The integrins are a large family of transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions (1–5). All known members of this superfamily are noncovalently associated heterodimers composed of an α and a β subunit. At present, 8 β(1–8) (See Ref. 6 and references therein) and 16 α subunits α(1–9), αv, αM, αL, αX, αIIb, αE, and αD) have been characterized (6–21), and these subunits associate to generate more than 20 different integrins. The β1 subunit has been shown to associate with 10 different α subunits, α1–9 and αv and to mediate interactions with extracellular matrix proteins such as collagens, laminins, and fibronectin. The major collagen binding integrins are α1β1 and α2β1 (22–25). The integrins αβ1 and αβ1 have also been reported to interact with collagen (26, 27), although this interaction is not well understood (28). The extracellular N-terminal regions of the α and β integrin subunits are important in the binding of ligands (29, 30). The N-terminal region of the α subunits is composed of a 7-fold repeated sequence (12, 31) containing FG and GAP consensus sequences. The repeats are predicted to fold into a β-propeller domain (32), with the last three or four repeats containing putative divalent cation binding sites. The α-integrin subunits α1, α2, αD, αE, αL, αM, and αX contain an ~200 amino acid inserted domain, the I-domain (A-domain), that shows similarity to sequences in von Willebrand factor, cartilage matrix protein, and complement factors C2 and B (33, 34). The I-domain is localized between the second and third FG-GAP repeats; it contains a metal ion-dependent adhesion site (MI-DAS), and it is involved in binding of ligands (35–38).

Chondrocytes, the only type of cells in cartilage, express a number of different integrins including α1β1, α2β1, α3β1, α5β1, α6β1, αβ3, and αβ5 (39–41). We have shown that α1β1 and α2β1 mediate chondrocyte interactions with collagen type II (25), which is one of the major components in cartilage. We have also shown that α2β1 is a receptor for the cartilage matrix protein chondroadherin (42). In the present study we have isolated a novel collagen type II binding integrin, α10β1, from bovine articular chondrocytes. Cloning and sequence analysis of the human homologue is described, and expression of α10 on chondrocytes is examined.

MATERIALS AND METHODS

Antibodies—A polyclonal antiserum was generated against the α10 cytoplasmic domain peptide CKRIPEEKREEKL. Peptide synthesis and conjugation to keyhole limpet hemocyanin, injection of rabbits and affinity purification were performed by Innovagen AB (Lund, Sweden). Monoclonal antibodies against human integrin subunit β1 (P4C10), α2 (P1E6), and α3 (P1B5) (unpurified ascites fluid) were from Life Technology Inc. The monoclonal antibody against human integrin subunit α1 (TS27; hybridoma supernatant) was a kind gift from Timothy Springer, Boston Blood Center, Boston, MA (43). Polyclonal antibody (serum) against the rat β1-integrin subunit was kindly provided by Staffan Johansson, Uppsala, Sweden (44). Polyclonal antibodies (serum) against human integrin subunit α2 (AB1936), α3 (AB1920), and polyclonal antibody (serum) against rat integrin subunits α1 (AB1934) were from Chemicon International Inc. (Temecula, CA). Polyclonal antibodies against the integrin subunit α9 (affinity-purified IgG) were a kind gift from Dean Sheppard, University of California San Francisco Lung Biology Center, San Francisco, CA (6).

Cell Isolation and Culture—Bovine chondrocytes were isolated by digestion of articular cartilage from 4–6-month-old calves with collagenase (CLS1; Worthington Biochemical Corp., Lakewood, NJ) as described elsewhere (45). Briefly, cartilage slices were digested by colla-
genase in Earle’s balanced salt solution (Life Technologies, Inc.) for 15–16 h at 37 °C. The tissue digest was filtered through a 100-μm nylon filter, and the isolated cells were then washed three times in Dulbecco’s modified phosphate-buffered saline (PBS).1 Life Technologies, Inc.). Human chondrocytes from articular cartilage were isolated by digestion with collagenase (Roche Diagnostics, Mannheim) for 15–18 h, as described by Häuselmann et al. (46). The cells were filtered and washed as described above. Human chondrocytes were cultured in Dulbecco’s minimum essential medium and F-12 (1:1) supplemented with 10% fetal calf serum, 25 μg/ml ascorbic acid, 50 IU of penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). To harvest the cells, the culture dish was washed three times with Ca2+/Mg2+-free PBS, and the cells were incubated with 0.5% trypsin and 1 mM EDTA (Life Technologies, Inc.) in Ca2+/Mg2+-free PBS for 5 min. Detached cells were suspended in medium containing 10% fetal calf serum or in PBS containing 1 mg/ml trypsin inhibitor (Sigma) and then washed in PBS.

**Coupling of Affinity Columns**—Collagen type II isolated from nasal cartilage by pepsin digestion (47) was coupled to CNBr-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the published procedure (25). A control column was produced by treating CNBr-Sepharose 4B in a similar manner but in the absence of protein. Bovine fibronectin (Sigma) was coupled to CNBr-Sepharose 4B according to instructions from the manufacturer. After blocking, the fibronectin-Sepharose was washed three times with PBS.

**Affinity Purification and Immunoprecipitation of Chondrocyte Membrane Proteins**—Human chondrocyte cell surface proteins were 125I-labeled and affinity-purified on collagen type II-Sepharose according to the published procedure (25). Cell lysates or affinity-purified samples were immunoprecipitated as described earlier (42). The following antibodies were used in immunoprecipitation experiments: monoclonal antibodies against the human integrin subunits β1,α1,α2, or α3 (unpurified ascites fluid, dilution 1/100), polyclonal antibody against the rat integrin subunit β1 (purified IgG, 50–100 μg/ml), polyclonal peptide antibodies against the integrin subunits α1,α2,α3, and α10 (serum, dilution 1/100). The immunoprecipitated proteins were separated by 4–12% SDS-PAGE and visualized by image analysis using the BioImaging Analyzer Bas2000 (Fuji Photo Film Co., Tokyo, Japan).

**Western Blot**—Human chondrocyte membrane proteins immunoprecipitated with polyclonal antibodies against α10 (10 μg/ml affinity-purified IgG) or β1 (100 μg/ml IgG) were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane essentially as described by Towbin et al. (48). The membrane was blocked with 3% dried milk in 10 mM Tris- HCl, pH 7.4, 0.15 M NaCl, and 0.2% Tween (blocking buffer) and was incubated with the β1 antibody (1) and α10 antibody (59) under reducing conditions, and stained with Coomassie Blue. The 160-kDa protein was excised from the gel and prepared for in-gel digestion (51).

**Isolation of Internal Peptides by In-gel Digestion and Peptide Sequencing**—Affinity-purified proteins were concentrated by precipitation using the methanol/chloroform protocol (49). After reduction/alkylation with dithiothreitol/iodoacetamide (50), the precipitated proteins were subjected to SDS-PAGE on a 4–12% polyacrylamide gel, and protein bands were visualized by Coomassie staining. The 160-kDa protein band was excised from the gel and prepared for in-gel digestion (51). Briefly, the gel slice was washed extensively to remove SDS and the dye, and after complete drying, protease was forced into the gel by rehydration with a solution of modified trypsin (Promega, Madison, WI) in 0.2 M NH4HCO3 buffer. After an overnight incubation, peptides were extracted and then isolated by narrow bore reversed phase liquid chromatography on a μRPC C2/C18 stainless steel 2.1/10 column operated in a SMART System (Amersham). Several peptides were analyzed by Edman degradation in a Perkin-Elmer Applied Biosystem Model 476 sequencer operated according to the manufacturer’s instructions.

**mRNA Purification and cDNA Synthesis**—mRNA from bovine or human chondrocytes were isolated using a QuickPrep Micro mRNA purification kit (Pharmacia). cDNA was synthesized at 42 °C for 1 h using the SuperscriptII RNase H− Reverse Transcriptase cDNA Synthesis system (Life Technologies, Inc.) random DNA hexamers and oligo(dT) (Promega, Madison, WI).

**PCR Amplification**—PCR reactions were performed in 50-μl reaction volumes and contained 1 μmol primer template DNA (bovine chondrocyte cDNA), and 0.1 μM each of dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim). PCR samples were heated to 94 °C for 5 min in a thermocycler and then subjected to 35 cycles consisting of 30 s at 94 °C (denaturation), 30 s at 48 or 52 °C (annealing) and 3 min at 72 °C (extension). The PCR products were re-amplified using 1 μl of each product for an additional 35 cycles. Amplified DNA was analyzed by 1% agarose gel electrophoresis. Small DNA fragments were analyzed using 4% Methaphor agarose (FMC BioProducts, Rockland, ME).

The degenerate primers GAY AAY ACI GCC CAR AC (DNTAQ, forward) and TIA TIS WRT GRT GIG GYT (EPHHISI, reverse) were used in PCR to amplify the nucleotide sequence corresponding to the bovine peptide 1 (Table I). A 900 base pair PCR fragment was then amplified from bovine cDNA using an internal specific primer TCA GCC TAC AT-

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1 The abbreviations used are: PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE (Race), rapid amplification of the cDNA end.
T CAG TAT (SAYIQY, forward) corresponding to the cloned nucleotide sequence of peptide 1 together with the degenerate primer ICT RTC-CCA RTG ICC IGG (PGHWDR, reverse) corresponding to the bovine peptide 2 (Table I). Mixed bases were used in positions that were 2-fold degenerate, and inosines were used in positions that were 3- or 4-fold degenerate.

To obtain cDNA that encoded the 5' end of α10, we designed the primer AAC TCG TCT TCC AGT GCC ATT CGT GGG (reverse; residues 1254–1280 in α10 cDNA) and used it for rapid amplification of the cDNA 5' end (RACE) as described in the Marathon™ cDNA amplification kit (CLONTECH INC., Palo Alto, CA).

Cloning and Sequencing of cDNA—PCR fragments were isolated and sequenced. The overlap between the two overlapping α10 clones hc1 and hc2 were obtained by screening a human articular chondrocyte library with a bovine α10 probe. The Race1 clone was obtained from human chondrocyte cDNA using the RACE technique. Arrows indicate the direction and extent of nucleotide sequencing.

### TABLE I

| Peptide | Amino acid sequence |
|---------|---------------------|
| 1       | DNTAQTSAYIQYEPHHSI |
| 2       | GPGHWDR             |
| 3       | AAFDDGSGQR          |
| 4       | FAMALFDP            |
| 5       | FTAASLNTAAAR        |
| 6       | VDASFRPQGXLAP       |

Peptides were isolated by in-gel digestion with trypsin and sequenced by Edman degradation.

![Fig. 3](image-url) Schematic map of the sequencing strategy. The overlapping α10 clones hc1 and hc2 were obtained by screening a human articular chondrocyte library with a bovine α10 probe. The Race1 clone was obtained from human chondrocyte cDNA using the RACE technique. Arrows indicate the direction and extent of nucleotide sequencing. kb, kilobases.

![Fig. 4](image-url) Nucleotide sequence and deduced amino acid sequence of the human α10 integrin subunit. The amino acid translation is under the first nucleotide of the corresponding codon. The signal peptide cleavage site is marked with an arrow, human homologues to bovine peptide sequences are underlined, and the I-domain is boxed. Metal ion binding sites are indicated with a dashed underline, potential N-glycosylation sites are indicated by an asterisk, and the putative transmembrane domain is double underlined. The normally conserved cytoplasmic sequence is indicated by a dot and dash underline. The sequence data is available from GenBank™ under accession number AF074015.

![TABLE I](image-url) Amino acid sequences of peptides from bovine α10-integrin

Peptides were isolated by in-gel digestion with trypsin and sequenced by Edman degradation.

| Peptide | Amino acid sequence |
|---------|---------------------|
| 1       | DNTAQTSAYIQYEPHHSI |
| 2       | GPGHWDR             |
| 3       | AAFDDGSGQR          |
| 4       | FAMALFDP            |
| 5       | FTAASLNTAAAR        |
| 6       | VDASFRPQGXLAP       |

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purified from agarose gels using Jet Sorb DNA extraction kit (Genomed Inc. Research Triangle Park, CA). Purified fragments were then cloned with the pCR Script® SK (+) stratagene, 2 mg/ml hyaluronidase (Sigma) in PBS, pH 5.0, for 15 min at room temperature, and then incubated overnight at 4 °C with the affinity-purified antibodies against the integrin subunit α9 or α10 (5 μg/ml in blocking buffer). For control, the α10 antibody was preincubated with the α10 peptide (0.1 mg/ml) for 30 min at 4 °C. After washing in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories Inc; diluted 1:200 in blocking buffer) at room temperature for 60 min. Washed sections were then incubated with VECTASTAIN® ABC reagent (Vector Laboratories, Inc. Burlingame, CA) for 1 h at room temperature and washed, and the color was developed using 1 mg/ml diaminobenzidine, 0.02% H2O2 and 0.1 M Tris-HCl, pH 7.2. Sections were rinsed in water for 5 min followed by 75, 95, and 99.5% ethanol for 5 min each and then three times in xylene for 3 min at room temperature. Samples were mounted in Perpx (Histolab Products AB, Gothenburg, Sweden) and examined by light microscopy.

**RESULTS**

Identification and Isolation of the Chondrocyte α10 Integrin Subunit—Affinity purification of 125I-labeled membrane proteins from human chondrocytes on collagen type II-Sepharose followed by immunoprecipitation showed that these cells, in addition to α1β1 and α2β1, express an unidentified β1-related α subunit (Fig. 1). This integrin subunit had an apparent molecular mass of approximately 160 kDa under reducing condition and was slightly larger than the α2 integrin subunit. This finding is in agreement with a previous study from our group showing that bovine chondrocytes also express an unidentified collagen binding β1-associated α subunit of similar molecular mass (25). To isolate this protein, we affinity-purified collagen type II-binding proteins from bovine chondrocytes. The chondrocyte lysate was first applied to a fibronectin-Sepharose precolumn, and the flow-through was then applied to a collagen type II-Sepharose column. As shown in Fig. 2, a number of proteins were eluted from the affinity columns. A protein with molecular mass of approximately 160 kDa was specifically eluted with EDTA from the collagen column but not from the fibronectin-Sepharose column. The molecular mass of this protein corresponded with the molecular mass of the unidentified β1-related integrin subunit (Fig. 1). The 160-kDa protein band was excised from the SDS-PAGE gel and digested with trypsin, and several of the isolated peptides were analyzed. Table I shows the amino acid sequence of six individual peptides.

**Cloning and Sequencing of the Human Integrin α Subunit Homologue**—The nucleotide sequence corresponding to peptide 1 (Table I) was obtained by PCR amplification, cloning, and sequencing of bovine cDNA. From this nucleotide sequence an exact primer was designed and applied in PCR amplification with degenerate primers corresponding to peptides 2–6 (Table I). Primers corresponding to peptides 1 and 2 amplified a 900-base pair PCR fragment from bovine cDNA that was cloned, sequenced, and used for screening of a human articular chondrocyte λZapII cDNA library to obtain the human integrin α subunit homologue. Two overlapping clones, hc1 and hc2 (Fig. 3), were isolated, subcloned, and sequenced. These clones

| Peptide | Amino acid sequence |
|---------|---------------------|
| α1      | KGFFKRPKLKKMKEK    |
| α2      | KGFFKRYKEMTKNPDO.IDETELLS |
| α10     | KGFFARHKIPKEKEKKEEQ |
| αM      | KGFFRQKYMMDMEGSGP.GAEQ |
| αX      | KGFFRQYKEMME4ANGQAPIENG10TPSFSSEKPSSEK |
| αL      | KGFFKRNKLKEMEA4GVFVPGIPAEDSDLALSQ.GEAGDGP.CLFLHEDSES.SGGKD |
| αE      | KGFFKRYQQLNLESLR1KAQLKSENLEEEN |
contained 2/3 of the nucleotide sequence, including the 3' end of the cDNA. A third clone (Race1; Fig. 3), which contained the 5' end of the a10 cDNA, was obtained using the RACE technique. From these three overlapping clones of a10 cDNA, 3884 nucleotides were sequenced (Fig. 4). The sequence contains a 3504-nucleotide open reading frame that is predicted to encode a 1167 amino acid mature protein. The predicted sequence included a signal peptide (22 amino acids), a long extracellular domain (1098 amino acids), a transmembrane domain (25 amino acids), and a short cytoplasmic domain (22 amino acids).

Sequence analysis of the 160-kDa protein sequence showed that it was a member of the integrin a-subunit family, and the subunit was named a10.

Comparison of a10 Integrin Subunit with Other a Subunits—Analysis of a10 with known a subunits showed that its structure follows the conserved pattern of integrin a subunits (Fig. 5). The extracellular domain contains a 7-fold repeated sequence including FG and GAP consensus sequences, three putative divalent cation binding sites (DXXD/NXXD), and an I domain of 199 amino acids. The protein contains 10 potential N-linked glycosylation sites (NX(T/S)). The calculated molecular mass is 153 kDa if carbohydrate chains with an average molecular weight of 2.5 kDa are assumed to attach to all 10 putative glycosylation sites. This is in agreement with the molecular mass of a10 as judged by SDS-PAGE where the molecular mass was estimated to approximately 160 kDa.

In contrast to most a-integrin subunits, the cytoplasmic do-
main of α10 does not contain the conserved sequence KKGFF(R/K)R (Table II). Instead, the predicted amino acid sequence is KLGFFAH. The deduced amino acid sequence of α10 showed the highest identity to the collagen-binding integrin subunits α1 (37%) and α2 (35%). The similarity of integrin α subunits are shown in Fig. 6.

Expression of the α10 Integrin Subunit on Chondrocytes—Northern blot analysis of mRNA from bovine chondrocytes showed that a human α10 cDNA probe hybridized with a single mRNA of approximately 5.4 kilobases (Fig. 7). As a comparison, a cDNA probe corresponding to the integrin subunit β1 was used. This cDNA probe hybridized a mRNA band of approximately 3.5 kilobases on the same filter. Translation of the α10 nucleotide sequence revealed an open reading frame of 3504 nucleotides (Fig. 4), which indicates that around 2000 nucleotides in the mRNA is not translated.

To study expression of α10 at the protein level, 125I-labeled membrane proteins from human chondrocytes were immunoprecipitated with polyclonal antibodies against the integrin subunits β1, α1, α2, α3, and α10 (Fig. 8). A polyclonal peptide antibody raised against the cytoplasmic domain of α10 precipitated two protein bands with molecular masses of approximately 160 and 125 kDa under reducing conditions. The α10-associated β-chain migrated as the β1 integrin subunit both under reducing and nonreducing conditions (Figs. 8, a and b). To verify that the α10-associated β-chain indeed is β1, chondrocyte lysates were immunoprecipitated with antibodies against α10 or β1 followed by Western blot using antibodies against the β1 subunit (Fig. 8c). These results clearly demonstrated that α10 is a member of the β1-integrin family.

Expression of α10 in cartilage was examined by immunostaining of human articular cartilage from the trochlear groove with the polyclonal α10 antibody. As shown in Fig. 9, this antibody specifically stained the chondrocytes in the cartilage tissue sections. The staining was completely ablated when the antibody was preincubated with the α10 peptide. A control antibody against the α9 integrin subunit did not stain chondrocytes in the tissue sections (Fig. 9).

**DISCUSSION**

The present study demonstrated that human chondrocytes express a novel, collagen type II-binding integrin in the β1 family. We have, in an earlier study, presented some evidence for that bovine chondrocytes and human chondrosarcoma cells also express this integrin (25). Because bovine chondrocytes are readily available in large amounts, we used these cells in the isolation of the integrin subunit α10. As shown in Fig. 2, several proteins were eluted from the columns in the affinity purification experiments. It was difficult to interpret the protein pattern in the eluate because typical integrin bands were not clearly distinguished on the SDS-PAGE gel. This may be explained by partial protein degradation, although a mixture of protease inhibitors were included in the lysate buffer. Based upon the finding that the β1 antibody immunoprecipitated an unknown collagen-binding integrin α subunit with a molecular mass of 160 kDa (Fig. 1), a protein with similar molecular mass that was specifically eluted with EDTA from the collagen type II column was excised from the gel and used for peptide sequencing. This 160-kDa protein was not eluted from the fibronectin-Sepharose, indicating that fibronectin is not a ligand for α10β1. However, this will be investigated in cell adhesion experiments using cells transfected with the α10 subunit.

The immunoprecipitation experiments showed that α2 and α10 integrin subunit have similar molecular masses under reducing conditions (Fig. 1). To avoid contamination of α2, the 160-kDa protein was excised from the SDS-PAGE gel as a very narrow band. This was apparently successful since human homologues to all six bovine peptides (Table I) that were isolated from the 160-kDa protein were found in the predicted amino acid sequence of human α10 subunit (Fig. 4).

The deduced amino acid sequence of α10 was found to share the general structure of the integrin α subunits described in previously published reports (6–21). The large extracellular N-terminal part of α10 contains a 7-fold repeated sequence that was recently predicted to fold into a β-propeller domain (32). The integrin subunit α10 contains three putative divalent cation binding sites (DxD/NxD/NXXXD) (53), a single spanning transmembrane domain, and a short cytoplasmic domain. In contrast to most α-integrin subunits, the cytoplasmic domain of α10 does not contain the conserved sequence KXGGF/R/KXR. The predicted amino acid sequence in α10 is KLGFFAH. Several reports indicate that the integrin cytoplasmic domains are crucial in signal transduction (54) and that membrane-proximal regions of both α- and β-integrin cytoplasmic domains are involved in modulating conformation and affinity state of integrins (55–57). It is suggested that the GFFKR motif in α-chains are important for association of integrin subunits and for transport of the integrin to the plasma membrane (58). The KXGFFKR domain has been shown to interact with the intracellular protein calreticulin (59), and interestingly, calreticulin-null embryonic stem cells are deficient in integrin-mediated cell adhesion (60). It is, in this context, tempting to speculate that the sequence KLGFFAH in α10 may have a key function in regulating the affinity between α10β1 and collagen.

Integrin α subunits are known to share an overall identity of 20–40% (61). Sequence analysis showed that the α10 subunit is most closely related to the I domain-containing α subunits (Fig. 6) with the highest identity to α3 (37%) and α2 (35%). The integrins α1β1 and α2β1 are known receptors for both collagens and laminins (24, 62, 63), and we have also recently demonstrated that α2β1 interacts with the cartilage matrix protein chondroadherin (42). Since α10β1 was isolated on a collagen type II-Sepharose, we know that collagen type II is a ligand for α10β1. We have also shown by affinity purification experiments that α10β1 interacts with collagen type I (data not shown), but it remains to be seen whether laminin or chondroadherin are also ligands for this integrin.

The peptide antibody that we raised against the cytoplasmic domain of α10 immunoprecipitated two proteins from human chondrocytes with molecular masses of approximately 125 and 160 kDa. The molecular mass of 160 kDa correlates with the unidentified β1-associated α subunit that was affinity-purified on collagen type II-Sepharose. The 125-kDa protein was in Western blot recognized by an antibody to the β1 subunit. This,
Integrin α1β1

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...with previous findings that α1β1 and α2β1 are present on isolated chondrocytes demonstrate that chondrocytes express at least three collagen-binding integrins in the β1 family (25). Further studies will answer the question whether these integrins have similar or different functions in cartilage.

Immunohistochemistry using the α10 antibody showed staining of the chondrocytes in tissue sections of human articular cartilage. The antibody staining was clearly specific because preincubation of the antibody with the α10 peptide completely abolished the staining. An antibody against the integrin subunit α9 did not stain the chondrocytes (6). This integrin is a receptor for tenascin C (64) and is not known to be present in cartilage.

Taken together, we have isolated and characterized a novel collagen type II-binding integrin designated α10β1. The α10 subunit was isolated from bovine chondrocytes, and the human homologue was cloned and sequenced. Antibodies against the α10-integrin subunit stained chondrocytes in tissue sections of articular cartilage, indicating that α10β1 indeed is expressed in cartilage. Further investigations including ligand interactions, tissue distribution, signal transduction, and knockout mutation will demonstrate the function of the integrin α10β1.

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