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Demonstration of the interaction of transforming growth factor beta 2 and type X collagen using a modified tandem affinity purification tag

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ABSTRACT

Like other members of the transforming growth factor beta (TGF-β) family of growth factors, the biological activity of TGF-β2 is believed to be regulated by the formation and dissociation of multiprotein complexes. To isolate the molecular complex formed by TGF-β2 secreted by hypertrophic chondrocytes we have used expression of TGF-β2 fused with the humanized, tandem affinity purification (hTAP) tag and mass spectrometry for the identification of interacting proteins. The hTAP synthetic gene was assembled by systematically replacing the rare codons of the original TAP tag with codons most preferred in highly expressed human genes to circumvent the poor translation efficiency of the original TAP tag in animal cells. TGF-β2 was shown to interact with Type X collagen and this interaction confirmed using V5 tagged TGF-β2. Functional interaction was suggested by the inhibition of TGF-β2 activity by type X collagen in culture and the influence of a mutation in type X collagen on the distribution of TGF-β2 in growth cartilage.

1. Introduction

Although the active forms of TGF-β1, TGF-β2 and TGF-β3 have very similar molecular structures and appear capable of stimulating widely overlapping effects in cell and organ culture they have distinct functions in vivo that appear to result from a combination of cell-specific expression and differences in protein interactions [1]. The TGF-β isotypes are synthesized as large inactive precursor proteins that are proteolytically processed in the Golgi into the active TGF-β peptide and an aminopropeptide (the latency associated peptide, LAP) that remains non-covalently linked. This complex, termed the small latent complex, associates both covalently and non-covalently via the LAP region with a variety of binding proteins. Although the mature TGF-β1, TGF-β2 and TGF-β3 are highly homologous, the sequences corresponding to their LAP regions are more divergent [2] and might provide differences in protein interaction that account for their functional differences. Although all transforming growth factor beta (TGF-β) isotypes have been shown capable of binding a family of latent TGF-β binding proteins (LTBPs) [3] the molecular interactions of TGF-β2 and TGF-β3 and the molecular composition of the large latent complexes formed by these isotypes remains largely unknown.

Tandem affinity purification (TAP) was successfully used to purify a large number of multiprotein complexes in yeast [4] and offers a promising approach to the isolation and analysis of large latent complexes formed by TGF-β isotypes. The TAP tag consists of a calmodulin binding peptide (CBP) fused via a specific tobacco etch virus (TEV) proteinase recognition sequence to the second affinity peptide; two IgG-binding domains of the protein A gene from *Staphylococcus aureus*. Isolation of protein complexes containing the TAP-tagged target protein was achieved by sequential IgG and calmodulin affinity purification. Close examination of the nucleotide sequences of the originally described TAP fusion tag cassette showed it contained many rare codons that should result in severely attenuated expression in cells of higher vertebrates. The success of codon optimization for enhanced expression of GFP and several other proteins prompted us to employ a similar strategy to enhance expression of the TAP-tagged proteins. Here we describe construction of the humanized TAP tag gene, termed hTAP. It was chemically synthesized from a set of long oligonucleotides with silent base mutations introduced according to the preferred codons of highly expressed human proteins.

The process of endochondral bone formation from a cartilaginous anlagen in the embryo or at the growth plate in the juvenile involves an ordered process of chondrocyte terminal differentiation

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encompassing chondrocyte division, hypertrophy and apoptosis prior to extensive cartilage dissolution and formation of trabecular bone and bone marrow [5]. Endochondral bone formation is complex and requires the co-ordination of many signaling pathways to maintain the cartilage architecture and generate the proper shape, length and structure of bones. TGF-β2 is one of several growth factors essential for this process [6]. It fills a multifunctional role that may link diverse local signaling pathways that regulate cartilage, bone and blood vessel formation and function. TGF-β2 is expressed by chondrocytes at a late stage of hypertrophic differentiation in culture [7,8] and can be detected in the last few hypertrophic chondrocytes of growth plates adjacent to the metaphyseal vasculature [9]. TGF-β2 inhibits chondrocyte hypertrophic differentiation, apparently by participating with parathyroid hormone-related peptide (PTHrP) in a common signal cascade [10,11]. TGF-β2 (in common with other TGF-β isotypes) is a potent inducer of both bone deposition by osteoblasts and bone turnover by osteoclasts [12] acting as a central component coupling bone formation to bone resorption [13]. TGF-βs also play a major role in vessel morphogenesis and maintenance of the vessel wall [14].

We report expression of the chick TGF-β2 fused with the hTAP tag and use of the tandem affinity purification strategy for isolation of TGF-β2 in combination with interacting proteins from the culture medium of hypertrophic chondrocytes. Analysis of the proteins that co-purify with TGF-β2 demonstrated the interaction of TGF-β2 with type X collagen. Reciprocal tagging experiments in cell culture confirmed the interaction. Functional interaction of type X collagen and TGF-β2 was also indicated by inhibition of TGF-β2 activity by type X collagen and the abnormal tissue distribution of TGF-β2 in growth cartilage from a patient expressing a mutation in type X collagen.

2. Experimental procedures

2.1. Codon-optimization

The TAP tag gene cassette originally used for expression in yeast contains many rare codons, which are not optimized for expression in mammalian or chick cells. For example, the TAP tag gene contains 17 of 18, 11 of 14 and 13 of 19 rare codons for leucine, glutamine and lysine residues (Table 1). We redesigned the DNA sequences of the TAP tag for efficient expression in mammalian systems and assembled the full-length TAP tag sequences using chemically synthesized long oligonucleotides with codons present in human highly expressed genes using PfuTurbo DNA polymerase (Table 1 supplementary material; Table 1; Fig. S1). Due to the repeat sequences within the IgG-binding domains, the hTAP had to be assembled in three consecutive steps. The CBP domain and TEV cleavage site were generated by two consecutive PCR amplifications with PfuTurbo DNA polymerase using long oligonucleotides TAPP2S and TAPP2A followed by use of primers TAPP1S and TAPP2A2. IgG binding domain one was amplified and cloned using long oligonucleotides TAPP3S and TAPP3A followed by primers TAPP3S2 and TAPP3A2. IgG domain two was amplified by using the cloned IgG domain one as template with primers TAPP4S and TAPP4A and cloned. A minimum of 12 clones of each fragment were sequenced. We have noticed that long oligonucleotides generated high rates of undesired mutations, particularly small deletions and point mutations within the fragments cloned. About one out of eight clones had correct sequences and were used to generate the full-length hTAP cassette. The CBP/TEV fragment was first ligated to the IgG binding domain one and amplified using TAPP1S and TAPP3A2 oligonucleotide. The PCR product was cloned and again sequenced. The correct fragment was cut out by digestion with SalI and ligated

Table 1
Codon usage of the original TAP tag and the optimized or humanized TAP tag (hTAP) compared with the optimized enhanced green fluorescent protein (EGFP) and the highly expressed (High) human genes. The most preferred codons are shown in bold.

Amino acid	Codon	Original TAP[a]	hTAP[a]	EGFP[a]	High (%)[b]
Ala	GCA	4	0	0	13
	GCC	5	**25**	**8**	**53**
	GCG	**8**	0	0	17
	GCT	**8**	0	0	17
Arg	AGA	**2**	0	0	10
	AGG	0	0	0	18
	CGA	**2**	0	0	6
	CGC	1	**4**	**6**	**37**
	CGG	0	1	0	21
	CGT	0	0	0	7
Asn	AAC	**15**	**20**	**13**	**78**
	AAT	5	0	0	22
Asp	GAC	5	**11**	**16**	**75**
	GAT	**6**	0	2	25
Gln	CAA	**11**	0	0	12
	CAG	3	**14**	**8**	**88**
Glu	GAA	**10**	0	1	25
	GAG	4	**14**	**15**	**75**
Gly	GGA	0	0	0	14
	GGC	0	**2**	**19**	**50**
	GGG	**2**	**2**	3	24
	GGT	**2**	0	0	12
His	CAC	1	**3**	**9**	**79**
	CAT	**2**	0	0	21
Ile	ATA	1	0	0	5
	ATC	**5**	**7**	**12**	**77**
	ATT	1	0	0	18
Leu	CTA	2	0	0	3
	CTC	1	5	3	26
	CTG	0	**13**	**18**	**58**
	CTT	**4**	0	0	5
	TTA	**10**	0	0	2
	TTG	1	0	0	6
Lys	AAA	**13**	0	1	18
	AAG	6	**19**	**19**	**82**
Phe	TTC	**7**	**9**	**12**	**80**
	TTT	2	0	0	20
Pro	CCA	**3**	0	0	16
	CCC	0	**7**	**10**	**48**
	CCG	**2**	0	0	17
	CCT	**2**	0	0	19
Ser	AGC	**5**	**10**	**7**	**34**
	AGT	2	0	0	10
	TCA	3	0	0	5
	TCC	3	0	3	28
	TCG	0	0	0	9
	TCT	0	0	0	13
Thr	ACA	0	0	0	14
	ACC	2	**4**	**15**	**57**
	ACG	0	0	0	15
	ACT	2	0	1	14
Tyr	TAC	0	**4**	**10**	**74**
	TAT	**4**	0	1	26
Val	GTA	**2**	0	1	5
	GTC	1	1	4	25
	GTG	1	**3**	**13**	**64**
	GTT	0	0	0	7

[a] The numbers of degenerate codons used for the corresponding gene.
[b] The percentage of codons used in highly expressed human genes.

to the second IgG domain fragment and cloned. The final full-length 552 bp hTAP gene cassette was cloned into pZero-2 (Invitrogen, Carlsbad, CA) and confirmed by sequencing. To further enhance the expression of genes tagged with the hTAP tag, we also introduced the eukaryotic leader sequence from the adapter plasmid Cla12Nco (kindly provided by Dr. Steven Hughes, National Cancer Institute, Frederick, MD) immediately before the initiator ATG codon of the target fusion gene.

2.2. Plasmid construction

2.2.1. hTAP TGF-β2

The chick replication-competent viral vector RCASBP(A) and its adapter plasmid Cla12Nco were kindly provided by Dr. Steven Hughes (National Cancer Institute, Frederick, MD). The adapter vector pLaxIg15 was constructed by subcloning the murine Igk-chain V-J2-C signal peptide cDNA fragment of pSecTag2A vector (Invitrogen, Carlsbad, CA) into the NcoI site of Cla12Nco adaptor vector. The chick TGF-β2 cDNA was amplified for 26 cycles by PfuTurbo DNA polymerase using chick chondrocyte reverse-transcribed cDNA as template and a pair of primers; (c TGF-β2SAfl) 5′-GCC TTA AGC CGC CAT GCA CTG CTA TCT CCT GAG-3′ and (c TGF-β2AXh) 5′-CGC TCG AGG CTG CAT TTG CAA GAC TTT A-3′. The cloned cDNA was verified using ABI BigDye Cycle Sequencing Kits. To make the hTAP-tagged TGF-β2 construct, the hTAP tag cassette (described above) in pZero-2 was subcloned into pLaxIg15 as pLaxIghTAP adaptor vector. The TGF-β2 cDNA fragment was ligated into pLaxIghTAP such that the hTAP cassette was fused in frame to the C-terminus of TGF-β2 (Fig. S1). The final RCASBP(A)TGFβ2–hTAP construct was generated by subcloning the ClaI fragment of TGF-β2 tagged with hTAP from pLaxIgTGFβ2–hTAP into RCASBP(A) vector.

2.2.2. TAP TGF-β2

To create TGF-β2/TAP the TAP sequence from the plasmid pBS1479 [4] was fused at the C-terminal end of TGF-β2, creating a chimeric TGF-β2/TAP RCASBP(A) vector like that described for the hTAP tag.

2.2.3. Flag-tagged TGF-β2

The chick TGF-β2 full-length cDNA (cTGF-β2) was cloned into a pCDNA3.1(+)/FLAG vector as an NcoI and XhoI fragment in frame fused to the FLAG tag (Fig. 1). The TGF-β2 fused with the FLAG tag was subcloned into pLaxIg15 and subsequently into RCASBP(A), generating the RCASBP(A)TGFβ2-FLAG construct.

2.2.4. V5-tagged mature TGF-β2

Fusion of TGF-β2 with any peptide, including V5, FLAG or hTAP at the C-terminus resulted in loss of biological activity. To generate epitope tagged TGF-β2 that retained activity the V5 tag was inserted at the N-terminus of the mature peptide (Fig. 1C). The RCASBP(A) LaxIgβ2hTAP, previously described, was used as a template for PCR. Two-step PCR was used to insert the V5 tag into the N-terminal end of the mature TGF-β2. The set of primers used to amplify the LAP region were: (SlaxAsc1S) 5′-CGT GGC GCG CCT CTA GAC CAC TGT GGC CAG G-3′ and (V5cLAPA) 5′-GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC ACG CTT CTT CCG CCG A-3′. The set of primers used to amplify the mature TGF-β2 were: (V5betaS) 5′-CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG GCT CTA GAT GCT GCC TAT TGT T-3′ and (cBetaAPme) 5′-GCA CGT TTA AAC TTT AGC TGC ATT TGC AAG AC-3′. PCR reaction was performed for 19 cycles and the products were purified by agarose gel electrophoresis. Products were combined at a 1:1 molar ratio and used as templates for amplification using SLaxAsc1S and cBetaAPme. The PCR products were gel-purified by agarose gel electrophoresis and digested with AscI and PmeI. Finally a V5-tagged mature TGF-β2 expression construct was generated by ligating the digested PCR products to pCEP4 expression vector precut with AscI and PmeI. Sequences of the construct were verified using ABI BigDye Cycle Sequencing Kits.

2.2.5. Type X collagen hTAP

Type X collagen/hTAP fusion proteins were generated to confirm the interaction of type X collagen with TGF-β2. hTAP for N-terminal expression (NhTAP) was constructed to avoid potential effects on type X trimer formation that might be expected if a large protein tag were fused to the C-terminal non-helical region. The NhTAP was reconstructed for expression at the N-terminal of the target protein by ligating the IgG binding domains from the hTAP cassette to the TEV and CBP binding domains derived from a pREP-NTAP vector kindly provided by Dr. Kathleen Gould (Dr. Kathleen L. Gould, Vanderbilt School of Medicine, Nashville, TN). Subsequently the N-terminal hTAP was placed immediately after the signal peptide BM40 (derived from the vector pCEP4-BM40-his-ColX, provided by Dr. Danny Chan, University of Hong Kong, Hong Kong, China), such that the fusion protein is targeted for secretion (Fig. 1D). A Kozak consensus sequences derived from the SLAX 12 NCO adaptor vector was also placed before the BM40 signal peptide. The Lax-BM40-NhTAP was subcloned into the pCEP4 expression vector (Invitrogen, Carlsbad, CA) and termed pCEP4BM40NhTAP secretion expression vector.

2.2.6. Cell culture

Chick DF-1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, CRL-12203). The cells were cultured in Dulbeco's modification of Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 5% fetal bovine serum as suggested by ATCC. Primary chick embryo fibroblasts were produced from 10 day old chick embryos (Charles River SPAFAS Laboratory, Storrs, CT) which were incubated for 10 days as described by Morgan and Fekete [15] and cultured in standard DMEM containing 5% FBS. Hypertrophic chondrocytes were isolated from the cephalic region of 18 day old chick embryo sterna (Charles River SPAFAS Laboratory, Storrs, CT) by collagenase digestion and cultured in DMEM as described previously [7].

2.2.7. Transfection and viral infection

DF1 fibroblasts or primary chick embryo fibroblasts were transfected using the Lipofectamine plus reagent according to the procedure recommended by the supplier (GIBCO-Life Technologies, Grand Island, NY). When replication competent RCASBP viral constructs were employed viral particles isolated from the culture medium of transfected fibroblasts were collected and used to infect freshly isolated chick embryo chondrocytes [16]. After overnight incubation, the cells were cultured for a further 5 days and assayed for viral production by immunostaining with anti gag monoclonal antibody (3C2, DSHB, Iowa, IA) to detect RCAS virus or with a peroxidase anti peroxidase complex (PAP Sternberger Monoclonals, Lutherville, MA) that detects the protein A component of the hTAP protein [17]. To monitor expression of the fusion transgene products and the processing of tagged proteins, conditioned media from viral-infected or transfected cells were harvested and concentrated using trichloracetic acid precipitation. Cultured cells were scraped from the plates and cell pellets lysed in SDS-sample buffer.

2.2.8. Purification of TGF-β2 complex

50 ml of conditioned medium from hypertrophic chondrocytes expressing hTAP tagged TGF-β2 was concentrated 5-fold using ultrafiltration (Millipore Life Science, Bedford, MA). TGF-β2 and interacting proteins were isolated according to the method described by Rigaut et al. [4] with the following modifications. 10 ml concentrated culture medium was cleared by ultracentrifugation at

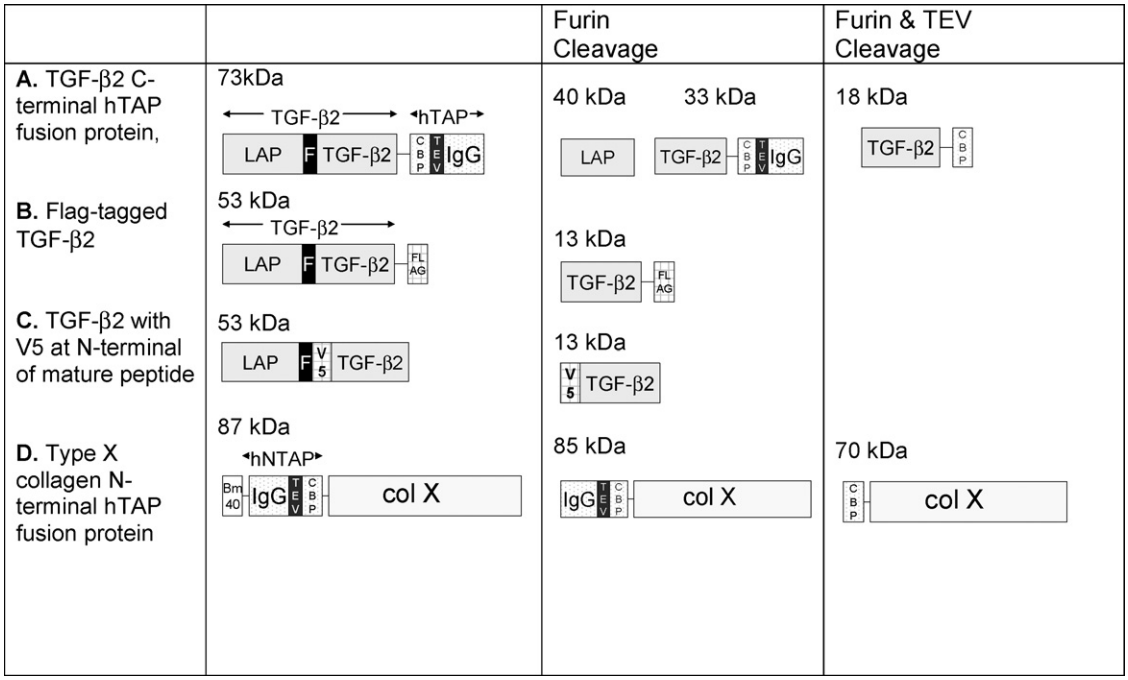

Fig. 1. Schematic presentation of hTAP and V5 constructs and their expected structures after intracellular furin processing or TEV digestion. LAP, TGF-β2 latency associated peptide. F, furin-type proteinases cleavage site in TGF-β2. CBP, calmodulin-binding peptide domain of hTAP. TEV, tobacco etch virus proteinases susceptible peptide in hTAP. Flag, flag peptide epitope. BM40 the bm40 signal peptide. V5 the V5 peptide epitope. Note that the BM-40 signal peptide show in D would be released after insertion into the ER rather than by Furin cleavage.

80,000 × *g* for 1 h and allowed to bind 200 μl IgG-agarose (Sigma, St. Louis, MO) at 4 °C in the presence of 0.1% NP-40 overnight in a disposable 15 ml centrifugation tube. The IgG beads were washed by re-suspension in IPP150 buffer (10 mM Tris pH8.0, 150 mM NaCl, 0.1% NP40) [4]. The mixture was loaded onto a disposable poly-Prep chromatography column (Bio-Rad, Hercules, CA) and washed with 3 × 10 ml IPP150 buffer followed by 10 ml TEV protease cleavage buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). The TGF-β2 complexes captured on the IgG beads were released after 2 h incubation at 20 °C with 100 U TEV (Invitrogen, Carlsbad, CA) in 1 ml TEV proteinase cleavage buffer. TEV-eluted proteins were collected and supplemented with $CaCl_2$ to a final concentration of 3 mM and diluted with 3 volume of calmodulin binding buffer (10 mM Tris pH8.0, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol, 0.1% NP40). 200 μl calmodulin-Sepharose beads (Stratagene, La Jolla, CA) pre-equilibrated in calmodulin binding buffer was added to the above diluted eluate, the mixture incubated for 2 h and then washed three times with calmodulin binding buffer. The TGF-β2 complexes were eluted using calmodulin elution buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM EGTA, 10 mM β-mercaptoethanol, 0.1% NP40). Proteins were concentrated by trichloracetic acid precipitation, washed with cold acetone and dried. Precipitated proteins were resolved on a 10% or 4–16% gradient SDS-PAG. Proteins were detected by silver staining [18] or by Western blot.

2.3. Mass spectrometry

Protein identification was performed on a Thermo LTQ-TF mass spectrometer outfitted with a nanoelectrospray ion source online with a nanoscale reverse phase HPLC system employing a 75 μm ID in-house prepared column and 100 μm ID trap packed with Magic C-18 (Michrom Bioresources, Inc., Auburn, CS). Protein bands excised from the SDS polyacrylamide gels were digested with trypsin using a Genomic Solutions ProGest robot and analyzed by data dependent tandem mass spectrometry for the top 6 most abundant ions. Raw data was processed into .xml peaklists using Thermo BioWorks v3.1 software (Thermo Scientific, Waltham, MA) and searched against Ensembl v35—November 2005 Gallus gallus database using X!Tandem software [19]. Database searching used default settings and included carbamidomethyl cysteine and one missed cleave for trypsin. Precursor mass tolerance was set to 15 ppm and fragment mass tolerance to 0.4 Da. Positive protein identification was determined using the default $\log(e) < 1$ cutoff and more than 2 peptides per protein [20].

2.3.1. Western blot

Samples from cell lysates, conditioned culture medium or protein purified by hTAP affinity chromatography were separated by SDS-PAGE, electrophoretically transferred to a PVDF membrane using a semi-dry electro-transfer method and incubated with primary antibody against; the Flag tag, the V5 tag (Invitrogen, Carlsbad, CA), a rabbit polyclonal antibody against recombinant chick TGF-β2 LAP protein generated in our laboratory [9], TGF-β2 (Santa Cruz Biotechnology, Santa Cruz, CA), type X collagen (using a polyclonal antibody kindly provided by Dr. Maurizio Pacifici [21], a monoclonal antibody against the N-terminal peptide of chick type X collagen kindly supplied by Dr. Gary Balian [22]), or the protein A domain of the hTAP tag using the peroxidase anti peroxidase complex [17]. Specific protein visualization was performed using peroxidase-conjugated second antibodies and chemiluminescence (NEN, Boston, MA).

2.4. Confirmation of TGF-β2 type X collagen interaction

To confirm the interaction of TGF-β2 and type X collagen 293 EBNA cells were co-transfected with type X collagen NhTAP and TGF-β2 V5 constructs at a ratio of 3:1 using Superfect transfection reagent (Qiagen, Valencia, CA) as described by the supplier. Cells

were cultured in DMEM containing 10% fetal calf serum with daily medium change. Culture medium collected at day 3 was subjected to calmodulin immunoaffinity chromatography as described above for tandem affinity chromatography but without prior purification and TEV elution on the IgG column. Proteins eluted using EGTA were separated by SDS polyacrylamide gel electrophoresis and identified by Western blot using V5 or LAP antibodies.

2.4.1. TGF-β bioassay

To examine the effects of type X collagen on TGF-β2 activity, EBNA 293 cells were co-transfected with chick or mouse type X collagen constructs and the V5 tagged TGF-β2 construct. Increasing concentrations of type X collagen DNA were transfected while maintaining a constant level of TGF-β2 DNA. Transfection efficiency of these cells was consistently greater than 80%.

TGF-β2 fused with V5 at the N-terminus of the mature peptide was secreted as the small latent complex. The N-terminal V5 tag appeared to have little effect on TGF-β2 activity as determined by bioassay. Culture medium samples were collected and activated at 75 °C for 20 min. The level of TGF-β2 activity in the presence of different levels of chick or mouse type X collagen was compared by bioassay using mink lung epithelial cells expressing the plasminogen activator inhibitor promoter fused with a luciferase reporter [23].

Type X collagen and TGF-β2 expression were confirmed and compared by Western blot analysis of the condition media used for bioassay and shown to be consistent with the level of DNA transfection or cell numbers expressing the construct.

2.5. Immunohistochemistry of TGF-β2 and type X

A block of iliac crest apophyseal growth plate cartilage, approximately 5 mm wide and 5 mm deep, extending from its superior tendinous surface to the underlying trabecular bone of the pelvis was obtained from a 13-year-old Schmid metaphyseal chondrodysplasia (SMCD) patient with a characterized mutation in the type X collagen gene [24] during hip reconstructive surgery. Similar tissue was obtained from a 10-year-old individual during the harvesting of iliac bone grafts for orthopedic procedures. This individual did not have skeletal dysplasias or disorders that were likely to affect the growth plate of the iliac crest. All samples were obtained with informed consent approved by the Institution's human Ethics Committee. The tissues were embedded in paraffin after decalcification in EDTA. 5 μm sections were rehydrated and digested with hyaluronidase (1 mg/ml, 1 h at room temperature). Sections were washed in NaCl (0.l5 M), Tris (0.05 M), pH 7.6 (TBS) and blocked in TBS containing bovine serum albumin (BSA, 10%, w/v, blocking buffer) for 30 min. After washing, sections were incubated with primary antibody (sc-90, Santa Cruz, CA diluted 1:100 in TBS containing BSA (1%, w/v). An alkaline phosphatase detection system (StrAviGen Super Sensitive Immunostaining System; Bio-Genex, San Ramon, CA) consisting of a biotinylated rabbit secondary antibody and a streptavidin alkaline phosphatase conjugate, was used. Color development was accomplished using a Vector Red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA).

3. Results

3.1. Construction of a synthetic hTAP gene cassette based on codons preferred in highly expressed human genes

Comparison of the expression of TGF-β2 fused with the hTAP tag with that fused with the original TAP tag demonstrated the effectiveness of tag optimization (Fig. 2A). The level of expression of TGF-β2 fused with the hTAP tag was similar to that of TGF-β2 fused with the efficiently expressed small hydrophilic octapeptide FLAG tag (Fig. 2B). TGF-β2 expression using the hTAP construct determined by serial dilutions of culture medium was approximately 30-fold greater than that observed with the unmodified TAP tagged TGF-β2. This is consistent with improved expression obtained with the codon optimized GFP protein [25]. Similar levels of expression were observed for several other proteins fused with the hTAP tag. These included MMP-13 and truncated constructs of this protein, MT1-MMP hemopexin domain, alkaline phosphatase, type X collagen and growth and differentiation factor 10.

The studies described in Fig. 2B show that the large hTAP tag had little effect on the processing and secretion of TGF-β2. Endogenous TGF-β2 protein has been shown to be expressed as a precursor of 53 kDa that is processed intracellularly by furin-type proteinases but remains associated as a latent complex of mature TGF-β2 (12.5 kDa) and latency associated peptide (LAP) of TGF-β2 (40 kDa). Western blot analysis of lysates of cells transfected with the TGF-β2–hTAP construct (Fig. 2B) demonstrated bands of approximately 73 kDa, consistent with that of hTAP tagged TGF-β2 precursor (53 kDa TGF-β2 precursor and 20 kDa hTAP tag) and 67 kDa. Western blot analysis of the culture medium using TGF-β2 specific antibodies showed a prominent band (33 kDa) of mobility consistent with fully processed TGF-β2/hTAP fusion protein (12.5 KDa TGF-β2 and 20 kDa hTAP tag) and a band of approximately 28 kDa. Loss of the faster migrating 67 and 28 kDa bands from cell lysate and culture medium after affinity purification suggested these proteins contained a proteolytically truncated hTAP tag rather than a glycosylation product.

3.2. Isolation and identification of proteins interacting with hTAP tagged TGF-β2

The conditioned medium from chondrocytes expressing the TGF-β2/hTAP gene was purified using two-step sequential IgG and calmodulin affinity chromatography. As seen in Fig. 3, combined IgG and calmodulin affinity chromatography resulted in the isolation of a mixture of proteins with migration and antibody affinity consistent with an 18 kDa TGF-β2/hTAP (the size of the hTAP portion of the fusion protein was reduced from 20 kDa to a 5 kDa CBP by TEV digestion, see also Fig. 1A); a 40 kDa TGF-β2 LAP; a cluster of faintly staining bands migrating with a kDa of approximately 66 kDa (shown by the bracket in Fig. 3A) and two slow mobility bands (shown by arrows 1 and 2 in Fig. 3A). Approximately equal amounts of TGF-β2 and LAP were observed, suggesting maintenance of association of the TGF-β2 small latent complex during purification. Western blot analysis using TGF-β2 and LAP antibodies confirmed the identity of the prominent protein bands as TGF-β2 and LAP and also demonstrated the presence of a small amount of TGF-β2 precursor (Fig. 3B).

Protein bands staining faintly with silver on SDS-PAGE (shown by the bracket in Fig. 3A) were present in all preparations including the tandem affinity purification of culture medium from hypertrophic chondrocytes not expressing the TGF-β2–hTAP fusion proteins. In several studies keratins were by far the predominant protein detected in these bands by mass spectrometry.

The two slow mobility bands 1 and 2 (shown by the arrows in Fig. 3A) were cut from polyacrylamide gels and identified by mass spectrometry after trypsin digestion. 28 tryptic peptides from band 1 and 18 tryptic peptides from band 2 were identified as peptides of chick type X collagen and established the primary component of these bands as type X collagen (Table S2). The only other peptides were identified as TGF-β2 (one tryptic peptide in band 1 and two tryptic peptides in band 2) and fibronectin (two tryptic peptides in band 1). Bands identified as type X collagen (bands 1 and 2, Fig. 3A) had a migration on reducing SDS-PAGE indicative of

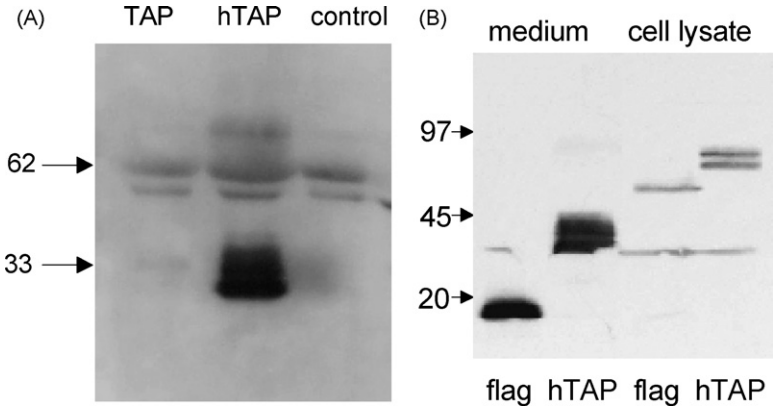

Fig. 2. Expression of TGF-β2 fused with the original and the codon-optimized TAP tags. (A) Chick fibroblasts were transfected with retroviral constructs containing the transgene TGF-β2 fused with the original (TAP) or the humanized TAP (hTAP) tags. Conditioned culture media were collected from infected fibroblasts as well as normal fibroblasts with no infection (control), separated by SDS-PAGE, immunoblotted using an antibody to TGF-β2 and detected by chemiluminescence. The 62 kDa band present in all culture media was due to a nonspecific reaction with the primary antibody and served to indicate equal protein loading in all samples. The same amount of protein was loaded on each lane and determined by BioRad protein assay. The 33 kDa TGF-β2/TAP fusion protein was prominent in conditioned medium of fibroblasts transfected with the hTAP construct and only weakly detected in fibroblasts transfected with the original TAP construct. (B) Chick chondrocytes were infected with the TGF-β2 fused with hTAP (hTAP) or TGF-β2 fused with the small Flag tag (flag). Culture medium and cell lysates were prepared, separated by SDS-PAGE and immunoblotted using the TGF-β2 antibody. Comparable protein loading was achieved by using the same volume of culture medium and cell numbers with greater than 90% transfection efficiency. Proteins detected in cell lysates had migration consistent with hTAP-tagged TGF-β2 precursor (73 kDa), a slightly faster migrating protein assumed to be a breakdown product and flag-tagged TGF-β2 precursor (53 kDa). Proteins detected in the culture medium were consistent with hTAP-tagged TGF-β2 (33 kDa) and flag-tagged TGF-β2 (13 kDa). Minimal contribution of the hTAP tag to signal intensity seen in these images is expected. Mouse IgG (TGF-β2 monoclonal antibody used) has weak interaction with the protein A component of the hTAP tag.

SDS stable trimers or higher multimers that have been reported previously [26,27]. Bands migrating in the position of the type X monomer were not processed for mass spectrometry because of high contamination with keratins in these regions but were identified by Western blot (in addition to type X collagen trimer and higher multimers) (Fig. 3C). Type X collagen could not be detected after tandem affinity purification of culture medium from chondrocytes that did not express the TGF-β2–hTAP fusion protein by either mass spectrometry (no peptides were detected in slices of SDS-PAG corresponding to the migration position of bands 1 and 2 from control culture not infected with the TGF-β2–hTAP construct, Fig. 3A lane 1) or Western blot (Fig. 3C, lane 1). Similarly when MMP-13 fused with the hTAP tag was expressed in chick chondrocytes type X collagen was not among the interacting proteins identified (G. Gibson and M. Yang, unpublished data).

3.3. Confirmation of TGF-β2 type X collagen interaction

Type X collagen fused with the hTAP tag was co-expressed with V5 tagged TGF-β2 in EBNA 293 cells to confirm the interaction of type X collagen and TFG-β2. As shown in Fig. 4, both TGF-β2 (detected using the V5 antibody) and LAP co-purified with the type X collagen hTAP fusion protein from the culture medium after affinity chromatography on the calmodulin column. Neither the V5

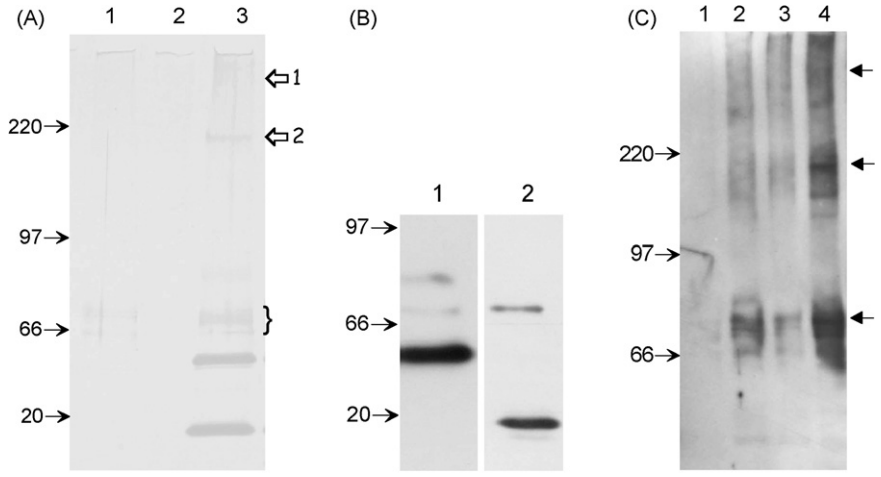

Fig. 3. Tandem affinity purification of the chick TGF-β2. (A) Culture medium from chondrocytes not infected with the plasmid (lane 1), DMEM, 10% FCS (lane 2), culture medium from chondrocytes infected with the RCASBP(A)cTGF-β2–hTAP construct (lane 3) was subjected to tandem affinity purification and analyzed for protein by sliver staining. Open arrows 1 and 2 indicate proteins bands analyzed by mass spectrometry. Protein bands shown by the bracket indicate contaminating keratins found in all samples. (B) Protein samples shown in A lane 3 were identified by Western blot using TGF-β2 LAP- (lane 1) and TGF-β2- (lane 2) specific antibodies after SDS-PAGE. The migration positions of protein molecular weight standards are shown at left. C, Detection of type X collagen in complex with TGF-β2–hTAP fusion protein. Protein complexes were isolated from conditioned culture medium from control chondrocytes (lane 1) or three separate preparations of chondrocytes infected with the RCASBP TGF-β2-TAP construct (lanes 2, 3, 4) by tandem affinity purification. The presence of type X collagen was detected by Western blot after SDS-PAGE using a type X collagen polyclonal antibody. Arrows to the right of the figure show predicted migration positions of type X collagen monomer, trimer and higher order multimers. The migration position of protein molecular weight standards is shown to the left.

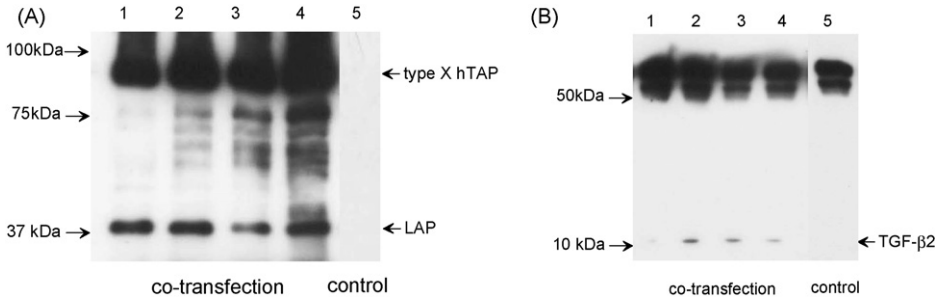

Fig. 4. Interaction of TGF-β2 and TGF-β2 LAP with hTAP-tagged type X collagen. Interaction of type X collagen with TGF-β2 was monitored by Western blot using LAP-specific (A) and V5-specific (B) antibodies after isolation by calmodulin affinity chromatography. Lanes 1–4 conditioned culture medium from 293 cells expressing both type X collagen fused with the hTAP tag and V5 tagged TGF-β2. Lane 5 conditioned culture medium from 293 cells expressing V5 tagged TGF-β2 only. The IgG binding domain of the type X collagen hTAP fusion protein was detected by the second antibody used to detect the LAP-specific rabbit polyclonal antibody in A. The bands migrating at 50–60 kDa in B are calmodulin binding proteins present in the bovine serum component of culture medium that react with the second antibody. Neither TGF-β2 LAP (A) nor TGF-β2 (B) bound to the calmodulin column in the absence of type X collagen (lane 2). Controls shown in lane 5 were separated on the same gel but lane sequences have been shifted to improve readability.

tagged TGF-β2 nor LAP bound the affinity columns in the absence of hTAP-tagged type X collagen. Although the levels of type X collagen and TGF-β2 released into the culture medium by the EBNA 293 cells was similar to that released by hypertrophic chondrocytes the possibility remains that the interaction observed was a result of abnormal expression of both type X collagen and TGF-β2 in these cells.

3.4. Inhibition of TGF-β activity by type X collagen

Co-expression of type X collagen with TGF-β2 resulted in a dose dependent inhibition of TGF-β activity in culture medium as measured by mink lung bioassay after prior heat activation (Fig. 5). Western blot analysis confirmed type X collagen and TGF-β2 expression and was consistent with the level of transfection (data not shown).

3.5. Distribution of TGF-β2 in growth cartilage from a SMCD patient

Immunohistochemical detection of TGF-β2 demonstrated clear differences between growth cartilage from a SMCD patient and a patient of similar age without deformity affecting growth plate structure (Fig. 6). TGF-β2 in growth cartilage of the SMCD patient was present in a diffuse area in the pericellular matrix surrounding hypertrophic chondrocytes. 51% of the 265 chondrocytes examined in the sections of SMCD growth cartilage showed predominant staining in the pericellular matrix like that shown in Fig. 6. The remaining 48% showed staining over the entire cell. This might reflect the same pericellular distribution but result from sectioning through the pericellular matrix. A faint diffuse distribution of TGF-β2 was also detected in the extracellular matrix between hypertrophic chondrocytes of the growth cartilage from the SMCD patient (Fig. 6). In the iliac crest growth cartilage of the control patient TGF-β2 was confined to the cytoplasm of the last few hypertrophic chondrocytes adjacent to the vascular interface (Fig. 6) similar to that seen in immature monkeys [28], rats and mice (Gibson unpublished data). Neither the pericellular (0% of cells) nor diffuse inter-territorial distribution of TGF-β2 was observed in control growth cartilage.

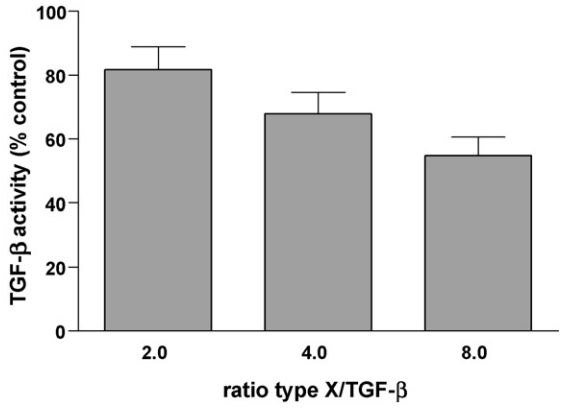

Fig. 5. Inhibition of TGF-β activity by type X collagen. EBNA 293 were co-transfected with hTAP-tagged type X collagen and V5-tagged TGF-β2. Concentrations of type X collagen DNA transfected as a ratio of TGF-β2 transfected ranged from 0 to 8 as indicated, while maintaining a constant level of TGF-β2 DNA. TGF-beta activity was measured in culture medium by mink lung cell bioassay, after heat activation. Average and standard deviation of TGF-β activity expressed as a percentage of control from 4 cultures is shown.

4. Discussion

Re-synthesis of the TAP tag gene cassette to optimize for codon usage prevalent in highly expressed human proteins enabled strong expression of exogenous transgene products in chick fibroblasts, chick chondrocytes and the EBNA 293 cell line. TGF-β2 fused with the optimized hTAP tag had an expression level similar to that seen with the efficiently expressed, small, commonly used, hydrophilic octapeptide, FLAG tag and contrasted the weak expression of TGF-β2 fused with the original TAP tag. The molecular size and immunoreactivity of the TGF-β2/hTAP fusion protein and co-purification of TGF-β2 with non-covalently associated LAP indicated that the additional 20 kDa N-terminal peptide of the hTAP sequence did not detectably alter the intracellular processing and secretion of TGF-β2. The original TAP tag has been widely used for isolation of protein complexes expressed by yeast [4,29,30] but has found more limited application in higher eukaryotes [31,32]. The studies presented here suggest depletion of rare tRNA as a result of expression of the original TAP gene that contains many rare codons resulted in attenuated expression of the TAP fusion proteins. Increased amino acid misincorporation and decreased quality of expressed proteins shown to result from expression of genes containing rare codons [33] might also have contributed to the poor expression of proteins fused with the original TAP tag.

The molecular interactions of the TGF-β family are critical to their function. These interactions affect secretion, growth factor activity and localization to the proper extracellular site [34,35]. The studies described here show TGF-β2 secreted by hypertrophic chondrocytes forms a previously unrecognized complex with type

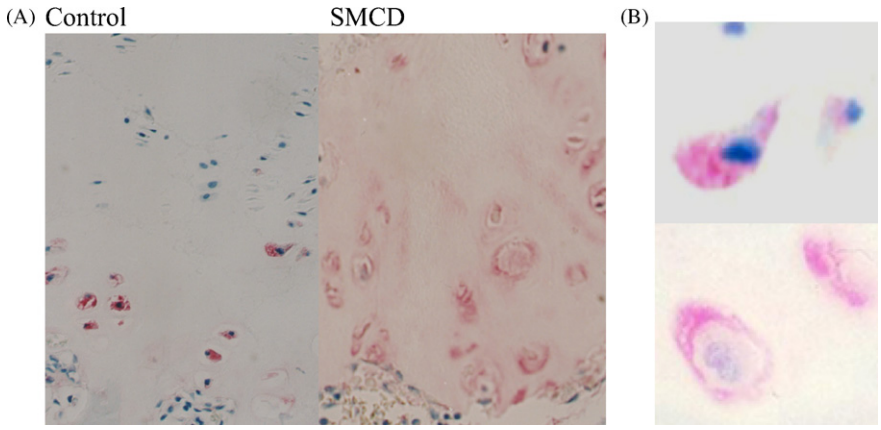

Fig. 6. TGF-β2 immunohistochemistry of growth cartilage from a SMCD patient. Paraffin sections of iliac crest growth cartilage were incubated with an antibody against TGF-β2 and the vector red substrate. (A) Low power view and (B) high power view top normal growth cartilage, bottom SMCD growth cartilage. TGF-β2 is localized in close association with hypertrophic chondrocytes adjacent to the resorbing vasculature in the unaffected child in contrast to a pericellular and faint diffuse inter-territorial distribution in the in the SMCD child.

X collagen. TGF-β2, based on the few published studies of its interactions, was expected to form complexes similar to those described for TGF-β1. TGF-β1 has been shown to interact with thrombospondin [36], fibronectin [37] and the latent TGF-β binding proteins (LTBPs) [38,39]. Abnormal skeletal development and osteopetrosis in LTBP 3 null mice suggested a role in cartilage development [40] and possible interaction with TGF-β2. Osteopetrosis in these animals, however, was attributed to defective osteoclastic bone turnover and a critical function for LTBP 3 in regulation of TGF-β (probably TGF-β1) activity during metaphyseal trabecular bone formation and remodeling rather than chondrocyte function [41]. In the current studies, we did not detect LTBPs after hTAP isolation of the TGF-β2 protein complex. This might be due to the absence of LTBPs in the culture medium conditioned by hypertrophic chondrocytes. Expression of LTBP1 and its deposition in the extracellular matrix has been reported in rat costochondral proliferative chondrocytes after subculture [42]. However, the phenotype of the cells isolated was not reported and since the chondrocyte phenotype is very sensitive to subculture LTBP1 expression was most likely associated with loss of the chondrocyte phenotype. We were unable to detect the expression of LTBP1 and LTBP2 or TSP1 by Western blot, however the affinity of the antibodies available (MAB 388 and MAB 3850, R&D Systems, Minneapolis, MN, or TSP Ab-11, Thermo Fisher Scientific, Fremont, CA) for the chick protein has not been demonstrated. Expression of LTPB by well-defined chondrocytes remains to be demonstrated. Levels of LTBPs in the TGF-β2 complex below the level of protein detected by silver staining of SDS-PAGE might have resulted in their failure to be detected since only visible protein bands were processed for mass spectrometric identification. Similarly co-migration of LTBPs with contaminating keratins would have resulted in their failure to be detected since protein bands in this region were not processed for mass spectrometry. However, LTBPs have molecular size of greater than 100 kDa and only degraded LTBPs would be expected to migrate at this mobility. Competition between endogenous TGF-β2 expressed by hypertrophic chondrocytes and the hTAP tagged TGF-β2 for binding proteins could have also affected the interactions observed, particularly if the hTAP tag reduced affinity for specific proteins. Co-expression of TGF-β2 with hTAP tagged type X collagen in EBNA 293 cells, however, confirmed the validity of this interaction and argued it is not a consequence of over expression in the presence of competing endogenous protein. Fibronectin was tentatively identified in the TGF-β2 complex isolated by tandem affinity chromatography consistent with previous reports [37]. However, the small number of tryptic peptides (two) identified demands further confirmation of the interaction of fibronectin with TGF-β2. The studies presented have examined only the interaction with proteins secreted by hypertrophic chondrocytes. It is expected that TGF-β2 secreted by hypertrophic chondrocytes would have the opportunity to interact with proteins, including LTBPs, secreted by the many cell types present in the region of hypertrophic cartilage resorption, vascular ingrowth and bone formation. Further studies are needed to determine if TGF-β2 expressed by hypertrophic chondrocytes interacts with binding proteins, expressed by other cell types from this region.

Co-expression of type X collagen with TGF-β2 suppressed the response of mink lung epithelial cell lines to TGF-β2. The observations suggest a physiologic role for the interaction of TGF-β2 with type X collagen. This data suggests expression of type X collagen in hypertrophic chondrocytes would act as a suppressor of the effects of TGF-β2. TGF-β2 plays a complex but critical role in regulation of bone formation. It has been shown stimulate osteoblast transition to osteocytes and osteoclastic activity in transgenic mice [13] and to inhibit osteoblast differentiation in cell culture [43]. Clearly tight regulation of TGF-β activity is necessary for the ordered formation of trabecular bone adjacent to the growth plate. TGF-β1 has been shown to interact with types I and III collagens [43,44], which in contrast to the effect of type X collagen on TGF-β2 activity, resulted enhancement of the activity of TGF-β1 in a mink lung epithelial cell [44]. In contrast type I collagen inhibited the effect of TGF-β1 on the differentiation of a murine osteoblast cell line [43].

Type X collagen has been recognized as a marker for chondrocyte hypertrophy for many years [45,46]; however, its physiologic function remains enigmatic. A number of mutations in type X collagen have been identified in patients with Shmid Metaphyseal Chondrodysplasia (SMCD) [47]. Recent characterization of transgenic mice expressing a type X collagen mutation found in SMCD patients showed misfolded collagen accumulation in the ER of hypertrophic chondrocytes and provided convincing evidence for subsequent ER stress as an explanation of the pathogenesis of SMCD [48]. Characterization of the pathology of SMCD, however, has shed little light on the function of type X collagen. The pericellular and diffuse interterritorial distribution of TGF-β2 seen in the SMCD patient compared with tight association with terminal hypertrophic chondrocytes in growth cartilage of control patients suggests the tissue distribution TGF-β2 is influenced by changes in the matrix distribution of type X collagen seen in these patients. Changes in type X collagen from an approximately uniform tissue

distribution in the hypertrophic zone to a largely pericellular distribution around hypertrophic chondrocytes were observed in both the growth cartilage of a SMCD patient and mouse SMCD model [48]. Changes in the distribution of TGF-β2 in growth cartilage and its association with type X collagen might be expected to affect its activity and function in regulation of vascular invasion, cartilage resorption and trabecular bone formation; all of which have be shown to be disrupted in growth cartilage from SMCD patients.

The present studies suggest a functional interaction of TGF-β2 and type X collagen. The effect of this interaction on chondrocyte differentiation; osteoblast differentiation and function; and vascular invasion and resorption of growth cartilage; associated with endochondral bone formation warrant further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.10.016.

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