Characterization of EHD4, an EH Domain-containing Protein Expressed in the Extracellular Matrix*

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To identify proteins that promote assembly of type VI collagen tetramers or stabilize type VI collagen filaments, a two-hybrid screen of a human placenta library was used and a new extracellular protein discovered. The cDNA sequence of the new protein encodes 541 amino acid residues. This cDNA sequence is identical to EHD4, a recently described member of the EH domain family of proteins. Two mRNAs of 4.4 and 3.0 kilobases were present in human skin fibroblasts and most tissues tested but were most prevalent in the heart. The chromosomal localization of the gene for this new protein was determined to be at 15q14-q15. Three polyclonal peptide antibodies were made against synthetic EHD4 peptides. The affinity-purified antibodies were used in immunofluorescent staining of developing limbs and matrices produced by human skin fibroblasts and mouse NIH3T3 fibroblasts in culture. Embryonic rat limb cartilage was strongly stained throughout development, and cultured fibroblasts deposited an extracellular filamentous network containing EHD4. In non-denaturing extracts of fetal bovine cartilage and in human skin fibroblast culture media, two components of ~220 and 158 kDa were observed, which, after reduction, migrated as a 56-kDa component on SDS-polyacrylamide gel electrophoresis. EHD4 is the first extracellular matrix protein described that contains an EH domain.

The EH domain (Eps15 Homology)1 was first defined by Wong et al. (1) when characterizing Eps15, a substrate for the epidermal growth factor receptor tyrosine kinase. Subsequently, screening a human fibroblast expression library with domains of proteins. Two mRNAs of 4.4 and 3.0 kilobases were present in human skin fibroblasts and most tissues tested but were most prevalent in the heart. The chromosomal localization of the gene for this new protein was determined to be at 15q14-q15. Three polyclonal peptide antibodies were made against synthetic EHD4 peptides. The affinity-purified antibodies were used in immunofluorescent staining of developing limbs and matrices produced by human skin fibroblasts and mouse NIH3T3 fibroblasts in culture. Embryonic rat limb cartilage was strongly stained throughout development, and cultured fibroblasts deposited an extracellular filamentous network containing EHD4. In non-denaturing extracts of fetal bovine cartilage and in human skin fibroblast culture media, two components of ~220 and 158 kDa were observed, which, after reduction, migrated as a 56-kDa component on SDS-polyacrylamide gel electrophoresis. EHD4 is the first extracellular matrix protein described that contains an EH domain.

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1 The abbreviations used are: EH, Eps15 homology; EHD1, Eps15 Homology Domain 1) was described (9). This protein was originally called Hpast (GenBank™ accession number AF001434), because it was the human analogue of a Drosophila protein called PAST (putative achaete scute target, GenBank™ accession number U70135). EHD1 is a slightly atypical family member, because it contains a single EH domain at the carboxyl-terminal end of a coiled-coil domain and is the only coiled-coil-containing member with a single EH domain. Other than a calcium binding EF hand within the EH domain, no other recognizable domains were found in this 62-kDa protein. From Northern blot analysis it was highly expressed in testis, intestine, spleen, and kidney. Immunohistochemical analysis of mouse embryos showed that EHD1 was expressed in cartilage of the ribs and spinal column at day 15.5 postcoitus. Whole mount in situ hybridization showed that EDH1 expression could be detected in limb buds and pharyngeal arches by day 9.5, in limb buds, sclerotomes, branchial arches, and occipital regions at day 10.5, but at day 17.5 there was no expression in the bones. Cellular localization experiments found EHD1 in several cytoplasmic vesicular structures, including the Golgi apparatus and endocytic vesicles. It is expressed by mesenchymal-derived cells or germ cells that are known to be induced by IGF1. Because of the structural similarities of EHD1 and Eps15, it was speculated that EHD1 is an insulin-like growth factor receptor substrate that mediates the endocytosis of the IGF1 receptor (9).

Exon-trapping experiments identified three new human genes designated EHD2, EHD3, and EHD4 because of their strong similarity to EHD1 (10). The sequence of EHD3 was subsequently corrected in an EMBL/GenBank™ submission (accession number NM014600). The levels of amino acid identity between EHD1 and EHD2, EHD1 and EHD3, and EHD1 and EHD4 are 71%, 86%, and 76%, respectively. They show distinct expression patterns on multiple-tissue Northern blot analysis. Comparison of the amino acid sequences of EHD1–4 revealed almost identical single EH domains containing a predicted calcium binding EF hand. In addition, a bipartite nuclear localization signal and an ATP/GTP binding motif were identified. Surprisingly, no mention was made of the putative coiled-coil region previously identified in EHD1.

We identified EHD4 during a search for proteins potentially involved in type VI collagen filament formation. Type VI collagen filaments are formed by end-to-end association of tetra-
mbers (11, 12). Attempts to reconstitute filaments in vitro from purified type VI collagen tetramers failed, so we proposed that other ligands might be required for type VI collagen fibrillogenesis (12). To find proteins that are potentially involved in type VI collagen filament assembly, the yeast two-hybrid system was used with selected amino- and carboxyl-terminal domains of each α(VI) chain to screen a human placenta library (13). EHD4 was found using a bait peptide composed of most of C1 and C2–C5 domains of the α3(VI) carboxyl terminus (14). Here we report the complete cDNA sequence of EHD4 and its characterization as an extracellular protein.

EXPERIMENTAL PROCEDURES

Materials—Bovine fetuses were obtained from the local slaughterhouse. A Superscript preamplification system for first-strand cDNA synthesis and the polymerase chain reaction (PCR) kits were from Life Technologies. The Micropolyp(A) Pure kit and the Northern Max-Gly kits were from Ambion. The Multiple tissue Northern blot and Protein Medleys (human heart, skeletal muscle, liver, lung, and placenta) were purchased from CLONTECH Laboratories, Inc. The QIAprep spin kit and Wizard PCR preparation kit were from Qiagen and Promega, respectively. Type II collagen monoclonal antibody I6B3 developed by T. F. Luke was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the Department of Biological Sciences (University of Iowa, Iowa City, IA). Type X (15) and type VI collagen antibodies have been described elsewhere (16). Secondary antibodies used, conjugated with fluorescein or Texas red were from (Sigma Chemical Co.) and Alexa 546 and Alexa 488 from Molecular Probes. The synthetic adjuvant Titermax was from Sigma. The Superpolyclonal antibody preparations. The peptides were linked to keyhole limpet hemocyanin via their carboxyl-terminal cysteine using the Imject acti-

C18 reverse phase support in 0.1% trifluoroacetic acid using an acetoni-
trite gradient, and their sequences and purity were checked by direct amino acid sequencing (Applied Biosystems 492) before use for antibody preparations. The peptides were linked to keyhole limpet hemocyanin via their carboxyl-terminal cysteine using the Imject acti-

RESULTS

Sequence Determination and Database Analysis—The entire assembled cDNA sequence of EHD4 and peptide translation, including the complete 3′-UTR is illustrated in Fig. 1A. Sequence analysis of clone 3C-110 revealed a short coding region of 294 bp which encodes 98 amino acid residues containing an EF hand calcium binding motif, followed by a 3′-untranslated region (Fig. 1, 1338–1339). The entire 3′-ORF sequence was obtained from the cDNA clone (Fig. 1, 1753–1772). The reading frame (sense: AAAGCCACTGGTGATGATGAGCG) and the known sequence (anti-
tense: TCTAGTCTGGCTCTCCACCCATCCCATGAGC, Fig. 1, position 1755–1772). The sequence was checked by sequencing the pro-
cut from a PCR amplification of human fibroblast cDNA using the primer set as follows: sense: ATGTTCAAGCTGATGGGCGGC, position 1–20; and antisense: CCAGGTCTGCCATCCATCCATCTAGAC, position 1755–1772 (Fig. 1).

Radiation Hybrid Mapping—Chromosomal localization of EHD4 was performed using the GeneBridge4 4 radiation hybrid panel (Research Genetics, Inc.) according to the protocol provided with the panel. The PCR primer sets used in the screening were designed from the open reading frame (sense: GGGGAGGGAGGCTAGTAAATAGTCC, position 1518–1546) and the 3′-UTR (antisense: ATGGGCGGCTGATGGGCGGCG, position 2261–2286) (17). This pair of primers generated a cDNA fragment of 767 bp. The results from screening all 93 templates in the hybrid panel were analyzed by the White Head Institute (www.genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl).

Polyclonal Antibody Preparation—Three peptides for antibody prepar-

ation, selected from the translated amino acid sequence of EHD4 were synthesized with an addition cysteine residue at the carboxyl-
terinus. The polyclonal antibodies were prepared in New Zealand White rabbits. The peptide sequences were QDLFRDIQSLQK

KAARVLKDNC (Fig. 1, 279–299 for pAb 1050), GTTEGFPNQGYGE

GAREGADEEEVVVAKDYPYDEC (Fig. 1, 418–453 for pAb 1051), and GKSISGVNKKEMVTSLKC (Fig. 1, 463–479 for pAb 1306). All the peptides were purified by high performance liquid chromatography on a
During the course of these investigations, a new group of EH domain-containing protein sequences were entered into the EMBL/GenBank™ data base that were highly similar to EHD1 and were consequently named EHD2, EHD3, and EHD4 (lacking part of the ORF and UTR) (10). EHD4 was identical to the protein described here; therefore, the name EHD4 was adopted. The completed EHD4 cDNA sequence reported here consists of an open reading frame of 1620 bp with the proposed translation-initiating methionine in the strong Kozak consensus sequence context RNNatgY (18). Chromosomal localization of the EDH4 gene on the physical map of chromosome 15 is illustrated in Fig. 2. Nearby markers WI-13757 and WI-11636 have been positioned on the radiation hybrid map enabling EDH4 to be assigned to 15q14-q15.

The domain analysis of the EHD4 protein amino acid sequence using the Simple Modular Architecture Research Tool (SMART; smart.embl-heidelberg.de) revealed a unique amino-terminal domain, a potential coiled-coil domain, and an EH domain with an embedded EF hand calcium binding motif (Fig. 1B). EHD4 contains four cysteine residues, two in the amino-terminal region and two in the calcium binding EF hand motif. Compared with the other EHD proteins, EDH4 has a low score for coiled-coil domain formation using the programs COILS (www.ch.embnet.org/software/COILS_form.html) and Multi-coil (nightingale.lcs.mit.edu/cgi-bin/multicoil). There is a predicted ATP/GTP binding site at the amino terminus and a possible bipartite nuclear recognition site in the center of a coiled-coil domain (PSORT program analysis psort.nibb.ac.jp). No signal peptide at the amino terminus was detected (SignalP program, www.cbs.dtu.dk/services/SignalP).

To determine the tissue distribution of the gene, a multiple tissue Northern blot and human skin fibroblast mRNA were probed using the cDNA insert of clone 3C-110. Two messages of 4.4 and 3.0 kb were detected, the smaller of the two being more abundant in all tested tissues and fibroblasts (Fig. 3). Heart had the highest message level followed by pancreas, kidney, placenta, and lung. Fibroblasts also expressed high levels of message.

**Immunolocalization of EHD4**—To identify and characterize EDH4 protein in tissues, three polyclonal peptide antibodies were made against peptides selected from the coiled-coil domain of EHD4.
The brain and the skeletal muscle. Hybridization with a probe for the two messages of 4.4 and 3.0 kb were visible in all of the tissue except skin fibroblast mRNA. The blots were probed using the entire insert of 3C-110 and developed after 72-h exposure at 70 °C. Two bands of 4.4 and 3.0 kb were visible in all of the tissue except the brain and the skeletal muscle. Hybridization with a probe for the \( \beta \)-actin gene indicated approximately equal mRNA loading for all lanes.

In 18-day-old rat embryo limb sections, EHD4 is confined to the cartilage matrix and co-distributed with type II collagen in the extracellular matrix except at the articular surface. Fig. 4 (A and D) shows the similar distributions of EHD4 and type II, respectively, in the elbow. Fig. 4B shows an enlargement of the ulna/humerus stained with EHD4, and Fig. 4C shows the same view stained with EHD4 antibodies preincubated with the peptide used to make the antibody. This negative control demonstrates the specificity of the observed staining. At the articular surface, there is a narrow zone that contains type II collagen, but EHD4 is absent. Enlargements of the ulna/humerus interface are shown in Fig. 4, E and F. In Fig. 4E, EHD4 is stained green and the cell nuclei red, and at the surface of the cartilage there is a region delineated by the presence of cell nuclei that does not contain EHD4. However, this same region is stained for type II collagen (Fig. 4F). Earlier in development at day 14, when EHD4 first appears, EHD4 and type II collagen do not co-distribute. In the clavicle, type II staining delineates the whole bone (Fig. 4H), whereas EHD4 is restricted more to the mid-diaphysis where chondrocytes differentiation is more advanced and where primary endochondral ossification will ultimately initiate (Fig. 4G). It appears while type II collagen is produced early in the differentiation of the condensing mesenchyme to chondroblasts, and EHD4 is produced slightly later in development by more differentiated chondrocytes. The next change in distribution observed occurs just prior to the formation of the secondary centers of ossification. Fig. 4I shows a bone epiphysis 5 days postpartum in which EHD4 labeling is diminished in the center but type II collagen staining is still uniform (Fig. 4L). Type X collagen, which is a marker for hypertrophic chondrocytes and endochondral ossification, is not expressed in this region (Fig. 4J), only in the metaphyseal growth plates. At this stage type VI collagen is concentrated at the articular surface of the epiphysial cartilage (Fig. 4K). Two days later, type X collagen appears in the secondary center of ossification (Fig. 4N). A large area devoid of EHD4 (Fig. 4M) still labels for type II collagen (Fig. 4P). It has a course appearance indicating a physical change in the structure of the cartilage matrix caused by the hypertrophy of chondrocytes and degradation of cartilage prior to calcification. Type VI collagen remains concentrated at the epiphysial surface (Fig. 4O) while EHD4 continues to be excluded from the developing articular cartilage.

Characterization of EHD4 Protein.—Because the Northern blot analysis indicated that EHD4 was expressed by fibroblasts, cultures of normal human skin fibroblasts and mouse NIH3T3 fibroblasts were grown to confluence and immunofluorescently labeled to better determine whether EHD4 is an extracellular or an intracellular protein. The proteins in the fibroblast culture medium and cell/matrix extracts were analyzed by Western blot analysis, and the distribution of EHD4 was examined by indirect immunofluorescent staining of fibroblasts grown on chamber slides. The results demonstrated that fibroblasts secreted EHD4 into the media (Fig. 5A). Western blot analysis with an affinity-purified antibody showed disulfide-linked EHD4 oligomers of 158 and 220 kDa were present in unreduced media and a monomer of 56 kDa after reduction. From the apparent molecular masses it would appear that the oligomers are trimers and tetramers, but, owing to the anomalous behavior of these proteins in SDS-PAGE, this is not certain. Two-dimensional gel electrophoresis revealed that both high molecular mass bands are reduced to a 56-kDa band recognized by an EHD4 antibody (data not shown). Therefore, it was concluded that both components are oligomers of EHD4. Furthermore, both mouse and human fibroblast cell cultures deposited an extracellular fibrillar network containing EHD4 (Fig. 5B).

To chemically confirm the presence of EHD4 in cartilage, fetal bovine femoral cartilage was extracted with a series of buffers with increasing matrix-solubilizing properties. It was found that most EHD4 was extracted in 20 mM Tris-HCl, pH 7.5, containing 1 M NaCl and 20 mM EDTA (Fig. 6). The bands recognized by EHD4 antibodies in low salt and high salt-containing Tris buffer without EDTA were thought to be degradation products of EHD4, because they varied in pattern and intensity from one preparation to the next (Fig. 6D). EHD4 protein was purified from the EDTA extract by separation on DEAE-cellulose. Bound material was further chromatographed over a Sephacryl S-400 column (Fig. 7). The apparent sizes of EHD4 protein isolated from culture media and cartilage are identical.

Interactions with Collagens.—The affinity of EHD4 for type VI collagen was confirmed by affinity blots of fetal bovine cartilage extract with biotinylated type VI collagen tetramers (Fig. 8). The results showed that the type VI collagen tetramers and EHD4 polyclonal antibody (pAb 1050) recognized the same 158- and 220-kDa components in the gel. Further binding studies (not shown) using EHD4 immobilized on enzyme-linked immunosorbent assay plates and fragments of type VI collagen demonstrated that the binding site was localized in the globular domain of type VI collagen and that the native domain is capable of binding EHD4. Similar assays showed that collagens I, II, III, and V had no affinity for EHD4. Thus, type VI collagen and EHD4 have an affinity for each other in vitro, which confirms the interaction detected in the two-hybrid screen.

As type VI collagen filaments formed in cultures of WI38, HT1080, and MG63 cells in the absence of EHD4 (not shown), EHD4 is not required for type VI collagen formation. Furthermore, the distributions of EHD4 and type VI collagen in cartilage are quite distinct, type VI collagen being concentrated at the periphery of epiphysial cartilage where EHD4 is at its

![Fig. 3. Multiple tissue Northern blot analysis of indicated tissues and skin fibroblast mRNA](image-url)
FIG. 4. Indirect immunofluorescence staining of EHD4 in developing rat limbs. Frozen sections of rat forelimbs were obtained from animals at stage E14 (G, H, double-labeled section), E18 (E, F), postnatal day 5 (I–L), and postnatal day 7 (M–P). Limb sections were labeled with antibodies to EHD4 (pAb 1050) (A–C, E, G, I, M), collagen type II (D, F, H, L, P), collagen type X (J, N), and collagen type VI (K, O). The EHD4 antibody, pAb 1050, in panel C was preincubated with the peptide antigen. Preincubation of pAb 1050 with either of the peptides used to generate two other EHD4 antibodies had no effect (not shown). Magnifications: A, D, I–P (40×); B, C, G, H (100×); and E, F (400×).

weakest or absent. However, there is a low concentration of type VI collagen in the inter-territorial cartilage matrix (19) where EHD4 is also found, and so an interaction is possible there.

DISCUSSION

EHD4 is a unique extracellular matrix protein in that it contains an EH domain with an embedded calcium binding EF hand. These are characteristics of a family of EH domain-containing proteins related to Eps15 that were previously thought to be exclusively intracellular. The four EHD proteins are a subset of this family with high sequence similarity. EHD4 was previously assumed to be an intracellular protein because of its structural similarity to EHD1. However, EHD4 has some unique characteristics that make this comparison unreliable. EHD1–3 have a predicted coiled-coil region, presumably used to assemble multimers in a similar manner to Eps15 (20). EHD4 has a very low score for a predicted coiled-coil region but, as we have shown, does assemble into disulfide-bonded multimers in tissues. Because the cysteine residues are not in, or immediately adjacent to, the predicted coiled-coil domain the interaction surfaces for association must involve other regions of the molecule. In addition, PSORT analyses reveal two conserved nuclear targeting sequences in the amino and carboxyl termini of EHD1–3, which are not present in EHD4.

All EHD proteins contain one conserved cysteine residue in the amino-terminal region of the molecules corresponding to C141 of EHD4 (Fig. 1B). EHD2 has a second cysteine located in the coiled-coil domain. EHD4 contains four cysteine residues, two in the calcium binding EF hand, which is rare in this motif (21), and two in the amino-terminal region (Fig. 1B). The third EH domain of Eps15 also contains an EF hand with two cysteine residues, one replacing a Ca2+-ligating Asp residue, which consequently does not bind calcium (22). The location of the two cysteine residues in the EF hand of EHD4, adjacent to the Asp residues involved in the ligation of calcium, is unique. Because they are in the sequence ’Asp-Cys-Asp-Cys-Asp’, they cannot form an intramolecular disulfide cross-link. They are probably involved in the intermolecular disulfide bond formation of polymers by interacting with adjacent EH domains. The calcium binding properties of this domain are unknown, but extraction of EHD4 from cartilage under non-denaturing conditions requires EDTA, indicating a metal ion-dependent interaction with the matrix.

Other notable structural features of the EHD proteins are that they contain a predicted bipartite nuclear localization signal, an ATP/GTP binding domain, and no signal sequence, which are characteristics of intracellular proteins (10). Although unusual, there are several secreted proteins without signal peptides, including fibroblast growth factor (23), interleukin-1 (24), galectins (25), thirodoxin (26), and annexin I (27). ATP/GTP binding domains are also present in extracellular bone and dentin proteins (28) and tubulointerstitial nephritis antigen (22), so the presence of these sequence motifs cannot be used to infer intracellular localization.

The genes for human EHD1–4 were localized to chromosomes 11q13, 19q13.3, 2q21, and 15q11.1, respectively (10). Here we report the localization of EHD4 to be 15q14-15. This discrepancy may result from using different radiation hybrid panels in the assays. However, the recent deposition of chromosome 15q15 genomic sequences into the data bank, which includes the entire sequence of EHD4, supports the latter localization (GenBank™ accession number AC039056).

Because of the high similarity of the EHD1–4 protein sequences, distribution data reported for EHD1 probably represents the sum of the distributions of EHD1–4, because the antigen used to make the antibody was full-length recombinant EHD1 protein. The regions used as epitopes to make the polyclonal antibodies described here are also similar in EHD1–4. Although the EHD2 and EHD3 proteins have not been characterized, their message levels were high in skeletal muscle and brain (10). The fact that the antibodies against EHD4 are negative on both of these tissues suggests that there is no antibody cross-reactivity with EHD2 and EHD3. In addition, recombinant EHD4 expressed in a cell-free rabbit reticulocyte system was recognized in Western blots by the three EHD4 antibodies, whereas EHD3 was not (not shown). It was also
reported from in situ hybridization results that EHD1 expression in developing mouse limbs peaked at day 15.5 in cartilage, preceding hypertrophy and ossification. However, EHD4 was found in the cartilage matrix of developing mouse limbs from day 14 up to postnatal day 7 indicating the antibodies were not recognizing EHD1. Furthermore, because EHD1 contains only one cysteine, it can only be monomeric or form covalently linked dimers. Neither of these was observed in Western blots of the EDTA extracts of bovine cartilage.

The EH domain is a protein-protein interaction motif and binds primarily to Asn-Pro-Phe (type I) sequences in target proteins (3, 8, 29). Other target sites, including FW, WW, SWG (type II), and H(S/T)F (type III) (30). The 3C-110 clone found in the two-hybrid screen contains the entire EH domain sequence lacking only the first two amino acid residues and should therefore recognize one of these sites. A search for the possible binding site on type VI collagen revealed only one FW type II target site, located in the Kunitz type inhibitor C5 domain of /H9251

FIG. 5. EHD4 protein in fibroblast cultures. A, Western blot of human fibroblast media proteins using EHD4 pAb 1306. The fibroblast media was collected from confluent cells, precipitated with 50% ammonium sulfate, dialyzed against 20 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl and analyzed on 7.5% SDS-PAGE gels. The mobilities of Bio-Rad prestained SDS-PAGE standards (205, 116, 82, and 47 kDa) are indicated on the left. The sizes of bands recognized by EHD4 antibody are also indicated. Two bands with molecular masses of 220 and 158 kDa appeared before reduction (lane 1) and a 58-kDa band after reduction (lane 2). B, indirect immunofluorescence staining of mouse NIH3T3 cell culture matrix. The NIH3T3 cells, 103 cell/chamber, were grown in the Dulbecco’s modified Eagle's medium media with 50 μg/liter ascorbate and stained with pAb 1050 when 3 days postconfluent. The EHD4 antibody recognizes an extracellular filamentous network.

FIG. 6. Western blot analysis of cartilage extracts. A stained gel (upper) and Western blot (lower) of sequential extracts from bovine fetal cartilage. An unreduced 10% PAGE gel was used for regular Coomassie Blue staining (upper gel), and an unreduced 6% PAGE was used for a Western blot using pAb 1051 (lower gel). The size and the positions of the EHD4 protein bands are indicated. The positions of the BENCHMARK standards protein ladder (182, 115, 84, 62, 51, and 38 kDa) are indicated on the left side of the gel. The extraction was done sequentially in 20 mM Tris-HCl, pH 7.5, buffer containing protease inhibitors and 0.5 mM NaCl (lane 1), 1.0 mM NaCl (lane 2), 1.0 NaCl and 20 mM EDTA (lane 3), 1.0 NaCl and 20 mM EDTA and 8 M urea (lane 4), and boiling 1% SDS (lane 5). A major portion of intact EHD4 was extracted with the addition of EDTA.
the lane 3 was applied to a DEAE anion-exchange column, and the bound fraction was collected. The chromatographic profile shown is from the separation of the EHD4 containing DEAE fraction on a Sephacryl S-400 molecular sieving column (2.6 × 110 cm) equilibrated with 40 mM Tris-HCl, pH 6.8, containing 2 mM urea and 0.2 mM sodium sulfate. The inset shows the Western blot (pAb 1306) on the left, and the Coomassie Blue-stained 7.5% polyacrylamide gel is on the right. The numbers of the lanes on the gel correspond to the numbered fractions on the profile. All samples were unreduced.

Because cartilage apparently contains EHD1 and EHD4, it also gives rise to the possible existence of heteropolymers, although this would severely limit the extent of disulfide bond formation because EHD1 contains only one cysteine.

The distribution of EHD4 is complex. We have demonstrated that fibroblasts in culture elaborate extracellular matrix filaments containing EHD4. It is, however, not known whether these filaments represent EHD4 deposited on pre-existing fibrils formed by other proteins such as fibronectin or collagens or whether EHD4 can independently assemble into filaments. In developing cartilage, EHD4 is expressed late in the differentiation of chondrocytes and is excluded from the developing articular cartilage similar to cartilage matrix protein (32, 33). EHD4 is present in the hypertrophic cartilage of the growth plate, but not of the secondary ossification center, similar to type XII collagen (32). The disappearance of EHD4 from the epiphyseal cartilage prior to the appearance of hypertrophic chondrocytes is evidence of extensive remodeling of the epiphyseal cartilage matrix prior to the onset of overt secondary ossification and has been also observed in the distribution of the exon 8-containing α1 chains of type XI collagen (34).

The extracellular localization of EHD4 in cartilage and in fibroblast culture suggests a function for this protein that is distinct from other members of the EH domain-containing family, all of which are intracellular proteins. Within the EHD group, the localization of EHD1 suggests that it too is intracellular, although the data presented did not rule out an extracellular location in some tissues such as cartilage. The localization of EHD2 and EHD3 have yet to be determined. We propose that EHD4 is a tightly bound component of the ECM and that the binding is calcium-dependent, similar to thrombospondin 4 (35) and mediated by the calcium-binding EH domain. This suggests the possibility of a structural role for EHD4, either through self-assembly or heterologous interactions, although other functions, including growth factor modulation, cannot be excluded at this time.

A New Extracellular Matrix Protein

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