Three heterotrimeric laminins produced by human keratinocytes

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Abstract

Laminins are a family of glycoproteins composed of α , β and γ chains. Five α ($\alpha 1$ – $\alpha 5$), three β ($\beta 1$ – $\beta 3$) and two γ ($\gamma 1$ and $\gamma 2$) chains have been cloned from human and their replaceable assembly into heterotrimers produces the variety of laminins. Reverse transcription-polymerase chain reaction of mRNAs showed that human keratinocytes express the $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$, $\gamma 1$ and $\gamma 2$ genes at high level among the ten cloned laminin chains. Western blot and immunoprecipitation of the cell lysate with antiserum directed against mouse laminin-1 ($\alpha 1\beta 1\gamma 1$) detected two trimers with the composition of $\alpha\beta 1\gamma 1$ (probably laminin-10 with the composition of $\alpha 5\beta 1\gamma 1$ and $\alpha\gamma\beta 1\gamma 1$). Meanwhile, antiserum directed against a synthetic peptide of human $\alpha 3$ detected only $\alpha 3\beta 3\gamma 2$ trimer (laminin-5). We thus show that keratinocytes produce three heterotrimeric laminins. We could not detect the assembly of $\alpha 3$ with $\beta 1$ and $\gamma 1$ chains to form $\alpha 3\beta 1\gamma 1$ (laminin-6) in keratinocytes.

Abbreviations: ECL, enhanced chemiluminescence; MEM, Eagle's minimal essential medium; NEM, N-ethylmaleimide; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl-fluoride; RIPA, radio-immunoprecipitation assay; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate.

Introduction

Laminins form a family of glycoproteins composed of α , β and γ chains (Burgeson et al., 1994; Timpl and Brown, 1994; Engvall and Wewer, 1996) assembled into triple coiled-coil structures supported by hydrophobic and ionic interactions of the long arm region of each chain (Beck et al., 1990). Many variants of laminin chains are reported in mouse and human. Five α ($\alpha 1$ – $\alpha 5$), three β ($\beta 1$ – $\beta 3$) and two γ ($\gamma 1$ and $\gamma 2$) chains have been cloned from both mouse and human (Engvall and Wewer, 1996; Miner et al., 1995; Liu and Mayne, 1996). Chain-replacement during the assembly produces laminin variants (Tokida et al., 1990). Laminin-5 ($\alpha 3\beta 3\gamma 2$) and laminin-6 ($\alpha 3\beta 1\gamma 1$), found in keratinocytes, are accepted as examples of such variants (Marinkovich et al., 1992a).

This understanding was based on the observation that $\alpha 3$ -specific monoclonal antibody (BM165) (Rousselle et al., 1991) precipitated both laminin-5 and 6 from the culture medium of radiolabeled human keratinocytes and that both laminins contained a polypeptide with similar migration distance of 200 kDa in sodium dodecyl sulfate (SDS)-electrophoresis (Marinkovich et al., 1992a).

Since keratinocyte is an important target of biotechnology for the design of artificial and reconstituted skin, we reexamined the laminin variants produced in human keratinocytes. Reverse transcription-polymerase chain reaction (RT-PCR) showed that $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 3$, $\gamma 1$ and $\gamma 2$ genes are transcribed actively among ten known laminin chains. Western blot and immunoprecipitation of the cell lysate with antiserum against a synthetic peptide of human $\alpha 3$ sequence

detected only laminin-5. Antiserum against mouse laminin-1 ($\alpha 1\beta 1\gamma 1$) detected two trimeric laminins ($\alpha x\beta 1\gamma 1$ and $\alpha y\beta 1\gamma 1$) which commonly have $\beta 1$ and $\gamma 1$ chains. Since anti- $\alpha 3$ antiserum failed to detect these trimers, we suggest that $\alpha 3$ in keratinocyte does not assemble with $\beta 1$ and $\gamma 1$ to form laminin-6 ($\alpha 3\beta 1\gamma 1$).

Materials and methods

Cell culture

Human keratinocyte Hk-f cells from Kyokuto Seiyaku Co. Ltd. (Tokyo) were cultured in 60 mm dishes (NUNC) by feeding K-110 (type II) medium (Kyokuto Seiyaku Co. Ltd.) every other day at 37 °C under humidified 95% air and 5% CO₂.

RT-PCR analysis

Total RNA from Hk-f cells was extracted by an acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomezyski and Sacchi, 1987). For reverse transcription of mRNAs, 2 μ g of total RNA extract was incubated for 10 min at 70 °C and chilled on ice, added with 4 μ l of 5 $\times$ first strand synthesis buffer (GIBCO BRL), 1 μ l of a 25 mM mixture of four deoxynucleotide triphosphates, 2 μ l of 0.1 M dithiothreitol and 100 ng of random primer to make the final volume of 20 μ l. The mixture was incubated for 2 min at 42 °C, added with 200 units of Super Script II RNase H-reverse transcriptase (GIBCO BRL) and incubated for 60 min at 42 °C. Reverse-transcript corresponding to 0.4 μ l of the reaction mixture was amplified by 20–30 cycles of PCR using recombinant Taq DNA polymerase (TAKARA) under a condition of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. PCR products were separated on 1.0% agarose gels using a λ -DNA fragments digested with *HindIII* and *EcoRI* as size markers. The sequences of paired primers used for amplification of laminin variant cDNAs are summarized in Table 1. To identify the amplified fragments, products were separated in low-melting temperature agarose gels and extracted. Purified fragments were digested with the following restriction enzymes and separated in 5% polyacrylamide gels to estimate the sizes of restriction fragments in the parentheses. $\alpha 3$ cDNA fragment was digested with *BamHI* (197, 297 and 125 bp), *HindIII* (48, 112 and 459 bp) and *SmaI* (225 and 394 bp). $\alpha 5$ cDNA fragment was digested with *SalI* (217 and 568 bp).

$\beta 1$ cDNA fragment was digested with *HapII* (464 and 226 bp) and *PstI* (243 and 447 bp). $\beta 3$ cDNA was digested with *PstI* (131 and 556 bp) and *SacI* (284 and 403 bp). $\gamma 1$ cDNA fragment was digested with *BglI* (54, 33, 21 and 567 bp), *AvaII* (416 and 259 bp) and *PstI* (232 and 443 bp). $\gamma 2$ cDNA fragment was digested with *PstI* (293 and 379 bp) and *PvuII* (177 and 495 bp).

Anti-laminin sera

Antiserum against mouse laminin-1 was raised in rabbits as described (Miki et al., 1987). For the preparation of anti-human $\alpha 3$ serum, rabbits were immunized with keyhole limpet hemocyanin-conjugated synthetic peptide having the sequence of QILTK-SETKEA which corresponds to 855–865 residues of human $\alpha 3$ chain (epiligrin α) (Ryan et al., 1994). In addition to the chains forming laminin-5, many cellular polypeptides were precipitated by the antiserum (see Figure 3). This was probably due to antibodies against various configurations of the peptide. Since affinity purification of the serum using a column conjugated with the synthetic peptide did not improve the result, we used the whole serum.

Western blot

Hk-f cells were harvested and homogenized with a buffer containing 20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 10 mM EDTA, 1 mM PMSF and 1 mM NEM. Cell extract corresponding to 3/4 volume of 60 mm dish culture was separated by SDS gel electrophoresis and blotted to Hybond ECL membrane. The membrane was blocked with 5% (w/v) skim milk in PBS containing 0.1% (v/v) Tween 20. As the second antibody, a horse radish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) was used with dilution of 1:1000. Development was done with ECL Western blotting detection reagents (Amersham).

Metabolic labeling of keratinocytes and immunoprecipitation

Culture of keratinocytes in 60 mm dish was pre-incubated in methionine-free Eagle's minimal essential medium (MEM; Sigma) for 30 min. Incubation was continued in 1 ml of methionine-free MEM containing 0.35 mCi ml⁻¹ Pro-mix (Amersham) for 4 h, labeled medium was removed, surface of cell layer was washed with PBS and cells were lysed by RIPA (radio immunoprecipitation assay) buffer

Table 1. The sequence of primer sets used for polymerase chain reaction

Laminin chain	Region (nucleotide number) (reference)	Sequence of paired primers (from 5' to 3')
$\alpha 1$	7175–7847 (Nissinen et al., 1991)	GGCAGAGTGAAGTTATGAC AATGCGCTTGTCATCACTG
$\alpha 2$	8759–9403 (Vuolteenaho et al., 1994)	ATAGCATTGATGGCTGCGTC ATGATACAGGTTGAACGCCC
$\alpha 3$	4524–5140 (Ryan et al., 1994)	GAAGAACTTTCAGCTGGATTC ACTGGTCAGGACAACCATTC
$\alpha 4$	4124–4769 (Richards et al., 1994)	GTCTCAGCAATCTCCAGCTC TTGGGCAGCTGTGCTCTGTC
$\alpha 5$	51–790 (Durkin et al., 1997)	CACTGCCCTCAAGTTCTACC CCCCAAAGTCATACAACAGC
$\beta 1$	4653–5340 (Pikkarainen et al., 1986)	CCAGGATAGTGCTGATTGG TTTGAGCAGTTGCAGCTTGC
$\beta 2$	4918–5595 (Iivanainen et al., 1995)	CAGCTACTGCAGGATGCACG GTGCTAGGAACTGGGGTAGG
$\beta 3$	2970–3654 (Gerecke et al., 1994)	CAAGCAGGACATTGCGCGTG AACGGGCTGGAACGTGTAGC
$\gamma 1$	4436–5108 (Pikkarainen et al., 1988)	GCCATCAACCAGACCATCAC CCTTCCAGCCCTAAAGACAC
$\gamma 2$	2709–3378 (Kallunki et al., 1992)	GTCCTTTCAGGTGGAAGAAG TCTGGTATCAACCTTCTGGG

(Marinkovich et al., 1992a). After centrifugation at $12\,000 \times g$ for 5 min, aliquots of labeled cell lysate corresponding to 5×10^8 dpm of trichloroacetic acid-insoluble radioactivity was diluted to 500 μ l by RIPA buffer and incubated with 1.5 μ l of anti-laminin-1 serum for 16 h and then with 50 μ l of 10% (w/v) Protein A-Sepharose CL-4B beads for 2 h. After centrifugation at $12\,000 \times g$ for 5 min, the supernatant was further incubated with 10 μ l of anti- $\alpha 3$ serum: and 50 μ l of 10% (w/v) Protein A-Sepharose CL-4B as above. Precipitated beads were washed three times with 500 μ l of RIPA buffer and suspended in SDS sample buffer containing 3% (w/v) SDS, 67 mM Tris-HCl pH 6.8, 5% (v/v) glycerol and 0.002% (w/v) bromophenol blue. Immunoprecipitation was also carried out reciprocally, first with anti- $\alpha 3$ serum and then with anti-laminin-1 serum.

Two-dimensional SDS-electrophoresis

After boiling immunoprecipitated complexes in SDS sample buffer, proteins were separated by the two-

dimensional SDS polyacrylamide gel electrophoresis (Morita et al., 1985; Aratani and Kitagawa, 1988). For the first dimension, the electrophoresis under non-reducing conditions was done with 4% (w/v) acrylamide disc gels. The gels were incubated in SDS sample buffer containing 2% (v/v) 2-mercaptoethanol for 3 h at 37 °C and applied to slab gel of 4% (w/v) acrylamide for the second dimension under reducing condition. After the electrophoresis, the gels were fixed in a solution containing 10% (v/v) trichloroacetic acid, 10% (v/v) acetic acid and 30% (v/v) methanol, and analyzed by a image analyzer Fuji BAS 2000.

Results and discussion

Expression of laminin chain genes in human keratinocytes

To explore the laminin genes expressed in human keratinocytes, mRNAs were amplified by RT-PCR using ten pairs of primers designed based on reported cDNA

Figure 1. Amplification of laminin chain mRNAs in human keratinocytes by RT-PCR. Total RNA extracted from Hk-f cells was amplified by 25 cycles of RT-PCR using ten paired primers designed based on reported cDNA sequences of human $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$ and $\gamma 2$. The products were electrophoresed on a 1% agarose gel with λ -DNA fragments digested with *Hind*III and *Eco*RI as a size marker (M).

sequences of human $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$ and $\gamma 2$. After 20 cycles of PCR, the primers for $\alpha 3$ and $\gamma 2$ mRNAs produced the DNA with expected sizes of 619 and 672 bp, respectively (not shown). The primers for $\alpha 5$, $\beta 1$, $\beta 3$ and $\gamma 1$ mRNAs showed the products of 742, 690, 687 and 675 bp, respectively, additionally after 25 cycles (Figure 1). The weak signals for $\alpha 1$ and $\beta 2$ observed after 25 cycles remained to be weak even after 30 cycles (not shown). The efficiency of amplification depends on many factors such as the G/C content of primers but the RT-PCR with increasing cycle numbers suggested that the amount of laminin mRNAs is in the order of $\gamma 2$, $\alpha 3$, $\gamma 1$, $\beta 3$, $\alpha 5$ and $\beta 1$. The identity of these products was confirmed by digesting them with restriction enzymes as described in the Section Materials and methods. $\alpha 1$ mRNA was hardly detected by Nomura et al. in their RT-PCR analysis on primary cultured human keratinocytes (Nomura et al., 1994). No or extremely low expression of $\alpha 1$ and $\alpha 2$ mRNAs was detected by the Northern analysis on 17 week old human skin (Vuolteenaho et al., 1994). RNase protection analysis on RNA isolated from skins of adult mouse and 17.5 days mouse embryo (Miner et al., 1997), showed no $\alpha 1$ mRNA nor $\alpha 2$ mRNA at the level above background, $\alpha 4$ mRNA at the moderate level and $\alpha 5$ mRNA at high level in addition to high level of $\alpha 3$ mRNAs. Ex-

cept for undetectable signal for $\alpha 2$ and $\alpha 4$ mRNAs, our semi-quantification of the laminin chain mRNAs in human keratinocytes gave a consistent result with those reported by other investigators.

Detection of heterotrimeric laminins assembled in human keratinocytes

To analyze the heterotrimeric assembly of laminin chains in human keratinocytes, we used two antisera, one against mouse Engelbreth Holm Swarm (EHS) laminin (laminin-1 $\alpha 1\beta 1\gamma 1$) and the other against a synthetic peptide of human $\alpha 3$ sequence. Probably due to the configurations conserved in laminin chains from different origin, the former antiserum can immunoprecipitate bovine (Tokida et al., 1990) and monkey (Niimi et al., 1997) $\alpha 1\beta 1\gamma 1$ heterotrimers and their constituting chains. When this antiserum was used in Western blot of the human keratinocyte lysate after separation by SDS electrophoresis under non-reducing condition, two heterotrimeric laminins $\alpha x\beta 1\gamma 1$ and $\alpha y\beta 1\gamma 1$: The chain composition will be discussed later) were detected in addition to $\beta 1\gamma 1$ dimer, $\beta 1$ and $\gamma 1$ monomers. Meanwhile, only $\alpha 1\beta 1\gamma 1$ heterotrimer was detected in the control lane of EHS laminin (left panel of Figure 2A). On the other hand, the antiserum against $\alpha 3$ chain (right panel of Figure 2A) detected a

Figure 2. Western blot of laminins produced in human keratinocytes. Laminins produced in Hk-f cells were extracted with a buffer containing EDTA and separated in parallel with EHS laminin (EHS) by SDS electrophoresis under non-reducing (A) or reducing (B) condition. After transferring the proteins to nitrocellulose membranes, the membranes were Western blotted with antiserum against EHS laminin (1:500) or against $\alpha 3$ synthetic peptide (1:500) as the primary antibody and then with horse radish peroxidase-conjugated anti-rabbit IgG (1:1000). Migration positions of heterotrimers of $\alpha \beta 1\gamma 1$, $\alpha \gamma \beta 1\gamma 1$, $\alpha 3\beta 3\gamma 2$, $\beta 1\gamma 1$ dimer, $\alpha 3$, $\beta 1$ and $\gamma 1$ chains are indicated in the margin.

band migrating faster than the heterotrimers detected by the antiserum against EHS laminin. Its migration distance is consistent with the size of 456 kDa estimated for $\alpha 3\beta 3\gamma 2$ heterotrimer (Marinkovich et al., 1992b). It is important to note that the band of $\alpha \gamma \beta 1\gamma 1$ recognized by antiserum against EHS laminin (left panel of Figure 2A) was not recognized by antiserum against $\alpha 3$ chain.

To know the chain composition of the heterotrimers, Western blot was done after cleaving the interchain disulfide-bonding by SDS electrophoresis under reducing condition (Figure 2B). Probably because the intrachain disulfide-bondings in EGF-like domain of the short arms were also cleaved, the antiserum against mouse EHS laminin could recognize neither human laminin chains nor mouse $\alpha 1$ chain but

recognized only mouse $\beta 1$ and $\gamma 1$ chains (left panel of Figure 2B). The antiserum against $\alpha 3$ chain recognized a 200 kDa peptide (Marinkovich et al., 1992b) consistent with the size of $\alpha 3$ chain (right panel of Figure 2B).

For further analysis of the laminin chain composition, human keratinocytes were metabolically labeled with [^{35}S] methionine/cysteine and the cell lysate was immunoprecipitated and separated by two-dimensional SDS-electrophoresis, in which non-reducing electrophoresis in the first dimension (left to right) was followed by reducing electrophoresis in the second dimension (top to bottom). Due to the interchain disulfide-bonding, laminin chains forming dimer and trimer can be detected in this electrophoresis as spots on vertical lines leaving monomeric poly-

Figure 3. Non-reducing and reducing two-dimensional SDS-electrophoresis of laminin complexes synthesized in human keratinocytes. Radiolabeled lysate of Hk-f cells was sequentially immunoprecipitated first with antibody against EHS laminin (A) then with antiserum against $\alpha 3$ (B), or first with antiserum against $\alpha 3$ (C) then with antiserum against EHS laminin (D). The precipitates were electrophoresed from left to right under non-reducing condition, and from top to bottom under reducing condition. The migration positions of laminin complexes and those of laminin chains are indicated at the bottom and left margins of each panel, respectively. Numbers at the right margins of each panel indicate migration positions of the molecular mass standard in kDa. The anti- $\alpha 3$ antiserum precipitated unidentified peptides migrating to the area of disulfide-bonded complex as typically indicated by stars in B and C. None of them had the migration rate of known laminin chains.

peptides on the diagonal line of the gel (Tokida et al., 1990).

Precipitation of the radiolabeled cell lysate with antiserum against EHS laminin showed that two heterotrimers observed in Figure 2A contained $\beta 1$ and $\gamma 1$ chains as the common components and a chain of 400 or 200 kDa as the third chains (Figures 3A and D). Precipitation of $\beta 1\gamma 1$ as only dimer suggests that these heterotrimers are formed by the assembly of either α chain to $\beta 1\gamma 1$ dimer. Among five α isoforms cloned from human (Engvall and Wewer, 1996), $\alpha 1$, $\alpha 2$ and $\alpha 5$ chains have the sizes in the range of 400 kDa. Considering the high level of $\alpha 5$ mRNA compared with

those of $\alpha 1$ and $\alpha 2$ mRNAs (Figure 1), it is highly possible that the a chain of 400 kDa is $\alpha 5$ chain. Without strong evidence for this identity, however, we will refer to it as αx . The heterotrimer containing α chain of 200 kDa is probably what was called 'K-laminin' by Marinkovich et al. (1992). The presence of laminin-6 with the composition of $\alpha 3\beta 1\gamma 1$ has been assumed based on the observation of such 'K-laminin'. Since the α chains of 200 kDa is not recognized by the antiserum against $\alpha 3$ chain (see below), however, we will refer to it as αy .

In the experiment in Figure 3B, the supernatant after precipitation with antiserum against EHS laminin

was further subjected to precipitation with antiserum against $\alpha 3$ chain. Three spots forming laminin-5 ($\alpha 3\beta 3\gamma 2$) are detected together with many other unidentifiable spots. Migration distance of the three spots agreed well with the reported sizes of $\alpha 3$ (200 kDa), $\beta 3$ (150 kDa) and $\gamma 2$ (105 kDa) (Marinkovich et al., 1992b). All spots including the components of laminin-5 disappeared in the precipitation to which the epitopic QILTKSETKEA peptide was added (not shown). This suggested that our antiserum against $\alpha 3$ chain contains antibodies recognizing various configuration of QILTKSETKEA peptide and cross-reacts with many cellular polypeptides. Most of such polypeptides were monomeric and migrated on the diagonal. Several unidentifiable spots remained in the complex area below the diagonal but none of them had the migration distance of known laminin chains.

Precipitation of $\beta 3$ and $\gamma 2$ by antiserum against $\alpha 3$ (Figure 3B) was due to their disulfide-bonding to $\alpha 3$ chains. If $\alpha \gamma$ is identical to $\alpha 3$, the $\beta 1$ and $\gamma 1$ in the assumed $\alpha 3\beta 1\gamma 1$ heterotrimer (laminin-6) should be precipitated for the same reason. Their absence in Figure 3B shows that $\alpha \gamma$ is distinct from $\alpha 3$.

To rule out the possibility that the assumed $\alpha 3\beta 1\gamma 1$ heterotrimer was depleted by antiserum against EHS laminin, we first precipitation with antiserum against $\alpha 3$. As shown in Figure 3C, the assumed $\alpha 3\beta 1\gamma 1$ complex was not precipitated where as $\alpha 3\beta 3\gamma 2$ complex was not precipitated where as $\alpha 3\beta 3\gamma 2$ was precipitated. Since further precipitation from the supernatant with antiserum against EHS laminin (Figure 3D showed $\alpha \gamma \beta 1\gamma 1$ trimer, we conclude that $\alpha 3$ chain does not assemble with $\beta 1$ and $\gamma 1$ chains to form $\alpha 3\beta 1\gamma 1$ heterotrimer (laminin-6). Concerning from the expression levels of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains (Figure 1), laminin-5 is one of the major laminin produced in keratinocytes. To test the possibility that antiserum against $\alpha 3$ failed to recognize $\alpha \gamma \beta 1\gamma 1$ because of excess laminin-5, the immunoprecipitation with antiserum against $\alpha 3$ was repeated after the first immunoprecipitation with antiserum against $\alpha 3$. Neither laminin-5 nor $\alpha \gamma \beta 1\gamma 1$ was precipitated in the second reaction whereas $\alpha \gamma \beta 1\gamma 1$ was immunoprecipitated by the following reaction with antiserum against EHS laminin (not shown). Plausible explanation for apparent discrepancy is that, in the experiment by Marinkovich, $\alpha \gamma \beta 1\gamma 1$ heterotrimer was precipitated by BM165 from the cultured medium due to association with $\alpha 3\beta 3\gamma 2$ heterotrimer (laminin-5) after the secretion. From the cell lysate, $\alpha \gamma \beta 1\gamma 1$ was

not immunoprecipitated with the antiserum against $\alpha 3$ since it was not associated with $\alpha 3\beta 3\gamma 2$ heterotrimer.

As the conclusion, we have shown that human keratinocytes produce three heterotrimeric laminins with the chain compositions of $\alpha \alpha \beta 1\gamma 1$ (probably $\alpha 5\beta 1\gamma 1$), $\alpha \gamma \beta 1\gamma 1$ and $\alpha 3\beta 3\gamma 2$ (laminin-5). Despite similar size of 200 kDa, $\alpha \gamma$ is immunochemically distinct from $\alpha 3$. Human keratinocytes actively synthesize $\alpha 3$, $\beta 1$ and $\gamma 1$ chains but do not assemble the $\alpha 3\beta 1\gamma 1$ heterotrimer. We thus question the existence of $\alpha 3$ chain forming $\alpha 3\beta 1\gamma 1$ heterotrimer (laminin-6) in keratinocytes.

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