The aggregating proteoglycans (aggrecan, versican, neurocan, and brevican) are important components of many extracellular matrices. Their N-terminal globular domain binds to hyaluronan, but the function of their C-terminal region containing a C-type lectin domain is less clear. We now report that a 90-kDa protein copurifies with recombinant lectin domains from aggrecan and versican, but not from the brain-specific neurocan and brevican. Amino acid sequencing of tryptic peptides from this protein identified it as fibulin-1. This extracellular matrix glycoprotein is strongly expressed in tissues where versican is expressed (blood vessels, skin, and developing heart), and also expressed in developing cartilage and bone. It is thus likely to interact with these proteoglycans in vivo. Surface plasmon resonance measurements confirmed that aggrecan and versican lectin domains bind fibulin-1, whereas brevican and neurocan do not. As expected for a C-type lectin, the interactions with fibulin-1 are Ca\textsuperscript{2+}-dependent, with $K_D$ values in the low nanomolar range. Using various deletion mutants, the binding site for aggrecan and versican lectin domains was mapped to the epidermal growth factor-like repeats in domain II of fibulin-1. No difference in affinity was found for deglycosylated fibulin-1, indicating that the proteoglycan C-type lectin domains bind to the protein part of fibulin-1.

The lectican family of large aggregating proteoglycans consists of versican, aggrecan, neurocan, and brevican (1). Their core proteins are organized with a central, extended, glycosaminoglycan-carrying part of variable length, flanked by globular domains (2–6). It has long been known that these proteoglycans are anchored to the hyaluronan meshwork through their N-terminal globular domains, in link protein-stabilized complexes (7–10). We have recently shown that the C-terminal globular domain also functions as a binding domain, in that it binds the brain-specific extracellular matrix protein tenascin-R (11, 12). Indeed, all these proteoglycans are expressed in the brain (1, 13). Although neurocan and brevican expression is restricted to the central nervous system, aggrecan is prominent in cartilage only (14), while versican is widely expressed in the extracellular matrix of many tissues, notably blood vessels and skin (15). Until now, no extra-neuronal ligand for the C-terminal globular domains of aggrecan and versican has been identified.

We now report that the fibrillar extracellular matrix protein fibulin-1 is such a ligand. Fibulin-1 was previously shown to be an elongated protein which consists of three domains (I-III) with different molecular structures. The C-terminal domain III can be varied by splicing (variants A-D) and this was shown to affect its binding properties to nidogen-1 (16–18). Here we show that fibulin-1 binds to the C-type lectin domains of versican and aggrecan with high affinity, but shows no binding to the brain-specific proteoglycans brevican and neurocan. We also map the binding site on the fibulin-1 molecule to the central stretch of calcium binding EGF-like repeats. The striking overlap in the expression pattern of fibulin and those of versican and aggrecan, suggests that this interaction is of physiological importance.

MATERIALS AND METHODS

Construction of Recombinant Lectin Domain Expression Plasmids—The construction of His-tagged versican and brevican lectin-like domain mammalian expression pCEP4 plasmids have previously been described (12). Plasmids for expression of aggrecan and neurocan lectin-like domains were constructed by transferring the expression cassette from the pcDNA3 vectors (12) into the pCEP4 plasmid as for the constructs above. All expression constructions were sequenced before use. The resulting expression vectors code for an immunoglobulin signal peptide, a C-type lectin-like domain and a hexahistidine tag. After cleavage of the signal peptide the resulting secreted recombinant proteins have an N-terminal Arg residue preceding the C-type lectin domain (amino acid residues 1909–2037, 659–787, 1024–1152, and 2177–2306 of aggrecan (2), brevican (5), neurocan (3), and versican (4), respectively), followed by the C-terminal shared sequence Ser-Thr-His$_6$.

Production of Recombinant Lectin Domains—The expression plasmids were transfected into human embryonal kidney 293-EBNA cells using LipofectAMINE. After 1 day of recovery, the cells were subjected to selection with 260 μg hygromycin and survivors were allowed to grow to confluency. For large scale production of recombinant His-tagged lectin domains, conditioned medium (Dulbecco’s modified Eagle’s medium with 10% fetal calf serum) was collected from 20 15-cm culture dishes of confluent producer cells every second day. After harvest, the medium was cleared of detached cells and debris through centrifugation, Tris-HCl (1 M, pH 8.0) was added to a final concentration of 50 mM, and 5 M NaCl was added to a final concentration of 0.5 M. Ni-NTA superflow-beads (Qiagen GmbH, Germany) were equilibrated in Buffer A (50 mM Tris-HCl (pH 8.0), 0.5 M NaCl), and added to the medium. After incubation at room temperature for 30 min the beads were transferred to a column, and washed with Buffer A until the A$_{280}$ of the flow-through was stable. The His-tagged proteins were eluted with 20 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl (pH 8.0), and further purified by ion exchange chromatography on a MonoQ HR 5/5 (Pharmacia Biotech, Uppsala, Sweden) column with salt elution (0–1 M NaCl gradient over 20 column bed volumes, in 20 mM Heps pH 7.5, 1 mM
Immobilization levels were between 250 and 2000 resonance units, in different flowcells of a CM5 sensorchip (BIAcore, Uppsala, Sweden). Fragments II8–9 CA). PentaGlu-6d was blocked by incubation in 3% bovine serum albumin overnight at 4 °C to remove glycosaminoglycans and unmask antigens. Unspecific binding was blocked by incubation on a Sephasil C8 SC 2 1/10 column (Pharmacia), using a 0–80% acetonitrile gradient over 170 min. Selected peaks were further purified on a Sepphasil C8 SC 2 1/10 column (Pharmacia) using a 0–80% acetonitrile gradient over 170 min. Peptides were sequenced on a ABI 476A automatic protein sequencer (Applied Biosystems Inc, Foster City, CA), according to the manufacturers instructions.

Antibodies and Immunohistochemistry—The affinity purified anti-mouse fibulin-1 antiserum has been previously described (20). An anti-aggrecan monoclonal antibody 12C5 (21) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Tissue was obtained from Sprague-Dawley rats and NMRI mice after CO2 eutanasia, fixed in 95% ethanol, 1% acetic acid (22), dehydrated, embedded in low melting point paraffin, and cut into 4 μm sections. After deparaffinization in xylene and rehydration, endogenous peroxidase activity was quenched through incubation for 15 min in 1% H2O2 in methanol. For some tissues the sections were then incubated with hyaluronidase (2 mg/ml, 30 min room temperature) to remove glycosaminoglycans and unmask antigens. Unspecific binding was blocked by incubation in 3% bovine serum albumin overnight at 4 °C. Antibodies were then detected through incubation with primary antibodies diluted in 0.3% bovine serum albumin for 1 h, biotinylated secondary antibodies, and avidin/biotin-horseradish peroxidase complex using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Recombinant Fibulin-1 Proteins—The production of most of the recombinant fibulin-1 proteins has previously been described (18, 23). The fragments IIR–9 II9 III, I8–16 II7–9 III, and I6–9 III, were produced by amplifying the corresponding regions of the cDNA, nucleotides 1324, 1450, and 1570 to 2241, respectively, in the mouse fibulin-1 sequence (17). Primers were used in the polymerase chain reaction that introduced a NheI site immediately upstream, and an XhoI site immediately downstream of the fibulin-1 fragment. The resulting products were then introduced into the pCEP4 mammalian expression vector (24). The recombinant proteins all contain the vector-derived amino acid sequence APLA preceding the first amino acid residue of the fibulin-1 fragment. The recombinant proteins were produced in 293-EBNA cells, purified, and characterized as described previously (23).

Surface Plasmon Resonance Measurements—The different proteoglycan rCLDs were diluted with 10 mM acetate (pH 4.0) and immobilized in different flowcells of a CM5 sensorchip (BIAcore, Uppsala, Sweden). Immobilization levels were between 250 and 2000 resonance units, giving responses of 50–600 resonance units, as described previously (12). In the affinity measurements, recombinant proteins, at different concentrations, were injected on the rCLD-coated surfaces at 35 μl/min (in 10 mM Heps pH 7.5, 150 mM NaCl, 1 mM CaCl2, 0.005% P20, 25 °C), and the binding and dissociation was registered in a BiaCore 2000 instrument. The lectin surfaces were regenerated by injection of a 200-μl pulse of 20 mM EDTA (in the same buffer) between each experiment. In control experiments with the same concentrations of recombinant proteins, but with 5 mM EDTA instead of CaCl2 in the buffer, no binding was seen. In experiments to map the interaction, a constant concentration of 300 nm recombinant fibulin, or its fragments was used. Bacterially expressed fibronectin type III repeats 3–5 from rat tenascin-R was used as a positive control (12). Association (k_a) and dissociation (k_d) rate constants were determined using the homogenous kinetic models (A + B ↔ AB type), and AB = A + B, respectively) in the BioEvaluation 2.1 program. The equilibrium dissociation constants (K_d) were calculated from these values.

Deglycosylation of Fibulin-1D—Recombinant fibulin-1D (5 μg) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, was digested with 0.2 units of N-glycosidase F (Roche Molecular Biochemicals, Germany) at 37 °C overnight. Samples (500 ng) of digested and undigested fibulin-1D incubated in parallel were separated on a 4–16% gel. Other aliquots of the material were analyzed by MALDI-TOF mass spectrometry. The affinities for the deglycosylated and control fibulin-1 were determined using surface plasmon resonance, as described above.

RESULTS

Fibulin-1 Copurifies with Recombinant Versican and Aggrecan Lectin Domains—We observed that a 90-kDa protein copurified with select rCLD proteins in Ni-NTA-agarose chromatography from conditioned medium of transfected human embryonal kidney 293-EBNA cells (Fig. 1). This protein notably copurifies with the rCLDs of aggrecan and versican, but not with those of the brain-specific neurocan or brevican (Fig. 1). It thus appears that it could be ligand-specific for the non-neuronal proteoglycan C-type lectin domains.

The 90-kDa protein from the Ni-NTA-agarose column eluate was purified to apparent homogeneity on a MonoQ ion exchange chromatography column. After separation on a SDS-PAGE gel, the band corresponding to the protein was digested with trypsin. The released peptides were separated using reverse phase high performance chromatography and the amino acid sequences of two of the peptides were determined. A BLAST homology search revealed that both peptides were apparently derived from the extracellular matrix protein fibulin-1 (Fig. 2). The amino acid sequence of peptide D was identical to the mouse and human fibulin sequence, but, as is clear from the sequence of peptide E, the isolated fibulin is neither the human nor the mouse homologue, but rather should represent copurified bovine fibulin-1 from the fetal calf serum in the culture medium.

Fibulin-1 and Versican or Aggrecan Are Expressed in an...
Versican and Aggrecan Bind Fibulin-1

Overlapping Pattern—Immunohistochemical stainings to identify the localization of the different proteins in various tissues demonstrated that versican and fibulin-1 show overlapping deposits both in skin and aorta. In addition, aggrecan and fibulin-1 both show a generalized presence in embryonic cartilage.

In rat skin, fibulin-1 is expressed in a fibrillar pattern throughout the dermis (Fig. 3A), reminiscent of the elastic fiber network. In particular, the hair follicles are surrounded by intense staining, and a thin layer at the dermal/epidermal border is distinctly stained. Versican staining in rat skin is very similar to the fibulin-1 staining (Fig. 3B), showing the same pattern in the dermis, at the dermal/epidermal border and in the hair follicles.

In rat aorta, fibulin-1 is found throughout the vessel wall, albeit the staining is more intense in the subintimal layers of the tunica media, in the tunica adventitia, and in the outer elastic capsule (Fig. 3C). Versican staining in the rat aorta overlaps with fibulin-1 (Fig. 3D), but is restricted to the subintimal layer of the media, and to the outer elastic capsule. No versican staining was found in the adventitia.

We did not detect any fibulin-1 epitopes in adult rat cartilage. As shown in Fig. 3E, the capsule surrounding tracheal cartilage stains strongly for fibulin-1, but no staining is seen in the cartilage, although good antibody penetration is evident from the aggrecan staining (Fig. 3F). In contrast, during development there is strong fibulin-1 expression in the cartilage templates and the newly formed bone during endochondral ossification (Fig. 3G), where aggrecan expression is also prominent (Fig. 3H).

FIG. 3. Immunohistochemical staining for fibulin-1 and versican or aggregan. Adult rat skin (A and B), aorta (C and D), tracheal cartilage (E and F), and mouse fetal femur proximal growth plate (embryonic day 17.5) were paraffin embedded and cut in 4-μm thick sections. The sections were stained with antibodies against fibulin-1 (A, C, E, and G), versican (B and D), or aggrecan (F and H). Panels A and B, the arrow indicates the border between dermis (d) and epidermis (ed). Panels C and D, lumen (lu), tunica media (tm), and tunica adventitia (ta) are indicated. Panels G and H, the zone of hypertrophic chondrocytes (ht) is indicated. Scale bar = 100 μm.

FIG. 4. Surface plasmon resonance measurements of lectin domain/fibulin-1 interactions. Versican (A, C, E, and F) and neurocan (B, D, and F) rCLDs were immobilized on a CM-5 biosensor chip, and in vivo recombinant fibulin-1C (panels A and B), or -1D (panels C and D), was injected over the surface. Tenascin-R FnIII3–5 (250 nM) was used as a positive control (panels E and F). Injection of the proteins started at 115 s and ended at 235 s. The response traces between 75 and 100 s were normalized to 0.

Versican and Aggrecan rCLDs Bind All Fibulin-1 Splice Variants with High Affinities—The interaction between different recombinant fibulin-1 splice variants, expressed in 293 cells (18), was measured with surface plasmon resonance technology using a BIACore 2000. The recombinant fibulins were flowed over the sensorchip with immobilized lectican rCLDs and bound material was measured over time. All fibulin-1 splice variants examined (A, C, and D) showed strong binding to versican (Fig. 4, Table I) and aggrecan rCLDs (Table I). This binding was completely dependent on calcium ions, as expected for a C-type lectin. None of the fibulins bound to the rCLDs of the brain-specific proteoglycans neurocan and brevican (Fig. 4, Table I). That these proteins showed adequate folding was verified in control experiments where they bound a recombinant fragment of tenascin-R, previously shown to bind all the proteoglycan rCLDs (12). The affinities for different fibulin-1 splice variants were in the low nanomolar range (Table I). The Versican and Aggrecan rCLD Interaction Maps to the Calcium-binding EGF Repeats of Fibulin-1—The interaction sites for the proteoglycan rCLDs on the fibulin-1C molecule were mapped using a large number of different distal and internal deletion mutants (Fig. 5), expressed in recombinant form in 293-EBNA cells (23). As can be seen from Table II, the central domain II (fragment II) is sufficient for undiminished aggrecan/versican rCLD binding. Deletion of the central calcium-binding EGF repeats (fragment ΔII2–8) completely abol-
In this paper we demonstrate that fibulin-1 is a high affinity ligand for the C-type lectin domains of the proteoglycans versican and aggrecan. This is the first example of a ligand for such CLDs that shows specificity for certain lectican proteoglycans, since it does not bind the brain-specific proteoglycans neurocan and brevican belonging to the same proteoglycan family. We found that fibulin-1 in solution bound to and coprecipitated with recombinant versican and aggrecan CLDs in cell culture supernatants where fetal calf serum was present (Fig. 1). The affinities for versican and aggrecan CLDs were in the low nanomolar range, as measured by surface plasmon resonance technology (Table I), whereas binding to neurocan or brevican CLDs could not be detected (Fig. 4, Table I). Analogous to the interaction with tenascin-R (12), this binding seems to be directed to the protein part of fibulin-1 (Fig. 6, Table III). Indeed, there are a number of C-type lectin domains that rather than using carbohydrate will bind to the protein part of their ligands. Examples are the low affinity IgE Fc receptor (25) and the natural killer cell receptor Ly-49A (26).

Mapping of the interaction site on the fibulin-1 molecule revealed that the proteoglycan CLDs bind to one or more calcium-binding EGF-like repeat(s) in the central rod-like domain of the molecule. As seen in Fig. 5 and Table II, the aggrecan rCLD clearly binds to EGF-like modules 8–9, and, although the versican rCLD shows a somewhat different binding profile, it also binds to similar modules in the central rod-like domain II, with no apparent involvement of domains I or III. In contrast, the nidogen-1 interaction with fibulin-1 (23) shows quite different characteristics, and was completely abolished by deletion of domain III, but also dependent on the presence of EGF-like repeats 6–9. The fibulin-1 self-assembly and fibronectin-binding sites, however, require only EGF-like repeats 5 and 6, but differ in their dependence on calcium (27).

It is interesting to note that although the proteoglycan CLDs bind to fibulin-1 through EGF-like repeats, they bind to a fibronectin type III repeat in tenascin-R, a brain-specific ligand for the CLDs of all the proteoglycans in this family (12). Since tenascin-R is a dimer or trimer (28) and fibulin-1 may self-assemble (17, 29), the binding in both cases seems directed to extended rod-like stretches of multimeric extracellular matrix molecules.

The tissue colocalization of fibulin-1 with versican or aggrecan immunoreactivity indicates that these interactions could be of physiological relevance. As shown in Fig. 3, both versican and fibulin-1 give strong signals in the extracellular matrix of rat dermis, particularly in the hair follicles, but also in a layer at the epidermal/dermal border, and in a reticular pattern throughout the dermis. Similarly, in human skin, both versican

### Table I

| Protein          | K_D (nM) |
|------------------|----------|
| Aggrecan         | 32       |
| Versican         | 14       |
| Brevican NB†     | 32       |
| Neurocan NB      | 31       |

* NB, no binding.

| Module     | K_D (nM) |
|------------|----------|
| I          | 1.3      |
| II         | 34       |
| III        | 8.9      |
| II8–9      | 1.2      |
| II4–5      | 1.0      |
| II6–8      | 20       |
| II2–5      | 17       |
| II2–8      | 12       |
| II9+III    | 9        |
| I18+9+III  | 9        |
| I17+9+III  | 12       |
| I16+9+III  | 31       |

**Fig. 5.** Schematic outline of the different recombinant mouse fibulin-1 proteins used for mapping the fibulin-1-binding site for aggrecan and versican C-type lectin domains. In addition to full-length fibulin-1 splice variants (A, C, and D), different combinations of domains (fragment II, II + III, different deletion mutants lacking various EGF repeats (ΔII2–3, ΔII4–5, ΔII6–8, ΔII2–5, and ΔII4–8), and different truncated fibulins (fragment II9+III, fragment I18+9+III, fragment I17+9+III, and fragment I16+9+III) were used. The K_D values of the interactions of the different recombinant proteins with aggrecan and versican CLDs, and for comparison, nidogen-1 (23) are indicated to the right. NB, no binding.
And fibulin-1 are found in the same locations, including the elastic fibers of the dermis (30–32). Indeed, mouse and human fibulin-2 was shown to bind to fibrillin-1, a molecule associated with the surface of elastic fibers (33). Although mouse fibulin-1 did not bind fibrillin-1 (33) it is unclear whether the human or rat homologues do, or whether any of the fibulins bind fibrillin-2. Interestingly, both versican and fibulin-1 are up-regulated in damaged skin, suggesting a functional association between the molecules (20, 34, 35).

As shown in Fig. 3, versican and fibulin-1 proteins both are found in the subintimal layer of the tunica media of rat aorta, as well as in the outer elastic capsule. Similar findings have been described in human blood vessels, where versican staining was found in all layers of the aortic wall, in the tunica adventitia of muscular arteries, in all veins examined and surrounding all capillaries (except in the kidney glomeruli) (15, 36). Strong fibulin-1 immunostaining was also found in the media of virtually all human blood vessels (32). Similarly to the situation in skin, versican expression in blood vessels is strongly up-regulated by different types of damage to the vessel wall. For example, intense versican staining was found in human vessels with split internal elastic lamina (15), and in restenotic neointima in human arteries after angioplasty (37). It is as yet unclear whether fibulin-1 is also up-regulated in response to damage in blood vessels.

During heart development, both versican and fibulin-1 are strongly expressed in the endocardial cushion tissue (38–42). An interaction between the molecules is supported by immunogold electron microscopy colocalization in the endocardial cushion tissue. Indeed, treatment of sections with hyaluronidase to degrade hyaluronic acid leads to removal of both versican and fibulin whereas fibronectin staining remains unaffected (42). Interestingly, disruption of the versican gene results in early embryonic death due to failure to form the endocardial cushion and the right cardiac chamber (43, 44). This indicates that the correct formation of a hyaluronan-versican-fibulin network may be important for heart development.

Despite the lack of fibulin-1 in adult cartilage, we found strong fibulin-1 staining in the growth plates of mouse embryo femur (Fig. 3G). This overlaps with aggrecan staining (Fig. 3H). Indeed, high levels of fibulin-1 expression have been previously reported in precartilaginous mesenchymal condensations and developing cartilage (40, 45). It may be that fibulin-1 binding by the aggrecan C-type lectin domain plays a special role during the development of cartilage and bone, perhaps assisting in organizing the early matrix. However, versican may also in this context be another relevant binding partner for fibulin-1, since versican expression precedes aggrecan in the development of cartilage (46, 47).

The biological functions of the interactions between the proteoglycan C-type lectin-like domains and fibulin-1 (or tenasin-R) remains unclear. Since both of these ligands form multimeric complexes in the extracellular matrix, it is probable that the proteoglycans act to bridge or organize other matrix constituents, e.g. connecting the hyaluronan meshwork with a matrix network represented by fibulin. As already alluded to above, there are several genes in the fibulin family: fibulin-1 (16, 17), fibulin-2 (48, 49), and S1–5/fibulin-3 (50). It remains to be seen whether these can interact with the lecticans also.

### Table II

**Mapping of the binding site on fibulin-1 for the C-type lectin-like domains of aggrecan and versican by surface plasmon resonance affinity measurements.**

| Ligand (300 nM) | Aggrecan lectin domain immobilized | Versican lectin domain immobilized |
|----------------|----------------------------------|-----------------------------------|
|                | $k_{\text{ass}}$ | $k_{\text{diss}}$ | $K_D$ | $k_{\text{ass}}$ | $k_{\text{diss}}$ | $K_D$ |
| Fibulin-1D     | 169,000 | $2.75 \times 10^{-3}$ | 16.2 | 84,700 | $2.75 \times 10^{-3}$ | 32.5 |
| Fibulin-1C     | 210,000 | $1.95 \times 10^{-3}$ | 9.3  | 72,000 | $0.91 \times 10^{-3}$ | 12.6 |
| Fibulin-1A     | 165,000 | $1.57 \times 10^{-3}$ | 9.5  | 82,100 | $1.23 \times 10^{-3}$ | 15.0 |
| Fragment II + III | 239,000 | $4.71 \times 10^{-3}$ | 19.7 | 181,000 | $3.58 \times 10^{-3}$ | 39.8 |
| Fragment II    | 124,000 | $1.06 \times 10^{-3}$ | 8.5  | 64,400 | $0.76 \times 10^{-3}$ | 11.8 |
| Fragment ΔII2–3 | 181,000 | $4.44 \times 10^{-3}$ | 24.6 | 40,100 | $1.45 \times 10^{-3}$ | 36.2 |
| Fragment ΔII4–5 | 196,000 | $3.43 \times 10^{-3}$ | 17.5 | 31,700 | $1.43 \times 10^{-3}$ | 45.1 |
| Fragment ΔII6–8 | 235,000 | $4.72 \times 10^{-3}$ | 20.1 | 82,200 | $1.54 \times 10^{-3}$ | 18.7 |
| Fragment ΔII2–5 | 176,000 | $2.99 \times 10^{-3}$ | 17.0 | 31,800 | $1.33 \times 10^{-3}$ | 41.8 |
| Fragment ΔII2–8 | No binding | No binding | No binding | No binding | No binding | No binding |
| Fragment ΔII9–9 | No binding | No binding | No binding | No binding | No binding | No binding |
| Fragment ΔII8–9+III | 143,000 | $1.24 \times 10^{-3}$ | 8.7  | 93,300 | $5.75 \times 10^{-3}$ | 616.3 |
| Fragment ΔII7–9+III | 90,800 | $1.10 \times 10^{-3}$ | 12.1 | 105,000 | $1.00 \times 10^{-3}$ | 95.2 |
| Fragment ΔII6–9+III | 36,000 | $1.10 \times 10^{-3}$ | 30.6 | 77,000 | $6.98 \times 10^{-3}$ | 90.6 |

$^a$ MALDI-TOF. Calculated mass: 74975.

$^b$ BIAcore.
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