of polymerized actin in the cells, either by treatment with drugs such as jasplakinolide or co-expressing actins that constitutively polymerize, caused a proportion of the protein to become localized to the cytoplasm. YY1 has previously been shown to interact strongly with the nuclear matrix,^{38,39} and we found that residual YY1 was almost always detectable in the nucleus. It is possible that cofilin causes an actin-independent localization of YY1 into the nucleus of cells with low levels of filamentous actin but that increased actin polymerization inhibits this activity. However, we could not demonstrate a direct interaction between YY1 and cofilin by immunoprecipitation (L.F., unpublished data).

Association between YY1 Localization and Muscle Cell Differentiation

Nuclear expression of YY1 without cytoplasmic expression, was associated with reduced expression of markers of SMC differentiation on the luminal side of elastic arteries, and this finding is consistent with our previous *in vitro* data showing that YY1 can inhibit the activation of the smooth muscle-specific promoters in VSMCs as well as in other cell types.^{7,12,40,41} Furthermore, we previously found that activation of the SM22 α by increased actin polymerization requires an intact YY1 binding site,⁷ suggesting that modification of YY1 function is integral to the regulation of SM22 α expression by actin. The data presented in this study suggest that the cytoskeleton modifies YY1 function by altering the localization of the protein. Furthermore the data suggest that the localization of YY1 is modified by the cytoskeleton *in vitro* and in the VSMCs of the elastic arteries *in vivo*. These changes in YY1 localization are associated with changes in SMC differentiation.

Association between YY1 Localization and Cell Proliferation

The nuclear, but not cytoplasmic, expression of YY1 found in the adluminal VSMCs co-localizes with a region in which SMC proliferation is known to increase after birth,² suggesting a role for YY1 in the proliferation of these cells. In this study we have shown that increased actin polymerization inhibits the *c-myc* promoter in

C2C12 cells, which have significant YY1 DNA binding activity, and that YY1 prevents this inhibition. These data are consistent with our previous work showing that increased actin polymerization reduces the ability of YY1 to bind to DNA⁷ and is also consistent with our studies on the regulation of the SM22 α promoter.⁷ The data therefore, support a role for the actin cytoskeleton in modifying YY1 activity. In C2C12 cells transfection of LIM kinase opposes the known activity of YY1 on both the *c-myc* and SM22 α promoters, and normal activity is restored by the addition of YY1. In the P19 cells, which do not have detectable YY1 DNA binding activity, LIM kinase does not have any effect on the same promoters in the absence of exogenous YY1 but opposes the activity of transfected YY1 on the *myc* promoter in this study and on the SM22 α promoter in our previous study.⁷

Taken together the data imply that inhibiting YY1 activity increases the expression of smooth muscle-specific genes and inhibits cell proliferation. Consistent with this suggestion, knockdown of YY1 caused a significant increase in SM22 α gene expression and a reduction in cell proliferation. Similar observations linking YY1 to muscle cell differentiation have been made using expression of exogenous YY1 and BrdU treatment.^{16,42}

In conclusion, our data suggest that after birth a reduction in filamentous actin in the inner medial SMCs of the elastic pulmonary arteries causes a relocation of YY1 from the cytoplasm to the nucleus. When in the nucleus and able to bind DNA, YY1 inhibits the activity of the SM22 α promoter and increases the activity of genes involved in the cell cycle, including *c-myc*. Under hypoxic conditions however, filamentous actin does not depolymerize so relocation of YY1 does not take place. Consequently, under these conditions YY1 neither inhibits the expression of differentiation markers nor activates the expression of cell cycle genes. Thus, YY1 is a central player in the regulation of pulmonary vascular remodeling under normal conditions. We therefore suggest that the failure of YY1 to inhibit contractile protein expression and promote the expression of cell cycle genes contributes to the failure of the pulmonary vasculature to remodel in neonatal pulmonary hypertension.

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