Collagen Triple Helix Repeat Containing 1, a Novel Secreted Protein in Injured and Diseased Arteries, Inhibits Collagen Expression and Promotes Cell Migration

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Abstract—Collagen triple helix repeat containing 1 (*Cthrc1*) was identified in a screen for differentially expressed sequences in balloon-injured versus normal arteries. Cthrc1 expression was not detectable in normal arteries. However, on injury it was transiently expressed by fibroblasts of the remodeling adventitia and by smooth muscle cells of the neointima. It was also found in the matrix of calcifying human atherosclerotic plaques. CTHRC1 is a secreted 28-kDa protein that is glycosylated and highly conserved from lower chordates to mammals. A short collagen motif with 12 Gly-X-Y repeats appears to be responsible for trimerization of the protein and this renders the molecule susceptible to cleavage by collagenase. Cthrc1 mRNA expression levels are increased in response to transforming growth factor-β and bone morphogenetic protein-4. Cell migration assays performed with CTHRC1-overexpressing fibroblasts and smooth muscle cells demonstrate that increased CTHRC1 levels are associated with enhanced migratory ability. Furthermore, CTHRC1 overexpression caused a dramatic reduction in collagen type I mRNA and protein levels. Our data indicate that the novel molecule CTHRC1 is transiently expressed in the arterial wall in response to injury where it may contribute to vascular remodeling by limiting collagen matrix deposition and promoting cell migration. (***Circ Res*. 2005; 96:261-268.**)

Key Words: calcification ■ fibrosis ■ remodeling ■ adventitia ■ intima

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Constrictive arterial remodeling has been identified as the major cause for the delayed failure of angioplasty procedures.[1] Using the balloon catheter injury model of the rat carotid artery, our own studies demonstrated that constrictive remodeling is also the most important contributor to lumen narrowing in this model.[2,3] Although the use of intravascular stents can control the impact of constrictive remodeling, their efficacy in preventing arterial lumen narrowing is diminished by the proliferation of smooth muscle cells (SMCs) within the stent. We have recently demonstrated that constrictive arterial remodeling in the rat model is largely a TGF-β–dependent process that is caused by fibrosis of the adventitia with abundant deposition of a collagenous matrix.[3] Furthermore, inhibition of TGF-β signaling also led to a significant reduction in neointimal lesion formation.[2,4] These studies demonstrated that the effects of TGF-β are not only responsible for constrictive arterial remodeling but also for the formation of intimal thickening composed of SMCs.

During the arterial response to injury, adventitial fibroblasts transdifferentiate into myofibroblasts with characteristic expression of smooth muscle α-actin.[2,5] As the healing process progresses, there is abundant deposition of extracellular matrix especially collagen type I, and at the same time, there is a decrease in cellularity and myofibroblasts disappear.[6] TGF-β has been implicated in myofibroblastic transdifferentiation[5] and inhibition of TGF-β signaling in this process did indeed block the adventitial myofibroblast differentiation process.

The present study originally emerged from a comparison of gene expression profiles of normal and 8 day balloon injured rat arteries using suppressive subtractive hybridization.[7] During this screen, collagen triple helix repeat containing 1 (*Cthrc1*) was identified as a novel gene regulated by TGF-β family members with expression restricted to the adventitia and neointima of injured arteries. In this study, we performed an initial biochemical and functional characterization of this novel molecule.

Materials and Methods

Animals

All protocols using rodents were approved by the Institutional Animal Care and Use Committee and were in compliance with all federal and local guidelines. Sprague-Dawley rats were obtained from Taconic (Germantown, NY).

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All surgical procedures were performed under general anesthesia by intraperitoneal injection of xylazine (2.2 mg/kg, AnaSed, Lloyd Laboratories) and ketamine (50 mg/kg body weight, Ketalar, Parke-Davis). The left carotid artery and the aorta were denuded with a 2F balloon catheter as described.[8] Completely deendothelialized segments of arteries were identified by intravenous injection of Evans blue dye (0.5 mL of 5% solution in saline) five minutes before harvest of the vessels.[9] Vessels used for immunoblot analysis were excised after perfusion of the animal with phosphate-buffered saline (PBS) and snap frozen in liquid nitrogen before protein extraction. For in situ hybridization, vessels were harvested after perfusion fixation with buffered 4% paraformaldehyde. For immunohistochemistry, the vessels were excised after perfusion fixation with Methyl Carnoy's fixative.

Subtractive Hybridization and Cloning of the Cthrc1 cDNA

Suppressive subtractive hybridization[7] was performed with a subtraction kit (PCR-Select cDNA Subtraction Kit, Clontech) following the manufacturer's instructions. cDNA was prepared from 2 μg of mRNA RNA extracted from normal rat carotid arteries and aortae as well as corresponding 8-day balloon-injured vessels.[8] For the purpose of isolating sequences overexpressed in injured arteries, cDNA from injured vessels was used as "tester" and cDNA from normal vessels as "driver" cDNA. The subtracted cDNAs were ligated into the pCRII cloning vector (Invitrogen). Partial sequences of approximately 300 clones were obtained by automated sequencing (ABI-PRISM 310 sequencer) and their identities were determined by searching Genbank databases including nonredundant and EST databases.

A cDNA library was prepared from RNA isolated from 8-day balloon-injured rat carotid arteries and aortae (Lambda ZAP Express, Stratagene). A 220-bp Cthrc1 cDNA clone obtained from the subtractive hybridization was used to prepare a ^{32}P-labeled probe for screening of the library to obtain a full-length Cthrc1 cDNA clone. Clones hybridizing with the probe were isolated and sequenced with sequence-specific oligonucleotides (Integrated DNA Technologies).

The human Cthrc1 cDNA was cloned by 5′-RACE (Roche) using RNA prepared from cultured human SMCs. Three sequence-specific primers, based on the human expressed sequence tag (EST) clone (AA584310) were used to amplify the 5′ region of Cthrc1 that was not present in Genbank at the time. The primer sequences were 5′-ATTTTAGCCGAAGTGAGC-3′, 5′-CACTGAACAAAACTCTTA-3′, and 5′-CTATTTGAACGCATCTTT-3′.

Cthrc1 Transcription and Translation

A full-length Cthrc1 cDNA was used to express protein with the TnT reticulocyte lysate system (Promega) in the presence of ^{35}S-labeled methionine (New England Nuclear). The translated products were resolved by 12% SDS-PAGE followed by autoradiography of the gel.

Regulation of Cthrc1 Expression

The regulation of Cthrc1 mRNA and protein levels in response to various growth factors (10 ng/mL FGF-2, 1 ng/mL TGF-β_1, 10 ng/mL BMP-4, 10 ng/mL PDGF-AB; all from R&D Systems) and 10% bovine serum was examined in NIH3T3 cells and MC3T3-E1 cells. Before stimulation, the cells were placed in serum free medium for 24 hours and harvested for Northern and Western blotting at the indicated times after stimulation.

Antibody Generation and Western Blotting

Recombinant rat CTHRC1 protein lacking the signal sequence was tagged with 6 histidine residues (6xhis) fused to the C terminus and expressed in *Escherichia coli.* Purified CTHRC1 was used for immunization of rabbits. The anti-CTHRC1 antiserum recognized less than 1 ng of recombinant protein on immunoblots. Immunoblotting was performed with anti-CTHRC1 serum at a 1:3000 dilution. Equal amounts of protein were loaded in each lane based on spectrophotometric determination of protein concentration. A monoclonal antibody recognizing soluble procollagen (clone SP1.D8, Developmental Studies Hybridoma Bank) was used at a 1:1000 dilution.

Biochemical Characterization of Cthrc1

Recombinant myc-6xhis–tagged CTHRC1 with the signal sequence was expressed in CHO cells. The recombinant protein was purified from conditioned medium with Ni-NTA-Agarose (Qiagen). The purified protein was subjected to SDS-PAGE and transferred to a PVDF membrane (Bio-Rad), and the N terminal protein sequence was determined by automated Edman degradation to identify the cleavage site of the predicted signal peptide. To determine whether CTHRC1 is glycosylated, CTHRC1 purified from CHO cell conditioned medium was treated with and without *N*-glycosidase F (PNGase F, New England Biolabs) in sodium phosphate buffer (50 mmol/L, pH 7.5) supplemented with 1% NP-40 after reduction and denaturation. The enzymatic treatment was performed at 37°C for 120 minutes and was followed by immunoblotting of the samples with anti-CTHRC1 antibody or anti-his tag antibody. CHO cell expressed CTHRC1 was cross-linked with disuccinimidyl suberate (DSS, 1 mmol/L, pH8.9 for 30 minutes at 25°C), and both native and cross-linked CTHRC1 were examined by immunoblot analysis. The presence of a short collagen domain within CTHRC1 prompted us to examine whether CTHRC1 is a substrate for collagenase. 500 ng of purified his-tagged CTHRC1 were incubated with 200 U of highly purified collagenase (Sigma C0773, collagenase type VII) at 37°C for 120 minutes in buffer (0.15 mol/L NaCl, 50 mmol/L Tris pH7.6, 30 mmol/L $CaCl_2$, 10 μmol/L $ZnCl_2$). The digested CTHRC1 was examined by immunoblotting with an anti-his tag antibody and with three different polyclonal antibodies raised against recombinant CHTRC1.

Immunohistochemistry

Tissues were fixed in Methyl Carnoy's fixative and embedded in paraffin. Sections were treated with 1% 2-mercaptoethanol for 30 minutes before incubation with anti-CTHRC1 antiserum (1:1000 dilution) overnight at 4°C. All other steps were performed as described.[10] Either diaminobenzidine or 3-amino-9-ethylcarbazole (AEC) were used to visualize the immunoreactivity. Controls with preimmune serum (1:1000 dilution) from the same rabbit were included for all tissue sections, and at this dilution, nonspecific staining was negligible.

In Situ Hybridization

^{35}S-UTP–labeled RNA probes corresponding to the sense and antisense strand of the coding region of rat *Cthrc1* were prepared, and in situ hybridization was performed on paraffin sections as previously described.[11]

Cell Lines

Cthrc1 expression constructs were generated by cloning the coding region of the rat Cthrc1 cDNA into the expression vector pTriEx-1.1 Neo (Novagen), and a stably transfected PAC1 smooth muscle cell line[12] was established using FuGene 6 transfection reagent (Roche). Because PAC1 cells express endogenous levels of Cthrc1, we also obtained stable PAC1 transfectants expressing Cthrc1 antisense RNA by cloning the coding region of Cthrc1 into pTriEx-1.1 Neo in reverse orientation. Empty vector transfected PAC1 transfectants were used as controls. Primary mouse embryonic fibroblast (MEF) cultures were established from 13-day-old wild-type and transgenic embryos expressing myc-tagged full-length CTHRC1 under CMV promoter control. Overexpression of CTHRC1 in the MEF from Cthrc1 transgenic embryos was verified by immunoblotting (data not shown) with an anti-myc antibody (clone 9E10, Zymed).

Northern Blotting

Northern blotting of total RNA (20 μg per lane) was performed as previously described.[2] The major ribosomal RNA bands were visualized by ethidium bromide staining, and a full-length rat Cthrc1 cDNA was used to probe the membrane. RNA from PAC1 transfectants was examined for collagen type I expression using rat cDNAs

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for both the α_1 and α_2 chain and expression was normalized to GAPDH expression. Quantification of mRNA expression levels was performed with a phophorimager, and the values are shown relative to control cells (set at 100).

Cell Migration and Adhesion Assays

The migratory properties of confluent PAC1 cell transfectants and MEF from wild-type as well as Cthrc1 transgenic mice were examined in a scratch wound assays by creating a denuded zone with a pipette tip. These assays were performed on tissue culture treated plastic with and without prior coating with collagen type I (5 μg/cm^2, BD-Biosciences). The area still devoid of cells was determined at the indicated time points by planimetry of digitized images used (NIH Image software). Three independent experiments with PAC1 transfectants and MEF derived from three different wild-type and Cthrc1 transgenic mice were performed and nearly identical results were obtained. ANOVA was used to determine whether significant differences between the means of groups were present ($P \leq 0.05$). Multiple comparisons between groups were then performed using the Scheffé test.

Results

Identification of Cthrc1 by Subtraction Cloning

We performed suppressive subtractive hybridization with the normal vessel providing the driver and the injured vessel providing the tester cDNA. Among the many differentially expressed sequences was Cthrc1, and we decided to pursue this sequence further because it was not expressed in normal but highly induced in injured arteries (Figure 1A). Cthrc1 expression was also examined by Northern blotting in a variety of organs from adult rats. In lung and brain, a 1.2-kb transcript was detected, although mRNA levels were much lower in other tissues examined (Figure 1B). Immunoblotting with an antibody raised against recombinant CTHRC1 demonstrated that expression of CTHRC1 in the injured arteries was transient with maximal levels seen at 4 and 8 days after injury (Figure 1C). At these time points the adventitia is undergoing extensive remodeling.[2] Similar to normal arteries, very little CTHRC1 was detectable at 4 weeks after injury.

Using the 230-bp *Cthrc1* cDNA as probe, a cDNA library prepared from 8-day balloon-injured rat arteries was screened to obtain a full-length cDNA clone. Several full-length clones were isolated and sequenced (GenBank accession nos. AY136824 and NM172333). The corresponding human cDNA homologue was cloned by 5′-RACE using RNA prepared from cultured human smooth muscle cells (SMCs) (GenBank accession no. AY136825). The sequences contained an open reading frame that predicted a 245 amino acid 26.5-kDa rat protein and a human homolog with 243 amino acids.

Database searches were performed to identify potential homologs of Cthrc1 in other species. With the *Caenorhabditis elegans* and *Drosophila* databases being more or less complete, it was very interesting to note that no homolog of *Cthrc1* was detectable in these species. Sequences of sea squirt (*Ciona intestinalis*), fish (*Fugu rubripes*), chicken (*Gallus gallus*), and mammals were extremely highly conserved, especially the C-terminal 200 amino acids (Figure 2A).

Localization of Cthrc1 Within Injured Arteries and Vascular Calcifications

We used in situ hybridization and immunohistochemistry to examine Cthrc1 RNA and protein expression at the cellular level in balloon-injured arteries and human atherosclerotic plaque. Cthrc1 mRNA was expressed at high levels predominantly in the adventitia at 8 and 14 days after injury (Figure 3B and 3C). Lower levels of expression were also seen in the developing neointima (Figure 3C). At 4 weeks after injury when the response to injury was complete, only very little Cthrc1 expression was still detectable in the adventitia (Figure 3D). Immunohistochemistry localized CTHRC1 protein to the cellular compartment and extracellular matrix of the adventitia and neointima in remodeling arteries (Figure 3F), whereas no immunoreactivity was detectable in normal arteries (not shown). Examination of en face preparations demonstrated that Cthrc1 mRNA and protein is not detectable in endothelial cells (data not shown). In endarterectomy specimens obtained from human carotid artery lesions, immunoreactive CTHRC1 was associated with vascular calcifications (Figure 3G). No immunoreactive CTHRC1 was detectable in noncalcified plaque regions. Most intense immunoreactivity in these specimens was found at the outer margins of the calcification fronts. Cartilaginous matrix surrounding the calcified tissue also revealed CTHRC1 expression by chondrocyte-like cells (Figure 3G).

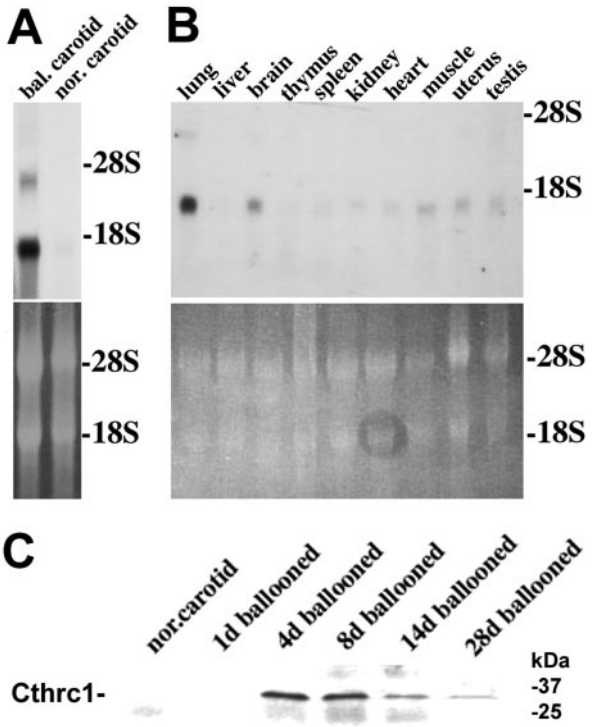

Figure 1. A, Northern blot of total RNA isolated from normal and 8-day balloon injured rat carotid arteries shows the Cthrc1 transcript only in injured arteries. B, Various organs from adult rats were examined for Cthrc1 mRNA expression by Northern blotting. A major 1.2-kb transcript was present in lung and brain, with significantly lower levels in other organs. Corresponding ethidium bromide stained membranes are shown below. C, Immunoblot analysis for CTHRC1 expression was performed on carotid artery lysates prepared from vessels harvested at the indicated time points. Note the transient expression of CTHRC1 after injury.

Regulation of Cthrc1 mRNA Expression

The expression of *Cthrc1* in the adventitial fibroblasts led us to examine the effects of TGF-β family members in NIH3T3

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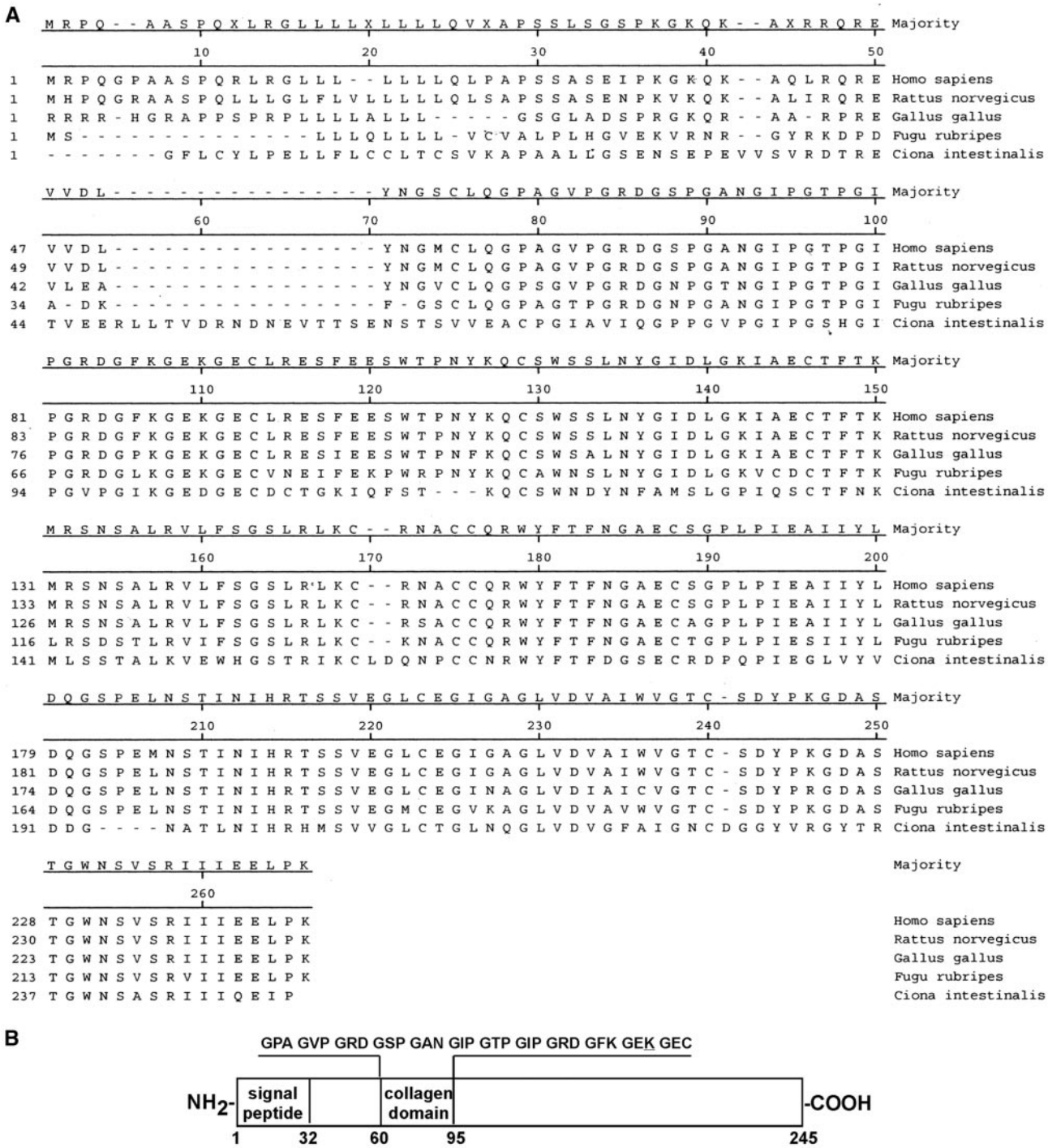

Figure 2. A, Alignment of CTHRC1 from various species. Sequences for *Gallus gallus*, *Fugu rubripes*, and *Ciona intestinalis* were assembled from various ESTs present in Genbank. Location of cysteine residues is conserved. B, Schematic of rat CTHRC1 protein showing location of the signal peptide and collagen domain. Potentially posttranslationally modified lysine residue (K) at the C terminal end of the collagen domain is underlined. Rat and human sequences were submitted to GenBank under the accession number NM172333 (AY136824) and AY136825.

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cells. Cthrc1 mRNA levels increased gradually in response to stimulation with BMP-4, with maximal levels seen at 24 hours after cytokine addition (Figure 4). Stimulation with TGF-β_1 also led to an increase in Cthrc1 mRNA levels with the highest levels observed 8 hours after growth factor addition (Figure 4). Platelet-derived growth factor (PDGF-AB) or fibroblast growth factor-2 (FGF-2) had no effect on Cthrc1 mRNA levels in NIH3T3 cells (data not shown).

Characterization of Recombinant Cthrc1

We performed in vitro translation with a rat Cthrc1 cDNA clone and the translated protein was resolved by 12% SDS-

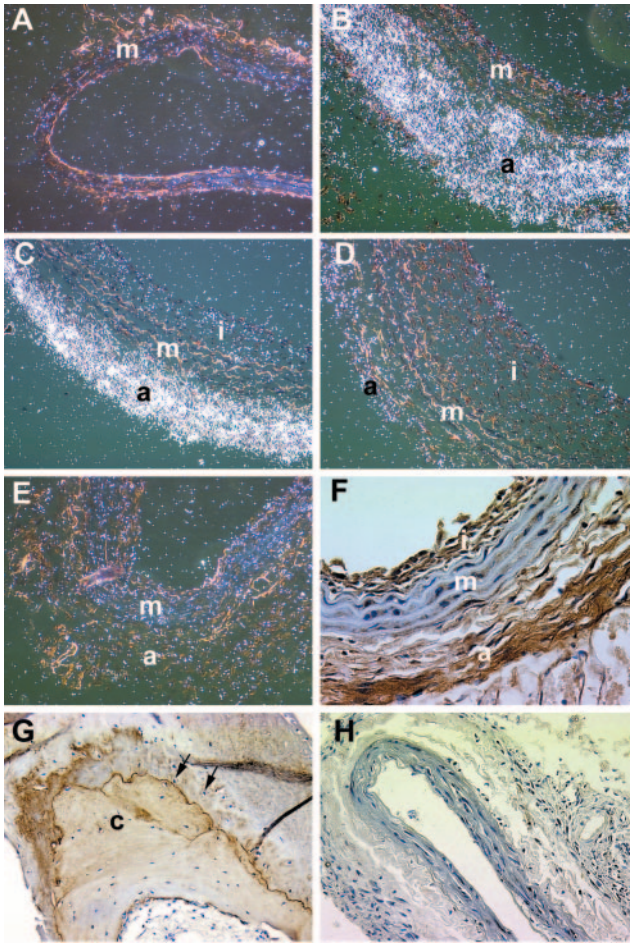

Figure 3. Expression of Cthrc1 in arterial tissues. Photomicrographs of in situ hybridization performed with ^{35}S-UTP labeled Cthrc1 antisense (A through D) and sense (E) riboprobes are seen under dark field illumination with silver grains appearing as white specks. Immunostaining with anti-CTHRC1 antibody (F and G) and preimmune serum (H) are shown. A, In the uninjured rat carotid artery, no Cthrc1 expression was detectable in the media (m). B, 8 days after balloon injury, increased Cthrc1 expression is detectable throughout the adventitia (a) where fibroblast proliferation and matrix production are prominent at this time. C, Two weeks after injury, there is still abundant expression of Cthrc1 mRNA in the adventitia (a) and lower levels are also seen in the neointima (i). D, At 4 weeks after denudation, only scattered cells in the adventitia still express Cthrc1 mRNA. E, A section of a carotid artery 8 days after balloon injury was hybridized with a Cthrc1 sense probe for evaluation of nonspecific background hybridization. F, Immunostaining with anti-CTHRC1 antiserum shows immunoreactive CTHRC1 in the extracellular matrix of the adventitia (a) and neointima (i) with little staining in the media (m) of 8 day balloon injured vessels. G, Human atherosclerotic plaque shows CTHRC1 associated with the calcification (c) and the surrounding matrix with chondrocyte-like cells (arrows). H, Preimmune serum from the same rabbit showed absence of nonspecific staining. Nuclear counterstain with hematoxylin. Original magnification 200× (A through E), 400× (F), and 100× (G and H).

PAGE followed by autoradiography. A single specific band with an approximate molecular weight of 28 kDa (Figure 5A) was seen, and this band was absent from the reaction performed with the vector lacking a cDNA insert (Figure 5A).

A leucine-rich hydrophobic region is located at the amino terminus (amino acids 1 to 32 of rat CTHRC1, MH-

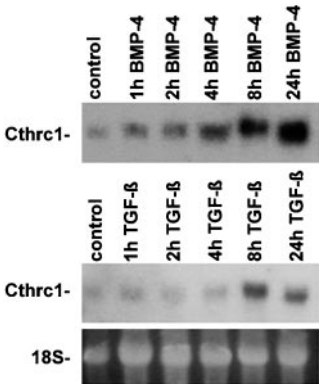

Figure 4. A, Northern blots of NIH3T3 cells stimulated with BMP-4 (10 ng/mL) and TGF-β (10 ng/mL) for indicated lengths of time were probed with a radiolabeled Cthrc1 cDNA.

PQGRAASPQLLLGLFLVLLLLLQLSAPSSAS) that serves as a signal sequence. This was verified by N-terminal protein sequencing of purified rat CTHRC1 expressed in CHO cells (data not shown). A short G-X-Y repeat domain (12 triplets)

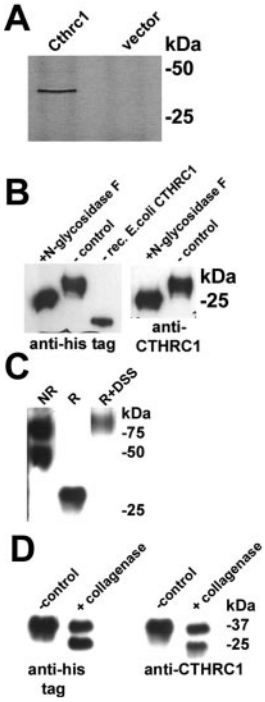

Figure 5. A, Using the rat Cthrc1 cDNA, in vitro translation was performed with the rabbit reticulocyte lysate method with ^{35}S-cysteine. Autoradiography showed a single protein band with an approximate molecular weight of 28 kDa, which was absent from the sample containing the empty vector. B, C-terminal myc-6xhis tagged CTHRC1 expressed in CHO cells had a reduced molecular weight after treatment with *N*-glycosidase F and immunoblotting with anti-his and anti-CTHRC1 antibodies (reducing conditions). Nonglycosylated CTHRC1 expressed in *E. coli* with only a 6xhis tag is shown for comparison. C, Immunoblots with anti-CTHRC1 antibody show CHO cell-derived CTHRC1 run under nonreducing conditions (NR) migrating as an apparent dimer and trimer; under reducing conditions (R) only the apparent monomeric forms is detected. Cross-linked CTHRC1 run under reducing conditions (*R*+DSS) showed only the trimer. D, Incubation of CTHRC1 with collagenase generates a C-terminal fragment that is detected with the anti-his antibody and the anti-CTHRC1 antibody.

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characteristic of collagens is located between amino acids 59 and 93 (Figure 2B). The molecule is further characterized by the presence of 10 cysteine residues, which amounts to a cysteine content of 4.7% in the mature protein. The position of the cysteine residues was conserved among species. Glycosylation was examined by treatment of his-tagged CTHRC1 purified from CHO cell conditioned medium with *N*-glycosidase F. *N*-glycosidase–treated CTHRC1 migrated with an apparent molecular weight decreased by approximately 4 kDa compared with untreated CTHRC1 (Figure 5B).

Immunoblotting of CHO cell–derived CTHRC1 with anti-CTHRC1 antibodies showed that under nonreducing conditions the recombinant CTHRC1 ran predominantly as a 50- and 75-kDa band (Figure 5C, lane 1, labeled NR). Under reducing conditions, however, the majority of the recombinant CTHRC1 migrated with an apparent molecular weight of 28 kDa as a single band (Figure 5C, lane 2, labeled R). Cross-linking of recombinant CTHRC1 with disuccinimidyl suberate (DSS) before reduction caused the majority of CTHRC1 to run with an apparent molecular weight of 84 kDa (Figure 5C, lane 3, labeled *R*+DSS).

The presence of a short collagen domain near the N-terminal portion of the mature protein is most likely responsible for the trimerization of the protein. This prompted us to examine whether CTHRC1 is susceptible to cleavage by collagenase. Recombinant CTHRC1 with a C-terminal his tag was incubated with highly purified collagenase and this generated a C-terminal fragment that was immunoreactive with the anti-his and anti-CTHRC1 antibodies (Figure 5D, apparent molecular weight approximately 20 kDa).

The localization of CTHRC1 to the extracellular matrix led us to investigate its effects on cell migration. PAC1 cells were stably transfected to generate CTHRC1 overexpressing cells and control vector transfectants. In addition, a stable antisense Cthrc1 transfected PAC cell line was established in which endogenous CTHRC1 levels were abolished (Figure 6A). It was interesting to note that the majority of endogenous CTHRC1 in PAC1 cells appeared to migrate in the form of two fragments with apparent molecular weights of 18 and 16 kDa (Figure 6A, arrowhead), whereas in CTHRC1-transfected PAC1 cells, full-length CTHRC1 (28 kDa) was the predominant form (Figure 6A, arrow). A scratch wound assay performed on confluent transfectants was used to determine the effect of CTHRC1 expression levels on cell migration. As shown in Figure 7A, migration and coverage of the scraped area was significantly increased in CTHRC1-overexpressing PAC1 cells compared with empty vector transfected PAC1 cells. This difference in migration was also observed when the same PAC1 transfectants were grown on collagen coated surfaces. The effect of increased CTHRC1 expression on cell migration was further verified using primary embryonic fibroblasts derived from wild-type and CTHRC1 transgenic mice and similar results were obtained (Figure 7B). These differences in cell migration were not attributable to differences in growth properties as growth curves established for the transfectants revealed no significant differences in cell growth (data not shown). Cell migration can be affected by large number of factors including alterations in extracellular matrix deposition. When we examined the PAC1 transfectants for levels of collagen type I, the predominant fibrillar collagen in these cells, it was found that mRNA levels for the α_1 and α_2 chain of collagen type I were reduced to 0.6% and 2%, respectively, to those observed in control PAC1 cells (Figure 6B). These dramatic differences in collagen type I mRNA levels were paralleled by equally dramatic differences in procollagen type 1 protein levels (Figure 6C). Procollagen type I levels were virtually undetectable both in the conditioned medium as well as cell lysates of CTHRC1-overexpressing PAC1 cells (Figure 6C).

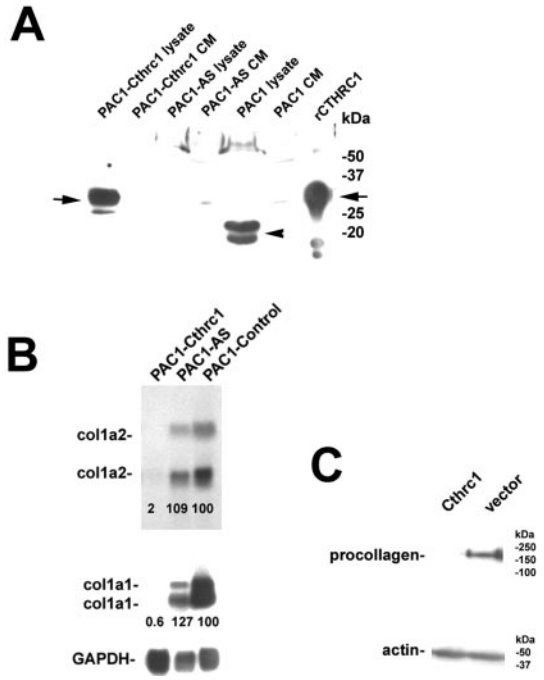

Figure 6. Overexpression of CTHRC1 in PAC1 cells inhibits collagen type 1 expression. A, CTHRC1 immunoblot analysis of conditioned medium (CM) and cell lysates prepared from PAC1 cells stably transfected with a Cthrc1 expression construct (PAC1-Cthrc1), an antisense Cthrc1 construct (PAC1-AS), and PAC1 control cells (PAC1al paral). Full-length CTHRC1 protein expressed in CHO cells (rCTHRC1) and PAC1-Cthrc1 cells is indicated with an arrow, whereas cleaved Cthrc1 is indicated with an arrowhead. Note the absence of CTHRC1 immunoreactive bands in the antisense Cthrc1 transfectants. B, Collagen type 1 mRNA expression was examined by Northern blotting. In CTHRC1 transfectants, levels of both the α1 (col1a1) and α2 (col1a2) chain transcripts were reduced to less than 2% of those observed in control cells. C, Immunoblotting with an anti-procollagen antibody demonstrates the absence of procollagen protein from lysates of confluent CTHRC1 overexpressing PAC1 cells compared with empty vector transfected cells. Equal loading of samples is demonstrated by anti-actin immunoblotting.

Discussion

Through suppressive subtractive hybridization, we have identified *Cthrc1* as a novel gene that is highly conserved among vertebrates and not found in invertebrates. The presence of a *Cthrc1* homolog in sea squirt, which is evolutionarily one of the first organisms to form a notochord during the larval stage, indicates that the evolution of the *Cthrc1* gene dates back at least 550 million years. Database searches have so far not revealed any other potential *Cthrc* family members. CTHRC1 is a glycosylated protein with a signal sequence,

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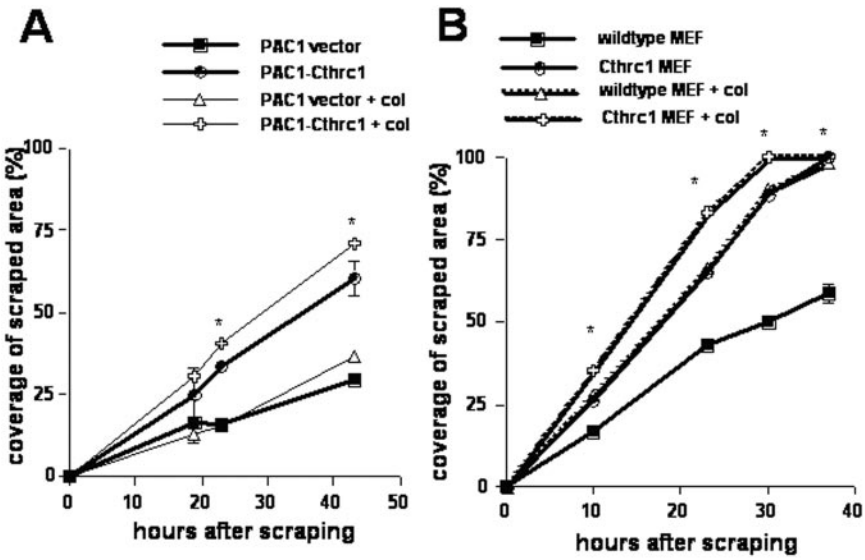

Figure 7. Scratch wound migration assay demonstrates increased migratory ability in (A) CTHRC1 overexpressing PAC1 smooth muscle cells (PAC1-Cthrc1) as well as (B) embryonic fibroblasts derived from transgenic mouse embryos overexpressing CTHRC1 (Cthrc1 MEF). Control PAC1 transfectants (PAC1-vector) and wild-type MEF served as controls. Values represent mean±SEM of measurements from three separate areas. *Statistically significant differences between PAC1 vector/wild-type MEF and the corresponding PAC1-Cthrc1/Cthrc1 MEF groups.

and this is consistent with the presence of CTHRC1 in the extracellular space of selected tissues. Although CTHRC1 is associated with the extracellular matrix and contains a short collagen-like motif, its transient expression in the vessel wall argues against a function as a structural protein unlike other members of the collagen family. Short collagen domains are also found in the C1q/tumor necrosis factor superfamily of proteins. Members of this family include EMILIN[13] and Acrp30/adiponectin with at least 7 functional paralogs,[14] termed C1q/tumor necrosis factor-α–related proteins (CTRPs). CTRPs are signaling molecules that all share the presence of a short collagen domain followed by a globular C1q domain located at the C terminus. CTHRC1 has a similar collagen domain; however, the conserved amino acids that define the C1q domain are not present in CTHRC1. Within the collagenous domain of adiponectin is the lysine containing motif GXKGE(D), where sequential hydroxylation and glycosylation of lysine residues occurs.[15] A similar motif is located within the collagen domain of CTHRC1 (GEKGEC, Figure 2B), and it is therefore likely that CHTRC1 undergoes a similar posttranslational modification; the deglycosylation experiments performed here (Figure 5B) are in agreement with this explanation. Cleavage experiments with collagenase support the notion that the collagen domain within CTHRC1 is responsible for triple helix formation and this is consistent with cross-linking studies of CTHRC1 (Figure 5C). As shown in Figure 5C, approximately only half of the CTHRC1 underwent cleavage by collagenase and this could potentially be explained by the fact that a similar portion of CTHRC1 in the sample is present as an apparent trimer, whereas the remainder represents a dimeric form (Figure 5C, lane 1) that would not be susceptible to cleavage by collagenase.

The transient expression of CTHRC1 in the vessel on injury and its presence in calcified atherosclerotic plaque indicate some similarities with other matrix proteins such as osteopontin. CTHRC1 resides in calcified atherosclerotic plaque particularly at the calcifying front which is the same location where osteopontin is found.[16] It was interesting to note that the chondrocyte-like cells surrounding the calcification also expressed CTHRC1 (Figure 3G). As was mentioned above, the adventitia is the major site of TGF-β signaling activity after vascular injury.[2] As Cthrc1 expression is inducible by TGF-β family members in fibroblasts, it was not surprising to see Cthrc1 expression induced mainly in the adventitial fibroblasts on injury.

Overexpression of CTHRC1 in PAC1 and MEF cells led to a significant increase in their migratory ability. Understanding the mechanism by which CTHRC1 increases cell migration requires further investigation. It is a possibility that the suppression of collagen type I matrix production by CTHRC1 is involved in this process, although migration on collagen-coated surfaces was increased in both control and CTHRC1 overexpressing cells. The nearly complete suppression of all collagen type I α chain transcripts is potentially of major therapeutic importance as this may provide an avenue to interfere with fibrotic processes that ultimately lead to organ failure and with regards to angioplasty to constrictive remodeling. The mechanism by which CTHRC1 inhibits collagen type 1 mRNA expression is presently not known and it raises the possibility that CTHRC1, similar to CTRPs, functions as a signaling molecule. Of interest was also the fact that the majority of endogenous CTHRC1 in PAC1 cells was present as a cleaved molecule. This observation indicates the CTHRC1 may undergo proteolytic processing and this in turn could have implications for the activity of the molecule.

In summary, this is the first report describing the novel gene *Cthrc1*, a highly conserved sequence among chordates. Our data indicate that this glycosylated protein is expressed at sites of tissue injury where it might participate in the regulation of collagen matrix deposition and cell migration. As a potent negative regulator of collagen matrix deposition, CTHRC1 may have therapeutic value in antifibrotic treatment strategies.

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