Developmental regulation and coordinate reexpression of FKBP65 with extracellular matrix proteins after lung injury suggest a specialized function for this endoplasmic reticulum immunophilin

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Abstract FKBP65 (65-kDa FK506-binding protein) is an endoplasmic reticulum (ER)-localized peptidyl-prolyl *cis-trans* isomerase predicted to play a role in the folding and trafficking of secretory proteins. In previous studies, we have shown that FKBP65 is developmentally regulated and associates with the extracellular matrix protein, tropoelastin, during its maturation and transport through the ER. In this study, we show that FKBP65 is expressed in the lung with the same developmental pattern as tropoelastin and other matrix proteins. To test the hypothesis that FKBP65 is upregulated at times when extracellular matrix proteins are being actively synthesized and assembled, adult mice were treated with bleomycin to cause reinitiation of matrix protein production during the ensuing development of pulmonary fibrosis. After bleomycin instillation, FKBP65 expression was reactivated in the lung with a pattern similar to that observed for tropoelastin and type I collagen. Using human lung fibroblast cultures, we showed that FKBP65 does not undergo the unfolded protein response, a response associated with an upregulation of resident ER proteins that occurs after increased ER stress. When fibroblasts were treated with transforming growth factor (TGF)- β 1, which is upregulated during the development of pulmonary fibrosis and known to induce matrix production, FKBP65 expression and synthesis was also increased. Similar to type I collagen and tropoelastin, this response was completely inhibited in a dose-dependent manner by GGTI-298, a geranylgeranyl transferase I inhibitor. Treatment of fibroblasts with an inhibitor of ribonucleic acid (RNA) polymerase II after TGF- β 1 treatment showed that the effect of TGF- β 1 was not because of increased stabilization of the FKBP65 messenger RNA. In summary, we have shown that FKBP65 is highly expressed in lung development, downregulated in the adult, and can be reactivated in a coordinated manner with extracellular matrix proteins after lung injury. The expression pattern of FKBP65, which is atypical for general ER foldases, suggests that FKBP65 has a distinct set of developmentally regulated protein ligands. The response to injury, which may be in part a direct response to TGF- β 1, assures the presence of FKBP65 in the ER of cells actively producing components of the extracellular matrix.

INTRODUCTION

FKBP65 (65-kDa FK506-binding protein) belongs to a highly conserved family of intracellular proteins called

immunophilins (Schreiber 1991; Sigal and Dumont 1992; Galat 1993). In addition to FK506-binding proteins (FKBPs), the immunophilin family also includes cyclophilins (CyPs), which bind cyclosporin A, and parvulins. All immunophilins possess the potential catalytic property of peptidyl-prolyl *cis-trans* isomerization and, although not well defined, appear to function in a wide range of cellular activities. Immunophilins are present in

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every compartment of the cell and exist as membrane-anchored, soluble, and even secreted species. FKBP65 is 1 of 4 FKBP's localized to the endoplasmic reticulum (ER) (Patterson et al 2000), the others being FKBP13 (Nigam et al 1993), FKBP23 (Nakamura et al 1998), and FKBP60 (Shadidy et al 1999). Because the ER is a major site of protein folding, it has generally been accepted that these FKBP's function in a ubiquitous manner to assist in *cis-trans* isomerization of X-proline bonds in newly synthesized proteins.

In previous work, using *in situ* chemical cross-linking of proteins in fetal bovine chondrocytes followed by immunoprecipitation, we identified tropoelastin as a ligand for FKBP65 in the ER (Davis et al 1998). Tropoelastin is an extracellular matrix protein that is highly expressed in elastogenic tissues during development (Mecham and Davis 1994). The protein is secreted as a monomer and assembled into insoluble elastic fibers that are critical for the normal formation and function of the lungs, skin, and vasculature (Li et al 1998; Wendel et al 2000). Because approximately 12% of the residues in tropoelastin are proline, it is clearly a good substrate for an enzyme that can catalyze *cis-trans* isomerization of X-proline bonds.

Tropoelastin is normally only expressed in developing tissues, with little or no expression in the adult unless reactivated by injury (Davis 1993; Capron et al 1997; Hoff et al 1999). The expression profile of FKBP65 was found to be similarly restricted to developing tissues (Coss et al 1995; Patterson et al 2000), suggesting that it may be a tropoelastin-specific foldase. Northern analysis of several developing mouse tissues, however, showed that FKBP65 is strongly expressed in tissues that have relatively little elastin, such as kidney (Patterson et al 2000), thus implying that additional, developmentally regulated ligands likely exist for FKBP65.

In this study, the hypothesis that FKBP65 expression is regulated in a coordinated manner with that of extracellular matrix proteins was investigated. To test this hypothesis, we sought to determine whether FKBP65 expression could be reactivated in a quiescent adult tissue on reinitiation of matrix protein production. To accomplish this, we used a well-characterized murine model of lung fibrosis, which uses an intratracheal instillation of bleomycin to cause pulmonary injury, upregulation of matrix proteins, and the formation of fibrotic lesions (Thrall and Scalise 1995). Because considerable evidence has implicated transforming growth factor (TGF)- β 1 in the pathogenesis of pulmonary fibrosis (Khalil and Greenberg 1991; Zhang and Phan 1996), we also determined the effect of TGF- β 1 on the expression and synthesis of FKBP65 in cultured human fetal lung fibroblasts. Finally, we investigated the ability of FKBP65 expression to respond to the quality and quantity of proteins in the ER by using tunicamycin treatment to induce the unfold-

ed protein response (UPR). Results from our study show that FKBP65 is not typical of many resident ER proteins and suggest that FKBP65 may serve a specialized function in the ER maturation and processing of a specific subset of proteins highly expressed during development and injury.

MATERIALS AND METHODS

Cells and reagents

Human fetal lung fibroblasts (GM05389, Coriell Institute for Medical Research, Camden, NJ, USA) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with L-glutamine, nonessential amino acids, antibiotics, and 10% calf serum (Hyclone Laboratories Inc., Logan, UT, USA). Tunicamycin (Sigma, St Louis, MO, USA) was stored at -20°C as a 5 mg/mL stock in dimethyl sulfoxide and used at a final concentration of 1 $\mu\text{g/mL}$. TGF- β 1 (R&D Systems Inc., Minneapolis, MN, USA) and GGTI-298 (generous gift from Dr Said M. Sebti, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, FL, USA) were used at the final concentrations indicated in the figures and figure legends. GGTI-298 was added to confluent cultures of lung fibroblasts 24 hours before TGF- β 1, and the incubations were continued for 4 hours. To determine message stability, media of confluent cultures were replaced with DMEM containing 1% serum before the addition of TGF- β 1 (3 ng/mL). After incubation with TGF- β 1 for 16 hours, 5,6-dichlorobenzimidazole riboside (DRB; Sigma) was added to a final concentration of 50 μM .

Antibodies and complementary deoxyribonucleic acid probes

Probes used for Northern analysis included a 2.3-kb bovine tropoelastin complementary deoxyribonucleic acid (cDNA) probe (Parks et al 1988), a 2.8-kb glucose regulated protein (Grp94) cDNA probe (generous gift from Dr Michael Green, Saint Louis University, MO, USA), a 1.5-kb human α 1(I) collagen cDNA probe covering the c-propeptide + 800 bp of helical region, a 700-bp cDNA probe to FKBP60 derived from a mouse expressed sequence tag (EST) (IMAGE:1852836) with homology to the 3' untranslated region, and a 1.7-kb, full-length FKBP65 cDNA probe (Patterson et al 2000). The primary antibody used for immunohistochemistry was a polyclonal FKBP65 antipeptide antibody raised against a synthetic peptide to the C-terminus of mouse FKBP65 (Davis et al 1998). The biotinylated secondary antibody and Avidin: Biotinylated enzyme complex (ABC) reagents were contained in the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) and were prepared according to

kit instructions. For Western blotting, a commercial anti-FKBP65 monoclonal antibody (BD Transduction Laboratories, Mississauga, ON, USA) was used at 1:500 followed by a Horseradish Peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Upstate, Lake Placid, NY, USA) at 1:5000.

Bleomycin treatment

To induce pulmonary fibrosis, C57Bl/6 mice were treated with 0.045 U bleomycin sulfate (Blenoxane, Bristol-Myers Squibb, New York, NY, USA) in 75 μ L of sterile saline solution (0.9% NaCl) by intratracheal administration, as described previously (Betsuyaku et al 2000). Control animals (shams) for each time point received saline only. At 6 time points, from 0.5 to 10 days after bleomycin exposure, mice were euthanized, and both lungs were removed for isolation of total ribonucleic acid (RNA) and Northern analysis. Lungs from additional mice at each time point, and from the sham animals, were prepared for paraffin embedding after fixation in 10% buffered formalin.

RNA isolation and Northern analysis

Mouse lungs dissected from postnatal and adult animals were immediately frozen in liquid nitrogen. Frozen tissues were homogenized in Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA), total RNA isolated according to manufacturers directions, and 10 μ g separated by electrophoresis through a 1% agarose gel containing 1 M formaldehyde. The RNA was transferred overnight to Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL, USA) and UV cross-linked using a Spectrolinker XL-100 UV Crosslinker (Spectronics Corporation, Lincoln, NE, USA). The cDNA probes were labeled with [32 P]dCTP using the Rediprime II random prime labeling system (Amersham), and unincorporated nucleotides were removed using the QIAquick Nucleotide Removal kit (Qiagen, Valencia, CA, USA). Membranes were hybridized overnight at 65°C using 250 mM Na₂PO₄ and 7% sodium dodecyl sulfate (SDS) or hybridized for 3 hours at 65°C using Rapid-hyb buffer (Amersham). Membranes were then washed once in 2 $\times$ SSPE (20 $\times$ SSPE = 3.6M NaCl, 0.2M NaH₂PO₄ - H₂O, 0.02M EDTA, pH 7.7), 0.1% SDS for 15 minutes at room temperature followed by 2 washes in 1 $\times$ SSPE, 0.1% SDS for 15 minutes at the hybridization temperature. Hybridized complexes were detected by exposure of the membrane to X-OMAT AR film (Kodak, Rochester, NY, USA). For reprobing, membranes were stripped using boiling 0.5% SDS.

For cultured cells, total RNA was extracted from replicate cultures by the acid guanidine isothiocyanate method (Chomczynski and Sacchi 1987) at the time points in-

dicated in the figure legends and text of this report. Formaldehyde-1.2% agarose gels were prepared, and 15 μ g of RNA was electrophoresed, then transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA, USA) and hybridized to either an FKBP65 or a procollagen cDNA probe labeled with 32 P by the random primer method (Indik et al 1987). Equivalent loading and transfer were verified by quantitative image analysis of ethidium bromide staining of ribosomal RNA in the blots. The hybridization signals were analyzed by phosphorimaging (Storm 840 PhosphorImager, Amersham Biosciences, Piscataway, NJ, USA) and the results quantified to determine the relative amounts of messenger RNA (mRNA) (ImageQuant V5.2, Amersham Biosciences).

Immunohistochemistry

Formalin-fixed sections of sham and bleomycin-treated lung, 10 days after tracheal instillation, were deparaffinized and hydrated through xylene and a graded series of ethanol. Sections were then incubated in 0.3% Triton X-100 in Tri-buffered saline (TBS) (10 mM Tris, 150 mM NaCl [pH 7.4]) for 15 minutes, rinsed in TBS followed by absolute MeOH for 2 minutes each, and then quenched of endogenous peroxidase activity in 0.6% H₂O₂ in absolute MeOH for 1 hour. After rinsing in absolute MeOH for 2 minutes, the sections were washed in 0.1% bovine serum albumin (BSA) in TBS thrice for 5 minutes. Sections were then incubated in a blocking solution consisting of 0.5% BSA in TBS with normal blocking serum from the Vectastain kit, added according to kit instructions. After 30 minutes, excess blocking solution was drained from the sections, and the sections were incubated in primary antibody diluted in blocking solution overnight in a humidity chamber at 4°C. The next day, the sections were washed in 0.1% BSA in TBS, incubated in diluted biotinylated secondary antibody for 1 hour at room temperature, washed again, and incubated in ABC reagent for 1 hour. After washing, the sections were incubated in Vector NovaRED substrate, counterstained with Vector hematoxylin, and mounted using VectaMount (Vector Laboratories Inc). Sections were viewed with a Zeiss Axioskop 2 microscope, and images captured with an AxioCam digital camera by AxioVision software (Carl Zeiss Inc, Thornwood, NY, USA).

Western blotting

Human fetal lung fibroblasts were grown to confluency in a 6-well plate. Each well was then incubated with 1 mL DMEM containing 1% calf serum with or without 3 ng/mL TGF- β 1 for 16 hours. After treatment, the cell layers were washed with phosphate-buffered saline (pH 7.4), then lysed in 1 mL of lysis buffer (25 mM Tris-HCl [pH

7.5], 5 mM ethylenediamine-tetraacetic acid, 250 mM NaCl, and 1% Triton X-100) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Laval, Quebec, Canada) with agitation at 4°C. After 30 minutes, lysates were transferred to 1.5-mL tubes and incubated end-over-end for an additional 30 minutes at 4°C. Samples were then acetone precipitated, and the precipitated proteins pelleted and resuspended in 70 μ L of Laemmli sample buffer containing 100 mM dithiothreitol (DTT). Samples were heated at 95°C for 5 minutes, and 25 μ L of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, nonspecific binding sites were blocked with 5% dry milk in TBS containing 0.5% Tween-20 overnight at 4°C. All remaining rinses, washes, and antibody dilutions were in 2% dry milk with 0.05% Tween-20 in TBS. After blocking, the blot was rinsed and incubated with an FKBP65 monoclonal antibody for 1.5 hours followed by three 10-minute washes. The blot was then incubated with HRP-conjugated secondary antibody for 1 hour, washed several times, rinsed in TBS, and incubated with chemiluminescent substrate (Pierce, Rockford, IL, USA) for 5 minutes before exposure to film. Films were scanned, and pixel density was quantified as described above for Northern blots.

RESULTS

FKBP65 expression shows developmental regulation in the lung

In previous work, we showed that FKBP65 is expressed in 12-day postnatal mouse lung at high levels but is virtually absent in adult lung. This finding was in direct contrast to another ER-localized resident protein, Grp94, which was found to be expressed at moderate levels in both developing and adult lung (Patterson et al 2000). To investigate the expression profile of FKBP65 in more detail, lungs from mice ranging in postnatal age from 0.5 to 56 days were collected and probed for FKBP65. At time points immediately after birth and during early development, FKBP65 was strongly expressed (Fig 1). By 14 days, the expression level was reduced, and by 26 days, FKBP65 message was very low, a level that persisted into young adulthood. To determine whether this expression pattern was distinct to FKBP65, the blot was reprobed for a second, closely related, ER-localized FKBP, FKBP60. In contrast to FKBP65, moderate expression of FKBP60 continued past postnatal development and was observed at all time points examined (Fig 1). These results indicate that developmental regulation of FKBP65 is not a universal feature of ER-localized FKBP.

Fig 1. FKBP65 is developmentally regulated in the lung. Northern analysis of 2 endoplasmic reticulum-localized FK506-binding proteins in mouse lung from 0.5 to 56 days of postnatal development. A high level of FKBP65 expression is observed in early development with a decrease to low levels in young adulthood. In contrast, FKBP60, which contains a similar domain structure as FKBP65, is steadily expressed throughout the time period examined. Graphic data of FKBP65 and FKBP60 expression normalized for loading are shown.

FKBP65 is upregulated in bleomycin-induced pulmonary fibrosis

The expression pattern of FKBP65 in lung suggests that it may play a role in the development of this organ. To date, tropoelastin has been the only ligand in the secretory pathway identified for FKBP65 (Davis et al 1998). In previous work, we showed that FKBP65 localizes to pulmonary blood vessel and airway smooth muscle cells, cells that produce and are surrounded by extracellular elastic fibers (Patterson et al 2000). Similar to FKBP65, tropoelastin expression also starts in late fetal life and continues during postnatal development until young adulthood is reached, after which time no new synthesis occurs (Fig 2A). In the lung, expression of tropoelastin and other matrix proteins can be reactivated after fibrotic injury, such as that induced by bleomycin treatment (Lucey et al 1996; Swiderski et al 1998). To determine whether FKBP65 would also be reactivated during the induction of pulmonary fibrosis, RNA from whole lungs, collected at several time points ranging from 0.5 to 10 days after

Fig 2. FKBP65 expression is reactivated in the adult by bleomycin treatment and localized to fibrotic lesions. (A) Northern analysis of the normal expression patterns for FKBP65, tropoelastin, $\alpha 1(I)$ procollagen, and Grp94 in 12-day postnatal and adult mice (control lanes). After bleomycin instillation, an initial, acute upregulation of the matrix proteins and FKBP65 is observed at 0.5 days, which subsequently subsides. At later time points, with the development of fibrosis, procollagen and tropoelastin expression are upregulated concomitant with a reactivation of FKBP65. (B) Immunohistochemistry for FKBP65 shows little, if any, protein in normal adult lung alveoli 10 days after a sham instillation (control). In contrast, at 10 days after bleomycin instillation, strong FKBP65 staining can be observed in the fibrotic lesions.

bleomycin instillation, was obtained and probed for FKBP65 expression. As shown in Figure 2A, bleomycin treatment leads to the reactivation of FKBP65 expression, with low levels observed at 3 days after instillation and increasingly higher levels observed at later time points. As expected, the expression levels of tropoelastin and

type I collagen were also induced during the development of pulmonary fibrosis (Fig 2A). Interestingly, the expression pattern for FKBP65 more closely paralleled that of type I collagen, both showing low levels of expression at 3 days after instillation, whereas that for tropoelastin lagged behind showing low levels at 5 days. An initial, acute upregulation at 0.5 days after instillation was observed for all 3 proteins. In contrast to FKBP65 and the matrix proteins, the expression of Grp94 remained relatively unchanged during the course of bleomycin treatment (Fig 2A).

Because the production of matrix proteins occurs primarily in the fibrotic lesions, immunohistochemistry was performed to determine whether the increased levels of FKBP65 message observed by Northern analysis correlated with increased FKBP65 protein in the areas of fibrosis. Lungs from control (sham) mice showed normal lung histology with little or no FKBP65 evident in the alveolar walls (Fig 2B, top). In contrast, the fibrotic lesions that had formed by 10 days after instillation of bleomycin showed dark staining of FKBP65 (Fig 2B, bottom). Areas outside the lesions, containing unaffected alveoli, were negative for FKBP65 immunoreactivity (data not shown).

FKBP65 does not undergo the unfolded protein response

The upregulation of extracellular matrix proteins observed in the days after bleomycin instillation and during the development of fibrotic lesions would argue that a significant increase in the trafficking of matrix proteins through the secretory pathway in the pulmonary fibroblasts must be occurring during this time. It is well known that increased production or accumulation (or both) of unfolded proteins in ER can trigger a signaling pathway from the ER to the cell nucleus to protect the cell. This pathway, known as the UPR, leads to the upregulation of a number of resident ER chaperones and folding enzymes in an effort to boost the folding capacity of the ER under stressed conditions (Lee 1992). To determine whether expression of FKBP65 can be influenced by ER stress, human lung fibroblasts were treated with tunicamycin, which inhibits *N*-glycosylation and causes an accumulation of unfolded proteins in the ER (Benedetti et al 2000). In contrast to the immunoglobulin heavy chain binding protein (BiP) and Grp94, which are indicators of a typical UPR by their steady upregulation in response to tunicamycin treatment, FKBP65 expression remained unchanged (Fig 3). These results demonstrate that the upregulation of FKBP65 that occurred in the bleomycin-treated lungs was not a consequence of generalized ER stress as a result of increased protein production.

Fig 3. FKBP65 does not undergo the unfolded protein response. Human lung fibroblasts were treated with vehicle alone, or with 2 μg/mL tunicamycin for 2 or 6 hours. Northern analysis of BiP and Grp94 shows an upregulation in response to the presence of unfolded proteins in the endoplasmic reticulum, whereas FKBP65 expression remains unchanged. Graphic data of messenger ribonucleic acid expression are normalized for loading. Values for each message are plotted relative to the untreated condition (% of control).

TGF-β1 modulation of FKBP65 expression

Considerable evidence suggests that TGF-β plays a central role in a variety of fibroproliferative diseases by inducing the production of extracellular matrix proteins (Sime et al 1997; Pittet et al 2001). Consistent with the

evidence, an increased expression of TGF-β isoforms has been shown to occur in bleomycin-induced lung injury during both the early inflammatory (days 1 and 3) and the later reparative (days 7 and 14) phases, particularly in areas of developing fibrosis (Santana et al 1995). To determine whether TGF-β can influence the expression of FKBP65, confluent cultures of human lung fibroblasts were incubated with 3 ng/mL of TGF-β1 for 16 hours, and Northern blots probed for FKBP65. A 2-fold increase in FKBP65 expression was observed with TGF-β1 exposure as compared with untreated cells (Fig 4A). As expected, the expression of type I collagen was also stimulated by TGF-β1 showing an approximate 3-fold upregulation (Fig 4B). A 10-fold upregulation of tropoelastin expression after TGF-β1 treatment of human lung fibroblasts has already been published (Kucich et al 1998).

To determine whether a similar signaling pathway was used in the upregulation of both FKBP65 and type I collagen, additional cultures were incubated with increasing concentrations of GGTI-298, a geranylgeranyl transferase I (GGTase I) inhibitor. For both FKBP65 and type I collagen, the GGTase I inhibitor blocked the upregulation of gene expression by TGF-β1 in a dose-dependent manner. Levels similar to control levels were observed at 30 and 10 μM of GGTI-298 for FKBP65 (Fig 4A) and α-1 procollagen I (Fig 4B), respectively. In previous work, the same GGTase I inhibitor was also shown to block the upregulation of tropoelastin expression in a similar dose-dependent manner (Kucich et al 1998). These results suggest that a signaling pathway involving one or more geranylgeranylated proteins is necessary for the TGF-β1-mediated response of all 3 proteins.

FKBP65 protein production correlates with mRNA levels

To confirm that the changes observed in expression of FKBP65 with exposure to TGF-β1 reflect changes in protein production, confluent cultures of human lung fibroblasts were incubated with 3 ng/mL of TGF-β1 for 16 hours, cell lysates collected, and Western blotted for FKBP65. As shown in Figure 5, TGF-β1 treatment results in a 2.6-fold increase in FKBP65 synthesis. A similar fold increase was observed for FKBP65 expression after TGF-β1 treatment (Fig 4A), indicating that changes in message are directly related to changes in protein for FKBP65.

Analysis of message stability

In previous work, upregulation of tropoelastin mRNA by TGF-β1 was determined to be as a consequence of increased stabilization of the message (Kucich et al 1998). To determine whether the increased expression of FKBP65 after TGF-β1 treatment was also because of in-

Fig 4. Transforming growth factor (TGF)- β 1 causes upregulation of FKBP65 expression. Confluent cultures of human lung fibroblasts were incubated for 24 hours with the geranylgeranyl transferase I inhibitor, GGTI-298, at the various concentrations indicated and then TGF- β 1 was added to a final concentration of 3 ng/mL. After 4 hours, total ribonucleic acid (RNA) was prepared from the cells and analyzed by Northern blotting for FKBP65 (A) and reprobed for type I collagen (B). Upregulation of message for both proteins is observed in the presence of TGF- β 1, which is inhibitable in a dose-dependent manner by GGTI-298. Graphic data represent the average of duplicate cultures. Control level refers to the value obtained in the absence of TGF- β 1. Equivalent loading and transfer was verified by ethidium bromide staining of ribosomal RNA (A).

creased stabilization of the FKBP65 transcript, message stability analysis was carried out. Cultures of confluent lung fibroblasts were pretreated with 3 ng/mL of TGF- β 1 for 16 hours, after which time DRB was added to the cultures to terminate transcription. Control, untreated cultures were similarly incubated with DRB. Analysis of mRNA levels determined by Northern hybridization showed that in the absence of TGF- β 1 stimulation, the

Fig 5. TGF- β 1 causes an increase in FKBP65 synthesis. Confluent cultures of human lung fibroblasts were incubated for 16 hours with or without TGF- β 1 added to a final concentration of 3 ng/mL. Cell lysates were collected, acetone precipitated, and resuspended in sample buffer. An equal volume of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for FKBP65 (top). The graphic data of pixel density represent the average $\pm$ SEM of 4 different samples for each condition (bottom). TGF- β 1 treatment resulted in a significant increase in FKBP65 production ($P = 0.001$, correlation coefficient = 0.899).

observed half-life was similar to that observed in the presence of TGF- β 1 (Fig 6). This result indicates that TGF- β 1 treatment has no effect on the stability of FKBP65 message and that the mRNA is regulated at the transcriptional level.

DISCUSSION

In this study, we have shown that FKBP65 is atypical of the many foldases and chaperones localized to the ER in that it is developmentally regulated, does not undergo the UPR, and can be reactivated in response to tissue injury. Because these features are unlike what has been reported for the 3 other ER-localized FKBP6s, our results support a specialized function for FKBP65.

Fig 6. Effect of TGF- β 1 on FKBP65 messenger ribonucleic acid (mRNA) stability. Confluent cultures of human lung fibroblasts were incubated for 16 hours in the presence or absence of 3 ng/mL of TGF- β 1. The 5,6-dichlorobenzimidazole riboside was then added to a final concentration of 50 μ M, and total RNA was prepared from duplicate or triplicate cultures at the indicated times and analyzed by Northern hybridization. Northern blots hybridized with FKBP65 probe, and graphic data show message decay with comparable half-lives in the presence or absence of TGF- β 1.

The first FKBP to be identified in the ER was FKBP13 (Jin et al 1991; Nigam et al 1993). Characterization of the expression pattern of this protein under conditions of protein misfolding in the ER, and analysis of the promoter region of the gene, showed FKBP13 to possess features similar to the classic ER chaperones, BiP, Grp94, and Erp72 (Bush et al 1994). As a proline isomerase located in an intracellular compartment dedicated to protein folding, it was logically inferred that FKBP13 likely plays a role as an ER-folding catalyst or molecular chaperone. Recently, FKBP13 has been identified to interact with brefeldin A-inhibited guanine nucleotide-exchange protein 1 (BIG1) by yeast 2-hybrid and confirmed by coimmunoprecipitation (Padilla et al 2003). Because BIG1 functions

to activate adenosine 5' diphosphate-ribosylation factors, which are essential components of intracellular vesicular transport, this finding further supports a role for FKBP13 in the secretory process. Subsequent to FKBP13, 3 other FKBP s were identified in the ER: FKBP23, FKBP60, and FKBP65 (reviewed in Davis 2000). On the basis of a motif structure that consists of single or multiple proline isomerase domains and a C-terminal ER-retention sequence or modified ER-retention sequence (Pelham 1990), these additional 3 FKBP s were also predicted to be ubiquitously expressed and to play generalized roles in ER protein folding and trafficking.

In 1997, Dolinski et al conducted an experiment that challenged the view that ER-localized FKBP s play general roles in protein folding. Research from this group showed that a mutant yeast strain lacking all the yeast CyP s and FKBP s was viable, with the only phenotype being the addition of the subtle phenotypes of the individual mutants. On the basis of their findings, the authors concluded that each immunophilin likely interacts with a unique set of partners to perform specific functions, which are nonessential for individual cell survival. To date, a limited number of ligands have been identified for FKBP s in the ER making it difficult to determine the potential specialized functions of these proteins. There are, however, 2 examples of such specialized functions in the CyP group of immunophilins. In *Drosophila*, the *ninaA* gene, which encodes a CyP homologue (Schneuwly et al 1989; Shieh et al 1989), colocalizes with its ligand, Rh1 rhodopsin, throughout the secretory pathway (Colley et al 1991). Significantly, mutations in the *ninaA* gene cause an intracellular accumulation of Rh1 in the ER and reduced transport of the protein to the cell surface (Colley et al 1991). A similar ligand-specific role has been reported for CyPB, which has been shown to facilitate procollagen I folding and exit from the ER (Smith et al 1995). The significance of this interaction was demonstrated by the observation of a reduced rate of folding, delayed secretion, and increased intracellular degradation of procollagen after cyclosporin A treatment of cells in culture (Steinmann et al 1991; Smith et al 1995). Clearly, as additional ligands are identified for the CyP s and FKBP s, the ability to determine the specialized functions of these proteins will be simplified.

To date, the only ligand that has been identified for FKBP65 in the secretory pathway is the extracellular matrix protein, tropoelastin (Davis et al 1998). Tropoelastin undergoes no posttranslational modifications in the ER, possesses no pre- or proform that requires processing, and is rapidly secreted from the cell. In the extracellular matrix, cross-linking domains on individual tropoelastin monomers must be aligned so that desmosine cross-links can be formed between adjacent lysine residues. Because proline residues are located immediately preceding, or

within, virtually all the cross-linking domains, the conformation of these peptidyl-prolyl bonds is likely critical for the proper folding, trafficking, and ultimate assembly of the tropoelastin monomers. In support of this hypothesis, elastic fiber-producing cells show a reduced rate of secretion and intracellular retention of tropoelastin when treated with FK506 in culture (E.C.D. unpublished data).

In this study, reactivation of tropoelastin and FKBP65 expression were observed after bleomycin treatment of mice and the subsequent development of pulmonary fibrosis. The reexpression of FKBP65, however, more closely followed that of type I collagen than tropoelastin, which lagged behind by several days. Although a direct interaction between procollagen chains and FKBP65 has yet to be shown, these results raise the intriguing possibility that FKBP65 may also act as a foldase or chaperone for type I collagen. Similar to tropoelastin, type I collagen has a high proline content with every third residue being a proline. *Cis-trans* isomerization of the peptidyl-prolyl bonds in procollagen chains is critical for assembly of the chains into collagen fibrils (Steinmann et al 1991). Although type I collagen has already been shown to interact with another member of the immunophilin family, CyPB (Smith et al 1995), and a specific molecular chaperone, Hsp47 (Nagata 1996; Tasab et al 2000), it is well established that members of the collagen family interact with a multitude of folding enzymes and chaperones during their transit time in the ER and along the course of the secretory pathway (Ferreira et al 1996; Lamande and Bateman 1999; Ko and Kay 2002).

Increasing evidence suggests that one of the main factors that mediates the reinitiation of extracellular matrix protein production after intratracheal instillation of bleomycin is TGF- β 1 (Santana et al 1995; Coker et al 1997). Consequently, we were interested to determine whether TGF- β 1 could directly influence FKBP65 expression. Using human lung fibroblasts, we showed that FKBP65 expression and protein synthesis increased after TGF- β 1 treatment. Stabilization of FKBP65 mRNA was relatively unchanged, indicating that the increased expression of FKBP65 elicited by TGF- β 1 was achieved primarily at the transcriptional level. These results, together with the fact that FKBP65 does not undergo the UPR, suggest that the upregulation of FKBP65 expression after bleomycin treatment is not because of a generalized increase in ER stress due to an overall increase in protein synthesis. Furthermore, the ability of a GGTase I inhibitor to modulate the TGF- β 1 regulation of FKBP65 in a manner similar to type I collagen and tropoelastin (Kucich et al 1998) provides supportive evidence that FKBP65 expression may be controlled by a similar signaling pathway as that of the matrix proteins.

Similar to FKBP65, the collagen-specific chaperone, Hsp47, has also been shown to be upregulated in bleo-

mycin-induced pulmonary fibrosis (Razzaque et al 1998a; Ishii et al 2003) and induced in a dose-dependent manner by TGF- β 1 in cultured collagen-producing cells (Yamamura et al 1998; Sasaki et al 2002). Consistent with this experimental data, both pulmonary and dermal fibrotic lesions in humans show an increased production of Hsp47 concomitant with that of collagen (Razzaque et al 1998b; Razzaque and Ahmed 2002). Interestingly, overexpression of Hsp47 has been reported to stimulate type I collagen synthesis and secretion, suggesting a novel pathway between the ER and the procollagen transcription (Rocnik et al 2002). In this study, we have shown that FKBP65 and type I collagen are both expressed by pulmonary fibroblasts; however, additional studies are undoubtedly necessary to determine whether FKBP65 can interact with procollagen chains, either directly or indirectly, and whether such an interaction has a mechanistic consequence on the secretion and assembly of type I collagen.

In summary, we have shown that FKBP65 expression is developmentally regulated in the lung and can be reinitiated in a coordinated manner with tropoelastin and type I collagen in experimentally induced lung injury. We further showed an upregulation of FKBP65 expression and protein production in response to TGF- β 1, possibly mediated by the same signaling pathway as type I collagen and tropoelastin. Recently, we have found that the only tissue that continues to express FKBP65 in the adult mouse is the ovary (Patterson CE, Davis EC, in preparation). This result is intriguing because the ovary is one of the few adult tissues that continually remodels with cycles of matrix production and angiogenesis. This result, together with the data presented in this study, supports the hypothesis that FKBP65 plays a specialized role in tissue growth and repair.

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