Primary Structure and Expression of Matrilin-2, the Closest Relative of Cartilage Matrix Protein within the von Willebrand Factor Type A-like Module Superfamily*

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A mouse cDNA encoding a novel member of the von Willebrand factor type A-like module superfamily was cloned. The protein precursor of 956 amino acids consists of a putative signal peptide, two von Willebrand factor type A-like domains connected by 10 epidermal growth factor-like modules, a potential oligomerization domain, and a unique segment, and it contains potential N-glycosylation sites. A sequence similarity search indicated the closest relation to the trimeric cartilage matrix protein (CMP). Since they constitute a novel protein family, we introduce the term matrilin-2 for the new protein, reserving matrilin-1 as an alternative name for CMP. A 3.9-kilobase matrilin-2 mRNA was detected in a variety of mouse organs, including calvaria, uterus, heart, and brain, as well as fibroblast and osteoblast cell lines. Expressed human and rat cDNA sequence tags indicate a high degree of interspecies conservation. A group of 120–150-kDa bands was, after reduction, recognized specifically with an antiserum against the matrilin-2-glutathione S-transferase fusion protein in media of the matrilin-2-expressing cell lines. Assuming glycosylation, this agrees well with the predicted minimum $M_r$ of the mature protein (104,300). Immunolocalization of matrilin-2 in developing skeletal elements showed reactivity in the perichondrium and the osteoblast layer of trabecular bone. CMP binds both collagen fibrils and aggrecan, and because of the similar structure and complementary expression pattern, matrilin-2 is likely to perform similar functions in the extracellular matrix assembly of other tissues.

Multidomain or mosaic proteins play an important role in the diverse functions of the extracellular matrix (ECM) in various tissues (1). Cartilage matrix protein (CMP) (2–4) is an abundant structural component of the ECM in some types of hyaline cartilage. It binds to aggrecan, the large cartilage proteoglycan (5, 6), and to collagen collagen fibrils (7), and thereby it may serve to connect the two major macromolecular networks. CMP is a homotrimERIC glycoprotein of about 50-kDa subunits (2–4), which appear as three connected ellipsoids on electron microscopy of the native mature protein (8). The primary structure of the monomer has been determined from the nucleotide sequence of chicken cDNA and genomic clones (3, 4), and it has also been confirmed in the human and mouse (9, 10).

After cleavage of the signal peptide, each subunit consists of two von Willebrand factor type A (vWFA)-like domains separated by an epidermal growth factor (EGF)-like module and followed by a COOH-terminal domain. The latter one has been shown recently to play a role in the trimer assembly via coiled coil formation (8, 11). CMP expression is restricted to particular zones in the growth plate (10, 12, 13).

CMP is one of the simplest members of the vWFA-like module superfamily, a diverse group of proteins sharing high sequence similarity over a segment, which was first identified as the repeated type A domain of von Willebrand factor and has since been found not only in plasma proteins but also in plasma membrane and ECM proteins (14). Crystal structure analysis of an integrin vWFA-like domain has revealed a classic $\alpha/\beta$ “Rossmann” fold and suggested a metal ion-dependent adhesion site, which is conserved in other vWFA-like modules and can be involved in binding protein ligands (15, 16).

Some of the major constituents of the cartilaginous matrix were found in structurally and genetically related forms in the ECM of other tissues. For example, versican, which is widely expressed in vascular and avascular connective tissue, and the brain-specific neurocan and brevican also bind hyaluronan and show structural similarity to the cartilage-specific aggrecan (reviewed in Ref. 17). Since one of our CMP-specific antisera showed immunostaining not only in cartilage but also in the perichondrium as well (12), it raised the question of whether a closely related gene product is functioning in other tissues. To test this hypothesis, we used a chicken CMP probe to isolate cross-hybridizing clones from a mouse epiphyses cDNA library. Here we report on the deduced primary structure of the cloned novel protein, which is a close relative of CMP. It is encoded by a distinct gene and differs from CMP both in structure and tissue specificity. The possible function of the novel protein is discussed.

MATERIALS AND METHODS
cDNA Library Construction and Screening—Poly(A)$^+$ RNA was prepared from the epiphyses and covering tissues of newborn BALB/c mice by affinity chromatography on oligo(dT)-cellulose (Invitrogen). The first cDNA strand was initiated by random hexamer primers and supplied with an oligo(dA) tail. The second strand was primed with an oligo(dA)-tailed XhoI linker. The double-stranded cDNA was supplied with an EcoRI adaptor, cleaved with XhoI, and inserted into the λ-ZAP II vector (Stratagene). After in vitro packaging, a $1 \times 10^6$ primary phages were amplified, and plaque lifts were hybridized to the insert of pCM6 (4) in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U69262 and U69263.

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‡ The abbreviations used are: ECM, extracellular matrix; vWFA, von Willebrand factor type A; CMP, cartilage matrix protein; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis.
were washed and replaced with Dulbecco's modified Eagle's medium
rabbits.

purified on a glutathione-Sepharose affinity adsorbent (Pharmacia Bio-

products were cloned in the
and sequenced to correct for ambiguities. Sequence alignments and phy-

Computer Group package (19). Human-expressed (w42930, f08289,

using synthetic primers or after subcloning in M13 phages. Nucleotide

ceeding clones were sequenced to correct for mutations during

Sequence Analysis—Both cDNA strands were sequenced with Seque-

variation of an mRNA of the same size both in mouse limb and the

Mouse Protein—In an attempt to clone CMP-related genes, a

RNA from L929 was amplified by Pyrococcus furiosus DNA polymerase. Then primer 2 was

isolation of agaurig acid, cleaved by SstI, and inserted into the SaII-

SmaI sites of pBluescript IISK'-. Another fraction of the first cDNA strand was supplied with a poly(A) tail using terminal deoxynucleotidyl transferase. The linker primer TLT was hybridized to the poly(A)-tailed cDNA and elongated for 40 min. The cDNA ends were amplified as

in the overlapping regions. Additional 5'-end cDNA clones were

Matrilin-2, a CMP-related Novel

Isolation of cDNA Clones for Matrilin-2, a CMP-related Novel

results

TABLE I

Table: Oligonucleotide primers used in this study

| Name               | Sequence                                             |
|--------------------|------------------------------------------------------|
| Primer 1           | 5'-TTCTGGAACACCTGAGGTCAGAAGCTCAAA-3'                 |
| Primer 2           | 5'-TTGAAATGCGCCACCAGGAA-3'                           |
| Primer 3           | 5'-AACACGGCAGCTCGATCCACCAT-3'                        |
| Primer 4           | 5'-GTTTGTGGCTGCAAGCTCTC-3'                           |
| Primer 5           | 5'-GCTGACCCATTGCGACCAT-3'                            |
| Primer 6           | 5'-CCACGCAGAGCTGACGGACTGCAAGCTCAAG-3'                |
| Linker primer TLT  | 5'-CCAGCGAGCAGGTGACAGGACTGCAAGCTCAAGCAAC-3'         |
| Linker primer L    | 5'-CGAGGAGTCAGGCTCAAGGC-3'                           |

0.9 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM EDTA, 0.1% SDS,

0.9 M NaCl, 90 mM trisodium citrate, pH 7.6, 0.1% SDS, and 0.05% sodium pyrophosphate at 53 °C.
The 5'-end of the cDNA was cloned following reverse transcription-
coupled polymerase chain reaction and rapid amplification of cDNA ends (18). Primer 1 (Table I) was annealed to poly(A)' RNA


together with a swine anti-rabbit IgG-peroxidase complex and 3-amino-

9269

924707, n52700, and a27272) and rat-expressed (r47063) sequence tags

CEL-7074, n36210, and r27272) and rat-expressed (r47063) sequence tags

and then products obtained by linker primer L and the gene-specific

were cloned in the SaII-SmaI sites of the vector. Several inde-

were cloned in two consecutive reactions, using first primer T and primer 5

and then products obtained by linker primer L and the gene-specific primer 5.

RCR12,527b) and human-expressed (h27272) and rat-expressed (r47063) sequence tags

sodium pyrophosphate at 53 °C.

0.9 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM EDTA, 0.1% SDS,

0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 100 μg/ml herring sperm DNA at 60 °C. Filters were washed with

several independent clones were sequenced to correct for mutations during

Sequence Analysis—Both cDNA strands were sequenced with Seque-

and then products obtained by linker primer L and the gene-specific primer 5.

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and then products obtained by linker primer L and the gene-specific primer 5.

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and then products obtained by linker primer L and the gene-specific primer 5.
result in a mature secreted protein with a minimum \( M_r \) of 104,300 and a more complex primary structure compared with that of CMP (Fig. 1). Thus the novel protein includes a pair of putative vWFA-like modules (vWFA1 and vWFA2) and a COOH-terminal domain, which have 47.4, 52.9, and 33.3% sequence identity, respectively, with the corresponding mouse CMP domains. Contrary to CMP, however, it carries: (i) 10 EGF repeats with an average sequence identity of 46% to the EGF module of CMP; (ii) a unique segment, which has not been identified in other proteins to date; and (iii) a group of positively charged amino acids between residues 24 and 39 preceding the first vWFA-like domain (Fig. 2A). The deduced amino acid sequence contains two NX(S/T) consensus sequences for potential N-glycosylation. One of them is located at the end of the first vWFA-like domain, whereas the other is in the unique segment. Apart from this, the unique segment contains an SG motif that matches the (E/D)\( \times \)SGXG consensus chondroitin sulfate attachment site proposed by Bourdon (25). The primary structure, including the lack of a transmembrane segment and the presence of a putative secretory signal peptide, suggests that the cDNA clones encode a CMP-related ECM protein that may also form oligomers via the potential COOH-terminal coiled-coil domain (see Fig. 3C). We therefore propose the name matrilin-2 for the novel protein and reserve the name matrilin-1 for CMP.

A database search indicated that this protein has not been identified previously. In the GenBank EST Division, however, several human-expressed sequence tags were found that had significant similarity to the coding region of mouse matrilin-2. An open reading frame of 313 amino acids (Fig. 2B) with 87% identity to the COOH-terminal region of mouse matrilin-2 was identified and confirmed by sequencing the longest cDNA clone. A short rat cDNA sequence tag encoding the putative oligomerization and unique modules of the protein was similarly found. These observations provide independent evidence that the gene is also expressed in human and rat cells.

Matrilin-2 is Related to Members of Other Protein Families—Multiple alignment of the vWFA-like domains between CMP and matrilin-2 confirmed a high degree of sequence similarity (Fig. 3A). A pair of cysteines located at both ends of the module, the five residues composing the metal ion-dependent adhesion site (16), and six hydrophobic residues reported to be highly conserved in other proteins at positions 17, 60, 100, 123, 153, and 163 (15) are also conserved in matrilin-2. The predicted secondary structures of both vWFA-like modules of matrilin-2 are in remarkably good agreement with the previously determined secondary structure of the vWFA-like domain (15, 16) (Fig. 3A). The potential N-linked oligosaccharide attachment site was found in the vWFA1 domain of matrilin-2 at a different position than in CMP.

The EGF-like modules in both CMP and matrilin-2 are of the B type, which do not contain potential Ca\(^{2+} \) binding motifs (28) and differ only by a single amino acid in length. Sequence alignment showed full conservation of each cysteine as well as a glycine and a lysine at positions 31 and 39, respectively, without insertion of gaps (Fig. 3B). Furthermore, from the 25 highly conserved residues, 20 are also present in CMP from three different species.

Although the sequence identity of the COOH-terminal domains between matrilin-2 and CMP is below the overall value (49%) for the two proteins, structural motifs characteristic of coiled-coil \( \alpha \)-helices (29) can clearly be recognized (Fig. 3C).

Within the heptad repeats, positions 1 and 4 are preferentially occupied by aliphatic moieties, and positions 5 and 7 are filled with polar residues. Alignment of this part of matrilin-2 with the trimerization modules of CMP and thrombospondin-1 indicates further conservation of residues in addition to the structural similarity. Immediately upstream of the heptad repeats, two closely spaced cysteines, which were shown to stabilize the homotrimers in thrombospondin-1 (30) and CMP (31), are also fully conserved in matrilin-2.

In mouse matrilin-2, the unique sequence of 75 residues located between the second vWFA-like module and the coiled-coil \( \alpha \)-helix contains a potential glycosaminoglycan attachment site, which is not conserved in humans and rats (Fig. 3D).

Sequence analysis of the expressed sequence tags data base has revealed variations among the human sequence tags. The stretch of 20 triplets missing from the rat sequence was also absent from one human tag. Apparently, this segment is subject to alternative splicing.

When the sequences of the vWFA1, vWFA2, EGF1, or COOH-terminal modules of matrilin-2 were used as a query in sequence similarity searches with two programs, BLASTP (20) and FASTA (32), it was found that the closest relatives of those are the corresponding modules in CMP. This suggests that the two proteins have evolved from a common ancestral gene. Computer analysis, using the Fitch-Margoliash algorithm of the Phylogeny Inference Package (22) for the construction of evolutionary trees, revealed a closer evolutionary relationship of the corresponding vWFA-like modules between matrilin-2 and CMP than between the vWFA1 and vWFA2 modules within either protein (data not shown). This indicates that the duplication of the vWFA-like modules preceded the separation of the genes for CMP and matrilin-2. Construction of the evolutionary tree for the EGF modules revealed that the first EGF repeat of matrilin-2 is more distantly related to the other repeats within the same molecule than to the EGF module of CMP (data not shown). However, the other EGF repeats of matrilin-2 have higher degrees of sequence similarity with each other, suggesting that the latter ones have started to diverge from each other after the separation of the ancestor genes of CMP and matrilin-2.

The Matrilin-2 Gene Is Expressed in a Variety of Organs and Cell Lines—Distribution of matrilin-2 mRNA in various mouse organs was studied by RNA blot hybridization. A strong band of 3.9 kilobases was detected in limbs of day 11 mouse embryos, but it was not visible in the epiphyseal cartilage samples of newborn mice (Fig. 4A). A transcript of the same size was also found in high abundance in the calvaria, uterus, and heart and in lower abundance in skeletal muscle, the brain, and skin (Fig. 4B). However, by this method the transcript was hardly or not
at all detectable in the trachea, femur, lung, spleen, and kidney. Since the mRNA for matrilin-2 was found in a wide variety of tissues, but not in cartilage, its expression pattern clearly differed from that of CMP. To test whether the broad tissue distribution is due to the expression in loose connective tissue cells present in different organs, mouse fibroblastic cell lines were also studied by Northern analysis. Cell lines L929, WEHI 164, and NIH 3T3, which originated from mouse C34/An, a BALB/c mouse fibrosarcoma, and a NIH Swiss mouse embryo, respectively, all expressed the 3.9-kilobase matrilin-2 mRNA, thus supporting this hypothesis (Fig. 4B). In addition to this, the mRNA was present in the rat osteoblast cell line, UMR-1 (Fig. 4B).

To gain information about the size and localization of the protein, two expressing cell lines, WEHI 164 and UMR-1, were selected for immunochemical studies. The antiserum raised against the matrilin-2-glutathione S-transferase fusion protein did in media from both cell lines specifically react with a group of bands migrating with a mobility of a molecular mass of 120–150 kDa after reduction (Fig. 5). These bands were not seen without prior reduction, indicating that they had been part of a high molecular mass complex, which under the conditions used did not enter the gel or transfer from the gel to the nitrocellulose (data not shown). The molecular mass of matrilin-2 monomers observed on SDS-PAGE is in approximate agreement with the predicted molecular mass, when the possibility of glycosylation is considered. Indeed, the staining of a group of bands between 120 and 150 kDa indicates a heterogeneity, which could be due to differences in glycosylation and/or alternative splicing.

The antiserum was used for immunohistochemical localization of matrilin-2 in sections of mouse tissues. In preliminary

FIG. 2. Nucleotide and deduced amino acid sequences of matrilin-2. A, complete sequence of mouse matrilin-2 precursor as determined from overlapping cDNA clones of Fig. 1. Arrohead, predicted propeptidase cleavage site. Positively charged amino acids at the amino terminus of the secreted protein are bold face. Potential N-linked glycosylation sites are underlined; a consensus motif for chondroitin sulfate attachment is double underlined. Dotted lines, putative polyadenylation signals. B, partial nucleotide and amino acid sequence of human matrilin-2.
experiments, expression was seen in the matrix adjacent to many mesenchymal but not muscle cells (data not shown), demonstrating that the expression pattern of matrilin-2 is distinct from that of CMP. Therefore, we focused our attention on the skeletal elements, in which CMP has a very characteristic distribution. In sections of tracheal cartilage the perichondrium but not the cartilage matrix proper was stained (Fig. 6, B and C). In sections of trabecular bone the osteoblast layer was positive (Fig. 6). This indicates that the expression pattern of matrilin-2 and CMP is complementary in the skeletal elements and agrees with the results from immunoblotting (Fig. 5), showing the presence of the protein in conditioned medium from fibrosarcoma cells and an osteoblastic cell line.

### DISCUSSION

This study reports on the molecular cloning and the complete coding sequence of the matrilin-2 gene from the mouse and a partial sequence from the human. Neither the gene nor the protein product has been described previously. Evidence is provided that the gene is expressed in a variety of mouse and human organs as well as mouse and rat cell lines, and it encodes a protein, which is secreted into the extracellular space. The 87% identity over a stretch of 313 amino acids between mouse and human matrilin-2 indicates that the protein is a functionally important novel component of the extracellular matrix in a broad range of mammalian tissues and organs.
Data base analyses both at nucleic acid and amino acid levels have revealed that matrilin-2 belongs to the vWFA-like superfamily. Several lines of evidence indicate that it is the closest relative of CMP: (i) the two proteins share three modules of a considerable degree of sequence similarity; although the vWFA-like domain has been shown to reshuffle with a great variety of modules in different proteins, no other members of the superfamily have been reported to contain the oligomerization module, and only the Caenorhabditis elegans ynx3 protein includes EGF modules (14–16); (ii) since the order of the related modules is also identical in CMP and matrilin-2, it is very unlikely that their genes have originated through convergent evolution; and (iii) data base searches indicate that the closest relatives of all of the three putative matrilin-2 modules are the corresponding ones of CMP; that is, the closest evolutionary relationship was found even for the least conserved putative oligomerization domains between matrilin-2 and CMP. From these data we conclude that the two proteins belong to the same protein family, which we now refer to as the matrilins.

The strikingly similar domain structures and the close evolutionary relationship supported by the construction of phylogenetic trees suggest that the genes for CMP (matrilin-1) and matrilin-2 have evolved via the duplication of a common ancestor gene encoding duplicated vWFA, single EGF, and putative oligomerization modules.

Our data show that the matrilin-2 gene is transcribed in a variety of mouse organs and cell types. Its expression level varies within a broad range, the mRNA being most abundant in the calvaria, uterus, and heart and less abundant or not detectable in others. Immunostaining revealed specific reactivity in the perichondrium and other connective tissue cells as well as osteoblasts. Preliminary in situ hybridization experiments also support a broad expression pattern. The human expressed sequence tags were derived from embryonic heart, lung, brain, senescent fibroblasts, and multiple sclerosis lesions of adult patients, which confirms expression in the con-

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**Fig. 4. Distribution of matrilin-2 mRNA in various mouse organs and cell lines.** A. Northern hybridization of 5 μg of total RNA samples from total limbs of day 11.5 mouse embryos (lane 1) and epiphyseal cartilage of newborn mice (lane 2). B. Relative abundance of matrilin-2 mRNA in mouse organs and various cell lines as determined by Northern analysis. 5 and 10 μg of total RNA were tested from adult mouse organs (lanes 1–11) and cell lines (lanes 12–15), respectively, as indicated above the lanes. Filters were hybridized consecutively with pCRII2 zDNA (MTR) and a chicken DNA fragment for rRNA (28S). Exposure times for autoradiography were 5 days with a screen for the matrilin-2 probe and 6 h without a screen (lanes 1–11) or 6 h with a screen (lanes 12–15) for the rRNA probe, respectively.

**Fig. 5. Immunoblot analysis of cell culture media.** Medium was collected from UMR-1 (lane 2) and WEHI 164 (lane 3) cells after 48 h of conditioning and applied to 4–15% SDS-PAGE gels after reduction. After transfer to nitrocellulose matrilin-2 was visualized by use of the antibody raised against the matrilin-2-glutathione S-transferase fusion protein. A heterogenous set of specifically stained bands is seen migrating between 120 and 150 kDa. A strong band at 50–70 kDa is seen also in the lane of molecular mass standards (lane 1) and represents a nonspecific titer, presumably directed to keratins. Autoantibodies to keratins are often found in rabbits, and keratins frequently contaminate the chemicals used in SDS-PAGE.

**Fig. 6. Immunolocalization of matrilin-2 in adult mouse tissues.** A, in the vertebral body (vb, upper right) matrilin-2 is detected in the osteoblast layer, which lines the bone trabeculae. The intervertebral disc (id, lower left) is devoid of staining. B, the perichondrium surrounding the tracheal cartilage is positive for matrilin-2, whereas the cartilage proper is negative. C, control section of tracheal cartilage treated with preimmune serum does not show any staining. Bar, 40 μm in A and 20 μm in B and C.
nective tissue of different organs. Although the gene activity was demonstrated in fibroblast and osteoblast cell lines, it requires further studies to identify all matrilin-2 expressing cell types in vivo clearly.

The strikingly similar domain structure and the complementary expression pattern of CMP and matrilin-2 suggest that the two proteins perform similar functions in the organization of different forms of ECM. Marked differences were noticed in the level of CMP gene expression depending on the chondrocyte differentiation stages both in vivo (10, 12, 13) and in various culture systems (33) and also depending on the forms of cartilage (34). The low level in articular cartilage and in the resting zone of the growth plate is in accordance with the low abundance of the CMP coding sequences in the RNA used for library construction in this article. Apart from cartilage, CMP expression has been reported only in the notochord and certain structures of the eye (12, 13, 35), suggesting a very specialized function. In fact, CMP was reported to bind both to aggrecan (5, 6) and type II collagen fibrils (7) as well as to form a collagen-independent filamentous network (36). Its binding to aggrecan apparently involves covalent cross-linking, which increases with age (6). If the interaction of CMP with the type II collagen fibrils and the aggrecan-hyaluronan network takes place simultaneously, it implies an important bridging function between the two major macromolecular networks. Such a role in the organization of the cartilaginous ECM may explain variations in the abundance of CMP in different forms of cartilage (34). It is not known which domain of CMP is involved in these interactions and what the molecular mechanism is. The vWF-like domains are major candidates for macromolecular interactions, since they have been shown to bind to a versatile group of ligands, including platelet glycoprotein Ib and collagen in von Willebrand factor (37), collagen, heparin, and hyaluronan in type VI collagen (14, 38), and ICAM-1, iC3b, and fibrinogen in integrins (16).

The primary structure of matrilin-2 indicates that it may play a similar role in the organization of the ECM in other tissues. The presence of a putative secretory signal peptide and the lack of a transmembrane domain in the coding region suggested an extracellular protein, which was confirmed by the immunological detection of the secreted protein in the cell culture media. Although other tissues do not have such a high abundance of ligands, including platelet glycoprotein Ib and collagen in von Willebrand factor (37), collagen, heparin, and hyaluronan in type VI collagen (14, 38), and ICAM-1, iC3b, and fibrinogen in integrins (16).

The expression pattern of matrilin-2 in mouse and the presence of the charged amino terminus may enable matrilin-2 to interact electrostatically with negatively charged polymers, e.g. hyaluronan or other glycosaminoglycans.

Its expression pattern in mouse and the presence of the matrilin-2 cDNA in sequence tags originating from various human and rat tissues indicate an important role in the ECM of mammals. If matrils perform an essential function in the organization of the ECM, then further members of this protein family with slightly different domain structures can be predicted to exist. These proteins may interact with the collagen and proteoglycan components of the specialized ECM of other tissues, such as bone, spleen, and lung.

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