THE JOURNAL OF IMMUNOLOGY

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J Immunol 2009;182;7916-7927
doi:10.4049/jimmunol.0801582
http://www.jimmunol.org/content/182/12/7916

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Thrombin-Induced Connective Tissue Growth Factor Expression in Human Lung Fibroblasts Requires the ASK1/JNK/AP-1 Pathway[1]

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Thrombin plays an important role in lung inflammatory diseases. Thrombin can induce connective tissue growth factor (CTGF) expression in lung fibroblasts. However, little is known about the signaling pathway in thrombin-induced CTGF expression. In this study, we investigated the role of apoptosis signal-regulating kinase 1 (ASK1) in thrombin-induced CTGF expression in human lung fibroblasts. Thrombin caused a concentration- and time-dependent increase in CTGF expression in WI-38 cells and primary lung fibroblasts. Thrombin-induced CTGF expression and CTGF-luciferase activity were inhibited by a protease-activated receptor 1 antagonist (SCH79797), the dominant-negative mutants (DNs) of ASK1 and JNK1/2, and an AP-1 inhibitor (curcumin). Thrombin caused ASK1 Ser^{967} dephosphorylation, the dissociation of ASK1 and 14-3-3, and a subsequent increase in ASK1 activity. Thrombin induced increases in JNK phosphorylation and kinase activity, which were attenuated by ASK1DN. Furthermore, SCH79797 diminished the thrombin-induced ASK1 and JNK activities. Thrombin-induced CTGF-luciferase activity was predominately controlled by the sequence −747 to −184 bp upstream of the transcription start site of the human CTGF promoter and was attenuated by transfection with the deleted AP-1 binding site construct. Thrombin caused increases in c-Jun phosphorylation, the formation of an AP-1-specific DNA-protein complex, and the recruitment of c-Jun to the CTGF promoter. Furthermore, thrombin-mediated AP-1 activation was inhibited by ASK1DN, JNK1/2DN, and SP600125. These results suggest for the first time that thrombin, acting through protease-activated receptor 1, activates the ASK1/JNK signaling pathway, which in turn initiates c-Jun/AP-1 activation and recruitment of c-Jun to the CTGF promoter and ultimately induces CTGF expression in human lung fibroblasts. ***The Journal of Immunology,*** **2009, 182: 7916–7927.**

Thrombin, a serine protease, is a well-known coagulation factor generated in vascular injury and which also plays an important role in lung inflammatory diseases (1). High thrombin levels exist in bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome, pneumonia, and asthma (2, 3). Thrombin also induces neutrophil migration and accumulation in the airway of mice (4) and contraction of bronchial rings in humans (5). The biological activities of thrombin are mostly transduced by the protease-activated receptors (PARs),[3] G protein-coupled receptors. To the present, four different PARs (PAR1–4) have been cloned (1) and determined to contribute to a variety of pathophysiological functions, such as chemokine and cytokine release and cellular proliferation (6–8). PAR1, PAR3, and PAR4 are activated by thrombin, whereas PAR2 is activated by trypsin (1). Several reports also demonstrated that thrombin exerts profibrotic cellular effects, and the levels of thrombin are increased in the bronchoalveolar lavage fluid of patients and in animal models of fibrotic lung disease (9, 10). Bleomycin-induced lung fibrosis in rats and mice is arrested by a thrombin proteolytic activity inhibitor (11). It was shown that thrombin can induce the release of fibrotic mediators, such as TGF-β (12), IL-8/CXCL-8 (13), and extracellular matrix proteins such as fibronectin and collagen (14, 15) in mesenchymal cells and lung fibroblasts. Recent studies showed that thrombin is also a very potent inducer of connective tissue growth factor (CTGF) production in lung fibroblasts and that PAR1 plays a key role during tissue repair, inflammation, lung injury, and the development of lung fibrosis (16, 17). However, little is known about the PAR1 signaling pathway of CTGF production in fibroblasts.

CTGF is a recently identified profibrotic agent. It is an immediate early gene and belongs to the CCN family (Cyr61 (CCN1), CTGF (CCN2), Nov (CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6)) of growth factors (18). The CTGF protein is a 38-kDa cysteine-rich, heparin-binding, secreted protein initially identified in the conditioned medium of cultured endothelial cells (19, 20). It is expressed by many human organs

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Received for publication May 16, 2008. Accepted for publication April 10, 2009.

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[1] This work was supported by grants (NSC96-2320-B-038-029-MY3 and NSC97-2320-B-038-014-MY3) from the National Science Council, Taiwan.

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[3] Abbreviations used in this paper: PAR, protease-activated receptor; CTGF, connective tissue growth factor; BCE-1, basal control element 1; Sp1, specificity protein 1; ASK1, apoptosis signal-regulating kinase 1; ActD, actinomycin D; CHX, cycloheximide; DN, dominant- negative mutant; NHALF, normal human adult lung fibroblast; MBP, myelin basic protein; NEAA, nonessential amino acid; ChIP, chromatin immunoprecipitation; MKK, MAPK kinase; MKKK, MAPK kinase kinase.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801582

and has various biological functions, including embryonic development, wound repair, and angiogenesis (21). Furthermore, CTGF has been shown to be involved in fibrosis in various organ systems including the lungs, kidneys, liver, and skin (22).

The promoter region of the human CTGF gene contains many transcription factor binding sites. These transcription factors include the AP-1, STAT, SMAD, basal control element (BCE) 1, NF-κB, specificity protein 1 (Sp1), and Elk-1 (23, 24). Previous reports demonstrated that TGF-β-induced CTGF expression is predominately mediated through SMAD signaling (25, 26). However, the role of AP-1 in regulating CTGF expression following thrombin stimulation is still unknown.

Apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPK kinase kinase (MKKK) family, is a serine/threonine protein kinase involved in regulating diverse cellular responses and is an upstream activator of JNK and p38 MAPK (27). ASK1 has been reported to be activated in response to many stress signals, including H_2O_2, TNF-α, endoplasmic reticular stress, and amyloid β peptide (28–31). However, the role of ASK1 in regulating thrombin-mediated CTGF expression in human lung fibroblasts is still unknown. Therefore, we investigated the role of ASK1 in thrombin-induced AP-1 activation and CTGF expression in human lung fibroblasts. In this study, we demonstrate for the first time that thrombin acts on PAR1 to activate the ASK1/JNK signaling pathways, which in turn initiate c-Jun/AP-1 activation and recruitment of the CTGF promoter, and ultimately induces CTGF expression in human lung fibroblasts.

Materials and Methods

Materials

Thrombin (from bovine plasma), myelin basic protein (MBP), SCH79797, curcumin, actinomycin D (ActD), and cycloheximide (CHX) were purchased from Sigma-Aldrich. SFLLRN-NH_2 (a PAR1 agonist peptide), TFRGAP-NH_2 (a PAR3 agonist peptide), and GYPGQV-NH_2 (a PAR4 agonist peptide) were purchased from Bachem Americas. SP600125 and tcY-NH_2 were purchased from Tocris Bioscience. ASK1 dominant-negative mutant (DN), JNK1DN, JNK2DN, and pcDNA were provided by Dr. M.-C. Chen (Taipei Medical University, Taipei, Taiwan). pBK-CMV-*LacZ* (*LacZ*) was provided by Dr. W.-W. Lin (National Taiwan University, Taipei, Taiwan). The human CTGF promoter (−747/+214) luciferase construct (pGL3-CTGF-Luc) and a series of human CTGF promoter deletion constructs (−408/+214, −184/+214, −119/+214, and −63/+214) were provided by Dr. M.-L. Kuo (National Taiwan University). MEM, FCS, penicillin/streptomycin, sodium pyruvate, L-glutamine, nonessential amino acids (NEAAs), and Lipofectamine Plus reagent were purchased from Invitrogen Life Technologies. An Ab specific for α-tubulin was purchased from Transduction Laboratories. An Ab specific for α smooth muscle actin was purchased from Abcam. Abs specific for JNK, JNK dually phosphorylated at Thr^{183} and Tyr^{185}, ASK1, and ASK1 phosphorylated at Ser^{967} were purchased from Cell Signaling Technology. Protein A/G beads, c-Jun-GST fusion protein (aa 1–79), rabbit polyclonal IgG, Abs specific for vimentin, cytokeratin 18, CTGF, c-Jun, c-Jun phosphorylated at Ser^{63}, c-Fos, and 14-3-3, and anti-mouse, anti-rabbit, and anti-goat IgG-conjugated HRP were purchased from Santa Cruz Biotechnology. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from GE Healthcare. A chromatin immunoprecipitation (ChIP) assay kit was purchased from Upstate Biotechnology. All materials for SDS-PAGE were purchased from Bio-Rad. All other chemicals were obtained from Sigma-Aldrich.

Cell culture

WI-38 cells, a normal human embryonic lung fibroblast cell line, were obtained from American Type Culture Collection. Cells were grown in MEM nutrient mixture containing 10% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified 37°C incubator with 5% CO_2. Cells were used between passages 18 and 30 for all experiments. After reaching confluence, cells were seeded onto 6-cm dishes for cell transfection, immunoblotting, kinase assays, and EMSA and onto 12-well plates for the cell transfection and luciferase assays.

Use of cultures of primary normal human adult lung fibroblasts (NHALFs) was approved by the Institutional Review Board of Taipei Medical University-Municipal Wangfang Hospital (Taipei, Taiwan). The human lung tissues were obtained from macroscopically normal nonpathogenic parts of the lung of patients undergoing lobar resection for lung cancer. Lung tissues were diced (1 × 1-mm pieces) and placed into 10-cm culture dishes. Cells were grown in an MEM nutrient mixture containing 10% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 100 μg/ml streptomycin and was replaced every 3 days. At 80% confluence, cells were trypsinized and seeded onto 6-cm dishes for the immunoblotting assays. To minimize phenotypic and other changes induced by culturing, all experiments were performed using fibroblasts at passages three to eight. The fibroblast phenotype was confirmed, and no myofibroblasts or airway epithelial cell contamination was confirmed by immunofluorescent staining with anti-α-smooth muscle actin (<1%), cytokeratin 18 (<1%), and vimentin (>99%).

Western blot analysis

Western blot analyses were performed as described previously (32). Briefly, WI-38 cells or NHALFs were cultured in 6-cm dishes. After reaching confluence, cells were treated with the vehicle and thrombin or pretreated with specific inhibitors as indicated followed by thrombin. Whole-cell lysates (50 μg) were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.02% Tween 20; pH 7.4) containing 5% BSA. Proteins were visualized using specific primary Abs and then incubated with HRP-conjugated secondary Abs. The immunoreactivity was detected using ECL according to the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Kodak).

Cell viability assay

Cell viability was measured by a colorimetric MTT assay (33). WI-38 cells (5×10^4 cells/well) were seeded onto 12-well plates and cells were incubated with the inhibitors (ActD, CHX, SCH79797, tcY-NH_2, SP600125, and curcumin) for 2.5 h or transfected with the DNs of ASK1, JNK1, and JNK2 for 24 h. After treatment, 0.5 mg/ml MTT was added to the culture plates and incubated at 37°C for another 2 h. Then cells were pelleted and lysed in 200 μl of DMSO. The absorbance at 595 nm was measured on a microplate reader. Each experiment was performed in duplicate and repeated three or four times.

DNA constructs

Two AP-1 site mutants of CTGF-Luc were constructed by introducing a point mutation using a two-step PCR process. The CTGF primer for the 3′ AP-1 site (−291 to −283, AP-1-1) mutant construct (CTGF mtAP-1-1-Luc) was 5′-TGATATGCATTCGGAGTGGTGCGA-3′ and that for the 5′ AP-1 site (−651 to −643, AP-1-2) mutant construct (CTGF mtAP-1-2-Luc) was 5′-TCCTGCGTTCCACGAGTCTTTG-3′.

Transfection and CTGF-luciferase assays

WI-38 cells (5×10^4 cells/well) were seeded onto 12-well plates and cells were transfected the following day using Lipofectamine Plus with 0.5 μg of CTGF-Luc, the CTGF promoter deletion constructs (−408/+214, −184/+214, −119/+214, or −63/+214), CTGF mtAP-1-1-Luc, CTGF mtAP-1-2-Luc, and 0.1 μg of *LacZ*. After 6 h, the medium was aspirated and replaced with basal medium devoid of FCS overnight and cells were stimulated with thrombin for another 16 h before being harvested. To assess the effects of the indicated inhibitors, drugs were added to cells 30 min before thrombin addition. To assay the effects of ASK1DN, JNK1DN, and JNK2DN, cells were cotransfected with CTGF-Luc, *LacZ*, and either ASK1DN, JNK1DN, or JNK2DN. Luciferase activity was determined and normalized on the basis of *LacZ* expression. The level of induction of luciferase activity was computed as the ratio of cells with and without stimulation. For the luciferase activity assay of CTGF promoter deletions, the inhibition rates (%) of the lost regions (−747 to −408, −408 to −184, −184 to −119, and −119 to −63 bp) of the CTGF promoter on thrombin induction were calculated as (CTGF luciferase activity of each deletion region caused by thrombin/CTGF (−747/+214) luciferase activity caused by thrombin) × 100%.

Immunoprecipitation and protein kinase assays

WI-38 cells were grown in 6-cm dishes. After reaching confluence, cells were treated with 0.3 U/ml thrombin for the indicated time intervals. After incubation, cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 125 mM

NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate and centrifuged. The supernatant was then immunoprecipitated with polyclonal Abs against ASK1 and JNK in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, and 2 mM DTT. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 20 μM ATP and 3 μCi of [γ-^{32}P]ATP at 37°C for 30 min. To assess ASK1 and JNK activities, 1 μg of MBP and 1 μg of c-Jun-GST fusion protein (aa 1–79) were, respectively, added as the substrates. The reaction mixtures were analyzed by 15% SDS-PAGE followed by autoradiography.

Coimmunoprecipitation

WI-38 cells were grown in 6-cm dishes. After reaching confluence, cells were treated with the vehicle or 0.3 U/ml thrombin for various time intervals. Cells were then harvested, lysed in 1 ml of PD buffer (40 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM sodium orthovanadate, 2 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM DTT) and centrifuged. The supernatant was then immunoprecipitated with a specific Ab against ASK1 in the presence of protein A/G beads at 4°C overnight. The immunoprecipitated beads were washed three times with PD buffer. Samples were fractionated on an 8% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblot analysis with Abs specific for ASK1 or 14-3-3.

Preparation of nuclear extracts and the EMSA

WI-38 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with the vehicle or 0.3 U/ml thrombin for various time intervals and then cells were scraped off and collected. In some experiments, cells were transfected with the ASK1DN for 6 h and replaced with basal medium devoid of FCS overnight before thrombin treatment. The cytosolic and nuclear protein fractions were then separated as previously described (7). Briefly, cells were washed with ice-cold PBS and pelleted. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 15 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 $\times$ *g* for 1 min. Supernatants containing the cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES (pH 7.6), 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 $\times$ *g* for 2 min and then stored at −70°C. A double-stranded oligonucleotide probe containing the AP-1 sequence (5′-CGCTTGATGAGTCAGCCGGAA-3′; Promega) was purchased and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (5 μg) was incubated with 1 ng of a ^{32}P-labeled AP-1 probe (50,000–75,000 cpm) in 10 μl of binding buffer containing 1 μg of poly(dI:dC), 15 mM HEPES (pH 7.6), 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 35°C for 20 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 5% polyacrylamide gels. Gels were vacuum dried and subjected to autoradiography with an intensifying screen at −80°C. For competition experiments, 1 ng of the labeled oligonucleotide was mixed with 50 ng of unlabeled competitor oligonucleotides before protein addition. For the supershift experiments, 2 μg of Abs specific for either c-Jun or c-Fos was mixed with the nuclear extract proteins.

ChIP assay

ChIP assays were performed using a ChIP assay kit according to the manufacturer's instructions. Briefly, WI-38 cells (2×10^6 cells) were incubated with 0.3 U/ml thrombin for 10 min and then cross-linked with formaldehyde at 37°C for another 10 min. Cell lysates were sonicated and then centrifuged for 10 min at 15,000 $\times$ *g* at 4°C to spin down the cell debris. Soluble cross-linked chromatins were immunoprecipitated with anti-c-Jun and anti-rabbit IgG Abs. DNA was purified and eluted with 50 μl of elution buffer using a spinning filter. PCR amplifications of AP-1-1 (−367/−239) and AP-1-2 (−746/-606) response elements on the CTGF promoter region were performed using the following primers: AP-1-1, 5′-GGATGTATGT CAGTGGACAGA-3′ (sense) and 5′-AAGCGCAGTATTTCCAG CACC-3′ (antisense) and AP-1-2, 5′-CTAGCCACTCGTCCCTTGT CC-3′ (sense) and 5′-CATTTGTCACTTGAGGTAACGG-3′ (antisense). Extracted DNA (2 μl) was used for 45 cycles of amplification in 50 μl of reaction mixture under the following conditions: 95°C for 30 s, 56°C for

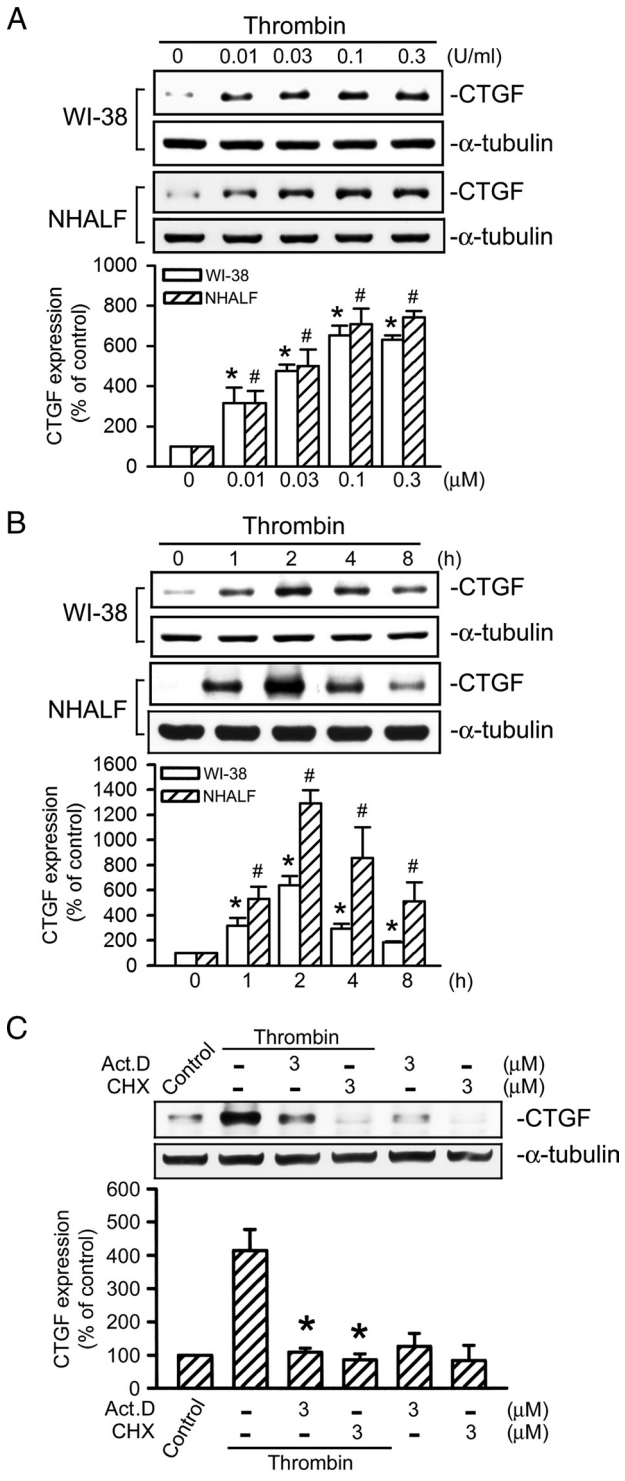

FIGURE 1. CTGF expression by thrombin in WI-38 cells and NHALFs. Cells were incubated with various concentrations of thrombin for 2 h (*A*) or with 0.3 U/ml thrombin for the indicated time intervals (*B*). Cells were lysed and then immunoblotted with Abs specific for CTGF or α-tubulin. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. ∗ and #, $p < 0.05$, respectively, compared with basal levels of WI-38 cells and NHALFs. *C*, Cells were pretreated for 30 min with 3 μM ActD or 3 μM CHX and then stimulated with 0.3 U/ml thrombin for another 2 h. Whole-cell lysates were prepared and then immunoblotted with Abs specific for CTGF or α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. ∗, $p < 0.05$ compared with the thrombin treatment group.

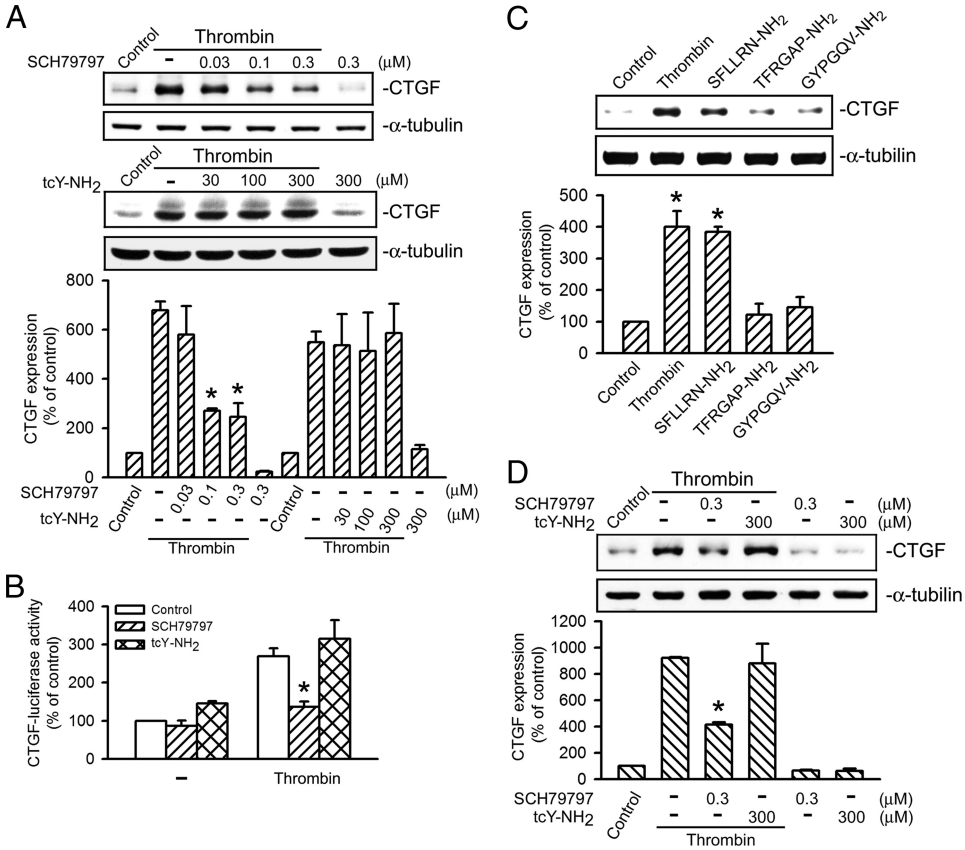

FIGURE 2. Involvement of PAR1 in thrombin-induced CTGF expression and CTGF-luciferase activity in WI-38 cells and NHALFs. *A*, WI-38 cells were pretreated with various concentrations of SCH79797 and tcY-NH_2 for 30 min and then stimulated with 0.3 U/ml thrombin for another 2 h. Whole-cell lysates were prepared and then immunoblotted with Abs specific for CTGF or α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. *, $p < 0.05$ compared with the group treated with thrombin alone. *B*, WI-38 cells were transiently transfected with 0.5 μg of CTGF-Luc and 0.1 μg of pBK-CMV-*LacZ* (*LacZ*) for 6 h. Cells were pretreated with 0.3 μM SCH79797 or 300 μM tcY-NH_2 for 30 min and then stimulated with 0.3 U/ml thrombin for 16 h. Cells were harvested for the luciferase activity assay. The level of induction of luciferase activity was compared with that of cells without thrombin treatment. Data represent the mean ± SEM of three experiments performed in duplicate. *, $p < 0.05$ compared with the control with thrombin treatment. *C*, WI-38 cells were incubated with 0.3 U/ml thrombin, 300 μM SFLLRN-NH_2 (a PAR1 agonist), 300 μM TFRGAP-NH_2 (a PAR3 agonist), and 300 μM GYPGQV-NH_2 (a PAR4 agonist) for 2 h. Cells were lysed and then immunoblotted with Abs specific for CTGF or α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. *, $p < 0.05$ compared with the control without agonist treatment. *D*, NHALFs were pretreated with 0.3 μM SCH79797 or 300 μM tcY-NH_2 for 30 min and then stimulated with 0.3 U/ml thrombin for another 2 h. Whole-cell lysates were prepared and then immunoblotted with Abs specific for CTGF or α-tubulin. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. *, $p < 0.05$ compared with the thrombin treatment group.

30 s, and 72°C for 60 s. The PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis

Results are presented as the mean ± SEM from at least three independent experiments. One-way ANOVA followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance of the difference between means. Values of $p < 0.05$ were considered statistically significant.

Results

Thrombin induces CTGF expression

Incubation of a human lung fibroblast cell line (WI-38) or NHALFs with thrombin (0.01–0.3 U/ml) for 2 h induced CTGF protein expression in a concentration-dependent manner, with maximum effects at 0.1–0.3 U/ml thrombin treatment (Fig. 1*A*). The thrombin (0.3 U/ml)-induced increases in CTGF expression were time dependent in WI-38 cells and NHALFs (Fig. 1*B*). After treatment, the induction of CTGF protein had begun by 1 h, reached a maximum at 2 h, and then gradually diminished to 8 h after thrombin treatment (Fig. 1*B*). Thrombin-induced CTGF expression obtained from WI-38 cells was similar to that of the primary NHALF response, supporting the use of WI-38 in further studies. In the following experiments, WI-38 cells were treated with 0.3 U/ml thrombin for 2 h. WI-38 cells were pretreated with either ActD (a transcriptional inhibitor) or CHX (a translational inhibitor) and then treated with 0.3 U/ml thrombin. As a result, the thrombin-induced elevation of CTGF expression was almost completely inhibited by ActD (3 μM) and CHX (3 μM) (Fig. 1*C*). In addition, treatment of cells with ActD (3 μM) and CHX (3 μM) did not affect cell viability (data not shown). These results suggest that the increase in CTGF protein in WI-38 cells responsive to thrombin was dependent on de novo transcription and translation.

Involvement of PAR1 in thrombin-induced CTGF expression

A previous study showed that PAR1, PAR2, PAR3, and PAR4 are expressed in WI-38 cells (34). PAR1, PAR3, and PAR4 are activated by thrombin, while PAR2 is activated by trypsin (1). To identify the PARs involved in thrombin-induced CTGF expression, the PAR1 antagonist SCH79797 and the PAR4 antagonist tcY-NH_2 were tested. As shown in Fig. 2*A*, pretreating WI-38 cells

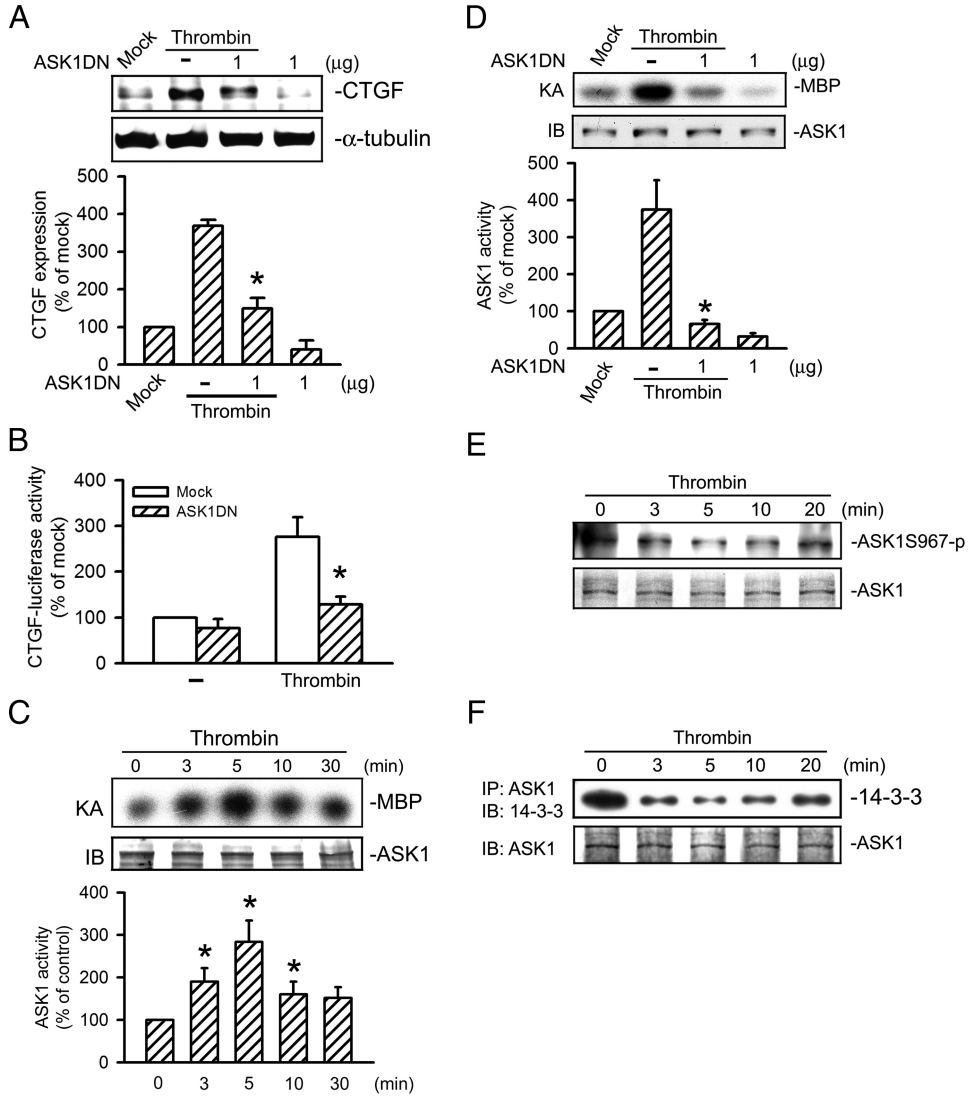

FIGURE 3. ASK1 is involved in thrombin-induced CTGF expression and CTGF-luciferase activity in WI-38 cells. *A*, Cells were transiently transfected with 1 μg of ASK1DN for 6 h and then stimulated with 0.3 U/ml thrombin for another 2 h. Whole-cell lysates were prepared and then immunoblotted with Abs specific for CTGF or α-tubulin. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean $\pm$ SEM. $*$, $p < 0.05$ compared with the thrombin treatment group. *B*, Cells were either transiently transfected with 0.5 μg of CTGF-Luc and 0.1 μg of *LacZ* or cotransfected with 0.5 μg of ASK1DN for 6 h and then stimulated with 0.3 U/ml thrombin for another 16 h. Cells were harvested for the luciferase activity assay. Data represent the mean $\pm$ SEM of three experiments performed in duplicate. $*$, $p < 0.05$ compared with the mock group with thrombin treatment. *C*, WI-38 cells were incubated with 0.3 U/ml thrombin for various time intervals and cell lysates were then immunoprecipitated with an Ab specific for ASK1. One set of immunoprecipitates was subjected to a kinase assay (KA) using MBP as a substrate. The other set of immunoprecipitates was subjected to 15% SDS-PAGE and analyzed by immunoblotting (IB) with an anti-ASK1 Ab. Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for ASK1. Traces represent results from three independent experiments, which are presented as the mean $\pm$ SEM. $*$, $p < 0.05$ compared with the basal level. *D*, Cells transiently transfected with 1 μg of ASK1DN for 6 h. Cells were then stimulated with 0.3 U/ml thrombin for another 5 min. Cell lysates were then immunoprecipitated with an Ab specific for ASK1 and subjected to the kinase assay. Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for ASK1. *E*, Cells were incubated with 0.3 U/ml thrombin for the indicated time intervals. Whole-cell lysates were prepared, and then immunoblotted with Abs specific for phospho-ASK1 Ser^{967} or ASK1. Equal loading in each lane is shown by the similar intensities of ASK1. Typical traces represent three experiments with similar results. *F*, Cells were incubated with 0.3 U/ml thrombin for the indicated time intervals. Cells were lysed and then immunoprecipitated with the anti-ASK1 Ab. The immunoprecipitated complex was then subjected to immunoblotting with an anti-14-3-3 Ab. Traces represent results from three or five (*C*) independent experiments, which are presented as the mean $\pm$ SEM. $*$, $p < 0.05$ compared with the group treated with thrombin alone.

with SCH79797 (0.03–0.3 μM) inhibited thrombin-induced CTGF expression in a concentration-dependent manner, while tcY-NH_2 (30–300 μM) had no effect. When cells were treated with 0.3 μM SCH79797, thrombin-induced CTGF expression was inhibited by 75 $\pm$ 16% (n = 3). In addition, treatment of cells with neither SCH79797 (0.3 μM) nor tcY-NH_2 (300 μM) affected cell viability (data not shown). Furthermore, WI-38 cells were transiently transfected with a human CTGF promoter (−747/+214)-Luc (CTGF-Luc) plasmid as an indicator of CTGF expression. As shown in Fig. 2*B*, WI-38 cells treated with thrombin (0.3 U/ml) for 16 h showed an increase in CTGF-luciferase activity by 269 $\pm$ 21% (n = 3). Thrombin-induced CTGF-luciferase activity was inhibited by 0.3 μM SCH79797 by 71 $\pm$ 7%, but not by 300 μM tcY-NH_2 (n = 3; Fig. 2*B*). Moreover, treatment of WI-38 cells with the

PAR1 agonist peptide SFLLRN-NH_2 (300 μM) also resulted in an increase in CTGF expression of 388 ± 2%, whereas the PAR3 agonist peptide TFRGAP-NH_2 (300 μM) and the PAR4 agonist peptide GYPGQV-NH_2 (300 μM) had no effects ($n = 3$; Fig. 2*C*). Similar to WI-38 cells, pretreating NHALFs with 0.3 μM SCH79797 inhibited thrombin-induced CTGF expression by 61 ± 2%, while 300 μM tcY-NH_2 had no effect ($n = 3$; Fig. 3*D*). These results suggest that thrombin-mediated CTGF expression in WI-38 cells and NHALFs may occur via activation of PAR1 signaling.

Involvement of ASK1 in thrombin-induced CTGF expression

To examine whether ASK1 activation is involved in the signal transduction pathway leading to CTGF expression caused by thrombin, an ASK1DN was used. As shown in Fig. 3*A*, transfection of WI-38 cells with 1 μg of ASK1DN markedly inhibited thrombin-induced CTGF expression by 59 ± 11% ($n = 3$). In addition, transfection of cells with ASK1DN (1 μg) did not affect cell viability (data not shown). Similarly, the thrombin-induced increase in CTGF-luciferase activity was also inhibited by 0.5 μg of ASK1DN by 70 ± 9% ($n = 3$; Fig. 3*B*). To further elucidate whether ASK1 activation is involved in the signaling cascade of thrombin-induced CTGF expression, ASK1 kinase activity was measured after thrombin exposure. Treatment of WI-38 cells with 0.3 U/ml thrombin resulted in an increase in ASK1 kinase activity in a time-dependent manner. The response had begun at 3 min, peaked at 5 min, and declined to 30 min after thrombin stimulation (Fig. 3*C*, *upper panel*). The protein level of ASK1 was not affected by thrombin treatment (Fig. 3*C*, *lower panel*; $n = 5$). These results suggest that ASK1 activation is involved in thrombin-induced CTGF expression in WI-38 fibroblasts. We confirmed the DN effect of ASK1DN on ASK1 kinase activity. As shown in Fig. 3*D*, the thrombin-induced increase in ASK1 kinase activity was completely inhibited by cells transfected with 1 μg of ASK1DN (Fig. 3*D*; $n = 3$). We next explored the mechanism by which thrombin induces ASK1 activation. Dissociation of ASK1 from the inhibitory protein 14-3-3 may lead to ASK1 activation. Phosphorylation of the ASK1 Ser^{967} residue was proposed as being a major mode for regulating ASK1 binding to 14-3-3 (35). We examined whether the extent of ASK1 Ser^{967} phosphorylation was altered after thrombin exposure. Thrombin had caused a marked decrease in ASK1 Ser^{967} phosphorylation at 5 min and this was sustained to 10 min after thrombin exposure (Fig. 3*E*, *upper panel*). The protein level of ASK1 was not affected in the presence of thrombin (Fig. 3*E*, *bottom panel*). Coimmunoprecipitation was then used to confirm the hypothesis that thrombin-induced ASK1 dephosphorylation was accompanied by dissociation of the ASK1-14-3-3 complex. As shown in Fig. 3*F*, the thrombin-induced dissociation between ASK1 and 14-3-3 had begun at 3 min, was sustained to 10 min, and then gradually recovered to 20 min after thrombin exposure. These results suggest that ASK1 Ser^{967} dephosphorylation and subsequent dissociation from 14-3-3 are required for thrombin-induced ASK1 activation.

JNK is involved in thrombin-induced CTGF expression

ASK1 belongs to the MAPK kinase kinase (MKKK) family and activates the JNK and p38 MAPK pathways via MAPK kinase (MKK) 4/7 and MKK3/6, respectively (27). JNKs, including the 46- and 54-kDa polypeptides of JNK1, JNK2, and JNK3, are activated in multiple cell types by mitogenic signals (36). We next attempted to determine whether JNK signaling events are involved in thrombin-induced CTGF expression using SP600125, a specific inhibitor of JNK. As shown in Fig. 4*A*, thrombin-induced CTGF expression was concentration-dependently attenuated by pretreating cells with SP600125 (3–30 μM). Pretreating cells with 30 μM

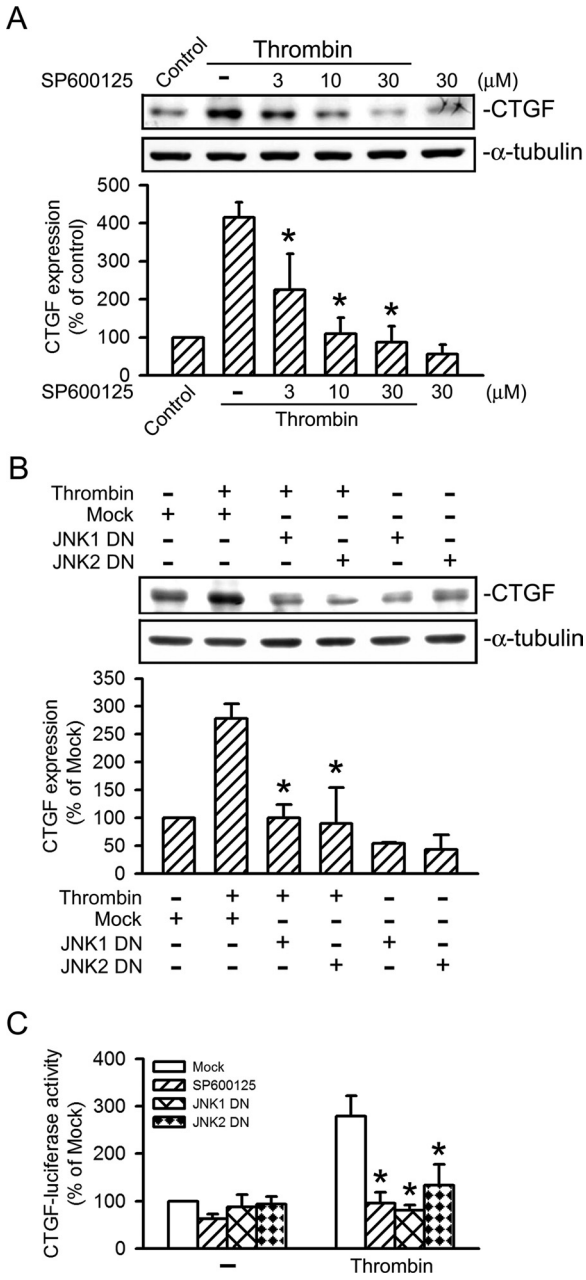

FIGURE 4. JNK is involved in thrombin-induced CTGF expression and CTGF-luciferase activity in WI-38 cells. Cells were pretreated with various concentrations (3–30 μM) of SP600125 for 30 min (*A*) or transiently transfected with 1 μg of JNK1DN or JNK2DN (*B*) for 6 h. Cells were then stimulated with 0.3 U/ml thrombin for another 2 h. Cells were lysed and then immunoblotted with Abs specific for CTGF or α-tubulin. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. *, $p < 0.05$ compared with the thrombin treatment group. *C*, WI-38 cells were either transiently transfected with 0.5 μg of CTGF-Luc and 0.1 μg of *LacZ* and then pretreated with 10 μM SP600125 for 30 min or cotransfected with 0.5 μg of JNK1DN or JNK2DN. Cells were then stimulated with 0.3 U/ml thrombin for 16 h and harvested for the luciferase activity assay. Data represent the mean ± SEM of three experiments performed in duplicate. *, $p < 0.05$ compared with the mock group with thrombin treatment.

SP600125 inhibited thrombin-induced CTGF expression by 90 ± 12% ($n = 3$). In addition, treatment of cells with SP600125 (30 μM) did not affect cell viability (data not shown). To further confirm that JNK mediates thrombin-induced CTGF expression,

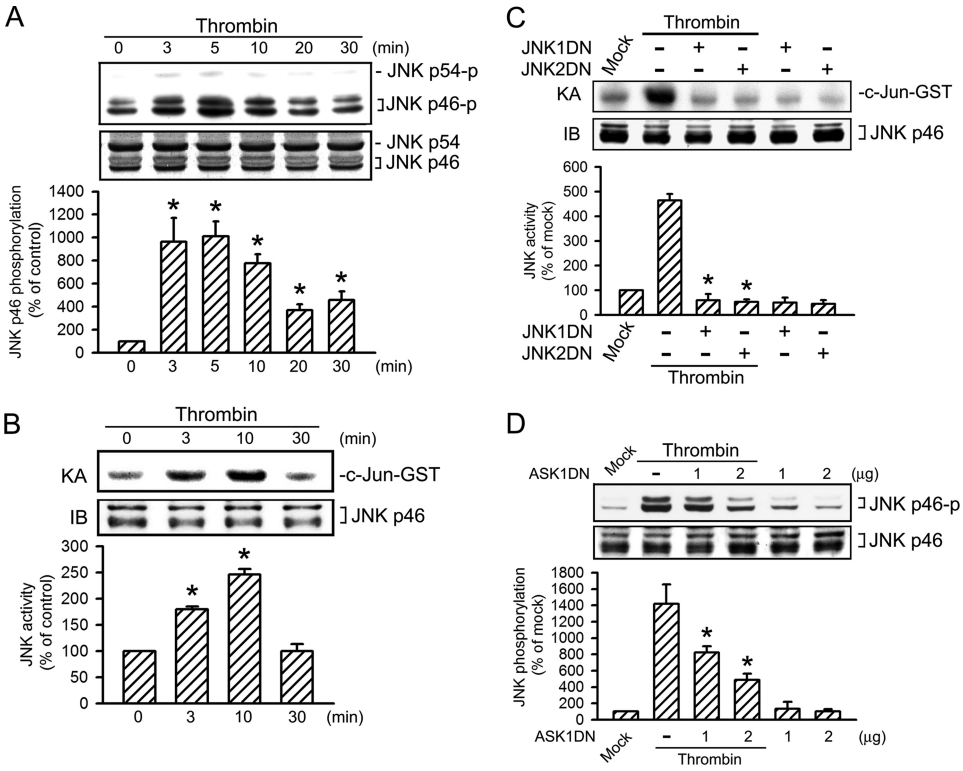

FIGURE 5. Involvement of ASK1 in thrombin-induced JNK activation in WI-38 cells. *A*, Cells were treated with 0.3 U/ml thrombin for different time intervals. Whole-cell lysates were prepared and then immunoblotted with Abs specific for phospho-JNK or JNK. Equal loading in each lane is shown by the similar intensities of JNK. *B*, WI-38 cells were incubated with 0.3 U/ml thrombin for the indicated time intervals and cell lysates were then immunoprecipitated with an Ab specific for JNK. One set of immunoprecipitates was subjected to the kinase assay (KA) using the c-Jun-GST fusion protein (aa 1–79) as a substrate. The other set of immunoprecipitates was subjected to 15% SDS-PAGE and analyzed by immunoblotting (IB) with the anti-JNK Ab. Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for JNK. Traces represent results from three independent experiments, which are presented as the mean ± SEM. ∗, $p < 0.05$ compared with the basal level. *C*, Cells were transiently transfected with 1 μg of JNK1DN and JNK2DN and then stimulated with 0.3 U/ml thrombin for 10 min. Cell lysates were prepared for the kinase assay with an Ab specific for JNK. *D*, Cells were transiently transfected with various concentrations of ASK1DN and then stimulated with 0.3 U/ml thrombin for 10 min. Cells were lysed and then immunoblotted with Abs specific for phospho-JNK or JNK. Equal loading in each lane is shown by the similar intensities of JNK. Traces represent results from three independent experiments, which are presented as the mean ± SEM. ∗ $p < 0.05$ compared with the basal level (*A* and *B*) or the thrombin treatment group (*C* and *D*), respectively.

DNs of JNK1 (JNK1DN) and JNK2 (JNK2DN) were used. As shown in Fig. 4*B*, transfection of WI-38 cells with 1 μg of JNK1DN and JNK2DN, respectively, inhibited thrombin-induced CTGF expression by 74 ± 17% and 73 ± 37% ($n = 3$). In addition, transfection of cells with neither JNK1DN (1 μg) nor JNK2DN (1 μg) affected cell viability (data not shown). Similarly, the thrombin-induced increase in CTGF-luciferase activity was also, respectively, inhibited by SP600125 (10 μM), JNK1DN (0.5 μg), and JNK2DN (0.5 μg) by 81 ± 18%, 89 ± 18%, and 77 ± 25% ($n = 3$; Fig. 4*C*). Taken together, these results imply that JNK may be responsible for thrombin-induced CTGF expression. We then examined whether thrombin is able to activate JNK. Treating WI-38 cells with 0.3 U/ml thrombin resulted in the time-dependent phosphorylation of JNK. The dual phosphorylation of p46JNKs at Thr^{183} and Tyr^{185} achieved a maximum at 3–5 min and was sustained to 30 min after thrombin stimulation (Fig. 5*A*, *upper panel*). However, the phosphorylation of p54JNK did not significantly increase. The protein level of JNK was not affected by thrombin treatment (Fig. 5*A*, *lower panel*). In parallel, using c-Jun-GST fusion protein as the JNK substrate, a time-dependent increase in JNK activity was also observed in thrombin-treated WI-38 cells. JNK activity had begun at 3 min, peaked at 10 min, and declined to 30 min after thrombin stimulation (Fig. 5*B*, *upper panel*). The protein level of JNK was not affected by thrombin treatment (Fig. 5*B*, *lower panel*). Confirming the effects of JNK1DN and JNK2DN, a marked reduction in JNK kinase activity caused by thrombin was observed in cells transfected with 1 μg of JNK1DN and JNK2DN ($n = 3$; Fig. 5*C*). To further ascertain the linkage between the ASK1 and JNK signaling cascade downstream of thrombin, we determined the effects of ASK1DN on thrombin-induced JNK activation. As shown in Fig. 5*C*, transfection of WI-38 cells with ASK1DN (1 and 2 μg), respectively, inhibited thrombin-induced JNK p46 phosphorylation by 45 ± 4% and 70 ± 10% ($n = 3$; Fig. 5*D*). Together these findings suggest that the ASK1/JNK pathway is required for thrombin-induced CTGF expression in WI-38 cells.

PAR1 mediates thrombin-induced ASK1 and JNK activation

To further examine whether PAR1 is involved in ASK1 and JNK activation caused by thrombin, activities of these kinases were tested by SCH79797. As shown in Fig. 6, when cells were treated with 0.3 μM SCH79797, thrombin-induced increases in ASK1 and JNK kinase activities were, respectively, inhibited by 77 ± 5% ($n = 3$) and 63 ± 5% ($n = 3$). These results suggest that the thrombin-induced increases in ASK1 and JNK activation were mediated by activation of PAR1 signaling.

The region between nucleotides −747 and −184 of the CTGF promoter is required for thrombin-induced CTGF expression

To determine which transcription factors are involved in thrombin-induced CTGF expression, WI-38 cells were transiently transfected

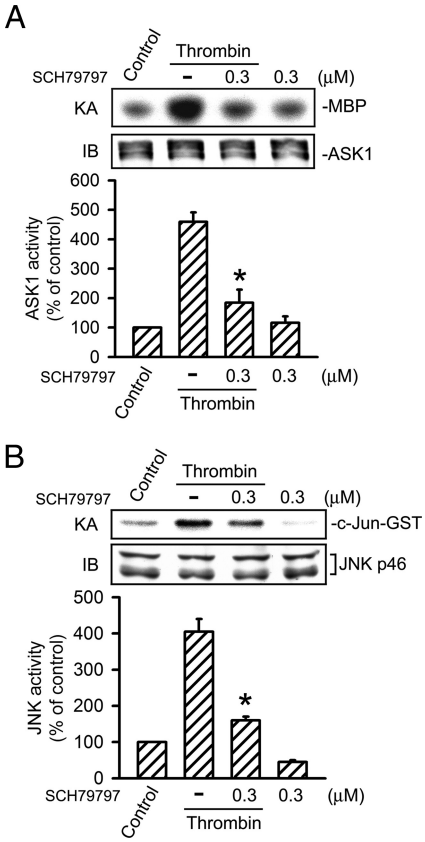

FIGURE 6. PAR1 is involved in thrombin-induced ASK1 and JNK activation in WI-38 cells. Cells were pretreated with 0.3 μM SCH79797 for 30 min and then stimulated with 0.3 U/ml thrombin for another 5 (*A*) or 10 min (*B*). Cell lysates were immunoprecipitated with an Ab specific for ASK1 or JNK and subjected to the kinase assay (KA). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting (IB) for ASK1 or JNK. Traces represent results from three independent experiments, which are presented as the mean ± SEM. *, $p < 0.05$ compared with the group treated with thrombin alone.

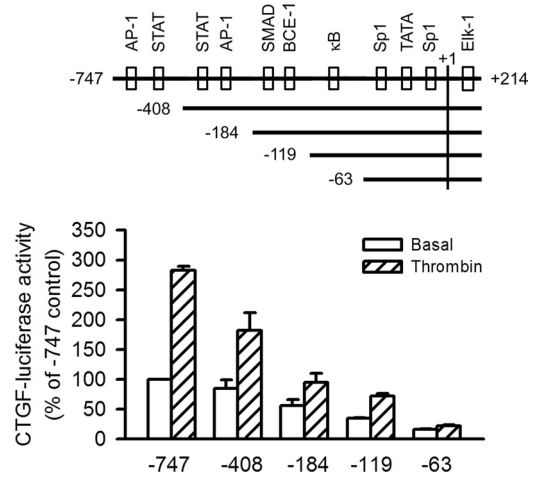

FIGURE 7. The region between nucleotides −747 and −184 of the CTGF promoter is required for thrombin-induced CTGF expression in WI-38 cells. Schematic diagram of human CTGF promoter deletion constructs. The region between −747 and +214 of the human CTGF promoter contains AP-1-, STAT-, SMAD-, BCE-1-, κB-, Sp1-, and Elk-1-binding elements. One CTGF-Luc (−747/+214) and four CTGF-Luc deletion constructs (−408/+214, −184/+214, −119/+214, and −63/+214) were used to examine the thrombin response elements of the CTGF promoter. Cells were transiently transfected with 0.5 μg of these CTGF-Luc constructs and 0.1 μg of *LacZ* for 6 h and then cells were incubated with 0.3 U/ml thrombin for 16 h. Cells were harvested for the luciferase activity assay. Data represent the mean ± SEM of three experiments performed in duplicate.

with CTGF-Luc (−747/+214) or four CTGF promoter deletion constructs (−408/+214, −184/+214, −119/+214, and −63/+214) and treated with 0.3 U/ml thrombin for 16 h. Fig. 7 shows that thrombin markedly induced an increase in CTGF (−747/+214) promoter luciferase activity by 283 ± 7%, which was reduced by the promoter deletion constructs of −408/+214, −184/+214, −119/+214, and −63/+214. To exclude a reduced effect on the basal level of these deletion constructs, the inhibition rate of each lost region on thrombin induction was calculated. Respective inhibition rates of the lost regions of −747 to −408, −408 to −184, −184 to −119, and −119 to −63 bp in the CTGF promoter on thrombin induction were 47 ± 8%, 32 ± 5%, 1 ± 0.5%, and 16 ± 2% ($n = 3$), respectively. These results indicate that thrombin-induced CTGF expression in WI-38 cells is predominately controlled by the segment −747 to −184 bp (79% inhibition rate) upstream of the transcription initiation start site on the human CTGF promoter.

AP-1 mediates thrombin-induced CTGF expression

The region between nucleotides −747 and −184 of the CTGF promoter contains two putative AP-1 binding sites (23, 24). Thus, we explored the role of AP-1 in thrombin-induced CTGF expression using the AP-1 inhibitor curcumin (37). As shown in Fig. 8*A*, thrombin-induced CTGF expression was markedly attenuated by pretreating cells with curcumin (1–10 μM) in a concentration-dependent manner. Curcumin at 10 μM suppressed thrombin-induced CTGF expression by 90 ± 26% ($n = 3$). In addition, treatment of cells with curcumin (10 μM) did not affect cell viability (data not shown). Furthermore, pretreating WI-38 cells for 30 min with 3 μM curcumin markedly inhibited the thrombin-induced increase in CTGF-luciferase activity by 69 ± 13% ($n = 3$; Fig. 8*B*). These results suggest that AP-1 activation is involved in thrombin-induced CTGF expression. Moreover, treating WI-38 cells with 0.3 U/ml thrombin resulted in the time-dependent phosphorylation of c-Jun. This response had reached a maximum at 3–10 min and declined to 30 min after thrombin stimulation (Fig. 8*C*, *upper panel*). The protein level of c-Jun was not affected by thrombin treatment (Fig. 8*C*, *lower panel*). Furthermore, AP-1 activation was directly evaluated by a gel shift DNA-binding assay using AP-1-specific oligonucleotides as the probes. As shown in Fig. 8*D*, the AP-1-specific DNA-protein complex formation time-dependently increased with a maximum effect at 10 min after thrombin treatment. However, after 20 min of treatment with thrombin, the intensities of these DNA-protein complexes had decreased. Formation of the DNA-protein complex was completely reduced by the addition of 50× cold AP-1 consensus DNA oligonucleotides (50× competitor), indicating that the DNA-protein interactions are sequence specific. To identify the specific subunits involved in the formation of the AP-1 complex after thrombin stimulation, supershift assays were performed using Abs specific for c-Jun and c-Fos. Incubation of nuclear extracts with Abs specific for c-Jun and c-Fos induced supershift bands and decreased the AP-1-specific DNA-protein complex ($n = 3$). Furthermore, incubation of nuclear extracts with normal IgG had no effect on the formation of AP-1-DNA complexes (Fig. 8*D*). These results suggest that the components of the AP-1 heterodimer are c-Jun and c-Fos. Pretreatment of WI-38 cells with SCH79797 (0.3 μM) for 30 min inhibited thrombin-induced formation of the AP-1-specific DNA-protein complex,

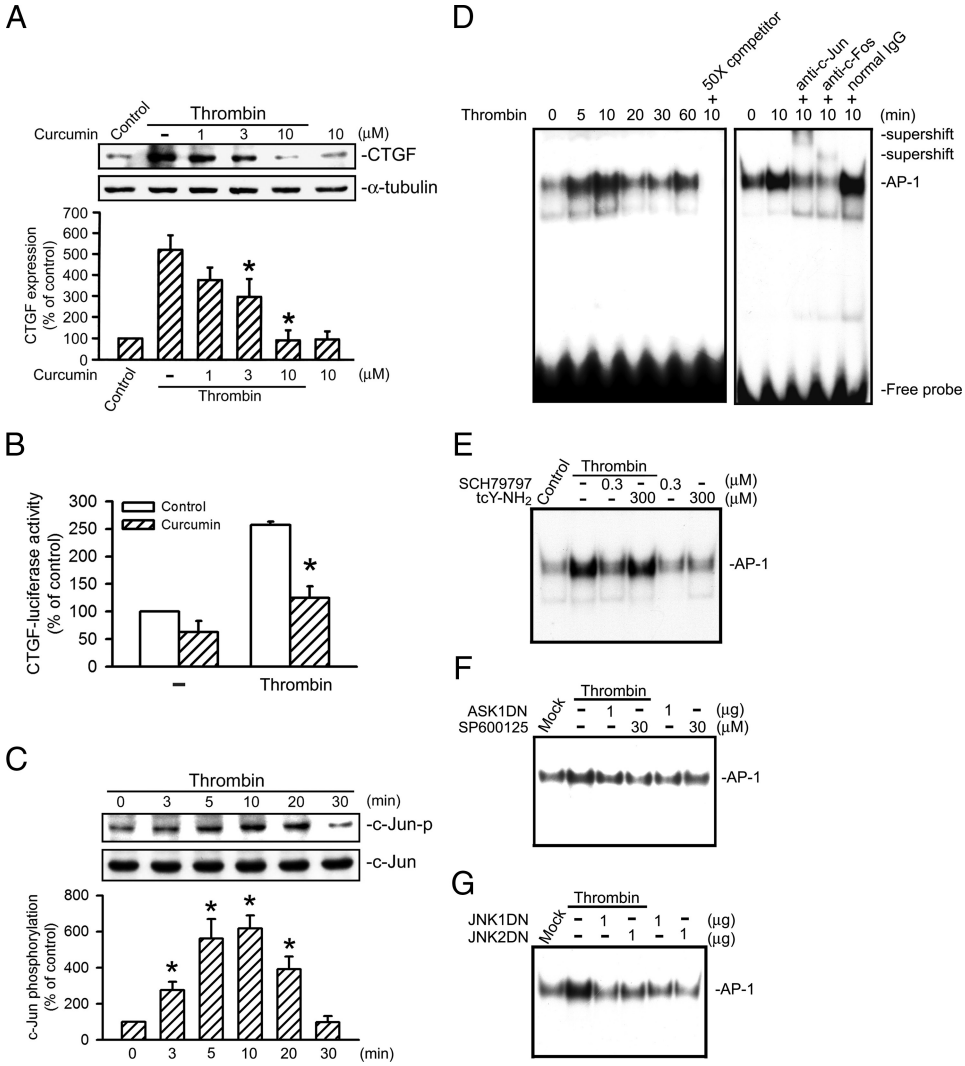

FIGURE 8. Involvement of the PAR1 and ASK1-JNK pathway in thrombin-induced AP-1 activation in WI-38 cells. *A*, Cells were pretreated with various concentrations (1–10 μM) of curcumin for 30 min and then stimulated with 0.3 U/ml thrombin for another 2 h. Cells were lysed and then immunoblotted with Abs specific for CTGF or α-tubulin. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SEM. ∗, $p < 0.05$ compared with the thrombin treatment group. *B*, WI-38 cells were transiently transfected with either CTGF-Luc or *LacZ* and then pretreated with 10 μM curcumin for 30 min. Cells were then stimulated with 0.3 U/ml thrombin for 16 h. Cells were harvested for the luciferase activity assay. Data represent the mean ± SEM of three experiments performed in duplicate. ∗, $p < 0.05$ compared with the control with thrombin treatment. *C*, Cells were incubated with 0.3 U/ml thrombin for the indicated time intervals. Whole-cell lysates were prepared, and then immunoblotted with Abs specific for phospho-c-Jun or c-Jun. Equal loading in each lane is shown by the similar intensities of c-Jun. Traces represent results from three independent experiments and are presented as the mean ± SEM. ∗, $p < 0.05$ compared with the basal level. *D*, WI-38 cells were incubated with 0.3 U/ml thrombin for 0–60 min and then the nuclear extract was prepared for EMSA. The top band represents AP-1. Results of a competition experiment using 50-fold unlabeled AP-1 oligonucleotide (50× competitor) and a supershift experiment with 2 μg of anti-c-Jun, anti-c-Fos, or normal IgG Abs performed on the nuclear extract from thrombin-stimulated cells for 10 min are also shown. *E–G*, Cells were pretreated with 0.3 μM SCH79797 (*E*), 300 μM tcY-NH_2 (*E*), or 30 μM SP 600125 (*F*) for 30 min or transiently transfected with 1 μg of ASK1DN (*F*), JNK1DN, and JNK2DN (*G*) for 6 h. Cells were then stimulated with 0.3 U/ml thrombin for another 10 min. The nuclear extract was prepared and EMSA was performed as described above. The top band represents AP-1. All typical traces are representative of three or four experiments (*D* and *E*) with similar results.

while tcY-NH_2 (300 μM) had no effect ($n = 4$;Fig. 8*E*). Therefore, thrombin-stimulated AP-1 activation is mediated through PAR1. We further examined the relationships among ASK, JNK, and AP-1 in the thrombin-mediated signaling pathway. As shown in Fig. 8, *F* and *G*, pretreating WI-38 cells for 30 min with 30 μM SP600125 or transiently transfecting them with 1 μg of ASK1DN, JNK1DN, or JNK2DN markedly inhibited thrombin-induced formation of the AP-1-specific DNA-protein complex. Based on these results, we suggest that activations of ASK1 and JNK occur upstream of AP-1 in the thrombin-induced signaling pathway.

Two AP-1 binding sites between −747 and −184 bp are required for thrombin-induced CTGF expression

To determine whether c-Jun is recruited to the endogenous CTGF promoter region in response to thrombin, we performed ChIP experiments on WI-38 cells stimulated with thrombin. Primer pairs were designed to correspond to the region of the putative AP-1-1 (−367/−239) and AP-1-2 (−746/-606) and were used to measure DNA fragments in immunoprecipitates by a PCR. Treatment of WI-38 cells with 0.3 U/ml thrombin for 10 min induced an

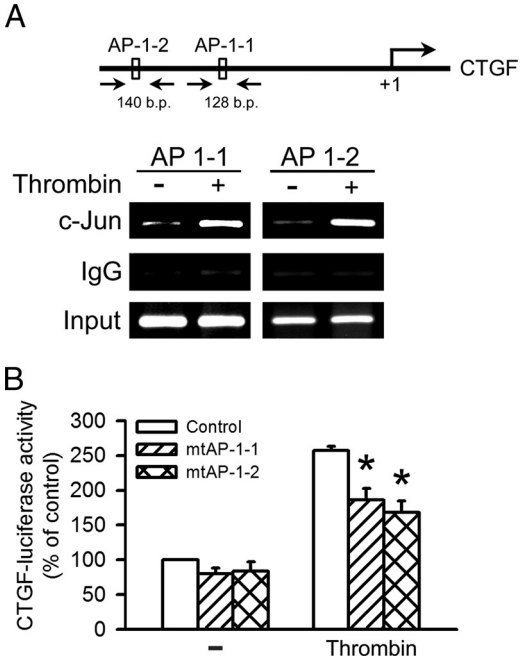

FIGURE 9. AP-1-mediated thrombin-induced CTGF transcription in WI-38 cells. *A*, Schematic diagram of AP-1-binding elements and ChIP primer locations on the CTGF promoter. ChIP primer pairs with 128- and 140-bp PCR products were designed to amplify DNA corresponding to the putative first (AP-1-1) and second (AP-1-2) AP-1 binding sites. Cells were incubated with 0.3 U/ml thrombin for 10 min and then cross-linked with formaldehyde at 37°C for another 10 min. Cell lysates were sonicated and prepared for the ChIP assay using an Ab specific for c-Jun as described in *Materials and Methods*. PCR amplification using primers designed against AP-1-1 and AP-1-2 binding sites was performed. Equal amounts of the soluble cross-linked chromatins present in each PCR were confirmed by the product for input. A rabbit polyclonal IgG was used as a negative control. Input, 1% of sonicated cross-linked chromatins. Typical traces are representative of three experiments with similar results. *B*, WI-38 cells were either transiently transfected with CTGF-Luc (control), CTGF mtAP-1-1-Luc (mtAP-1-1), or CTGF mtAP-1-2-Luc (mtAP-1-2) and *LacZ* and then stimulated with 0.3 U/ml thrombin for 16 h. Cells were harvested for the luciferase activity assay. Data represent the mean ± SEM of three experiments performed in duplicate. *, $p < 0.05$ compared with thrombin treatment in cells transfected with the CTGF-Luc plasmid.

increase in recruitment of c-Jun to AP-1-1 and AP-1-2 response elements on the promoter region of CTGF (Fig. 9*A*). In addition, regions of AP-1-1 (−367/−239) and AP-1-2 (−746/-606) in the CTGF promoter were detected in the cross-linked chromatin sample before immunoprecipitation (Fig. 9*A*, "Input," positive control). These results therefore establish that thrombin induces the recruitment of c-Jun to two AP-1 binding sites on the promoter region of the endogenous CTGF gene. To further examine whether the two AP-1 binding sites are involved in thrombin-induced CTGF transcriptional activation, the AP-1 binding site mutants of the CTGF promoter, CTGF mtAP-1-1 and CTGF mtAP-1-2, were used. As shown in Fig. 9*B*, transfection of WI-38 cells with either CTGF mtAP-1-1 or CTGF mtAP-1-2 inhibited thrombin-induced CTGF-luciferase activity by 32 ± 3% and 47 ± 5%, respectively ($n = 3$). The results indicate that the two AP-1 binging sites are required for thrombin-induced CTGF expression in WI-38 cells.

Discussion

The findings of our study demonstrate that thrombin might act on PAR1 to activate the ASK1/JNK signaling cascade, which in turn initiates c-Jun/AP-1 activation and recruitment to the CTGF promoter, and finally induces CTGF expression in human lung fibroblasts. It is well known that thrombin plays a critical role in lung inflammatory diseases (1). Thrombin generated in the lungs not only induces the expression of proinflammatory factors, such as IL-6, IL-8/CXCL-8, and PGE_2 (6–8), but also modulates the production of profibrotic mediators, such as CTGF, fibronectin, and collagen (14–16). In this study, we found that thrombin induced an increase in CTGF-luciferase activity and CTGF protein expression in a human lung fibroblast cell line (WI-38) and NHALFs. These results may be significant in understanding the role of thrombin in the development of lung fibrosis.

Thrombin is known to activate three PARs: PAR1, PAR3, and PAR4 (1). Previous studies demonstrated that WI-38 cells express PAR1, PAR2, PAR3, and PAR4 receptors (34). We found that the PAR1 antagonist SCH79797 inhibited thrombin-induced increases in CTGF-luciferase activity, CTGF expression, activation of ASK1 and JNK, and AP-1-specific DNA-protein complex formation, while the PAR4 antagonist tcY-NH_2 had no such effect. Previous studies indicated that SFLLRN-NH_2, TFRGAP-NH_2, and GYPGQV-NH_2 are respective agonist peptides for PAR1, PAR3, and PAR4 (7, 8, 34). In this study, we found that use of the PAR1 agonist peptide SFLLRN-NH_2 resulted in CTGF expression, whereas the PAR3 and PAR4 agonist peptides had no effect. These results suggest that PAR1, but not PAR3 or PAR4, is involved in thrombin-induced activation of ASK1, JNK, and AP-1 as well as CTGF expression in human lung fibroblasts. Our results are in agreement with the finding that PAR1 signaling plays a key role in bleomycin-induced lung inflammation and fibrosis (17).

Activated ASK1 plays a key role in regulating diverse cellular responses, including cell differentiation and apoptosis, through stimulating downstream signaling proteins including MKK4/7 and JNK (38, 39). JNK is well known to play an important role in AP-1-mediated transcriptional activation of target genes (40, 41). However, whether the ASK1/JNK/AP-1 signaling pathway participates in thrombin-induced CTGF expression has not previously been demonstrated. In this study, we found that treatment of WI-38 cells with thrombin caused sequential activations of ASK1, JNK, c-Jun, and AP-1 and that the DNs of ASK1, JNK1, and JNK2, as well as a JNK inhibitor (SP600125) and an AP-1 inhibitor (curcumin) attenuated the thrombin-induced increases in CTGF-luciferase activity and CTGF expression. Furthermore, ASK1DN suppressed thrombin-induced JNK1/2 activation. Moreover, the thrombin-induced formation of the AP-1-specific DNA-protein complex was inhibited by ASK1DN, JNK1/2DN, and the JNK inhibitor. These results suggest that thrombin might activate ASK1 to induce JNK activation, which in turn causes AP-1 activation, ultimately leading to CTGF expression in human lung fibroblasts.

ASK1 activity is regulated by multiple mechanisms including phosphorylation and interactions with various proteins. One of the essential ASK1 activation processes is dephosphorylation of the ASK1 Ser^{967} residue which binds to the inhibitory protein 14-3-3 (35). In this study, we found that the thrombin-induced increase in ASK1 activity was accompanied by ASK1 Ser^{967} dephosphorylation and dissociation of the ASK1-14-3-3 complex. However, the signaling pathways that control ASK1 function through Ser^{967} remain unresolved. A previous study showed that activation of an unknown protein phosphatase is required for TNF-α-induced ASK1 activation by dephosphorylating ASK1 Ser^{967} (35). Goldman et al. (42) further demonstrated that an okadaic acid-sensitive phosphatase is required for H_2O_2-induced ASK1 Ser^{967} dephosphorylation in COS7 cells. Recently, we also found that protein phosphatase 2A may play a pivotal role in amyloid β-induced ASK1 Ser^{967} dephosphorylation in cerebral endothelial cells (31). Therefore, activation of protein phosphatase via PAR1 might be

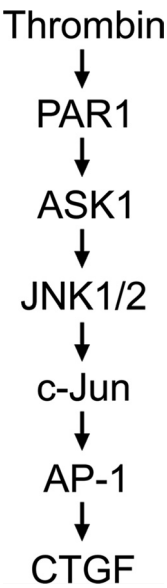

FIGURE 10. Schematic summary of the signaling pathway involved in thrombin-induced CTGF expression in human lung fibroblasts. Thrombin acts on PAR1 to activate the ASK1/JNK signaling pathway, which in turn initiates c-Jun/AP-1 activation and recruitment to the CTGF promoter, and ultimately induces CTGF expression in human lung fibroblasts.

involved in thrombin-induced ASK1 activation in WI-38 cells. However, the precise mechanism involved in thrombin-induced ASK1 activation needs to be further elucidated.

Activation of JNK family activity is involved in various physiological and pathological processes, like cell apoptosis, the inflammatory response, and cytokine production (43). JNKs are activated after their dual phosphorylation of threonine and tyrosine by the specific MKK4 and MKK7 (44, 45). Members of the JNK family are encoded by three distinct genes (*jnk1*, *jnk2*, and *jnk3*), the transcripts of which are alternatively spliced to yield four JNK1 isoforms, four JNK2 isoforms, and two JNK3 isoforms (36). Some studies reported that splicing at the C terminus of the JNK isoforms yields 46- and 54-kDa polypeptides of JNK1 and JNK2. Isoforms of JNK3 (48 and 57kDa) were yielded in the presence of an extended NH2 terminus in the sequence of JNK3, which are larger than the corresponding JNK1 and JNK2 proteins (36, 46). Interestingly, in this study, we found double phospho-JNK bands near 46 kDa in the blot induced by thrombin in lung fibroblasts. We suggest that the double phospho-JNK may be phospho-p46-kDa JNK1/2 and phospho-p48-kDa JNK3. Consistent with our study, thrombin also induced double phosphorylation of JNK bands near 46 kDa in airway epithelial cells (47).

The promoter region of the human CTGF gene contains many transcription factor binding sites which include AP-1, STAT, SMAD, BCE-1, NF-κB, Sp1, and Elk-1 (23, 24). The results from the ChIP assay indicated that c-Jun directly binds to the endogenous CTGF promoter region caused by thrombin signaling. Furthermore, a reporter assay showed that thrombin-induced CTGF-luciferase activity was attenuated by the transfection of two constructs, the AP-1 sites of which were deleted into human lung fibroblasts. Taken together, these data suggest that the two AP-1 sites on the CTGF promoter play crucial roles in enhancing CTGF induction by thrombin. In the CTGF promoter deletion assay, we also demonstrated that the region from -747 to -184 contains two AP-1 and two STAT binding sites in the CTGF promoter and is the major element for thrombin-mediated CTGF-luciferase activity. This result suggests that STAT, in addition to AP-1, might be involved in thrombin-induced CTGF expression. Previous reports showed that STAT-3-dependent cytosolic phospholipase A_2 expression and STAT-5B-dependent heat shock protein 27 and fibroblast growth factor 2 expressions are required for thrombin-induced vascular smooth muscle cell motility (48, 49). Furthermore, several reports demonstrated the physical and functional interactions between AP-1 and several STAT family members (50, 51). Whether AP-1 cooperates with other transcription factors such as STAT in thrombin-stimulated CTGF expression in lung fibroblasts needs to be further investigated.

In conclusion, the present study for the first time shows that thrombin, acting through PAR1, activates the ASK1/JNK signaling pathway, which in turn initiates c-Jun/AP-1 activation and recruitment to the CTGF promoter, and finally induces CTGF expression in human lung fibroblasts. This is the first study to demonstrate that thrombin-induced ASK1 activation may occur through dephosphorylating ASK1 Ser^{967}, which in turn causes dissociation of the ASK1-14-3-3 complex in lung fibroblasts. Fig. 10 is a schematic representation of the signaling pathway involved in the expression of CTGF in response to thrombin in human lung fibroblasts. Our results provide a mechanism linking thrombin and the profibrotic protein CTGF and provide support for the development of therapeutic strategies to reduce lung fibrosis caused by thrombin.

Acknowledgments

We thank Dr. M.-L. Kuo for the gift of the CTGF promoter Luc plasmids and Dr. Robert F.-C. Chen for the lung tissue of lung cancer patients provided by the Department of Thoracic and Cardiovascular Surgery, Taipei Medical University-Municipal Wangfang Hospital, Taipei, Taiwan.

Disclosures

The authors have no financial conflict of interest.

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