Mutations in *FKBP10*, which result in Bruck syndrome and recessive forms of osteogenesis imperfecta, inhibit the hydroxylation of telopeptide lysines in bone collagen

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Although biallelic mutations in non-collagen genes account for <10% of individuals with osteogenesis imperfecta, the characterization of these genes has identified new pathways and potential interventions that could benefit even those with mutations in type I collagen genes. We identified mutations in *FKBP10*, which encodes the 65 kDa prolyl *cis–trans* isomerase, FKBP65, in 38 members of 21 families with OI. These include 10 families from the Samoan Islands who share a founder mutation. Of the mutations, three are missense; the remainder either introduce premature termination codons or create frameshifts both of which result in mRNA

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instability. In four families missense mutations result in loss of most of the protein. The clinical effects of these mutations are short stature, a high incidence of joint contractures at birth and progressive scoliosis and fractures, but there is remarkable variability in phenotype even within families. The loss of the activity of FKBP65 has several effects: type I procollagen secretion is slightly delayed, the stabilization of the intact trimer is incomplete and there is diminished hydroxylation of the telopeptide lysyl residues involved in intermolecular cross-link formation in bone. The phenotype overlaps with that seen with mutations in PLOD2 (Bruck syndrome II), which encodes LH2, the enzyme that hydroxylates the telopeptide lysyl residues. These findings define a set of genes, FKBP10, PLOD2 and SERPINH1, that act during procollagen maturation to contribute to molecular stability and post-translational modification of type I procollagen, without which bone mass and quality are abnormal and fractures and contractures result.

INTRODUCTION

During the last several years nine genes have been identified in which biallelic mutations give rise to autosomal recessive forms of osteogenesis imperfecta (OI) and related conditions. We have found that recessively inherited forms of OI account for ≈5% of all mutations in the >2,500 individuals we have characterized (unpublished data). Although >90% of individuals with OI have dominant mutations in the type I collagen genes, COL1A1 and COL1A2, the recessively inherited forms of OI hold out the promise that the identification of the minimal alteration that results in the phenotype could identify better and more directed forms of intervention than currently available. The genes targeted in the recessive forms of OI and related disorders fall into six groups: first, a transcription factor involved in osteoblast differentiation (SP7, which encodes osterix) (1); second, CRTAP (2–4), LEPRE1 (2,5) and PP1B (6–8) which encode the components of the prolyl 3-hydroxylation complex, cartilage associate protein (CRTAP), prolyl 3-hydroxylase (P3H1) and cyclophilin B (CYPB), respectively; third, SERPINH1 (9) and FKBP10 (10–16) which encode two chaperone-like proteins, heat shock protein 47 (HSP47) and FKBP65, respectively; fourth, PLOD2 (17,18) which encodes lysyl hydroxylase 2 (LH2) that can hydroxylate lysyl residues outside the major triple helix of type I collagen crucial to formation of mature intermolecular cross-links in bone, cartilage and other tissues and, fifth, BMP1 (19,20), which encodes the protease that removes the carboxyl-terminal propeptide of type I procollagen. Mutations in SERPINF1 (21,22), which encodes pigment epithelium-derived factor, form a separate group with the mechanism yet to be clearly defined.

Mutations in CRTAP, LEPRE1 and PP1B appear to affect the rate of chain association or the efficiency of folding of the triple helix of the chains of type I procollagen so that the chains can become overmodified because of prolonged residence in an unfolded form. The effect mimics that of mutations in type I collagen genes (COL1A1 and COL1A2) that disrupt chain association or helix propagation. A complex of these three proteins in the rough endoplasmic reticulum (RER) is responsible for 3-hydroxylation of the prolyl residue at position 986 of the triple-helical domain in the procI(1) chain (3,5,6). In contrast, mutation in SERPINH1 does not disrupt the initial phases of folding but the final details of helix formation appear to be left unattended so that apparently intact molecules remain unexpectedly protease sensitive (9,23). In the absence of HSP47 (encoded by SERPINH1), type I procollagen molecules have a shorter residence time in the RER and are quickly transported to the Golgi (9). Although mutations in FKBP10 appear to effect a subtle delay in the rate of type I procollagen secretion (10), neither type I procollagen molecules made by those cells nor those made by cells from individuals with mutations in PLOD2 (24) are overmodified, indicating that the actions of FKBP65 and LH2 are independent of CRTAP, P3H1 and CYPB in the assembly and secretory pathway of type I procollagen molecules.

FKBP65 is an RER resident protein and is a member of the family of prolyl cis–trans isomerases that are inhibited by FK506, a drug that has effects on immunological integrity (25). It is not clear that FKBP65 has an immune system function but, instead, has been found to associate with the extracellular matrix protein tropoelastin during its transit through the secretory pathway (25). Recently, Alanay et al. (10) identified a set of families from Turkey that had the combination of a recessively inherited form of epidermolysis bullosa simplex that resulted from mutations in KRT14, located on chromosome 17 and a co-segregating recessive form of OI. A region of 4 Mb on chromosome 17q21.2 was identical by descent in all affected members in multiple families. This region contained FKBP10, which had become a candidate because of its ability to bind gelatin (denatured collagen) sepharose (26). All affected members of these families were homozygous for a 33 bp deletion in exon 2 of FKBP10. Analysis of DNA from a consanguineous Mexican family identified a 1 bp insertion that led to a frameshift, a downstream termination codon and nonsense-mediated mRNA decay with no residual protein. Subsequent studies suggested that the phenotypic range in those with FKBP10 mutations overlapped that of individuals with mutations in the PLOD2 gene and a clinical picture of Bruck syndrome (12).

We have now identified missense, nonsense and frameshift mutations in FKBP10 all of which are associated with a phenotype that overlaps with Bruck syndrome, characterized by congenital contractures and fractures, that results from mutations in PLOD2 (18). We have confirmed that some individuals thought to have Bruck syndrome have mutations in FKBP10, we have found that members of a previously published Bruck syndrome family in which bone collagen cross-linking was abnormal (24) have missense mutations in FKBP10, and we have demonstrated that bone collagen cross-links are abnormal in individuals with null mutations in
FKBP10. Given this clinical overlap and biochemical analysis of bone collagen from one of the subjects studied here that demonstrated abnormal hydroxylation of cross-links, it is clear that FKBP10 represents a Bruck syndrome locus. The absence or marked diminution of FKBP65 appears to mediate the phenotype, at least in part, through a failure of LH2, encoded by PILOD2, to fully hydroxylate telopeptide lysines in type I collagen molecules in bone.

RESULTS

Clinical presentations: phenotype and clinical findings

We identified 33 subjects with FKBP10 mutations from 21 unrelated families; a further five subjects from three of these families were identified on clinical grounds to bring the total number of affected individuals to 38 (Table 1). Seventeen subjects (from 10 families) originated from Samoa (or nearby islands) in the South Pacific, and 17 subjects (from 7 families) from different Middle Eastern countries and the remaining 4 were identified in the USA. Clinical data on four subjects from two families (Families K and R, Table 1), thought on clinical grounds to have Bruck syndrome, were previously published (27,28).

Twenty-eight of the subjects were identified in infancy because they presented with contractures (14 subjects) or fractures (5 subjects) or both (9 subjects). Flexion contractures were evident at birth, and variably affected the elbows and knees (preventing full extension) and the ankle, preventing dorsiflexion and presenting as talipes (Fig. 1A). Contractures of the wrists or digits were uncommon. Fractures were most common in the femurs (Fig. 1B), were often multiple and occasionally were detected in utero.

A smaller group of seven subjects presented later in childhood, typically with pain on walking or long bone fractures (most commonly the femur between the ages of 4 and 18 years). The difficulty with ambulation was the result of progressive acetabular protrusion (unilateral or bilateral), which often produced artifacts from scoliosis, fractures and acetabular protrusion. In five subjects from the Samoan group, the mean age-related SD (Z-score) at the radial shaft site was normal at 0.0 (range −0.9 to +0.5). The mean Z-score for the cortical width at the mid-point of the second metacarpal measured from hand radiographs in six subjects was −0.1 (range: −1.0 to +1.3).

Undecalified sections from a transiliac bone biopsy showed trabecular osteopenia, but the cortices were of above average width. There was no mineralization defect and under polarized light the bone had a normal lamellar structure (Fig. 2).

FKBP10 mutations identified

We identified 9 separate mutations in these 21 families (Fig. 3 and Table 2), five of which resulted in frameshifts and unstable mRNA, three were missense mutations and one was a nonsense mutation. All but two (C1, C2) of the 33 affected individuals tested were homozygous for a mutation seen in the family. In 9 of the 10 families that derived from the Samoan or nearby islands (families A, B and D–J), the affected individuals were homozygous for a single basepair duplication (c.948dupT, p.Ile317Tyrfs*56) that resulted in a translational frameshift and mRNA instability (data not shown) so that no protein was produced from the mutant allele (Fig. 4B). Individuals from family C were heterozygous for the c.948dupT, p.Ile317Tyrfs*56 mutation (inherited from their Samoan mother) and for another frameshift mutation (c.831dupC, p.Gly278Argfs*95, inherited from the European father) that also resulted in mRNA instability. We identified the second mutation seen in family C in members of three other families (N, T and U), all of whom were homozygous for the alteration. Two additional frameshifts that resulted from duplication of single nucleotides were seen in a Family P from East Africa/Somalia (c.288dupG, p.Arg97Alafs*101) and Family M from the Middle East (c.743dupC, p.Gln249Thrfs*12). At the time of ascertainment, all these
Table 1. Clinical characteristics of individuals with *FKBP10* mutations

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Fractures at birth</th>
<th>Fracture site (and age)</th>
<th>Mobility</th>
<th>Scoliosis</th>
<th>Acetabular protrusion</th>
<th>Long bone deformity</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Birth to age 1 year</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>A1</td>
<td>F</td>
<td>No</td>
<td>Femur (18y)</td>
<td>Yes (1y)</td>
<td>Yes</td>
<td>Unaided (22y)</td>
<td>(13y)</td>
<td>+0.5</td>
</tr>
<tr>
<td>A2</td>
<td>F</td>
<td>Talipes</td>
<td>Humerus (16y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Two crates (18y)</td>
<td>(12y)</td>
<td>+1.3</td>
</tr>
<tr>
<td>B1</td>
<td>M</td>
<td>No</td>
<td>Femur (5y), Tibia (15y), Radioulna (2(3)y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Never walked (44)</td>
<td>(15y)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>M</td>
<td>Wrist</td>
<td>Femur (4y)</td>
<td>Yes (1y)</td>
<td>Yes</td>
<td>Two crates (14y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>F</td>
<td>No</td>
<td>Tibia (20y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Never walked (22y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>M</td>
<td>No</td>
<td>Femur (10y), Tibia (20y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Unaided (34y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td>F</td>
<td>No</td>
<td>Femur (4y), (14y, 18y)</td>
<td>Yes</td>
<td>Yes</td>
<td>One crutch (19y)</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>C1</td>
<td>F</td>
<td>No</td>
<td>Femur (11y, 13y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Unaided (35y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>M</td>
<td>Talipes, multiple</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Two crates (26y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>M</td>
<td>Talipes, multiple</td>
<td>No (3y)</td>
<td>No</td>
<td>No</td>
<td>Unable to stand (2y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>M</td>
<td>Talipes, multiple</td>
<td>Fibula (2y)</td>
<td>No</td>
<td>Yes</td>
<td>Unable to walk (3y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>M</td>
<td>Talipes, multiple</td>
<td>Clavicle, scapula</td>
<td>No</td>
<td>Yes</td>
<td>Stopped walking (3y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>M</td>
<td>Talipes, multiple</td>
<td>Scapula (2y), clavicle (3y), femur (5y), vertebral</td>
<td>No</td>
<td>Yes</td>
<td>Stopped walking (3y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>M</td>
<td>No</td>
<td>Kyphosis</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
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<tr>
<td>G1</td>
<td>F</td>
<td>Talipes, knee</td>
<td>Femur (5y), forearm, ribs</td>
<td></td>
<td>Yes</td>
<td>'Acetabular dysplasia'</td>
<td>0.0 (5m)</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>F</td>
<td>Talipes, knee</td>
<td>Tibia and others (from age 3y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Wheelchair (39y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>M</td>
<td>Yes</td>
<td>Tibia and others</td>
<td>Yes</td>
<td>Wheelchair (37y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>F</td>
<td>No</td>
<td>Vertebral, femora, shoulder (from age 8y)</td>
<td>Yes</td>
<td>Yes</td>
<td>Never walked</td>
<td></td>
<td></td>
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<tr>
<td>H2</td>
<td>F</td>
<td>No</td>
<td>Tibia, forearm, humerus</td>
<td>Yes</td>
<td></td>
<td>Wheelchair (37y)</td>
<td></td>
<td></td>
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<tr>
<td>I1</td>
<td>F</td>
<td>No</td>
<td>L. ilium (12y)</td>
<td>Yes</td>
<td>Unable to walk (12y)</td>
<td></td>
<td>(24,28)</td>
<td></td>
</tr>
<tr>
<td>I2</td>
<td>M</td>
<td>Talipes, knee</td>
<td>Tibia, forearm, humerus</td>
<td>Yes</td>
<td>Unable to walk (7y)</td>
<td></td>
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<tr>
<td>J1</td>
<td>M</td>
<td>Yes</td>
<td>Femur, recurrent (7y)</td>
<td>Yes</td>
<td>Unable to walk (7y)</td>
<td></td>
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<tr>
<td>J2</td>
<td>F</td>
<td>No</td>
<td>Femur, recurrent (7y)</td>
<td>Yes</td>
<td>Unable to walk (7y)</td>
<td></td>
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<tr>
<td>J3</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No (2y)</td>
<td>No</td>
<td>Able to stand (2y)</td>
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Continued
<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Contractures at birth</th>
<th>Fractures</th>
<th>Scoliosis</th>
<th>Acetabular protrusion</th>
<th>Height Z-score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Skull Circum&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Wormian bones</th>
<th>Platyschia</th>
<th>Cortical width</th>
<th>Radial shaft BMD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Comment</th>
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<tr>
<td>Family L, FKBP10: c.1330C &gt; T, p.Gln444Ter</td>
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<tr>
<td>L1 F Both elbows</td>
<td>2 fractures at birth</td>
<td>Multiple long bones</td>
<td>Yes (8y)</td>
<td>Yes, tibiae</td>
<td>−4.5 (8y)</td>
<td>−0.3 (8y)</td>
<td>Yes</td>
<td></td>
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<tr>
<td>L2 M L elbow</td>
<td>Femur x2 by 14d</td>
<td>No (4y)</td>
<td>No (4y)</td>
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<tr>
<td>L3 L elbow, both knees</td>
<td>No (3y)</td>
<td>R femur and tibia</td>
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<tr>
<td>L4 Talipes, knee, elbow</td>
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<tr>
<td>Family M, FKBP10: c.743dupC, p.Gln249Thrfs*12</td>
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<td>M1 F</td>
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<td>M2 F</td>
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<tr>
<td>Family N, FKBP10: c.831dupC, p.Gly278Argfs*95</td>
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<tr>
<td>N1 F Yes (knees)</td>
<td>Arm fracture (8m)</td>
<td>Several long bones (humeri, femora)</td>
<td>Yes</td>
<td>Yes (femora, tibiae)</td>
<td>Walks with difficulty</td>
<td>−3.9 (13y)</td>
<td>−0.0 (13y)</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N2 F Yes (knees)</td>
<td>Several long bones (humeri, femora, vertebrae)</td>
<td>Yes</td>
<td>Yes (femora, tibiae)</td>
<td>Walks (6y)</td>
<td>−3.8 (6y)</td>
<td>−0.1 (6y)</td>
<td>Yes</td>
<td></td>
<td></td>
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<tr>
<td>O1 F Yes</td>
<td>R foot at 4m</td>
<td>3–4 every year</td>
<td>Yes</td>
<td>Yes</td>
<td>Wheelchair (11y)</td>
<td>Yes</td>
<td>−5.7 (13y)</td>
<td>+2.0 (11y)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
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<tr>
<td>O2 F Yes (elbows, knees, ankles)</td>
<td>At 6m</td>
<td>7 fractures by age 13</td>
<td>Yes (11y)</td>
<td>Yes (13y)</td>
<td>Wheelchair (13y)</td>
<td>Yes</td>
<td>−3.9 (13y)</td>
<td>+3.3 (13y)</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>P1 F 'Multiple neonatal'</td>
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<tr>
<td>P2 F Yes</td>
<td>Several - long bones, ribs, vertebrae</td>
<td>No (2y)</td>
<td>Yes</td>
<td>Not walking (2y)</td>
<td>−4.0 (2y)</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Family Q, FKBP10: c.337G &gt; A, p.Glu113Lys</td>
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<tr>
<td>Q1 M Yes (arms and legs)</td>
<td>Multiple- long bones, ribs</td>
<td>Yes</td>
<td>Yes</td>
<td>Mostly wheelchair</td>
<td>Yes</td>
<td>−10.0 (20y)</td>
<td>Yes</td>
<td>−0.7 (20y)</td>
<td>Linear growth failure (27)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Q2 F No</td>
<td>Several (femora, tibiae)</td>
<td>No</td>
<td>Non-ambulatory (3y)</td>
<td></td>
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<tr>
<td>R1 M Elbows, thumbs, R femur, ribs bilateral talipes</td>
<td>'Many fractures' incl. vertebrae</td>
<td>Yes</td>
<td>Yes (femora)</td>
<td>Never walked</td>
<td>−9.0 (19y)</td>
<td>−0.6 (19y)</td>
<td>Yes</td>
<td>Died of restrictive lung disease</td>
<td></td>
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<tr>
<td>R2 M</td>
<td></td>
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<tr>
<td>S1 M Yes (elbows, knees)</td>
<td>Long bones, ribs, vertebrae</td>
<td>Yes (tibiae, R femur)</td>
<td></td>
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<tr>
<td>Family T, FKBP10: c.831dupC, p.Gly278Argfs*95</td>
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<tr>
<td>T1 F Yes (arms)</td>
<td>None</td>
<td>'many' from age 2y</td>
<td>Yes</td>
<td>Some bowing - not severe</td>
<td>Walker at home, wheelchair outside</td>
<td>−6.5 (30y)</td>
<td>Basilar invagination (8y)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Family U, FKBP10: c.831dupC, p.Gly278Argfs*95</td>
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<tr>
<td>U1 M No</td>
<td>Yes (14y)</td>
<td>Walks with difficulty</td>
<td>Yes, severe (14y)</td>
<td>'Mild short stature'</td>
<td>'OI type IV'</td>
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Numbers in parentheses indicate age in years (y), months (m), or days (d) at which assessment was made or event occurred. A, adult. In the case of scoliosis the age at which corrective spinal surgery was undertaken is shown. Blank fields, no information available.<br><br><sup>a</sup>Standard deviation scores.<br><br><sup>b</sup>Phenotypic information only (no genetic confirmation).<br><br><sup>c</sup>BMD, bone mineral density, measured by DEXA scanning; Circum, circumference; L/R, left/right; MC, metacarpal.<br><br>Some details of families K and R have been previously published—references are shown in the last column.
affected individuals were thought to have either an unusual form of OI or a variant form of OI type IV. Because of known familial consanguinity, recessive inheritance was assumed in the last two families. The affected families from the Samoan islands were not known to each other and consanguinity was not recognized in those families.

The homozygous nonsense mutation (c.1330C > T, p.Gln444Ter) that we identified in Family L occurred in

Figure 1. Clinical and radiographic variability in patients with FKBP10 mutations. (A) Subject D1 (age 2½ years). He is of short stature (<3rd centile) and is unable to plantar flex either foot or extend the knees because of contractures. By the age of 3 he had sustained no fractures. (B) Radiograph from subject E1 age 3 months. Fractures were noted \textit{in utero} and limb contractures were present at birth. At age 3 months he had a skull fracture and multiple rib and long bone fractures (arrows). (C) Development of severe acetabular protrusion during adolescence in A2. (D) The development of scoliosis in A2. Her first fracture occurred at the age of 16 years. (E) Subject C1 age 35. Note marked acetabular protrusion on the right. She sustained femoral fractures at age 11 and 13, but has had no fractures since. (F) Skull findings of playbtasia, relative macrocephaly and Wormian bones (arrow) in A1.
and very little was present in the cell (Fig. 4B). Alanay et al. (data not shown; Fig. 4A) and produced no protein when identified in families A, M and N had very unstable mRNA assessed by western blot analysis (Fig. 4B). In those with missense mutations, the protein was unstable (families Q and K), the protein was unstable compared with control fibroblasts (Fig. 5C and D). Secreted type I procollagen molecules had subtle and localized regional instability (Fig. 6A and B) similar to but less marked than that seen with a mutation in the SERPINH1 gene (see Fig. 6A) (9).

Effects on hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) cross-linking

Fibrillar collagen molecules in tissues are connected through lysyl- and hydroxylysyl-derived cross-links. The mature trifunctional cross-links (pyridinolines) join three chains from three different collagen molecules and involve two telopeptide lysines and one from the triple-helical domain. The involved lysyl residues are located in the amino-terminal and carboxyl-terminal telopeptides and two sites in the triple-helical domain. LH2, encoded by PLOD2, is thought to be the major modifying enzyme for the telopeptide residues and LH1, encoded by PLOD1, is largely responsible for hydroxylation of the triple-helical cross-linking lysyl residues (24,29).

Pyridinoline cross-links were decreased in bone collagen from two individuals in family K (K1 and K2) thought to have Bruck syndrome [described in (24)], in whom no PLOD2 mutation was identified (17) but who we found were homozygous for a missense mutation in FKBP10 (c. 344G > A, p.Arg115Gln; Table 2 and Fig. 3). Those cross-linking findings indicated that there was underhydroxylation of telopeptide lysines in bone type I collagen.

To extend those studies and compare the effect of null mutations in FKBP10, we measured hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) cross-links in bone from A1 and in urine from N1, N2, B1, B4, B5 and D1 (Fig. 7). The content of total pyridinolines in bone from A1, an adult with the Samoan FKBP10 mutation, was about one-tenth that of normal bone (moles/mole of collagen) (Fig. 7A and B). HP is formed by the condensation of three hydroxylated lysyl residues, from two telopeptides and one helical site. LP is formed by condensation of two hydroxylated telopeptide lysyl residues and a non-hydroxylated helical lysyl residue. The abnormally low ratio of HP/LP and very low total pyridinoline content of the bone suggest that there was underhydroxylation of helical domain cross-linking lysines as well as telopeptide lysines. The reversed HP/LP ratio in the FKBP10 bone collagen (Fig. 7B) required the molecular sieve clean-up step to observe on HPLC because pyridinoline levels were low. Without the first step, background fluorescence overshadowed the HP peak giving a falsely high HP/LP ratio. This may explain why such low ratios were not previously reported for bone from an FKBP10 case of Bruck syndrome (24).

The HP/LP ratio in urine was higher than normal and did not match the inverse ratio found in the FKBP10 mutant bone collagen. Most of the HP found in FKBP10 mutant urine, however, appears to be derived from cartilage collagen, a conclusion supported by the structure of the cross-linked peptide from the C-telopeptide domain of type II collagen present in FKBP10 mutant and normal urines (see Fig. 9D).
Mass spectral peptide analysis of the effects of FKBP10 mutations on bone and cartilage collagen cross-linking

On electrophoresis type I collagen extracted from FKBP10 mutant bone had a cross-linked β-dimer pattern similar to that seen in normal skin rather than bone (Fig. 8A). There was an increase in the relative amount of the α1(I)–α2(I) dimer (B12) species. The β-dimers from skin are derived from intramolecular cross-links and the increased amount is consistent with failure to progress along the pathway to form the hydroxylysine-based cross-links seen in normal bone. To determine whether the effect on pyridinoline formation reflected changes in telopeptide hydroxylation, the α1(I) chains from bone were fragmented with trypsin and the peptides were identified by tandem mass spectrometry. A fragment found in the sample from the bone from A1 but not the control contained an N-telopeptide lysyl residue that was not hydroxylated (Fig. 8B). All four species of a peptide that contained the cross-linking lysyl residue at position 87 of the triple helix of α1(I) were identified: those with no hydroxylation, the hydroxylated residue, and the mono- and di-glycosylated species (Fig. 8C). Tandem mass spectrometric
FKBP65. Western blot analysis showed loss of the FKBP65 protein in individuals with missense mutations (Q1 and K2). Compound heterozygosity for a missense and nonsense mutation in PLOD2 has no effect on the amount of FKBP65 present.

Analysis of peptides prepared by bacterial collagenase digestion of bone collagen identified a prominent peptide in patient A1 and control (Fig. 9A and B). The peptide contained the triple helical domain of the proα1(I) chains (Lys-930). From the mass spectral MS/MS data (not shown), this residue was significantly more hydroxylated in the A1 sample than the same lysine residue in the control. The same bacterial collagenase digest also contained a peptide in the control sample representing the FKBP10 amplification, and the second lane, A, represents actin. In the presence of the missense mutation (Q1), the mRNA is stable and similar in abundance to that in the control. In the M1 sample there is residual mRNA, and there is no measurable stable mRNA from the cells from N1. (B) Mutations in FKBP10 result in unstable or diminished FKBP65 protein. Western blot analysis showed loss of the FKBP65 protein in individuals with frameshifts due to single base duplications in FKBP10 (A1 [c.948dupT, p.Ile317Tyrfs*56], M1, and N1), and marked reduction of protein in individuals with missense mutations (Q1 and K2). Compound heterozygosity for a missense and nonsense mutation in PLOD2 has no effect on the amount of FKBP65 present.

**DISCUSSION**

FKBP10 encodes a member of the FK506-binding protein family known as FKBP65, a reflection of its molecular mass of 65 kDa. Originally identified as a tropoelastin-binding protein, it clearly has additional ligands (25). We identified biallelic mutations in FKBP10 in members of 21 families who were ascertained because they were thought to have a variety of OI even though most of the usual causes of OI had been excluded. Among these are 10 families in which the probands are residents of or have strong family links to the Samoan islands or the nearby northern Tongan islands; all have the same mutation (c.948dupT, p.Ile317Tyrfs*56). It is likely that this is a founder mutation brought in the initial migration of the early Polynesian settlers to these islands some 3 millennia ago. In four other families that are from different global regions, we identified a common mutation (c.831dupC, p.Gly278Argfs*95) that reflects slippage during replication in a sequence that harbors seven consecutive cytosines and highlights the hazard of extended runs of a single nucleotide to the coding elements in the genome. Sequence analysis of the region indicated that the mutation in each family had occurred on a different allelic background. The same mutation was identified in other studies in families of Mexican, Turkish, South African and Caucasian origin (10,12) which argues for independent origins of the mutation. This is compatible with findings in many other disorders in which this type of mutation is recurrent. The majority of families we identified have mutations that result in mRNA instability and loss of measurable FKBP65 protein in the cell. Since the identification of mutations in FKBP10 by Alanay et al. in 2010 (10), mutations have been identified in individuals originally thought to have Bruck syndrome, characterized by congenital contractures and fractures with apparent recessive inheritance (11–14,16). In the families we studied, this includes families R and K (17,27,28). Families restudied by Kelley et al. (12) include those originally described by Viljoen et al. (30) and by Mokete et al. (31).

There is striking phenotypic diversity in individuals with mutations in FKBP10. About half of the affected individuals sustained fractures in the neonatal period or the first year of life. The remainder had a pattern of fractures that was unusual for a moderate OI phenotype in that the first fractures (typically of the femur) occurred between the age of 2 and 18 years. Bone that was not immobile had normal or increased cortical width (Fig. 2) and normal bone density indicating that low bone mass was not the only factor in the etiology of fractures. The consistent finding of platybasia and acetabular protrusion suggest that the bone was unusually soft and malleable. A little more than half of the individuals we identified, regardless of type of mutation, had a clinical picture with neonatal contractures that overlaps substantially with that seen in individuals with Bruck syndrome who have
biallelic mutations in PLOD2. PLOD2 encodes a long-splice form of lysyl hydroxylase (LH2b) that includes an alternative-spliced exon, has a preference for the non-triple helical lysyl residues located in the telopeptides of the type I collagen chains and is highly expressed in bone.

The genetic heterogeneity in individuals with congenital contractures and fractures was recognized by van der Slot et al. (17) who identified PLOD2 mutations in two of three families they studied with similar phenotypes. In the third, they obtained bone and demonstrated that the content of mature pyridinoline cross-links was markedly reduced, but they were unable to identify a mutation in PLOD2. That family is included in this study as family K, the affected members of which are homozygous for a missense mutation in FKBP10 (c.344G>A, p. Arg115Gln) that results in the production of a protein that is only partially stable.

In bone from one of the Samoan individuals, there was a marked reduction in pyridinoline cross-links and inversion of the ratio (HP/LP) of the two isoforms in the pyridinoline cross-links that did form (Fig. 7). While this could be consistent with underhydroxylation of both telopeptide and helical site lysines, the peptide analyses (Figs 8C and 9A and B) showed that the level of hydroxylation at the helical sites was normal or higher. In bone the triple-helical lysyl residues at the Lys-87 and Lys-930 cross-linking sites in type I collagen chains are usually hydroxylated by LH1 (29) whereas the telopeptide lysines are hydroxylated by LH2. Our findings point to a selective effect in osteoblasts on the ability of LH2 to hydroxylate telopeptide lysines in the chains of type I collagen that alters cross-linking (Fig. 10). Some PLOD2 missense mutations cause enzyme inactivity through misfolding of LH2 (32). It is reasonable to suspect that the

Figure 5. Effects of different mutations in FKBP10 on synthesis and intracellular transport of type I procollagen through the cell. (A) Type I procollagen is not overmodified in cells from individuals with frameshift (A1, M1, N1), missense (Q1) mutations in FKBP10 or biallelic mutations in PLOD2. Cells were labeled with [3H]-proline for 16 h and macromolecules in the culture medium and in the cell layer were harvested and separated after reduction of disulfide bonds (top panels) or treated with pepsin to remove the amino- and carboxyl-terminal propeptides and separated under non-reducing conditions. (B) There is a modest delay in secretion of type I procollagen from cells with mutations in FKBP10 and PLOD2. Cells were labeled for 1 h with [3H]-proline then chased for up to 2 h with unlabeled proline. At 60 min, the rate of secretion of the trimers of type I procollagen showed a delay in the patient cells compared with control (standard t-test, P-value of 0.046), quantitated in the graph to the right. (C and D) Distribution of type I procollagen between the RER (C) and the Golgi (D). Complete loss of FKPB65 as a result of frameshift mutations in FKBP10 (A1, M1, N1) and partial loss of protein that results from missense mutations (Q1, K2) all lead to slight retention of type I procollagen in the RER. Aggregates of type I procollagen reported by Alanay et al. (10) are not evident in these images.
underhydroxylation of telopeptide lysyl residues caused by 
FKBP10 mutations could result from misfolding of LH2 
because the enzyme is a substrate for the cis–trans isomerase. 
Alternatively, the telopeptide substrates may need to be folded 
into an accessible form. The reversed HP/LP ratio in the bone 
collagen (Fig. 7) might result if the few hydroxylysine alde-
hydes formed in telopeptides showed preferential bonding to 
lysines over hydroxylysines at the partially hydroxylated 
triple-helical sites.

The effect of loss of FKBP65 is probably more complex 
than just the marked diminution of lysyl hydroxylation. The 
screted type I procollagen appears to have regions of instabil-
ity, similar to those seen in the context of mutations in 
SERPINH1 (9,23), which encodes the molecular chaperone 
HSP47. Although we have not been able to demonstrate 
stable interactions between FKBP65 and LH2 or HSP47, the 
effect on the activities of these proteins suggests that either 
they are substrates for FKBP65 and that their activity 
depends on the isomerization of the peptide bonds involving 
at least one prolyl residue or that the region in the telopeptide 
of type I collagen chains is a substrate. Structural analysis of a 
unique mutation in equine PPIB (which encodes cyclophilin 
B) that results in fragile skin alters interaction with LH1 and 
leads to alterations in collagen cross-links in skin that reflect 
diminished hydroxylation of the triple-helical lysyl residues 
in type I collagen (33). Cyclophilin appears to act in the 
early phases of collagen formation by an effect on folding of 
the carboxyl-terminal propeptides into conformations that 
can form trimers (8), and a later phase of isomerization to the 
trans form of the peptidyl-prolyl bonds in the triple-helical 
domains to permit the formation of a stable triple helix (34). 
That function is not altered by the equine mutation. The 
absence of cyclophilin B results in slowing of chain associ-
ation and of triple-helix formation with a consequent delay 
in exit from the RER. FKBP65 seems to act further down-
stream in the biogenesis of the collagen molecules and in 
tissue specific ways. It facilitates the hydroxylation of telopep-
tide lysyl residues involved in cross-link formation, and con-
tributes to the final stages of triple-helix maturation. The 
clinical effects of mutations in these two isomerases are differ-
ent.

The overlapping spectra of phenotypic consequences of 
FKBP10 and PLOD2 mutations appear to be causally linked 
though mechanisms that alter collagen cross-link formation 
by diminution of telopeptide lysyl hydroxylation. The vari-
ation seen in families with the same FKBP10 mutation no 
doubt reflects other genetic influences that, in large families 
or in limited populations like the Samoans, might be identified 
by current technologies. The overlap of the clinical effects of 
mutations in FKBP10 and PLOD2 may lead to diagnostic
Figure 6. Mutations in FKBP10 affect protease sensitivity of the type I collagen triple helix. (A) Secreted type I procollagen molecules from cells with FKBP10 mutations are more sensitive to proteolytic digestion than those from control cells, with the apparent cleavage sites similar to those with mutations in SERPINH1. Proteins in medium from cultured dermal fibroblasts labeled overnight with [3H]-proline were digested for 1 or 5 min with a combination of trypsin and chymotrypsin at 37°C without prior cooling of the sample. Procollagens secreted into the culture medium by control cells and then pretreated at 50°C for 15 min were completely degraded following treatment with trypsin/chymotrypsin while procollagens from medium left at 37°C had protease-resistant triple-helical domains. A subset of type I procollagen molecules secreted from patient cells were cleaved asymmetrically at 37°C (the black arrows indicate fragments seen in affected cells and either not seen or in low abundance in the control cells). (B) Characterization by cyanogen bromide cleavage of products of type I procollagen following trypsin/chymotrypsin treatment. The gel lanes from runs equivalent to those in (A) were excised and the bands cleaved with cyanogen bromide. The larger of the new bands, which migrated just below α2(I) was derived from α1(I). The α1(I)CB7 fragment (residues 551–821 of the triple-helical domain) is missing and replaced by fragment A. The size of the fragment indicates that the parent peptide had been cleaved at or near the mammalian collagenase cleavage site (775–776 in the triple-helical domain). The smaller fragment in the parent gel was derived from α2(I). The α2(I)CB3-5 fragment (residues 357–1014 in the triple-helical domain) was shortened, fragment B and the estimated size indicated that it had been cleaved in the region of the collagenase cleavage site (residues 776–777 of the triple-helical domain).
confusion and should lead to analysis of mutations in both genes until discrete elements of clinical presentation can be identified.

METHODS AND MATERIALS

Identification of families for study

We identified mutations in FKBP10 with two strategies. First, because we participated with Alanay et al. (10) in the analysis of the effects of mutations in FKBP10, we screened for mutations in FKBP10 in 142 individuals selected because they had apparently recessively inherited forms of OI, or their cells, when available (84 of 142) made type I procollagen the chains of which had normal electrophoretic mobility, or they had no mutations in type I collagen genes. A number of these had mutations in another recessive OI gene [PLOD2 (1), SERPINF1 (2), SERPNIH1 (1), CRTAP (1), LEPRE1 (2), PPBP (1), FBLN4 (1) (35)] or in type I collagen genes, COL1A1 (5), COL1A2 (1). Because one of the individuals in whom we identified an FKBP10 mutation had the clinical diagnosis of Bruck syndrome (27), we extended the search to include individuals thought to have Bruck syndrome. Second, homozygosity mapping (by M.R.H., A.A., K.C.) in families that originated from Samoa with a unique apparently recessively inherited form of OI identified a 4.8 Mb region of shared homozygosity on 17q in one family (A), in which, fortuitously, the gene had been sequenced as part of the first strategy. All samples screened were in the IRB approved Repository for Inherited Connective Tissue Disorders at the University of Washington. Once mutations were identified in individuals from Samoa, additional samples were obtained with appropriate consent from individuals with compatible phenotypes hospitalized for care in Honolulu (L.S.) or carried in practices in Auckland (T.C.), where the study was approved by the Auckland Regional Ethics Committee.

Mutation identification in FKBP10

Genomic DNA was extracted either from cultured dermal fibroblasts using the QIAamp® DNA Mini Kit (Qiagen), or from peripheral blood using the Puregene® DNA purification kit (Gentra). DNA was extracted from a Guthrie card for one individual (C2) who had died and was used only for specific mutation analysis. The 10 exons of FKBP10 and flanking intronic sequences were amplified in eight reactions (primer sequences and amplification and sequencing conditions are available on request). Sequencing reactions were done using Big Dye Terminators, version 3.1 (Applied Biosystems), and run on an AB 3130XL or AB 3730XL Genetic Analyzer. Sequences were analyzed using Mutation Surveyor® (Softgenetics).

Analysis of collagen produced by cultured fibroblasts

Cells were cultured and type I procollagen and collagen were analyzed as previously described (36).

Western blot analysis of proteins

Fibroblasts (250,000) from affected individuals and controls were plated at confluence in 35 mm tissue culture dishes, allowed to attach overnight, incubated for 18 h in the presence of 50 μg/ml ascorbic acid, and proteins were harvested from the cell layer by ethanol precipitation. Proteins were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes and visualized using standard western blot techniques with a
polyclonal antibody to FKBP65 (25); GAPDH (Santa Cruz, sc-25778) was used as an internal control.

Pulse-chase studies
Pulse-chase studies were performed as previously described (9).

Immunocytochemical analysis of proteins in cultured fibroblasts
Fibroblasts (75 000) from affected individuals and controls were plated onto sterile coverslips in 12-well tissue culture plates, allowed to attach overnight and then incubated in the presence of 50 μg/ml ascorbic acid for 18 h. Immunofluorescence was performed using a modified protocol from Abcam (http://www.abcam.com/index.html?pageconfig=resource&rid=11417). Briefly, cells were rinsed with cold 1× PBS, fixed with 4% paraformaldehyde in 1× Sorenson’s for 15 min at room temperature and permeabilized with 1X PBS + 0.25% Triton X-100 at room temperature for 10 min. Following a
1-h treatment in block solution at room temperature, primary polyclonal antibodies to type I procollagen (LF9) (37) were added in sets of two with either an antibody to the KDEL ER-marker protein (Abcam ab12223), or the 58 K Golgi protein (Abcam ab6284) and allowed to hybridize at room temperature for 2 h. Secondary antibodies conjugated to fluorophores (Invitrogen A11032, A11034) were incubated with cells for 1 h at room temperature. Coverslips were mounted in Prolong® DAPI and cells were examined using a Nikon microphot-SA microscope. Images were captured using a Photometrics sensys monochrome digital camera.

Protease digestion assay
Protease sensitivity studies were performed as previously described (9).

Cyanogen bromide mapping
Cleavage of collagens with cyanogen bromide and peptide mapping was performed as previously described (36).

Sample preparation and separation of collagens in bone
An iliac crest biopsy was obtained from A1 with appropriate consent. Control adult bone was obtained from Northwest Tissues Services, Renton, WA. Bone was defatted in chloroform/methanol (3:1 v/v) and demineralized in 0.5 M EDTA at 4°C by established methods (38). Collagen was then solubilized from an aliquot of each tissue preparation by heat denaturation in SDS–PAGE sample buffer and the chains were separated in 5% SDS–PAGE gels (39).

Cross-link analysis
Bone and urine samples were hydrolyzed in 6 N HCl, dried and the material redissolved in 10% acetic acid. The soluble material was loaded on a Bio-Gel P-2 (Bio-Rad) column (1.5 cm × 30 cm) and then eluted with 10% acetic acid. The fractions that contained pyridinolines were pooled for quantitative analysis by C18 reverse-phase HPLC (40).

Mass spectrometry of collagen peptides
In-gel trypsin digests of collagen chains cut from SDS–PAGE gels were carried out as described (3,5). Demineralized bone was digested with bacterial collagenase (38) and the resulting collagen-derived peptides were separated by reverse-phase HPLC (C8, Brownlee Aquapore RP-300, 4.6 mm × 25 cm) with a linear gradient of acetonitrile:n-propanol (3:1 v/v) in aqueous 0.1% (v/v) trifluoroacetic acid (4). The pyridinoline cross-linked type II collagen C-telopeptide previously identified in urine (41,42) was enriched by reverse-phase and ion-exchange cartridge extraction and resolved by C8 HPLC for mass spectrometric analysis.

Electrospray MS was performed on in-gel trypsin digests and individual HPLC column fractions using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (LC) (ThermoFinnigan) using a C8 capillary column (300 μm × 150 mm; Grace Vydac 208MS5.315) eluted at 4.5 μl/min. The LC mobile phase consisted of buffer A (0.1% formic acid in MilliQ water) and buffer B (0.1% formic acid in 3:1 acetonitrile:n-propanol v/v). The LC sample stream was introduced into the mass spectrometer by electrospray ionization (ESI) with a spray voltage of 3 kV. The Sequest search software (ThermoFinnigan) was used for linear peptide identification using the NCBI protein database. Cross-linked peptides and glycosylated variants were identified manually by calculating theoretical parent ion masses and possible ms/ms ions and matching these to the actual parent ion mass and ms/ms spectrum.

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Conflict of Interest statement. None declared.

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