Genetic bases of severe junctional epidermolysis bullosa presenting spontaneous amelioration with aging

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Change of the clinical picture with aging is noted in some patients suffering from junctional epidermolysis bullosa (JEB), an inherited blistering disorder caused by extensive disadhesion of the epithelia. We have studied a patient born with severe JEB associated with absent expression of laminin 5. A remarkable reduction of the blistering tendency was observed with aging that correlated with a restored expression of immunoreactive laminin 5 molecules. Genetic analysis of the gene LAMB3 detected compound heterozygosity for the nonsense mutation R635X and a novel 2 bp deletion (1587delAG) resulting in a downstream premature termination codon. RT–PCR amplification of total RNA purified from skin biopsies demonstrated that the mutated \( \beta3 \) mRNA transcribed a new internally shortened \( \beta3 \) transcript with advancing age. Our genetic and biochemical data show that (i) the illegitimate splicing of the \( \beta3 \) pre-mRNA results in synthesis and secretion of a laminin 5 heterotrimer with an internally deleted \( \beta3 \) polypeptide, (ii) expression of the mutated \( \beta3 \) polypeptide is up-regulated in the basal keratinocytes with high proliferative potential, (iii) absence of the N-terminal region of the \( \beta3 \) rod domain II thought to stabilize the tertiary structure of the laminin 5 is not required for the assembly of the protein and (iv) the mutant laminin 5 retains its adhesive potential. Our results demonstrate that mRNA rescue may underlie the evolution of the clinical phenotype in inherited skin conditions.

INTRODUCTION

The term epidermolysis bullosa (EB) refers to a group of inherited blistering diseases of the skin characterized by epithelial fragility to mechanical friction (1). EB is associated with molecular defects of the dermal–epidermal junction and has been divided into three major clinical subtypes according to the level of the blister plane as determined by ultrastructural and immunohistochemical analysis of the involved skin (2). In EB simplex, tissue separation occurs within the basal keratinocytes and is caused by genetic mutations in keratins 5 and 14 or in the hemidesmosomal component plectin. In dystrophic EB, the split localizes within the papillary dermis below the lamina densa of the basement membrane zone (BMZ), and results from mutations in collagen type VII. In junctional EB (JEB), blisters occur within the lamina lucida of the BMZ and are caused by mutations in the extracellular adhesion ligand laminin 5 in integrin \( \alpha6\beta4 \) and collagen type XVII, the two transmembrane components of hemidesmosomes (HD).

Laminin 5 constitutes the major adhesion ligand of the basal cells in the stratified and transitional epithelia exposed to the external environment (3). In the epidermis, laminin 5 co-localizes with the anchoring filaments, the threadlike adhesion structures that span the lamina lucida and link the HD of the basal keratinocytes to the lamina densa of the BMZ (4). By bridging the cellular receptor integrin \( \alpha6\beta4 \) and collagen type VII, the major component of the anchoring fibrils of the papillary dermis, laminin 5 mediates the stable adhesion of the basal keratinocytes to the underlying chorion (5). Furthermore, interaction of the C-terminal G domain of laminin 5 with integrin \( \alpha6\beta4 \) triggers formation of HD (6).

Laminin 5 is synthesized by the basal keratinocytes as a heterotrimeric molecule composed of \( \alpha3 \) (200 kDa), \( \beta3 \) (140 kDa) and \( \gamma2 \) (155 kDa) chains encoded by the genes \textit{LAMA3}, \textit{LAMB3} and \textit{LAMC2}, respectively (7). The \( \alpha3\beta3\gamma2 \) heterotrimer assembles by formation of a stable \( \beta3\gamma2 \) heterodimer that integrates the \( \alpha3 \) chain via labile interactions (8). Upon secretion in the extracellular matrix, the \( \gamma2 \) chain undergoes proteolytic processing that reduces the size of the polypeptide from 155 to 105 kDa (9,10). A distinct proteolytic processing also reduces the size of the \( \alpha3 \) chain from 200 to 165 kDa (10). The assembled molecule displays the cross-shaped structure characteristic of laminins, with a globular domain formed by the C-terminal region of the \( \alpha3 \) chain, a long rod-like domains formed by a coiled-coil helix resulting from interaction of domains I and II of each chain, and three N-terminal domains that in laminin 5 are truncated (11). Interchain ionic interactions and distal disulfide bonds stabilize the rod-like domain, while the N-terminal domains of the \( \beta3 \) and \( \gamma2 \) chains form two short arms visible by rotary shadowing analysis of purified
laminin 5. The short arm of the β3 chain consists of an intermediate domain III/V and an N-terminal globular domain VI. Domain III/V contains multiple cysteine-rich epidermal growth factor (EGF) modules the functions of which remain unknown. Domain VI is believed to mediate the interaction of laminin 5 with laminin 6 and/or collagen type VII (5,12,13).

In Caucasian patients with JEB, most of the genetic mutations so far identified in laminin 5 affect the LAMB3 gene. Mutation database analysis indicates that nonsense or frameshift mutations on both LAMB3 alleles that abolish laminin β3 expression result in the severe and often lethal form of JEB [Herlitz JEB (H-JEB)]. Conversely, the combination of nonsense mutations with missense mutations or mutations in the splice site consensus sequences of LAMB3, which reduce the expression levels of the β3 polypeptide, result in the mild manifestations of the condition [non-Herlitz JEB (n-HJEB)] (14). At the protein level, the clinical severity of JEB correlates well with the extent of laminin 5 assembly and deposition in the BMZ (15,16). It also correlates with the structural shape of the HD that, in H-JEB, appear either absent or reduced in number and structurally disorganized, while in n-HJEB they appear normally shaped (17).

In this study, we report the genetic basis of an uncommon form of JEB characterized by a marked improvement of skin adhesion with advancing age. Our results show that the general amelioration of the clinical picture is linked to activation of an illegitimate splicing event leading to expression of an aberrant laminin β3 pre-mRNA that results in the synthesis of a partially functional laminin 5.

RESULTS

Clinical features

The proband, a 7-year-old Caucasian female, is the product of the union of unrelated and clinically unaffected parents that have an older healthy daughter. Shortly after birth, the patient developed extensive cutaneous blisters and erosions at sites exposed to friction (Fig. 1A). Electron microscopy of the skin revealed paucity of dysplastic HD in non-involved areas, and tissue separation at the basal cell/lamina lucida interface in the blisters (not shown). Immunostaining of non-blistered skin revealed an absence of reactivity to monoclonal antibody (mAb) GB3, specific to native laminin 5 (Fig. 1Cb) and mAb K140, specific to laminin β3 chain (Fig. 1Ce). Staining of the laminin α3 and γ2 chains and that of the major components of the HD were attenuated (not shown). Based on these findings the diagnosis of JEB was made.

During the first months of life, the patient developed severe erosions of the skin and oral mucosa. Ulceration of the cornea and nail dystrophy was also observed. Nails were definitely lost at 9 months. The involvement of the patient’s skin then decreased progressively. At the age of 4, the proband presented an excellent general state and a normal growth rate (Fig. 1B). Sporadic blistering affecting the groin and acral areas submitted to mechanical stress. (C) Immunofluorescent analysis of patient’s skin at birth and 4 years old. Skin specimens from a healthy control (a and d) and non-involved areas of patient’s integument at birth (b and e) and at the age of 4 years (c and f) were analyzed using mAb GB3, directed against the native laminin 5 (a–c), and mAb K140 directed against the laminin β3 chain (d–f). At birth, the patient’s skin was neither reactive to mAb GB3 (b) nor to mAb K140 (e). At the age of 4 years, immunoreactivity to the laminin 5 was enhanced (c and f). Brightly fluorescent patches found at the tips of the dermal papillae alternated with areas of weak fluorescence at the rete ridges. (D) Double staining of patient’s skin at 4 years using pAb SE144 against the laminin γ2 chain (a) and mAb IOT29 directed against the integrin β1 (b). The irregular laminin 5 localization correlates with the patchy expression of integrin β1 in highly proliferative cells. Bar, 50 μm.

Search for mutations in LAMB3

Because immunostaining of the laminin β3 chain was undetectable in the patient’s skin at birth, we searched for genomic mutations in LAMB3. A search for sequence variations was initiated by PCR amplification of the exonic
sequences of the gene (18). Direct sequencing of the PCR products covering all exons revealed a heterozygous AG deletion at position 1587 of exon 13 (GenBank accession no. L25541) (Fig. 2A). This 1587delAG deletion leads to a shift of the reading frame and results in a downstream premature termination codon (PTC) in exon 14. Presence of the PTC predicts a truncated β3 polypeptide terminating at residue 524. The mendelian segregation of mutation 1587delAG in the proband’s family was assessed by allele-specific oligonucleotide (ASO) analysis of the genomic DNA obtained from the members of the proband’s family and unrelated controls. The proband, the father and the sister were heterozygous carriers for the mutation (Fig. 2B).

Direct sequencing of the PCR-amplified fragment of DNA corresponding to exon 14 detected a heterozygous C→T transition (Fig. 2C). This base substitution corresponds to nucleotide 1903 of the β3 cDNA sequence and leads to the nonsense mutation R635X in which the termination codon TGA replaces an arginine codon (CGA). The presence of transition 1903C→T in the members of the proband family was verified by ASO analysis of the genomic DNA (Fig. 2D). The proband, the proband’s mother and the maternal grandfather were heterozygous for this mutation.

Expression of β3 transcripts in the proband’s cultured keratinocytes

To examine the consequences of the mutations at the mRNA level, total RNA was purified from the proband’s cultured keratinocytes and used for RT–PCR amplification. The coding sequence of the β3 mRNA was reverse transcribed and PCR-amplified using a pair of primers allowing production of a 738 bp cDNA fragment spanning the 3’ end of exon 12 (120 bp), the entire exons 13 (112 bp) and 14 (379 bp) and the 5’ region of exon 15 (127 bp). The agarose gel electrophoresis of the PCR products detected a slow migration band of the expected size and an additional fast migrating band of ~350 bp (Fig. 3A). Subcloning and amplification of these cDNAs into a bacterial vector, and direct nucleotide sequencing, identified four distinct species of laminin β3 transcripts expressed in patient’s keratinocytes. Two cDNAs (736 and 738 bp, respectively) are produced by legitimate splicing of the aberrant β3 pre-mRNAs transcribed from the two LAMB3 alleles. Both transcripts retain exon 14 (data not shown). The 738 bp cDNA identifies the maternal transcript carrying mutation 1903C→T that leads to PTC, while the 736 bp cDNA identifies the paternal transcript that carries deletion 1587delAG leading to a downstream PTC at nucleotide 1602 (Fig. 3C). The other two β3 cDNAs (359 and 357 bp, respectively) correspond to mRNA species internally deleted of exon 14, and generated by illegitimate splicing involving the acceptor splice site of exon 13 and the donor splice site of exon 15 of the LAMB3 alleles (Fig. 3B). Amplification of the 359 bp cDNA reveals that the maternal transcript carries an out-of-frame deletion of exon 14 which creates a downstream PTC within exon 15 (nucleotide 2117) (Fig. 3C), while amplification of the 357 bp cDNA shows that in the paternal transcript carrying deletion 1587delAG, the internal deletion of exon 14 restores the open reading frame (ORF) and allows expression of a shortened β3 polypeptide (Fig. 3C). Further analysis by RT–PCR amplification of the β3 RNA transcripts using a series of primer pairs sited between exons 11 and 16 excluded skipping of other exonic sequences (data not shown).

Expression of an internally deleted laminin β3 transcript in the proband’s skin

The expression products of the LAMB3 gene were then assessed using skin biopsy specimens obtained from the proband at birth and at the age of 4 (Fig. 4). Total RNA was extracted from skin sections and used for RT–PCR amplification of the 738 bp β3 cDNA fragment spanning from exons 12 to 15 that identifies the species of laminin β3 transcripts synthesized by the proband’s keratinocytes ex vivo. Agarose gel electrophoresis of the PCR amplification products detected the 738 bp cDNA fragment in the skin of an unrelated healthy control, while no band was detectable in the proband’s skin at birth (Fig. 4). A unique fast-migrating cDNA fragment with an apparent mobility of 350 bp was amplified from the proband’s skin samples obtained at the age of 4. This fast-migrating cDNA fragment was cloned and amplified in bacteria. Direct DNA nucleotide sequencing of plasmid cDNAs isolated from
30 independent colonies revealed that all the bacteria had amplified the 357 bp \( \beta_3 \) cDNA that identifies the paternal \( \beta_3 \) transcript carrying mutation 1587delAG and the downstream in-frame skipping of exon 14 (Fig. 3B). These results indicate that no laminin \( \beta_3 \) transcript is readily detected in the proband’s skin at birth, while an mRNA encoding an aberrant laminin \( \beta_3 \) polypeptide is found at the age of 4. The abnormal polypeptide is internally shortened by 127 amino acids within domains II and III, and harbors a methionine and a proline residue that substitute the glycine and cysteine at positions 530 and 531, respectively.

The proband’s keratinocytes express a mutant laminin 5 comprising an internally shortened laminin \( \beta_3 \) chain

To establish a possible correlation between the restored expression of the aberrant laminin \( \beta_3 \) chain and the favorable evolution of the condition, we assessed whether the shortened \( \beta_3 \) transcript encoded by the paternal \( \text{LAMB3} \) allele incorporates into laminin 5 heterotrimers. Cell lysates and spent medium from cultures of the proband’s keratinocytes were immunoprecipitated using polyclonal antibodies (pAb) specific to each single chain of laminin 5 and mAb GB3. All the antibodies co-precipitated a polypeptide with the expected size (130 kDa) of the mutant \( \beta_3 \) chain internally deleted of 127 amino acids, and two polypeptides with the apparent molecular mass of 190 and 155 kDa characteristic of the unprocessed \( \alpha_3 \) and \( \gamma_2 \) chain, respectively (Fig. 5A). No band corresponding to the wild-type \( \beta_3 \) chain (145 kDa) was detected in the patient’s cells. In the spent medium, the \( \beta_3 \) mutant of 130 kDa co-precipitated with the processed \( \alpha_3 \) chain (165 kDa) and the unprocessed and processed forms of the \( \gamma_2 \) chain (155 and 105 kDa, respectively). By quantification of the autoradiograms, the mutant \( \beta_3 \) polypeptide was estimated to represent 15% of the amount of the wild-type counterpart secreted by the healthy control keratinocytes. These results demonstrate that the proband’s keratinocytes synthesize and secrete reduced amounts of a mutant laminin 5 comprising the aberrant 130 kDa \( \beta_3 \) chain.

To determine whether the mutated laminin 5 exerts a functional effect on cell attachment to the culture substrate, we used a cell-detachment kinetic assay to evaluate the adhesive capacity of the patient’s keratinocytes compared to wild-type
and laminin β3-null keratinocytes (19). Exponentially growing cell cultures were treated with a trypsin/EDTA solution and the number of cells dislodged at increasing intervals of time was evaluated by direct counting (Fig. 5B). The results showed that attachment of the proband’s keratinocytes is sensibly enhanced compared with laminin 5-deficient counterparts. Indeed, 12% of the proband’s keratinocytes were detached, compared with 40% of the wild-type cells. Taken together, these observations show that expression of the mutant laminin β3 results in the secretion of partially functional laminin 5 molecules that enhance cell adhesion.

DISCUSSION

We have investigated the genetic background of a rare form of JEB characterized by evolution from extreme skin fragility at birth to a blistering tendency that sensibly reduced with aging. The favorable course of the blistering condition, initially diagnosed as a lethal form of JEB, correlated with enhanced reactivity of the skin to antibodies directed against laminin 5.

Genetic analysis based on nucleotide sequencing of the cDNA encoding the laminin β3 polypeptide revealed that the proband is a compound heterozygote for mutations R635X and 1587delAG in the LAMB3 gene. Both mutations hamper expression of the laminin β3 polypeptide. R635X is a recurrent mutation detected in 45% of the LAMB3 alleles in Caucasian patients affected by the lethal form of JEB (20). In our proband, this mutation is generated by the base substitution 1903 C→T in exon 14 of the maternal LAMB3 allele. Conversely, the 2 bp deletion 1587delAG disclosed in exon 13 of the paternal LAMB3 allele is a novel genetic mutation. This mutation induces a shift of the reading frame that results in a downstream PTC within the mRNA sequence encoded by exon 14. Consistent with the notion that the presence of a PTC causes the decay of aberrant RNA transcripts (21), the steady state level of the laminin β3 messenger RNAs was extremely low in skin biopsies obtained shortly after the birth of the patient. The drastic reduction in the expression of LAMB3 explains the lack of immunoreactivity of the skin to the antibodies specific to the laminin β3 chain and the native laminin 5. Absence of laminin 5 accounts for the severity of the condition.

By analyzing skin biopsy specimens obtained from the patient at the age of 4, we found that the paternal Lamb3 allele contributes to the favorable course of the disease. In fact, with advancing age, mutation 1587delAG in exon 13 becomes leaky, and illegitimate skipping of exon 14 restores the ORF of the mutant β3 pre-mRNA and generates an internally shortened messenger RNA. Skipping of exons containing nonsense mutations has been observed in several conditions (22–24). Such a mechanism, which is thought to involve ribosomemediate scanning of the pre-mRNAs transcripts for PTCs, allows translation of a functional polypeptide by rescuing the reading frame of mutant mRNA molecules (25). Indeed, in-frame skipping of exons containing PTCs in the LAMB3 gene has been currently observed in patients with mild JEB (26). In our proband, however, absence of mutant β3 transcripts at birth suggests that activation of the cryptic splice site allowing skipping of exon 14 from the paternal β3 pre-mRNA takes place a relatively long time after birth. A similar phenomenon of age-dependent activation of illegitimate mRNA splicing has been observed in analbuminemic rats carrying a mutation at a 5′ splice site of the albumin gene (27). Age-dependent splicing modulation has also been found in a case of transient EB associated with pyloric atresia where the functional restoration of a mutated splice site of integrin β4 pre-mRNA leads to expression of a wild-type β4 polypeptide (28).

External signal modulating gene expression may act on the mRNA splicing machinery. We could not determine the nature of the factors that with age activate the illegitimate splicing of the paternal β3 transcripts in our patient. We observed, however, that the deregulation of the splicing process occurs when the patient’s keratinocytes are expanded in culture. Intriguingly, immunostaining of the laminin β3 polypeptide in the dermal–epidermal junction is discontinuous and corresponds to the staining pattern of integrin β1. High expression of integrin β1 is required for maintenance of epidermal stem cells, while progressive loss of its expression characterizes the transit-amplifying keratinocytes endowed with limited proliferative capacity and committed to differentiation (29,30).

In light of these observations, expression of the mutated laminin 5 in our patient appears to correlate with the proliferative potential of the basal keratinocytes. The fact that corneal erosions do not take a favorable course with aging could be consistent with this idea. The persistent susceptibility to ocular lesions may in fact reflect a defective expression of laminin 5 in the transit amplifying basal cells with a central and para-central localization in the cornea, i.e. the cells that have left the stem cell compartment (the limbus) and have lost their proliferative capacity (31).

It cannot be formally excluded that the irregular distribution of laminin 5 in the patient skin results from an enhanced local degradation of the protein in the extracellular matrix underlying the transit amplifying cells. The laminin 5 synthesized by the patient harbors a mutant 126 kDa β3 polypeptide carrying a substitution of two amino acids at position 530–531 and an internal in-frame deletion of 127 amino acids spanning the N-terminus of the long arm and the proximal EGF-like repeat of the polypeptide (Fig. 6). We have shown that the proband’s keratinocytes synthesize the 126 kDa β3 chain in vitro, and that the mutant β3 chain associates with the α3 and γ2 chains to form functional laminin 5 heterotrimeric chains which are secreted in the spent medium. The altered structure of this mutant

**Figure 4.** Expression of LAMB3 transcripts in the proband’s skin. Total RNA extracted from patient and control skin biopsies were amplified by RT–PCR. Analysis of the PCR products by agarose gel failed to detect β3 cDNA fragments in the proband skin at birth (lane 1) and revealed a fast-migrating cDNA band of 357 bp in the proband skin at the age of 4 years (lane 2). A unique wild-type cDNA fragment of 738 bp was amplified from the control skin biopsy (lane 3).
laminin 5 may modify the turnover of the protein in vivo, and/or affect its stability. Laminin 5 heterotrimers assembly proceeds intracellularly by formation of stable $\beta_3\gamma_2$ intermediates that associate with the $\alpha_3$ chain via labile interactions (8). The $\alpha_3\beta_3\gamma_2$ heterotrimer associates by specific interactions between the domains I and II of each chain to form the triple-helix coiled-coil long arm of laminin 5 (32). Disulfide bonds at each end of the long arm are thought to stabilize the heterotrimer. The mutant $\beta_3$ chain expressed by our patient lacks the two N-terminal cysteines of domain II that are required for binding to the $\alpha_3$ and $\gamma_2$ chains. Our results demonstrate that these cysteine residues are not required for the assembly of functional laminin 5 molecules, but do not provide information on the stability of the mutant laminin 5.

In this patient, de novo synthesis of the laminin $\beta_3$ chain raised no detectable immune reaction against either the $\beta_3$ polypeptide or laminin 5. This observation implies that transfer of a curative $\beta_3$ transgene may present a limited risk of immune rejection in patients with mild JEB associated with expression of a defective laminin $\beta_3$ chain, which therefore opens interesting perspectives for a therapeutic approach of JEB based on gene transfers (19,33,34). However, this issue deserves further investigation, because transient synthesis of the laminin $\beta_3$ chain during the fetal life of our patient cannot be excluded, which could account for the tolerance to this protein expressed a relatively long time after birth.

**MATERIALS AND METHODS**

**Samples**

The keratinocytes obtained from newborn skin biopsies were cultured on a feeder layer of irradiated mouse J2-3T3 fibroblasts (35). Total RNA was purified from frozen skin biopsies and from cultured keratinocytes using the RNable extraction kit (Eurobio, Les Ulis, France). Genomic DNA was extracted from the peripheral blood following standard techniques (36).

**Immunohistochemistry**

Indirect immunofluorescence analysis of frozen skin samples was performed as previously described (37). Expression of laminin 5 was investigated using the mAb GB3 raised against...
the native laminin 5 (38), mAB K140 specific to the laminin β3 chain (9), and pAB SE85 and SE144, specific to the laminin α3 and γ2 chains, respectively (15). Reactivity to integrin β1 was assessed using mAB IOT29 (Immunotech, Marseille, France). Immunomapping of the HD components was performed using mAB HD121 directed against plectin (39), mAB GoH3 to integrin α6 (40), mAB 3E1 to integrin β4 (Gibco BRL - Life Technologies, Cergy Pontoise, France), pAB FP1 and mAB 1A8C, specific to bullous pemphigoid antigen of 230 kDa (41), and mAB collagen XVII (42), respectively. Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Dako S.A., Trappes, France), goat anti-rat IgG (Cappel, ICN Biomedicals, Orsay, France) or swine anti-rabbit Ig (Dako S.A.). Double labeling was carried out using Texas red-conjugated goat anti-rabbit IgG (Cappel) and FITC-conjugated goat anti-mouse Ig (Dako S.A.). Tissue sections and cell cultures were analyzed using a Zeiss Axiophot microscope.

Detection of genetic mutations

PCR reactions using 100 ng of genomic DNA as a template were performed following standard conditions (43). The pairs of primers were synthesized on the basis of the DNA sequences corresponding to the intron–exon boundaries of the gene LAMB3 (18) (GenBank accession nos U17744–U17759). Direct DNA sequence analysis of the amplification products was performed using the ABI Prism 310 automated sequencing system (Applied Biosystems, Foster City, CA). To detect mutation 1587delAG, the primers were (L) 5′-ACGTGGCCACGATCGCTG-3′ (mutant) and 5′-ACGTGGCCACGATCGCTG-3′ (wild-type) and 5′-GAGCAGATCGCTGAGCTT-3′ (mutant).

RT–PCR analysis of laminin β3 transcripts

Total RNA was purified from proband and control cultured keratinocytes and from frozen skin biopsies obtained from the proband at birth and at the age of 4 years. Total RNA was reverse-transcribed in a volume of 20 µl as recommended by the manufacturer (Promega, Madison, WI). The reaction products were used for PCR amplification of the 737 bp fragment of laminin β3 cDNA (nucleotides 1366–2103; Genbank accession no. L25541) (12) comprising exons 13 and 14 of LAMB3. The primers were (L) 5′-AAGTCGAGGTTGGTCCTCTG-3′ and (R) 5′-GTCCCTCTCTGATAC-3′, and the PCR cycling conditions were: 94°C for 5 min, followed by 94°C for 45 s, 56°C for 45 s, 72°C for 50 s (35 cycles) and 72°C for 10 min. An actin cDNA fragment (nucleotides 363–1100) (GenBank accession no. AB004047), used as an internal control, was amplified using primers (L) 5′-ATCTGGCACCACCACTTCTACTATTG-3′ and (R) 5′-GAGTGCTGAGATCGCTGAGCT-3′, and the PCR cycling conditions of 94°C for 5 min, 94°C for 45 s, 56°C for 45 s, 72°C for 50 s (35 cycles) and 72°C for 10 min. Five micrograms of the reaction products was run on 1% agarose gels. The amplified fragments of the β3 cDNA were subcloned into the Topo cloning vector according to the recommendations of the manufacturer (Stratagene, La Jolla, CA) and submitted to direct DNA sequence analysis.

Immunoprecipitation

Radioimmunoprecipitation of cell extracts and spent culture medium has been described elsewhere (45). SDS–PAGE of the immunoprecipitates was performed using a 6.5% polyacrylamide gel under reducing conditions (46). The dried gels were exposed to X-ray films, using an intensifying screen. Quantitation of the autoradiograms was performed by densitometric scanning using the Bioprofil software program (Vilbert Lourmat, France).
REFERENCES


