

Thrombin alters the synthesis and processing of CYR61/CCN1 in human corneal stromal fibroblasts and myofibroblasts through multiple distinct mechanisms

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Purpose: Previous research in our laboratory indicated that prothrombin and other coagulation enzymes required to activate prothrombin to thrombin are synthesized by the cornea and that apoptotic human corneal stromal cells can provide a surface for prothrombin activation through the intrinsic and extrinsic coagulation pathways. The purpose of the work reported here is to study the role of thrombin activity in the regulation of matricellular protein Cyr61 (CCN1) produced by wounded phenotype human corneal stromal fibroblasts and myofibroblasts.

Methods: Stromal cells from human donor corneas were converted to defined wounded phenotype fibroblasts and myofibroblasts with fetal bovine serum, followed by basic fibroblast growth factor (bFGF) and transforming growth factor beta-1 (TGFβ-1), respectively, and stimulated with varying concentrations (0–10.0 units (U)/ml) of thrombin from 1-7 h. Cyr61 transcript levels were determined using reverse transcriptase-PCR (RT-PCR) and quantitative PCR (qPCR) while protein forms were analyzed using western blot data. Protease activities were characterized via protease class-specific inhibitors and western blot analysis. Thrombin activity was quantified using the fluorogenic peptide Phe-Pro-Arg-AFC. Protease-activated receptor (PAR) agonist peptides-1 and -4 were used to determine whether cells increased Cyr61 through PAR signaling pathways. The PAR-1 antagonist SCH 79797 was used to block the thrombin cleavage of the receptor. PCR data were analyzed using MxPro software and western blot data were analyzed using Image Lab™ and Image J software. Student *t* test and one- and two-way ANOVA (with or without ranking, depending on sample distribution), together with Dunnett's test or Tukey comparison tests for post-hoc analysis, were used to determine statistical significance.

Results: Full-length Cyr61 is expressed by human corneal stromal fibroblasts and myofibroblasts and is significantly upregulated by active thrombin stimulation at the message ($p < 0.03$) and protein ($p < 0.03$) levels for fibroblasts and myofibroblasts. Inhibition by the allosteric thrombin-specific inhibitor hirudin prevented the thrombin-associated increase in the Cyr61 protein expression, indicating that the proteolytic activity of thrombin is required for the increase of the Cyr61 protein level. PAR-1 agonist stimulation of fibroblasts and myofibroblasts significantly increased cell-associated Cyr61 protein levels ($p < 0.04$), and PAR-1 antagonist SCH 79797 significantly inhibited the thrombin stimulated increase of Cyr61 in fibroblasts but not in myofibroblasts. In the fibroblast and myofibroblast conditioned media, Cyr61 was detected as the full-length 40 kDa protein in the absence of thrombin, and mainly at 24 kDa in the presence of thrombin at ≥ 0.5 U/ml, using an antibody directed toward the internal linker region between the von Willebrand factor type C and thrombospondin type-1 domains. Although known to undergo alternative splicing, Cyr61 that is synthesized by corneal fibroblasts and myofibroblasts is not alternatively spliced in response to thrombin stimulation nor is Cyr61 directly cleaved by thrombin to generate its 24 kDa form; instead, Cyr61 is proteolytically processed into 24 kDa N- and 16 kDa C-terminal fragments by a thrombin activated leupeptin-sensitive protease present in conditioned media with activity distinct from the proteolytic activity of thrombin.

Conclusions: In cultured human corneal stromal fibroblasts and myofibroblasts, thrombin regulates Cyr61 through two mechanisms: 1) thrombin increases the Cyr61 expression at the message and protein levels, and 2) thrombin increases the activation of a leupeptin-sensitive protease that stimulates the cleavage of Cyr61 into N- and C-terminal domain populations in or near the thrombospondin type-1 domain. Generation of Cyr61 peptides during corneal injury stimulation may reveal additional functions of the protein, which modulate corneal wound healing activities or decrease activities of the full-length Cyr61 form.

Corneal cells synthesize proteins that facilitate wound healing during injury resolution. Biochemical analysis of

corneal proteins in our laboratory indicates that the normal human cornea produces the entire complement of proenzymes and cofactors responsible for the prothrombin activation to thrombin and subsequent generation of fibrin in vitro [1]. Therefore, the avascular cornea represents a coagulation system that is isolated from the vascular system and can

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orchestrate the entire suite of coagulation-related wound healing activities without vascular involvement.

Prothrombin present in the cornea is probably activated upon corneal injury to generate fibrin from fibrinogen. Fibrin is a component of the provisional extracellular matrix that is generated in response to injury at the surface of and within the injury site of the cornea [2,3]. Corneal stromal fibroblasts migrate through 3-D fibrin matrices, secrete fibronectin at the leading edge that facilitates cell migration and spreading, and form an interconnected cell-fibronectin network [4-6]. Thrombin not only cleaves fibrinogen to form fibrin [7] but also alters cell function through multiple mechanisms that require proteolysis, including activation of proenzymes and other proteins, such as protease-activated receptors (PARs) [8-14], and through non-proteolytic mechanisms [14-19] that involve thrombin peptides such as TP508 [14-16,19]. In the absence of fibrin/fibrinogen, thrombin induces actomyosin contraction of corneal fibroblasts and Rho kinase-dependent cell clustering when plated on top of collagen matrices [5]. These results suggest that thrombin controls corneal stromal fibroblast migration after corneal injury, through both the formation of fibrin and direct effects on stromal cells.

Many of thrombin's direct effects on cells are mediated through the cleavage and activation of PARs [8-10,20]. These receptors are a subfamily of heterotrimeric G-protein coupled receptors activated in response to protease activity [10,13,20]. Of the four PAR family members, PARs -1, -3, and -4 are activated in response to thrombin cleavage at low concentrations [9,20,21], although the signaling mechanism of PAR-3 in humans is unknown [10-12,20].

One of the genes upregulated in response to thrombin-mediated PAR-1 signaling [22-25] and downstream activation of the mechanosensitive Hippo-yes-associated protein (YAP)/ transcriptional coactivator with PDZ binding motif (TAZ) pathway [26] is the Cysteine-rich secreted protein-61 (Cyr61), also known as CCN1. Cyr61 is a matricellular protein related to the connective tissue growth factor (CTGF/CCN2), a protein involved in corneal fibrosis [27]. Cyr61 binds to extracellular matrix (ECM) molecules [28-31] and cell-surface receptors, including integrins and heparan sulfate proteoglycans [28-30,32], to regulate proliferation [30,32,33], adhesion [29,30,34], migration [29,30,32], apoptosis [28,30], survival [30,33,34], and senescence [30,35,36] in a cell-contextual manner. This matricellular protein stimulates angiogenesis [37,38] and is required for placental and cardiovascular development during embryogenesis [39]. CYR61 is also essential for vascular integrity [39] and is a regulator of the inflammatory process in the ECM [30].

Cyr61 exerts its biologic effects by selective receptor or receptor-ECM binding of its four domains, which include, from the N- to C-terminal, insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C repeat (vWFC), thrombospondin type-1 (TSP-1), and cysteine knot/C-terminal domain (Knot) [27]. Each domain is separated by linker regions that allow the domains to move relative to one another and are prone to proteolysis. The largest linker region of Cyr61 is between the vWFC and the TSP-1 domains and is composed of 72 amino acid residues.

Smaller forms of Cyr61 at the transcript and protein levels are produced in various cell types under normal [40,41] and pathological [38,42] conditions. Truncated forms of the Cyr61 protein are either produced through proteolysis [38,41-47] or alternative splicing [40,48]. These forms can support cellular activities similar to full-length Cyr61 [38,44,45] or alter cellular activities in a distinct manner [38,44,46]. Therefore, different Cyr61-mediated signaling effects may be revealed by the coordinated generation and downstream signaling response of the lower-molecular weight forms of this protein during injury resolution.

In contrast to CTGF, very little is known about Cyr61 in the cornea, including whether it is synthesized by corneal cells. The only published work on Cyr61 related to the cornea is connected to the ability of Cyr61 to induce angiogenesis in a rat corneal pocket assay [37]. Aside from this initial characterization of Cyr61 as a proangiogenic molecule, endogenous Cyr61 in the cornea has not been studied.

The purpose of this study was to determine whether cultured human corneal stromal fibroblasts and myofibroblasts produce and release Cyr61. If so, the aim was to determine whether these cells respond to thrombin stimulation with respect to Cyr61 at the message and protein levels. Since Cyr61 can undergo proteolysis [38,41-47] or alternative splicing [40,48] in response to external stimuli, the ability of thrombin to regulate Cyr61 expression and to control the Cyr61 forms present via these mechanisms was also assessed in vitro.

METHODS

Human corneas and cell culture: Anonymized human donor corneas were obtained from the Wisconsin Lions Eye Bank, Madison, WI. Donors were 36 to 74 years of age, and 65% of them were male. Stromal cell keratocytes were extracted from human corneal tissue following the method described in Jester et al. [49]. To summarize, corneas were washed in dibasic phosphate buffered saline (DPBS) from the Sigma Chemical Company, St Louis, MO, and the epithelial and endothelial layers were removed by scraping. The corneas

were cut into sections and incubated with 150 units (U)/ml of collagenase (Sigma or Gibco, Gaithersburg, MD) for 18–24 h to release keratocytes from the surrounding ECM. Collagenase was inactivated using Dulbecco's Modified Eagle Medium (DMEM) from Gibco containing 5% fetal bovine serum (FBS; Sigma or HyClone, Logan, UT), and the cells were seeded in a T-25 flask containing high glucose, high glutamine DMEM (Gibco) with 5% FBS, 0.1% MITO+ serum extender (Corning Life Sciences, Corning, NY), and 10 µg/ml ciprofloxacin (Sigma). The cells were fed every 48 h until confluent, washed twice with Hank's balanced salt solution (HBSS; Gibco), and then passed into a T-75 flask via trypsin-EDTA (Life Technologies, Grand Island, NY) dissociation. Trypsin was inactivated by the addition of DMEM containing 5% FBS. Epithelial contamination decreased with increasing cell passage number due to the differing sensitivity of epithelial cells to trypsin dissociation in comparison to stromal cells. Cell passages 3–5 were used for all experiments.

To produce defined wounded phenotype stromal corneal cells, the FBS cultured cells were seeded onto plates coated with Type I collagen (PurCol, Advanced BioMatrix, San Diego, CA) at a density of 100,000 cells/well in a 24-well plate or 300,000 cells/well in a 6-well plate and incubated at 37 °C for 24 to 48 h before the initiation of the differentiation protocol as described by Jester et al. [49]. Briefly, the FBS-derived stromal cells were fed a serum-free medium with a 1% RPMI vitamin mix (Invitrogen, Grand Island, NY), 100 µM non-essential amino acids, 1 mM pyruvate, 100 µg/ml ascorbic acid, and 10 µg/ml ciprofloxacin (Sigma) that contained either 10 ng/ml of FGF-2 (Invitrogen) or 1 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) to generate FGF-2 fibroblasts [50] or TGF-β1 myofibroblasts [49,50], respectively. The cells were incubated at 37 °C in 5% CO₂ and fed a serum-free medium with growth factors for seven days before all experiments.

Thrombin treatment of cells: Defined human corneal stromal fibroblasts and myofibroblasts were fed a serum-free medium with growth factors and then incubated for 3 or 4 h. After the preincubation period, cells were treated with thrombin (Haematologic Technologies Inc., Essex Junction, VT) for a final concentration of 0.01 to 10.0 U/ml or an untreated control medium from 1–24 h. The conditioned media were collected and the cellular debris removed by centrifugation at 19,540 × g or, for volumes >2 ml, at 1,430 × g for 10 min. For recombinant Cyr61 (rCyr61, PreproTech, Rocky Hill, NJ) degradation experiments, the centrifuged media were used directly. For western blot analysis, the clarified conditioned media were concentrated by trichloroacetic acid (TCA) precipitation and resuspended in 1X reduced sample

buffer for a final concentration of 10X before loading on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. Cells were either lysed in 0.5 M sodium hydroxide or modified radioimmunoprecipitation assay (RIPA) buffer (Invitrogen) [51]. Cell lysates collected for western blot analysis were flash frozen in dry ice, thawed on ice, and vortexed to break up cellular debris. Lysates were clarified by centrifugation at 19,540 × g for 10 min and the supernates removed for downstream protein analysis.

Western blot analysis: Proteins present in the conditioned media and cell lysates from the corneal stromal fibroblasts and myofibroblasts were electrophoresed under reducing conditions through 12% SDS–PAGE gels (BioRad, Hercules, CA). The proteins were transblotted to polyvinylidene difluoride (PVDF) membranes (BioRad). The blots were probed for Cyr61 using specific antibodies raised against the peptides corresponding to the N-terminal region (goat polyclonal, N-16: epitope near the N-terminus), the linker region between the vWFC and TSP-1 domains, which is the most unique amino acid sequence of the protein [27] (rabbit polyclonal, H-78: generated to amino acids 163–240), or the C-terminal region (mouse monoclonal, H-2: epitope to amino acids 345–381) of human Cyr61 from Santa Cruz Biotechnology, Inc., Dallas, TX. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies matched to the species of the primary antibodies from BioRad. Non-specific binding by the secondary antibody was determined by direct exposure of the blots to the secondary antibody with omission of the primary antibody. The bands were visualized using a Western Blot Detection Kit from Amersham, Piscataway, NJ, and detected with either the Amersham Hyperfilm or ChemiDoc™ XRS+ molecular imager (BioRad). Image analysis was performed using Image Lab™ software version 2.0 (BioRad) or ImageJ 1.51j8 [52,53]. Protein loading was either normalized to cellular GAPDH or total cell protein. GAPDH levels were determined on the same blots that were used for Cyr61 visualization. The bound antibodies were dissociated using 5% acetic acid, and then, the blots were reprobed with a mouse monoclonal antibody raised against human GAPDH from Chemicon, Billerica, MA. Total cell protein was determined via the modified Bradford Coomassie assay using reagents from Pierce, Rockford, IL.

Transcript analysis: Defined corneal stromal fibroblasts and myofibroblasts cultured in the serum-free medium containing growth factors were treated with either medium alone or medium containing thrombin for a final thrombin concentration of 1.0 U/ml for 60 min and lysed with RNeasy Mini RLT buffer + β-mercaptoethanol (Qiagen, Valencia, CA). One microgram of mRNA was converted to cDNA with the

First-Strand-SuperScript™ III reverse transcriptase kit (Invitrogen). PCR amplification was performed using exon-exon bridging primer sets (Table 1) in order to ensure specificity and to detect any contaminating genomic DNA. To probe for full-length Cyr61 mRNA and for alternative splicing isoforms of Cyr61, the cDNA from control and thrombin-treated cells was PCR amplified using exon-exon bridging primer sets from Invitrogen across the entire Cyr61 transcript (Table 1). Controls for contamination included samples without added template or reverse transcriptase. GAPDH amplification was used to normalize samples. PCR products were electrophoresed through a 1.5% agarose gel (Invitrogen) with 0.33 mg/ml ethidium bromide (BioRad) and visualized using a ChemiDoc™ XRS+ molecular imager (BioRad). For real-time PCR, specific primers in exons 2 and 3 were used (Table 1). Real-time PCR was performed with a Stratagene Mx3005P fluorescent thermal cycler (Santa Clara, CA) and an SA Biosciences RT² SYBR Green/ROX qPCR Master Mix (SA Biosciences, Valencia, CA). Data were calculated using the MxPro qPCR Software version 3.20 (Stratagene).

PAR-1 and -4 signaling analyses: Cells were treated with 111 μM of PAR-1 or -4 peptide agonists (TFLLR or AYPGKF, AnaSpec, Fremont, CA), corresponding scrambled PAR-1 or -4 peptides (RLLFT or YAPGKF, Tocris Bioscience, Minneapolis, MN), 1.0 U/ml thrombin, or cell naïve medium for 1 h. Lysates were prepared, and the media collected, clarified, and concentrated. Then, the proteins were separated by SDS-PAGE and analyzed using western blot data generated using the H-78 antibody specific for the linker region between the vWF and TSP-1 domains of Cyr61. For PAR-1 inhibition experiments, cells were first pretreated with or without the PAR-1 antagonist, SCH 79797 (Tocris Bioscience), using a concentration of 25 nM for fibroblasts and 50 nM for myofibroblasts for 30 min. Then, either an additional serum-free medium or a medium containing thrombin at a final concentration of 1.0 U/ml was added to the cultures. Cells were further incubated with the combination treatment for 3 h. Lysates and supernates were collected, prepared, and

analyzed for Cyr61 using western blot data generated using the H-78 antibody.

Cellular protease inhibition assays: Corneal stromal fibroblasts and myofibroblasts were preincubated for 3 h in a serum-free medium containing growth factors. Vehicle (dimethyl sulfoxide [DMSO], water, or PBS [Gibco]), the thrombin-specific inhibitor hirudin (4.4 Antithrombin Units/ml [AT-U/ml] in water, Sigma, St. Louis, MO), the serine protease inhibitor aprotinin (0.3 μM, in water, Roche, Indianapolis, IN), the cysteine protease inhibitor E-64 (10 μM, in water, Sigma), the serine/cysteine protease inhibitor leupeptin (10 and 100 μM, in PBS, Sigma), the chymotrypsin-like serine and cysteine protease inhibitor chymostatin (100 μM, in DMSO, Sigma), the metalloproteinase inhibitor marimastat (10 μM, in DMSO, Tocris Bioscience), or the aspartic protease inhibitor pepstatin A (1 μM, in DMSO, Sigma) were added at least 5 min before the addition of 1.0 U/ml of thrombin or additional medium. The concentrations given were the final concentrations in the cell media. The cells were incubated with thrombin at a final concentration of 1.0 U/ml for 2 or 3 h. The lysates and media were processed for SDS-PAGE and transferred to PDVF membranes. Cyr61 was visualized on western blots using the central linker region-specific Cyr61 antibody.

Thrombin assay: Thrombin was incubated (1.0 U/ml) with hirudin (4.4 AT-U/ml), leupeptin (100 μM), or DMEM using the same thrombin to inhibitor ratios as those used under stromal fibroblast and myofibroblast culture conditions. Aliquots of these solutions were added to 50 μM labeled substrate peptide D-Phe-Pro-Arg-AFC (7-amido-4-trifluoromethylcoumarin, Anspec, Fremont, CA) at room temperature and transferred to a fluorescent plate reader preheated to 37 °C. Relative fluorescence was measured as a function of time over 60 min using a fluorescent plate reader that was programmed for excitation at 380 nm and for emission at 500 nm using Gen 1.5 software (BioTek Instruments, Inc., Winooski, VT). The fluorescence data was used to calculate cleavage rates.

TABLE 1. EXON-EXON BRIDGING PRIMER SETS (5' – 3') ACROSS THE ENTIRE CYR61 TRANSCRIPT LENGTH USED FOR ALTERNATIVE SPLICING ANALYSIS AND REAL-TIME PCR.

Primer Name	Primer sequence (5' → 3')	Use
Cyr61 Exon 1 Forward	ACACCAGCTTGTTGGCGTCT	PCR
Cyr61 Exon 5 Reverse	GTCATTGAACAGCCTGTAGA	PCR
Cyr61 Exon 2 Forward	TGCTGTAAGGTCTGCGCAA	PCR and qPCR
Cyr61 Exon 3 Reverse	ACAGGGTCTGCCCTCTGACTGA	PCR and qPCR
Cyr61 Exon 4 Reverse	TTCAGGCTGCTGTACTGG	PCR

rCyr61 cleavage and inhibition assays: A total of 1.25 µg/ml (31.6 nM) rCyr61 was added to high glucose, high glutamine DMEM/serum-free medium or aliquots of non-concentrated conditioned media from the fibroblasts and myofibroblasts cultured in either the presence or absence of 1.0 U/ml thrombin for 3 h. These mixtures were further incubated in the presence or absence of an additional 1.0 U/ml of thrombin and in the presence or absence of proteinase inhibitors for 2 h at 37 °C. The final concentrations of protease inhibitors were 4.4 AT-U/ml hirudin to inhibit thrombin activity, 100 µM leupeptin to inhibit arginine/lysine specific serine and cysteine proteases, 0.3 µM aprotinin to inhibit some serine proteases but not thrombin, or 10 µM E-64 to inhibit cysteine and some trypsin-like serine proteases. Reactions were stopped by the direct addition of 6X reduced sample buffer and boiled. Equivalent volumes of each non-concentrated sample were loaded on SDS-PAGE gels, transblotted, and probed for Cyr61 on western blots using N-terminal (N-16), central linker region (H-78), or C-terminal (H-2) antibodies.

Data analysis: Band densities of Cyr61 and GAPDH were calculated with Image Lab™ software (BioRad) or ImageJ 1.51j8 [52,53], using equivalent areas and the local subtraction method to control for variation within the blot. Each band was normalized to the average band density of the total blot to control for differences in exposure between blots. The Cyr61 band density was normalized to the GAPDH band density or total cell protein. Transcript levels of Cyr61 were normalized to GAPDH using MxPro ver. 3.20 software (Stratagene). Statistical analysis was performed using SigmaPlot versions 12.25–13.0 (Systat Software Inc., San Jose, CA). For one-variable experiments, p values were calculated using the Student *t* test, one-way ANOVA, and one-way ANOVA on ranks for skewed sample distributions, while p values from two-variable experiments were calculated using the two-way ANOVA. Post-hoc analyses include Dunnett's test and Tukey multiple pairwise comparison test for one- and two-variable analyses. Results are expressed as means ± standard error of mean (SEM). The p values of <0.05 were considered to be statistically significant.

RESULTS

Thrombin increases the expression of cell-associated Cyr61 in corneal stromal fibroblasts and myofibroblasts: Cyr61 is expressed in many non-ocular tissues [22-26,30,32-40,54] and some ocular tissues, including the retina during development, trabecular meshwork, iris, and ciliary smooth muscle [38,55,56]. To determine whether the wounded phenotype human corneal stromal cells synthesize Cyr61 and whether thrombin treatment increases Cyr61 at the transcript and

protein levels, human corneal fibroblasts and myofibroblasts were treated both without and with 1.0 U/ml thrombin. The transcripts were analyzed for the presence of the full-length Cyr61 message using unique primers located in exons 1 and 5 (Figure 1A, Table 1). Incubation of cells in the presence of thrombin increased the mRNA product, and the products were observed at about the expected size of 1,185 bp. To quantify this increase more accurately, real-time PCR was used with specific primers in exons 2 and 3 (Table 1). Thrombin increased Cyr61 transcripts approximately threefold, relative to untreated controls in both fibroblasts and myofibroblasts (Figure 1B,C).

To determine whether the Cyr61 protein is present and to evaluate whether thrombin also increases the Cyr61 expression at the protein level, human corneal stromal fibroblasts and myofibroblasts were treated with thrombin (1.0 U/ml) or additional medium from 1-7 h, and the supernates of cell lysates were probed with an antibody specific to the central linker region of Cyr61 (Figure 1D). Thrombin treatment increased the 40 kDa cell-associated Cyr61 protein level two- to threefold in comparison to untreated controls at all time points in both cell types with a peak at 3 h (Figure 1E,F). For most subsequent cell experiments, 3 h of thrombin stimulation were used. The observed protein was about 40 kDa, close to the calculated expected size of 39.5 kDa for full-length Cyr61. To further assess the Cyr61 protein expression in response to thrombin stimulation, the fibroblast and myofibroblast cultures were treated with 0.01 to 10 U/ml thrombin or additional medium for 3 h, and the cell lysate supernates were probed with the central linker region Cyr61 antibody (Figure 1G,H). Thrombin treatment at a concentration of 1.0 U/ml increased Cyr61 protein levels about threefold in fibroblasts and myofibroblasts. At 10.0 U/ml of thrombin, the level of cell-associated Cyr61 decreased, relative to cells stimulated with 1.0 U/ml thrombin, to levels similar to those of untreated cells. Based on the fact that fibrin is deposited in the injured cornea [2,3] and that the level of thrombin present must be at least 10 nM (approximately 1.2 U/ml) to form a visible clot [7], at least 1 U/ml (approximately 9 nM) thrombin is expected to be present in the cornea upon wounding. This level is also consistent with the amount of inactive prothrombin present in the stroma (325 ± 35 ng/mg protein or about 54 nM) [1]. Therefore, most cell-based experiments were performed with a thrombin concentration of 1.0 U/ml.

Thrombin activity is required to increase the synthesis of Cyr61: Since inactive thrombin and thrombin peptides can alter some cellular activities independently of proteolytic activity [15-19], human corneal fibroblasts and myofibroblasts were treated with the allosteric thrombin-specific

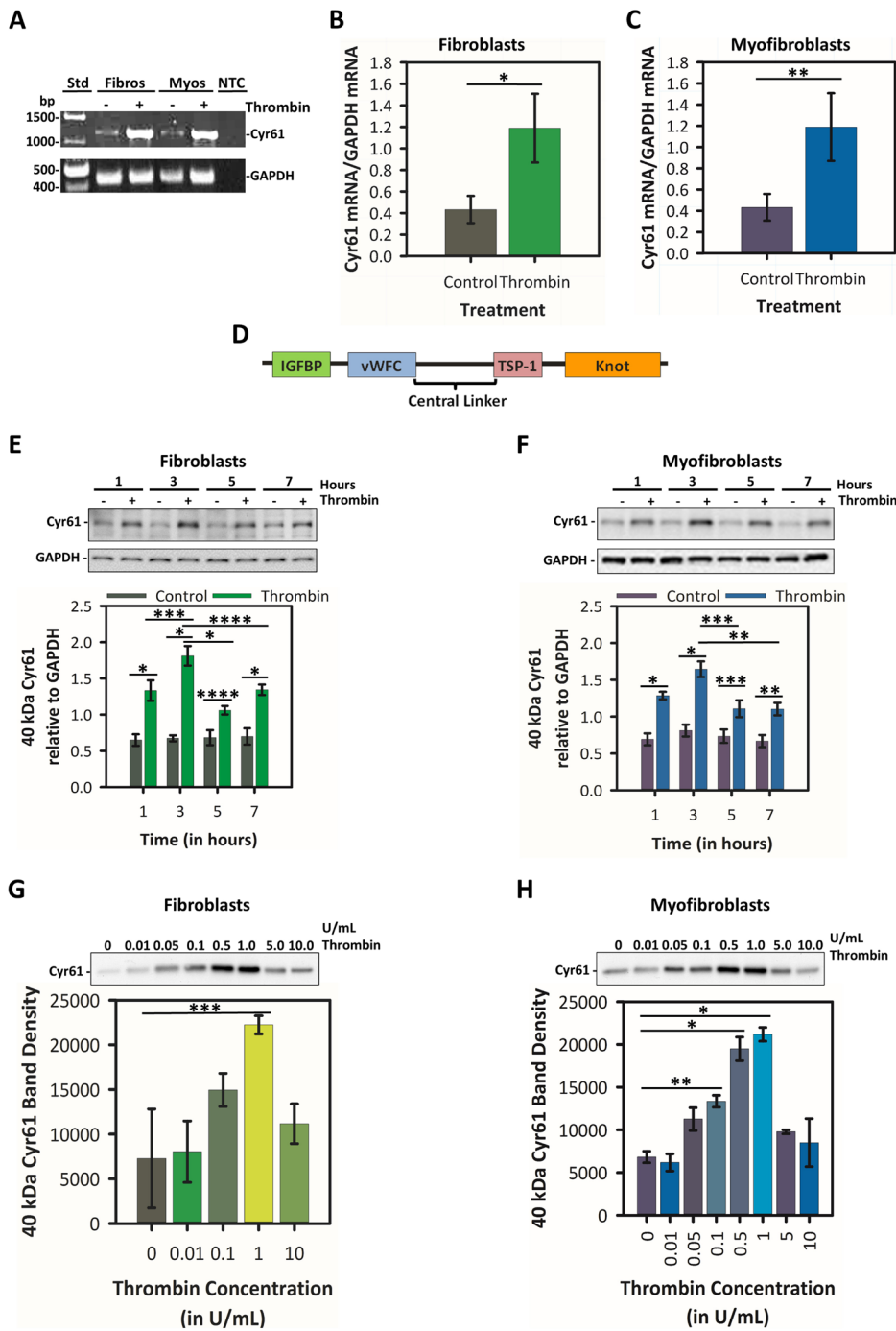


Figure 1. Thrombin increases synthesis of Cyr61 by human corneal stromal fibroblasts and myofibroblasts. **A-C**: Human corneal fibroblasts (Fibros) and myofibroblasts (Myos) were incubated with a serum-free medium alone (-) or a serum-free medium containing 1.0 U/ml thrombin (+) for 60 min. Alterations in the Cyr61 mRNA levels were detected using agarose gels (**A**) to visualize the reverse transcriptase-PCR (RT-PCR) products of primers in exons 1 and 5 (Table 1) and were quantified by real-time PCR using primers in exons 2 and 3 (Table 1). GAPDH was used for the normalization. Controls were negative. For both types of experiments, n = 4 using cells from individual donors. A representative gel is given in **A**. The real-time PCR data (**B, C**) were analyzed with a one-tailed Student *t* test. Fibroblasts: $t = -2.271$, $df = 14$, $*p = 0.020$, $n = 8$; myofibroblasts: $t = -2.205$, $df = 10$, $**p = 0.026$, $n = 6$. Error bars represent standard error of means (SEM). **D**: Location of the epitope for the central linker region-specific Cyr61 polyclonal antibody used in **E-H**. **E-H**: Fibroblasts (**E, G**) and myofibroblasts (**F, H**) were incubated in a serum-free medium from 1-7 h with (+) or without (-) thrombin (1.0 U/ml) for **E** and **F**. For **G** and **H**, the cells were incubated in a serum-free medium (Control) or thrombin (0.01 to 10 U/ml) for 3 h. Cell lysates were collected, processed, and evaluated for Cyr61 via western blot analysis. GAPDH was used as the loading control in **E** and **F** and the modified Bradford Coomassie assay was

used for **G** and **H**. These methods gave equivalent results. Representative blots are shown with corresponding mean band densities from independent experiments (**E, F**: n = 5 and **G, H**: n = 3) of different cell donors. **E, F**: The two-way ANOVA for Time: $F = 4.872$, $df = 3, 32$, $p = 0.007$ for fibroblasts, and $F = 6.042$, $df = 3, 32$, $p = 0.002$ for fibroblasts; for Treatment: $F = 102.569$, $df = 1, 32$, $p < 0.001$ for fibroblasts and $F = 79.511$, $df = 1, 32$, $p < 0.001$, $n = 5$ for myofibroblasts, and the Time and Treatment Interaction factor for fibroblasts: $F = 5.051$, $df = 3, 32$, $p = 0.006$, $n = 5$ and for myofibroblasts: $F = 2.658$, $df = 3, 32$, $p = 0.065$, $n = 5$. Significant Tukey multiple pairwise comparisons are $*p < 0.001$, $**p < 0.005$, $***p < 0.010$, $****p = 0.011$. Error bars displayed are SEM. **G, H**: One-way ANOVA for fibroblasts: $F = 3.605$, $df = 4, 10$, $p = 0.046$, $n = 3$ and for myofibroblasts: $F = 17.669$, $df = 7, 16$, $p < 0.001$, $n = 3$. Significant Dunnett's multiple pairwise comparisons are $*p < 0.001$, $**p = 0.017$, $***p = 0.026$. Error bars displayed are SEM.

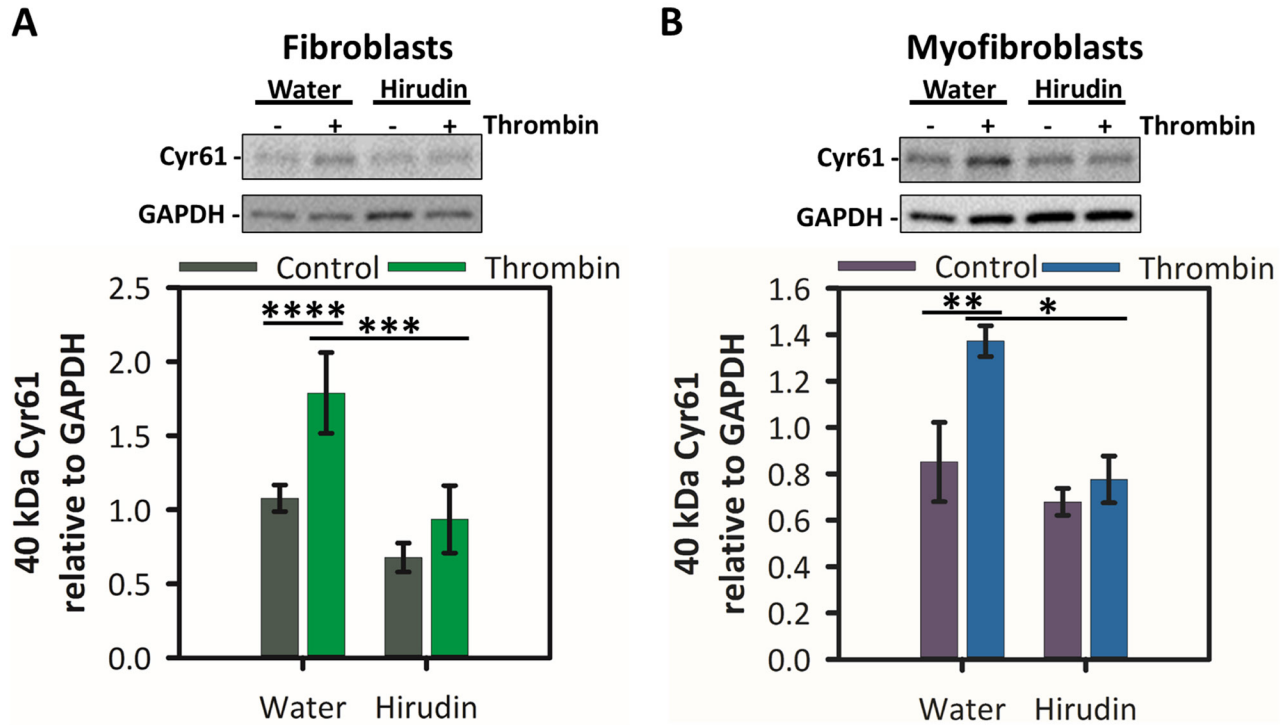


Figure 2. The proteolytic activity of thrombin is required for increasing the Cyr61 protein synthesis in human corneal stromal fibroblasts and myofibroblasts. **A** and **B**: Fibroblasts (**A**) and myofibroblasts (**B**) were treated with a serum-free medium alone (-), a medium with 1.0 U/ml thrombin (+), a medium with 4.4 AT-U/ml hirudin, or a medium with 1.0 U/ml thrombin + 4.4 AT-U/ml hirudin. Cell lysates were collected, processed, and evaluated via western blot analysis for Cyr61 using the central linker-specific Cyr61 antibody. Representative blots are shown. GAPDH was used for normalization. Intracellular band densities from four independent experiments using different cell donors were analyzed via the two-way ANOVA. For fibroblasts, Hirudin Inhibitor factor: F: 10.973, df: 1, 12, $p = 0.006$; Thrombin factor: F: 6.527, df: 1, 12, $p = 0.025$; Interaction factor: F: 1.423, df: 1, 12, $p = 0.256$; $n = 4$. For myofibroblasts, Hirudin Inhibitor factor: F: 12.536, df: 1, 12, $p = 0.004$, $n = 4$; Thrombin factor: F: 8.116, df: 1, 12, $p = 0.015$, $n = 4$; Interaction factor: F: 3.816, df: 1, 12, $p = 0.074$; $n = 4$. Statistically significant comparisons and corresponding p values for the Tukey multiple comparison tests are denoted in the figure as * $p = 0.002$, ** $p = 0.005$, *** $p = 0.008$, **** $p = 0.021$, given above the bars. Error bars displayed are SEM.

inhibitor hirudin [57]. Hirudin significantly prevented the thrombin-induced increase in cell-associated Cyr61 protein levels in fibroblasts (Figure 2A) and myofibroblasts (Figure 2B). Therefore, the proteolytic activity of thrombin is required for increasing the expression of cell-associated Cyr61.

PAR-1 agonist peptide increases cell-associated Cyr61, but PAR-4 agonist peptide does not: As thrombin regulates protein levels through PAR signaling in other cell types [8-10,14,20,22-26], the role of PAR-1 and PAR-4 signaling in altering Cyr61 protein expression was evaluated in human corneal fibroblasts and myofibroblasts. Cell cultures were treated for 1 h with 111 μ M PAR-1 and -4 specific peptide agonists and corresponding scrambled peptides in order to control for off-target signaling effects. Similar to thrombin stimulation, the PAR-1 peptide agonist significantly stimulated an increase in the cell-associated Cyr61 protein expression, relative to the corresponding scrambled peptide (Figure

3A,B). The PAR-4 peptide agonist did not significantly alter the cell-associated Cyr61 protein expression with respect to its scrambled peptide in either cell type. Therefore, the results indicate that intracellular Cyr61 protein levels are increased through stimulation of PAR-1 signaling in human corneal fibroblasts and myofibroblasts.

Thrombin stimulation of Cyr61 synthesis is partially inhibited by the PAR-1 antagonist SCH 79797: The potent and selective PAR-1 antagonist SCH 79797 was used to probe the involvement of PAR-1 in the thrombin-induced increase of Cyr61 levels. This antagonist is a peptide mimetic that binds to PAR-1 and prevents the binding of the thrombin-exposed tethered cryptic activator and PAR-1 agonist activity via competitive inhibition [58]. A level of 25 nM SCH 79797 was chosen for fibroblasts and a level of 50 nM SCH 79797 was chosen for myofibroblasts based on initial experiments over a concentration range of 7.5 nM to 1.7 μ M as the best levels

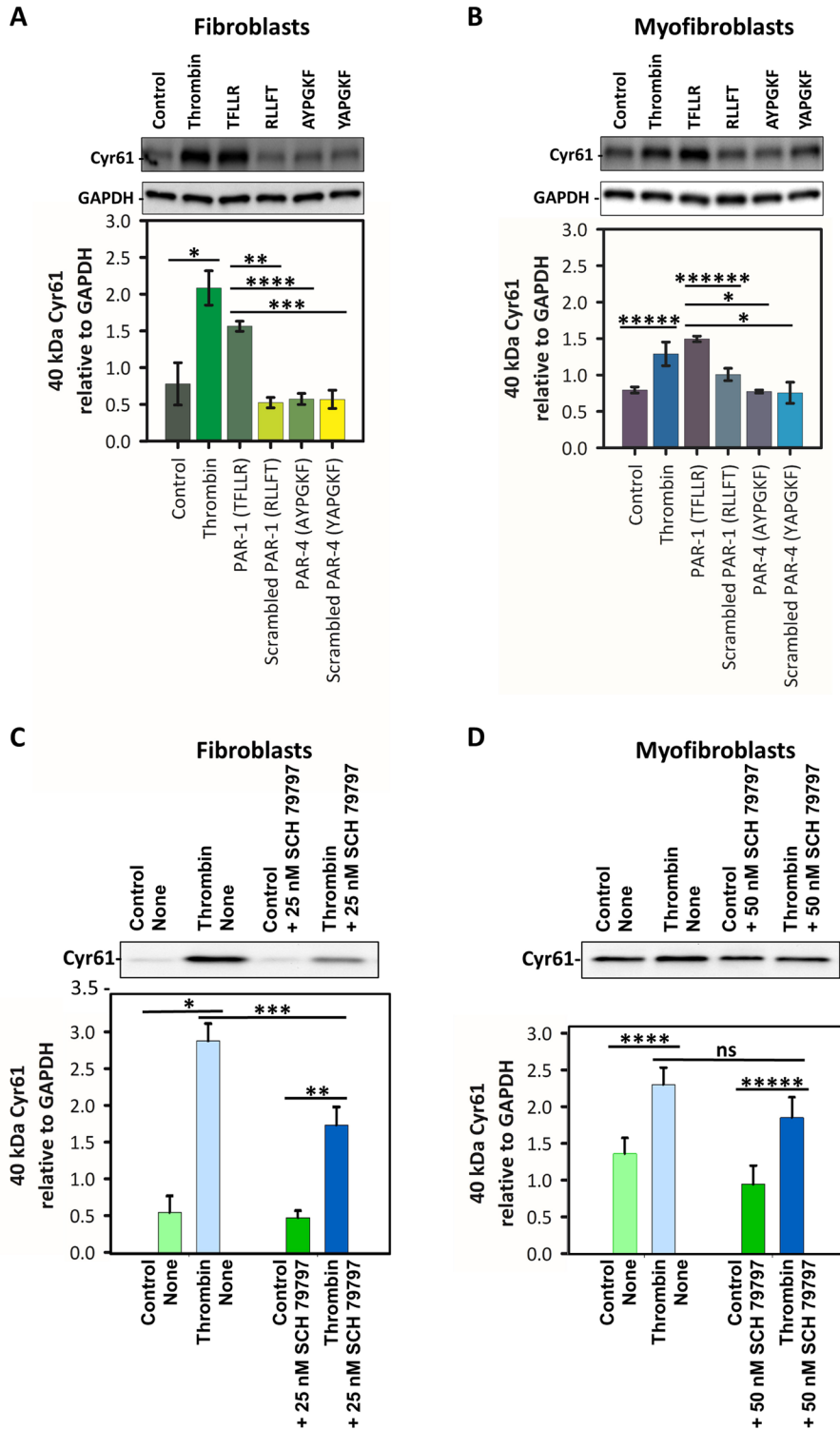


Figure 3. PAR-1 agonist increases the cell-associated Cyr61 protein levels in human corneal stromal fibroblasts and myofibroblasts, and PAR-1 antagonist decreases the thrombin stimulated increase of Cyr61 in human corneal stromal fibroblasts. **A** and **B**: Fibroblasts (**A**) and myofibroblasts (**B**) were treated with control (a serum-free medium alone), thrombin (1.0 U/ml as a positive control), 111 μ M PAR-1 or PAR-4 agonist peptides (TFLLR and AYPGKF, respectively), or 111 μ M scrambled PAR-1 or PAR-4 agonist peptides (RLLFT and YAPGKF, respectively) for 1 h. Cell lysates were collected and evaluated via western blot analysis for Cyr61 using a specific antibody to the central linker region of Cyr61 (Figure 1D); representative blots are shown. GAPDH normalized Cyr61 band densities were calculated for fibroblasts and myofibroblasts, respectively, and analyzed using a one-way ANOVA: $F = 15.183$, $df = 5, 12$, $p < 0.001$, $n = 3$ for fibroblasts and $F = 9.928$, $df = 5, 12$, $p < 0.001$, $n = 3$ for myofibroblasts. Tukey pairwise multiple comparisons are denoted as * $p = 0.002$, ** $p = 0.009$, *** $p = 0.012$, **** $p = 0.013$, ***** $p = 0.030$, ***** $p = 0.039$; $n = 3$ independent experiments using different cell donors. Error bars displayed are SEM. **C** and **D**: Fibroblasts (**C**) and myofibroblasts (**D**) were treated with a serum-free medium alone (Control, None), a medium containing 1.0 U/ml thrombin (Thrombin, None), a medium containing a PAR-1 antagonist, SCH 79797, at 25 nM for fibroblasts (**C**, Control + 25 nM SCH 79797) and 50 nM SCH 79797 for myofibroblasts (**D**, Control + 50 nM SCH 79797), or a medium containing 1.0 U/ml thrombin and 25 nM SCH 79797 (**C**, Thrombin + 25 nM SCH 79797) for fibroblasts and 50 nM SCH 79797 for

myofibroblasts (**D**, Thrombin + 50 nM SCH 79797) for 3 h. Representative blots are shown. Protein normalized Cyr61 band densities were calculated and analyzed. For fibroblasts, the two-way ANOVA results are: Treatment: $F = 75.540$, $df = 1, 8$, $p < 0.001$; Inhibitor: $F = 8.650$, $df = 1, 8$, $p = 0.019$; Interaction between Treatment and Inhibitor: $F = 6.733$, $df = 1, 8$, $p = 0.032$. For myofibroblasts, the two-way ANOVA results are: Treatment: $F = 6.280$, $df = 1, 8$, $p = 0.037$; Inhibitor: $F = 0.00104$, $df = 1, 8$, $p = 0.975$; Interaction between Treatment and Inhibitor: $F = 0.0185$, $df = 1, 8$, $p = 0.895$. Significant Tukey pairwise multiple comparison are denoted as * $p < 0.001$, ** $p = 0.003$, *** $p = 0.005$, **** $p = 0.026$, ***** $p = 0.030$ and non-significant as ns; $n = 3$ independent experiments using different cell donors. Error bars displayed are SEM.

for the inhibition of the thrombin increase of intracellular Cyr61 (data not shown). For both fibroblasts and myofibroblasts pretreated with either the medium alone or with the SCH 79797 antagonist, the addition of thrombin resulted in a significant increase in intracellular Cyr61 (Figure 3C,D) in comparison to untreated cells, indicating that SCH 79797 did not totally inhibit thrombin stimulation of Cyr61. However, further analysis of Cyr61 protein expression indicated that the antagonist inhibited the thrombin stimulated increase of Cyr61 by about 40% in fibroblasts and about 19% in myofibroblasts. In fibroblasts, the inhibition was statistically significant but did not reach significance for myofibroblasts. The smaller amount of SCH 79797 inhibition of myofibroblast Cyr61 synthesis, relative to that of fibroblasts (Figure 3C versus Figure 3D), may be related to the observed three-fold increase in fibroblast Cyr61 synthesis versus the 0.5 fold increase in myofibroblast Cyr61 synthesis in the presence of the PAR-1 agonist to increase Cyr61 protein expression relative to that for the scrambled peptide (Figure 3A versus Figure 3B). These results suggest that thrombin activation of PAR-1 increases Cyr61 to a greater extent in fibroblasts than in myofibroblasts and that thrombin increases Cyr61 through mechanisms other than cleavage and activation of PAR-1.

In the presence of thrombin, extracellular Cyr61 is detected mainly in the 24 kDa form using an antibody to the central linker region: Because Cyr61 is a secreted matricellular protein, conditioned media from the control and thrombin-treated human corneal fibroblasts and myofibroblasts were also investigated via western blot analysis using an antibody to the central linker region of Cyr61. In both cell types, Cyr61 accumulated over time in the conditioned media from cells treated with 1.0 U/ml thrombin at a molecular weight form of 24 kDa, as detected by the central linker region-specific Cyr61 antibody (Figure 4A,B). This band was lower in molecular weight than what was observed for cell-associated Cyr61 (Figure 4C,D, lower panels). A 40 kDa form of extracellular Cyr61 is visible without the addition of thrombin and at low thrombin concentrations in the conditioned media of some donors (Figure 4C,D, upper panels versus 4A,B). With increasing amounts of thrombin, the 40 kDa secreted form of Cyr61 decreased while the 24 kDa form increased (Figure 4C,D). Substantial amounts of the 24 kDa form of Cyr61 were observed at thrombin levels of 0.5 U/ml and above. The 24 kDa form of Cyr61 was not detected in cellular extracts; only the 40 kDa form was observed. These results suggest that, at higher thrombin concentrations, more extracellular Cyr61 is present in the 24 kDa form than in the 40 kDa form.

The 24 kDa extracellular Cyr61 form generated in the presence of thrombin is not produced by alternative splicing:

The appearance of lower molecular weight forms of a protein and Cyr61, in particular, can be caused by two processes: 1) the alternative splicing of the Cyr61 mRNA [40,48], which generates a transcript that codes for the 24 kDa form that does not accumulate in the cells but is immediately released, or 2) the proteolysis [38,41-46] of the 40 kDa full-length form of Cyr61. If thrombin induces alternative splicing of mRNA to produce the 24 kDa extracellular form of Cyr61, not only would the full-length Cyr61 mRNA be synthesized in the presence of thrombin but shorter forms would be synthesized as well. Transcripts from control and thrombin-treated fibroblasts and myofibroblasts were further analyzed using a series of exon-exon bridging primer sets over the entire transcript length of Cyr61 in order to detect the splicing isoforms of Cyr61 produced in the presence of thrombin (Table 1, Figure 5C). Identically sized products were observed for the mRNA of both the control and thrombin-treated fibroblasts and myofibroblasts with all primer sets and with both cell types; all PCR products were of the predicted size for the native transcript (Figure 5). No additional bands were generated over a 50 to 900 nM range of primers at different ratios for the primers given in Figure 5A,B (data not shown). Thus, thrombin stimulation of fibroblasts and myofibroblasts does not appear to induce alternative splicing of the Cyr61 mRNA.

The 24 kDa extracellular Cyr61 form generated in the presence of thrombin is produced by a leupeptin-sensitive protease: If the 24 kDa form of extracellular Cyr61 detected by the central linker region-specific antibody is the product of proteolysis, then multiple distinct Cyr61 peptides should be detected in the conditioned media of thrombin-treated cells using antibodies specific to the N-terminal, central linker, and C-terminal regions of human Cyr61. Multiple peptides were observed in the conditioned media of thrombin-treated cells with similar Cyr61 fragmentation patterns for stromal fibroblasts and myofibroblasts (Figure 6A-F). Molecular weight analysis of the Cyr61 protein forms that were detected with the N-terminal-, central linker-, and C-terminal-specific antibodies indicate several possible cleavage sites within Cyr61. Both the N-terminal and central linker antibodies detected a band at 24 kDa (Figure 6A,B,D,E), which suggests that the detected protein spans the N-terminal region to the central linker region (Figure 6G,H). In contrast, the C-terminal antibody (Figure 6I) did not detect a 24 kDa band; instead, the C-terminal antibody detected fragments at 16 kDa and 13 kDa (Figure 6C,F). Based on the detected molecular weight forms of Cyr61 and the epitope location of each antibody, the products could be formed from an initial cleavage site near the TSP-1 domain to generate a 24 kDa peptide that includes the N-terminal end, the IGF1R domain, the vWFC domain, and the entire central linker region with a corresponding 16 kDa

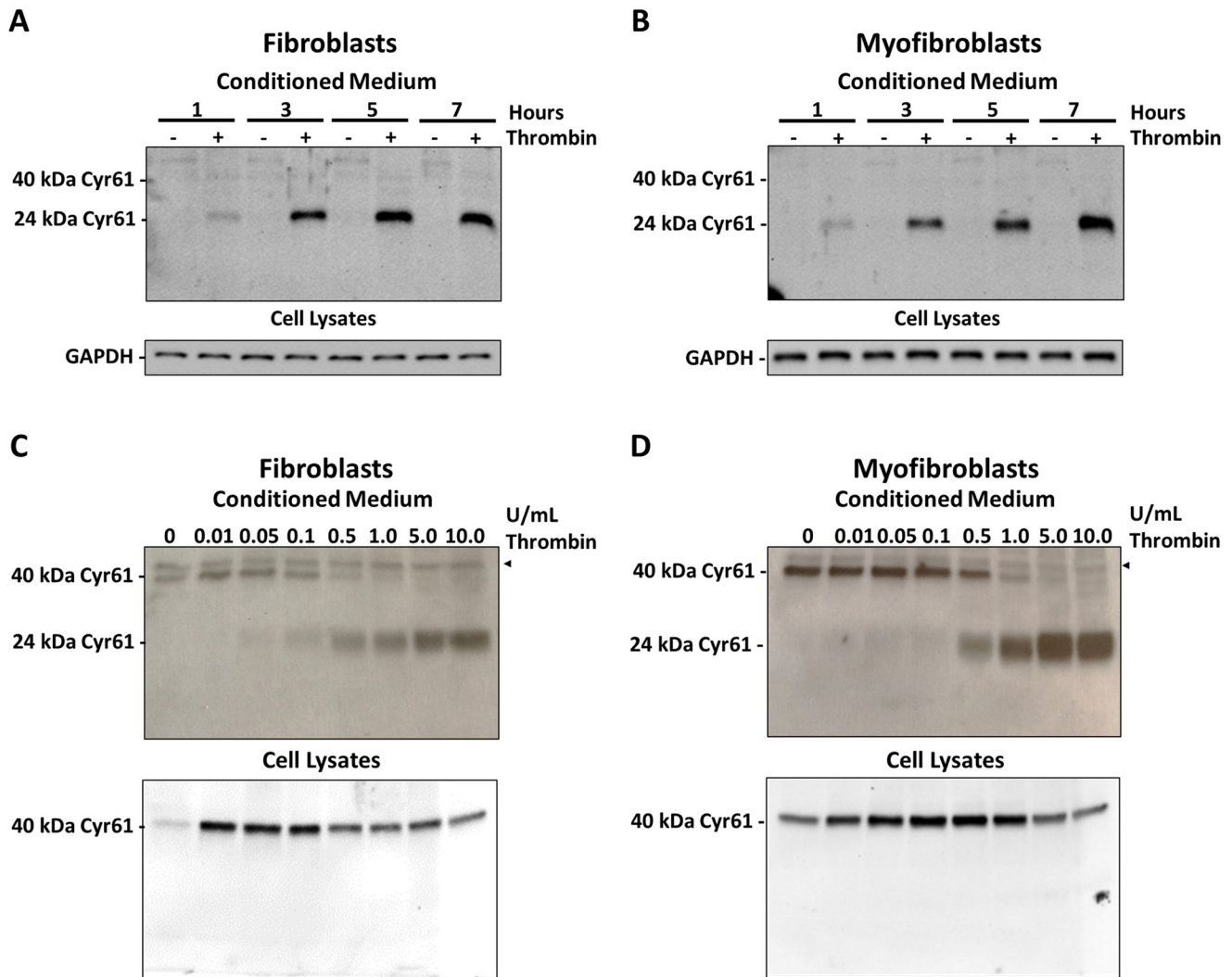


Figure 4. Thrombin induces a molecular weight shift in extracellular Cyr61 at all time points and with increasing concentrations of thrombin in human corneal stromal fibroblasts and myofibroblasts. Fibroblasts (A, C) and myofibroblasts (B, D) were treated with a serum-free medium alone (-) or containing 1.0 U/ml thrombin (+) from 1-7 h (A, B), or with 0 to 10.0 U/ml thrombin for 3 h (C, D). Conditioned media and cell lysates were collected, processed, and evaluated via western blot analysis for Cyr61 using an antibody against the central linker region of Cyr61 (Figure 1D). Samples were normalized to GAPDH (A, B) or total cell protein (C, D). Representative paired blots are shown. Arrowheads denote non-specific bands detected on secondary antibody only blots; n = 2 independent experiments with different cell donors.

peptide that contains the TSP-1 domain and the entire knot domain (Figure 6G-I). The smaller 13 kDa C-terminal band is probably derived from the 16 kDa C-terminal peptide (Figure 6C,F,I). Low levels of the 24 kDa Cyr61 cleavage product generated in the absence of added thrombin were observed for some donor cells with the central linker antibody (Figure 6B,E, Figure 4C,D) but were not seen with other donor cells (Figure 4A,B). In addition, low levels of the 16 kDa Cyr61 cleavage product were observed with the C-terminal antibody (Figure 6C,F). This suggests that the conditioned media from corneal fibroblasts and myofibroblasts generated

in the absence of added thrombin can, for some donors, contain an active protease that cleaves Cyr61. In addition to Cyr61 fragments, a doublet of bands at 40 and 42 kDa were observed with the C-terminal antibody (Figure 6C,F). The higher molecular weight band of Cyr61 observed with the C-terminal antibody may be a post-translationally modified form of Cyr61 to which the other two antibodies cannot bind while the lower band is the full-length Cyr61 form.

To explore the properties of the Cyr61-cleaving enzyme, the stromal fibroblasts and myofibroblasts were treated with a panel of cell compatible protease inhibitors in the

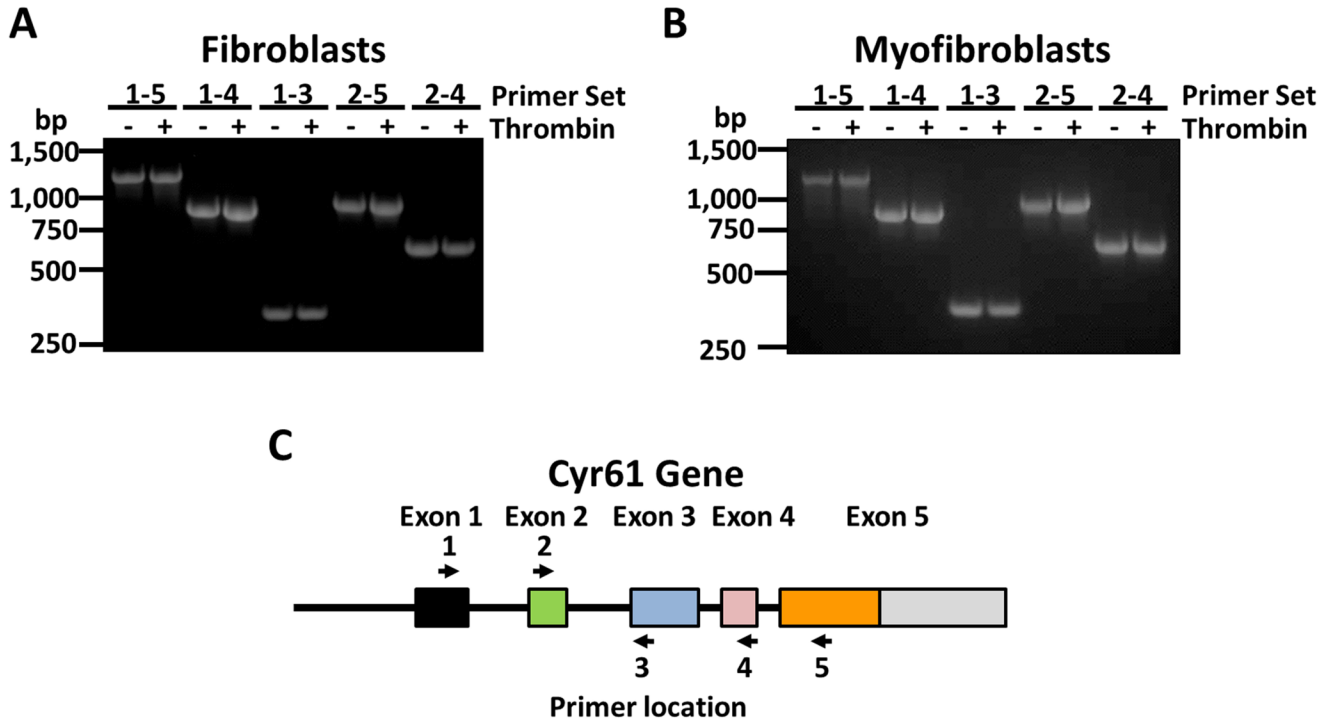


Figure 5. Thrombin stimulation of human corneal stromal fibroblasts and myofibroblasts does not induce alternative splicing of the Cyr61 transcripts to produce the 24 kDa form of Cyr61. **A** and **B**: Fibroblasts (**A**) and myofibroblasts (**B**) in a serum-free medium were treated with (+) or without (-) 1.0 U/ml thrombin for 60 min. Total transcripts were isolated from the cells and PCR amplified using exon-exon bridging primer sets across the entire transcript length of Cyr61 (PCR program optimized for the full-length transcript) and were electrophoresed in 1.5% agarose gels with ethidium bromide. Samples were loaded for detection of PCR products rather than normalized for GAPDH. Representative gels are shown; n = 3 independent experiments with different cell donors. **C**: Diagram of human Cyr61 gene and locations of each primer set listed in Table 1. Black box = 5' untranslated region, Green box = IGFBP domain, Blue box = vWFC, Pink box = TSP-1, Orange box = Knot, Gray box = 3' untranslated region. Expected size of PCR products: 1-5 = 1,185 bp, 1-4 = 902 bp, 1-3 = 360 bp, 2-5 = 972 bp, and 2-4 = 689 bp.

presence and absence of thrombin stimulation (Figure 7). In both cell types, the generation of the 24 kDa Cyr61 product decreased in the presence of the thrombin-specific inhibitor hirudin (Figure 7A,D, top blots) and the serine/cysteine protease inhibitor leupeptin with an arginine/lysine cleavage specificity (Figure 7B,E, top blots). However, the appearance of the 24 kDa form of Cyr61 was not inhibited by aprotinin (Figure 7A,D, top blots). This protein inhibits many serine proteases but does not inhibit thrombin. Neither was the generation of this 24 kDa form inhibited by E-64, which inhibits most cysteine proteases (Figure 7A,D, top blots), chymostatin, which inhibits chymotrypsin-like serine proteases and some cysteine proteases, marimastat, which inhibits metalloproteinases, or pepstatin A, which inhibits acid aspartic proteases (Figure 7C,F, top blots).

When added to cell cultures, hirudin not only inhibited the generation of the 24 kDa extracellular form of Cyr61, but it also inhibited the increase in cell-associated 40 kDa Cyr61

in both cell types (Figure 2A,B; Figure 7A,D). This decrease in the 24 kDa form of Cyr61 in the presence of hirudin is probably primarily due to the decreased synthesis of the full-length Cyr61 (Figure 2A,B and 7A,D). However, this does not rule out the possibility that active thrombin may also be important for the proteolysis of the 40 kDa Cyr61 form.

Thrombin does not directly cleave rCyr61: Thrombin may directly cleave Cyr61 or activate an extracellular zymogen form of a protease that cleaves Cyr61. Direct cleavage of Cyr61 is a possibility because the primary sequence of Cyr61 contains at least two potential thrombin-specific cleavage sites. These predicted cleavage sites have proline-arginine in P2-P1 sites and two non-charged amino acids in P1'-P2' sites; however, they deviate in the P3 and P4 sites from the hydrophobic amino acids in the thrombin consensus sequence [59,60]. To determine whether thrombin can directly cleave Cyr61, 31.6 nM of rCyr61 was incubated in cell naïve medium both with and without 1.0 U/ml thrombin for 2 h (Figure 8A).

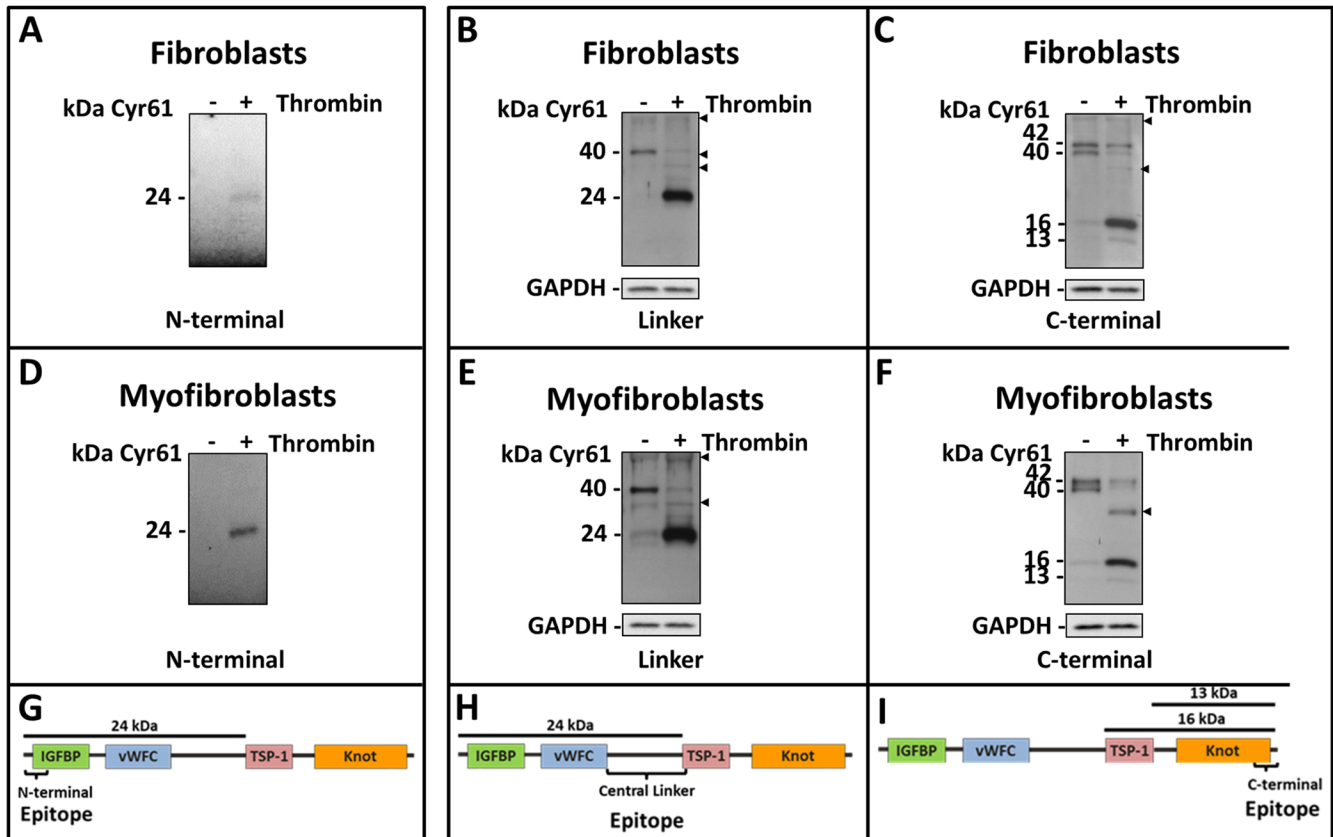


Figure 6. Thrombin stimulation of human corneal stromal fibroblasts and myofibroblasts induces the generation of multiple distinct extracellular N- and C-terminal Cyr61 forms. **A-F**: The conditioned media from fibroblasts and myofibroblasts cultured in serum-free medium alone (-) or 1.0 U/ml thrombin (+) for 24 h (**A, D**) or 3 h (**B, C, E, F**) were collected, processed, concentrated, and subjected to western blot analysis for Cyr61 with antibodies specific to the N-terminal (**A, D**), central linker (**B, E**), and C-terminal (**C, F**) regions of Cyr61 with antibodies specific to the N-terminal (**A, D**), central linker (**B, E**), and C-terminal (**C, F**) regions of Cyr61. Equivalent amounts of total protein were loaded in **A** and **D**, while samples in **B, C, E, F** were normalized based on GAPDH. Arrowheads indicate non-specific bands detected on secondary antibody only blots. Representative blots from two independent experiments using two different donors are shown. **G-I**: Approximate location of cleavage products based on epitope location of the Cyr61 antibodies.

However, no smaller products of Cyr61 were generated, suggesting that thrombin does not directly cleave Cyr61 under our experimental conditions. Similar results were previously reported by Pendurthi et al. [44].

Thrombin activates an extracellular leupeptin-sensitive zymogen that cleaves rCyr61: To address the possibility that thrombin might not only stimulate the synthesis of Cyr61 in fibroblasts and myofibroblasts but also activate a zymogen form of a protease present in the fibroblast and myofibroblast conditioned media, rCyr61 was incubated with conditioned media from cells cultured in the presence and absence of 1.0 U/ml thrombin. For this donor, the conditioned media from unstimulated stromal fibroblasts and myofibroblasts contained very little, if any, of the 24 kDa Cyr61 product when probed with the central linker region Cyr61 antibody

(Figure 8B,C, lane 1). Addition of thrombin to the conditioned media and incubation under the same conditions used in Figure 8A resulted in the appearance of a small amount of the 24 kDa band, most visible for fibroblasts (Figure 8B,C, lane 2). Generation of this band was inhibited when the thrombin-specific inhibitor hirudin was added at the same time as thrombin (Figure 8B,C, lane 4). This suggests that thrombin activates a protease present in an inactive form in the cell conditioned media that is capable of cleaving Cyr61 and generating a 24 kDa peptide.

To further analyze the presence of a thrombin activated protease, the conditioned media from fibroblasts and myofibroblasts treated with 1.0 U/ml thrombin were incubated in both the absence and presence of hirudin and with and without additional thrombin followed by incubation in the

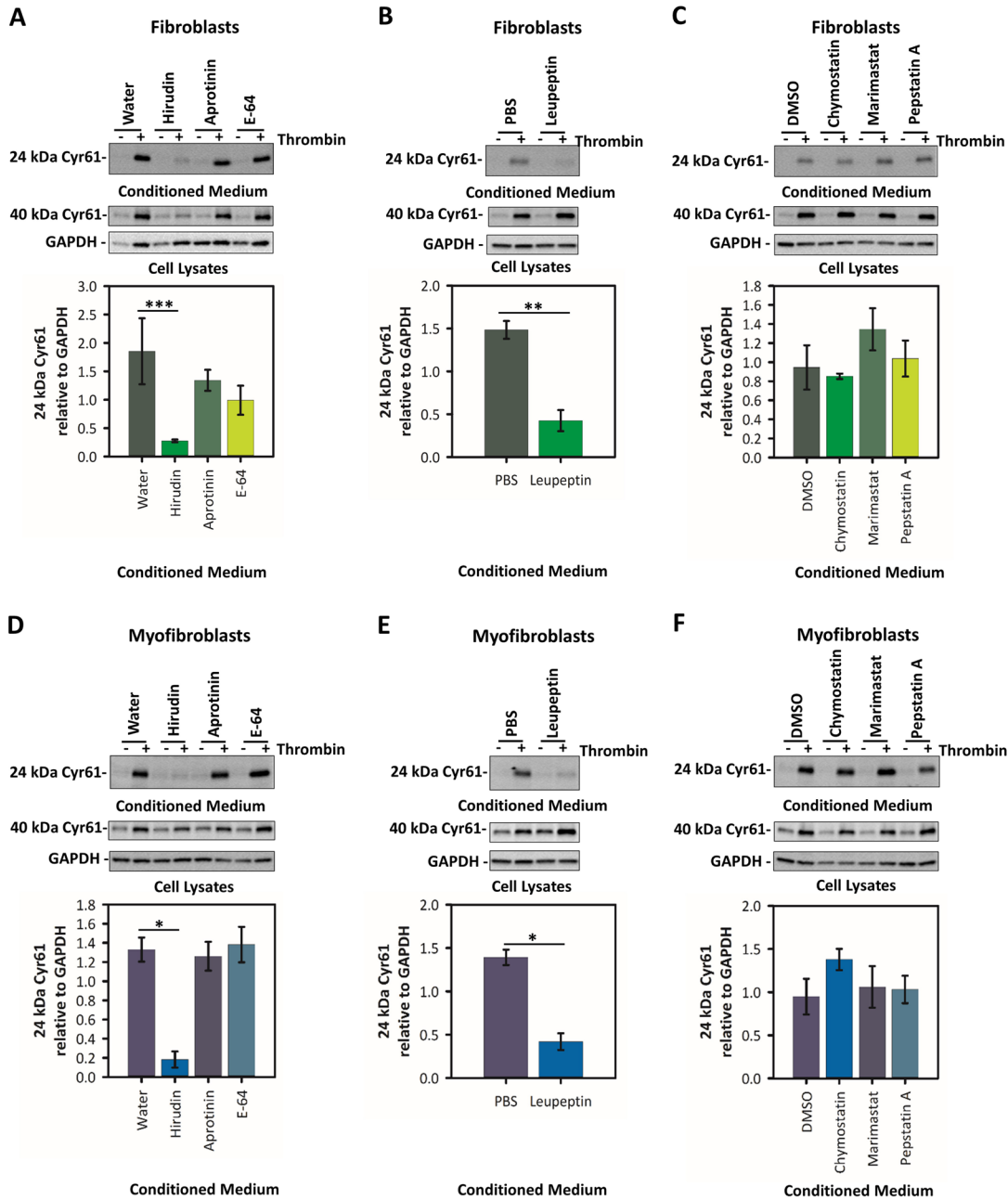


Figure 7. Leupeptin and hirudin inhibit the appearance of the 24 kDa Cyr61 form in the fibroblast and myofibroblast conditioned media. Human corneal stromal fibroblasts (A-C) and myofibroblasts (D-F) were preincubated for at least 5 min with a panel of protease inhibitors (4.4 AT-U/ml hirudin, 0.3 μ M aprotinin, 100 μ M leupeptin, 10 μ M E-64, 100 μ M chymostatin, 10 μ M marimastat, 1 μ M pepstatin A, or vehicle) before the addition of 1.0 U/ml thrombin (+) or additional medium (-). Medium or thrombin (1.0 U/ml) was added and the cells were incubated for an additional 3 h. The conditioned media and lysates were collected, processed, and evaluated via western blot analysis for Cyr61 using an antibody against the central linker region of Cyr61 (Figure 1D). Band densities for the 24 kDa Cyr61 form produced under differing culture conditions were normalized to GAPDH. These values were statistically analyzed using a one-way ANOVA or a one-way ANOVA on ranks (Kruskal–Wallis test) for skewed sample distributions. A one-way analysis was used to compare the effect of the inhibitors in the presence of thrombin because reliable band densities could not be obtained in the absence of thrombin. For fibroblast sample groups: (A) Water soluble inhibitors: F: 10.147, df: 3, $p = 0.017$; $n = 4$; (B) PBS soluble inhibitors: F: 43.452, df: 1, 4, $p = 0.003$; $n = 3$; (C) DMSO soluble inhibitors: F: 1.312, df: 3, 8, $p = 0.336$; $n = 3$. For myofibroblast sample groups: (D) Water soluble inhibitors: F: 16.480, df: 3, 12, $p < 0.001$; $n = 4$; (E) PBS soluble inhibitors: F: 54.801, df: 1, 4, $p = 0.002$; $n = 3$; (F) DMSO soluble inhibitors: F: 1.011, df: 3, 12, $p = 0.422$; $n = 4$. Statistically significant p values per Dunnett’s test are indicated as * $p < 0.001$, ** $p < 0.002$, *** $p < 0.050$ in the figure above the bars. All error bars displayed are SEM. Band densities analyzed were from at least three independent experiments from different cell donors for each inhibitor type. Representative blots are shown.

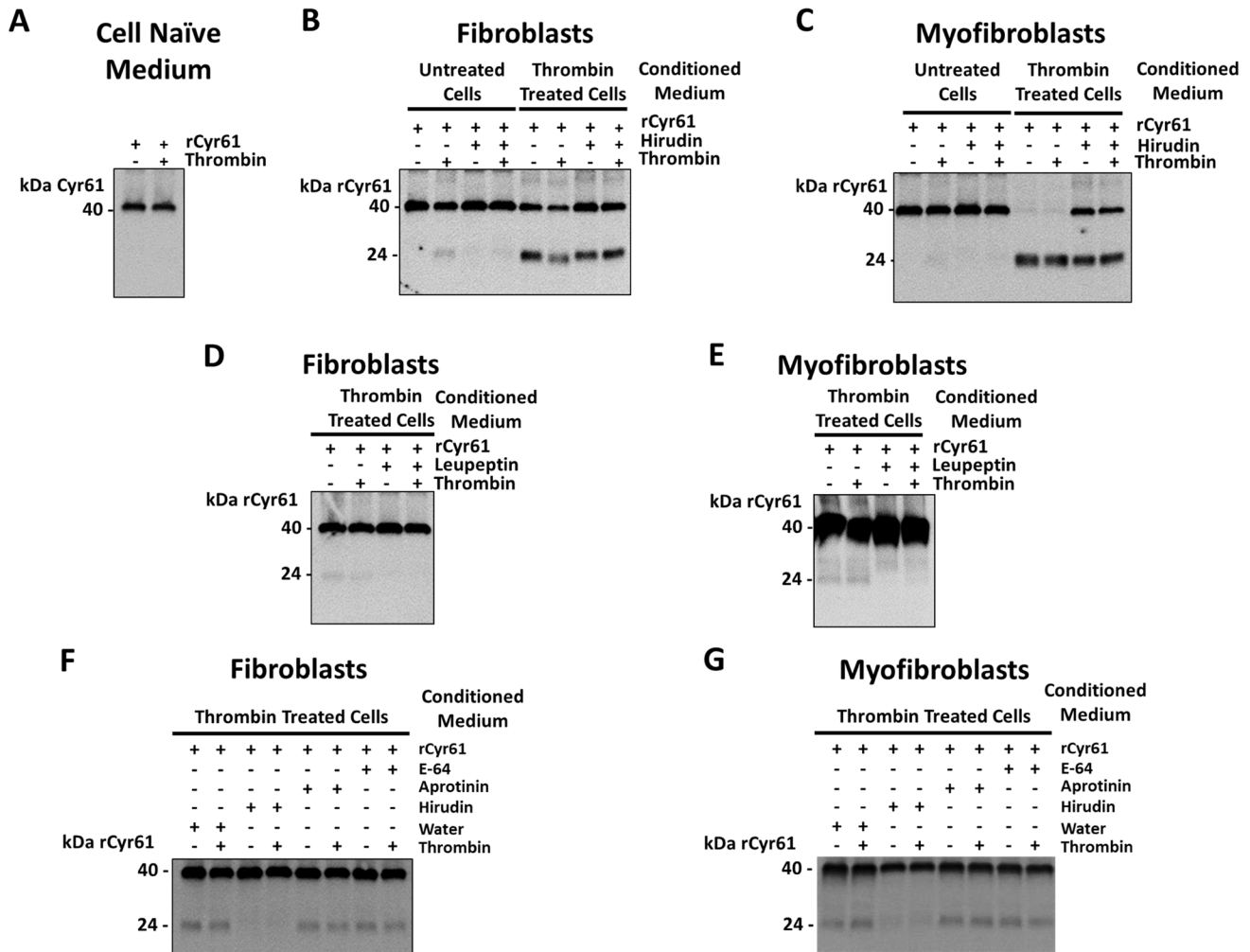


Figure 8. Thrombin does not directly cleave Cyr61 but activates a leupeptin-sensitive Cyr61 cleaving enzyme. **A**: The culture medium (cell naïve) was incubated with recombinant Cyr61 (rCyr61, 31.6 nM) in the presence (+) or absence (-) of thrombin (1.0 U/ml) for 2 h at 37 °C; n = 2 independent experiments. **B** and **C**: The conditioned media from 3 h untreated or 1.0 U/ml thrombin-treated fibroblast (**B**) and myofibroblast (**C**) cultures were incubated with rCyr61 (31.6 nM) in the presence of additional medium, additional thrombin (1.0 U/ml), hirudin (4.4 AT-U/ml), or additional thrombin plus hirudin for 2 h at 37 °C; n = 3 independent experiments. **D** and **E**: The conditioned media from 3 h thrombin-treated fibroblast (**D**) and myofibroblast (**E**) cultures were incubated with rCyr61 (31.6 nM) in the presence of additional medium, additional thrombin (1.0 U/ml), leupeptin (100 µM), or additional thrombin plus leupeptin for 2 h at 37 °C; n = 2 independent experiments. The conditioned media from 3 h thrombin-treated fibroblast (**F**) and myofibroblast (**G**) cultures were incubated with rCyr61 (31.6 nM) in the presence of additional medium, additional thrombin (1.0 U/ml), hirudin (4.4 AT-U/ml), hirudin plus thrombin, aprotinin (0.3 µM), aprotinin plus thrombin, E-64 (10 µM), or E-64 plus thrombin for 2 h at 37 °C; n = 2 independent experiments. **A-G**: The aliquots, without concentration, on western blots were probed for Cyr61 using a specific antibody against the central linker region of Cyr61 (Figure 1D). All conditioned media plus inhibitor experiments were repeated at least twice using the conditioned media from cells of independent donors.

presence of rCyr61 (Figure 8B,C, lanes 5–8). The 24 kDa Cyr61 cleavage product was generated under all conditions, including when the thrombin-specific inhibitor hirudin (4.4 AT-U/ml) was present at a two- or fourfold excess needed to completely inhibit the thrombin present (Figure 8B,C, lanes 5 and 6 versus lanes 7 and 8). This indicates the presence

of an active protease that is distinct from thrombin in the conditioned media from fibroblasts and myofibroblasts cultured in the presence of thrombin. More of the 40 kDa form of rCyr61 was retained during the incubation period in the presence of hirudin, relative to the uninhibited samples (Figure 8B,C, lanes 5 and 6 versus lanes 7 and 8), suggesting

that thrombin, in the absence of hirudin, activates additional zymogen molecules during the incubation period.

The second protease inhibitor to decrease the generation of the extracellular 24 kDa form of Cyr61 in the presence of cells was leupeptin (Figure 7B,E). Since leupeptin inhibits proteases with an arginine/lysine specificity and work by Brass and Shattil indicates that leupeptin at high concentrations inhibits thrombin [61], leupeptin could potentially inhibit thrombin and thus prevent the synthesis or activation of the second protease in a similar manner to that observed for hirudin. If leupeptin significantly inhibits thrombin under the experimental conditions used here, then it should also inhibit the thrombin-stimulated increase in the 40 kDa cell-associated Cyr61, which was observed in thrombin-treated fibroblasts and myofibroblasts. Fibroblasts and myofibroblasts incubated in the presence of thrombin and leupeptin retained the increase in cell-associated 40 kDa Cyr61 (Figure 7B,E, 40 kDa Cyr61). This suggests that leupeptin does not inhibit thrombin activity in the cell culture experiments. To further confirm that thrombin is not inhibited by leupeptin under the experimental conditions used, thrombin alone—or with leupeptin at the same concentrations used for all cellular and conditioned media experiments—was incubated with the thrombin fluorogenic peptide substrate Phe-Pro-Arg-AFC. Three independent experiments resulted in the generation of 564 ± 69 fluorescence units/min in the presence of thrombin alone (1.0 U/ml, approximately 9 nM) and 519 ± 21 fluorescence units/min in the joint presence of thrombin (1.0 U/ml, approximately 9 nM) and leupeptin (100 μ M). This difference was not statistically significant using the Student *t* test, suggesting that leupeptin does not inhibit thrombin activity under the conditions used in the current experiments and that leupeptin inhibits the generation of the 24 kDa Cyr61 peptide through the inhibition of a protease other than thrombin.

To determine whether the conditioned media from thrombin-treated corneal stromal fibroblasts and myofibroblasts contained a leupeptin-sensitive Cyr61 cleaving protease, rCyr61 was incubated with vehicle alone, leupeptin alone, or with leupeptin and supplemental thrombin (1.0 U/ml) added to the cell conditioned media generated in the presence of thrombin. The generation of the 24 kDa Cyr61 product from rCyr61 was inhibited in the presence of leupeptin (Figure 8D,E, lanes 3 and 4) in a similar manner to that observed when leupeptin was added to the fibroblast and myofibroblast cultures (Figure 7B,E, 24 kDa Cyr61). This indicates that leupeptin inhibits the protease that generates the 24 kDa peptide in the stromal fibroblast and myofibroblast conditioned media.

In the absence of cells, E-64 inhibits leupeptin-sensitive cysteine cathepsins [62]. However, because E-64 can stimulate inhibitor-induced feedback in the presence of cells—causing suppression of active cathepsin L and increased active cathepsin S [63]—the leupeptin-sensitive protease in the cell conditioned media was further probed with protease inhibitors. The corneal stromal fibroblast and myofibroblast conditioned media that were generated in the presence of thrombin were incubated with rCyr61 and vehicle (water) or additional thrombin (1.0 U/ml), plus vehicle, hirudin (4.4 AT-U/ml), aprotinin (0.3 μ M), or E-64 (10 μ M; Figure 8F,G). The lack of inhibition by the cysteine protease inhibitor E-64 in the absence of cells (Figure 8F,G, lanes 7 and 8) suggests that the leupeptin-sensitive protease is not a cysteine cathepsin protease. These results suggest thrombin stimulates human corneal stromal fibroblast and myofibroblast synthesis and release of a thrombin-activated, leupeptin-sensitive protease that cleaves Cyr61.

Proteases in the thrombin-treated fibroblast and myofibroblast cell conditioned media generate similar peptides from rCyr61 to those produced by the cells in culture: To explore whether the thrombin-activated protease present in the cell conditioned media generates similar N- and C-terminal Cyr61 fragments to those observed in the presence of cells (Figure 6), aliquots of conditioned media from the 3 h thrombin-treated and untreated cultures of stromal fibroblasts and myofibroblasts were incubated with rCyr61 for an additional 2 h in the presence of 4.4 AT-U/ml hirudin (fourfold excess) in order to inhibit thrombin (Figure 9). The forms of Cyr61 produced were detected without concentration of the media using antibodies specific for the N-terminal, central linker, and C-terminal regions. The observed forms were from rCyr61 because the cell-generated forms of Cyr61 present in the conditioned media were undetectable via western blot analysis without first concentrating the samples (data not shown). The cell conditioned media, produced in the presence of thrombin, generated more Cyr61 cleavage products than the cell conditioned media produced in the absence of thrombin (Figure 9A-F, right lane [+] versus left lane [-] for each blot). Cyr61 peptides produced from rCyr61 by enzymatic components in fibroblast and myofibroblast conditioned media were similar to those generated by endogenously produced Cyr61 (Figure 6A-F versus Figure 9A-F). The 24 kDa N-terminal form and the 16 and 13 kDa C-terminal forms were generated in the cell conditioned media regardless of Cyr61 source (i.e., produced by cells or exogenously added rCyr61). In addition to the 24 kDa N-terminal form, the N-terminal antibody detected a 16 kDa form of Cyr61 produced when rCyr61 was incubated with the conditioned media from thrombin stimulated cells (Figure 9A,D). This varying pattern could be due

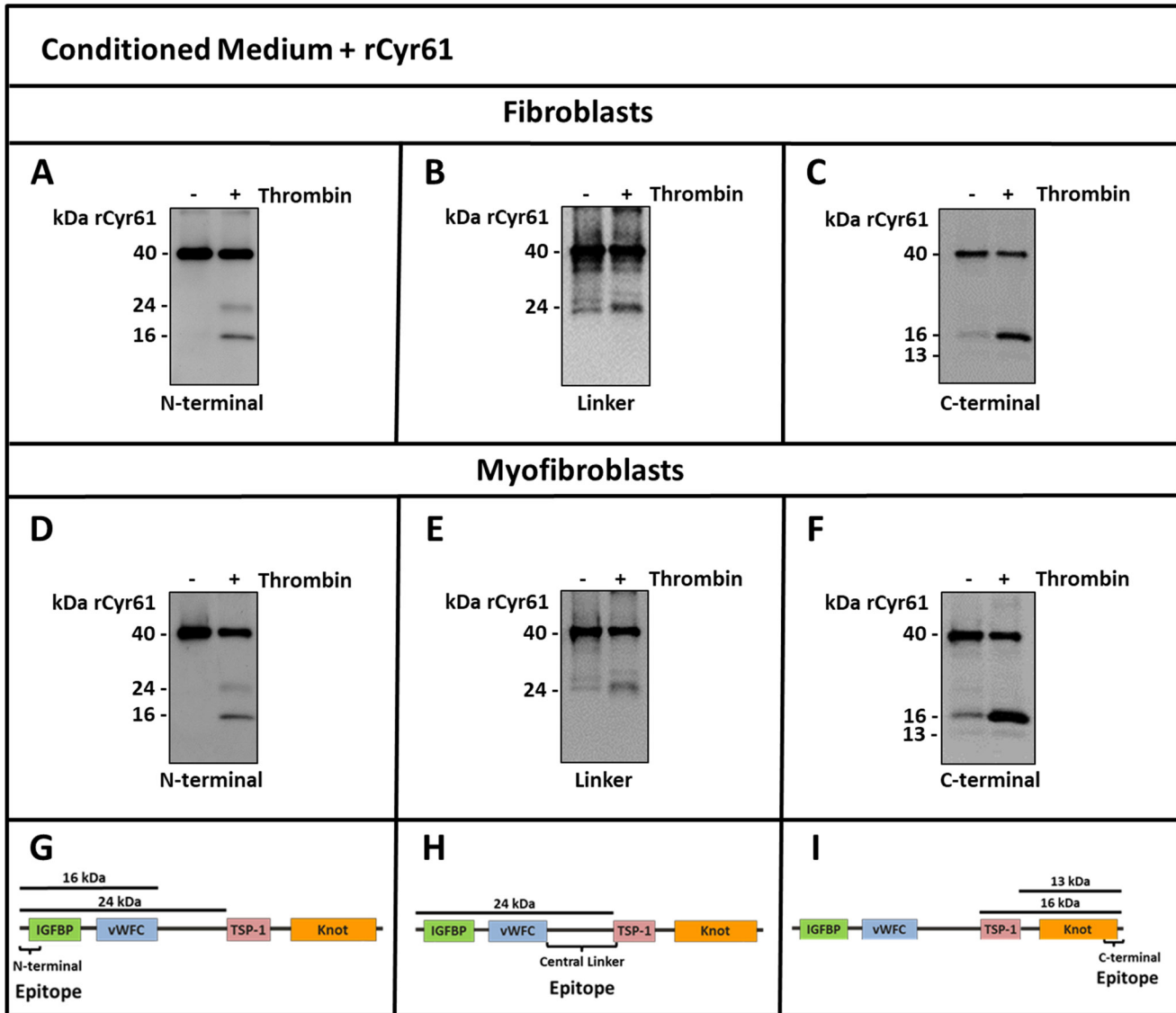


Figure 9. The thrombin conditioned media contain rCyr61 cleavage enzymes. **A-F**: The conditioned media without concentration from 3 h control (-) and 1.0 U/ml thrombin-treated (+) fibroblasts (**A-C**) and myofibroblasts (**D-F**) were incubated with rCyr61 (31.6 nM) for 2 h at 37 °C with 4.4 AT-U/ml hirudin to inhibit thrombin. The aliquots of each reaction were probed for Cyr61 using N-terminal (**A** and **D**), central linker (**B** and **E**), and C-terminal (**C** and **F**) region-specific antibodies. Equivalent reaction volumes were loaded, as the conditioned media were collected from pooled control and thrombin-treated cultures with equivalent cell number seeded per well. Representative blots are shown for one of two experiments using independent donors. **G-I**: Approximate location of cleavage products based on epitope locations of Cyr61 antibodies.

to the further processing of the 24 kDa form in the absence of cells or to the subsequent reuptake of the smaller forms of Cyr61 in the presence of cells. Cell conditioned media components, produced by cells in the absence of added thrombin, were capable of cleaving rCyr61 to form 24 and 16 kDa products similar to those observed in the cell culture of some cell donors (Figure 9B,C,E,F versus Figure 6B,C,E,F). These

results suggest that active components capable of converting full-length Cyr61 to peptides are present in the fibroblast and myofibroblast conditioned media of some donors and that the presence of thrombin during cell culture increases the amount of Cyr61 peptides generated. Furthermore, the continued presence of thrombin activity is not required for rCyr61 cleavage in the conditioned media.

DISCUSSION

Human corneal stromal fibroblasts and myofibroblasts produce ECM components [64-66] and corresponding ECM remodeling enzymes [64,66-69] to heal an injured cornea. Until now, the 38 kDa CTGF was the only member of the cysteine-rich extracellular matrix-associated protein family (CCNs) detected in human corneal stromal fibroblasts and myofibroblasts [70,71]. Here, we report the presence and synthesis of Cyr61, the CTGF homologous protein, by cultured human corneal stromal fibroblasts and myofibroblasts and demonstrate the role of thrombin in regulating Cyr61 expression and processing.

The experiments reported here were performed using 1.0 U/ml (approximately 9 nM concentration equivalent based on varying specific activity by lot number) thrombin, which is a level that is close to the physiologic levels expected to be present in the wounded cornea based on fibrin localization in wounded corneas [2,3], the amount of thrombin required for coagulation [7], and the presence of coagulation pathway enzymes and cofactors in the cornea [1,3,72,73]. The corneal stroma contains the components required for both the intrinsic and extrinsic coagulation pathways in inactive forms, including prothrombin at 325 ± 35 ng/mg protein or about 54 nM [1,72]. Following the photokeratectomy of mouse eyes, fibrin is deposited initially within the stroma near the wound edge and is then diffusely observed throughout the cornea [3]. Initial activation of corneal prothrombin to thrombin required for fibrinogen cleavage probably occurs in the wounded area because the corneal stromal cells undergoing apoptosis can serve as a surface for activation of prothrombin to thrombin [1]. Depending on the type of corneal wound, prothrombin and other coagulation factors from tears [74,75] and aqueous humor [76], in addition to the cornea, may also be present. These sources of prothrombin are more than adequate to supply the 1 nM (0.14 U/ml) thrombin required for initiation of fibrinogen cleavage to generate fibrin peptides A and B and the 10 nM (1.4 U/ml) thrombin required to form a visible fibrin clot [7]. Based on this information, the use of 1.0 U/ml (approximately 9 nM) active thrombin corresponds to a level attainable in the ECM of the human corneal stroma to initiate the conversion of fibrinogen to fibrin within the cornea following injury.

Cyr61 synthesis is stimulated by the PAR-1 agonist, TFLLR, in the corneal stroma fibroblasts and myofibroblasts, suggesting involvement of PAR-1 signaling for the thrombin-induced increase of this protein. However, treatment with the PAR-1 antagonist SCH 79797 significantly inhibited Cyr61 synthesis in fibroblasts but not in myofibroblasts, where the observed decrease did not reach statistical significance. In

both cases, the PAR-1 antagonist did not completely inhibit the thrombin-induced increase in intracellular Cyr61. This suggests that thrombin not only acts through PAR-1 cleavage to increase Cyr61 synthesis in corneal stromal cells but also through other mechanisms, such as thrombin cleavage of other proteins [9,77] or non-catalytic binding to other molecules [14-19], leading to stimulation of Cyr61 synthesis. It is known that thrombin peptides and inhibited thrombin can stimulate colonic myofibroblast synthesis of cyclooxygenase-2 (COX-2) and PGE₂ [14], and that PGE₂ can stimulate Cyr61 synthesis and protein expression [54]. Non-catalytic thrombin peptides increase the IL-1 β release from human peripheral blood mononuclear cells [19], and the IL-1 β levels could then stimulate Cyr61 synthesis and protein expression [54]. Furthermore, thrombin can proteolytically release and activate FGF-2 and TGF- β from the extracellular matrix of bovine corneal endothelial cells [77], and FGF-2 and TGF- β can stimulate Cyr61 synthesis and protein expression [78,79]. Based on these examples of non-PAR-1 mediated mechanisms in non-corneal cells through which thrombin can induce the release, activation, or synthesis of molecules that stimulate Cyr61 synthesis, it is likely that thrombin increases Cyr61 synthesis in human corneal stromal fibroblasts and myofibroblasts through cleavage of PAR-1 and other mechanisms.

In the fibroblast and myofibroblast media, thrombin stimulated Cyr61 was observed in the 24 kDa form detected by the antibody that recognized a portion of the linker region between the vWFC and the TSP-1 domains. In other culture systems, Cyr61 can undergo alternative splicing in response to serum stimulation [40,48]. However, thrombin treatment of cultured corneal stromal fibroblasts and myofibroblasts did not induce alternative splicing of Cyr61 nor did it directly cleave Cyr61. Instead, thrombin activated the zymogen of a leupeptin-sensitive protease present in the conditioned media of fibroblasts and myofibroblasts that cleaved Cyr61 near the TSP-1 domain. This cleavage produced populations of 24 kDa N-terminal and 16 kDa C-terminal extracellular forms of Cyr61, as detected by the N-terminal-, central linker-, and C-terminal-specific antibodies. Although some full-length Cyr61 was observed in the conditioned media from fibroblasts and myofibroblasts cultured in the presence of 0.5 U/ml or greater thrombin, most protein was present in the form of a 24 kDa N-terminal peptide. This peptide is predicted to contain the IGFBP domain, the vWFC domain, and the central linker region with a corresponding 16 kDa C-terminal peptide, which is predicted to contain the TSP-1 and knot domains. However, accurate molecular weight determination of all endogenous populations of extracellular Cyr61 cleavage products is challenging using immunoblot-based methods.

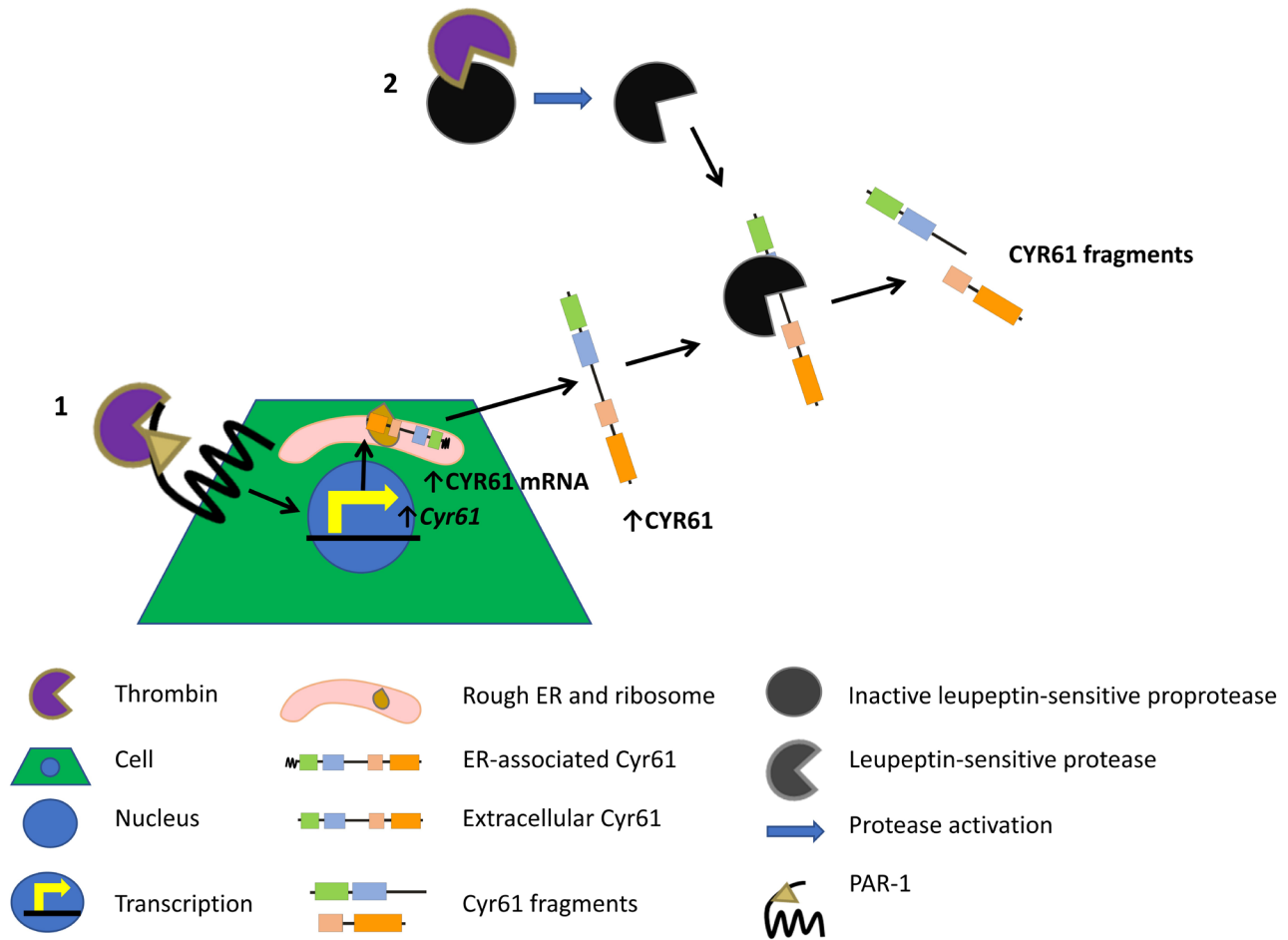


Figure 10. The proposed model for thrombin regulation of the Cyr61 protein in cultured human corneal stromal fibroblasts and myofibroblasts. Thrombin regulates the Cyr61 protein through two mechanisms: 1) Thrombin stimulates stromal fibroblast and myofibroblast Cyr61 synthesis in part through PAR-1, at least for fibroblasts; 2) Thrombin induces, activates, or sequentially induces and activates at least one extracellular leupeptin-sensitive protease capable of cleaving Cyr61.

Initial protease analysis with a panel of inhibitors indicated that a leupeptin-sensitive protease is present in the cell conditioned media produced in the presence of thrombin and that it is capable of cleaving extracellular Cyr61. Although leupeptin does inhibit thrombin at high leupeptin to thrombin molar ratios [61], at the 11:1 ratio used in the present experiments, thrombin is not inhibited by leupeptin as demonstrated by the retention of the ability of thrombin to stimulate Cyr61 synthesis and no significant inhibition in the thrombin assay using a peptide substrate. This lack of inhibition of thrombin by leupeptin under the present conditions additionally supports the presence of a second enzyme, which is leupeptin-sensitive and is a thrombin-induced or activated protease. This thrombin-activated leupeptin-sensitive proteinase may

also activate PAR-1 because multiple proteases can cleave and unmask the N-terminal PAR-1 tethered ligand [13].

Multiple metalloproteinases and serine proteases cleave Cyr61 [38,41-45,47]. Although matrix metalloproteinases (MMP)-1 [47], -2 [38,47], -8 [47], -9 [47] -10 [41], and -14 [38,42,47] are capable of directly cleaving and inducing Cyr61 proteolysis in vitro and are present in the cornea [64,80], MMPs are not targeted by leupeptin, as leupeptin inhibits cysteine and serine proteases with arginine/lysine specificity [81].

Several leupeptin-sensitive serine proteases are synthesized by corneal cells, including kallikrein 14 (KLK-14) [82], plasmin [83,84], urokinase type plasminogen activator (uPA) [67,68,84,85], and tissue type plasminogen activator (tPA)

[67,69]. Leupeptin-sensitive serine proteases known to cleave Cyr61 include plasmin [44] and kallikreins 1, 7, 12, and 14 [43,45]. However, preliminary Cyr61 proteolysis assays in our system indicates that KLK-14 and plasmin do not produce cleavage products at similar molecular weights to the Cyr61 cleavage products produced by thrombin-stimulated corneal stromal fibroblasts and myofibroblasts (data not shown). Neither uPA nor tPA are likely candidates for the leupeptin-sensitive, thrombin-activated protease because thrombin does not activate in our system but inactivates both urokinase and tissue type plasminogen activators instead (unpublished data). Additional leupeptin-sensitive, E-64 insensitive serine protease candidates have been identified by mass spectral studies of human corneal stromal amyloid deposits [82] and activated stromal cells [66]. These include trypsin-like enzymes, PRSS-1, 3, and 8, hepsin, and acrosin, which can be released from cells.

Although there are no reports of cysteine proteases cleaving Cyr61, it is possible that the thrombin-activated protease is a leupeptin-inhibited cysteine protease. Most cysteine proteinases, including the cysteine cathepsin proteinases [62] and the serine protease trypsin [86], are inhibited by E-64, but this inhibitor stimulates the production of cathepsin S and decreases cathepsin L through inhibitor-induced feedback in the presence of breast cancer cells [63]. In the experiments reported here, E-64 did not inhibit thrombin induced cleavage of Cyr61 in the presence of stromal fibroblasts and myofibroblasts nor did it affect the cleavage of rCyr61 by the leupeptin-sensitive protease in the conditioned media in the absence of cells. These results suggest that the leupeptin-sensitive protease is not a cysteine cathepsin protease. Although protease inhibitors are used to explore protease classes, the exact class of the leupeptin-sensitive protease is unknown because some inhibitors, including leupeptin [81] and E-64 [86], can inhibit proteases of more than one class.

Proteolytically processed forms of Cyr61 are bioactive molecules, often with altered levels of activity or distinct activities from those of the parent protein due to exposure of amino acid motifs that become uncovered upon cleavage and react with receptors or other proteins [38,42,44,46]. Cyr61 fragments generated by KLK-12 do not enhance the migration of A549 lung cancer cells; however, these fragments decreased HUVEC survival under serum starvation conditions, relative to untreated cells [45]. The TSP-1-knot domains of Cyr61 are about four times as active at stimulating ERK activation in MCF-7 cells than full-length Cyr61 [46]. Proteolysis can also produce fragments that lose activities that are observed with full-length Cyr61 [38,45,46]. Endogenously produced corneal proteases may cleave Cyr61 in response

to injury in order to produce lower molecular weight forms of Cyr61 to modulate stromal fibroblast and myofibroblast activities during corneal wound healing.

The experiments presented here utilize a model system in which human corneal stromal keratocytes were converted into defined fibroblasts and myofibroblasts using FGF-2 and TGF- β 1, respectively, and then cultured in defined serum-free media in the presence or absence of added thrombin and in the presence or absence of protease inhibitors, PAR-1 and -4 agonists, or PAR-1 inhibitors. By working in a simple culture system with a defined cellular medium, we were able to decouple the thrombin-mediated activities of fibroblasts from myofibroblasts and other cell types present in the wounded stroma in order to study thrombin effects within and external to these cells. Although there is information gained about corneal stromal fibroblasts and myofibroblasts using this or any model system, extrapolation to the in vivo wounded cornea must be cautioned because the in vivo environment is quite different than the model system used in the reported experiments. For example, epithelial, stromal, or endothelial cells [72], the corneal extracellular matrix [72,73], tears-lachrymal fluid [74,75], or aqueous humor [76], may contribute proteases and protease inhibitors to the injury site that could modify the active levels and types of proteases present thus altering the synthesis and processing of Cyr61.

In summary, cultured human corneal stromal fibroblasts and myofibroblasts endogenously produce Cyr61 at the transcript and protein levels that are increased two- to threefold in response to thrombin stimulation, possibly due to PAR-1 signaling. With increasing amounts of thrombin, Cyr61 is cleaved extracellularly near the TSP-1 region to produce an N-terminal fragment of about 24 kDa and a C-terminal peptide of about 16 kDa. Although thrombin does not directly cleave Cyr61, it activates a leupeptin-sensitive protease, which cleaves Cyr61 to produce the 24 and 16 kDa fragments. This proenzyme is present mainly in an inactive form in the conditioned media from cells cultured in the absence of thrombin, and it is activated in response to thrombin treatment. Thus, thrombin regulates the Cyr61 protein levels in human corneal stromal fibroblasts and myofibroblasts through two distinct mechanisms: 1) by increasing cell-associated Cyr61 protein levels, partially through PAR-1, at least for fibroblasts, and 2) by inducing and activating an extracellular leupeptin-sensitive protease that cleaves extracellular Cyr61 into N-terminal and C-terminal domain populations (Figure 10). We predict that thrombin-mediated induction of limited proteolysis of extracellular Cyr61 by a leupeptin-sensitive protease may enhance or reduce selective functional attributes of Cyr61

domains in order to enhance wound healing and regulate neovascularization during corneal injury resolution.

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