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The Specific Regulation of Type I Collagen Synthesis in Fibrosis

Le Cai
THE SPECIFIC REGULATION OF TYPE I COLLAGEN SYNTHESIS IN FIBROSIS

By

LE CAI

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The members of the committee approve the dissertation of Le Cai defended on March 15, 2010.

__________________ ________________
Branko Stefanovic
Professor Directing Dissertation

__________________ ________________
Hengli Tang
University Representative

__________________ ________________
Myra M. Hurt
Committee Member

__________________ ________________
Yanchang Wang
Committee Member

Approved:

__________________ ________________
Richard Nowakowski, Chair, Biomedical Sciences

__________________ ________________
John P. Fogarty, Dean, College of Medicine

The Graduate School has verified and approved the above-named committee members.
To my family
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ABSTRACT

Fibrosis is characterized by excessive synthesis of type I collagen which impedes the normal function of an affected organ. Type I collagen is the most abundant protein in the human body, produced by the folding of two α1(I) polypeptides and one α2(I) polypeptides into the triple helix. Expression of collagen 1(I) gene is predominantly regulated at the level of mRNA stability and translation. A conserved stem–loop structure is found in the 5’ untranslated region of collagen mRNAs. The 5′ stem-loop structure which is critical for the coordinated translation encompasses the start codon in collagen mRNAs and regulates collagen synthesis by the binding of RNA binding proteins. Assembly of collagen heterotrimer occurs on the membrane of endoplasmic reticulum (ER) while α1(I) and α2(I) mRNAs are associated with polysomes. I have found that the binding of 5’stem-loop binding proteins LARP6 and nonmuscle myosin II to the 5′ stem-loop regulates coordinated translation of collagen mRNAs and is required for collagen triple helix formation. My work describes that the coordinated translation of collagen mRNAs increases local concentration of the chains necessary for productive folding.

Chapter 1 presents the identification of 5’ stem loop binding protein and its function in regulating collagen synthesis. To identify 5’stem-loop binding proteins we performed expressional cloning and cloned La ribonucleoprotein domain family member 6 (LARP6) as the protein which binds to both 5′ stem-loop of collagen α1(1) and α2(1) mRNAs a sequence specific manner. LARP6 has a distinctive bipartite RNA binding domain (amino acids 32-45 and 218-300) which is not found in other members of the La super family. The RNA binding domain of LARP6 interacts with the two single-stranded regions of the 5’ stem–loop. The $K_d$ for binding of LARP6 to the 5′ stem–loop is 1.4 nM. The binding is to the single stranded regions of the bulge of 5’ stem-loop RNA, in both the nucleus and the cytoplasm. Recombinant LARP6 has a similar binding affinity and specificity to the 5’stem-loop as the endogenous LARP6. The C-terminal region of LARP6 has a nuclear localization signal, which allows LARP6 to accumulate in the nucleus.
Combination of gain of function by adenoviral delivery and loss of function by using either dominant negative forms of LARP6 or siRNA directly against the RNA sequence of LARP6 were employed to determine the function of LARP6. Overexpression of LARP6 decreased synthesis of collagen protein, however it didn’t change collagen mRNA steady state level. Also, collagen mRNAs were redistributed from polysomal fractions to the fractions representing the free polysomes in LARP6 overexpressed cells. This suggests that overexpression of LARP6 blocked ribosomal loading on collagen mRNAs and inhibited collagen mRNA translation. Endogenous LARP6 and overexpressed LARP6 were not associated with polysomes. These results suggested that LARP6 prevents premature translation of collagen mRNAs. Knocking down LARP6 by small interfering RNA also decreased steady state level of collagen polypeptide in the cell as well as the section rate of the protein. However, collagen mRNA stability was not affected, nor was the degradation of collagen protein by proteasome. Therefore, it is likely that collagen mRNA translation had been inhibited by the depletion of LARP6.

We describe that collagen protein is synthesized at discrete regions of the endoplasmic reticulum. Using a collagen–GFP (green fluorescent protein) reporter protein, we could reproduce this focal pattern of synthesis, but it was observed only when the reporter was encoded by mRNA with the 5′ stem–loop and in the presence of LARP6. When the reporter was encoded by mRNA without the 5′ stem–loop, or in the absence of LARP6, it accumulated diffusely throughout the endoplasmic reticulum. This indicates that LARP6 activity is needed for focal synthesis of collagen polypeptides. We postulated that the LARP6-dependent mechanism increases local concentration of collagen polypeptides for more efficient folding of the collagen heterotrimer.

In Chapter 2, we describe that nonmuscle myosin IIB plays an important role in regulating collagen synthesis. First, we identified nonmuscle myosin II B as LARP6 binding protein by tobramycin affinity purification and confirmed the binding specificity of myosin II B to LARP6 and to collagen mRNAs. We showed that the C terminus of LARP6 was required for the binding to nonmuscle myosin IIB, and that this binding was not RNA dependent. Secondly, we identify the role of nonmuscle myosin IIB in regulating type I collagen synthesis. Nonmuscle myosin II filaments are required for the
secretion of collagen α2(I) peptide and the colocalization of α1(I) and α2(I) peptides in the cell. The motor activity of myosin II as well as the integrity of the filaments is involved in this process. We also discovered that the effect of myosin II on type I collagen synthesis was mediated by 5’ stem-loop of LARP6 and through the protein-protein interaction with LARP6. At the end, we determined that nonmuscle myosin IIB filaments are involved in directing collagen mRNAs to polysomes for translation. Therefore, we concluded that nonmuscle myosin II interacts with LARP6 and collagen 5’stem-loop to regulates coordinate translation of collagen mRNAs.

In Chapter 3, we explain the role of LARP6 and nonmuscle myosin II in collagen synthesis stimulated by cytokines and growth factors TGF-beta and ouabain. Cytokines and growth factors including TGF-beta and ouabain are stimulators of fibrosis and they increase collagen synthesis in scleroderma skin fibroblasts, hepatic stellate cells, kidney fibroblasts and cardiac fibroblasts. When we down regulated LARP6 or disrupted nonmuscle myosin II filaments, the stimulation of type I collagen secretion by TGF-beta 1 and ouabain was diminished. Therefore, we concluded that LARP6 and nonmuscle myosin II regulate inducible collagen synthesis in fibrosis.

This dissertation describes the specific regulation of type I collagen synthesis in fibrosis. The first finding was that collagen protein synthesis took place of discrete regions on the ER membrane through by the binding of LARP6 to 5’stem-loop. The second finding was that coordinated synthesis of type I collagen polypeptide requires nonmuscle myosin II. The third finding discovered that profibrotic cytokines like TGF-beta and ouabain induced collagen synthesis through LARP6 and nonmuscle myosin II mechanism in human scleroderma skin fibroblasts and rat cardiac fibroblasts. In conclusion, we have discovered that LARP6 and nonmuscle myosin II regulated collagen synthesis. This pathway may contribute to excess collagen deposition in fibrosis. This information will help to find future anti-fibrotic therapy.
INTRODUCTION

Fibrosis is a significant health problem worldwide. Nearly 45% of all death in the developed world is caused by chronic fibro proliferative disease. Excess type I collagen deposition is the cause of fibrosis. Fibrosis affects different organs such as lung, kidney, heart, vascular, eye, skin, pancreas, intestine and bone marrow. It can lead to organ failure and death. There are multiple causes of fibrosis including autoimmune reactions, persistent infections, chemical insults and radiation. But fibrosis can also be idiopathic. Until now, there is only indirect treatment of fibrosis; however, the existing treatment has many controversies and limitations. For instance, under prolong injury, inflammatory response leads to fibrogenesis and treatment focusing on inflammation has been used for treating fibrosis. However, some studies suggested that the inhibition of inflammation is preventing the reversal of fibrosis (Wynn 2008). Another treatment is organ transplantation which is very expensive and there is a limited supply of organs. The proposed treatment with inhibition of growth factors and cytokines like TGF-beta could cause side effects. This treatment is not highly specific due to the multi-function of growth factors and cytokines in the cell.

There is a difference between fibrosis and wound healing. Wound healing is the physiological process which ends in the formation of a limited scar. When epithelial and/ or endothelial cells are damaged, they release inflammatory mediators and recruit fibroblasts and other epithelial and endothelial cells. When fibroblasts migrate to the wound, they activate into myofibroblasts and increase synthesis of extracellular matrix proteins, mostly type I collagen and type III collagen. Collagen synthesis exceeds collagen degradation and form the provisionally ECM (extracellular matrix). Myofibroblasts contract from the edge of wound to the center. At the same time, epithelial and/or endothelial cells also migrate to the wound and repopulate the tissues. Inflammatory cells such as neutrophils and macrophages migrate to the injury site. Macrophages produce growth factors and tumor necrosis factor to stimulate the forming of new blood vessels and ECM. In the final process of wound healing, collagen synthesis returns to normal and TGF-beta (transforming growth factor-beta) which is only activated transiently to promote wound healing is turned off (Branton and Kopp 1999).

Fibrogenesis is like wound healing, but after the prolonged injury cells are no longer under normal regulatory control and synthesize excessive extracellular matrix (ECM) proteins. The
upregulation of ECM proteins and the down regulation of metalloproteinase that degrade ECM proteins disrupt organ structure and function and lead to fibrosis (Mutsaers, Bishop et al. 1997; Kisseleva and Brenner 2008). There are several stages during fibrogenesis after chronic injury. The damage of epithelial and endothelial barrier causes blood clotting and the recruiting of inflammatory cells. Inflammation promotes fibrosis through facilitating cytokine secretion and generation of reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radicals. ROS promotes the release of the major fibrogenic cytokine including TGF-beta which activate fibroblasts and induce epithelial to mesenchymal transition. Activated fibroblasts express excess ECM proteins and leads to fibrosis (Kisseleva and Brenner 2008).

Fibroblasts, myofibroblasts and hepatic stellate cells are the cells responsible for collagen production. Differentiated fibroblasts are derived directly from embryonic mesenchymal cells and can proliferate as the resident fibroblasts. A recent study suggested that the origin of fibroblasts can also be epithelial cells and bone marrow stem cells which populate in lung, kidney and liver. The process of differentiation of epithelial cells into fibroblast is named epithelial to mesenchymal transition (EMT), this occurs when epithelial cells morphology changes from a cuboidal to a fibroblastic shape and the cells invade into ECM. The biochemical changes occur at the same time as loss of epithelial markers (E-cadherin and cytokeratin) and gain of mesenchymal markers (fibroblast specific protein-1, α smooth muscle actin) (Zeisberg and Kalluri 2004). Moreover, another type of EMT is EnMT (endothelial-messenchymal transition). Endothelial-messenchymal transition is another source of fibroblasts besides the residential fibroblasts, which occurs in heart and kidney (Zeisberg, Tarnavski et al. 2007; Zeisberg, Potenta et al. 2008; Kizu, Medici et al. 2009). TGF-beta (transforming growth factor beta) has been discovered to induce EMT in kidney (Willis and Borok 2007). In retina, lens, all organs, EMT depends on smad pathway (Valcourt, Kowanezt et al. 2005) (Saika, Kono-Saika et al. 2004). Moreover, TGF-beta was identified to be a key regulator in cardiac fibrosis since it induces endothelial-to-messenchymal transition in cardiac endothelial cells (Goumans, van Zonneveld et al. 2008). Also, Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury (Saika, Kono-Saika et al. 2004; Roberts, Tian et al. 2006) Other growth factors such as epidermal growth factor (EGF), hepatocyte growth factor, fibroblast growth factors (FGF) are involved in stimulating this transition as well (Willis, duBois et al. 2006; Zeisberg, Tarnavski et al. 2007). In addition to
fibroblasts and myofibroblasts which contribute to fibrosis, other residential cells such as hepatic stellate cells (HSCs) are responsible for collagen synthesis. In the liver, HSCs are the primary myofibroblasts responsible for liver fibrosis.

Myofibroblasts are defined as activated fibroblasts which synthesize a large amount of ECM proteins. It is reported that the transition of fibroblasts to myofibroblasts is mediated by growth factors and cytokines. For instance, in a healthy liver, hepatic stellate cells store vitamin A and stay quiescent. When liver is injured, the macrophages --- Kuffer cells release cytokines and ROS which activate HSCs. The major mediator in liver fibrosis is TGF-beta which plays an important role in activating HSCs. Activated HSCs synthesize α-smooth muscle actin (α-SMA), lose vitamin A stores and upregulate ECM genes expression (collagen I, III, IV, Fibronectin, undulin, elastin, laminin, hyaluronan and proteoglycans) (Li, Liao et al. 2008; Wallace, Burt et al. 2008). In vivo studies have shown that TGF-beta1 promotes transcription of collagen α1(I) I in hepatic stellate cells. When ECM protein such as collagen and fibronectins are highly increased in connecting tissue, the feedback loop stimulates fibroblast activation. (Kisseleva and Brenner 2008). In vitro activation of hepatic stellate cells has been used as a model for liver fibrosis. Quiescent HSCs cultured on plastic tissue culture plate undergo the similar process of activation as in liver fibrosis (Friedman 2008; Wallace, Burt et al. 2008).

Although there are many cytokines and growth factors in different tissues, the molecular mechanism how they induce collagen expressions not clear. TGF-beta not only promote endothelial/epithelial to mesenchymal transition, but also promotes activation of fibroblasts through induction of ECM protein synthesis, including collagen. The detailed mechanism will be explained in the introduction of chapter 3. Moreover, TGF-beta upregulates the genes expression of both TIMP-1 (tissue inhibitor of metalloproteinase) and PAI (plaminogen-activator inhibitor 1). The upregulation of TIMP and PAI inhibits the degradation of collagen and other ECM proteins. The accumulation of ECM eventually progresses to scar formation and fibrosis (Bonniaud, Kolb et al. 2004; Gauldie, Bonniaud et al. 2007).

Can fibrosis be reversed? There is evidence that advanced fibrosis cannot be reversed. The only way to slow down fibrosis is to eliminate the profibrotic stimulates. (Schnabl, Scholten et al. 2008). This was shown successfully in the removal of both hepatitis B and C viruses. Drug treatment to remove fibrogenic cells through apoptosis was shown to be a way to reverse
fibrosis. Apoptosis of activated HSCs allows for normal degradation of the fibrous scar in liver and can reverse liver fibrosis in patients. Reducing inflammation reverse fibrosis as well (Brenner 2009). Current approaches are also focusing on inhibition of TGF-beta and other growth factors and cytokines which promote fibroblast proliferation and activation to slow down fibrosis progression. Moreover, transplantation of bone marrow stem cells to the liver favors regeneration of the damaged hepatocytes, but the effect is trivial in reversal of fibrosis, since some bone marrow stem cells can proliferate into myofibroblasts. Therefore, stem cell transplantation may promote fibrosis (Alison, Islam et al. 2009).

Understanding Type I collagen synthesis is very important to identify potential fibrosis treatment. Type I collagen is the most abundant protein in skin, tendon, bones, and it plays an important role in keeping the connective tissue integrity (Li, Artlett et al. 1995). Collagen mRNAs for α1(I) and α2(I) are translated on the membrane of the endoplasmic reticulum (ER). The N terminus of the polypeptides has signal sequences which direct the polypeptide to the rough ER. During translation elongation, hydroxylases, prolyl 4-hydroxylase and prolyl-3-hydroxylase hydroxylate proline residues, while three different lysyl hydroxylases hydroxylates lysyl residues (Lamande and Bateman 1999). Furthermore, galactosyltransferase and a glucosyltransferase add these sugars to hydroxylysyl of the carboxy-terminal propeptides (Prockop 1984). Type I collagen triple helix is composed by two α1(I) and one α2(I) peptides. (Rossert, Terraz et al. 2000) Disulfide-bonds link the three peptides together through the C-terminus. These modifications are critical for stabilizing and folding of the triple helix. Chaperon Hsp47 is required for folding of the triple helix as well (Fessler and Fessler 1978; Asano, Markiewicz et al. 2009). After secretion, procollagen amino-proteinase removes the amino-propeptides from the three procollagen chains; procollagen carboxy-proteinase removes the carboxy-propeptides. This gives rise to mature collagen triple helix, which polymerizes into fibers. The fibers are cross-linked by lysyl oxidase and covalently linked together (Prockop 1984) (Prockop and Kivirikko 1995).

Incomplete modification of residues in collagen triple helix leads to the intracellular degradation of the collagen triple helix. This degradation is likely through lysosomal proteases. This indicated that the conformation of the triple helix is an important quality control and allows secretion only if collagen is properly folded and modified (Berg, Schwartz et al. 1980). In osteogenesis imperfecta, α1(I) collagen gene has certain mutations that decrease the rate of
assembly of type I collagen into triple helix. Unassembled collagen α1(I) polypeptides are hyper modified on proline and lysine residues and degraded in the cell (Tajima, Takehana et al. 1994). In the same disease, when α2(I) chains contain mutations, they do not incorporate into collagen molecules. In this case, type I collagen is composed of three α1(I) peptides to form a homotrimer instead of the regular heterotrimeric. The homotrimers are less efficient to polymerize into fibrils than heterotrimer, and collagen fibers are less stable and less strong physically. This results in osteogenesis imperfecta. In normal tissue a small percent of homotrimer synthesized, but the amount is well controlled and not more than 10%, mostly in the skin. A 10-15% synthesis of homotrimer leads to osteoporosis (Han, McBride et al. 2008).

The synthesis of type I collagen is first regulated by the transcription of α1(I) and α2(I) gene. The regulatory regions on collagen α1(I) and α2(I) includes both promoter and first intron. The functional promoter regions are utilized differently in different tissues and cell types for collagen α1(I) transcription. For instance, DNA sequence between -376 and -108 bp of human α2(I) is involved in transcription in scleroderma skin fibroblasts. DNA sequence from -376 to +58 bp of collagen α2(I) promoter is sufficient to activate transcription in scleroderma lung fibroblasts. For collagen α1(I), the region between +390 and +1440 bp of the first intron of the gene is necessary for enhancing transcription and to mediate overexpression of collagen gene in scleroderma skin fibroblasts (Ghosh 2002). DNA sequences from -2.3 to -1.7 kb is essential for α1(I) promoter expression in bone and teeth. In the tendon, both sequence -3.5 to -1.7 kb in the promoter and downstream sequence of -1.7kb, are needed for α1(I) gene transcription. Transgenic mice which have 3.2 kb of the promoter region expressed at high levels of the transgene in tendon and fascia fibroblasts. With 2.3kb of promoter, transgenic mice express high levels of transgenic α1(I) gene in osteoblasts and odontoblasts. With 900bp of promoter, transgene was expressed only in skin and was a low level (Jiang and Stefanovic 2008).

Multiple transcription factors interact with the promoter or an upstream regulatory sequence to mediate type I collagen transcription. CBF, NF1, C/EBP, SP1, smads, CBP/p300, C-Myb, c-Krox, BTEB, PP1, PP2A and CBFA1 are transcription factors that promote type I collagen genes transcription, while, IF1, IF2, AP1, NF-KB, B-Myb and MYc inhibit transcription (Ghosh 2002). For example, transcription of collagen α1 (I) is positively regulated by binding of Sp1 to the G C rich sequence of the promoter region (Li, Artlett et al. 1995). Transcription
factor Fli1 regulates collagen synthesis by binding to the α(I) promoter region and repress transcription in mouse skin (Asano, Markiewicz et al. 2009). Homeobox transcription factors Prx1 promotes type I collagen transcription of collagen α1(I) gene in hepatic stellate cells. In hepatic stellate cells, collagen α2(I) response to TGF-beta induction is dependent on the interaction of SP1, Sp3 and Smad3 proteins (Inagaki, Nemoto et al. 2001). Methylation on the promoter region of collagen α1(I) gene and first exon of collagen α(I) gene repress transcription by affecting transcription factor binding on the regions (Ghosh 2002).

Although transcription of type I collagen gene is upregulated in fibrosis, another mechanism appears to be the major regulation. Posttranscriptional regulation is the predominant regulation of type I collagen in hepatic stellate cells and activated fibroblasts. Processing/transport of the mRNA and translation and stability of the mRNA are the example of posttranscriptional regulation. The two collagen chains are synthesized in a 2:1 ratio in embryonic chicken calvaria. Because the proc1(I) mRNA level in the cells is twice as much as proc2(I) level (Vuust, Sobel et al. 1985). Prockop and others presented that posttranscriptional mechanism to maintain the steady state ratio of collagen α1(I) to α2(I) 2:1 (Olsen and Prockop 1989). In liver fibrosis, hepatic stellate cells are the primary cells that synthesize type I collagen. Hepatic stellate cells are activated in fibrosis and increase the synthesis of type I collagen 100 fold. There is 60-70 fold of increase in type I collagen mRNA steady state level in activated hepatic stellate cells. The steady state of the mRNA is determined by the rate of transcription, percentage of transcripts that are processed and transported to the cytoplasm, and the half life of the mRNA in the cytoplasm. Since there is only three fold increase in the rate of type I collagen gene transcription. The half life of the mRNA increases 16 fold, suggests that posttranscriptional regulation is predominant responsible for 100 fold increase in collagen protein synthesis.

The sequences in the 3’ UTR play an important role in collagen mRNA half life. A protein named α-CP binds to C-rich sequence located 23nt at the 3’ end to the stop codon and stabilizes the messages by diverting them from the degradation pathway. It is reported that the binding take place only in activated hepatic stellate cells, but not in the quiescent cells. α-CP belongs to the K-homology domain protein family; it is a RNA binding protein which shuttles between nucleus and cytoplasm. Phosphorylation of α-CP inhibits the binding to the collagen mRNA. α-CP may also promote collagen mRNA translation by interaction with PABP (poly A
binding protein). PABP stimulates translation of all mRNAs by binding with eIF4E. It is possible that α-CP is the bridge for the interaction of eIF4E and PABP, this stabilizes the mRNA into a circular form and facilitates for ribosome recycling which leads to efficient translation on the message (Lindquist, Marzluff et al. 2000).

The translation of collagen mRNA is also regulated at the translation initiation level. Translation initiation starts from the interaction of translation initiation factors with the 5’cap of mRNAs. The 40s ribosome subunit is recruited to the cap and starts scanning from the cap towards the start codon. The large subunit then associates with the 40s subunit to form the ribosome which continues with translation elongation. Since translation initiation factors are limited in the cell, different mRNAs compete with each other for the translation initiation. Therefore, different mRNAs are translated with different efficiency. The RNA that contains the sequences which favor translation initiation is preferentially selected.

There are four types of sequences in collagen mRNA that can affect translation initiation: two short upstream open reading frames (uORF), a start codon, the 5’ stem-loop and the 3’UTR. 5’UTR can block initiation complex formation and inhibit translation (Lindquist, Marzluff et al. 2000). There are many studies which showed that mRNAs with long and highly structured 5’ UTRs are inefficiently translated. Some stem-loops with $\Delta G > 50$ kcal/mol are stable; some stem-loops bind RNA binding proteins. Both of types can block ribosome scanning to inhibit translation initiation if the stem loops are adjacent to the cap. The uORF has been reported to inhibit the translation of several mRNAs by attenuating scanning of ribosomes toward to the start codon. This decreases the rate of translation initiation. All type I collagen mRNA all have two uORF proceeding the start codon in all the vertebrates, but the sequences of these ORFs are not conserved. However, deletion of collagen uORFs did not increase translation in reticulocyte lysate and in vivo experiments are needed to further elucidate the function of uORFs. The mechanism by which the 3’UTR affects translation initiation is described above for αCP. Since 3’UTR binds to PABP and PABP interact with eIF4G, mRNAs appear in a circular form. Specific proteins binding to the 3’UTR may affect the interaction of PABP to poly-A tail or to eIF4G, or the interaction of eIF4G with the 5’ cap. This effect may mediate translation initiation efficiency.

The 5’stem-loop is conserved in collagen mRNAs of all vertebrates, such as Xenopus, chicken, fish and human with only two nucleotides difference (Lindquist, Marzluff et al. 2000).
The collagen mRNA 5’ stem-loop structure is about 75nt from the cap of collagen α1(I) α2(I) and α1(III) mRNAs. Start codon of type I collagen mRNAs is within the 5’stem –loop. The stability of the type I collagen 5’ stem-loop is ΔG=25-30kcal/mol (Stefanovic, Hellerbrand et al. 1999). Therefore, it is unlikely to completely block ribosomal scanning. However, reporter mRNA with collagen 5’ stem-loop was translated in vitro 3 fold less efficient than the mutant stem-loop reporter mRNA. This suggested that potential 5’stem-loop binding proteins may be involve in blocking ribosomal scanning on collagen mRNA to inhibit translation. Mov13 homozygous mice does not produce collagen α1(I) (Dziadek, Timpl et al. 1987). When collagen α1(I) genes with and without 5’ stem-loop were transfected into Mov13 fibroblasts, collagen α1(I) synthesized from WT gene was secreted in triple helix form. Without the 5’ stem-loop, collagen α1(I) synthesized tracing amount of disulfide bond collagen trimmer. This suggested that the 5’stem-loop and its binding proteins may regulate the coordinated translation of α1(I) and α2(I) polypeptides (Stefanovic and Brenner 2003). In culture, hepatic stellate cells are activated; the 5’stem-loop prevented the expression of reporter genes in quiescent HSCs by decreasing the mRNA stability. However, 5’ stem-loop allowed for expression in activated HSCs. In activated HSCs, there is/are 120 KDa cytosolic proteins that bind to 5’ stem loop. The function of these proteins may be to promote translation and/or stabilize the collagen mRNAs. In quiescent HSCs, there are no proteins that bind to 5’stem-loop in vitro. Culturing HSCs in three-dimensional matrix revert them from activated to quiescent phenotype. The binding of cytosolic proteins to 5’ stem-loop was greatly reduced when the cells were cultured in the matrix. It is suggested that collagen mRNA maybe inefficiently translated or target for degradation without these binding proteins. The 5’stem-loop binding proteins are also present in the nucleus. The nuclear binding proteins may assist in the transport of collagen mRNA into the cytoplasm (Lindquist, Marzluff et al. 2000). Since 5’ stem-loop binding proteins are important for regulation, 5’stem-loop RNA was made as a molecular decoy and introduced into HSCs to titrate away the 5’stem-loop binding proteins. The decoy decreased the level of collagen α1(I) peptide and the disulfide-bond collagen trimmer by50%-60%. This suggested that 5’stem-loop binding proteins interact with 5’stem –loop to control type I collagen synthesis, proper folding and the stability of the collagen triple helix (Stefanovic, Schnabl et al. 2002; Stefanovic and Brenner 2003).
Early studies showed, type I collagen mRNAs are brought to a distinct endoplasmic reticulum compartment to ensure the coordinated synthesis of pro-collagen peptides. This suggests that the organization of triple helix begins with the organization of translation of collagen mRNAs. Judging from the similarity of signal peptides sequences and the initiation codons in the mRNAs of pro-α1(I) and pro-α2(I), Vogeli and Yamada groups suggested that the chains might be secreted into the endoplasmic reticulum from the common entry site. Furthermore, Kirk provided the evidence that type I collagen triple helix is formed while the mRNAs are still associated with polysomes bound to the endoplasmic reticulum membrane (Brownell and Veis 1976; Vogeli, Ohkubo et al. 1981; Veis, Leibovich et al. 1985; Veis and Kirk 1989). Therefore, type I collagen posttranscriptional regulation may be the essential step in regulating triple helix formation.

In this dissertation, I first describe the cloning and characterization of the collagen 5’ stem-loop binding protein, LARP6, and its effects on collagen synthesis. The detailed regulation of 5’ stem-loop dependent collagen synthesis and the role of nonmuscle myosin II in regulation of coordinated translation of collagen mRNAs through its motor activity are described in chapter 2. In chapter 3, we describe that LARP6 and nonmuscle myosin II regulation of collagen synthesis is universal in different primary cells such as cardiac fibroblasts, lung fibroblasts and skin fibroblasts and that this mechanism regulates cytokine and hormone induced collagen synthesis.
CHAPTER 1
BINDING OF LARP6 TO THE CONSERVED 5’ STEM-LOOP REGULATES TRANSLATION OF MRNAS ENCODING TYPE I COLLAGEN

Introduction

Type I collagen, the most abundant protein in the human body, is highly expressed in skin, bone, and tendon (Kivirikko 1998; Canty and Kadler 2002). Fibroproliferative disorders are characterized by excessive and persistent production of type I collagen in parenchymal organs, and 45% of all deaths in the United States are attributable to fibroproliferative disorders (Bitterman and Henke 1991). There is no cure for uncontrolled collagen synthesis and molecular details of its regulation are incomplete. The regulation is complex, because type I collagen is a heterotrimeric protein that requires synthesis, modification, and folding of two \( \alpha_1(I) \) chains and one \( \alpha_2(I) \) chain (Kivirikko 1998). The rate of posttranslational modifications and the rate of folding are coupled, because the mutations that delay folding result in hypermodifications of the chains and severe forms of osteogenesis imperfecta (Tajima, Takehana et al. 1994; Lamande and Bateman 1999).

Posttranscriptional regulation of collagen expression by stabilization of the \( \alpha_1(I) \) mRNA is an important mechanism that regulates collagen expression in various cell types (Focht and Adams 1984; Mauch, Hatamochi et al. 1988; Penttinen, Kobayashi et al. 1988; Mauch, Kozlowska et al. 1992; Eckes, Mauch et al. 1993; Maatta, Ekholm et al. 1995; Eckes, Mauch et al. 1996). Destabilization of collagen \( \alpha_1(I) \) mRNA is one of the features associated with quiescence of fibroblasts (Mauch, Kozlowska et al. 1992; Eckes, Mauch et al. 1993; Stefanovic, Lindquist et al. 2000), while transforming growth factor \( \beta \), the major profibrotic cytokine, induces collagen synthesis by increasing the half-life of the transcripts several fold (Penttinen, Kobayashi et al. 1988; Rishikof, Kuang et al. 1998; Sato, Shegogue et al. 2004). \( \alpha \)-CP, also known as PCBP or hnRNP-E (heterogeneous nuclear ribonucleoprotein E), binds to the C-rich sequence located 23 nt 3’ to the stop codon of collagen \( \alpha_1(I) \) mRNA (Stefanovic, Hellerbrand et al. 1997) and stabilizes collagen \( \alpha_1(I) \) mRNA (Lindquist, Kauschke et al. 2000).
α-CP stabilizes several other long-lived mRNAs, including α globin mRNA (Wang, Kiledjian et al. 1995), 15-1ipoxygenase mRNA, and tyrosine hydroxylase mRNA (Holcik and Liebhaber 1997), by binding to similar C-rich sequences.

In the 5’ untranslated regions (UTRs) of the three collagen mRNAs, α1(I), α2(I), and α1(III), there is a stem–loop structure encompassing the translation initiation codon (Yamada, Mudryj et al. 1983). The 5’ stem–loop structure is located 75–85 nt from the cap and has a stability of \( \Delta G = 25–30 \text{ kcal/mol} \). The 5’ stem–loop is well conserved in evolution, differing by only 2 nt in Xenopus and human collagen mRNAs (Su, Suzuki et al. 1991). The sequence constraints required to maintain the 5’ stem–loop dictate the sequence around the start codon of collagen mRNAs. Therefore, the start codon is not in the optimal sequence context for translation initiation (Kozak 1987). In addition, collagen α1(I), α2(I), and α1(III) mRNAs have two short upstream open reading frames (ORFs) preceding the coding region, which are usually inhibitory for translation (Kozak 2005). Thus, it seems that the three collagen mRNAs are designed to be inefficiently translated.

Our previous work indicated that collagen polypeptides have to be encoded by the mRNA with the 5’ stem–loop to be properly assembled into a triple helix (Stefanovic and Brenner 2003). This suggests that the 5’ stem–loop couples translational machinery to the collagen assembly pathway and is the first example of an RNA element that affects protein folding.

Here, we describe the cloning and characterization of the collagen 5’ stem–loop binding protein and its effects on translation of collagen mRNAs.

Results

5’ Stem–loop in the 5’ UTR of collagen mRNAs

Three collagen mRNAs, α1(I), α2(I), and α1(III), encoding collagens type I and III, have a conserved 5’ stem–loop structure in their 5’ UTRs (Yamada, Mudryj et al. 1983). The sequence conservation of the 5’ stem–loop from three distant species is shown in Fig.1.1(a). The translation initiation codon is buried in stem 2. We hypothesized that the 5’ stem–loop,
being unique for collagen mRNAs, may regulate coordinated translation of collagen mRNAs by binding specific RNA binding protein(s).

Figure 1.1

(a)

\[
\begin{align*}
\text{CAAAAGAGTCTACATGTCCTGATAAAGAGACATGT} & \quad \text{TCAGCTTTT} & \quad \text{FISH a1(I)} \\
\text{CAAAAAGAGTCTACATGTCCTAATATTAGACATGT} & \quad \text{TCAGCTTTT} & \quad \text{CHICK a1(I)} \\
\text{CCACAAAAGAGTCTACATGTCCTAAGGTCTAGACATGT} & \quad \text{TCAGCTTTTG} & \quad \text{HUMAN a1(I)} \\
\text{CAAAAGAGGAGTCTGACATGCTCTAATATTAGACATGT} & \quad \text{TCAGCTTTTG} & \quad \text{FISH a2(I)} \\
\text{CAAAAGAGTCTGACTTCATGCTCAAGTAGACATGC} & \quad \text{TCAGCTTTTG} & \quad \text{CHICK a2(I)} \\
\text{CAAAAGAGGAGTCTGACTTCATGCTCAAGTAGACATGC} & \quad \text{TCAGCTTTTG} & \quad \text{HUMAN a2(I)} \\
\text{CAAAAGAGGAGTCTCATTACATGCTAATATTACATGA} & \quad \text{TGAGCTTTT} & \quad \text{CHICK a1(III)} \\
\text{CAAAAGAGGAGTCTCATTACATGCTAATATTACATGA} & \quad \text{TGAGCTTTT} & \quad \text{HUMAN a1(III)} \\
\end{align*}
\]
Sequence-specific binding of a protein to the collagen 5′ stem–loop

To identify binding activity to the 5′ stem–loop RNA, we used nuclear and cytosolic extracts of human lung fibroblasts in gel mobility assays. In nuclear extracts, an RNA–protein complex was detected (Fig. 1.1b, lane 2), the binding of which was effectively competed with 50- and 250-fold molar excess of the same unlabeled RNA (lanes 3 and 4). When the RNA with the inverted sequence of the 5′ stem–loop (inverted 5′ stem–loop RNA) was used as competitor, only a weak competition was observed at a molar excess of 250-fold (lanes 6). In cytosolic extracts (Fig.1.1c), an RNA–protein complex with similar electrophoresis mobility was seen (lane 2). While the specific RNA effectively competed for its binding (lanes 3 and 4), the inverted 5′ stem–loop RNA did not compete at all (lanes 5 and 6). The inverted 5′ stem–loop RNA also did not bind any protein factors in the same extract (lanes 7 and 8). To verify that the complex formation on the 5′ stem–loop RNA is due to binding of proteins and not to binding of RNA, DNA, or carbohydrates, the cytosolic extract was treated with SDS and proteinase K prior to loading on the gel. Both treatments completely abolished the complex formation (not
shown). Similar, but weaker, 5’ stem–loop binding activity was also found in extracts of mouse embryonic fibroblasts, human skin fibroblasts, and rat liver myofibroblasts (not shown).

To obtain information about the molecular mass of the protein that directly binds to the 5’ stem–loop RNA, we performed UV cross-linking experiments using cytosolic extracts (Fig. 1.1d). A cross-link of 65 kDa was obtained with 5’ stem–loop RNA (lane 2) and no cross-linking was seen with the inverted 5’ stem–loop RNA. A similar cross-link was observed using nuclear extracts (not shown). Since this cross-link contains a protein and a small piece of RNA that resisted digestion with RNase T1, it represents only an approximation of the actual size of the protein.

Single-stranded bulge as target for protein binding

Evolutionary conservation of the 5’ stem–loop suggests that not only the structure, but also the sequence of both the stems and the bulge may be important for protein binding. We made a series of mutant probes wherein we mutated a single U in the B1 segment of the bulge (U mut), 4 nt in the B2 segment (B2 mut), or we inverted the sequence of the stems S1 or S2 but maintained the base-pairing (S1 mut and S2 mut, respectively). The sequences of these probes are shown in Table 1. Each mutant probe was analyzed for binding using cytosolic extracts (Fig. 1.1e). Changing a single U nucleotide into an A in the B1 segment completely abolished protein binding (compare lanes 4 and 2). Changing 4 nt in the B2 segment also completely abolished the binding (compare lanes 6 and 2). Inverting the sequence of the bottom stem diminished the binding (compare lanes 8 and 2), while the inversion of the top stem had a weaker effect (compare lanes 10 and 2). From this experiment, we concluded that the single-stranded regions of the bulge are important for binding of the detected protein to the 5’ stem–loop RNA.

Expression cloning of the 5’ stem–loop binding protein

We used a gel mobility shift assay as a readout for expression screening of a human fibroblast cDNA library. The library was amplified in pools containing 100 plasmids and each pool was transfected into human embryonic kidney (HEK) 293 cells. Cell lysates were
analyzed by gel mobility shift assay and one of the 130 pools screened yielded an increased binding to the 5′ stem–loop. This pool was divided into subpools containing 20 clones, which were identically screened until a single clone was isolated. This clone encoded a full-sized ORF identical to human La ribonucleoprotein domain family member 6 (LARP6) protein (Valavanis, Wang et al. 2007). The predicted molecular mass of LARP6 is 55 kDa. This is similar to that of the protein cross-linked to the 5′ stem–loop (Fig. 1.1 d), taking into account the inaccuracy of UV cross-linking.

Table 1. Sequence of the probes. Mutant 5′ stem–loop RNAs used as probes in gel mobility shift experiments. WT probe is shown at the top and the changed nucleotides in each mutant RNA are underlined.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCACAAAGAGUCUACAUGUCUAGGGUCUAGACAUGUUCAGCUUUGUGG</td>
<td>U MUT</td>
</tr>
<tr>
<td>CCACAAAGAGACUACAUGUCUAGGGUCUAGACAUGUUCAGCUUUGUGG</td>
<td>U MUT</td>
</tr>
<tr>
<td>CCACAAAGAGUCUACAUGUCUAGGGUCUAGACAUGUUGGACUUUGUGG</td>
<td>B2 MUT</td>
</tr>
<tr>
<td>AUCGUGCAAGUCUACAUGUCUAGGGUCUAGACAUGUUCAGUGCACGAU</td>
<td>S1 MUT</td>
</tr>
<tr>
<td>CCACAAAGAGUCUGCUAGACUUCGGUGAAGUCUAGCUCAGCUUUGUGG</td>
<td>S2 MUT</td>
</tr>
</tbody>
</table>

**LARP6 as the 5′ stem–loop RNA binding protein**

LARP6 is an uncharacterized RNA binding protein (Valavanis, Wang et al. 2007). To characterize its RNA binding properties, the binding to the 5′ stem–loop RNA was analyzed by gel mobility shift experiments (Fig. 1.2). First, we verified that endogenous LARP6 is in the complex bound to the 5′ stem–loop RNA. To this goal, we added anti-LARP6 antibody to the cytosolic extracts of lung fibroblasts and compared the mobility shift to that obtained with the control antibody (Fig. 1.2a). LARP6 antibody reduced binding of the endogenous protein to the
5’ stem–loop (lane 3). Second, we overexpressed LARP6 in cells expressing very little of the endogenous LARP6 (HEK293 cells). Binding to the 5’ stem–loop was dramatically enhanced in HEK293 cells when LARP6 was overexpressed compared to that in control cells (Fig.1.2). Two RNA–protein complexes were seen, probably representing the monomer and dimmer of LARP6 bound to the 5’ stem–loop RNA. A 50-fold excess of specific competitor RNA significantly reduced the binding, while a 250-fold excess completely abolished it (lanes 2 and 3). Nonspecific competitor (inverted RNA) had no effect (lanes 4 and 5).

Figure 1.2
To verify that LARP6 primarily binds the bulge of the 5′ stem–loop, we analyzed its binding to the mutant 5′ stem–loop probes. Fig. 1.2c shows that changing a single U nucleotide into an A in the B1 segment of the bulge almost completely abolished the binding (lane 2). Likewise, changing 4 nt in the B2 segment of the bulge dramatically reduced the binding (lane 3). Inverting the top or the bottom stem, but preserving the structure, resulted in stronger formation of the monomer complex and attenuation of the putative dimmer, but the overall binding was not significantly affected (lanes 4 and 5). From these experiments, we concluded that LARP6 binds the collagen 5′ stem–loop bulge in a sequence-specific manner and with specificity similar to that of the endogenous 5′ stem–loop binding activity detected in fibroblasts (Fig. 1.1).

To verify that LARP6 is sufficient for binding 5′ stem–loop, we purified the bacterially expressed glutathione S-transferase (GST)–LARP6 fusion protein. Recombinant LARP6 strongly bound wild-type (WT) 5′ stem–loop RNA (Fig. 1.2d, lane 1), while the binding was almost completely abolished when the U mutation of B1 was used (lane 2). The B2 mutant showed a decreased but significant binding (lane 3), in contrast to LARP6 expressed in mammalian cells, which does not bind this probe (Fig. 1.1 and Fig. 1.2). Two RNA–LARP6 complexes are indicated.
complexes were resolved, indicating that the purified protein can form dimmers. This experiment verified that LARP6 is sufficient for binding the 5′ stem–loop and that posttranslational modification of the protein is not absolutely necessary for binding.

To analyze if LARP6 interacts in vivo with collagen α1(I) and α2(I) mRNAs, we expressed hemagglutinin (HA)-tagged LARP6 in HEK293 cells and performed immunoprecipitation from the cytosolic extracts. The immunoprecipitated material was analyzed by reverse transcription–PCR (RT-PCR) for pull down of collagen α1(I) and α2(I) mRNAs. As shown in Fig. 1.2e, collagen α1(I) and α2(I) mRNAs coimmunoprecipitated with LARP6, while fibronectin mRNA did not (lane 1). Control RNA binding protein, RBMS3 (Fritz and Stefanovic 2007), did not pull down any mRNA (lane 2). Therefore, we concluded that both collagen mRNAs associate with LARP6 in vivo.

High affinity of binding of LARP6 to the 5′ stem–loop

Purified recombinant RNA binding proteins are commonly used to assess the affinity of binding to their targets (Lindquist, Kauschke et al. 2000; Katsamba, Park et al. 2002; Metzinger, Hallier et al. 2008). These results may not reflect the binding in the cellular environment, where the proteins are complexed with their interacting factors and where other proteins limit the accessibility to the target. To provide an estimate for the binding affinity of LARP6 to the 5′ stem–loop RNA, LARP6 was overexpressed in HEK293 cells and a fixed amount of cytosolic extract was added to the increasing amounts of radiolabeled 5′ stem–loop RNA probe (Fig. 1.3a). The fractions of the bound and unbound RNA were resolved by gel mobility shift assay, and Scatchard plot analysis was performed to determine the $K_d$ (Fig. 1.3b), as described (Meisterernst, Gander et al. 1988; Lindquist, Kauschke et al. 2000; Ohman, Kallman et al. 2000). From three independent experiments, we estimated the $K_d$ to be 1.4 nM (SD = 0.7). This is comparable to that of other sequence-specific RNA binding proteins (Singh and Valcarcel 2005; Lunde, Moore et al. 2007).

To assess if LARP6 binds the 5′ stem–loop of collagen α2(I) and α1(III) mRNAs with an affinity similar to that of α1(I) mRNA, we competed the binding of LARP6 to the α1(I) 5′ stem–loop probe with an excess of unlabeled 5′ stem–loop RNA derived from α2(I) and α1(III) mRNAs. As seen in Fig. 1.3c, the α2(I) 5′ stem–loop seemed to compete with the binding of
LARP6 to the α1(I) 5’ stem–loop slightly better than the α1(I) 5’ stem–loop (compare lanes 6–9 to lanes 2–5). The competing effect of the α1(III) 5’stem–loop was similar to that of the α1(I) 5’ stem–loop (lanes 10–13). We repeated this experiment three times and plotted the percentage of bound probe versus the molar excess of competitor (Fig. 1.3d). Although the competition by the α2(I) 5’ stem–loop was stronger than that of α1(I) and α1(III) 5’ stem–loops, the effect did not reach statistical significance at \( p < 0.05 \). Therefore, we concluded that LARP6 recognizes collagen α1(I), α2(I), and α1(III) 5’ stem–loops with similar affinity.

Unique RNA binding domain of LARP6

Figure 1.4a shows the predicted domains of LARP6. Amino acids 85–183 have a similarity to the La domain found in the members of the La-related protein (LARP) superfamily (Wolin and Cedervall 2002; Markert, Grimm et al. 2008). Adjacent to the La domain there is a domain with a weak similarity to a generic RNA binding domain (RBD) (amino acids 183–296) (Nagai, Oubridge et al. 1995). The other regions of LARP6 have no homology to known proteins. To assess which region of LARP6 is required for binding the collagen 5’ stem–loop RNA, we derived several deletion mutants. Figure 1.4a summarizes the mutants and their ability to bind 5’ stem–loop RNA and Fig. 1.4b shows the binding. When the C–terminal 300–491 amino acids were deleted (mutant A), the protein still had the ability to bind the 5’ stem–loop RNA (Fig. 1.4b, lane 3), however, deletion of most of the predicted RBDs (mutant B) completely abolished the binding (lane 4). Deletion of the N-terminal 183 amino acids, including the La domain (mutant C), also completely abolished the binding (lane 5). To assess if reintroduction of the La domain would restore the binding, we constructed a protein that had only the La domain and RBD (mutant D). This mutant was unable to bind the 5’ stem loop (lane 6), suggesting that amino acids N-terminal to the La domain are needed. When we added 40 amino acids N-terminal to the La domain, the binding was restored (mutant E, lane 7). Next, we made two internal deletions adjacent to the La domain (mutants F and G). While the mutant F, which contained four amino acids upstream of the boundary to the La domain was able to bind (lane 8), the mutant in which these amino acids were deleted (mutant G) lost the ability to
Figure. 1.3. High-affinity binding of LARP6 to collagen 5′ stem–loop. (a) Gel mobility shift assay with increasing amounts of 5′ stem–loop RNA probe and a fixed amount of cellular extract expressing LARP6. Femtomoles of probe used in the binding reactions is indicated at the bottom and migration of the bound and unbound probe is indicated at the right. (b) A representative Scatchard plot from the data shown in (a). The fractions of the bound and unbound RNA were quantified by phosphoimaging and Scatchard plot is shown. $K_d$ of binding was estimated from three independent experiments. (c) Gel mobility shift assay in the absence and presence of competitor RNA. WT α1(I) 5′ stem–loop RNA probe and cytosolic extract of HEK293 expressing LARP6 were used in all lanes. Lane 1, binding in the absence of competitor; lanes 2–5, binding in the presence of indicated molar excess of α1(I) 5′ stem–loop RNA; lanes 6–9, binding in the presence of molar excess of α2(I) 5′ stem–loop RNA; lanes 10–13, binding in the presence of molar excess of α1(III) 5′ stem–loop RNA. (d) Statistical evaluation of the degree of competition in three independent experiments. Bound and unbound probe from experiments in (c) were measured by phosphoimaging and the percentage of probe bound is shown for each molecular excess of competitor RNA added. Error bars indicate ± 1SD.

interact with the 5′ stem–loop (lane 9). From these experiments, we concluded that LARP6 needs the RBD and several amino acids adjacent to the N-terminus of the La domain to interact with the 5′ stem–loop. These amino acids are not present in the other La-containing proteins.

$LARP6$ is a nuclear and cytosolic protein

Immunostaining of the endogenous protein showed that LARP6 accumulates in both nucleus and cytoplasm (Fig. 1.4c). This is consistent with the presence of the endogenous 5′ stem–loop binding activity in the nuclear and cytosolic extracts of fibroblasts (Fig. 1.1). LARP6 has a predicted nuclear localization signal (NLS) between amino acids 293 and 303 (Valavanis, Wang et al. 2007). To assess if this signal is required for nuclear localization of LARP6, we expressed full-size LARP6, mutant A, lacking part of NLS, and mutant B, lacking the whole NLS, and prepared cytosolic and nuclear fractions. The fractions were analyzed by Western blot for distribution of LARP6 (Fig. 1.4d). Full-size LARP6 was equally distributed between nuclear and cytosolic fractions, consistent with immunostaining of the endogenous protein, while mutants A and B were predominantly cytoplasmic. Tubulin, which is exclusively a
cytosolic protein (McKean, Vaughan et al. 2001), was absent from the nuclear preparations, indicating that there was no cross-contamination. From these experiments, we concluded that

Figure 1.4
LARP6 accumulates in the nucleus and the cytosol and that a functional NLS resides in the C-terminal domain.

**Excess of LARP6 is inhibitory to translation of collagen mRNAs**

To test the hypothesis that LARP6 may regulate translation of collagen mRNAs, we constructed adenoviruses expressing full-size LARP6, mutant A (Fig. 1.4a), and control protein lysyl oxidase (LOX). Using adenoviruses, we could express these proteins in 100% of lung
fibroblasts, allowing studies on their effects on the endogenous collagen mRNAs. When we expressed full-size LARP6 and mutant A (both of these proteins can bind the 5’ stem–loop; Fig. 1.4b), there was a dramatic decrease in the cellular level of type I collagen protein (Fig.1.5a, lanes 1 and 2) and in the secretion of procollagen into the cellular medium (lanes 4 and 5). The cellular level and secretion of fibronectin were not significantly affected, indicating that the effect of LARP6 was collagen specific. Since the steady-state level of collagen mRNAs was unchanged (Fig.1.5b), we concluded that the excess of LARP6 as well as of mutant A inhibited translation of collagen mRNAs.

**Figure 1.5**

**Figure1.5.** Excess of LARP6 is inhibitory to translation of collagen mRNAs. (a) Effect of excess of LARP6 on collagen protein expression. Full-size LARP6 (FS, lane 1), mutant A (lane 2), and control protein LOX (lane 3) were expressed in lung fibroblasts. Cellular level of collagen α1(I) polypeptide (COLL) was assessed by Western blot. Expression of fibronectin (FIB) is shown as control. Lanes 4–6, medium from the same cells analyzed by Western blot. (b) Effect of excess of LARP6 on collagen mRNA expression. Total RNA from cells in A was analyzed by RT–PCR for expression of collagen α1(I) mRNA (COL1A1), α2(I) mRNA (COL1A2) and α1(III) mRNA (COL3A1). Actin is shown as loading control.
To verify this, we fractionated polysomes from LARP6-overexpressing cells and analyzed the polysomal loading of collagen α1(I) and α2(I) mRNAs. Figure 1.6a shows the distribution of ribosomal RNA in sucrose fractions of polysomes after treatment of cells with cycloheximide and puromycin, while Fig.1.6b shows the OD$_{260}$ profile of sucrose fractions from cycloheximide-treated cells. On the basis of the sensitivity to puromycin and on the fractionation of 80S ribosomes into fraction 13 and ribosomal subunits into fractions 14 and 15, we estimated that fractions 1–12 represent polysomes. Figure 1.6c shows that when control protein was overexpressed, collagen α1(I) mRNAs were found in all polysomal fractions (fractions 1–12) and to a lesser extent in fractions 13 to 15. However, when LARP6 was overexpressed, collagen α1(I) mRNA was reduced in polysomal fractions and accumulated predominantly in fraction 15 (Fig. 1.6c, top panel). A similar result was obtained with collagen α2(I) mRNA. It was distributed throughout the polysomal fractions in control cells and confined predominantly into fractions 13–15 in LARP6-overexpressing cells (Fig. 1.6c middle panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was distributed in all fractions and not affected by LARP6 overexpression (Fig. 1.6c, bottom panel). Similar polysomal distribution of GAPDH mRNA was described by other investigators (Laurent, Madjar et al. 1998) and allowed us to use this mRNA as a control for loading. We concluded that the redistribution of collagen mRNAs into fractions lighter than polysomes is responsible for the decrease of collagen protein synthesis in cells overexpressing LARP6 (Fig. 1.5a).

A similar result was obtained in the presence of excess mutant A, which can bind the 5′ stem–loop but is lacking the C-terminus (Fig.1.6d). Overexpression of this mutant showed even stronger exclusion of collagen mRNAs from the polysomes and their shift into the lightest fraction (fraction 15). This explained the lower level of collagen synthesis in the cells expressing mutant A than that in LARP6 (Fig.1.5a).

The presence of the endogenous LARP6 in polysomal fractions was assessed by Western blot. LARP6 was absent from all sucrose fractions and it was detected only in the supernatant above the sucrose gradient (Fig.1.6e, top panel), indicating that LARP6 does not associate with polysomes or ribosomal subunits. Similarly, the overexpressed LARP6 was found only in fraction 15 and in postpolysomal supernatant (bottom panel).

*Knockdown of LARP6 results in decreased collagen protein synthesis*
To assess the effect of loss of function of LARP6 on collagen expression, we inactivated LARP6 by small interfering RNA (siRNA). We used adenoviruses expressing one effective

Figure 1.6

(a)  

(b)  

OD260 vs. FRACTIONS

CHX

PUR

80S
60S
40S
**Figure 1.6.** Overexpression of LARP6 precludes polysomal loading of collagen mRNAs. (a) Analysis of ribosomal RNA. Polysomes were isolated from cells treated with cycloheximide (CHX, upper panel) or puromycin (PUR, lower panel) and distribution of ribosomal RNA in 45% (lane 1) to 15% (lane 15) linear sucrose gradient is shown. (b) Polysomal profile of cells treated with cycloheximide. OD$_{260}$ profile of sucrose fractions. The position of ribosomal subunits is indicated. (c) Polysomal distribution of collagen mRNAs in cells overexpressing LARP6. Top, distribution of collagen α1(I) mRNA in control cells (COL1A1 CON) and LARP6-overexpressing cells (COL1A1 LARP6). Middle, distribution of collagen α2(I) mRNA in control cells (COL1A2 CON) and LARP6-overexpressing cells (COL1A2 LARP6). Bottom, distribution of GAPDH mRNA. Polysomal fractions were analyzed by RT-PCR. (d) Polysomal distribution of collagen mRNAs in cells overexpressing mutant A. Experiment as in (b), except mutant A was overexpressed. (e) Distribution of LARP6 in polysomal fractions. Upper panel, endogenous LARP6 was analyzed in sucrose fractions and postpolysomal supernatant (SUP) of control cells by Western blot. Lower panel, analysis of overexpressed HA-tagged LARP6.

siRNA gene targeting LARP6 (D2) and one scrambled siRNA gene (CON). The depletion of LARP6 protein was assessed by Western blot (Fig.1.7a) and by gel mobility shift assay (Fig. 1.7b). Both assays revealed that LARP6 was depleted by about 70%. When LARP6 was knocked down, there was no change in the steady-state level of collagen α1(I) and α2(I) mRNAs (Fig.1.7c, lanes 1 and 4) and no difference in their decay rates after a transcription block with actinomycin D (lanes 2, 3, 5, and 6). However, when we measured collagen protein in LARP6-depleted cells, there was a reduction in the intracellular level (Fig.1.7d, top panel) and in the rate of secretion into the medium (bottom panel). The cellular level and secretion of fibronectin were not significantly altered. This suggested that the normal cellular level of LARP6 is needed for high steady-state level of collagen protein in lung fibroblasts. A similar result was obtained for skin fibroblasts (not shown). Taken together, these results indicate that LARP6 can be a translational repressor if expressed at high levels; however, at physiological levels, it acts to stimulate collagen synthesis.

The decreased level of collagen protein upon inactivation of LARP6 may be due to either increased protein turnover or decreased synthesis. To distinguish between these two possibilities, we first determined collagen protein level after treatment of cells with proteosome inhibitor MG132 (Fig.1.7e). The cells expressing LARP6-specific siRNA (D2) or control siRNA were treated with MG132 and intracellular collagen level was compared to that of the untreated
D2 siRNA decreased collagen expression in control cells (compare lanes 3 and 4), as well as in MG132-treated cells (compare lanes 1 and 2). Thus, we concluded that increased protein degradation is not likely to be responsible for the decrease in collagen protein level, but since protein degradation can also be by a nonproteosomal mechanism, we cannot completely exclude this possibility.

**LARP6 targets collagen synthesis to discrete regions in the cell**

Collagen, as extracellular protein, is synthesized on the membrane of the endoplasmic reticulum (ER) and folded within the lumen. Immunostaining of lung fibroblasts with collagen-specific antibody revealed that the collagen protein found intracellularly is not uniformly distributed throughout the ER. It colocalized with the ER marker calnexin, and while calnexin showed diffuse immunostaining, collagen was found at discrete foci (Fig. 1.8a). This suggests that its synthesis or accumulation in the ER is not random. This prompted us to analyze if LARP6 regulates this focal synthesis. To this goal, we made a collagen–GFP reporter gene, where we fused GFP to the C-terminus of collagen α1(I) ORF. The reporter protein was expressed from the mRNAs with or without the 5′ stem–loop (Fig. 1.8b) in a HEK293 cell line stably expressing LARP6 and in control cells. These cells were chosen because they express very little endogenous LARP6. When LARP6 was expressed in HEK293 cells, collagen–GFP protein encoded by the mRNA with the 5′stem–loop accumulated in a granular pattern, showing discrete spots of increased accumulation (Fig. 1.8c, upper left panel). The protein encoded by the mutant mRNA accumulated in a diffuse pattern resembling the ER and lacking the spots of concentration (upper right panel). The focal enrichment of collagen–GFP protein was also lost when the 5′ stem–loop mRNA was expressed in cells lacking LARP6 (bottom left panel), suggesting that the subcellular accumulation of the reporter protein is both 5′ stem–loop- and LARP6-dependent. To exclude the possibility that focal subcellular accumulation of the reporter protein is due to the transfected LARP6, we transfected the reporters into lung fibroblasts that express only endogenous LARP6 (Fig. 1.8d). A similar focal accumulation of COLL–GFP protein was observed when it was encoded by mRNA with the 5′ stem–loop (upper panel), compared to diffuse accumulation of COLL–GFP protein encoded by an mRNA without the 5′ stem–loop (lower panel).
Figure 1.7

(a) Depletion of LARP6 protein by siRNA. LARP6 expression was analyzed by Western blot; lane 1, LARP6-specific siRNA (D2); lane 2, scrambled siRNA (CON). Control for loading, fibronectin (FIB). (b) Depletion of LARP6 5’ stem–loop binding activity by siRNA analyzed by gel mobility shift assay. (c) Steady-state level and decay rate of collagen mRNAs in LARP6-depleted cells (lanes 1–3) and control cells (lanes 4–6). Steady-state level of collagen \( \alpha_1(\text{I}) \) and \( \alpha_2(\text{I}) \) mRNAs (lanes 1 and 4) and their decay after transcription block with actinomycin D (lanes 2, 3, 5, and 6) were analyzed by RT-PCR. (d) Depletion of LARP6 decreases collagen protein expression. Cellular extracts (top) and medium (bottom) were analyzed for collagen protein by Western blot. Fibronectin is shown as loading control. Lane 1, LARP6-depleted cells; lane 2, control cells. (e) Effect of proteosome inhibitor MG132. Cells expressing LARP6-specific siRNA (D2, lanes 1 and 3) or control siRNA (lanes 2 and 4) were treated with MG132 (lanes 1 and 2) or left untreated (lanes 3 and 4) and cellular level of collagen was analyzed by Western blot.

Figure 1.7. Inactivation of LARP6 decreases collagen synthesis. (a) Depletion of LARP6 protein by siRNA. LARP6 expression was analyzed by Western blot; lane 1, LARP6-specific siRNA (D2); lane 2, scrambled siRNA (CON). Control for loading, fibronectin (FIB). (b) Depletion of LARP6 5’ stem–loop binding activity by siRNA analyzed by gel mobility shift assay. (c) Steady-state level and decay rate of collagen mRNAs in LARP6-depleted cells (lanes 1–3) and control cells (lanes 4–6). Steady-state level of collagen \( \alpha_1(\text{I}) \) and \( \alpha_2(\text{I}) \) mRNAs (lanes 1 and 4) and their decay after transcription block with actinomycin D (lanes 2, 3, 5, and 6) were analyzed by RT-PCR. (d) Depletion of LARP6 decreases collagen protein expression. Cellular extracts (top) and medium (bottom) were analyzed for collagen protein by Western blot. Fibronectin is shown as loading control. Lane 1, LARP6-depleted cells; lane 2, control cells. (e) Effect of proteosome inhibitor MG132. Cells expressing LARP6-specific siRNA (D2, lanes 1 and 3) or control siRNA (lanes 2 and 4) were treated with MG132 (lanes 1 and 2) or left untreated (lanes 3 and 4) and cellular level of collagen was analyzed by Western blot.
Figure 1.8

(a)

(b)

5' SL COL1A1/GFP

\[ \text{CMV PROM} \rightarrow \text{COL1A1 5' UTR} \rightarrow \text{COL1A1} \rightarrow \text{GFP} \]

ΔSL COL1A1/GFP

\[ \text{CMV PROM} \rightarrow \text{COL1A1} \rightarrow \text{GFP} \]
From these experiments, we concluded that LARP6 targets the synthesis of collagen reporter protein to discrete subcellular sites in a 5’ stem–loop-dependent manner.

Discussion

The 5’ stem–loop has emerged as the key cis-acting element regulating expression of type I collagen (Stefanovic, Hellerbrand et al. 1999; Stefanovic, Lindquist et al. 2000; Stefanovic, Schnabl et al. 2002; Stefanovic and Brenner 2003), but the trans-acting factors have been unknown. Here, we show that LARP6 is the protein that binds the 5’ stem–loop of collagen α1(I) and α2(I) mRNAs. So far, LARP6 has been an uncharacterized RNA binding protein, belonging to the La-domain-containing protein superfamily (Maraia and Bayfield 2006). As the collagen 5’ stem-loop RNA binding protein, LARP6 could be involved in multiple roles in regulating collagen protein synthesis. LARP6, also known as Acheron (ACHN, FLJ11196), contains two domains that have similarity to other proteins, the LA domain and the RNA binding domain. LA domain is well conserved in all La family proteins. The RNA binding domain (RBD) in LARP6 is diverse from the RBD found in other members of the La family. Very little research has been done on LARP6 and the function of the protein has been unknown. One report showed that LARP6 is induced during the programmed cell death of skeletal muscles in the moth Manduca sexta. (Valavanis, Wang et al. 2007) It may also
regulate integrin expression, adhesion, and motility in differentiating myoblasts. (Glenn, Wang et al.)

Other La family proteins are involved in many aspects of RNA metabolism. LARP1 localizes to gremline p bodies and functions in RNA degradation. (Nykamp, Lee et al. 2008) LARP2 has several isoforms in human, chimpanzee, dog, red junglefowl, and fish. The exact function of LARP2 is unknown. In LARP3 (La autoantigen), the RNA binding domain is adjacent to the La domain and promotes LARP3 binding to the 3’ poly U sequence of polymerase III primary transcripts. (Bousquet-Antonelli and Deragon 2009). LARP3 protein is also involved in termination of transcription by RNA polymerase III and in formation of the splicing complex of U6 snRNA (Yoo and Wolin 1994). Nonphosphorylated LARP3 is cytoplasmic and associate with mRNAs that contain 5’-terminal oligopyrimidine(5’TOP) motifs, this binding stimulates mRNAs translation. Phosphorylation on serine 366 of LARP3 in the La domain targets LARP3 into the nucleus and assists processing of pret-RNA. (Intine, Tenenbaum et al. 2003) The function of LARP4 (c-Mpl binding protein) and LARP5 is still unknown. LARP7 binds to the conserved 3’ U rich sequence of 7SK RNA and stabilize the RNA. The binding of 7SK RNA to HEXIM protein inhibits p-TEFb (positive transcriptional elongation factor b) and inhibits RNA polymerase II transcription elongation (Diribarne and Bensaude 2009) (Markert, Grimm et al. 2008).

The binding of LARP6 to the collagen 5’ stem–loop is strictly sequence-specific, because changing of a single nucleotide U completely abolishes the binding (Fig.1.1 and Fig.1.2). The binding affinity is in the nanomolar range and with similar affinity to collagen α1(I), α2(I), and α1(III) 5’ stem–loops (Fig.1.3). High affinity and specificity of binding indicate that the function of LARP6 is to regulate expression of type I (and possibly type III) collagen. LARP6 has a unique bipartite RNA binding domain not found in the other members of the La superfamily (Fig.1.4). This domain recognizes the two regions of the 5’ stem–loop predicted to have single-stranded conformation, while double-stranded stems are dispensable for binding. However, the predicted single-stranded regions may be folded into a complex tertiary structure.

Binding of LARP6 to the collagen 5’ stem–loop is likely to occur in the nucleus, before the export of collagen mRNAs into the cytosol. LARP6-binding activity was detected in nuclear extracts (Fig.1.1), and the protein has an NLS and accumulates in the nucleus (Fig.1.4). Thus,
it is possible that LARP6 shuttles between the nucleus and the cytoplasm and participates in nuclear export of collagen mRNAs. However, we provide evidence that its cytoplasmic role is to regulate translation of collagen mRNAs.

An excess of LARP6 can prevent the formation of polysomes on collagen mRNAs; they co-fractionated with ribosomal subunits when LARP6 was overexpressed. The same result was obtained when LARP6 mutant lacking the C-terminus was overexpressed (Fig.1.6). Since both of these proteins bind the 5′ stem–loop, it is possible that they can mask collagen mRNAs. This suggests that when collagen mRNAs emerge into the cytoplasm bound by LARP6 they may be inaccessible for translation until LARP6 dissociates. This repression is transient, and we postulate that it is needed to prevent independent translation of collagen α1(I) and α2(I) mRNAs and random synthesis of collagen polypeptides. Assembly of the triple helix of type I collagen has a slow step of registration of α1(I) and α2(I) chains and a fast step of propagation of the triple helix (Boudko, Frank et al. 2002; Bachmann, Kiefhaber et al. 2005). The slow registration step is concentration-dependent, and a mechanism that can increase local concentration of the chains in vivo has been suggested (Beck, Boswell et al. 1996; Gura, Hu et al. 1996; Stefanovic, Lindquist et al. 2000). The coordination of the synthesis of collagen α1(I) and α2(I) chains is also underscored by the fact that > 99% of naturally synthesized type I collagen is a heterotrimer of two α1(I) chains and one α2(I) chain and not a homotrimer of α1(I) chains (Uitto 1979). Collagen α1(I) homotrimers readily form a triple helix in humans having a complete absence of α2(I) chain (Malfait, Symoens et al. 2006) and in knockout mice in which the α2(I) gene had been inactivated (Sims, Miles et al. 2003). Therefore, α1(I) chains have the propensity for folding into a functional triple helix, but some coordination of translation of α1(I) mRNA and α2(I) mRNA must occur to ensure almost 100% formation of the heterotrimer. Binding of LARP6 may serve this purpose. This can explain why collagen synthesis decreases when LARP6 is knocked down (Fig.1.7).

Using collagen–GFP reporter protein, we visualized the subcellular regions that may represent the sites of collagen synthesis or accumulation. In cells expressing LARP6, a focal or granular pattern of collagen–GFP localization was observed, but only when this reporter was encoded by the mRNA with the 5′ stem–loop (Fig.1.8b). This is similar to the subcellular localization of endogenous collagen protein. Since COLL–GFP protein has a signal peptide, the granular pattern represents either the regions on the ER membrane where the synthesis of
the reporter protein takes place or the regions in the ER lumen where the protein accumulates after translocation. Since the formation of these granules was dependent on both the presence of 5′-stem–loop in the mRNA and LARP6 expression, and not on the protein sequence, we favor the hypothesis that these are the sites where translation of collagen mRNAs takes place. The LARP6-dependent aggregation of collagen–GFP protein synthesis is consistent with the hypothesis that LARP6 prevents independent translation of collagen mRNAs and targets them to discrete regions on the ER membrane. This can increase the local concentration of chains for preferential folding of the heterotrimer.

Inactivation of LARP6 resulted in a decrease of total collagen protein (Fig. 1.7), suggesting that physiological levels of LARP6 are needed for efficient collagen synthesis. This implies that the level of LARP6 in the cell has to be tightly regulated; overexpression may overwhelm the mechanism needed to dissociate LARP6 from the 5′ stem–loop. Since endogenous LARP6 is not found in polysomal fractions (Fig. 1.6d), it must dissociate from the 5′ stem–loop once collagen mRNAs are committed for translation. Dissociation of LARP6 can be achieved either by posttranslational modifications, which would reduce its affinity for the 5′ stem–loop, or by the ubiquitin-dependent degradation. Treatment of cells with proteasome inhibitors (Meng, Mohan et al. 1999) increased the level of LARP6 protein (not shown), suggesting that it is regulated, at least in part, by proteasomal degradation. Our preliminary experiments also suggest that LARP6 is phosphorylated. Since bacterially expressed LARP6 has the ability to bind the 5′ stem–loop (Fig. 1.3d), phosphorylation is not absolutely required for binding, but may regulate the affinity.

The cloning and characterization of LARP6 as a collagen 5′ stem–loop RNA binding protein is a critical step toward understanding the synthesis of the most abundant protein in the human body, type I collagen. The 5′ stem–loop is not absolutely required for collagen synthesis, because triple helical collagen is made, albeit at a reduced level, by the embryonic fibroblasts that have a mutated 5′ stem–loop in the endogenous α1(I) gene (L.C., D.F., L.S. and B.S., unpublished results). We propose that the 5′ stem–loop/LARP6 mechanism is activated when large amounts of type I collagen are produced, as in wound healing or in pathologic fibrosis (Leask, Denton et al. 2004; Abraham, Eckes et al. 2007).

Other stem-loop binding proteins were discovered having multiple functions in mRNA regulation. Histone 3′ stem-loop mRNA binding protein facilitates 3′-end formation through its
interaction with U7 snRNA. (Dominski, Zheng et al. 1999). The phosphorylation of histone stem loop binding protein promotes high-affinity binding to the RNA. (Borchers, Thapar et al. 2006) Therefore, it is possible that LARP6 is involved in other metabolic steps of collagen mRNAs such as localization, processing and splicing by interacting with other proteins or through its posttranslational modifications.

Materials and Methods

Expression cloning of the 5′ stem–loop binding protein

Human fibroblast cDNA library cloned in the pSPORT6 plasmid was obtained from Invitrogen. The library was amplified in pools containing approximately 100 colonies and plasmid preparations were made from these pools. Each pool was transfected into HEK293 cells and cytosolic extracts were used in the gel mobility shift assay using 5′ stem–loop RNA as probe (Stefanovic, Lindquist et al. 2000). The complexity of one positive pool was reduced by retransforming the plasmids from the pool and collecting 20 colonies into subpools. The subpools were screened as above until a single clone was isolated.

Construction of clones and adenoviruses

HA-tagged LARP6 was constructed by PCR amplification of the clone isolated from the library and cloning of the PCR product into EcoRV–XbaI sites of pCDNA3 vector (Stratagene). C-terminal deletion mutants were made by cutting with XcmI and HindIII restriction sites and blunting; N-terminal deletions were made by removing KpnI–EcoRV or EcoRI–EcoRV fragments, while internal deletions were made with Bal31 nuclease. In-frame deletions were confirmed by Western blot and sequencing. Collagen–GFP fusion protein was made by cloning human collagen cDNA (Geddis and Prockop 1993) lacking 10 C-terminal amino acids in pCDNA3 vector (Stratagene). The GFP cassette was then cloned in-frame at the C-terminal end. The 5′ stem–loop was deleted from the above clone by cutting with HindIII and XbaI, blunting, and religating the plasmid.

Adenoviruses were constructed by recloning of LARP6 pCDNA3 constructs into the pAdCMVTRACK vector, followed by recombination with the pAdEasy vector, as described (He, Zhou et al. 1998). Control adenoviruses expressed either a truncated version of LOX (Kagan
and Li 2003) or an unrelated RNA binding protein, RBMS3 (Fritz and Stefanovic 2007). Adenoviruses were amplified in HEK293 cells and purified by a Virapure kit (Clontech). Expression of each construct was verified by Western blot.

siRNA genes were made by cloning several small hairpin double-stranded oligonucleotides targeting human LARP6 into pSuper vector (Oligoengine). The siRNA genes were cotransfected with HA-tagged LARP6 into 293 cells and the efficacy of LARP6 knock down was assessed by Western blot. One effective siRNA (D2) was found and the cassette from pSuper vector containing H1 promoter and D2 siRNA sequence was recloned into the pAdTRACK vector and adenovirus was constructed as above. Control adenovirus contained an siRNA gene with a scrambled sequence.

Cells and transfections

HEK293 cells and human lung fibroblasts immortalized by expression of telomerase reverse transcriptase (Yamada, Castro et al. 2003) were grown under standard conditions. HEK293 cells were transfected with 1 μg of plasmid per 35-mm dish using 293TransIT reagent (Mirus). Stable cell lines expressing LARP6 were developed by transfecting the HEK293 cells as above and selecting with G418 for 3–4 weeks.

Transduction of lung fibroblasts with adenoviruses was done by adding adenoviruses at a multiplicity of infection (MOI) of 100. With this MOI, between 95% and 100% of the cells were transduced, as judged by expression of the viral marker, GFP. The cells were harvested for analysis 2-5 days after the viral delivery.

Proteosome inhibitor MG132 was used at concentration of 100 μM for 18 h prior to analysis of collagen protein.

Purification of recombinant LARP6

The coding region of LARP6 cDNA was amplified by PCR and cloned into BamHI and XbaI of pGEX3Ti vector in frame with GST. The protein was expressed in E. coli and purified on a glutathione Sepharose column, as described (Hengen 1996).

Cellular fractionation
Cytosolic extracts were prepared by hypotonic lysis of cells in 10 mM Tris–HCl (pH 7.6), 5 mM MgCl₂, 10 mM KCl, and 0.4% NP-40, and after removal of nuclei by centrifugation, the supernatant was used as cytosolic extract. For nuclear extract, the pelleted nuclei were washed several times in the above buffer and nuclear proteins were extracted by the method of Dignam et al (Dignam, Lebovitz et al. 1983). Protein concentration was measured by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

**Gel mobility shifts**

Double-stranded oligonucleotides with the sequence of wt and various mutant 5′ stem–loops were cloned into the SmaI site of pGEM3 vector (Promega). The RNA probes were prepared by *in vitro* transcription from these templates after linearization with BamHI (Stefanovic, Hellerbrand et al. 1999). The sequence of the probes is shown in Table 1. Total protein (40 μg) from cytosolic or nuclear extracts was incubated with 20 fM of the probes in the presence of 5 mM MgCl₂ and 10 μg of tRNA for 10 min on ice. The RNA–protein complexes were resolved on a 6% native gel and visualized by autoradiography. For competition experiments, the unlabelled RNA was transcribed from the 5′ stem–loop template cloned into the correct orientation (specific competitor) or cloned in an inverted orientation (nonspecific competitor). For supershift experiments, 2 μl of LARP6-specific antibody or control antibody (anti-GFP) was added prior to addition of RNA probes. For quantitative assessment of binding, the amount of bound and unbound probe was measured by phosphoimaging. Scatchard plot was constructed as described (Lindquist, Kauschke et al. 2000).

**UV cross-linking**

The RNA probe was mixed with cytosolic extracts as described for gel mobility shift assay. The samples were irradiated with UV light at 230 nm for 15 min, treated with 2 U of RNase T1 for 10 min at room temperature, and analyzed by SDS-PAGE and autoradiography.

**RT-PCR**

RT-PCR reactions were done with 100 ng of total RNA according to the published procedure (Stefanovic, Hellerbrand et al. 1995; Stefanovic, Hellerbrand et al. 1999; Stefanovic,
The primers used are listed in Table 2. Tth DNA polymerase and 3′ gene-specific primer were used for reverse transcription, while PCR amplification was done in the subsequent step by adding 5′ primer and [α-32P]deoxy-ATP. The radiolabeled PCR products were resolved on sequencing gels and visualized by autoradiography. Radiolabeling of PCR products allowed the use of a minimal number of cycles for detection of the products; for analysis of total RNA, 16–18 cycles were used, while for analysis of polysomal fractions, 25–30 cycles were used.

<table>
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<th>Primer</th>
<th>Forward Primer (F)</th>
<th>Reverse Primer (R)</th>
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<td>h-collagen α1(I) (122 nt)</td>
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<td>h-collagen α2(I) (160 nt)</td>
<td>F: CAGCAGGAGGTTTCTGGCTAA</td>
<td>R: CAACAAAGTCCGCGTATCCA</td>
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<tr>
<td>h-collagen α1(III) (120 nt)</td>
<td>F: ATCTTGGTCAGTCCTATGG</td>
<td>R: GCAGTCTTAATTCTTGATCGTCA</td>
</tr>
<tr>
<td>h-fibronectin (220 nt)</td>
<td>F: ACCAACCTACGGATGACTCG</td>
<td>R: GCTCATCATCTGGCCATT</td>
</tr>
<tr>
<td>h-LARP6 (246 nt)</td>
<td>F: TTACACGGGACTGGAGAACC</td>
<td>R: GTCCCCAAAAGCTTGAGCAG</td>
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<td>h-GAPDH (74 nt)</td>
<td>F: ACCGGTTCCAGTAGGTACTG</td>
<td>R: CTCACCGTCACTACCGTACC</td>
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Table 2-continued

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<th>h-actin (213 nt)</th>
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<tr>
<td></td>
<td>R: 5′-GAAGG TAGTTTCGTTGGATGCC</td>
</tr>
<tr>
<td>D2 siRNA</td>
<td>5′-UCCAACUGTCCACGTCGCU</td>
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</table>

**Fractionation of polysomes**

Polysomes were fractionated from $3 \times 10^7$ cells on linear 15–45% sucrose gradients, as described (Chen, Xu et al. 1995). Fractions (0.5 ml) were collected and RNA was extracted with phenol–chlorophorm and precipitated with isopropanol. RNA samples were run on 1% agarose gel to estimate the distribution of ribosomal RNAs. Expression of collagen mRNAs and GAPDH mRNA was analyzed by RT-PCR. For protein analysis the proteins from sucrose fractions were precipitated with 0.05% deoxycholate and 6.5% trichloroacetic acid, washed with acetone, and analyzed by Western blot. When the cells were treated with puromycin, the treatment was for 1 h prior to isolation of polysomes.

**Western blots**

Western blots were done under reducing conditions, as described (Stefanovic, Lindquist et al. 2000; Stefanovic, Schnabl et al. 2002; Stefanovic and Brenner 2003; Stefanovic, Stefanovic et al. 2004; Stefanovic, Stephens et al. 2005). Antibody against type I collagen was from Rockland and has been used before (Stefanovic, Lindquist et al. 2000; Stefanovic, Schnabl et al. 2002; Stefanovic and Brenner 2003). Collagen α2(I)-specific antibody (C19) was from Santa Cruz Biotechnology, anti-LARP6 antibody was from Abnova, anti-fibronectin antibody was from BD Transduction Laboratories, anti-tubulin antibody was from Zymed, and anti-HA antibody was from Sigma.

To analyze collagen secretion into cellular medium, after expression of LARP6, its derivatives, or siRNA, fresh serum-free medium was added to the cells and incubation continued for 3 h. This allowed for accumulation of the secreted collagen in the fixed time and without contamination by collagen present in calf serum.
Immunostaining and confocal microscopy

Immunostaining was done by fixing the cells in 4% paraformaldehyde and, after blocking with 5% goat serum for 1 h, anti-LARP6 antibody (Abnova) or anti-collagen antibody (Rockland) together with anti-calnexin antibody (Cell Signaling) was added at 1:10 dilution and incubated overnight at 4 °C. After washing, Alexa Fluor- or Cy2-labeled secondary antibody was added at 1:50 dilution for 1 h. For control cells the primary antibody was omitted. Immunostained cells were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) stain (Vector Laboratories) and the images were taken with a Leica TCS SP2 AOBS laser confocal microscope under 110-fold magnification. Images through one of the focal planes are shown.

For detection of collagen–GFP fusion proteins, the cells were fixed in paraformaldehyde and mounted in Vectashield, and images were taken as above.
CHAPTER 2
NONMUSCLE MYOSIN FILAMENTS ARE REQUIRED FOR SYNTHESIS
OF TYPE I COLLAGEN

Introduction

Type I collagen is the most abundant protein in human body (Rippe, Schrum et al. 1999). It is composed of two α1(I) and one α2(I) polypeptides which fold into a triple helix (Prockop 1984). The biosynthesis of type I collagen has multiple steps, but the majority of work to elucidate regulation of collagen expression was concentrated on transcriptional regulation. Recently it became evident that regulation of stability of collagen mRNAs is the predominant mechanism for high level of synthesis in multiple cell types. Collagen mRNAs have long half-lives, which can be modulated by growth conditions and profibrotic cytokines, like TGFβ. Two sequence elements were implicated in regulating collagen mRNA stability (Varga, Rosenbloom et al. 1987); the C-rich region in the 3’ UTR of collagen α1(I) mRNA and a stem-loop structure found in the 5’ UTR of collagen α1(I), α2(I) and α1(III) mRNAs (Stefanovic, Schnabl et al. 2002). αCP is the protein which binds the C-rich sequence and regulates the stability of collagen mRNAs (Lindquist, Parsons et al. 2004).

We have cloned La ribonucleoprotein domain family member 6 (LARP6), as the protein which binds 5’ stem-loop of collagen α1(I), α2(I) and α1(III) mRNAs with high affinity and specificity (chapter 1). LARP6 is a 54 KD protein containing a La domain (amino acid 85-183) which is conserved in La-related protein super family. The RNA binding domain of LARP6 is between amino acids 183-296, while the nuclear localization signal is between amino acids 293-303, the predicted nuclear export signal is in amino acids 186-193 (chapter 1).

The binding of LARP6 to the 5’ stem-loop is necessary for high level of expression of type I collagen (chapter 1). We postulated that LARP6 binding serves to prevent premature translation of collagen mRNAs, allowing for their subsequent coordinated translation on the membrane of the endoplasmic reticulum (ER). This coordination is evidenced by localization of collagen synthesis into discrete subcellular sites (Fig. 1.8). Translation of collagen α1(I) and α2(I) mRNAs in close proximity at these sites may be needed to increase the local
concentration of the polypeptides, which favors formation of $\alpha_{1(I)}/\alpha_{2(I)}/\alpha_{1(I)}$ heterotrimers. In support of this, heterotrimers of type I collagen are almost exclusively synthesized in all tissues, although the homotrimers of $\alpha_{1(I)}$ polypeptides readily form in the absence of $\alpha_{2(I)}$ polypeptide (Han, McBride et al. 2008).

Nascent collagen polypeptides undergo glycosylation and hydroxylation, as they are cotranslationally translocated into the lumen of the ER (Lamande and Bateman 1999). Folding of the collagen triple helix starts with disulfide bonding of two $\alpha_{1(I)}$ and one $\alpha_{2(I)}$ polypeptides at the C-terminal end, with subsequent folding into a triple helix. Disulfide bonded collagen polypeptides were found associated with polysomes, suggesting that interchain bonding starts before the release of polypeptides from the polysomes (Veis and Kirk 1989). Folding and posttranslational modifications of collagen polypeptides are in kinetic equilibrium, slow folding results in hypermodifications of the polypeptides. Hypermodified collagen peptides fold into unstable triple helix, resulting in a phenotype of osteogenesis imperfect (Han, McBride et al. 2008). Therefore, translational elongation, the rate of modifications and the rate of folding are coordinated. TRAM2 protein, as a part of translocon, associates Ca$^{++}$ pump Serca2b to the translocons where collagen chains are elongated. It has been proposed that this increases local Ca$^{++}$ concentration to stimulate collagen specific molecular chaperones to facilitate folding of the heterotrimer (Stefanovic, Stefanovic et al. 2004).

Nonmuscle myosin II is a hexamer which is formed by two heavy chains, two regulatory light chains (RLC) and a pair of essential light chains. Globular head domain contains ATP and actin binding sites that are required for its motor activity. There are three isoforms of nonmuscle myosin II, A, B and C (Sandquist, Swenson et al. 2006). Each of them contain two unique heavy chains which can interact with actin and cause contractility (Bresnick 1999). All of them are regulated by phosphorylation of regulatory light chain (Tan, Ravid et al. 1992; Watanabe, Hosoya et al. 2007) (Smith, Cande et al. 1983). MLCK (myosin light chain kinase) phosphorylations of RLCs (regulatory light chains) mediate polymerization of myosin II into bipolar filaments which are the active form of myosin (Totsukawa, Yamakita et al. 2000). Nonmuscle myosin filaments have motor activity in the head domain of the heavy chains and can pull actin into different directions. Myosin hydrolyses ATP when actin is not attached. Actin binding triggers the phosphate to release from myosin. The releases of phosphate promote the force generation of myosin II. Then ADP release from actin bound myosin, and ATP binds to
myosin again and this cycle repeats (Murphy, Rock et al. 2001). As a cytoskeleton protein, nonmuscle myosin II is involved in cytokinesis, cell locomotion (Conti and Adelstein 2008; Goeckeler, Bridgman et al. 2008). In wound healing, nonmuscle myosin II is needed for migration of fibroblasts to the wound where fibroblasts secret collagen to facilitate the healing process (Maciver 1996). The other functions of nonmuscle myosin are not well understood yet. Recent studies in chick fibroblast, mouse brain and yeast indicated that myosin is involved in actin based movement of mRNAs. Myosin-Va, plays a role in mRNP (messenger ribonucleoprotein) transport in neuronal dendrites and spines. Myosin IIB facilitates actin mRNA transport to restrict the spatial distribution of actin protein synthesis and augments cell asymmetry and motility in fibroblasts. Myosin 4p interacts with She2p to transport Ash mRNA in Saccharomyces Cerevisiae. mRNA localization is important in protein synthesis (Lopez de Heredia and Jansen 2004). Microtubules are also involved in mRNA transport and localization. Microtubules dependent motors, dynein and kinesins interact with protofilaments and transport mRNAs in eukaryotic cells. Studies showed that dynein transports Drosophilar Gurken mRNA to antero-dorsal position in oocytes and Bicoid mRNA to the anterior end in the oocytes (MacDougall, Clark et al. 2003; Weil, Forrest et al. 2006). Disruption of kinesin heavy chain affected the localization of oskar mRNA in Drosophila oocytes (Brendza, Serbus et al. 2000).

Despite cloning and characterization of LARP6, the mechanism which coordinates synthesis of type I collagen is poorly understood. In this manuscript we describe one key step in coordinated synthesis of type I collagen; the interaction of collagen mRNAs with filaments composed of nonmuscle myosin. It is likely that actin and myosin transport collagen mRNAs to distinct locations to control protein synthesis.

Results

*Nonmuscle myosin copurifies with 5’ stem-loop RNA.*

LARP6 was previously cloned as the protein which directly binds 5’ stem-loop RNA, however, other proteins which associate in the complex with LARP6 and 5’ stem-loop are unknown. To identify these proteins we performed tobramycin affinity purifications by attaching a tobramycin aptamer to 5’ stem-loop RNA (Fig 2.1). Tobramycin aptamer is a short RNA with
high affinity to antibiotic tobramycin and affinity purifications using tobramycin aptamer have been described for purifications of splicing complexes (Hartmuth, Urlaub et al. 2002; Hartmuth, Vornlocher et al. 2004). After incubation of the RNA bait which composed of collagen 5’ stem-loop and tobramycin aptamer in cytosolic extracts of human lung fibroblasts, the bound proteins were pulled-down with tobramycin agarose and eluted with an excess of free tobramycin. As control, inverted 5’ stem-loop fused to the aptamer was used. The two most prominent proteins specifically pulled down with 5’ stem-loop were identified as nonmuscle myosin IIB and vimentin (Dillon Fritz Dissertation).

The identification of nonmuscle myosin IIB as the protein which associates in the complex with 5’ stem-loop was unexpected and there have been no reports on the role of nonmuscle myosin in synthesis of type I collagen.

*The interaction of collagen mRNAs with nonmuscle myosin*

Since LARP6 is the only discovered protein which directly binds 5’ stem-loop, it is possible that in the tobramycin affinity purifications nonmuscle myosin had been tethered to the 5’ stem-loop by the protein-protein interaction with LARP6. To verify if LARP6 and nonmuscle myosin interact we performed co-immunoprecipitation experiments. (Dillon Fritz Dissertation) LARP6 has 4 domains, the N-terminal domain of unknown function, the La-homology domain found in other LARPs, the unique RNA binding domain (RBD) necessary for binding 5’ stem-loop and the C-terminal domain of unknown function (Fig 2.2A). To identify which domain is needed for the interaction with myosin IIB we expressed HA-tagged full size LARP6 and HA-tagged LARP6 lacking the C-terminal domain (ΔC-LARP6) and performed immunoprecipitations with anti-HA antibody. While myosin IIB co-immunoprecipitated with the full size LARP6 (Fig 2.2 B, lane 1), myosin IIB failed to co-immunoprecipitate with ΔC-LARP6 (lane 2) or the control protein RBMS3 (lane 3). Myosin IIA was also co-immunoprecipitated with full size LARP6, but not with ΔC-LARP6 (Fig 2.2 C), suggesting that LARP6 interacts with the both major isoforms of nonmuscle myosin through LARP6 C-terminal domain.
We also tested if the interaction between LARP6 and nonmuscle myosin is dependent on intact of RNA. To investigate this, we digested the samples with RNase A prior to analysis (Fig 2.2 D). Immunoprecipitation of LARP6 pulled-down myosin IIB, regardless of RNase A digestion (lanes 1 and 2), suggesting that these proteins form a complex by protein-protein interactions. These interactions were specific, because fibronectin was not coimmunoprecipitated and the control RNA binding protein, RBMS3, failed to interact with any of the proteins (lane 3).

Collagen α1(I) and α2(I) mRNAs were also found immunoprecipitated with nonmuscle myosin in a 5’ stem-loop dependent manner. α2(I) mRNA cannot associate with nonmuscle
myosin independently of α1(I) mRNA and the intact of 5’ stem-loop on α1(I) mRNA is needed for this process. (Dillon Fritz Dissertation)

*Integrity of nonmuscle myosin filaments is necessary for secretion of type I collagen*

To assess the role of nonmuscle myosin in collagen synthesis, we disrupted nonmuscle myosin filaments in two different cell types, primary human lung fibroblasts and scleroderma skin fibroblasts. Nonmuscle myosin filaments were disrupted by treatment of cells with ML-7, or by overexpression of kinase-dead (KD) myosin light chain kinase mutant (KD-MLCK, a kind gift of Dr. P. Gallagher, Indiana University) (Gallagher, Herring et al. 1993). ML-7 is a specific

Figure 2.2
Figure 2.2. Interaction of LARP6 and myosin IIB requires LARP6 C-terminus and the interaction is not RNA dependent. A. Domains of LARP6. N-TER; N-terminal domain, LA; La domain, RBD: RNA binding domain, C-TER: C-terminal domain which excludes amino acid 300-491 on the C-terminus of LARP6. B. C-terminal domain of LARP6 is needed for interaction with nonmuscle myosin. Immunoprecipitation with anti-HA antibody from cells expressing HA-tagged LARP6 (lane 1), HA-tagged ΔC-LARP6 (lane 2) and HA-tagged RBMS3 (lane 3), analyzed with anti-myosin IIB antibody. C. Same as in B, but analyzed with anti-myosin IIA antibody. D. RNA independent interaction of LARP6 and myosin. After expression of HA-tagged LARP6 immunoprecipitation was done with anti-HA antibody and analyzed by western blot with (lane 1) and without (lane 2) digestion with RNase A. MYOIIB; nonmuscle myosin IIB, FIB; fibronectin. Lane 3; control precipitation with HA-tagged RBMS3.

inhibitor of myosin light chain kinase (MLCK) and inhibition of MLCK leads to disassembly of myosin IIA and IIB filaments (Saitoh, Ishikawa et al. 1987; Xu, Gao et al. 2008). Fig 2.3 A shows immunostaining of human lung fibroblasts and scleroderma fibroblasts for myosin IIB without (CON) and with treatment of ML-7 (ML-7). In untreated cells the myosin filaments are clearly visible, while in the ML-7 treated cells the myosin staining is confined around the nucleus, indicating the disruption of nonmuscle myosin IIB filaments. ML-7 affected human lung fibroblasts and scleroderma skin fibroblasts in a similar way. The total level on myosin IIB remained unchanged, as judged by western blot (figure 2.3 B) and by the similar staining intensity of the cells.
KD-MLCK isoform was expressed using adenovirus vector for efficient delivery into the primary fibroblasts. Overexpression of KD-MLCK resulted in the disassembly of the nonmuscle myosin IIB filaments (Fig. 2.3C) in both human lung fibroblasts and scleroderma skin fibroblasts, suggesting that this mutant acts as a dominant negative protein for the filament assembly in fibroblasts, as previously described. Therefore, we used both approaches to assess the role of nonmuscle myosin filaments in collagen expression. Control staining without primary anti-myosin IIB antibody (Fig. 2.3 D) in HLF and SCL indicated the specificity of the staining. DAPI stained DNA, representing the nucleus.

To assess collagen synthesis upon ML-7 treatment, we analyzed the levels of intracellular and secreted collagen α1(I) and α2(I) polypeptides by western blots using the chain specific antibodies. For medium analysis we seeded equal number of cells and incubated them in serum free medium for 3 hours. This resulted in accumulation of collagen protein into serum free medium for a fixed period of time, allowing us to determine the efficacy of this process. The ML-7 treatment profoundly affected the secretion of type I collagen, but the effect differed in the two types of the primary fibroblasts used. In lung fibroblasts, the intracellular level of collagen α1 and α2 polypeptides was not significantly affected by ML-7 treatment (Fig 2.4A, lanes 1 and 2). In medium, collagen α1(I) polypeptide was found in similar amounts in control and ML-7 treated cells, however, collagen α2(I) polypeptide was barely secreted after ML-7 treatment (lanes 3 and 4). The absence of α2(I) polypeptide in the medium suggests that ML-7 uncoupled secretion of α1(I) and α2(I) chains. When we probe α1(I) chains under nonreducing condition (figure 2.4 B), it appeared as triple helix, dimmer and monomer. Our experience with multiple cell lines is that cell culture in vitro secrete unassembled and perfectly assembled collagen molecules, therefore we see a large fraction of monomers and dimmers. As α2(I) is not secreted, collagen α1(I) was secreted as a homotrimer. Secretion of fibronectin was not affected, suggesting that the general machinery for protein secretion was unaffected.
Figure 2.3 A

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In scleroderma skin fibroblasts, the intracellular level of collagen $\alpha_1(I)$ polypeptide was not changed with ML-7 treatment; however, the level of $\alpha_2(I)$ polypeptide was greatly reduced (Fig. 2.4.C, lanes 1 and 2). In multiple experiments, we have noticed the appearance of bands indicated by arrows in the ML-7 treated samples (Fig. 2.4 C). Since these bands were immunoreactive to the antibodies, they may represent degradation products of $\alpha_1(I)$ and $\alpha_2(I)$ polypeptides. Importantly, collagen $\alpha_1(I)$ polypeptides were barely detectable in the medium, while collagen $\alpha_2(I)$ polypeptide was completely absent. Thus, ML-7 treatment almost
completely abolishes collagen secretion, with the minimal amounts of the homotrimer being secreted in scleroderma fibroblasts. The secretion machinery seemed to be intact in ML-7 treated cells because the fibronectin secretion was not affected. Also, immunostaining for the ER marker protein calnexin did not show a difference in ML-7 treated cells and control cells, suggesting that this organelle was not affected (Figure 2.4 D). From the data shown, it appears that ML-7 disrupts the integrity of myosin II filaments which are necessary for collagen synthesis.

Figure 2.4 A

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Since collagen α2(I) polypeptide is not secreted and does not accumulate to a high level intracellular upon ML-7 treatment in both human lung fibroblasts and scleroderma fibroblasts, we tested whether collagen α2(I) is subjected to accelerated intracellular degradation in the cell upon ML-7 treatment. We treated human lung fibroblast and scleroderma skin fibroblasts with a proteasome inhibitor, exopomycin (100nM overnight) (Meng, Mohan et al. 1999), and with and without ML-7 (Fig 2.5). The smaller molecular weight band (arrow) which can be recognized by collagen antibody, did not disappear in either cell type. This indicated that the degradation of cellular collagen upon ML-7 might be through mechanism other than proteasome. It is possible that collagen was cleaved upon disruption of myosin II filaments.

**Figure 2.4.** Integrity of nonmuscle myosin II filaments is necessary for secretion of type I collagen. A. Human lung fibroblasts (HLF) were treated with ML7 (lanes 1 and 3). The intracellular level of collagen α1(I) and collagen α2(I) polypeptides (CELL) (lanes 1 and 2) and secretion rate into the medium (MED, lanes 3 and 4) were analyzed by western blot. Untreated control cells are in lane 2 and 4. Fibronecin (FIB): loading control. B. Western blot of the medium of human lung fibroblasts was analyzed by western blot for collagen α1(I) peptide under reducing condition (lanes 1 and 2) (RED) and nonreducing condition (Lanes 3 and 4) (NONRED). ML7 treated cells (lanes 1and 3), control untreated cells (lane 2 and 4) (CON). The migration of collagen α1(I) monomer (COL1A1), dimmer and trimmer are indicated. C. Human scleroderma skin fibroblasts (HLF) were treated with ML7 (lanes 1 and 3). The intracellular level of collagen α1(I) and collagen α2(I) polypeptides (CELL) (lanes 1 and 2) and secretion rate into the medium (MED, lanes 3 and 4) are analyzed by western blot. Untreated control cells are in lane 2 and 4. Fibronecin (FIB): loading control. Possible degraded collagen α1(I) and collagen α2(I) peptides are indicated by the arrows. D. Immunostaining on calnexin (red) in control HLF cells (CON) and ML7 treated cells (ML-7). DAPI stained nucleus.
Figure 2.5. Collagen peptides are likely not degraded by the proteasome after ML-7 treatment. Human lung fibroblasts (lanes 1 and 2) and scleroderma fibroblasts (lanes 3 and 4) intracellular collagen α1(I) and α2(I) levels were analyzed by western blot. The cells all were treated with epoxomycin (EPO) (lanes 2 and 4) and ML-7 (lanes 1 and 3).

To exclude that nonspecific effects of ML-7 are responsible for the perturbation in collagen synthesis we repeated the analysis after overexpressing the kinase-dead isoform of MLCK (KD-MLCK). This isoform disrupted the nonmuscle myosin IIB filaments similar to ML-7 (Fig 2.3 C). The effects on collagen synthesis were also similar to that of ML-7. In lung fibroblasts, KD-MLCK dramatically reduced the secretion of α2(I) polypeptide, with no effect on secretion of α1(I) polypeptide (Fig. 2.6 A, lane 3 and 4). Intracellular collagens were not affected (lane 1 and 2). In scleroderma fibroblasts, KD-MLCK reduced the intracellular level of collagen α1(I) and α2(I) polypeptides (Fig 2.6 B, lane 1 and 2). This time we did not observe the characteristic degradation products seen after ML-7 treatment. In the medium α1(I) polypeptide was reduced, while α2(I) polypeptide was absent (Fig. 2.6 B, lanes 3 and 4).
These results suggested that it is possible to uncouple secretion of collagen α1(I) and α2(I) polypeptides by disrupting filaments composed of nonmuscle myosin. We concluded from these experiments that intact nonmuscle myosin filaments are needed for secretion of type I collagen composed of α1(I) and α2(I) polypeptides. Different cell types have different abilities to compensate for the loss of α2(I) secretion by secreting α1(I) homotrimer.

**Figure 2.6.** Overexpression of KD-MLCK decreased secretion of type I collagen. A. Western blot analysis of collagen α1(I) (COL1A1) and α2(I) (COL1A2) polypeptides. In human lung fibroblasts (HLF) KD-MLCK was overexpressed by adenovirus (lanes 1 and 3); control cells were treated with control adenovirus (lanes 2 and 4). Cellular collagen levels (lane 1 and 2) and secreted collagen levels (lanes 3 and 4) are shown. Fibronectin was loaded as control. B. Same experiment as in A was done in scleroderma cells (SCL).

When normal collagen heterotrimer is folded, the α1(I) and α2(I) polypeptides must show colocalization in the lumen of the ER. Since ML-7 treatment did not affect the cellular level of the individual collagen polypeptides, just their secretion in lung fibroblasts (Fig 2.4 A), we used this cell type to assess if their subcellular localization was affected by ML-7. If their
colocalization had been affected, this could explain the low level of α2 polypeptide in the medium, which cannot be efficiently secreted without folding with α1 polypeptide. Using the chain specific antibodies for immunostaining, we observed the strict colocalization of α1(I) and α2(I) polypeptides in control cells (Fig 2.7A, top panel, MERGE). However, in ML-7 treated cells, collagen synthesis was disorganized and a significant fraction of α2(I) polypeptide (red) was not colocalized with α1(I) polypeptide (green) (Fig 2.7A, bottom panel, MERGE). This is consistent with lack of their coordinated synthesis upon disruption of nonmuscle myosin filaments. Control cells were stained just with secondary anti-rabbit cy2 and showed the specificity of collagen α1(I) antibody.

Figure 2.7 A
Collagen triple helix formation starts while the mRNAs still associate with polysomes. We discovered that intact myosin II is responsible for collagen peptide colocalization. It is very likely this regulation is through regulating collagen mRNA localization. Since myosin IIB interacts with LARP6 and collagen mRNAs, we tested whether this association was dependent on the intact of myosin II (Dillon Fritz Dissertation). Our results indicated that intact nonmuscle myosin II B filaments were required for the LARP6--myosin interaction and for the nonmuscle myosin and collagen α1(I) and collagen α2(I) mRNA interaction. From these experiments we concluded that tethering of collagen mRNAs to the nonmuscle myosin filaments by LARP6 coordinates synthesis of α1(I) and α2(I) polypeptides for productive secretion of the heterotrimeric type I collagen.
Myosin II has motor activity and is able to carry mRNAs to designated locations. We discovered that Myosin IIB associate with collagen mRNAs (Fig. 2.2), and regulate collagen synthesis. We tested whether myosin motor activity is involved in collagen synthesis. Blebbistatin binds to the ATPase of myosin heavy chain and inhibits the ATPase activity by slow down phosphate release. This decreases the affinity of myosin and actin, but does not affect the binding and disassociation of myosin and actin (Kovacs, Toth et al. 2004). Therefore, we treated cells with Blebbistatin to inhibit nonmuscle myosin motor activity. To assess if the myosin motor is required for collagen synthesis, we treated lung fibroblasts and scleroderma fibroblasts with blebbistatin and analyzed for the production of collagen α1(I) and α2(I) polypeptides as well as their secretion rate in the medium. A similar result as disruption in the integrity of myosin filaments was obtained. Blebbistatin did not significantly affect intracellular collagen α1(I) or secretion rate in human lung fibroblasts. However, intracellular and secretion of collagen α2(I) was decreased (Fig. 2.8A). Fibronectin as a control was affected in that both cellular and secretion rate was decreased. Upon blebbistatin treatment, the decreasing rate of intracellular collagen α2(I) was less than fibronectin, but the percent of secretion of collagen α2(I) was less than fibronectin. There was a small effect on the expression of fibronectin, in that intracellular level and secretion rate were slightly reduced. Therefore, we included another control for the cellular fractions, tubulin, which showed equal loading. These results indicated myosin II motor activity is needed for collagen α2(I) secretion in human lung fibroblasts. This is consistent with ML-7 and KD treated human lung fibroblast results (Fig. 2.4A and Fig 2.6A). In scleroderma fibroblasts, collagen α1(I) intracellular level was not affected by blebbistatin, but secretion was slightly decreased. Collagen α2(I) intracellular level was not affected, but secretion was greatly affected. Fibronectin intracellular level and secretion rate were both slightly decrease (Fig. 2.8B). Tubulin as a loading control was not affected. These results indicated that collagen α1(I) and collagen α2(I) secretion were both decreased through the inhibition of myosin motor activity. The results corresponded to ML-7 and KD treated
scleroderma results (Fig. 2.4 C and Fig. 2.6B). We concluded that the motor function of myosin is indispensable for the coordination of synthesis of type I collagen.

Figure 2.8 A

In order to further test if collagen synthesis is dependent upon actin filaments, we treated cells with cytochalasin B to disrupt actin filaments and analyzed the effect of collagen secretion. Cytochalasin B is a cell permeable drug which inhibits actin polymerization and the interaction between myosin and actin (MacLean-Fletcher and Pollard 1980). Human lung

Figure 2.8. Motor activity of nonmuscle myosin II is required for the type I collagen synthesis. A. Human lung fibroblasts (HLF) cellular extracts (CELL) and medium (MED) were analyzed for collagen α1(I) (COL1A1) and collagen α2(I) (COL1A2) protein by western blot. Cells treated with blebbstatin (BLEB) are lanes 1 and 3, and control cells without treatment (CON) are in lanes 2 and 4. Fibronectin (FIB) and tubulin (TUB) are shown as the loading control. B. The same experiment as in A was done in scleroderma skin fibroblasts (SCL).
fibroblasts and scleroderma skin fibroblasts were treated with and without cytochalasin B (50uM over night). Upon disruption of the actin filaments, the effect of collagen α1(I) and collagen α2(I) secretion were analyzed by western blot (Figure 2.9). In human lung fibroblasts, the secretion of collagen α1(I), collagen α2(I) and fibronectin were all inhibited upon treatment with cytochalasin B (lanes 1 and 2). This suggested that the disruption on actin filaments had a general effect to protein secretion pathway and not only on collagen secretion. In scleroderma skin cells, the secretion of collagen α1(I) was not affected, while that collagen α2(I) was minimally affected (lanes 3 and 4). Fibronectin as a loading control was not significantly affected. This is in great contrast with the effect of ML-7 which almost completely prevented secretion of α1 (I) and α2(I) polypeptides from scleroderma fibroblasts. From this result, we concluded that the integrity of actin filaments does not specifically regulate collagen synthesis, but may have a general role in protein synthesis.

Figure 2.9

![Image of western blot results showing COL1A1, COL1A2, and FIB proteins in HLF and SCL conditions with cytochalasin B (CYTOB) and control (CON) treatments.](image-url)
Figure 2.9. Disruption of actin filaments has different effect on collagen synthesis than disruption of myosin filaments. The effect of actin disruption on collagen was analyzed by western blot in human lung fibroblasts (HLF) and scleroderma skin fibroblasts (SCL). Collagen α1 (I) peptide (COL1A1) and collagen α2(I) peptide (COL1A2) were probed in cytochalasin B treated cells (lanes 1 and 3) (CYTOB), and control untreated cells (lanes 3 and 4) (CON). Fibronectin (FIB) was a control.

The effect of nonmuscle myosin on collagen synthesis is mediated by the 5’ stem-loop

To investigate the role of conserved 5’ stem-loop of collagen mRNAs in regulation of collagen synthesis, we obtained mouse embryonic fibroblasts (MEFs) from mice in which 5’ stem-loop was disrupted in the context of the endogenous collagen α1(I) gene. The mutation of the 5’ stem-loop did not change the coding region of the α1(I) gene (Fig. 2.10A). The 5’ stem-loop of α2(I) gene was not changed. The exclusive expression of α1(I) mRNA with the mutated 5’ stem-loop (Δ5’SL) in mouse embryonic fibroblasts (MEFs) from homozygous mutant animals was verified by RT-PCR (Dillon Fritz Dissertation). Control animals expressed only wt collagen α1(I) mRNA and homozygous mutant animals expressed only Δ5’SL collagen α1(I) mRNA. Collagen α1(I) mRNA steady state level was decrease to half due to the decreased stability of the mRNA in stem-loop mutant MEF cells. The secretion rate of collagen α1(I) polypeptide was slower when it was encoded by the mRNA without 5’ stem-loop compared to wt cells (Dillon Fritz Dissertation). Nonmuscle myosin II was initially identified through pull down with 5’ stem-loop RNA. Dillon Fritz verified the myosin II and collagen α1(I) and α2(I) mRNA interaction. He also discovered that nonmuscle myosin did not pull down collagen α1(I) or α2(I) mRNAs in α1(I) 5’stem-loop mutant MEFs. This suggested that collagen α1(I) and collagen α2(I) interact with myosin II in a α1(I) 5’ stem-loop dependent manner in vivo (Dillon Fritz Dissertation).

We treated MEFs with ML-7 and to test the effect on intracellular collagen α1(I) polypeptide level and in the medium (Fig. 2.11A). In wt MEFs ML-7 treatment did not change the cellular level of α1(I) polypeptide (Fig. 2.11 A, lanes 1 and 2), however, its secretion into the medium was drastically reduced (lanes 3 and 4). This result is similar to the result obtained with scleroderma fibroblasts (Fig 2.3C). In Δ5’SL MEFs, the treatment with ML-7 did not affect secretion of α1(I) polypeptide (Lanes 5 and 6). Similar amounts were found in the medium of
cells treated with ML-7 and untreated cells (Fig 2.11, lanes 7 and 8). These results indicated that ML-7 treatment affects collagen secretion only if it is encoded by the mRNA with 5' stem-loop. Therefore, we concluded that the 5' stem-loop couples synthesis of type I collagen to the integrity of nonmuscle myosin filaments. If collagen polypeptides are encoded by the mRNA without the 5' stem-loop, they are secreted by a myosin independent pathway. Due to the limitation of our antibody which could not efficiently recognize rodent α2(I) chain, we could not probe for collagen α2(I) peptide.

**Figure 2.10**

![Diagram](image)

**Figure 2.10.** Disruption of 5' stem-loop in the context of the endogenous collagen α1(I) gene in mouse embryonic fibroblasts (MEFs). Left, sequence of wild type 5' stem-loop (WT) in collagen α1(I) gene. Right, sequence of mutant 5' stem-loop (Δ5'SL).
Figure 2.11

![Figure 2.11](image)

**Figure 2.11.** The effect of nonmuscle myosin II on type I collagen synthesis is mediated by the α1(I) 5’ stem-loop. Collagen α1(I) level (COL1A1) was analyzed by western blot in wild type mouse embryonic fibroblasts (WT MEFS) and MEFs with mutation of collagen α1(I) 5’ stem-loop (Δ5’SL MEFS). Cellular level of collagen α1(I) peptides (CELL) (lanes 1, 2, 5 and 6) and medium level (MED) (lanes 3, 4, 7 and 8). Wild type and mutant MEFs treated with ML-7(ML7) (lanes 1, 3, 5 and 7) and control cells (CON) (lanes 2, 4, 6 and 8). Fibronectin (FIB) was indicated as loading control.

Discussion

Nonmuscle myosin has classically been implicated in promoting cell contractility, motility and karyokinesis with two major isoforms, IIA and IIB, having different roles in these processes. The finding that collagen mRNAs associate with cytoskeletal proteins is novel, although association of other mRNAs with cytoskeleton has been reported. There has been no report on the involvement of nonmuscle myosin in translation. During the physiological need for high collagen expression, like wound healing, there is activation and migration of fibroblasts to the site of injury. To accommodate this process activated fibroblasts and myofibroblasts upregulate nonmuscle myosin expression. Our results suggest that nonnucle myosin filaments are also a prerequisite for secretion of type I collagen, which follows after the arrival of the cells to the wound. Thus, the migration and the ability to make type I collagen are coupled processes in fibroblasts.
In the fibrotic stage, type I collagen is synthesized excessively and the protein is not actively degraded. Collagen triple helix forms while collagen mRNAs are still associated with polysomes. Therefore, collagen mRNAs need to be regulated for the coordinated translation of two $\alpha_1$(I) and one $\alpha_2$(I) peptides, this leads to efficient synthesis of collagen trimmers. Most human tissues synthesize exclusively heterotrimer of type I collagen, although in the absence of $\alpha_2$(I) chains homotrimers of $\alpha_1$(I) chains readily form. So, the cells have the ability to fold and secrete homotrimer of type I collagen, but there must be a mechanism that normally prevents this. If the translation of individual collagen chains is random and their registration and folding is not strictly coordinated, the formation of homotrimers would inevitably happen to a significant extent. One way to assure predominant synthesis of the heterotrimer would be to prevent independent translation of $\alpha_1$(I) mRNA and couple it to that of $\alpha_2$(I) mRNA.

We have shown previously that LARP6 is the protein which specifically binds 5’ stem-loop of collagen $\alpha_1$(I) and $\alpha_2$(I) mRNA. The binding of LARP6 is of high affinity to prevent translation, suggesting that one of the roles of LARP6 may be to prevent random translation of collagen mRNAs. Here we show that: 1. LARP6 associates collagen mRNAs with nonmuscle myosin filaments. 2. Disruption of nonmuscle myosin filaments results in either, lack of secretion collagen $\alpha_2$(I) polypeptide or diminished secretion of both $\alpha_1$(I) and $\alpha_2$(I) polypeptides due to the mislocalization of the polypeptides. 3. Disruption of nonmuscle myosin motor activity decreased collagen $\alpha_2$(I) secretion and or diminished collagen $\alpha_1$(I) secretion. 4. Disruption on actin filaments has a different effect from disruption of myosin filaments. 5. The effect of nonmuscle myosin on collagen synthesis depends on the presence of 5’ stem-loop in the mRNA (Fig 2.11).

Here, we have identified nonmuscle myosin as one of the proteins which interacts with the 5’ stem-loop using tobramycin affinity purification. This method has been developed to purify loosely associated splicing complexes. Other purification methods using 5’ stem-loop as bait failed to isolate nonmuscle myosin, suggesting that it is weakly associated in the complex. LARP6 and nonmucle myosin interact through the C-terminal domain of LARP6 (Fig. 2.2). Since LARP6 is the only protein we know which binds 5’ stem-loop with high affinity (Chapter 1) and LARP6 and myosin II interaction is RNA independent (Dillon Fritz Dissertation), it is likely that 5’ stem-loop of collagen mRNAs associate with nonmuscle myosin through LARP6. The binding of nonmuscle myosin to collagen $\alpha_2$(I) mRNA is dependent on the intact of 5’stem
–loop in collagen α1(1) mRNA (Dillon Fritz Dissertation). Other results from our lab indicate that nonmuscle myosin filaments are needed for loading of collagen mRNAs on a subset of polysomes (not shown). This suggested that nonmuscle myosin II binds collagen α1(1) and α2(1) together for their coordinated translation.

When nonmuscle myosin filaments were disrupted by two independent methods, ML-7 and KD-MLCK adenovirus, we observed the effect on collagen synthesis in two cell types. In human lung fibroblasts, collagen α2(1) secretion was dramatically decreased, but collagen α1(1) secretion was not affected (Fig. 2.4). In scleroderma cells, the result was different in that collagen α1(1) polypeptides secretion was also inhibited and α2(1) secretion was abolished. Since α1(1) polypeptide has the propensity to form homotrimers, it seems likely that lung fibroblasts can readily form and secrete the homotrimers in the absence of myosin filaments, while scleroderma fibroblasts cannot. The only way collagen α2(1) can fail to be secreted is in the case that it is not incorporated into the collagen triple helix. We observed the accumulation of degradation products of both collagen α1(1) and collagen α2(1) polypeptides, they were predominant when proteasome was inhibited by epoxomycin in lung fibroblasts. Since the integrity of nonmuscle myosin II is required for the colocalization of collagen α1(1) and α2(1) peptides (Fig.2.7), the degradation of collagen peptides may due to the mislocation of the polypeptides. Although collagen α1(1) can secret as homotrimer, the degradation of collagen α1(1) indicated the low efficiency for the secretion of collagen α1(1) homotrimer. Thus, without intact nonmuscle myosin filaments fibroblasts cannot synthesize normal type I collagen. The effect of collagen synthesis with ML-7 and KD virus was similar. Since ML-7 may affect cells in other ways besides disruption of myosin filaments, overexpression of dominant negative myosin light chain kinase (KD) is more specific in disrupting nonmuscle myosin filaments than treating cells with ML-7. The intracellular steady state level of collagen polypeptides were also decreased in KD treated scleroderma fibroblasts (Fig.2.6). This may be due to the inhibition of translation and/or collagen peptide degradation. We did not detect the similar degradation product in KD treated cells (Fig. 2.4 C arrows). This suggested the ML-7 involved degradation pathway could be unique. But the experiment with epoxomycin was not done.

The Motor activity of nonmuscle myosin II is required for collagen synthesis (Fig.2.8). Disrupt myosin motor activity by blebbistatin uncouple the secretion of collagen α1(1) and α2(1) polypeptides. This indicated that nonmuscle myosin II is involved in transport collagen mRNAs
to the same place by interaction with actin filaments for coordinated translation to form collagen triple helix. However, the effect of disrupting myosin filaments is different from disrupting actin filaments (Fig.2.9). When we disrupted actin filaments, collagen α1(I) and α2(I) and fibronectin secretion were all decreased in human lung fibroblasts. Also, in scleroderma fibroblasts, the secretion of collagen α1(I) polypeptides was not affected; the secretion of collagen α2(I) polypeptides was not affected as much as in ML-7 treated cells. This indicated actin filaments are not specific in regulating collagen synthesis, instead, they have a general role in protein synthesis. Myosin filaments have a specific role in regulating collagen synthesis. Whether LARP6-collagen mRNAs directly associate with actin filaments or nonmuscle myosin filaments are not known.

We postulate that normal synthesis of type I collagen requires coordination of translation of collagen α1(I) and α2(I) mRNAs. This is achieved by binding of LARP6 to the 5’ stem-loop and interaction of LARP6 with nonmuscle myosin. mRNA localization is a powerful way to regulate the local concentration of mRNA and local protein synthesis (Czapinski and Singer 2006). Many ribonucleoprotein particles which localize RNAs are formed in the nucleus. LARP6 localizes in both cytoplasm and nucleus, Collagen mRNPs maybe formed in the nucleus and prevent the premature translation before collagen mRNAs are transported to the designated location. Collagen mRNAs may be aggregated by LARP6 binding to 5’stem-loop. The LARP6-collagen mRNA complex is transported by nonmuscle myosin II through its motor activity. Once the mRNAs are transported on the certain location on ER, LARP6 leaves and this promotes collagen mRNAs translation initiation. We believe this process contributes to increase the local concentration of collagen mRNAs of certain locations on the ER, and the stimulation on heterotrimer formation of type I collagen.

Our hypothesis is that nonmuscle myosin II is required for coupling synthesis of collagen polypeptides to certain location on the ER for heterotrimer formation. Myosin II associates with collagen mRNAs in collagen mRNA 5’ stem-loop α1(I) dependent manner, and possibly the binding of myosin II to collagen mRNAs is through LARP6. Myosin II delivers the collagen mRNAs to polysomes on the ER through actin filaments. Triple helix collagen forms while collagen mRNAs are being translated. Disruption on the intact of nonmuscle myosin II decreased myosin II interaction with collagen mRNAs, possibly due to the disassociation of myosin and LARP6. Thus, collagen mRNA translational pathway is abolished. Collagen
mRNAs are randomly translated. The synthesized collagen peptides cannot form heterotrimer collagen since collagen α1(I) and α2(I) mRNAs are not translated in the same location. Cells synthesize collagen α1(I) homotrimers are formed to compensate the loss of heterotrimer.

How these events are regulated is still not known. Phosphorylation of LARP6 could be involved this process. Based on my preliminary results, LARP6 is phosphorylated. I compared the phosphorylation of LARP6 mutants A-G (Fig. 1.4) by western blot and narrowed down the phosphorylation sites which are in amino acid 48-81 and 218-300. It is reported that serine 56, 58 are phosphorylated on LARP6 (Olsen, Blagoev et al. 2006), but the function of these phosphorylation and the kinase that are responsible for phosphorylation are still unknown. In order to test if phosphorylation or dephosphorylation of these sites affect the localization of LARP6, I mutated 56, 58 serine into alanine (A) and aspartic acid (D) to mimic the dephosphorylation and phosphorylation state of the serines. Human lung fibroblasts stable cell lines that express wild type LARP6, 56A, 58A, 56D, 58D mutants were developed. Western blots confirmed the expression of LARP6 mutants. I stained these cells with LARP6 specific antibody. My confocal microscopy results indicated that 56 and 58 serine phosphorylation and dephosphorylation did not affect LARP6 localization in the nucleus or cytoplasm. To test whether the phosphorylation or dephosphorylation of 56, 58 serines affect the binding of LARP6 to 5’stem-loop, I overexpressed wild type LARP6 and 56 and 58 mutant LARP6 in human kidney fibroblasts. Gel shift experiment showed that the binding of wild type LARP6 and mutant LARP6 to 5’stem-loop RNA were the same. This indicated that phosphorylation on 56, 58 serine may be not responsible to LARP6 binding to 5’stem-loop collagen mRNA or multiple phosphorylation on different sites are involved in the binding. Whether phosphorylation of LARP6 regulates LARP6 and myosin II interaction is not known.

Materials and Methods

Chemicals and cells.

ML-7 was from Sigma and was used at 40 μM overnight incubation. Epoxomycin from Sigma was used at 100nM, over night incubation. Cytochalasin B was from sigma was used at 50uM overnight.
Mouse embryonic fibroblasts were derived from knock-in mice in which 5’ stem-loop of collagen α1(I) gene has been mutated. Fibroblasts from wt littermates were used as control. Several independent cell isolates were used throughout the study. The cells were cultured in DMEM supplemented with 10% FBS for up to 10 passages. Immortalized human lung fibroblasts were described before.

**Plasmid constructs and adenovirus preparation.**

The HA-tagged LARP6 clone and deletion mutants derived from it were described. Adenoviruses were constructed by recloning of the constructs into pAdCMVTRACK vector followed by recombination with pAdEasy vector, as described. Adenoviruses were amplified in HEK293 cells and purified by virapure kit (Stratagene). Expression of each construct was verified by western blot. The resulting viruses express both the full-size LARP6 or ΔXCM and GFP from an independent transcription unit, which served as a control of infection. Control adenovirus expressed a truncated form of lysyl oxidase (LOX).

**RT-PCR analysis of gene expression**

Total cellular RNA was isolated using an RNA isolation kit (Sigma). RT-PCRs were done with 100 ng of total RNA or using rTth reverse transcriptase (Boca Scientific, Boca Raton, FL) and [^32P]dCTP was included in the PCR step to label the products, as described. When RNA from polysomal fractions was analyzed an equal aliquot of each fraction was used. PCR products were resolved on sequencing gels and visualized by autoradiography. The number of cycles was adjusted to be in the linear range of the reaction. The following primers were used for RT-PCR:

m-β-actin  
5’ primer CGTGCCTGACATCAAAGAGAAGC  
3’ primer TGGATGCCACAGGATTCCATACC

m-collagen α1(I)  
5’ primer AGAGGCGAAGGGAACGTGC  
3’ primer GCAGGGCCAATGTCTAGTCC

m-collagen α2(I)  
5’ primer CTTCGTGCCTAGCAACATGC
3’ primer TCAACACCATCTCTGCCTCG

**Determinations of mRNA stability**

Wild type and 5’ SL -/- MEFs were treated with actinomycin D (10 μg/ml of medium) for 0 h, 12 h or 24 h, after which total RNA was extracted and analyzed by RT-PCR. Three independent determinations were performed and gels were quantified by phosphoimaging. The level at time 0 was set as 100%.

**Antibodies**

Antibodies used in Western blot analysis and immunoprecipitation reactions were from: anti-HA antibody and anti-GFP antibody from Sigma, anti-MYH10 antibody from University of Iowa hybridoma bank, anti-nucleolin antibody and anti-vimentin antibody from Santa Cruz, anti-type I collagen antibody from Rockland, anti-fibronectin antibody and anti-tubulin antibody from BD biosciences and anti-LARP antibody from Abnova.

**Western blot analysis and immunostaining**

Protein concentration was estimated by the Bradford assay with BSA as the standard. Western blots were done using between 10 and 100 μg of protein, however, proteins isolated by immunoprecipitation and subjected to western blotting did not have protein concentration estimated by Bradford assay. Equal loading of these proteins was confirmed by additional methods.

For immunostaining, cells were seeded onto glass coverslips and after treatment the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. After blocking with 10% goat serum/5% BSA in PBS for 1 hr at room temperature, the cells were incubated with primary antibody overnight at 4°C, washed 3 X 5 min in PBS, and visualized with Alexafluor 594-conjugated secondary antibody. The cells were mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories) and images taken by Leica TCS SP2 AOBs laser confocal microscope equipped with a Chameleon Ti:Sapphire multiphoton laser. Optical sections were processed with LCSLite software.
Immunoprecipitation

Cell extracts were prepared in lysis buffer (10 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 0.5% NP-40, 170 μg/ml phenylmethylsulfonyl fluoride) and, after removal of nuclei by centrifugation, the clear lysate was incubated with 1 μg of antibody for 1 hour at 4 °C. 20 μl of washed protein A/G plus-agarose (Santa Cruz Biotechnology) was added, and incubation continued for an additional 3 hours. After washing the beads three times in PBS, immunoprecipitated complexes were dissolved in SDS-PAGE loading dye or RNA was extracted.

In reactions in which RNase A was added after immunoprecipitation, 0.2μg/μl of RNase A was incubated with the A/G plus-agarose beads for 15 minutes at room temperature, followed by washing two times in phosphate-buffered saline.

5' stem-loop affinity pull-downs

Biotin labeled collagen α1(I) and α2(I) 5’ stem-loops (Integrated DNA Technologies) were incubated with cellular extract from WT and 5’ SL-/- MEFs for 1 hour at 4 °C. Streptavidin agarose (Novagen) was added, and incubation continued for an additional 3 hours. After washing the beads three times in phosphate-buffered saline, complexes associated with the biotin-labeled 5’ stem-loop were analyzed by SDS-PAGE and mass spectroscopy, or total RNA was isolated and used in reverse-transcriptase PCR reactions.

Tobramycin affinity pull-downs were done as described. A chimeric RNA bait was made by in vitro transcription from a template having a tobramycin aptamer fused 3’ to the collagen α1(I) 5’ stem-loop. The same binding conditions were used as above, with the exception that the complexes associated with the tobramycin aptamer-5’ stem-loop were purified by the addition of a tobramycin matrix and eluted with excess tobramycin.

Mass Spectroscopy

The purified proteins were visualized on Coomassie or SYPRO Ruby (Bio-Rad) stained SDS-PAGE or 2-D SDS-PAGE gels. The proteins were excised and in-gel digested with trypsin. Eluted peptides were sequenced by LC-coupled ESI tandem MS (LC-MS/MS) on an LTQ XL instrument (Thermo Scientific). The corresponding proteins were identified by
searching against all entries in the National Center for Biotechnology Information nonredundant database by using MASCOT (Matrix Science) as a search engine.

**Mutagenesis of LARP6 serine 56 and 58**

QuickChange Multi Site-Directed Mutagenesis Kit was used to mutate the LARP6 56, 58 serines to alanine and aspartic acid. The primers are indicated as follows:

- 56 serine mutated into alanine: 5'-ccggctggggcgccgagcgaggg-3'
- 56 serine mutated into aspartic acid: 5'-cccggctggggcgacgcgagcgaggg-3'
- 58 serine mutated into alanine: 5'-tggggcagcgcggccgaggagccg-3'
- 58 serine mutated into aspartic acid: 5'-ctggggcagcgcggccgaggagccg-3'
- Both 56 and 58 serines mutated into alanine: 5'-cccggctggggcgccgagcgaggg-3'
- Both 56 and 58 serines mutated into aspartic acid: 5'-cccggctggggcgacgcgagcgaggg-3'
CHAPTER 3
CYTOKINES AND HORMONES REGULATE TYPE I COLLAGEN SYNTHESIS IN MULTIPLE CELL TYPES BY LARP6 DEPENDENT MECHANISM

Introduction

Systematic sclerosis (scleroderma, SSc) is a chronic connective tissue disease which is caused by an imbalance of cellular and humoral immune system, vascular dysfunction and activation of resident fibroblasts in multiple organs (Geyer and Muller-Ladner). The disease is associated with skin thickening and many complications, such as skin fibrosis, pulmonary fibrosis, pulmonary hypertension and renal disease (Hachulla and Launay; Denton and Black 2000). The characteristic of SSc is an increase of collagen deposition which results in all manifestations of the disease. Until now, there is no cure for scleroderma, because the molecular mechanism of collagen synthesis in scleroderma is not clear (Moore and Desantis 2008).

Cytokines are inducers of ECM proteins and TGF-beta is the most effective profibrotic cytokine which upregulates the expression of collagen genes. There are three mammalian isoforms of TGF-beta, 1, 2 and 3 and they regulate a wide range of biological events such as: cell growth, cell death, cell differentiation and ECM synthesis (Ihn 2002). TGF-beta is secreted as a dimer bound to the latency-associated protein (LAP), as LAP is needed for efficient secretion, the correct folding of the TGF-beta (Saharinen, Hyytiainen et al. 1999) and TGF-beta activation. How TGF-beta is activated is not completely understood, the studies have shown that thrombospondin (TSP1) can activate TGF-beta through conformational changes of the TGF-beta inactive complex (Keski-Oja, Koli et al. 2004). Once TGF-beta is activated, it binds to TGF-beta receptor I and II (serine/threonine kinases) on the membrane of the cell, this induces phosphorylation of the Gly-Ser residues in type I receptor by the type II receptor which is a kinase. TGF-beta receptor I is activated after phosphorylation and phosphorylates Smad2 and Smad3 proteins at the C-terminal serines. After phosphorylation, these smad proteins associate with Smad4 reform a complex. This complex translocates from cytoplasm to the
nucleus and regulates transcription of the target genes by interacting with the other nuclear transcription factors such as CBP and p300 coactivators. Smad 7 has been reported to inhibit TGF-beta signaling pathway by preventing both smad 2 and 3 phosphorylation and complex formation of smad 2, 3 and 4. Smad 7 also appears to compete with smad2 and smad 3 for binding to TGF-beta receptor I (Asano, Ihn et al. 2004). Both smad 6, 7 can interact with E3 ubiquitin ligases Smurf1 and Smurf2 and mediate ubiquitination and degradation of Smad 2, 3 and 4 proteins (Derynck and Zhang 2003). Therefore, smad 6 and 7 are the negative regulators of smad 2, 3 and 4 and TGF-beta1 signaling pathway.

Most of the studies on TGF-beta induced collagen synthesis in skin fibrosis are focused on transcriptional regulation of collagen genes (Leask 2006). When TGF-beta was applied to fibroblasts, TGF-beta induced the expression of the mRNAs encoding ECM proteins (Cotton, Herrick et al. 1998). TGF-beta induce transcription of collagen genes through AP1 protein binding to the collagen \( \alpha 1(I) \) promoter region (Cho, Kim et al. 2006). Elevated levels of TGF-beta 2 and type I collagen mRNAs were found in scleroderma biopsy samples (Denton and Abraham 2001). Varga's group identified that TGF-beta stimulates collagen \( \alpha 2(I) \) transcription in skin fibroblasts. They discovered that smads stimulate collagen \( \alpha 2(I) \) promoter activity, especially under the treatment with TGF-beta (Chen, Yuan et al. 1999). TGF-beta also stimulates transcription of collagen \( \alpha 2(I) \) gene promoter through the binding of Sp1 containing protein complex to collagen promoter (Inagaki, Truter et al. 1994). Blockage of TGF-beta signaling pathway with TGF-beta antibodies or with TGF-beta1 antisense oligonucleotide abolish the upregulated transcription of collagen \( \alpha 2(I) \) gene in SSc dermal fibroblasts (Ihn, Yamane et al. 2001). Transcription factor sp1 interact with smad proteins and activate collagen \( \alpha 2(I) \) gene in response to TGF-beta (Verrecchia and Mauviel 2002). In addition, TGF-beta pathway mediates collagen synthesis by inducing other signaling pathway as well. Protein kinase C stimulates the effect on connecting tissue growth factor mediated collagen induction. TGF-beta activates PKC\( \delta \) in mesangial cells, this promotes Smad3 transcription activity and increases collagen \( \alpha 2(I) \) transcription (Holmes, Abraham et al. 2001; Pannu and Trojanowska 2004).

There is little research done on the effect of TGF-beta on posttranscriptional regulation of collagen mRNAs. LARP6 regulated collagen synthesis may play an important role in induced collagen synthesis by TGF-beta. Future study of cytokine pathways in scleroderma
skin fibroblasts is needed to discover of cytokine signaling pathway, pathology of scleroderma skin disease and novel anti-fibrosis therapy.

Cardiac disease is the number one leading cause of death in the western world. Heart failure is the number one cause of death among all cardiac diseases (Heineke and Molkentin 2006). Cardiac fibrosis is directly related to heart failure. Cardiac fibrosis is a morphological change of the heart accompanied with diastolic dysfunction, which is abnormal filling of heart during diastole. Diastolic dysfunction associated with preserved systolic function is an important cause of heart failure. Cardiac fibrosis is the end result of acute myocardial infarction (myocardial infarction, MI or heart attack) which is another cause of heart failure (van Rooij, Sutherland et al. 2008). Cardiac fibrosis is related to ventricular hypertrophy which is also a main risk factor associated with myocardial failure. The characteristic of hypertrophied left ventricle in hypertensive heart disease is the deposition of fibers. There are no anti-fibrotic therapies available in the clinic right now (McMurray and Pfeffer 2005; Zeisberg, Tarnavski et al. 2007).

Causes of cardiac fibrosis could be myocyte necrosis, inflammation, enhanced workload and hypertrophy. Two main cytokines, TGF-beta and angiotensin II, play an important role in regulating cardiac fibroblasts to be activated into myofibroblasts and also upregulate collagen synthesis (Samuel, Unemori et al. 2004). Cardiac fibroblasts are the only cells in the heart that are responsible for synthesis of type I collagen. A significant fraction of fibroblasts are derived from endothelial cells. TGF-beta is an inducer for endothelial to mesenchymal transition in cardiac fibrosis, the transition increases the number of fibroblasts (Goumans, van Zonneveld et al. 2008). Collagen fibers are part of connecting tissue in the myocardium which connect myocytes and capillaries to prevent the slippage of adjacent muscle fibers (Diez 2007). However, an excess of collagen fibers increases tensile strength; causing the increase of the stiffness of the tissue and damage to the diastolic function of the heart (Butt, Laurent et al. 1995).

Ouabain belongs to cardiotonic steroids that are also called endogenous digitalis like factors. Ouabain physically binds to Na\(^+\)/K\(^+\)-ATPase which inhibits Na\(^+\) pump activity. The inhibition increases Ca\(^{2+}\) entry to the cell and decreases Ca\(^{2+}\) exit through Na/Ca exchange. These effects increase contractility and indirectly affect blood pressure (Blaustein, Zhang et al. 2009; Manunta, Ferrandi et al. 2009). In rat ventricular myocytes, ouabain was discovered as
promoter of Ca\(^{2+}\) influx. The signaling transduction pathways of ouabain include the stimulation of Src kinase following by activation of Ras. The activation of Ras leads to the activation of mitogen-activated protein kinases (MAPKs) or the increased production of reactive oxygen species (ROS) by mitochondria. These pathways increase contractility and hypertrophy of the heart (Tian, Gong et al. 2001).

In mammals, ouabain is produced in adrenal cortex and hypothalamus (Bagrov and Shapiro 2008) (Bagrov, Shapiro et al. 2009). It affects mostly heart, blood vessels and kidneys (Nicholls, Lewis et al. 2009). The raising of Ca\(^{2+}\) resulting from inhibition of Na\(^+\) pump by ouabain, contributes to vasoconstriction and the elevation of blood pressure (Manunta, Ferrandi et al. 2009). Ouabain is highly elevated in both human hypertension as well as several animal models of hypertension. In the rat model, treatment with ouabain increased total type I collagen in mesenteric resistance arteries vascular wall (Briones, Xavier et al. 2006). Also, zhang’s group discovered that ouabain induces collagen deposition in rat arterial walls which contributes to hypertension (Zhang, Hamlyn et al. 2009). Ouabain promotes collagen synthesis in Bovine chondrocytes through inhibition of Na\(^+\) pump (Natoli, Skaalure et al.). Another study indicated that administration of ouabain (10-50ug/kg/day) in rats increased arterial pressure and induced cardiac hypertrophy. The details on how ouabain regulates collagen synthesis are not known.

We identified LARP6 and nonmuscle myosin II as the regulators of collagen synthesis. Since triple helix formation of type I collagen is dependent on LARP6-nonmuscle myosin pathway, it is important to find out if cytokines and hormones induce collagen synthesis through this pathway. So far, little has been discovered on posttranscriptional regulation of type I collagen by cytokines, yet, type I collagen is mostly regulated by TGF-beta at the posttranscriptional level.

In this chapter, I will show that TGF-beta induces collagen synthesis in scleroderma skin fibroblast by LARP6 and nonmuscle myosin dependent mechanism and I will extend these results to cardiac fibroblasts. My finding indicates that LARP6 and nonmuscle myosin function in different cell types and confirm the importance of this pathway in TGF-beta induced fibrosis.
Results

*Knock down of LARP6 abolishes TGF-beta induced collagen secretion*

Figure 3.1

![Western blot image showing depletion of LARP6 by siRNA in scleroderma fibroblasts. Lane 1, LARP6 specific siRNA (D2); lane 2, scrambled siRNA (CON). Control for loading, calnexin.](image)

**Figure 3.1.** Depletion of LARP6 by siRNA in scleroderma fibroblasts. LARP6 expression was analyzed by western blot. Lane 1, LARP6 specific siRNA (D2); lane 2, scrambled siRNA (CON). Control for loading, calnexin.

Figure 3.2

![Western blot image of collagen secretion.](image)

**Figure 3.2.** Collagen secretion with different conditions: Cellular and Medium, D2 and CON.
To test the hypothesis that TGF-beta1 induced collagen synthesis is LARP6 dependent, we inactivated LARP6 in scleroderma skin fibroblasts using LARP6 specific siRNA D2 and treated the cells with TGF-beta. D2 siRNA was delivered by adenovirus to the cells (as mentioned in Chapter 2). Control siRNA was encoded by a scrambled siRNA gene. The depletion of LARP6 in scleroderma fibroblasts was assessed by western blot (Fig. 3.1). Western blot revealed that LARP6 depletion was more than 50%. Calnexin was probed as a loading control and was unaffected. Its level in the cell was not affected by either LARP6 siRNA or the scrambled control siRNA (Fig. 3.1). We concluded that D2 siRNA specifically knocked down LARP6. We treated the cells with TGF-beta1 after the inactivation of LARP6 and assessed both the cellular level and the secretion rate of collagen α1(I) peptide. Cells were serum starved for 24 hours before treating with TGF-beta1 to eliminate the cytokines from the serum. Collagen α1(I) intracellular level did not change in LARP6 depleted cells (lane1), or with TGF-beta treatment (Fig. 3.2, top panel, lanes 2-4). However, cells treated with TGF-beta secreted more collagen α1(I) then the untreated control cells (lanes 6 and 8). In the untreated cells, upon LARP6 depletion, the secretion of collagen α1(I) decreased compared to the control cells (lanes 5 and 7). When we depleted LARP6 in TGF-beta treated cells, the induction of collagen α1(I) was abolished (Fig. 3.2 upper panel lanes 5-8). Fibronectin, as a control, was not affected significantly by any treatment indicating the effect is specific to collagen and that TGF-beta1 stimulation on collagen α1(I) secretion is LARP6 dependent. This suggested that LARP6 mediates TGF-beta induced type I collagen secretion.

*Overexpression of dominant negative LARP6 decreased TGF-beta1 induced collagen secretion.*
Another approach to inactivate LARP6 is to overexpress a dominant negative form of the protein. In order to determine if overexpression of dominant negative isoform of LARP6 would affect TGF-beta induced collagen α1(I) synthesis in scleroderma cells, we overexpressed LARP6 mutant A and assessed intracellular level and secretion rate of collagen. LARP6 mutant A is previously discussed in chapter 2. It does not contain the C-terminus domain and binds to 5’ stem-loop (see chapter 2), but does not interact with nonmuscle myosin IIB. (Dillon Fritz Dissertation). The mutant A and control protein (truncated version of lysyl oxidase protein) were delivered by adenoviruses into scleroderma cells. Overexpression of LARP6 mutant A did not change intracellular collagen α1(I) level in both TGF-beta1 treated and untreated cells (Fig.3.3 upper panel, lanes1 and 3). TGF-beta1 induced collagen α1(I) secretion as in the previous experiment (upper panel, lanes 6 and 8), however, overexpression

Figure 3.3

![Figure 3.3](image)

**Figure 3.3.** Overexpression of a dominant negative LARP6 decreased TGF-beta1 induced collagen secretion. Lane 1-4 scleroderma cellular extracts, lane 5-8 medium were analyzed for collagen α1(I) and fibronectin by western blot. LARP6 dominant negative mutant (A) overexpressed cells (lanes 1, 3, 5 and 7). Lanes 2, 4, 6 and 8, control cells without treatment. Lanes 3 and 7, in LARP6 dominant negative (A) overexpressed cells with TGF-beta1 treatment. Lanes 4 and 8, in TGF-beta1 treated cells. Fibronectin (FIB) is shown as a loading control.
of LARP6 mutant A abolished this induction (lanes 7 and 8). Fibronectin cellular and secretion level was not significantly affected (Lower panel). This suggested that overexpression dominant negative form of LARP6 diminishes TGF-beta1 induced collagen α1(I) secretion. We concluded that TGF-beta induced type I collagen synthesis is dependent on LARP6. The effect of ML-7 on TGF-beta induced collagen synthesis remains to be done.

Expression of LARP6 in cardiac fibroblasts.

Figure 3.4

![Immunostaining of LARP6 in mouse cardiac fibroblasts.](image)

**Figure 3.4.** Immunostaining of LARP6 in mouse cardiac fibroblasts. Left, confocal images of LARP6 localization in mouse cardiac fibroblasts. Control staining without LARP6 antibody is shown on the right.

To analyze the expression of LARP6 in the cardiac fibroblasts and assess if it is the same as in lung fibroblasts (chapter 2), we did immunostaining of mouse cardiac fibroblasts with LARP6 antibody and with control antibody. Staining with LAPRP6 antibody (Figure 3.4) indicated that endogenous LARP6 accumulates in both nucleus and cytoplasm (left panel), but predominantly in the cytoplasm. Control staining (right panel) showed no signal. We concluded that the subcellular distribution of LARP6 is similar in lung fibroblasts and mouse cardiac fibroblasts.
Nonmuscle myosin filaments are required for ouabain induced type I collagen synthesis.

It is important to understand how profibrotic cytokines, like TGF-beta, increase collagen synthesis in cardiac fibroblasts. To assess if LARP6 dependent mechanism is involved in TGF-beta mediated stimulation of collagen expression we treated rat and mouse cardiac fibroblasts with TGF-beta. However, we could not see a significant increase in collagen protein secretion in cardiac fibroblasts, even though they express TGF-beta receptors. Similarly, lack of stimulation was observed by other investigators (Akiyama-Uchida, Ashizawa et al. 2002). When fibroblasts are cultured on cell culture plates, they undergo similar activation as in vivo, and become myofibroblasts and synthesize high level of collagen. Therefore, they cannot respond to TGF-beta. To transform the cells to quiescent, we cultured them on laminin coated plates and stimulated them with TGF-beta. Even then we did not see a significant increase in expression of type I collagen by western blot. Therefore, we tried another profibrotic compound ouabain. Ouabain and other cardiotonic steroids increase cardiac fibrosis (Bagrov, Shapiro et al. 2009), so we surmised that ouabain stimulation in vitro can be a good model of cardiac fibrosis.

To test if ouabain can induce type I collagen synthesis in rat cardiac fibroblasts, we serum starved the cells over night and treated them with ouabain (100um, 24hours) and then assessed collagen synthesis by western blot. Both collagen α(I) and collagen α2(I) intracellular levels were not changed (Fig 3.5 lane 1 and 2). The secretion of collagen α1(I) and α2(I) polypeptides are significantly increased by ouabain comparing to the control cells (Figure 3.5 lane 3 and 4). Fibronectin secretion was not affected by ouabain, this indicated that ouabain specifically induces collagen secretion. We concluded that ouabain treatment is an acceptable model to study inducible collagen synthesis in cardiac fibroblasts.

To verify whether nonmuscle myosin II filaments are involved in ouabain regulated collagen synthesis, we assessed ouabain induced collagen secretion upon disrupting of nonmuscle myosin II filaments with ML-7. Upon ML-7 treatment, collagen α2(I) secretion was significantly decreased (Fig.3.6, lane 3), however, collagen α1(I) secretion rate was minimally affected (lane 3). This is consistent with the result in human lung fibroblasts (Figure 2.5, lanes 3 and 4). When the cells were treated with ouabain, collagen α1(I) and collagen α2(I) secretion was induced (lane 2). However, when cells were treated with ML-7, this induction was decreased to
the control level (lane1). Fibronectin was not significantly affected by any treatment. This suggested that ouabain mediated collagen secretion is depend on the integrity of nonmuscle myosin II filaments.

Figure 3.5

Figure 3.5. Ouabain induces collagen secretion by rat cardiac fibroblasts. The effect of ouabain (OU) on collagen α1 (1) (COLL1A1) and collagenα2 (1) polypeptides (COLL 1A2) synthesis was assessed by western blot. Lanes 1and 3 ouabain (OU) treated cells; lanes 2 and 4, control untreated cells (CON). Lanes 1 and 2, cellular level of collagen. Lanes 3 and 4, secretion collagen level. Expression of fibronectin (FIB) is shown as the loading control.

Figure 3.6
We also overexpressed LARP6 mutant A by adenovirus in rat cardiac fibroblasts, however, this did not affect intracellular or secretion rate of type I collagen. This indicated that human LARP6 A may not be a dominant negative protein in cardiac fibroblasts. Alternatively, it is possible that cardiac fibroblasts do not require LARP6 for ouabain induced collagen synthesis. More work is needed to verify if LARP6 is involved in ouabain induced collagen synthesis.

Discussion

Hormones and cytokines play important roles in regulating collagen synthesis in wound healing and fibrosis. The effect of TGF-beta1 and ouabain on posttranscriptional regulation of type I collagen has not been defined. In scleroderma skin fibroblasts, LARP6 depletion decreased the secretion rate of collagen α1(I) (Fig.3.2). This result is similar to what we observed from human lung fibroblasts where depletion of LARP6 decreased both the intracellular level of collagen α1(I) and its secretion rate (chapter 2). However, the effect of LARP6 depletion on the intracellular collagen α1(I) level is different. In scleroderma skin fibroblasts, there was no change in intracellular level upon LARP6 depletion. One possibility is that the degradation rate of unsecreted collagen polypeptides is different in the two cell types. LARP6 cannot regulate collagen α1(I) polypeptide localization under LARP6 depletion, this could cause collagen α1(I) polypeptides localize randomly in the ER. They are not properly modified or folded; they may be degraded in the cell instead of secreted. Therefore, there will be more intracellular collagen α1(I) polypeptides in scleroderma fibroblasts if the degradation rate is slower than in lung fibroblasts. Without LARP6, collagen α1(I) polypeptides may have been secreted as a homotrimer which is inefficient in scleroderma fibroblasts. This could
explain the decrease in collagen $\alpha 1(I)$ secretion. (Fig 3.2 lane 5). When cells were treated with TGF-beta1, TGF-beta induced collagen $\alpha 1(I)$ secretion about 2 fold (Fig 3.2 lane 8), while intracellular collagen $\alpha 1(I)$ was not changed (Fig 3.2 lane 2 and 4). Depletion of LARP6 abolished the induction of collagen by TGF-beta (Figure 3.2 lane 7) and the level was even lower than in untreated cells (Fig 3.2. lanes 6 and 7). This suggested that LARP6 might be a downstream regulator of collagen synthesis in scleroderma skin disease controlled by TGF-beta 1 signaling. There is a possibility that TGF-beta regulates LARP6 is by posttranslational modifications of LARP6, since LARP6 expression level does not change in normal or fibrotic cells. Our preliminary date suggested that LARP6 is phosphorylated. Phosphorylation or dephosphorylation of LARP6 may be regulated through TGF-beta signaling. One possibility is that the modification affects LARP6 binding to the 5'stem-loop collagen mRNAs, and regulates collagen synthesis. Another possibility is that the modifications may affect the localization of LARP6 to the nucleus or cytoplasm. The posttranslational modification may also regulate the interaction of LARP6 with other proteins such as nonmuscle myosin. However, more work need to be done to test these possibilities.

LARP6 mutant A lacks the nuclear localization signal. It was showed in chapter 2 that this protein is predominately cytosolic. Since mutant A binds to both collagen $\alpha 1(I)$ and $\alpha 2(I)$ 5'stem-loop, it can inhibit translation of type I collagen. As mutant A does not interact with nonmuscle myosin II like wild type LARP6, it acts as a dominant negative protein. Overexpression of mutant A decreased both the collagen $\alpha 1(I)$ polypeptides steady state level in the cell and its secretion rate (Fig. 3.3). This is similar to the result with human lung fibroblasts (Chapter 2). Overexpression of the dominant negative LARP6 may inhibit collagen mRNA translation, and collagen $\alpha 1(I)$ polypeptides could be made in trace amount by another pathway independent of myosin II and secreted out. This pathway seems to be inefficient. Overexpression of mutant A also abolished TGF-beta1 induced collagen synthesis to more than 50% (Fig. 3.3), indicating TGF-beta stimulated collagen synthesis is dependent on LARP6. The mechanism by which mutant A abolishes TGF-beta induced collagen synthesis could be the same as we described above. These results suggested that LARP6 mechanism is involved in TGF-beta induced type I collagen in scleroderma skin cells.

In cardiac fibroblasts, ouabain induced collagen synthesis is nonmuscle myosin II dependent (figure 3.6). The decreased secretion of collagen may be due to the dislocation of
collagen mRNAs upon disruption of myosin II filaments. When the collagen mRNAs are not transported for coordinated translation, formation of collagen heterotrimer triple helix is inhibited. Collagen α2(I) polypeptides are not secreted because they are not incorporated into triple helix. The secreted triple helix collagen is most likely formed by collagen α1(I) polypeptides. These results showed that the nonmuscle myosin II regulated type I collagen synthesis is functional in different cell types and suggested that ouabain upregulates collagen expression in cardiac fibrosis through the mechanism including nonmuscle myosin filaments.

We postulate that LARP6, nonmuscle myosin and 5' stem-loop regulated type I collagen synthesis is activated when the cells are stimulated by cytokines in wound healing or fibrosis and that is a general mechanism of inducible collagen synthesis.

Material and Methods

Antibodies

Antibodies used in Western blot analysis were from: anti-type I collagen antibody from Rockland, anti-fibronectin antibody was from BD biosciences and anti-LARP antibody from Abnova. 1:1000 dilution was used in the experiments.

Western blot analysis and immunostaining

Protein concentration was estimated by the Bradford assay with BSA as the standard. Western blots were done using between 10 and 100 μg of protein. Medium was loaded in equal amount on the gel.

For immunostaining, cells were seeded onto glass coverslips and after treatment the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. After blocking with 10% goat serum/5% BSA in PBS for 1 hr at room temperature, the cells were incubated with primary antibody overnight at 4˚ C, washed 3 X 5 min in PBS, and visualized with Alexafluor 594-conjugated secondary antibody. The cells were mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories) and images taken by Leica TCS SP2 AOBS laser confocal microscope equipped with a Chameleon Ti:Sapphire multiphoton laser. Optical sections were processed with LCSLite software.
Cellular fractionation

Cytosolic extracts were prepared by hypotonic lysis of cells in 10 mM Tris–HCl (pH 7.6), 5 mM MgCl₂, 10 mM KCl, and 0.4% NP-40, and after removal of nuclei by centrifugation, the supernatant was used as cytosolic extract. For nuclear extract, the pelleted nuclei were washed several times in the above buffer and nuclear proteins were extracted by the method mentioned in Dignam group (Dignam, Lebovitz et al. 1983). Protein concentration was measured by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

Cell culture

ML-7 was purchased from Sigma, the working concentration was 40uM, incubate overnight. Ouabain was purchased from Sigma, we added 100nm to the culture medium after 24 hours of serum starvation of cardiac fibroblast and incubated over night. hTGF-beta1 was from R&D system. Scleroderma fibroblasts were serum starved over night and added 5ng/ml hTGF-beta1 and incubated overnight.

Construction of clones and adenoviruses

Construction of D2 virus and LARP6 A mutant see chapter 2.

Isolation of cardiac fibroblasts

Mouse and rat hearts were cut into 1-2mm² pieces and digested in Dulbecco’s Modification of Eagle’s Medium (Mediatech, Inc) with 0.25% collagenase (sigma) in 37°C shaker for 30mins.

The cells were spun down and plated cells on uncoated plates. Cells were cultured for 2 weeks until fully differentiated. Other than fibroblasts, other types of cells were not attached and were washed away by changing the culture medium (Borg, Rubin et al. 1984; Burgess, Terracio et al. 2002).
GENERAL CONCLUSION

Fibrosis is a fatal disease which can affect several organs. Current treatment for fibrosis is very limited and there is not specific cure yet. Fibrosis is characterized by deposition of collagen and other extracellular matrix proteins. Uncontrolled type I collagen synthesis is the cause of mortality in fibrosis. Cytokines and hormones induce collagen synthesis to promote wound healing. In fibrosis, due to prolonged injury, wound healing no longer respond to the control. In order to discover anti-fibrotic drugs, we need to discover the molecular mechanism of type I collagen regulation in fibrosis. In my dissertation, I identified the specific regulators of type I collagen synthesis. LARP6 and nonmuscle myosin II and their function were characterized.

In the first chapter, we identified La ribonucleoprotein domain family, member 6 (LARP6) which directly binds to the 5' stem-loop regulatory region of collagen α1(I) and α2(I) mRNAs in a sequence specific manner. LARP6 is sufficient to bind to 5'stem-loop, and no posttranslational modifications of the protein are needed. The high affinity binding aggregates collagen mRNAs into a complex with nonmuscle myosin to target collagen mRNAs to certain locations in the cell. Overexpression of LARP6 inhibits collagen mRNA translation, while depletion of LARP6 decreases collagen protein steady state level in the cell and its secretion rate. This indicates tightly regulated levels of LARP6 are needed for optimal collagen synthesis.

How LARP6 regulates collagen mRNA translation was described in the second chapter. Since the 5'stem-loop is the cis element which regulates collagen synthesis, we identified another protein that interacts through 5'stem-loop. Nonmuscle myosin II B was discovered by 5’ stem-loop tobramycin affinity purification. Nonmuscle myosin interacts with LARP6 and indirectly with collagen mRNAs. It may associate the collagen α1(I) and α2(I) mRNAs in a complex, since the interaction of α2(I) mRNA with nonmuscle myosin II is dependent on the intact of α1(I) mRNA. We postulated that nonmuscle myosin filaments are required for collagen mRNAs coordinated translation, this insures that collagen α1(I) and α2(I) peptides are synthesized in certain subcellular regions and fold into heterotrimeric triple helix of type I collagen.
The next question addressed was whether LARP6 and nonmuscle myosin II regulated synthesis of type I collagen is involved in fibrosis (chapter 3). In the fibrotic stage, type I collagen is highly upregulated by cytokines and hormones signaling. We showed that fibrotic cytokines and hormones such as TGF-beta1 and ouabain induce collagen synthesis in LARP6 and nonmuscle myosin II dependent pathway in scleroderma skin fibroblasts and rat cardiac fibroblasts. This finding suggested a common pathway in fibrosis that is shared by skin fibroblasts, lung fibroblasts and cardiac fibroblasts.

Future research will be focused on the regulation of LARP6 such as phosphorylation. The phosphorylation sites, the kinases which phosphorylate these sites and the corresponded function of the phosphorylation all need to be discovered. We suspect that LARP6 phosphorylation regulates LARP6 interaction with 5’stem-loop mRNA, or its interaction with myosin II, or its localization, or its degradation. Since LARP6 is tightly regulated in collagen synthesis, these findings will elucidate the mechanism of collagen gene regulation and the findings will contribute to anti-fibrotic drug discovery.
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Le Cai grew up in Beijing, China. She obtained her B.S. degree from Beijing Institute of Technology in 2004. She continued with her graduate education in department of biomedical sciences, Florida State University since 2004.