The Short Arm of the Laminin γ2 Chain Plays a Pivotal Role in the Incorporation of Laminin 5 into the Extracellular Matrix and in Cell Adhesion

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Abstract. Laminin 5 is a basement membrane component that actively promotes adhesion and migration of epithelial cells. Laminin 5 undergoes extracellular proteolysis of the γ2 chain that removes the NH2-terminal short arm of the polypeptide and reduces the size of laminin 5 from 440 to 400 kD. The functional consequence of this event remains obscure, although lines of evidence indicate that cleavage of the γ2 chain potently stimulates scattering and migration of keratinocytes and cancer cells. To define the biological role of the γ2 chain short arm, we expressed mutated γ2 cDNAs into immortalized γ2-null keratinocytes. By immunofluorescence and immunohistochemical studies, cell detachment, and adhesion assays, we found that the γ2 short arm drives deposition of laminin 5 into the extracellular matrix (ECM) and sustains cell adhesion. Our results demonstrate that the unprocessed 440-kD form of laminin 5 is a biologically active adhesion ligand, and that the γ2 globular domain IV is involved in intermolecular interactions that mediate integration of laminin 5 in the ECM and cell attachment.

Key words: keratinocyte • epithelial adhesion • cell migration • basement membrane • epidermolysis bullosa

Introduction

The epithelial basement membranes consist of a complex network of extracellular matrix (ECM) molecules that mediate tissue integrity and homeostasis, and control morphogenesis as well as tissue repair and tumorigenesis. Laminins are multifunctional glycoproteins of the ECM that contribute to the architecture of the basement membranes and play a crucial role in cell adhesion, growth, migration, and differentiation (Colognato and Yurchenco, 2000). These cross-shaped molecules consist of three subunits, classified as α, β, and γ chains on the basis of their primary structure deduced from sequence data. Laminin isoforms are expressed at various stages of development and in specific tissue locations in different species. In the human epidermis, basal keratinocytes express laminin 5 (α3β3γ2), laminin 6 (α3β1γ1), and laminin 10 (α5β1γ1) (Champliaud et al., 1996).

Laminin 5 is found in the basement membrane of stratified and transitional epithelia, prevalently associated with the extracellular anchoring filaments of the lamina lucida that connect the hemidesmosomes to the anchoring fibrils of the underlying stroma (Verrando et al., 1987; Rousselle et al., 1991; Masunaga et al., 1996). Laminin 5 is a specific substrate for adhesion in proliferating and migrating keratinocytes (Rousselle et al., 1991; Rousselle and Aumailley, 1994). The biological relevance of laminin 5 in dermal–epidermal adhesion has been emphasized by the identification of genetic mutations affecting laminin 5 in patients with junctional epidermolysis bullosa (JEB), an inherited genodermatosis characterized by fragility of the skin and blistering in response to minor trauma (Aberdam et al., 1994a; Pulkkinen et al., 1994). The adhesive role of laminin 5 was also confirmed by the detection of circulating autoantibodies against epitopes of laminin 5 in patients with acquired blistering skin disorders which are characterized by dermal–epidermal cleavage (Yancey et al., 1995; Lazaroa et al., 1996). Laminin 5 has been reported to influence morphogenetic events (Baker et al., 1996; Stahl et al., 1997), to affect cell motility (Zhang and Kramer, 1996; O’Toole et al., 1997), to enhance invasiveness in human cancers (Kikkawa et al., 1994; Pyke et al., 1994, 1995; Gianelli et al., 1997; Koshikawa et al., 1999), and to control cell growth (Ryan et al., 1999).

Current models propose that laminin 5 mediates epithelial cell adhesion via integrin α3β1 in focal adhesions and integrin α6β4 in hemidesmosomes (Carter et al., 1991; Sonnenberg et al., 1993; Mainiero et al., 1995; Baker et al.,)
It has been shown that the domain(s) interacting with the cell surface receptors reside within the COOH-terminal G domains of the protein (Baker et al., 1996; Mizushima et al., 1997; Hiroasaki et al., 2000). Conversely, the precise contribution of the NH2 terminus of the three chains of laminin 5 to cell adhesion is not yet well understood. In laminins, in general, the NH2-terminal domains are thought to mediate polymerization into a network that stabilizes the basement membrane (Colognato and Yurchenco, 2000). Compared with the other laminin isoforms, the short arms of the α3, β3, and γ2 chains are substantially truncated and do not conserve the domains known to direct integration of laminins into the basement membrane architecture (Cheng et al., 1997; Colognato and Yurchenco, 2000).

However, it has been proposed that the NH2 terminus of laminin 5 can interact with laminin 6 and 7, and also with the NC-1 domain of type VII collagen, the major component of anchoring fibrils (Marinkovich et al., 1992; Champliaud et al., 1996; Chen et al., 1997; Rousseau et al., 1997). On these bases, it has been suggested that monomeric laminin 5 molecules within the anchoring filaments bridge integrin α6β4 to type VII collagen and provide the significant force that forms of 440 and 400 kD. The 440-kD heterotrimer is generated by proteolytic processing of the α2 short arm excised by the g2 chain (Gerecke et al., 1994; Amano et al., 2000). It has also been proposed that binding of the basement membrane component fibulin 2 through the globular domain VI of the chain β3 and the domain IIIa of the α3 chain (Gerecke et al., 1994; Amano et al., 2000).

Materials and Methods

cDNA Constructs

Mutated laminin γ2 constructs were obtained by site-directed mutagenesis of a full length γ2 cDNA clone in the expression vector pYWT (Gagoux-Palacios et al., 1996) in which the antigenic peptide sequence MASMTG-GQQMG (T7-Tag) of T7 bacteriophage was inserted 3’ to the coding sequence of the γ2 cDNA clone (see Fig. 1 A, B). The presence of the T7-Tag does not affect the laminin 5 function (data not shown). To construct mutant pYN C, two γ2 cDNA fragments of 1,308 and 476 bp (comprising nt 1–1293 and 1305–1767 of the laminin γ2 cDNA, respectively; EMBL/GenBank/DDBJ accession no. X73920) were PCR amplified using plasmid pYWT as a template. The 1,308-bp cDNA fragment was amplified using primers A, left and B, right (Table I). The reaction mixture (25 μl) contained 10 ng of the template, 400 nM of each primer, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 0.1% Triton X-100, 100 μg/mL nuclelease-free BSA, 200 mM dNTP, and 2.5 U of Pfu DNA polymerase (Stratagene). For amplification of the 476-bp cDNA fragment, primers were C, left, and D, right. The primary PCR products were mixed and used as templates for a secondary PCR program using primers A and D in a reaction mixture of 25 μl. The PCR products were electrophoresed on a 1% agarose gel, eluted, mixed together, cleaved with the restriction endonucleases BsmBI and EcoRI, and cloned into a BsmBI-EcoRI-digested pYWT vector. To generate pY C, a γ2 cDNA with an internal deletion of 1,208 bp (nt 1302–94) was prepared by PCR amplification of two γ2 cDNA fragments: a 107-bp γ2 cDNA fragment (nt 1–93) using primer A, left, and E, right, and a 478-bp γ2 cDNA fragment (nt 1,303–1,766) using primers F, left, and D, right. The amplification products were subjected to a secondary PCR amplification using primers A and D. The resulting cDNA products were cloned into a BsmBI-EcoRI-digested pYWT plasmid as described above. To obtain plasmid pYN E, a 1,081-bp (nt 1–1065) and a 496-bp (nt 1286–1766) cDNA fragments were amplified using the primer pair A, left, and G, right, and pair H, left and D, right, respectively. After a secondary PCR amplification using primers A and D, the resulting amplimers were cloned into plasmid pYWT as described above. To generate the mutant pYN H, which bears an internal deletion of 101 bp, two γ2 cDNA fragments of 1,159 and 536 bp were PCR amplified using the primers pair A, left and L, right, respectively, and primer pair J, left and D, right. After a secondary PCR amplification using primers A and D, the resulting amplimers were cloned into plasmid pYWT as described above. To construct pYN V, which contains an internal deletion of 242 bp, pYWT was double digested with Bsa36I and BspEl (equals AcclII), blunted, and then religated. Plasmid pYN F was generated using the QuikChange™ site-directed mutagenesis kit (Stratagene) and primers K, left and L, right to substitute the amino acid residues SVHKI (residues 203–207) with five alanines. Plasmid pYN F was generated from pYN F1, using the QuikChange™ kit and the oligonucleotide pair M, left and N, right to substitute the peptide sequence SAEYVSVKI (residues 199–207) with nine alanines. Plasmid pYN GP was generated using the QuikChange™ kit and primers O, left and P, right to delete the amino acid residues YS (residues 432–433) and substitute the amino acid residues GD (residues 434–435) with the amino acid residues GP. The conformational changes of the γ2 chain introduced by the GP substitution were assessed using the programs Biopolymer (Molecular Simulations, Inc.) and Antheprot (http://www.ibtcp.fr).

To generate plasmid pYN SP, the 1,302-bp γ2 cDNA fragment (nt 1–1302) coding for the NH2-terminal domain of the laminin γ2 chain was subcloned into the BamHI-EcoRV sites of the expression vector pCDNA3, upstream the T7-Tag. To construct plasmid pYN GP, the 1,574-bp cDNA sequence encod-
Table I. Nucleotide Sequence of the Primers Used in Site-directed Mutagenesis of the Laminin γ2 cDNA

| Primer | Nucleotide sequence |
|--------|---------------------|
| A      | 5'-AACAGCTATGACCATG-3' |
| B      | 5'-ATTGTCAGATTTCACATTCCTCTTGTGTCG-3' |
| C      | 5'-GAGCACGAGGAGTTGAGAACTCCTGACATTGAGTGTG-3' |
| D      | 5'-CCATCCATTCGCACTTCTAC-3' |
| E      | 5'-TCAGATTCTTCTCATCTGGCAATCATACAGATCT-3' |
| F      | 5'-GTCTGTATGCAATGTGAGAATCTCTGAGATTTGAG-3' |
| G      | 5'-CTGAAATAAACCTCTGTATCTCCTACATGAGTACGTC-3' |
| H      | 5'-CATATGGAGATACAGATGTATGTTCAGGATG-3' |
| I      | 5'-CCCTTGCAGCTATCAGTTCACAACCAAGG-3' |
| J      | 5'-CCCGGTTGGAACAGTGTAACCCTGACATTGAG-3' |
| K      | 5'-AGCTCGTGAGATACGAGAGCGCGAGCTACTCTACTTCTCAT-3' |
| L      | 5'-ATGAAAGTGATAGGTAGCTGCGGCTGCTGCTGTTCTTACGACT-3' |
| M      | 5'-TCAGCAGCTGGCTGCCGCGCTGGAGCAGCCGCAGCTGAGCCTG-3' |
| N      | 5'-AGTGGCGCTGCTGCCTGCTCAGCGGGCGGCTGCTGCTGCTGAGTGA-3' |

Backgrounding the NH₂-terminal domains of the laminin β3 chain (nt 1–1574, EMBL/GenBank/DDBJ accession no. L25541) was subcloned into pcDNAs upstream of the hemagglutinin (HA) Tag sequence (YPYDVPDYA).

The plasmids were amplified in E. coli XL1-blue (Stratagene), purified using a plasmid purification kit (QIAGEN), and analyzed by nucleotide sequencing using an ABI Prism 310 genetic analyzer.

**Antibodies**

mAbs used in the study were: K140, specific to laminin β3 (Marinkovich et al., 1992); GB3, directed against native laminin 5 (Verrando et al., 1993); T7 bacteriophage (Novagen); and anti-HA (clone 12CA5), specific to the peptide sequence (YPYDVPDYA) of the influenza HA protein (Santa Cruz Biotechnology, Inc.).

Expression of laminin γ2 chain was examined using polyclonal antibody (pAb) SE144 (Valiull et al., 1994). pAb SE1097 was raised against the fusion protein corresponding to the laminin γ2 short arm. To prepare the fusion protein, the γ2 cDNA fragment was excised from plasmid pγ50 and subcloned into the EcoRI site of the procaroytic expression vector pGEX-5X-3 from Amersham Pharmacia Biotech. The purified GST fusion protein was used for rabbit immunization. pAb SE1097 was then purified by immunoraffinity chromatography against the corresponding fusion protein immobilized on nitrocellulose filters after SDS-PAGE. The second antibodies coupled to tetramethylrhodamine isothiocyanate or to fluorescein isothiocyanate were obtained from Dako. Actin cytoskeleton was labeled using phallloidin Texas red (Molecular Probes, Inc.).

**Cells and Organotypic Cell Cultures**

The human keratinocytes cell lines HKSV and LSV5 were grown in a 3:1 mixture of DME and Ham’s F-12 medium (Life Technologies and GIBCO BRL) containing FCS (10%), hydrocortisone (0.4 µg/ml), cholera toxin (0.1 nM), and EGF (10 ng/ml) (Miquel et al., 1996). Swiss mouse 3T3-J2 cells and the African monkey kidney cell line COS-7 were cultured in DME containing 10% calf serum. Secondary cultures of human keratinocytes HNks were grown on irradiated feeder layers of mouse 3T3-J2 cells in DME/ Ham’s F12 medium as described above and supplemented with insulin (5 µg/ml), adenine (0.18 mM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml) (Rheinwald and Green, 1975). To construct artificial epithelia, cultured keratinocytes (1.6 × 10⁶ cells) were seeded in stainless steel rings (0.6 cm²), laid on acetate cellulose filters (Millipore), and maintained immersed in growth medium changed every other day. 4 d later, the medium in the rings was removed and the keratinocyte culture was brought at the air–liquid interface for 4 d to induce differentiation (Rosdy et al., 1993). The artificial epithelia were then included in OCT compound Tissue-Tek (Miles Laboratories) and immediately frozen in liquid nitrogen. Vertical 4-µm sections were used for indirect immunofluorescence studies.

**DNA Transfections**

For transient expression, subconfluent keratinocyte cultures were transfected using the polycationic lipid Dospor (1,3-Di-Oleoylsoyxy-2-[carboxy-spermyl]-propyl-amide) from Roche. In brief, before transfection the cell cultures were incubated at 37°C with medium deprived of serum and growth factors. DNA and Dospor were separately diluted in Hepes-buffered saline (20 mN Hepes, 150 mN NaCl, pH 7.4), then mixed at a final concentration of 0.2 and 0.1 µg/ml, respectively, and added dropwise to the cell layers. After 6 h at 37°C, cells were fed with medium containing serum and growth factors two times concentrated. Transfection efficiency and expression of the transfected genes was monitored 48 h later using indirect immunofluorescence assays.

**Table cell lines Lync, LyC, LyGP, LyF1, and LyF2 using plasmids prpNC, prpC, prpGP, prpF1, and prpF2, respectively, calcium-phosphate transfection of LSV5 cells was performed using the MBS™ mammalian transfection kit (Stratagene). 1.5 × 10⁵ cells were seeded in 100-mm petri dishes and incubated for 24 h at 37°C. 20 µg of plasmid DNA precipitated for 15 min at room temperature in BES buffer (50 mN Na₂HPO₄, pH 6.95) containing 125 mN CaCl₂ was added dropwise to the cell layers. The cell cultures were incubated for 3 h in DME supplemented with 5% modified bovine serum provided with the transfection kit, then rinsed and fed with DME/Ham’s F12 medium. Selection for neoreistance to genetin (400 µg/ml; Sigma-Aldrich) was started 48 h later.

**Immunofluorescence Microscopy**

Immunofluorescence analysis of subconfluent cell layers grown on glass coverslips and fixed in PBS, pH 7.4, containing 1 mN CaCl₂, 1 mN MgCl₂, and 3% formaldehyde were processed as reported (Gagnoux-Palacios et al., 1996). Semithin sections of frozen samples of organotypic cultures were fixed and analyzed as described (Rosdy et al., 1993). Cells monolayers and sections were analyzed using a ZEISS Axioskop microscope.

**Immunoprecipitation and Immunoblotting Studies**

Immunoprecipitation and immunoprecipitation analysis of the cell extracts and samples of culture medium were analyzed using an ECL Western blotting kit (Amersham Pharmacia Biotech).

**Cell Detachment Assay**

Cells (2 × 10⁵/cm²) were seeded in tissue culture flasks and incubated for either 12 or 48 h at 37°C to reach 50 and 80% confluence, respectively. The monolayers were then treated with a solution of trypsin/EDTA (Versene and BioWhittaker) diluted 1:70 in PBS. The number of cells detached at increasing time of incubation was determined by collecting the supernatants and direct cell counting. Each experiment was repeated six times. A representative experiment is shown.
Quantification of Laminin 5 Deposited in the ECM by Cultured Keratinocytes

Laminin 5 deposition was quantified both on plastic cell culture substrate and on plastic coated with ECM components. To coat the plastic substrate, multiwell plates (96 wells; TPP) were incubated with solutions of collagen type I or IV, vitronectin, fibronectin, laminin 1, or ECM (all from Sigma-Aldrich) dissolved in PBS at a concentration of 10 µg/ml. After 1 h of incubation, cells were seeded and incubated for either 12 or 48 h at 37°C in humidified atmosphere in the presence of 5% CO2. The cells were then washed twice in PBS and detached as devised by Delwel et al. (1993). In brief, the cells were incubated overnight at 4°C in PBS containing 20 mM EDTA, leupeptin (10 µg/ml), aprotonin (10 µg/ml), phenylmethanesulfonyl fluoride (1 mM), and soybean trypsin inhibitor (10 µg/ml) and then dislodged by pipetting. The matrices were washed with PBS, incubated for 10 min with PBS-0.2% Triton X-100 to remove cell debris, and saturated with 1 h at room temperature in PBS-1% BSA. Each well was incubated for 2 h at room temperature in 50 µl PBS-1% BSA containing mAb GB3 (10 µg/ml). The plates were washed seven times with PBS and incubated for 1 h at room temperature with 50 µl of PBS-1% BSA containing 1 µg/ml of anti-mouse HRP mAb (Dako) per well. After extensive washing, 50 µl of a solution of 0-phenylenediaminedihydrochloride (Sigma-Aldrich) was added to each well for 10 min in the dark as devised by the supplier. Color yields were determined at 490 nm in an ELISA reader (Dynatech). Values were expressed as percentage of the values obtained with mutant LγCNC cells.

Adhesion Assay

Multiwell plates containing the ECM secreted by the different mutant cell lines were prepared as described. Wells were saturated for 1 h at room temperature with a solution of 0.5% heat-denatured BSA. Wild-type HNKs (3 x 10⁴ cells per well) were plated and fed with serum-deprived medium containing 0.25% heat-denatured BSA. After 1 h at 37°C, the medium and the cells in suspension were removed and the wells were washed with PBS. Adherent cells were fixed in 3.7% formaldehyde, stained with 0.5% crystal violet dissolved in 20% methanol, and washed three times in PBS. The dye was eluted with 50% ethanol/0.1 M sodium citrate, pH 4.2. Absorbance at 540 nm was determined using a microplate reader. Values were expressed as a percentage of the values obtained with mutant LγNC cells.

Results

The functional role of the short arm domains of the laminin γ2 chain was investigated by transfecting a series of mutant γ2 cDNA clones into keratinocyte cell line LSV5. Cell line LSV5 is derived from the keratinocytes of an H-JEB patient with a homozygous nonsense mutation (R95X) in the gene (LAMC2) coding for the laminin γ2 chain (Miquel et al., 1996). LSV5 cells do not synthesize the laminin γ2 chain, but express the full repertoire of the laminin chains found in HNKs, including the laminin α3 and β3 chains (Miquel et al., 1996). Transfection of a wild-type γ2 cDNA restores production of functional α3β3γ2 laminin 5 molecules (Gagnoux-Palacios et al., 1996). The complementary DNA sequences coding for mutant laminin γ2 chains were generated by directed mutagenesis of the expression vector pγWT which encodes the full length γ2 polypeptide (Gagnoux-Palacios et al., 1996). As depicted in Fig. 1 B, the mutant cDNA pγNC expresses a recombinant γ2 polypeptide with an internal deletion encompassing the four amino acids (YSGD) within domain III that constitute the proteolytic cleavage site of the γ2 chain (Vailly et al., 1994; Amano et al., 2000). In mutant pγGP, the GlyPro residues substitute the YSGD cleavage site and introduce a structural modification from “sheet” to “coil” configuration of the γ2 chain domain III. Mutants pγIII and pγV carry a deletion affecting the EGF-like repeat 1 of domain III and repeats 2 and 3 of domain V, respectively. To investigate the functional role of the laminin γ2 chain short arm, we generated a cDNA clone encoding a polypeptide lacking the NH2-terminal domains IV and V that are excised in the extracellular processing of laminin 5 (plasmid pγC). Further, a polypeptide with an internal deletion encompassing the EGF-like repeat 1 of domain III and the COOH-terminal portion of domain IV (plasmid pγM) was also constructed. Plasmid pγM corresponds to a mutated γ2 chain missing 73 amino acids of the polypeptide sequence detected in a patient suffering from JEB (Pulkkinen et al., 1994). The deletion was detected to interfere with the extracellular processing of the polypeptide (Amano et al., 2000).

The different mutant cDNAs were transiently transfected into actively growing LSV5 keratinocytes. Immunocytochemical analysis of the transfected cultures using pAb SE144 (not shown) and mAb GB3 indicated that all the mutant γ2 polypeptides were actively synthesized and incorporated into laminin 5 heterotrimers (Fig. 2). The ECM deposited on the culture support by cells LγWT, LγNC, LγΔIII, LγΔV, and LγGP transfected with plasmin γ2WT, γ2NC, γ2ΔIII, γ2ΔV, and γ2GP was immunoreactive, whereas the ECM deposited by the cell cultures LγC and LγM transfected with plasmin γ2C and γ2M was not labeled. These results indicate that deletions within the globular domain IV of the γ2 chain prevent secretion and/or deposition of laminin 5 into the ECM, but do not limit laminin 5 synthesis.

To confirm the role of the globular domain of the γ2 polypeptide in deposition of laminin 5 to the cell culture substratum, plasmin γ2NC, γ2GP, and γ2C were stably transfected into LSV5 keratinocytes. Cell lines LγNC and LγGP were generated that are expected to secrete a mutated 440-kD form of laminin 5 with a full length γ2 chain (155 kD), whereas cell line LγC is expected to produce a 400-kD molecule corresponding to laminin 5 with a processed γ2 chain (105 kD). Consistent with the results obtained with transiently transfected LSV5 cells, immunocytochemical analysis of LγNC, LγGP, and LγC cells using pAb SE144 and mAb GB3 detected a strong cytoplasmic labeling in all cell lines. These experiments also confirmed that only the ECM laid down by LγNC and LγGP keratinocytes contains immunoreactive laminin 5 epitopes (not shown). These results indicate that presence of the γ2 short arm is required for deposition of laminin 5 to the culture substratum, but do not rule out the possibility that laminin 5 harboring a γC recombinant chain is secreted into the media without being incorporated into ECM. Expression and secretion of the recombinant γC, γGP, and γC polypeptides were examined by Western blot analysis of spent medium of LγNC, LγGP, and LγC cells. A unique 105-kD migration band was detected in the LγC medium using pAb SE144, whereas a specific 155-kD band was observed with LγNC and LγGP cells (Fig. 3 A). The intensity of these bands was comparable and was estimated to be threefold weaker than the intensity of the corresponding bands detected in the medium conditioned by wild-type keratinocytes. Thus, although the γ2 short arm is required for incorporation into the ECM, it is not needed for secretion of γ2 chain.

Incorporation of the recombinant γ2 polypeptides into extracellular laminin 5 molecules was further verified by
immunoprecipitation of LγC and LγNC cell culture medium using the mAb K140 and the anti–T7-Tag mAb. As shown in Fig. 3 B, comparable amounts of 400- and 440-kD laminin 5 molecules were immunoprecipitated from the LγNC and LγC cell medium, respectively, which attests to the assembly of the 105- and 155-kD recombinant γ2 polypeptides into laminin 5. These data demonstrate that the absence of the γ2 NH2-terminal domains does not hinder the intracellular processing of laminin 5 and its secretion into the culture medium. They also confirm that the tetrapeptide YSGD is the unique physiological cleavage site of the extracellular processing of the γ2 chain.

Artificial epithelia constructed either with HNKs or LSV5 cells expressing a recombinant wild-type γ2 chain have been shown previously to lay down laminin 5 at the interface between the basal cells and the cell culture support (Rosdy et al., 1993; Gagnoux-Palacios et al., 1996; Miquel et al., 1996). Because truncation of the γ2 short arm appeared to prevent deposition of laminin 5 on monolayer submersed cultures, LγC and LγNC keratinocytes were grown to confluence on cellulose acetate filters and exposed to air to obtain stratification into multilayered epithelia. Immunofluorescence analysis of the artificial epithelia using mAb GB3 detected a strong reactivity in the case of LγNC and HKSV cells (Fig. 3 C, b and d), and no reactivity with LSV5 and LγC keratinocytes (Fig. 3 C, a and c). Therefore, these observations confirm that the 400-kD form of laminin 5 produced by LγC keratinocytes is not incorporated into the ECM, and underscore the importance of the γ2 short arm plays in the deposition of laminin 5 at the epithelial–ECM interface.

Because our results suggested that the laminin γ2 short arm is essential for the incorporation of laminin 5 into the ECM secreted by LSV keratinocytes, we verified whether human skin and the matrix secreted by wild-type keratinocytes contain the NH2-terminal γ2 polypeptide generated by the extracellular processing of the 440-kD laminin 5. Specific antiserum pAb SE1097 was generated against

Figure 1. Schematic representation of laminin 5 and the γ2 chain mutants used in this study. (A) The EGF-like repeats (□) and the globular domains (○) of the α3, β3, and γ2 chains of laminin 5 are depicted. Full gray squares represent the extracellularly cleaved NH2-terminal domain of the γ2 chain. (B) The different γ2 mutant constructs were obtained by directed mutagenesis of the expression vector pγWT that contains a full-length laminin γ2 cDNA carrying the antigenic sequence T7-Tag at the 3′ end. Plasmid pγNC encodes a γ2 chain bearing an internal deletion of the four amino acids (YSGD) that constitute the proteolytic cleavage site of the γ2 chain short arm (Vailly et al., 1994). Plasmid pγGP encodes a γ2 chain in which a glycine and a proline substitute the tetrapeptide YSGD. Plasmid pγC encodes a γ2 chain truncated of the NH2-terminal domains that are excised in the extracellular processing of the laminin 5. Plasmid pγM encodes a γ2 chain with an internal amino acid deletion within the EGF-like repeats 2 and 3 of domain V. Plasmid pγIII corresponds to a γ2 chain with an internal deletion (33 amino acids) of the first EGF-like repeat of domain III. Plasmids pγF1 and pγF2 encode γ2 chains harboring a substitution of the amino acid sequence SVHKI into five alanines, and a substitution of the amino acid sequence SAEYSVHKI into nine alanines, respectively. Plasmid pγ50 encodes for the 50-kD NH2-terminal cleaved polypeptide of the laminin 5 γ2 chain. Plasmid pβ60 encodes for the first 526 amino acids of the laminin 5 β3 short arm chain. Secretion (Secr.) in the culture medium and deposition (Dep.) on the culture support of the mutant laminin 5 heterotrimers were investigated by Western blotting and immunofluorescence observations. Cell attachment (Adh.) was determined by detachment assays.
recombinant fragment γ50, which is cleaved from the short arm of the γ2 chain (Fig. 1 B). The antibody stained the human epidermal basement membrane in a strong linear fashion (Fig. 4, A and B), comparable to the labeling observed with antibody SE144 (not shown). Samples of the ECM produced by cultures of wild-type keratinocytes were then collected from culture dishes and analyzed by immunoblotting using pAb SE144 and pAb SE1097. As shown in Fig. 4 C, pAb SE144 recognized the 155- and 105-kD migration bands corresponding to the unprocessed and processed γ2 polypeptide, respectively. pAb SE1097 identified the unprocessed 155-kD γ2 chain and an additional fast-migrating band with the expected mass (50 kD) of the NH2-terminal domains, which are extracellularly excised from the γ2 chain (Fig. 4 D). The finding that only unprocessed γ2 chain is found intracellularly and that the processed γ2 chain and its cleavage fragment are found in the ECM indicate that the 440-kD laminin 5 molecules are proteolytically cleaved into the 400-kD form after incorporation into the ECM. This strengthens the idea that the γ2 short arm plays a role in the integration of laminin 5 in the matrix.

To verify this hypothesis, wild-type keratinocytes were transfected with the construct py50 encoding the NH2-terminal domains IV and V of γ2 carrying a T7-Tag peptide (Fig. 1). Control cultures were transfected with an expression vector (p60) that encodes the NH2-terminal domains III, V, and VI of the laminin β3 chain tagged with the HA epitope (Fig. 1). As shown by immunofluorescence microscopy, mAb T7-Tag reacted with the cytoplasm of the keratinocytes transfected with plasmid py50 and labeled the ECM deposited by these cells (Fig. 4 E, a and b). Conversely, the anti-HA mAb stained the cytoplasm of the keratinocytes expressing the recombinant cDNA p60, but not the ECM they deposited (Fig. 4 E, c and d). Similar results were obtained by transfection experiments performed using LSV5 cells (not shown). These observations suggest...
that the short arm of the laminin γ2 chain carries domains essential to the incorporation of laminin 5 into the matrix.

It has been suggested that the globular domain IV of the mouse laminin γ2 chain binds to the ECM protein fibulin 2 (Utani et al., 1997). However, residue Phe-202 of the γ2 amino acid sequence, which is essential to the interaction, is not conserved in humans. In addition, we were unable to demonstrate interactions between human laminin 5 and fibulin 2 in our experimental conditions (data not shown). Intriguingly, the amino acid sequence of the NH₂-terminal region of the γ2 domain IV is highly conserved in mammals (Table II), which may reflect a relevant physiological role of this portion of the polypeptide. Indeed, it was suggested that disruption of the fibulin 2 binding site could hamper the proteolytic processing of γ2 (Utani et al., 1997). Therefore, we constructed plasmids pγF1 and pγF2 that encode γ2 polypeptides in which the amino acid sequence SADFS-VHKI (residues 199–207) homologous to the active site of the mouse fibulin 2 binding site was partially (pγF1) and totally (pγF2) substituted by alanine residues (Fig. 1). LSV5 keratinocytes transfected with constructs pγF1 and pγF2 were examined by immunofluorescence analysis using mAb GB3. Expression of the mutant laminin 5 molecules resulted in a strong staining of the cytoplasm, and also of the ECM deposited onto the tissue culture support (Fig. 5 A, a and c). Western blot analysis of medium collected from cultures of LγF2 keratinocytes using pAb SE144 detected hybridization bands corresponding to the uncleaved (155 kD) and the proteolytically cleaved (105 kD) pγF2 chain (Fig. 5 B). According to these observations, immunofluorescence examination of frozen sections of artificial epithelia constructed using LγF1 and LγF2 keratinocytes detected a strong reactivity of the basement membrane zone to mAb GB3 (Fig. 5 C, a and b). These re-

Figure 2 (Continued)
The results attest to the incorporation of the mutant laminin 5 molecules into the ECM deposited on the cell culture substrate and show that disruption of the region homologous to the putative fibulin 2 binding site of the mouse g2 short arm does not interfere with the processing and deposition of laminin 5 to the matrix.

Reexpression of wild-type laminin 5 restores adhesion of LSV5 cells and H-JEB keratinocytes (Gagnoux-Palacios et al., 1996; Vailly et al., 1998). Because the 400-kD molecules of laminin 5 produced by LgC keratinocytes are not deposited into the ECM, LgC cells are expected to retain the poor adhesion capacity of the parental cell line LSV5. Indeed, epidermal sheets of stratified epithelium generated by confluent cultures of LgC, LgGP, and LgF1 keratinocytes spontaneously detached from the culture vessel. In contrast, when LgNC keratinocytes became confluent and stratified, the epidermal sheet firmly adhered to the plastic dish and detachment required enzymatic treatment (not shown). Therefore, attachment of LgC keratinocytes was quantified in detachment kinetic assays in the presence of trypsin/EDTA (Vailly et al., 1998). The attachment capacity of LgNC keratinocytes was also compared with that of wild-type keratinocytes. Cell suspensions were seeded on petri dishes to obtain exponentially growing cultures, 12 h after plating, the percentage of the adhering LgNC and LgC cells was similar to that of parental LSV5 cells (Fig. 6 A). Although the number of LgNC and LgWT cells resistant to trypsinization increased 48 h after seeding (50% of LgNC cells were dislodged after 14 min), that of LgC and LSV5 cells remained low (50% of dislodged cells after 8 min; Fig. 6 B). Therefore, the progressive enhancement of adhesion of LgNC and LgWT...
keratinocytes appeared to correlate with accumulation of laminin 5 molecules harboring the $\gamma_2$ short arm in the matrix. Detachment of L$\gamma$GP and L$\gamma$F1 keratinocytes was also assessed in similar experimental conditions. As demonstrated in Fig. 6 B, in all these cells other than L$\gamma$NC synthesis of mutated laminin 5 molecules had no appreciable effect on the strength of cell adhesion.

To confirm that resistance to trypsinization of L$\gamma$NC cells correlates with laminin 5 incorporation in the ECM, the amount of laminin 5 layered down by the different $\gamma_2$ mutant keratinocytes was determined by ELISA assay using mAb GB3. As shown in Fig. 6 C, laminin 5 produced by L$\gamma$NC cells was efficiently deposited on the plastic culture substrate and accumulated with increasing time, whereas mutant $\gamma_C$ laminin 5 was inefficiently layered down. L$\gamma$WT, L$\gamma$GP, or L$\gamma$F1 cells layered down comparable amounts of laminin 5. In addition, by seeding the mutant keratinocytes in a plastic vessel coated with differ-
ent components of the ECM, we assessed the deposition rate of the wild-type and mutant laminin 5 molecules to be independent from the nature of the cell culture substrate on which the keratinocytes are grown (Fig. 7). Plating on a feeder of irradiated mouse 3T3-J2 cells did not modify the deposition pattern of laminin 5 (not shown). Because the trypsinization assay provides information on the effect of laminin 5 on the strength of cell attachment, the functional role of the laminin γ2 short arm in cell adhesion was further assessed by seeding wild-type primary human keratinocytes on the ECM deposited by the different LSV5 γ2 mutants. The mutant cells were seeded in a range of concentrations leading to deposition of equivalent amounts of laminin 5 48 h after plating. The ECM was prepared after detachment of the cells by EDTA treatment, and concentration of laminin 5 in the ECM was checked by ELISA assays using mAb GB3. Primary wild-type keratinocytes were then seeded and allowed to adhere for 60 min at 37°C. Results show that adhesion of keratinocytes on the matrix secreted by L-γNC

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Figure 5. Secretion and deposition on the ECM of mutants LγF1 and LγF2 laminin 5 molecules. (A) Immunofluorescence staining of LSV5 keratinocytes transiently transfected with plasmids pγF1 (a and b) and pγF2 (c and d). Double immuno-fluorescence labeling was performed using mAb GB3 (a and c), specific to native laminin 5 and phalloidin (b and d). (B) Western analysis of spent medium of LγF2 keratinocytes. 50 μg of proteins from spent medium collected from cultures of LγF2 (lane 1), LSV5 (lane 2), and HKSV (lane 3) were loaded on a 7.5% SDS-polyacrylamide gel, transferred onto nitrocellulose filter, and reacted with pAb SE144. The mass of molecular markers is indicated on the left of the gel. Exposure time was 5 min. (C) Immunofluorescence analysis of artificial epithelia generated using LγF1 (a) and LγF2 (b) keratinocytes. The substitution of the fibulin 2 binding site of the γ2 short arm does not prevent deposition of the mutant laminin 5 to the epithelial–ECM interface. Bars, 50 μm.
Table II. Alignment of the Laminin γ2 cDNA Sequences Homologous to the Active Site of Fibulin 2 Binding in the Mouse

| species | CHA | SADFVHKITSTFSQDV | SADFVHKITSTFSQDV | CHA | SADFVHKITSTFSQDV | CHA | SADFVHKITSTFSQDV |
|---------|-----|------------------|------------------|-----|------------------|-----|------------------|
| Mouse  |     | SADFVHKITSTFSQDV | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |
| Human  |     | SADFVHKITSTFSQDV | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |
| Dog    |     | SADFVHKITSTFSQDV | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |     | SADFVHKITSTFSQDV |
| Horse  |     | SADFVHKITSTFSQDV |

The active site of fibulin 2 binding in mouse laminin 5 is underlined. The residues essential to binding are in bold. The dog and horse cDNA sequences are available from EMBL/GenBank/DDBJ under accession nos. AF236864 and AF292647, respectively.

Discussion

Transfection of a wild-type laminin γ2 cDNA in LSV5 keratinocytes rescues expression of functional laminin 5 molecules (Gagnoux-Palacios et al., 1996). Similar to laminin 5 produced by wild-type keratinocytes, the recombinant laminin 5 secreted by the reverted LSV5 cells undergoes extracellular maturation, including proteolytic excision of the NH2-terminal domain of the γ2 short arm. In this study, we examined the functional role of the laminin γ2 short arm by complementing the genetic defect of LSV5 cells by transfer of mutant γ2 cDNAs. Expression of the mutant cDNAs demonstrated that all the different recom-
Figure 8. The unprocessed form of laminin 5 promotes cell adhesion. Wild-type keratinocytes (NHK) were seeded on ECM secreted by LSV5 cell expressing laminin 5 harboring the mutant γ2 chains. Cell were left to adhere for 1 h at 37°C. Cell adhesion was measured using the colorimetric reaction described in Materials and Methods. The ECM deposited by LγWt, LγNC significantly increased adhesion of NHKs compared with the ECM produced by laminin 5-null LSV5 cells. The matrix secreted by LγF2 and LγGP keratinocyes also enhanced adhesion. Each point is the average of triplicates from three independent experiments and represents the mean ± SD of the optical density values. Values obtained with LγNC cells were taken as 100%.

Binary γ2 chains associate with the endogenous laminin α3 and β3 polypeptides to assemble into α3β3γ2 heterotrimers, and that the NH2-terminal domains of the γ2 chain are required for deposition and incorporation of laminin 5 in the ECM.

Our results, for the first time, define a physiologic function for the short arm of the γ2 chain. We demonstrate that mutant laminin 5 molecules lacking the γ2 chain NH2-terminal sequences that are excised in the extracellular processing are found in the spent media of LγC cells, but are not layered on the cell culture substrate. Specifically, deposition of laminin 5 in the ECM produced by the LSV5 keratinocytes requires integrity of the γ2 globular domain IV, whereas preservation of the tightly folded structure of the EGF-like rich domains III and V is not essential. Indeed, transfection of LSV5 cells with γ2 cDNA mutants carrying a deletion within the EGF-like repeats of either domain III or V results in deposition of laminin 5. Consistent with these observations, laminin 5 molecules carrying a deletion of the γ2 domain V EGF-like repeat 2 has been found in the dermal–epidermal junction of a patient with mild epidermolysis bullosa consequent to in-frame skipping of LAMC2 exon 4 (Castiglia, D., P. Posteraro, M. Pinola, C. Angelo, P. Puddu, and A. Zambruno, manuscript submitted for publication). Therefore, removal of EGF-like repeats weakens the functionality of laminin 5, but may not prevent the polarized secretion and deposition of the protein to the basement membrane. Conversely, transfection of mutant pgM, in which the deleted fragment encompasses the γ2 domains III and IV, results in lack of deposition of laminin 5 that correlates well with the epidermolysis bullosa phenotype of the patient carrying this mutation (Pulkkinen et al., 1994). We did not assess whether absent deposition of the mutant γM chain was consequent to lack of secretion. Conversely, by deletion of the proteolytic cleavage site YSGD of the γ2 polypeptide, we show that laminin 5 with an unprocessed γ2 chain (mutant plasmid pgNC) is efficiently layered down and enhances adhesion of LSV5 and wild-type keratinocytes. These findings imply that the extracellular 440-kD form of laminin 5 is an active adhesion ligand and not a mere precursor of the processed 44-kD form.

Basement membrane components are coordinately deposited in the ECM and subsequently assemble to form a supramolecular network sustaining multiple functions, including cell adhesion (Colognato and Yurchenco, 2000 and references therein). It has been shown that laminin 5 is one of the first basement membrane components laid down by migrating keratinocytes (Rousselle et al., 1991; Ryan et al., 1994; Lampe et al., 1998). However, the intermolecular interactions of laminin 5 in the basement membrane remain poorly understood. Laminin 5 binds the NC-1 domain of collagen type VII (Cheng et al., 1997; Rousselle et al., 1997) and is found associated with laminin 6 in the skin and laminin 7 in amnion (Rousselle et al., 1991; Gerecke et al., 1994; Champliaud et al., 1996). Utani et al. (1997) have proposed that the ECM component fibulin 2 interacts with the short arm of the mouse γ2 chain. Apart from Phe-202, which is required for the full binding activity of the mouse γ2 chain, the remaining active residues of the consensus sequence mediating interaction between the laminin γ2 chain and fibulin 2 are conserved in humans and other mammals (Table II). We could not demonstrate a direct interaction between the γ2 chain and fibulin 2 in human keratinocytes. In addition, our results show that in mutants γF1 and γF2, substitution of the putative fibulin 2 binding site with alanine residues that modify the NH2-terminal region of the γ2 short arm domain IV does not affect the cleavage of the γ2 chain and deposition of laminin 5. This argues against the participation of this region of the γ2 globular domain IV in the extracellular processing of the γ2 polypeptide (Utani et al., 1997). However, the adhesive function of laminin 5 harboring the mutant γF1 or the γF2 chain is diminished, as attested by the weak resistance to trypsinization of the cells expressing these molecules. The relevance of the γ2 short arm in cell adhesion is confirmed by the fact that substitution of the YSGD site with the amino acids GlyPro, where the proline residue modifies the orientation on the plane of the NH2-terminal domains, also results in efficient deposition of 440-kD laminin 5 molecules exerting a reduced adhesive function. Although further investigations at the molecular level are needed to clarify the mechanisms underlying these observations, it is likely that integrity of the globular domain IV and the correct orientation of the γ2 NH2 terminus determine the appropriate arrangement of laminin 5 in the ECM produced by LSV5 cells and contribute the functional activity of this adhesion ligand.

Laminin 5 purified from mouse epidermis is a mixture of molecules harboring a cleaved (105 kD) or an uncleaved (155 kD) γ2 chain (Aberdam et al., 1994b). In organ-cultured bovine skin, conversion of the 155-kD γ2 chain to 105 kD was not observed, whereas laminin 5 isolated from amnion was found to contain the 105-kD γ2 chain only (Marinkovich et al., 1992). It has been suggested that the proteolytic truncation of the γ2 short arm may facilitate the interaction between the β3 short arm of laminin 5 and other basement membrane components, such as laminins 6 and 7 and collagen type VII, and consequently may acti-
vate the adhesion function of laminin 5 (Amano et al., 2000). This hypothesis is not verified in our system, because the mutant laminin 5 molecules harboring a fully processed γ2 short arm are secreted in the culture medium, but are not found in the matrix produced by LSV5 cells. Conversely, we demonstrate that the progressive accumulation of the 440-kD form of laminin 5 on tissue culture plastic and the dermal equivalent of organotypic cultures enhances adhesion of LSV5 cells. Our data confirm previous observations showing that a threshold level of laminin 5 accumulation is required for efficient cell adhesion (Hormia et al., 1995; Gagnoux-Palacios et al., 1996). They also indicate that cell adhesion mediated by laminin 5 is independent of the extracellular processing of the γ2 chain. However, these results do not argue against a role of the processed 400-kD form of laminin 5 in cell adhesion. Proteolysis of the γ2 chain, which may occur after interaction of laminin 5 with laminins 6 and 7 or collagen type VII, could trigger additional or distinct adhesion functions to laminin 5.

Proteolysis regulates ECM assembly, editing the excess ECM components and release of active polypeptides during morphogenesis, growth, tissue repair, and pathological processes (for review see Werb, 1997). Proteinases negatively regulate the function of a variety of ECM components and cell surface receptors. It has recently been reported that collagen XVII, a transmembrane component of the hemidesmosomes that contributes to formation of the anchoring filaments, undergoes furin-mediated proteolysis resulting in excision of the large extracellular collagenic domain (Hirako et al., 1998). Integrin β4 also undergoes a proteolytic processing resulting in the cleavage of the cytoplasmic and extracellular domains, probably mediated by calpain and matrilysin (Giancotti et al., 1992; Potts et al., 1994; von Bredow et al., 1997). Therefore, proteolysis of the major components of the anchoring filaments may constitute an effective and rapid mechanism for regulating keratinocyte anchorage. The conversion of laminin 5 into a freely shedding product may thus account for the abundance of processed laminin 5 in spent medium of keratinocyte cell cultures and in body fluids (amniotic, cerebrospinal, and inner ear fluids; Aberdam et al., 1994b).

Proteolytic digestion of the laminin γ2 chain by BMP1 at the physiologic YSGD cleavage site has recently been demonstrated in vitro (Amano et al., 2000). Evidence has also been provided that the 155-kD laminin γ2 chain is cleaved in vitro by the matrix metalloprotease (MMP) MT1-MMP to yield the shortened 105-kD γ2 polypeptide and a 80-kD γ2 chain with a further truncated NH2 terminus (Koshikawa et al., 2000). This observation implies two MT1-MMP cleavage sites on laminin 5. The 80-kD γ2 chain is not found in skin extracts and in cultures of human keratinocytes, whereas the 105-kD γ2 polypeptide and the excised 50-kD NH2-terminal domain are easily detectable. In this study we demonstrate that the γ2 chains missing the tetrapeptide YSGD do not undergo proteolytic steps, which confirms in vitro studies and suggests the involvement of BMP-1 in the processing of laminin 5 at this cleavage site (Amano et al., 2000). On the other hand, although BMP-1-null mice display an ultrastructurally altered cutaneous basement membrane, they do not suffer from epidermal adhesion defects (Suzuki et al., 1996). This may indicate either that the extracellular cleavage of the γ2 chain is not essential to keratinocyte adhesion, or that other proteases, including MT1-MMP, can compensate for the absence of BMP-1 in the extracellular processing of the laminin γ2 chain. Indeed, cleavage of the laminin γ2 chain by MT1-MMP has been associated with the migratory behavior of a variety of transformed epithelial cell lines, and inhibition of MT1-MMP partially downregulates cell migration induced by laminin 5 in tumoral cells. Therefore, MT1-MMP may cleave the γ2 short arm and release the interaction between laminin 5 and the ECM components. Since the proteases of the MMPs family are involved in tissue remodeling in various physiological and pathological conditions, it is tempting to speculate that MT1-MMP cleaves laminin 5 and activates cell migration in pathologic circumstances, whereas BMP-1 governs proteolysis of laminin 5 in a physiological context.

Several proteolytic fragments of ECM proteins maintain a biological function (Sage, 1997). A pentapeptide generated by proteolysis of collagen type I is known to regulate transcription of the COL1A1 gene (Katayama et al., 1993), and proliferation of endothelial cells induced by specific growth factors is inhibited by the 16-kD prolactin cleavage fragment (D’Angelo et al., 1995). Laminin γ2 chain and its proteolytic fragments are found at the invasion front of tumors, where they may play positive roles in neoplastic invasion (Pyke et al., 1994, 1995; Koshikawa et al., 1999). Monomeric laminin γ2 chain is also secreted by carcinoma cells in vitro, but the biological relevance of these observations is unclear (Koshikawa et al., 1999; Maatta et al., 1999; Ono et al., 1999). After proteolytic processing, the γ2 chain short arm is detected in the epidermal basement membrane and in the matrix produced in vitro by normal keratinocytes. Deposition of the recombinant γ2 short arm (γ50) in ECM by cultured keratinocytes confirms the adhesive potential of the polypeptide. Therefore, it is possible that after cleavage from laminin 5 the γ2 short arm sustains interactions with specific basement membranes components and cell receptors and participates in basement membrane assembly. In this regard it has been reported that keratinocyte-specific knock-out of mouse integrin β1 results in disorganization of the cutaneous basement membrane and induces skin blistering that correlates with a reduced immunoreactivity and an altered staining pattern of a pAb antibody directed against the γ2 short arm (Brakebush et al., 2000).

Laminin 5 promotes adhesion, migration, and scattering of several types of cultured cells more efficiently than all the other ECM proteins. We demonstrate that the extracellular deposition of laminin 5 is mediated by the short arm of the γ2 chain that then steers intermolecular interactions with the basement membrane components and promotes cell adhesion. The functional role of the proteolytic processing of the laminin γ2 chain in the formation of the epithelial basement membranes remains unclear. The construction of knock-in mice expressing either a mutant γNC or a truncated γC chain will clarify the specific biological functions of the extracellular forms of laminin 5.

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