PrematureTermination Codon Mutations in the Type VII Collagen Gene in Recessive Dystrophic Epidermolysis Bullosa Result in Nonsense-Mediated mRNA Decay and Absence of Functional Protein

Angela M. Christiano,* Satoshi Amano,† Lawrence F. Eichenfield,‡ Robert E. Burgeson,† and Jouni Uitto*§

Departments of *Dermatology and Cutaneous Biology, and §Biochemistry and Molecular Pharmacology, Jefferson Medical College, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, †Cutaneous Biology Research Center, Department of Dermatology, Harvard Medical College, Charlestown, Massachusetts, and ‡Division of Dermatology, Departments of Pediatrics and Medicine, Children's Hospital and Health Center, University of California San Diego, San Diego, California, U.S.A.

The severe mutilating Hallopeau-Siemens type of recessive dystrophic epidermolysis bullosa (HS-RDEB) is characterized by the absence of anchoring fibrils that consist of type VII collagen. We have previously identified premature termination codon (PTC) mutations in both alleles of the type VII collagen gene (COL7A1) in HS-RDEB patients. In this study we have defined the mechanism by which these mutations elicit their phenotypic consequences in a family. The extent of nonsense-mediated mRNA decay induced by these mutations was assessed by quantification of the level of expression of the corresponding mRNA from each of the mutant alleles by RT-PCR of parental RNA. The level of expression of the paternal mutant allele with a PTC in exon 2 was ~30% of that of the wild-type allele whereas that of the maternal mutant allele with a PTC in exon 104 was reduced to about 80% of the normal allele. Immunoprecipitation of newly synthesized type VII collagen with a monoclonal antibody revealed reduced quantities of α1(VII) polypeptides in both parents' cells, whereas their synthesis was entirely absent in the proband's keratinocytes. Thus, a consequence of these premature termination codon mutations in COL7A1 is nonsense-mediated mRNA decay, with a dramatic reduction in type VII collagen synthesis, and the absence of anchoring fibrils in the proband. These results establish a mechanistic link between the presence of premature termination codon mutations in both alleles of COL7A1 and the clinical phenotype of HS-RDEB. Key words: blistering skin diseasesigenodermatoses/ cutaneous basement membrane zone. J Invest Dermatol 109:390-394, 1997

Type VII collagen, a member of the collagen family of proteins, is a homotrimer consisting of three identical polypeptides, α(1(VII)) chains, encoded by a 9.2-kb mRNA (Christiano et al, 1994a). The corresponding human gene COL7A1 consists of a total of 118 exons, the largest number of exons reported in any gene thus far (Christiano et al, 1994b). Type VII collagen is the major, if not the exclusive, component of anchoring fibrils, attachment structures stabilizing the association of the dermal-epidermal basement membrane to the underlying mesenchyme (Uitto and Christiano, 1992; Burgeson, 1993). The dystrophic forms of epidermolysis bullosa (EB) make up a group of heritable diseases with the ultrastructural hallmark of abnormalities in the anchoring fibrils (Uitto and Christiano, 1993). As detected by diagnostic transmission electron microscopy, the morphology of the anchoring fibrils can be perturbed, their number can be reduced, or they may be entirely absent (McGrath et al, 1993). These morphologic observations, initial immunofluorescence studies (Bruckner-Tuderman et al, 1988), and genetic linkage data (Uitto and Christiano, 1994) suggested that COL7A1 is the candidate gene for mutations in the dystrophic forms of EB (DEB).

We have demonstrated a number of COL7A1 mutations both in dominantly and recessively inherited forms of DEB (Uitto and Christiano, 1994; Christiano and Uitto, 1996). In the most severe form of recessive dystrophic EB, the Hallopeau-Siemens type (HS-RDEB), the characteristic genetic lesion is a premature termination codon mutation, resulting either from small insertions or deletions that lead to a frameshift and downstream premature termination codon or from nucleotide substitutions creating a nonsense mutation. Although premature termination codons in both alleles of the proband would predict the synthesis of truncated α(1(VII)) collagen polypeptides, previous studies on different experimental systems have suggested that the major consequence of premature termination codon mutations is the degradation of the corresponding mRNA transcripts, a phenomenon known as nonsense-mediated mRNA decay (Baserga and Benz, 1988; Cooper, 1993).

In this study, we report a family with a proband with HS-RDEB...
that has premature termination codons for translation in both alleles of COL7A1. By using keratinocytes from the proband and his parents, we have examined the consequences of the premature termination codon mutations at the mRNA and protein levels.

MATERIALS AND METHODS

Clinical The proband (J.L.M.; birth date, February 11, 1992) was a 3-year-old male at the time of initial examination with severe fragility of the skin and the mucous membranes that had resulted in extensive scarring of the skin particularly on the hands and feet. He also had esophageal strictures that had required several dilatation procedures. The family was enrolled in the National Epidermolysis Bullosa Registry at the Stanford University Clinical Site, which confirmed the diagnosis of the proband as HS-RDEB. A skin biopsy specimen from the proband revealed complete absence of anchoring fibrils, when examined by electron microscopy. The proband was the only child of unaffected unrelated parents, and there was no history of a similar blistering disease in the family. The proband’s father had three clinically unaffected children with another partner.

Mutation Detection Strategy We have recently devised a strategy to amplify all 118 exons of COL7A1 directly from genomic DNA by polymerase chain reaction (PCR) using 72 primer pairs placed on flanking intronic sequences (GenBank accession no. L23982; Cristiano et al., in press). The PCR products are screened for sequence variations by heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE; Ganguly et al., 1993). If evidence for a heteroduplex is noted, the corresponding PCR product is subjected to nucleotide sequencing. When a putative mutation is identified, its inheritance in the family is verified either by allele-specific oligomer hybridization (ASO) or by restriction enzyme digestion.

In this study, genomic DNA was isolated from the peripheral blood leukocytes from the proband, his parents, and the clinically unaffected relatives. For amplification of exons 2 and 104 of COL7A1, which were shown to contain the deletion mutations (see Results), the following primer pairs were used. For exon 2, a 375-bp product, the sense primer was 5'-ACCATCCCAAGTCCAGTG-3' and the anti-sense primer was 5'-GTGTTTGGTGAAAGACCTGG-3'. For exon 104, a 513-bp product, the sense primer was 5'-CGGGCCTGTTGTATTAAG-3' and the anti-sense primer was 5'-CAAAAGCTACCACACTGGTG-3'.

The PCR amplification conditions were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. The PCR products were examined on 2% agarose gels and subjected to heteroduplex analysis. In the event of evidence for a heteroduplex, both alleles of the PCR product were subcloned into a PCR compatible vector (TA, Invitrogen, San Diego, CA) and sequenced manually by using the diodeoxyribonucleotide chain-termination method (Sanger et al., 1977).

The mutation in exon 2, a nucleotide deletion (see Results), was confirmed at the DNA level by ASO hybridization with the following oligomers: wild-type oligomer, 5'-CTTTCTCTAGGAAGCTTGGT-3'; mutant oligomer, 5'-CTTTCTCTAGGAAGCTTGGT-3'.

The PCR product containing the mutation was subjected to digestion with this restriction enzyme. In the event of a heteroduplex, both alleles of the PCR product were subcloned into a PCR compatible vector (TA, Invitrogen, San Diego, CA) and sequenced manually by using the diodeoxyribonucleotide chain-termination method (Sanger et al., 1977).

RESULTS

Identification of the Paternal Mutation in Exon 2 As indicated above, we have designed a strategy to identify mutations in COL7A1 in patients with the dystrophic forms of EB (Christiano et al., in press). In this study, CSGE analysis of the PCR product corresponding to exon 2 revealed a heteroduplex with DNA from the proband and his father, but not from his mother’s DNA. Subsequent sequencing of the mutant allele revealed the deletion of a G nucleotide in the position 189, corresponding to COL7A1 cDNA (GenBank accession no. L02870), in comparison with the sequence in the normal allele (Fig 1B). This mutation, 189delG, causes a frameshift and results in a premature termination codon of translation (TGA) 121 nucleotides downstream from the site of the deletion.

The presence of the nucleotide deletion mutation in the proband and his father was demonstrated by ASO. Both the mutant and the wild-type oligomers clearly hybridized to the patient’s and father’s DNA (Fig. 1C). In contrast, the mother’s DNA and that from an unrelated healthy control hybridized with the wild-type oligomer only. These results indicated that the proband was heterozygous for the mutation 189delG that had been inherited on the paternal allele.

Identification of the Maternal Mutation in Exon 104 CSGE analysis with the PCR amplification product corresponding to exon 104 of COL7A1 revealed a distinct heteroduplex with proband’s and his mother’s DNA (Fig. 2A). The father demonstrated the presence of a homoduplex only. Sequencing of the mutant allele revealed a deletion of a G nucleotide in position 7786, in comparison with the sequence in the normal allele (Fig 2B). This mutation also results in a frameshift and downstream premature termination codon of translation (TGA) 105 bp downstream from the site of the nucleotide deletion.
The mutation 7786delG deleted a restriction enzyme site for HphI. Digestion of the control or paternal DNA revealed the presence of 180-, 150-, 93-, 60-, and 30-bp bands, indicating that the PCR product was cleaved by this enzyme (Fig 2A). In contrast, the PCR product from the proband and the mother demonstrated the presence of an additional uncleaved band of 210 bp, indicating that they were heterozygous for this mutation, as verified by the loss of the HphI site (Fig 2C). Collectively, the mutational analyses indicated that the proband in this family is a compound heterozygote for 189delG/7786delG mutations. Both mutations result in a frameshift and a premature termination codon for translation downstream from the site of the mutation, and the parents were heterozygous carriers of the respective mutations.

mRNA Transcript Levels Corresponding to the Mutant Alleles Because premature termination codon mutations have been shown to result in reduction of the corresponding mRNA transcript levels (Baserga and Benz, 1988; Cooper, 1993), we determined the level of expression of mRNA from each of the mutant alleles in keratinocytes established from the parents, who were heterozygous carriers for the respective mutations. The transcript-targeted PCR and subsequent hybridization of the wild-type and the mutant allele from the father revealed ~30% level of expression of the allele containing the mutation 189delG in exon 2, in comparison to the wild-type allele in the same cells (Fig 3A). Similar hybridizations indicated that the allele containing the maternal mutation 7786delG in exon 104 was expressed at about 80% level of the wild-type allele in the mother’s keratinocytes (Fig 3B). Furthermore, we performed PCR on the heterogeneous nuclear RNA pool by using intron-based primers and restriction endonuclease digestion specific for the maternal mutation and showed that the relative amount of heterogeneous nuclear RNA was approximately equal for both the wild-type and mutant maternal alleles (data not shown). Thus, a consequence of both the maternal and paternal premature termination codon mutations is a reduction in the level of the corresponding mRNA transcript, either at the time of splicing or immediately after.

Consequences of the Mutations at the Protein Level The synthesis of type VII collagen in keratinocyte cultures established from the proband, his parents, and an unrelated control individual was examined by immunoprecipitation with a specific monoclonal antibody. The specificity of the α1(VII) band was demonstrated by pre-clearing the samples with antibody binding prior to immunoprecipitation. In this case, the α1(VII) collagen band was entirely removed (Fig 4). Examination of the immunoprecipitated radiolabeled material by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the father’s and mother’s keratinocytes clearly synthesized full-length type VII collagen polypeptides, but the level of the protein was reduced in comparison to the amount of α1(VII) chains noted in normal human keratinocytes (Fig 4). In contrast to the parents, who were heterozygous for the premature termination codon mutations, the HS-RDEB patient’s keratinocytes did not synthesize any full-length type VII collagen polypeptides (Fig 4).

DISCUSSION

The Hallopeau-Siemens variant of recessive DEB presents with extreme fragility of the skin and mucous membranes and is ultrastructurally characterized by the absence of anchoring fibrils (McGrath et al, 1993; Uitto and Christiano, 1993). In several of these cases, premature termination codon mutations have been demonstrated in both alleles of the gene encoding type VII collagen (COL7A1; Uitto and Christiano, 1994, 1996). Previously, the pathomechanisms by which these mutations elicit their effects at the phenotypic level had not been examined in detail. In this study, we examined a family with an affected individual who is a compound heterozygote for two different premature termination codon mutations. The paternal mutation was shown to reside within exon 2, and the maternal mutation was in exon 104.

Since nonsense mutations have previously been shown to result in markedly reduced levels of the corresponding mRNAs, we
expression has been previously noted in different heritable disease and in experimental systems (Cooper, 1993). For example, nonsense mutations in the dihydrofolate reductase gene located upstream from the terminal exon have been shown to result in markedly reduced mRNA levels, whereas nonsense mutations within the last exon of the gene yielded essentially normal levels of the corresponding mRNA transcript (Urlaub et al., 1989). Nuclear run-on assays and experiments using an inhibitor of transcription (actinomycin D) suggested that the reduced mRNA levels were not a result of reduced rate of transcription and did not directly reflect instability of the fully transcribed mRNA (Urlaub et al., 1989). On the basis of these observations, two possible models explaining the reduced steady-state levels of mRNA transcripts containing nonsense mutations were suggested (Urlaub et al., 1989; Cooper, 1993). One of them, the translational translocation model, implies coupling between the processing of the pre-mRNA transcript and the translational machinery. Specifically, it has been proposed that translation commences during or shortly after transport of the transcript into the cytoplasm has been initiated and that translation is physically coupled with translocation of the pre-mRNA from splicing complex through the nuclear pores. In the event of a premature termination codon for translation, the translocation would be arrested leaving the mRNA transcript vulnerable to RNase digestion (Cooper, 1993). Nonsense mutations occurring in the last exon would not be recognized until translocation of the entire mRNA was nearly complete, thus explaining the lack of a profound effect of 3'—end mutations at the mRNA level.

An alternate model, the nuclear scanning of translation frames, suggests the existence of a mechanism that scans for the presence of open reading frames within the pre-mRNA. Detection of a premature termination codon would slow down the mRNA processing and translocation of the transcript into the cytoplasm (Cooper, 1993). In support of the latter mechanism is an observation that presence of a nonsense mutation within an in-frame exon can result in selective skipping of this exon, resulting in the synthesis of a shortened mRNA and polypeptide (Dietz et al., 1993b). Thus, there appears to be two possibilities, either intranuclear or cytoplasmic, for accelerated decay of the mRNA transcripts with nonsense mutations (Peltz et al., 1993).

Irrespective of the precise mechanism resulting in marked reduction of the COL7A1 steady-state mRNA levels in the keratinocytes of the proband presented in our study, it is clear that his cells were unable to produce functional type VII collagen. Specifically, immunoprecipitation of protein with an antibody recognizing epitopes within the amino-terminal non-collagenous (NC-1) domain of type VII collagen failed to reveal the synthesis of any full-length polypeptides in the proband's cell cultures, and the amount of the α1(VII) polypeptides in the parents was clearly reduced. As a consequence of the lack of type VII collagen synthesis, no anchoring fibrils could be demonstrated in the proband's skin by transmission electron microscopy, leading to severe fragility and easy blistering in the skin of the proband as a result of minor trauma. These observations attest to the importance of anchoring fibrils in securing the association of the lower portion of the cutaneous basement membrane to the underlying dermis.

In summary, detailed examination of the family with an affected individual with severe HS-RDEB has provided novel mechanistic insights implicating accelerated mRNA decay as a consequence of the premature termination codon mutations in COL7A1. The functional consequences of the reduced mRNA levels were also demonstrated by the absence of type VII collagen and anchoring fibrils. In the future, understanding the mechanism of nonsense-mediated mRNA decay (Peltz et al., 1993), coupled with ribosome-mediated repair of defective mRNA by targeted trans-splicing (Sullenger and Cech, 1994), could provide an alternative strategy to conventional gene replacement therapy for this devastating group of genodermatoses.
Figure 4. Detection of type VII collagen synthesis in keratinocyte cultures by immunoprecipitation. Cells from the proband, his parents, and an unrelated control individual were cultured and radiolabeled, as described in the Materials and Methods. Type VII collagen was precipitated with a specific monoclonal antibody and detected by autoradiography (>). The specificity of the immunoprecipitation was demonstrated by pre-clearing parallel samples with the antibody prior to immunoprecipitation (corresponding lanes C).

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