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Cloning, expression, and characterization of chicken transforming growth factor β4

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Abstract

Transforming growth factor β4 (TGF-β4) is unique to avian species, though its roles in vivo have not yet been well established. In this paper we describe the expression and partial characterization of recombinant chicken TGF-β4. By using a GC-rich PCR system in a modified 5′RACE methodology we generated the 5′-end of cDNA sequence encoding the TGF-β4 precursor, which was in-frame cloned into pcDNA3.1/V5-His-TOPO and transfected into the Chinese hamster ovary cell line (CHO-K1). A cell line stably expressing TGF-β4 precursor protein was established from CHO-K1 cells. Acid-activated mature TGF-β4 inhibited the growth of mink lung epithelial (Mv1Lu) cell line. TGF-β4 also stimulated the expression of type I procollagen and enhanced heat shock protein 47 (Hsp47) expression in chicken tendon fibroblasts. Hsp47 expression by TGFβ4 is likely regulated through activation of heat shock transcription factor 1 (HSF1). Because the presence of TGF-β1 has not been documented in avian cells and our data show that TGF-β4 elicits biological activities in chicken tendon cells, which closely parallel that of TGF-β1, we propose that TGF-β4 plays roles in avian species similar to what TGF-β1 plays in mammalian species.

Keywords: cDNA of TGF-β4 precursor; CHO-K1 cell line; pcDNA3.1/V5-His-TOPO; Hsp47 induction; HSF1 activation; Chicken tendon fibroblasts; Inhibition of Mv1Lu cell line

The transforming growth factor-β (TGF-β) family is a group of multifunctional cytokines that control growth, differentiation, and apoptosis of cells, and have important functions during embryonic development [1,2]. They are synthesized as large inactive precursor molecules containing a proteolytical cleavage site R–X–X–R which can be cleaved by extreme pH [3], increased temperature, and furin [4,5], or plasmin to release a carboxyl terminal peptide of 110–140 amino acids. The mature peptide contains 7–9 cysteine residues that are necessary for the assembly of biologically active dimers [6,7]. All members of this family share the Cys–X–Cys–X–COOH carboxyl terminus. The associated pro-protein region is called the Latency Associated Peptide. Latent TGF-β can also contain a protein of variable size called the Latent TGF-β Binding Protein [8]. Both peptides must be removed for TGF-β activation. Three isoforms, TGF-β1–3, are present in mammalian cells with sequence homology among them being greater than 65% and with a similar biological activity [9]. TGF-β is a potent chemoattractant for inflammatory cells and fibroblasts. It is an important regulator of the extracellular matrix (ECM) formation, stimulating collagen deposition, inhibiting ECM degrading proteases, and upregulating the synthesis of protease inhibitors [7].

Four avian TGF-β isoforms (1–4) have been described; TGF-β4 is unique to avian species [10]. Chicken TGF-β1 and TGF-β4 share 100% and 82% amino acid identity to human TGF-β1, respectively. The reported sequence of chicken TGF-β1 might have been contaminated with a portion of porcine TGF-β1 sequence during screening of the chicken cDNA library with porcine cDNA [11,12]. We have shown that human TGF-β1 exhibited only moderate stimulation of collagen synthesis in chicken tendon cells and have hypothesized that chicken TGF-β4 plays the role of mammalian TGF-β1 in avian species [13]. We obtained a complete cDNA

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sequence of TGF-β4 using the 5′RACE method and successfully expressed TGF-β4 in the CHO-K1 cell line. In vitro assays confirmed that this TGF-β4 is biologically active and that its activities are comparable to those of TGF-β1, an isoform whose presence in the avian system is uncertain. We, therefore, propose that TGF-β4 plays the role of TGF-β1 in avian species.

Materials and methods

Chicken tendon cell cultures. Chicken embryonic tendon fibroblasts were isolated from gastrocnemius tendons removed from 18-day old chicken embryos and grown in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) as described [14].

Chicken fibroblast cDNA library screening. The Uni-ZAP XR chicken fibroblast lambda cDNA library derived from a 9 to 10 day old chicken embryo was screened as recommended by the manufacturer (Stratagene, La Jolla, CA). The cDNA probes were based on the available sequence of chicken TGF-β4 cDNA (GenBank Accession No. M31160) using PCR DiG Probe Synthesis Kit (Roche–Boehringer–Mannheim, Indianapolis, IN) by two primers [forward primer: GACCTCGACACCGACTACTG (bp position 780–799); reverse primer: AAATCCTCTCGTTGTGGG (bp position 1194–1212)].

Plasmid construction and expression in bacteria. The PCR-amplified cDNA encoding the mature TGF-β4 protein was ligated in-frame into a pET-28 expression vector (Novagen, Madison, WI) which has 6× histidine (His-tag) attached to the amino terminus of the fusion protein. For recombinant protein expression, pET28-β4 constructs were transformed into the BL21 (DE3) strain of *Escherichia coli*. To express TGF-β4 in bacterial periplasmic space, pBAD/gIIIA expression vector (Invitrogen) and the bacterial strain TOP10 were used under the induction of L-arabinose. The periplasmic protein preparation was subjected to osmotic shock as recommended by the manufacturer.

Purification and refolding of TGF-β4 fusion protein from inclusion bodies. TGF-β4 fusion protein expressed in bacteria was purified using a Ni–NTA column (Invitrogen, Carlsbad, CA) under denatured conditions as recommended by the manufacturer. Amino acid sequencing by Edman degradation of TGF-β4 was done by the Molecular Genetics Instrumentation Facility at the University of Georgia. The refolding of fusion protein was done as described by Han et al. [15].

5′RACE method. We made two modifications to the methodology described in the 5′/3′RACE kit (Roche). One is that THERMOSCRIPT reverse transcriptase (Gibco–Invitrogen, Grand Island, NY) and random-hexamer primers were utilized for the reverse transcription. The other is that both the first and nested PCRs were performed using a GC-rich PCR system (Roche). mRNA was purified from total RNA using mRNA isolation kit (Roche). The two reverse primers [primer 1 (from 675 to 703 bp position) and primer 2 (from 72 to 99 bp position)] were based on the cDNA sequence of chicken TGF-β4 (GenBank Accession No. M31160). The second PCR product was ligated into pGEM-T vector (Promega, Madison, WI). The plasmids were screened with digoxigenin-dUTP labeled oligonucleotides (derived from bp position 1–35, GenBank Accession No. M31160). The sequences obtained from positive plasmids were analyzed with a Sequencer program (Gene Codes, Ann Arbor, MI). mRNA structures were analyzed with the MFOLD program (Version 3.1; http://bioinfo.math.rpi.edu/ mfold/rna/form1.cgi).

Establishment of the TGFβ-4 precursor-producing CHO-K1 cell line. We generated the 5′end of chicken TGF-β4 cDNA (GenBank Accession No. AF395834) using the 5′RACE method. The complete open reading frame of cDNAs was produced by RT-PCR. The following primers were used: forward CCCATGGATCCGTCGCCGCTGCTG and downstream GCTGCACTTGCAGGCACGGACC. cDNAs were in-frame cloned into the expression vector pcDNA3.1/V5-His-TOPO. Constructed plasmid DNA (5 μg) was introduced into CHO-K1cells using 30 μg liposome (NovaFECTOR, VennNova, Pompano Beach, FL) and selected in F10-nutrient mixture (Ham's) medium with 10% FBS and 600 μg/ml G418 (Geneticin). Serum-free media (15 μg protein aliquots) conditioned by individual positive clones were harvested and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with a goat polyclonal anti-human TGF-β1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody was developed to the carboxy terminus of TGF-β1 and did not cross react with either TGF-β2 or 3. Recombinant TGF-β4 (rTGF-β4) from conditioned medium was concentrated using Centricon plus-80 (Amicon Bioseparations, Millipore, Bedford, MA), purified using ProBond Resin column (Invitrogen) under native conditions, and then activated by dialysis against 0.004 M HCl for 24–48 h.

Northern blotting. Total RNA was extracted from confluent cell cultures, kept in serum-free DMEM for 24 h, fractionated in a 1.5% agarose gel (5 or 10 μg per lane), and transferred to a nylon membrane. Blots were prehybridized in Dig Easy Hyb solution (Roche) for 1 h at 68 °C and hybridized overnight at 68 °C in Dig Easy Hyb containing indicated probes. The signal was detected with the NBT/BCIP or CSPD (Roche) system. Relative transcription was normalized with 18S or 28S rRNA, or hybridized with chicken β-actin RNA probe. Both methods gave closely corresponding results. Each experiment was performed at least twice.

Probe preparation. The complete chicken Hsp47 (GenBank Accession No. X57157), β-actin (GenBank Accession No. L08165), and partial type I procollagen α1(I) (GenBank Accession No. J00836) cDNA sequences were used to design primers utilizing Generunner program (Hastings Software): Hsp47, forward primer 5′-ATCCAA CGTCTTCCATGCC-3′ (bp position 1062–1080) and reverse 5′-AAT CCCCCCCTAAAAAACAC-3′ (1304–1323); type I procollagen α1(I), forward 5′-AACGAGATCGAGATCAGG (1207–1226) and reverse 5′-TTACTCTCTCCTGTCACGC (1477–1496) primers. Chicken β-actin, forward 5′-GCTACGTCGACATGGATTTCG (718–738) and reverse 5′-TAGAAGCATTTGCGGAC (1176–1195) primers. Dig-labeled mRNA probes were produced by Digoxigenin-RNA Labeling Kit (Roche–Boehringer–Mannheim). Labeling efficiency was confirmed by a dot-blot test.

Mv1Lu cell proliferation inhibition assay. The mink lung epithelial cell (Mv1Lu) growth inhibition assay was used to test TGF-β4 biological activity [16]. Mv1Lu cells were plated at a density of 1×10^4 cells/well (24-well plate) in DMEM with 10% FBS and grown overnight at 37 °C. The cells were incubated with TGF-β for 48 h in serum-free medium, trypsinized, and counted. The activity of rTGF-β4 was compared in this and the other assays with the biological activity with human TGF-β1 extracted from platelets (R&D Systems, Minneapolis, MN).

Western blotting. Cell cultures were lysed in a Nonidet P-40 lysis buffer. For heat shock factor 1 (HSF1) detection nuclear protein was extracted as described [17]. Protein aliquots (15 μg) transferred from 12% SDS–polyacrylamide gels onto nitrocellulose membranes were incubated with either the mouse monoclonal anti-Hsp47 antibody (StressGen Biotechnologies, Vancouver, BC, Canada) or the anti-human HSF1 polyclonal antibody (Santa Cruz). Antigen–antibody complexes were detected using the ABC Kit (Vector Laboratories, Burlingame, CA).

Results

cDNA library screening

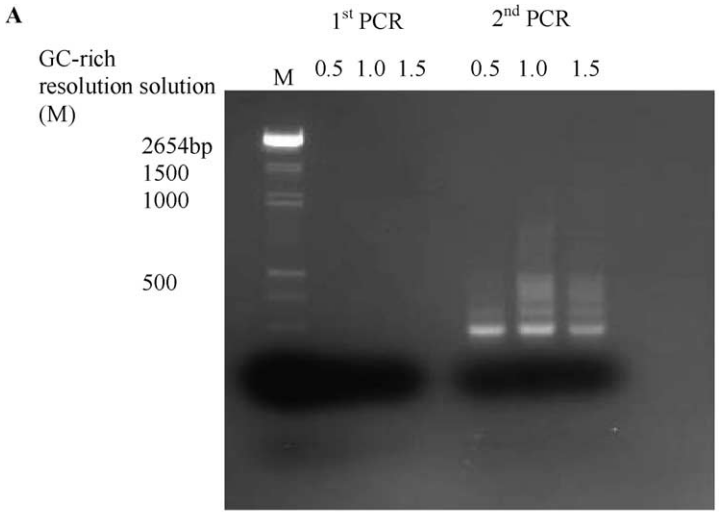
The screening of the chicken fibroblast λ cDNA library yielded 60 positive clones. Digestion with *Eco*RI and *Xho*I enzymes determined the correct insert size.

The sequencing of positive plasmids containing inserts larger than 500 bp showed that most of the inserts represented fragments of chicken TGF-β3. None of the sequences aligned with sequences of any of the three other TGF-β isoforms (1, 2, or 4).

Recombinant chicken TGF-β4 expressed in E. coli lacks biological activity

Next we used RT-PCR methodology of Han et al. [15] to generate cDNA encoding the mature TGF-β4 protein with *Nde*I and *Xho*I restriction sites. After ligation of the cDNA into the pET-28 vector, the pET28-TGF-β4 plasmid was transformed into the BL21 (DE3) strain of *E. coli*. In the presence of 1 mM IPTG, the level of rTGF-β4 expression approached 40% of cellular protein content (data not shown). Since this recombinant protein contains His-tag, we used Ni–NTA column to purify it. Bound TGF-β4 was eluted using a stepwise gradient elution with imidazole. The presence of TGF-β4 in preparations and fractions was determined by SDS–PAGE and by Western blotting using the human TGF-β1 polyclonal antibody (data not shown). The solubilized protein was diluted (<100 μg/ml) to prevent precipitation, dialyzed against buffer containing 0.1 mM dithiothreitol for 3 h, and continued to dialyze without dithiothreitol for three more hours. For refolding or disulfide bond formation, the sample was dialyzed against a redox system. Dimerization was confirmed by non-reduced SDS–PAGE (data not shown). The fusion protein was digested with thrombin to remove the His-tag. N-terminal sequencing revealed H–M–D–L–D–T–D–Y–C–F–G–P–X–T–D–E–K–N–X–X sequence ("X" = weak or undetermined signal). The first two amino acids H and M came from the plasmid and the remaining sequence corresponded to the predicted amino acid sequence of chicken TGF-β4 [12,18]. The rTGF-β4 expressed in bacteria displayed little inhibition of proliferation of Mv1Lu cells or stimulation of procollagen α1(I) mRNA expression in chicken tendon fibroblasts as determined by Northern blotting (data not shown). We hypothesized that rTGF-β4 synthesized by *E. coli* did not fold properly.

Because formation of disulfide bonds occurs during folding of proteins in the endoplasmic reticulum of



B

1 CTGGGGGTGGTGGGGGTGTCCCATACCATTTAGAGAAGACCCCCCCGCCCC

52 CCCTCACACTTAATGGGGAACCCCGTTGGATTGAAGGGGGAATCCCTCTGC

103 GTTAAAGGGGGAATCCCGCAGCCGCGCCTACGGGGGATCCCGGCCCCTTTG

154 CGGGGGGGGAGGGGT CCCGGTGCCCCATCGGAGTTATTTGGGGGGGGGGTC

206 CCTCCGCCCCCC CCCATGGATCCGTCGCCGCTGCTGGCGCTGCTGCTGCTGC

257 TGCTGGGGGCCGCC

Fig. 1. Recovered sequences of TGF-β4 cDNA in the 5′ end. The 5′RACE methodology is described in Materials and methods. (A) Ethidium bromide staining of first and second PCR products obtained using the GC-rich system and separated in an agarose gel. First lane on the left: markers; 0.5, 1, and 1.5 M: molarity of the GC-rich resolution solution. (B) The newly recovered 271 oligonucleotides contained several poly-G or C stretches and ATG initiation site (underlined).

eukaryotes and the periplasmic space of prokaryotes, we then chose a modified *E. coli* system enabling protein secretion into the periplasmic space. To facilitate the secretion of rTGF-β4 into the periplasmic space cDNA encoding mature chicken TGF-β4 was in-frame cloned into pBAD/gIIIa plasmid and expressed in the bacterial strain TOP10. TGF-β4 was expressed in bacterial cytoplasm as confirmed by SDS–PAGE. However, even under osmotic shock conditions TGF-β4 was not secreted into the periplasmic space (data not shown).

Generation of 5′end of TGF-β4 cDNA using 5′RACE method

Chicken mRNA (25 μg) was purified from the total RNA isolated from embryonic chicken tendon cells. Though it was not possible to visualize the products from the first PCR, the second PCR, in which the first PCR products were used as templates, showed a strong band of 250 bp in size and several weak bands of about 300 or 400 bp in size (Fig. 1A). All bands in each lane were excised and pooled. cDNA was purified from this pooled material and cloned into the pGEM-T vector. Southern blotting of plasmid cDNA from 10 positive colonies digested with *Apa*I and *Pst*I showed that all 10 plasmids were positive (data not shown). Sequence alignment analysis revealed the presence of an additional, previously not sequenced, region of 271 nucleotides in the 5′end of cDNA of chicken TGF-β4 (Fig. 1B) that included the ATG initiation site. The composition of this new region was about 70% GC bases. The complete open reading frame (ORF) of the chicken TGF-β4 is shown in Fig. 1B, together with the partial chicken TGF-β4 sequence (GenBank Accession No. M31160). Analysis by the MFOLD program predicted the formation of multiple stem-loop secondary structures in the mRNA in this region. To verify that this sequence was an integral part of the TGF-β4 cDNA, a forward primer was designed from the middle of the newly sequenced region. A specific band (1173 bp) was obtained using this forward primer and a reverse primer derived from the downstream region (data not shown).

Establishment of the TGF-β4 precursor-producing CHO-K1 cell line

cDNA encoding the chicken TGF-β4 precursor protein was in-frame cloned into pcDNA3.1/V5-His-TOPO (pcDNA3.1-β4), and pcDNA3.1-β4 was transfected into CHO-K1 cells with liposome [19,20]. Sixteen colonies of transfected CHO-K1 cells expressed the chicken precursor TGF-β4 molecule (about 55 kDa). TGF-β4 expression was verified by Coomassie blue staining (Fig. 2A, lanes 3–8) and Western blot analysis of the conditioned medium (Fig. 2B, lanes 3–8). Only transfected cells expressed TGF-β4 as no immunoreactive band was

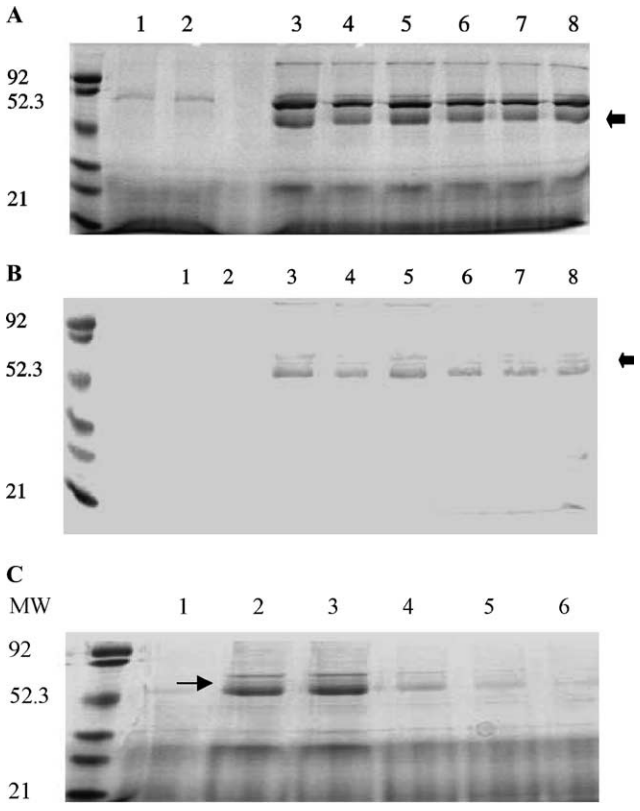


Fig. 2. CHO-K1 cell line expressing TGF-β4 precursor. Plasmid construction and positive colony selection are described in Materials and methods. Chicken TGF-β4 precursor cDNA was in-frame cloned into pcDNA3.1/V5-His-TOPO, transfected into CHO-K1 cells, and selected by G418. Protein aliquots (15 μg) were separated by SDS–PAGE. (A) Coomassie blue staining showed an additional band (←) in extracts from selected colonies (lanes 3–8), but not in extracts from non-transfected cells (lanes 1 and 2). (B) Immunoblotting using TGF-β1 antibody confirmed the presence of TGF-β4 in transfected colonies (←, lanes 3–8), but not in untransfected cells (lanes 1 and 2). (C) rTGFβ4 (→) was enriched on a Probond Resin column and eluted with the gradient of imidazole (lanes 2 and 3).

detected by Western blotting in non-transfected cells (Fig. 2B, lanes 1 and 2). rTGFβ4 was purified from conditioned medium using Probond Resin Column under native conditions (Fig. 2C, lanes 2 and 3). TGF-β4 was activated by pH 1.5 for 48 h or by furin treatment and it was biologically active. TGF-β4 strongly inhibited the proliferation of Mv1Lu cells (Fig. 3A). Its stimulation of type I procollagen α1(I) mRNA expression in chicken tendon fibroblasts was quite strong (Fig. 3B), approximately 60% higher than that of human TGF-β1 [13].

Recombinant chicken TGF-β4 stimulates HSP47 expression and activates HSF1 in chicken tendon fibroblasts

Because our previous data indicated that human TGF-β1 stimulated chicken Hsp47 expression at both mRNA and protein levels, and activation of HSF1 [13]

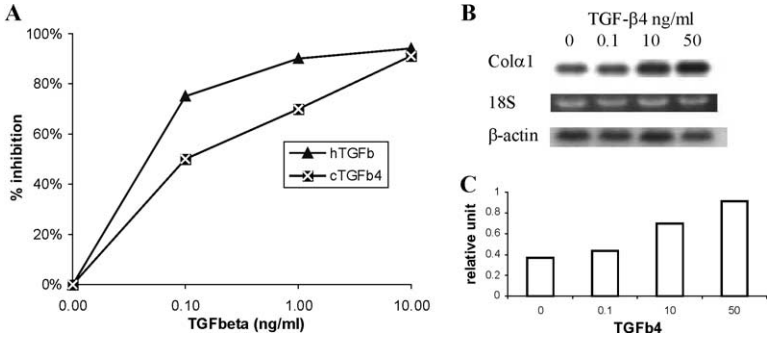


Fig. 3. Activity of chicken rTGF-β4. (A) rTGF-β4 inhibited the growth of Mv1Lu cells in an assay described in Materials and methods. Human TGF-β1 was used as a positive control. (B) rTGF-β4 stimulated procollagen mRNA expression in chicken tendon cells in a dose-dependent manner as shown by Northern blotting and a graph obtained by densitometric conversion of the Northern blot (C). 18S tRNA and a β-actin probe were used as internal controls. cTGF-β4, chicken TGF-β4; hTGF-β1, human TGF-β1; and Colα1, procollagen α1(I) mRNA.

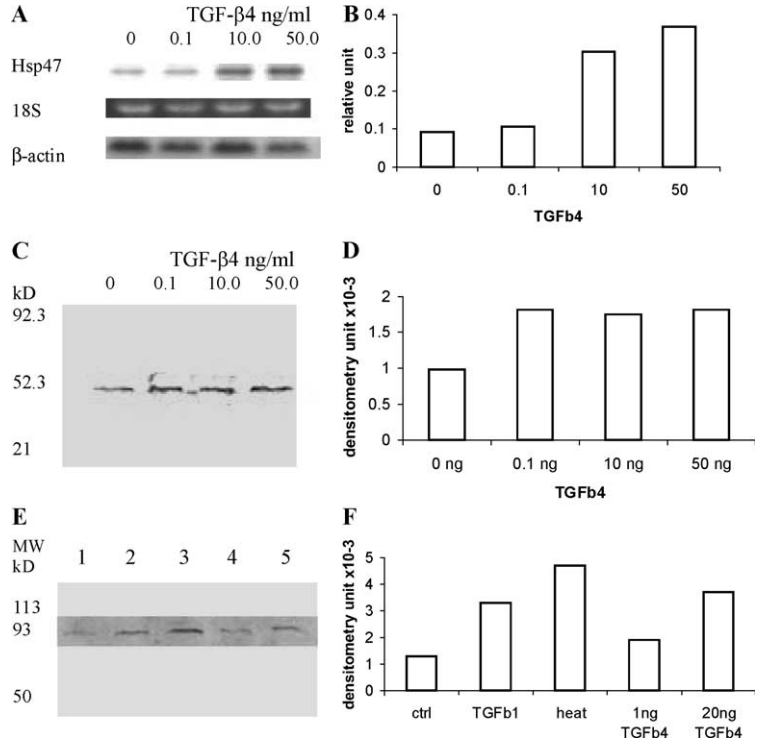


Fig. 4. Northern blot (A) and a graph (B) obtained by densitometric conversion of the Northern blot showed rTGF-β4 stimulation of Hsp47 mRNA expression in chicken tendon cells. 18S tRNA and a β-actin probe were used as internal controls. For Western blotting equal aliquots (15 μg) of extracted protein were loaded. rTGF-β4 increased the Hsp47 level in chicken tendon fibroblasts as determined by Western blotting (C) and a graph obtained by densitometric conversion of the Western blot (D). rTGF-β4 induced HFS1 in chicken tendon cells as determined by immunoblotting (E) and a graph obtained by densitometric conversion of the immunoblot. Lane 1, control; lane 2, human TGF-β1; lane 3, 43 °C for 3 h; and lanes 4 and 5, 1 ng and 20 ng/ml rTGF-β4 for 24 h, respectively.

we examined whether rTGF-β4 would exhibit the same activity in chicken tendon cells. The addition of rTGF-β4 to the quiescent cultures of chicken tendon cells increased Hsp47 mRNA expression and Hsp47 protein synthesis in a dose-dependent manner (Figs. 4A–D). Western blot analysis also showed that chicken TGF-β induced an increase of HSF1 protein in the cell nuclear preparation and that this increase was dose-dependent (Figs. 4E and F, lanes 4 and 5).

Discussion

In this study we sequenced complete cDNA of TGF-β4 precursor, expressed rTGF-β4 in a mammalian system, and partially characterized its biological activities. Because this TGF-β isoform was not only commercially unavailable, but also its cDNA sequence was not fully elucidated [12], it was necessary to obtain the full cDNA of TGF-β4 first before studying its biological activity.

Following the example of Han et al. [15] who succeeded in the expression of biologically active human TGF-β2 fusion protein in *E. coli*, we expressed mature TGF-β4 in *E. coli*. In spite of the formation of dimers this TGF-β4 lacked biological activity, most likely due to faulty formation of disulfide bridges in the reducing cytosol of bacterial cells. Alternatively, we tried to express mature TGF-β4 in bacterial strain (TOP10) with pBAD/gIIIA vector [21]. However TGF-β4 was not secreted into the periplasmic space, even after osmotic shock. It is likely that the presence of the precursor protein of TGF-β4 and/or Latent TGF-β Binding Protein is necessary to facilitate secretion of this protein [22,23].

Biologically active human TGF-βs have been successfully expressed in CHO cells or insect cells [19,20]. Only a partial chicken TGF-β4 cDNA gene sequence (GenBank Accession No. M31160) was available, lacking the 5′end in its open reading frame. We successfully generated the missing 5′end of this gene using the modified 5′RACE. Due to the rich GC content in the 5′end of this gene we did not obtain any PCR results using standard reaction conditions. We, therefore, utilized the THERMOSCRIPT reverse transcriptase, which is an avian RNase H-minus one and is engineered for higher thermal stability and the GC-rich PCR system from Roche–Boehringer–Mannheim that contains other organic solvents besides DMSO. The sequence alignment analysis showed that the 5′end of the TGF-β4 precursor gene contains 70% GC with several poly-G or C or GC stretches. The possibility of formation of multiple stem-loops by TGF-β4 precursor mRNA as predicted by MFOLD program would explain the failure of conventional reverse transcription enzymes to read through this region and the failure of a standard PCR protocol to amplify the corresponding cDNA [24].

Our last attempt consisting of in-frame cloning of TGF-β4 precursor cDNA into pcDNA3.1/V5-His-TOPO led to the successful establishment of CHO-K1 cell line expressing the TGF-β4 precursor. The molecular mass of ~55 kDa (plus tag) of this fusion protein is in line with the molecular mass (52.5 kDa) of human TGF-β 2 precursor protein expressed in CHO [18]. The biological activity of mature TGF-β4 was comparable to that of human TGF-β1 in several assays in chicken tendon fibroblasts and in mink lung epithelial cells. The attached His-tag did not hinder the activity. Most likely this active TGF-β represents a TGF-β4 homodimer as Western blotting indicated undetectable levels of TGF-β1 in non-transfected CHO cells.

Induction of Hsp47, a molecular chaperone facilitating the folding and assembly of procollagen molecules [25], by TGF-β1 has been previously reported in several cell systems [13,26–28]. TGF-β1 also induces trimer formation of HSF1 which in turn enhances Hsp47 expression [28]. We demonstrated that rTGF-β4 enhanced Hsp47 protein and mRNA expression, and HSF1 protein level in chicken embryo tendon fibroblasts.

It is of interest that the presence of TGF-β1 has not been documented in avian cells. TGF-β1and TGF-β4 show 82% identity in their amino acid sequences and contain the active motif WXXD in their mature region (human TGF-β1 WSLD versus chicken TGF-β4 WSAD) [18]. The biological activity of TGF-β4 comparable to TGF-β1 raises the possibility that chicken TGF-β4 may play roles in avian species similar to roles of TGF-β1 in mammalian species. Our findings are bolstered by Jakowlew et al. [10] claim that TGF-β4 together with TGF-β3 is the main TGF-β isoform expressed in chicken embryo fibroblasts and chondrocytes. Future experiments are needed to determine whether TGF-β4 binds to the same receptor system as TGF-β1 and what its other biological roles are.

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