A human chondrodysplasia due to a mutation in a TGF-β superfamily member

J. Terrig Thomas[1], Keming Lin[1], Maithily Nandedkar[1], Mauricio Camargo[2], Jaroslav Cervenka[3] & Frank P. Luyten[1]

The TGF-β superfamily comprises a number of functionally diverse growth factors/signalling molecules[1] which elicit their response upon binding to serine-threonine kinase receptors[2]. We recently reported the isolation and characterization of two new members of the family, designated cartilage-derived morphogenetic protein (CDMP) 1 and 2 (ref. 3) which are closely related to the sub-family of bone morphogenetic proteins. *CDMP-1* is predominantly expressed at sites of skeletal morphogenesis[3], and we now show that a mutation in h*CDMP-1* is associated with a recessive human chondrodysplasia (acromesomelic chondrodysplasia, Hunter-Thompson type[4,5]). The disorder, characterized by skeletal abnormalities restricted to the limbs and limb joints, is phenotypically similar to murine brachypodism (*bp*) which is due to mutations in growth/differentiation factor-5 (*Gdf-5*)[6], the mouse homologue of h*CDMP-1*. Affected individuals are homozygous for a 22-bp (tandem-duplication) frameshift mutation in the mature region of *CDMP-1*. The resulting phenotype provides direct evidence for the involvement of CDMP-1 in human skeletal development and represents the first human disorder attributable to a mutation in a TGF-β superfamily member.

The sub-family of bone morphogenetic proteins (BMP's) was originally described as an activity isolated from demineralised bone matrix[7]. Characterization by protein purification and cDNA cloning led to the discovery of a family of TGF-β-related molecules[8–13] which are soluble factors able to initiate *in vivo* the process of endochondral ossification when implanted on a suitable carrier at an ectopic site. It should be noted, however, that the term 'BMP' has now become somewhat misleading because BMP's are also expressed by a number of other tissues (reviewed in ref. 1) and homologues have been isolated from species such as drosophila (dpp and 60A) and sea urchin (univin) which do not have a bony skeleton[14–16]. In contrast, the physiological role of some BMP's in skeletal development is undoubted. For example, the mouse genetic disorders short ear (*se*) and brachypodism (*bp*) are due to null mutations in the *BMP-5* and growth differentiation factor-5 (*GDF-5*) genes respectively[6,17]. Only a limited number of bones are affected by the absence of each gene product, substantiating the hypothesis that the size, shape and form of each bone in the skeleton is controlled by the composite expression pattern of a number of different factors.

The human osteochondrodysplasias comprise a large and heterogeneous group of inherited disorders affecting skeletal morphogenesis[18]. Some members of the acromesomelic group of chondrodysplasias[19] exhibit similarities to the mouse *bp* mutation[6,20] and, in particular, individuals from a previously described family from the Choco district of Columbia affected with Hunter-Thompson type chondrodysplasia[5]. Hunter-Thompson type chondrodysplasia[4,5] and *bp*[6,20] are typified by abnormalities restricted to the limbs, and the severity of

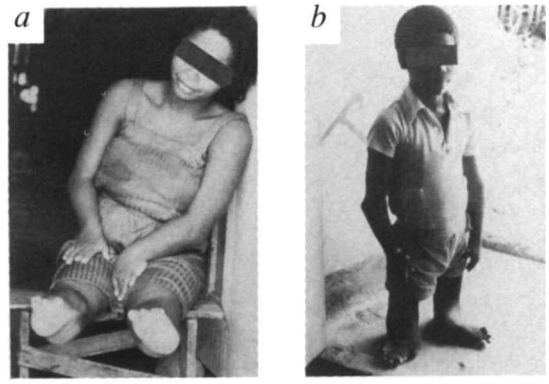

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Fig. 1 Morphological characteristics of affected individuals and X-ray analysis. *a*, Patient 1: female, 20 years; abnormalities are limited to the limbs, the craniofacial and axial skeleton is normal. The forearms and hands are relatively shorter than the upper arms and the shanks are relatively shorter than the thighs. The feet are very short and the third, fourth and fifth toes are ball shaped and functionless. The ankles are dislocated and the feet are everted. Finger and toe nails are normal. *b*, Patient 2, male 13 years; shows the same short-limbed dysplasia as his sister but with additional dislocations of the right hip, elbows and knees. Again, the hands and feet are most severely affected, but the pattern of finger shortening is different. Craniofacial and axial skeleton are normal. *c*, X-ray of elbow of patient 2: posterior dislocation of the radial head. Compared to the humerus, both radius and ulna are short and there is bowing of the radius. *d*, X-ray of lower limbs of patient 2: severe dislocations of both knees; hypo-plastic femoral condyles and shortened tibiae. Very short fibulae adjacent to the distal tibial shafts. *e*, X-ray of the left hand[26] of an age and sex matched control of patient 2. *f*, X-rays of hands of patient 2 at age 13 years: defects in both hands are symmetrical. Nine instead of eight carpal bones, all of which are abnormally shaped. All metacarpals are short and cuboidal and the growth plates of metacarpals and phalanges are prematurely closed (compare to *e*). The proximal and medial phalanges are short. The proximal phalanges increase in size from first to fifth finger, whereas the opposite is seen for the medial phalanges. There is a fusion or absence of the medial phalanx in the fifth finger. Distal phalanges are relatively normal. Patient 1 refused radiographic examination. X-ray images shown in *c*, *d* and *f* are reproduced, with kind permission, from ref. 1. Image *e* was reproduced from ref. 26.

[1]*Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892, USA*
[2]*Department of Biology, University of Antioquia, Medellin, Columbia*
[3]*Department of Oral Sciences, Dental School, University of Minnesota, Minneapolis, Minnesota 55455, USA*

Correspondence should be addressed to J.T.T.

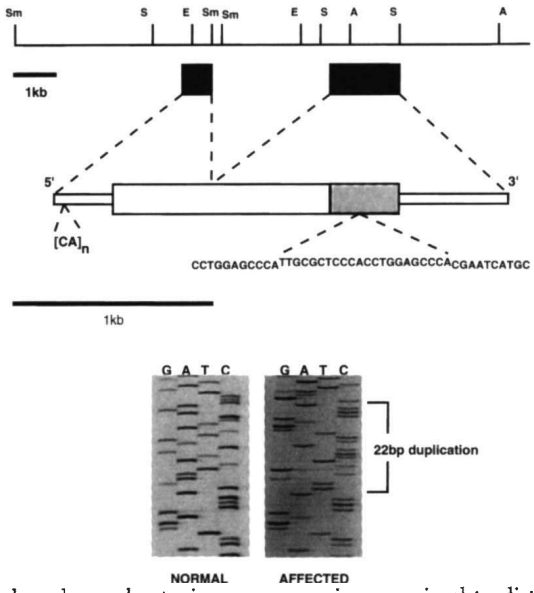

Fig. 2 Individuals affected by Hunter-Thompson type chondrodysplasia have a frame shift mutation in the mature region of h*CDMP-1*. *a*, Partial restriction map and schematic representation of h*CDMP-1* showing the exon–intron locations, the domain structure of the derived cDNA and the position and sequence of the 22-bp tandem-duplication within the biologically active mature region (shaded box). The open reading frame (ORF) of h*CDMP-1* is encoded by two exons. One exon encodes 5'-untranslated region (UTR) and 681 bp of ORF. The second exon encodes the remaining 873 bp of ORF and 557 bp of 3'-UTR. The length of the intron separating the two exons is approximately 3 kb. The $[CA]_n$ dinucleotide repeat, 5' of the transcription start site, is highly polymorphic and was used to map h*CDMP-1* to chromosome 20 q11.2 (manuscript submitted for publication). S-*Sac*I; Sm-*Sma*I; A-*Apa*I; E-*Eco*RI. *b*, Sequences of the normal and mutant h*CDMP-1* alleles.

long bone shortening progresses in a proximal to distal direction. The hands and feet are most severely affected but the distal phalanges are relatively normal (Fig. 1). X-ray analyses show long bone shortening, in the metacarpals and possibly in the phalanges, to be coupled with an apparent premature closing of the growth plates (Fig. 1). Affected individuals have joint dislocations but the number of joints involved is not consistent. The reason for the variability is not known but the same phenomenon has also been noted for *bp* mice[20]. Significant differences exist between the mouse and human phenotypes in the abnormalities observed in the phalangeal bones. In digits 2–5 of *bp* mice, the proximal and medial phalanges are replaced by a single bone, whereas in Hunter-Thompson type chondrodysplasia there is considerable variation in the extent of shortening of the phalangeal bones (Fig. 1) with only digit 5 being replaced by a single bone. It is unclear whether the single phalangeal bones arise from a fusion of the proximal and medial phalanges or whether the medial phalange is simply absent.

To analyse patient DNA for possible mutations in h*CDMP-1*, the genomic structure (Fig. 2) and the sequences of the exons and exon-intron junctions were determined. Exon-intron junctions were deduced by comparison of the genomic sequence with that of the previously reported cDNA[15] and the presence of splice site consensus sequences at the predicted boundaries. Intron sequences at the 3' end of exon 1 and the 5' end of exon 2 were also determined to allow for the production of polymerase chain reaction (PCR) primers for use in subsequent analyses of patient DNA. Genomic DNA was isolated from whole blood or blood spots and h*CDMP-1* was directly sequenced from PCR products. Sequence differences were observed between affected and non-affected individuals within the mature region of h*CDMP-1* at nucleotide positions 1448 and 1475. Subsequent sequence analysis of subcloned PCR products revealed the existence of a frameshift mutation resulting from a 22-bp insertion (Fig. 2). The insertion is a tandem duplication at position 1475 of 1453–1474 resulting in an altered open reading frame of 43 amino acids before a stop codon is reached. PCR analysis of genomic DNA revealed both affected individuals to be homozygous for the 22 bp duplication and their non-affected children to be heterozygous (Fig. 3).

The mutation observed in affected individuals' *CDMP-1* probably results in an altered structure of the biologically active domain of this protein. CDMP-1 is closely related to the BMP sub-family of TGF-β-like signalling molecules and is synthesized in a pro-form which subsequently dimerize by a single interchain disulfide bond. The mature biolgically active region is formed following cleavage at a characteristic Arg–X–X–Arg site[1]. The overall structure of the mature protein is determined by the invariable spacing of seven cysteine residues, one of which is involved in the formation of the interchain disulfide bond. The importance of the cysteine residues in determining the structure and ultimate function of TGF-β superfamily members has been inferred from X-ray and nuclear magnetic imaging (NMR) studies[21,22] and demonstrated by site-directed mutagenesis[23,24]. Replacement of any one of the seven conserved cysteine residues of TGF-β1 by a serine produces a dramatic reduction of activity[23,24]. The mutation in Hunter-Thompson type chondrodysplasia occurs in the mature region at position 1475, 11 amino acids after the third cysteine, disrupting the highly con-

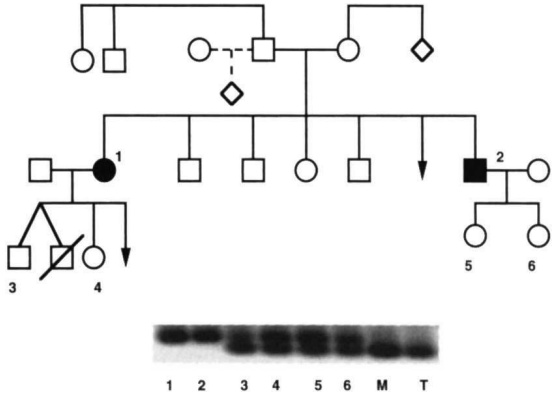

Fig. 3 *a*, Pedigree of Hunter-Thompson type acromesomelic dysplasia family. Affected individuals are indicated by black boxes. Both affected individuals and their children [1–6] were analysed for mutations in h*CDMP-1* as described in Fig. 1. Other family members were unavailable for study. Preliminary linkage analysis, on family members 1–6, demonstrated that both affected individuals exhibited the same CA dinucleotide repeat banding pattern (results not shown). *b*, Second round PCR products obtained from genomic DNA of affected individuals (1, 2), their non-affected children (3–6) and control DNA (M,T). A 139-bp sequence flanking the mutation site within the mature region of h*CDMP-1* was amplified by PCR. Both affected individuals are homozygous for the 22-bp duplication as indicated by the single PCR product of 151 bp and the four non-affected children are heterozygous.

served seven cysteine pattern and results in a mature protein where only the first 62 out of 120 amino acids are in frame. The 43 out of frame amino acids share no identity to the normal protein and therefore, in all probability, the mutation results in a loss of function. The differences observed between Hunter-Thompson type chondrodysplasia and the *bp* mouse phenotype may be due to genetic background, differences between human and mouse skeletal development or interspecies variation in expression patterns of other compensatory gene products. An alternative explanation would be that some residual activity still remains in the mutated hCDMP-1. The nature of the mutation makes this unlikely and, in addition, given that the TGF-β superfamily are dimeric proteins, if a mutated and a normal molecule were able to associate together one would expect to observe a phenotype in heterozygous individuals. However, all the heterozygous family members were normal.

It is becoming apparent that the development, size and shape of every bone in the skeleton is controlled by the composite expression pattern of a number of genes. From studies on the human osteochondrodysplasias and both naturally occurring and gene targeted mouse mutations, much can be learned about the specific contribution of any one gene product to overall skeletal form. From the present investigation it is clear that the development of the appendicular skeleton is influenced by factors distinct from those of the craniofacial and axial skeleton. h*CDMP-1* is involved in determiing the size and shape of the digits and the observation of joint dysplasia alludes to an additional function in peripheral joint morphogenesis.

Methods

Genomic clone isolation and detection of h*CDMP-1* mutation. Genomic clones [λ-Fix (Stratagene)] encoding h*CDMP-1* were isolated using a 690-bp α^{32}P-dCTP-labelled cDNA encoding part of the pro-region of h*CDMP-1* (position 601–1291 in ref. 2). Genomic DNA was isolated from whole blood (Puregene DNA isolation kit; Gentra Systems) or from blood spots [25]. Primer sets were designed to amplify the entire ORF and exon–intron boundaries and unless otherwise indicated, the numbers refer to nucleotide positions in ref. 3. PCR products were cycle sequenced using ^{35}S-dATP and the AmpliTaq kit (Perkin Elmer). First round PCR amplifications used the primers 97–133 and 5'–CCCTCCATTCATGCAGATGCC–3' (61–41 bp from the 5' end of the intron) and 5'–GAATGGGGCAGAGGTGAAAG–3' (124–105 bp from the 3' end of the intron) and 1732–1710 under the following conditions; 30 cycles at 94 °C for 5 s, 56 °C for 10 s and 72 °C for 50 s. Second round amplifications used the same 5' primers and primers 5'–ATGCCCCTCCCTCTGAGCCGT–3' (45–25 bp from the 5' end of the intron) and 1680–1662. Sequencing primers were 105–130, 316–336, 384–366, 5'–ATGCCCCTCCCTCTGAGCCGT–3' (as above), CGAAGTGACTGGCTCCCTTGG–3' (68–39 bp from the 3' end of the intron), 830–855, 1243–1226, 1168–1188, 1378–1395 and 1680–1662. PCR products containing the mutation region were sub cloned into PCR-Script (Stratagene) and individual subclones were sequenced using the primer sets 1168–1188, 1378–1395 and 1680–1662.

Demonstration that affected individuals are homozygous for the h*CDMP-1* mutation. Due to the high degree of identity within the mature region of CDMP family members first round PCR amplifications were carried out using primers in the pro-region (830–852) and in the 3'-UTR (1680–1661) under the following conditions: 4 cycles at 94 °C for 5 s, 45 °C 10 s and 72 °C for 60 s followed by 26 cycles at 94 °C for 5 s, 56 °C for 10 s and 72 °C for 60 s. Second round PCR amplifications used the mature region primers 1378–1395 and 1517–1500 under the following conditions: 25 cycles at 94 °C for 5 s, 56 °C for 10 s and 72 °C for 10 s. PCR products were electrophoresed on a 2.5% agarose gel and stained with ethidium bromide (0.5 μg/ml).

Acknowledgements

We thank G. Wallis for her expert advice and opinions in identifying possible candidate disorders, C. Francomano and M. Kuehn for their critical review of the manuscript and A. Tovar of Quibdo, Columbia.

Received 14 September; accepted 8 December 1995.

1. Kingsley, D. M. What do BMPs do in mammals? Clues from the mouse short-ear mutation. *Trends Genet.* **10**, 16–21 (1994).
2. Massagué, J., Attisano, L. & Wrana, J.L. The TGF-β family and its composite receptors. *Trends Cell. Biol.* **4**, 172–178 (1994).
3. Chang, S. C. *et al.* Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-β superfamily predominantly expressed in long bones during human embryonic development. *J. Biol. Chem.* **269**, 28227–28234 (1994).
4. Hunter, A.G.W. & Thompson, M.W. Acromesomelic dwarfism: description of a patient and comparison with previously reported cases. *Hum. Genet.* **34**, 107–113 (1976).
5. Langer, L.O., Cervenka, J. & Camargo, M. A severe autosomal recessive acromesomelic dysplasia, the Hunter-Thompson type, and comparison with the Grebe type. *Hum. Genet.* **81**, 323–328 (1989).
6. Storm, E. E. *et al.* Limb alterations in brachypodism mice due to mutations in a new member of the TGFβ superfamily. *Nature* **368**, 639–643 (1994).
7. Urist, M. R. Bone: formation by autoinduction. *Science* **159**, 893–899 (1965).
8. Celeste, A. J. *et al.* Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* **87**, 9843–9847 (1990).
9. Luyten, F. P. *et al.* Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J. Biol. Chem.* **264**, 13377–13380 (1989).
10. Özkaynak, E. et al. OP-1 cDNA encodes an osteogenic protein in the TGF-beta family. *EMBO J.* **9**, 2085–2093 (1990).
11. Sampath, T.K., Muthukumaran, N. & Reddi, A.H. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. *Proc. Natl. Acad. Sci. USA* **84**, 7109–7113 (1987).
12. Wang, E. A. *et al.* Purification and characterization of other distinct bone-inducing factors. *Proc. Natl. Acad. Sci. USA* **85**, 9484–9488 (1988).
13. Wozney, J.M. *et al.* Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528–1534 (1988).
14. Padgett, R. W., St. Johnston, D. & Gelbart, W.M. A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. *Nature* **325**, 81–84 (1987).
15. Wharton, K. A., Thomsen, G. H. & Gelbart, W. M. Drosophila 60A gene, another transforming growth factor β family member, is closely related to human bone morphogenetic proteins. *Proc. Natl. Acad. Sci. USA* **88**, 9214–9218 (1991).
16. Stenzel, P., Angerer, L.M., Smith, B.J., Angerer, R.C. & Vale, W.W. The univin gene encodes a member of the transforming groth factor-β superfamily with restricted expression in the sea urchin embryo. *Develop. Biol.* **166**, 149–158 (1994).
17. Kingsley, D.M. *et al.* The mouse short ear skeletal morphogenesis locus is associatedwith defects in a bone morphogenetic member of the TGF beta superfamily. *Cell* **71**, 399–410 (1992).
18. Spanger, J. International classification of osteochondrodysplasias. *Eur. J. Padiatr* **151**, 407–415 (1992).
19. McKusick, V.A. in *Mendelian Inheitance in Man: A Catologue of Human Genes and Genetic Disorders* (ed. McKusick, V.A.) (The Johns Hopkins University Press, Baltimore and London, 1994).
20. Grüneberg, H. & Lee, A.J. The anatomy and development of brachypodism in the mouse. *J. Embryol. Exp. Morph.* **30**, 119–141 (1973).
21. Venkataraman, G., Sasisekharan, V., Cooney, C.L., Langer, R. & Sasisekharan, R. Complex flexibility of the transforming growth factor β superfamily. *Proc. Natl. Acad. Sci. USA* **92** 5406–5410 (1995).
22. Griffith, D.L., Keck, P., Sampath, T.K., Rueger, D.C. & Carlson, W.D. Three dimensional structure of recombinant human osteogenic protein 1: Structural paradigm for transforming growth factor β superfamily proteins *Proc. Natl. Acad. Sci. USA* (in the press)
23. Amatayakul-Chantler, S. *et al.* [Ser^{77}] transforming growth factor-β1. Selective biological activity and receptor binding in mink lung epithelial cells. *J. Biol. Chem.* **269**, 27687–27691 (1994).
24. Brunner, A.M. et al. Site-directed mutagenesis of glycosylation sites in the transforming growth factor-β1 (TGFβ1) and TGFβ2 (414) precursors and of cysteine residues within TGFβ1: effects on secretion and bioactivity. *Molec. Endocrinol.* **6**, 1691–1700 (1992).
25. Walsh, P.S., Metzger, D.A. & Higuchi, R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506–513 (1991).
26. Greulich, W.W. & Pyle, S.I. in *Radiographic Atlas of Skeletal Development of the Hand and Wrist* (eds Greulich, W.W. & Pyle, S.I.) 108–109 (Stanford University Press, Stanford, CA, 1983).