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Genetics

Dominant dystrophic epidermolysis bullosa: Identification of a Gly → Ser substitution in the triple-helical domain of type VII collagen

(genodermatoses/bullous skin diseases/cutaneous basement membrane zone)

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ABSTRACT **Epidermolysis bullosa (EB) represents a group of genodermatoses characterized by fragility and easy blistering of the skin. In the dystrophic forms of EB, blisters occur below the basement membrane of the skin, at the level of the anchoring fibrils. We have recently demonstrated tight genetic linkage between the type VII collagen gene (*COL7A1*) and both the dominant and recessive forms of dystrophic EB. We searched for mutations in dominant dystrophic EB by PCR amplification of genomic segments of *COL7A1*, followed by heteroduplex analysis. Examination of the PCR fragment corresponding to exon 73 of *COL7A1* revealed a marked shift in the electrophoretic pattern in patients from a large Finnish dominant dystrophic EB family with genetic linkage to the *COL7A1* locus ($\hat{Z} = 5.37$, $\theta = 0$). Sequence analysis revealed a G → A transition at nucleotide 6118 in the triple helical domain of *COL7A1*, which converted a glycine residue to a serine (GGT → AGT). This mutation occurs between interruptions 11 and 12 of the triple helix, in the seventh of a series of 24 uninterrupted Gly-Xaa-Yaa repeats. Pathogenetic glycine substitutions that disrupt the triple helix have been shown to exert a deleterious effect on the protein in several other disorders involving collagen genes. The clinical phenotype in this family probably arises due to a dominant negative mutation in type VII collagen, resulting in the formation of structurally abnormal anchoring fibrils.**

Epidermolysis bullosa (EB) is a group of mechanobullous diseases characterized by blistering of the skin and the mucous membranes as a result of minor trauma (1, 2). Based on the specific level of tissue separation within the cutaneous basement membrane zone, as determined by diagnostic electron microscopy, the heritable forms of EB have been divided into three major categories: the simplex, the junctional, and the dystrophic forms. The simplex forms are due to tissue separation at the level of basal keratinocytes of the epidermis as a result of mutations in the basal keratin genes, *KRT5* and *KRT14* (3, 4). The junctional forms display blister formation within the cutaneous basement membrane at the level of the lamina lucida, and specific mutations in the genes *LAMB3* and *LAMBC2* encoding the kalinin B1 and B2 chains, respectively, have recently been detected (5, 6). The dystrophic (scarring) forms of EB demonstrate tissue separation below the dermal–epidermal basement membrane, and mutations in the gene encoding type VII collagen (*COL7A1*) have been identified in families with the recessively inherited forms of the disease (7–9).

The diagnostic hallmark of the dystrophic forms of EB is abnormalities in the anchoring fibrils, attachment structures at the dermal–epidermal junction, including morphologic alterations, scarcity, or even complete absence (10). Type VII collagen has been demonstrated to be the major, if not the exclusive, component of the anchoring fibrils (11, 12). We have recently cloned the human type VII collagen cDNA and mapped *COL7A1* to the chromosomal locus 3p21 (refs. 13–15 and A.M.C., unpublished data). Using intragenic as well as flanking restriction fragment length polymorphisms, we demonstrated strong genetic linkage between *COL7A1* and both the dominantly and recessively inherited forms of dystrophic EB, without evidence for locus heterogeneity (7, 15–18). In this study, we report a mutation in *COL7A1* in a family with dominant dystrophic EB (DDEB).

MATERIALS AND METHODS

PCR Amplification and Heteroduplex Analyses. A large Finnish family with clinical, histopathologic, and ultrastructural features of DDEB was studied (for details, see ref. 15). DNA isolated from peripheral blood lymphocytes was used as template for amplification of genomic sequences within *COL7A1*. For this purpose, oligonucleotide primers were synthesized on the basis of intronic sequences (A.M.C., unpublished data) to generate ≈300-bp products. The primers used to amplify the 287-bp fragment containing exon 73 were an upstream primer (5′-GGGTGTAGCTGTACAGCCAC-3′) and a downstream primer (5′-CCCTCTTCCCTCACTC-TCCT-3′). For PCR amplification, ≈500 ng of genomic DNA was used as template, and the amplification conditions were 94°C for 7 min, followed by 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec for 40 cycles, in an OmniGene thermal cycler (Marsh Scientific, Rochester, NY). Amplification buffer contained 1.5 mM $MgCl_2$ and 2 units of *Taq* DNA polymerase (GIBCO/BRL), in a total volume of 50 μl. Aliquots of 5 μl were analyzed by agarose gel electrophoresis in 1.0% gels and 10 μl of the sample was prepared for heteroduplex analyses according to the manufacturer's recommendations (MDE; AT Biochem, Malvern, PA). Heteroduplexes were visualized by staining with ethidium bromide. Bands of altered mobility detected in heteroduplex analysis were subcloned into the pT7 Blue T-vector (Novagen) and sequenced by standard techniques (19).

Verification of the Mutation. The mutation detected in the PCR product containing exon 73 was verified at the genomic level. For this purpose, a search for potential change in restriction endonuclease sites as a result of the mutation was performed, and a restriction site for *Bfa* I was detected

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Abbreviations: EB, epidermolysis bullosa; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB.

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(CTGG to CTAG). DNA in the region of the mutation was amplified by PCR using the primers indicated above, the products were cleaved with *Bfa* I according to the manufacturer's recommendations (New England Biolabs), and electrophoresed on a 3% agarose gel. Individuals bearing the G2040S mutation (see *Results*) were heterozygous for the undigested fragment (287 bp) and the cleaved fragments (186 and 101 bp), whereas unaffected individuals showed no cleavage by *Bfa* I.

RESULTS

A large Finnish pedigree with DDEB, consisting of 20 affected and 22 unaffected living individuals in four generations, was studied (Fig. 1*A*; for extended pedigree, see ref. 15). Most affected individuals demonstrated a clinical phenotype consistent with the Cockayne–Touraine type, characterized by localized involvement of the skin that primarily affected the extremities (1, 2). However, some individuals within this family also demonstrated features of the Pasini type, which is somewhat more generalized but specifically distinguished from the Cockayne–Touraine type by the presence of albopapuloid lesions (1, 2). The inheritance of the mutated gene in this family was previously linked to the *COL7A1* locus on 3p21 ($\hat{Z} = 5.37$ with $\theta = 0$) (15). This tight genetic linkage and ultrastructural abnormalities in the anchoring fibrils (16) suggested that type VII collagen is the candidate gene for mutations in this family.

To identify the mutation in this family, genomic DNA segments were amplified by PCR and the products were subjected to heteroduplex analysis. Examination of a PCR product spanning exon 73 of *COL7A1* demonstrated a band of altered mobility in affected individuals, as compared to unaffected family members or unrelated healthy individuals (Fig. 1*B*). Sequencing of the subcloned PCR products from all normal individuals of the family demonstrated the presence of guanosine in nucleotide position 6118 (Fig. 2). In contrast, sequencing of the subcloned PCR products containing the mutant allele from affected individuals of the family demonstrated a G → A transition. This nucleotide change resulted in a Gly → Ser substitution at amino acid position 2040 in one of the *COL7A1* alleles. All affected individuals were heterozygous for this mutation, designated G2040S. Examination of the adjacent sequences demonstrated that G2040S occurred within a segment encoding 24 uninterrupted Gly-Xaa-Yaa repeats in the *COL7A1* gene. The Gly → Ser substitution occurred within the seventh Gly-Xaa-Yaa triplet, causing an imperfection in this collagenous domain.

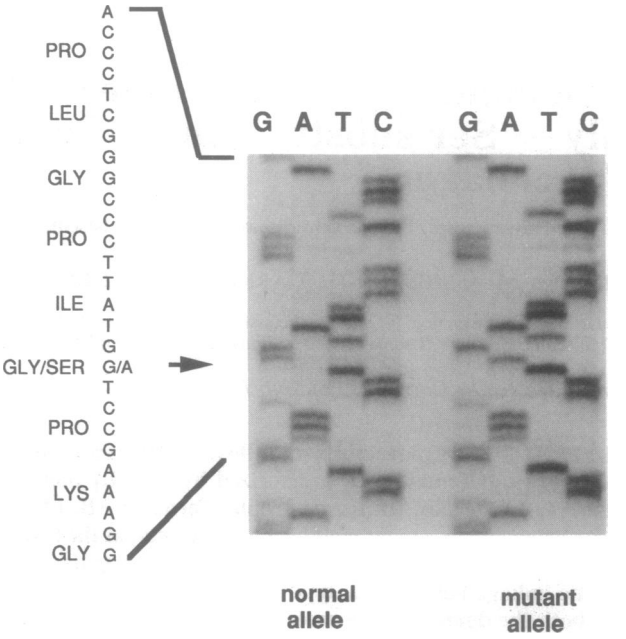

FIG. 2. Sequence analysis of the mutation in exon 73. Nucleotide sequencing of subcloned PCR fragment containing the mutation revealed a G → A transition at nucleotide 6118 in the *COL7A1* cDNA. This resulted in the Gly-2040 → Ser substitution (GGT → AGT) in the triple-helical domain of type VII collagen.

G → A substitution of the first guanosine in the sequence CTGG generated a restriction enzyme site for the endonuclease *Bfa* I (CTAG). To examine the cosegregation of this mutation in the family and to verify its presence in genomic DNA, *Bfa* I digestions were performed on 10 family members (Fig. 1*C*). The results indicated that individuals with an

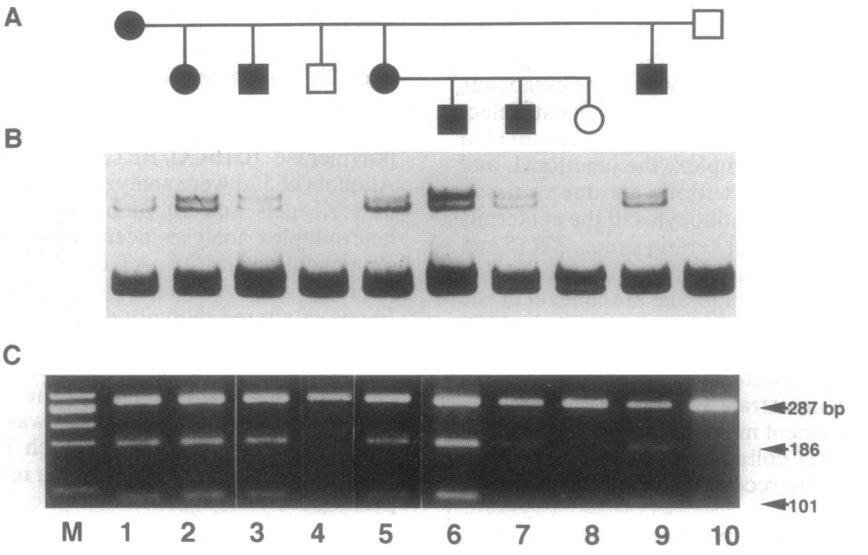

FIG. 1. Cosegregation of heteroduplexes and verification of the mutation G2041S in a family with DDEB. The pedigree under examination is depicted in *A*. Solid circles and squares represent affected females and males, respectively (lanes 1–3, 5–7, and 9), and open symbols represent unaffected individuals (lanes 4, 8, and 10). Heteroduplex analysis shows a markedly shifted band (*B*) in lanes 1–3, 5–7, and 9, corresponding to affected individuals. Verification of the mutation was performed by digestion of the PCR-amplified genomic DNA fragment with *Bfa* I and electrophoresis in 3% agarose (*C*). Affected individuals in lanes 1–3, 5–7, and 9 are all heterozygous for the normal allele (287-bp band) and the mutated allele (186- and 101-bp bands), whereas normal individuals show only the 287-bp uncleaved fragment (lanes 4, 8, and 10).

altered mobility of this PCR product in MDE heteroduplex analysis were heterozygous for an allele digestible with *Bfa* I, bearing the G → A transition. In addition, *Bfa* I digestion of PCR-amplified DNA from 36 *COL7A1* alleles from unrelated ethnically matched individuals demonstrated the absence of the G2040S mutation. Furthermore, this mutation was not present in 73 unrelated patients of different forms of dystrophic EB with varying ethnic backgrounds. These observations suggest that G2040S is not a common polymorphism, nor is it a prevalent mutation in other DDEB families.

DISCUSSION

The results of this study demonstrate that DNA from affected individuals in a large pedigree with DDEB contains a heterozygous G2040S mutation in *COL7A1*, the locus to which it had been linked (15). This mutation interrupts a collagenous region consisting of 24 contiguous Gly-Xaa-Yaa repeats. It was detected only in the affected family members, whereas unaffected family members, 18 ethnically matched healthy individuals, and 73 unrelated dystrophic EB patients of various ethnic backgrounds did not demonstrate this mutation. These observations suggest that the mutation G2040S is the underlying cause of DDEB in this family.

Previously, more than 100 missense mutations that result in a Gly → Xaa substitution have been described in the genes encoding type I, II, III, and IV collagens, manifesting clinically as osteogenesis imperfecta, chondrodystrophies, Ehlers–Danlos syndrome type IV, and Alport syndrome, respectively (20, 21). These observations further emphasize the likelihood that the Gly → Ser substitution is the underlying cause of DDEB. Specifically, this substitution is predicted to destabilize the triple-helical domain within type VII collagen extending from amino acids 2022 to 2093. It should be noted, however, that the collagenous domain of type VII collagen contains 19 imperfections or interruptions in the characteristic collagenous Gly-Xaa-Yaa amino acid repeat (refs. 13, 14, and 22, and A.M.C., unpublished data). Furthermore, the interruption caused by the Gly → Ser substitution is 62 amino acids downstream from the major nonhelical interruption in the collagenous domain, consisting of 39 amino acids of noncollagenous sequences. We speculate that the stability of the triple-helical segment adjacent to the unstable nonhelical interruption is critical for the function of type VII collagen. In support of this suggestion are preliminary observations that the amino acid sequences of type VII collagen are not well conserved through evolution, yet the positions of the imperfections and interruptions are well conserved (22, 23). Thus, a specific domain organization appears to be necessary for the functionality of type VII collagen. It should also be noted that several mutations in *COL4A5* replace glycine with another amino acid in the collagenous domain that also contains interrupted triple-helical segments, and these substitutions are the genetic bases of Alport syndrome (21, 36).

In contrast to the apparent dominant negative nature of the missense mutation demonstrated in this study in DDEB, the severe forms of the mutilating (the Hallopeau–Siemens) type of recessive dystrophic EB (RDEB) are frequently due to premature termination codons in type VII collagen (9, 18). In such cases, the heterozygous carriers, which have one normal allele encoding the full-length α1(VII) chain and one mutated allele encoding a truncated polypeptide (see Fig. 3), synthesize reduced amounts of type VII collagen, and the number of anchoring fibrils is reduced by ≈50% (24). For dominant negative mutations in type VII collagen, which is a homotrimer consisting of three identical α1(VII) chains (12), only 1 out of 8 collagen molecules is expected to be normal (25). This is consistent with the electron microscopic observations of the anchoring fibrils at the dermal–epidermal junction in the family with DDEB studied here (15, 16). The presence of a few thin anchoring fibrils, as opposed to the complete absence of anchoring fibrils observed in the Hallopeau–Siemens type of RDEB (10), would explain the milder phenotype in the dominantly inherited forms of dystrophic EB.

As indicated above, we have demonstrated (18) several mutations in *COL7A1* underlying RDEB, and here we demonstrate a dominantly inherited mutation in the same gene. There are several previous examples of structural proteins in which mutations in the same gene can result in a dominant negative phenotype or be clinically manifested as a recessive trait, including missense mutations in the keratin 14 gene that result in EB simplex inherited either in a dominant or recessive fashion (3, 4, 26). In contrast, in other diseases, such as Marfan syndrome, both premature termination codons and missense mutations have been observed in the fibrillin gene (*FBN1*), yet the pathogenesis consistently results via a dominant negative mechanism (27). This difference may reflect alternate mechanisms of macromolecular assembly and suggests that only full-length mutant polypeptides, which are incorporated into the type VII collagen trimer molecule, exert a dominant negative effect, whereas truncated molecules fail to become incorporated and do not result in a clinical phenotype in heterozygotes (9, 18) (Fig. 3). Thus, elucidation of a large number of mutations in *COL7A1* will enhance our understanding of the structure–function relationships within different domains of type VII collagen.

Identification of mutations in type VII collagen in the dystrophic forms of EB has several implications. First, characterization of mutations will allow classification of these diseases on a molecular basis, rather than based on clinical, and often subtle, differences (2). In this context, it is of interest to note that DDEB has been clinically divided into two subtypes, the Cockayne–Touraine and the Pasini types. Although most of the members of this family demonstrated clinical features of the Cockayne–Touraine type, several individuals had also features of the Pasini (albopapuloid) type (15, 29). The intrafamilial heterogeneity in the clinical phenotype manifesting from the same underlying genetic lesion suggests that the classification of DDEB patients into Cockayne–Touraine and Pasini subtypes may be arbitrary, although one must also consider the potential influence of genetic background and modifying genes.

The second consequence of the identification of mutations is that patients with the dominant dystrophic form of EB in the family can elect DNA-based prenatal diagnosis during the first trimester of gestation (30). We have recently performed prenatal analysis of the fetal genotype using chorionic villus sampling in three families at risk for the recurrence of RDEB (30, 31). In two cases, the predictions were made by intragenic restriction fragment length polymorphisms, and in one case, a specific mutation was used as the marker of the genotype. In each case, the fetus was predicted to be a heterozygous clinically normal carrier of a mutation, and these predictions were fulfilled by the birth of clinically normal infants.

Finally, identification of mutations in *COL7A1* in the dystrophic forms of EB provides the foundation for the development of gene therapy for these patients. For patients with RDEB who do not synthesize full-length α1(VII) chains due to presence of premature termination codons, introduction of normal type VII collagen genes using transfection of keratinocytes with the type VII collagen gene *ex vivo*, followed by replantation of the keratinocytes to the skin, is being contemplated (28, 32). Alternatively, direct introduction of the *COL7A1* gene into the skin using a biolistic microprojectile accelerator could potentially be useful in the recessive dystrophic forms of EB (33). However, for dominant negative mutations, these strategies would not be expected to be successful, since the product of the mutated

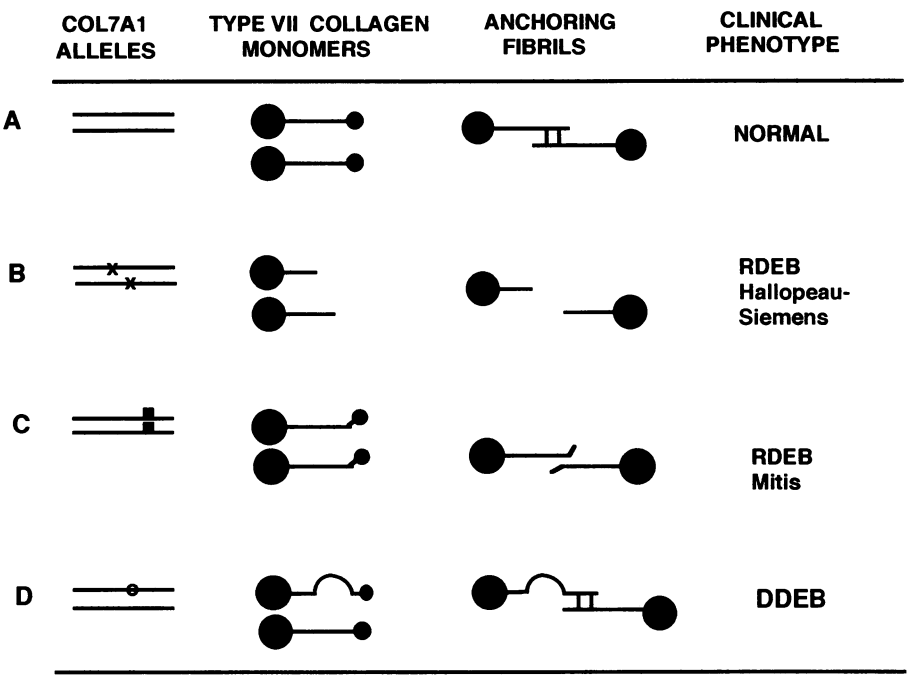

FIG. 3. Schematic representation of consequences of mutations in the type VII collagen gene, *COL7A1*. (*A*) In normal individuals, the *COL7A1* alleles encode polypeptides that associate into a homotrimeric type VII collagen monomer molecule consisting of a central collagenous domain (——) flanked by a large amino-terminal (NC-1, large solid circle) and a smaller carboxyl-terminal (NC-2, small solid circle) noncollagenous domain. In the extracellular milieu, the monomers form antiparallel dimers, a portion of the NC-2 domain is proteolytically cleaved, and the association of the monomers is stabilized by intermolecular disulfide bonds (‖) in the overlapping carboxyl-terminal regions. These dimer molecules laterally aggregate to form normal anchoring fibrils (see refs. 12 and 28). (*B*) Premature termination codons for translation (x) in *COL7A1* result in synthesis of truncated polypeptides that in either homozygous or compound heterozygous state result in the severe mutilating (Hallopeau–Siemens) type of RDEB, whereas the heterozygous carriers are clinically normal (9, 18, 24). (*C*) A homozygous missense mutation (■) within the NC-2 domain has been shown to result in a relatively mild (mitis) type of RDEB (8), probably due to interference in the association of the monomers and/or subsequent stabilization of the dimer molecules by disulfide bonds. The heterozygous carriers were shown to be clinically normal (8). (*D*) A missense mutation (○) resulting in a Gly → Ser substitution in the triple-helical portion of the type VII collagen causes a dominant negative interference in the formation of stable homotrimers (see *Results*). Since one out of eight homotrimer molecules consists of three normal α1(VII) chains, the clinical phenotype of DDEB patients is milder than that in RDEB.

allele apparently interferes with the function of the polypeptide encoded by the normal allele. One approach could involve the use of antisense technology that would selectively deactivate the expression of the mutated allele (34). In the future, this strategy, combined with the upregulation of the normal allele by cytokines, such as transforming growth factor β, which is known to upregulate type VII collagen gene expression *in vitro* (35), could potentially ameliorate the clinical symptoms in patients whose phenotype results from a dominant negative mutation.

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