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Identification of a Mammalian Angiopoietin-Related Protein Expressed Specifically in Liver

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Based on searches of EST databases for signal sequences and amphipathic helices, we have identified and cloned an angiopoietin-like gene, ANGPTL3. Multiple tissue Northern blots show that ANGPTL3 is expressed principally in the liver. ANGPTL3 is expressed early during liver development, and expression is maintained in adult liver. Human ANGPTL3 is a 460-amino-acid polypeptide with the characteristic structure of angiopoietins: a signal peptide, an extended helical domain predicted to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen homology domain (FHD). Murine ANGPTL3 is a 455—acid polypeptide encoded by seven exons on mouse chromosome 4, spanning about 11 kb of DNA. ANGPTL3 contains the four conserved cysteines implicated in the intramolecular disulfide bonds within the FHD, but it does not contain two other cysteines that are found within the FHD of angiopoietins 1, 2, and 4. ANGPTL3 also does not contain the characteristic calcium binding motif found in the other angiopoietins. By radiation hybrid mapping and the use of surrounding genes, human ANGPTL3 maps to the 1p31 region.

INTRODUCTION

The angiopoietins are a new family of growth factors specific for vascular endothelium. They bind to the Tie2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptor and include the agonists ANGPT1 (Davis *et al.*, 1996) and ANGPT4 (Valenzuela *et al.*, 1999) and the antagonist ANGPT2 (Maisonpierre *et al.*, 1997). The domain structure characteristic of the angiopoietin family comprises a signal peptide, an extended helical domain predicted to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen homology domain (FHD). The principle sequence homology in this family of proteins is within the FHD, which is also thought to be the receptor binding domain (Valenzuela *et al.*, 1999). As with other FHD-containing proteins, the FHD of the angiopioetins is a carboxyl-terminal domain. While FHDs are found in many other proteins, the combination of a coiled-coil and a FHD domain is a signature of the angiopoietin family. In the angiopoietins, the interactions leading to oligomer formation have been ascribed to the amino-terminal coiled-coil domains. Both ANGPT1 and ANGPT2 have been shown to form dimeric and oligomeric structures (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). *Scabrous,* a related protein from *Drosophila,* also forms secreted, disulfide-linked dimers (Lee *et al.*, 1996). The protein–protein interactions ascribed to the coiled-coil domain are important to the function of the molecule since the angiopoietins appear to require oligomerization for function. The oligomeric nature of the functional protein is also a common feature of many of the proteins that contain a FHD.

Both the Tie1 receptor (whose ligand is unknown) and the Tie2 receptor are found on endothelial cells, and targeted disruption of the Tie2 gene in mice leads to defects in angiogenesis and embryonic lethality (Suri *et al.*, 1996). Both ANGPT1 and ANGPT2 have significant biological function with regard to angiogenesis (hence their designations as angiopoietins). The biology of ANGPT1 and ANGPT2 has been elucidated in some detail. ANGPT1 acts through the Tie2 receptor and regulates later stages of vascular development, stabilization, and maturation (Hanahan, 1997). ANGPT2 is up-regulated at sites of angiogenesis and vascular regression (Goede *et al.*, 1998), acts as an antagonist of ANGPT1, and destabilizes vascular networks. Both the targeted disruption of the ANGPT1 gene and the overexpression of ANGPT2 lead to phenotypes similar to that observed when Tie2 is disrupted. ANGPT4

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exhibits a high level of expression in lung tissue and also binds the Tie2 receptor.

Recently there have been reports of other genes that carry the signature coiled-coil and FHD structure of the angiopoietin family: CDT6 (Peek *et al.*, 1998), ARP-1 (Kim *et al.*, 1999a), and ARP-2 (Kim *et al.*, 1999b). These proteins do not bind to the Tie2 receptor (Valenzuela *et al.*, 1999; Kim *et al.*, 1999b). In this paper we report on the identification of another angiopoietin-related protein, ANGPTL3,[2] that is expressed specifically in the liver.

MATERIALS AND METHODS

Computational biology. Assembled expressed sequence tag (EST) databases were searched for translated sequences matching a pair of weight matrices. A weight matrix of dimension $n \times 20$ provides the score of each amino acid to each position of a polypeptide segment of length n. The two weight matrices used, both of length 15, were the von Heijne signal sequence matrix (von Heijne, 1986) and a matrix tailored to recognize amphipathic helices (data not shown). The potential of a protein segment to form a coiled-coil (Lupas *et al.*, 1991) was determined using the Paircoil program (Berger *et al.*, 1995). Preference of a coiled-coil segment for dimeric versus trimeric oligomerization was determined using the Multicoil program (Wolf *et al.*, 1997). Multiple alignment of the fibrinogen homology domains was performed using the Clustal algorithm (Thompson *et al.*, 1994). Three-dimensional structure modeling was performed using Biosym Insight II software (Biosym/MSI, San Diego, CA).

Northern blot analysis. Analysis of tissue distribution was performed by the Northern blotting technique using Human Multiple Tissue blots (MTNI, MTNII, and MTNIII) and Master Dot blots (Clontech, Palo Alto, CA). A probe was obtained by restriction digestion of the EST clone EST135867 with *Eco*RI and *Xho*I to remove the insert from the vector. Northern analysis was also performed on a Mouse Multiple Tissue Northern blot and Mouse Embryo blot (Clontech). A probe was obtained from a commercially available mouse cDNA clone EST917237. Labeling, hybridization, and detection protocols were as described previously.

Isolation of murine ANGPTL3 genomic clones and sequencing. A genomic λ KOS phage library from a murine Lex-1 129/SvEv substrain was screened as described by Wattler *et al.* (1999). In brief, 12 of 94 library subpools containing the ANGPTL3 locus were identified by a PCR-based screening using primer ZG-1 (5′-AAT TGA GAC AAA AAA TGC ACA C-3′) and primer ZG-6 (5′-GTT GAG CTT CTG AAA TAT GTC G-3′) contained in the first exon of the ANGPTL3 gene. The amplified product of 242 bp was purified by S300 and G50 spin column centrifugation as described by Nehls and Boehm (1993) and verified with dye terminator cycle sequencing using AmpliTaq FS DNA polymerase (Perkin–Elmer/ABI, Foster City, CA) with ZG-1 and ZG-6 as primers. Four library subpools were plated and hybridized using the ZG-1/6 PCR product. All four independent λ KOS phages were isolated, converted into pKOS plasmid clones as described by Nehls *et al.* (1994), and directly sequenced by primer walking.

Chromosomal mapping. Human ANGPTL3 was mapped using the commercially available GeneBridge 4 and Stanford G3 Radiation Hybrid (RH) panels (Research Genetics, Inc., Huntsville, AL). Publicly available WWW servers (http://carbon.wi.mit.edu:8000/cgi-bin/contig/rhmapper.pl and http://shgc-www.stanford.edu/RH/rhserverformnew.html) allowed chromosomal localization in relation to the respective chromosomal framework markers to be performed.

Murine ANGPTL3 was mapped on the commercially available mouse T31 whole genome radiation hybrid panel (Research Genetics, Inc.). Linkage analysis was carried out using the Map Manager QT linkage analysis program together with mapping panel data sets obtained from The Jackson Laboratory (http://lena.jax.org/resources/documents/cmdata).

RESULTS

Identification and Characterization of ANGPTL3

Based on searches of assembled EST databases as described under Materials and Methods, we identified an assembly containing the human ESTs EST135867 and EST319951. This assembly contained an open reading frame with the predicted signal peptide MFTIKLLLFIVPLVISSR followed by a peptide, IDQDNSSFDSLSPEPKS, and the predicted amphipathic helical peptide RFAMLDDVKILANGL. A full-length cDNA for ANGPTL3 was obtained from a plasmid containing an insert corresponding to EST319951 from a fetal liver/spleen library. The insert was sequenced and found to comprise 1496 bases with an open reading frame of 1383 bases. We named this gene ANGPTL3 (GenBank Accession No. AF152562).

The full-length human ANGPTL3 cDNA codes for a polypeptide of 460 amino acids having the angiopoietin signature: a signal peptide, an extended helical domain, a short linker peptide, and a globular fibrinogen homology domain (FHD) (Fig. 1).

Subsequently we identified the mouse EST EST744974 from a kidney library, by database searching using the human ANGPTL3 open reading frame. This EST had 87% identity to the human ANGPTL3 polypeptide and contained the analogous predicted translation initiation codon. We also identified mouse EST917237 with 75% identity to the human ANGPTL3 polypeptide. The inserts for both ESTs were sequenced and determined to represent the same gene. Interestingly, EST744974 was missing a segment of 64 amino acids, from Asn232 through Phe295. Neither the N- nor the C-terminus of this deleted segment is on an intron junction (see below), and alternative intron donor and acceptor sites could not be located near these residues. Furthermore, EST917237 did not contain this deletion. A virtual full-length mouse ANGPTL3 was created by splicing the sequences from EST744974, before the 64-amino-acid deletion, and EST917237. This revealed a segment with an overall 80% identity to the human ANGPTL3 cDNA, encoding a full-length polypeptide of 455 amino acids with an overall 76% identity to the human ANGPTL3 polypeptide (Fig. 1) (GenBank Accession No. AF162224). Subsequently we identified about 10 different mouse ESTs, from liver and kidney libraries, that extend the 5′ UTR of our transcript. The mouse EST EST2080932 provides the longest extension, with the 33 bases 5′-CAG GAG GGA GAA GTT CCA AAT TGC TTA AAA TTG-3′. This extended sequence exactly matches the murine ANGPTL3 gene sequence and likely represents the extreme 5′ end of the murine ANGPTL3 transcript.

[2] The human gene symbol ANGPTL3 has been approved by the HUGO Nomenclature Committee.

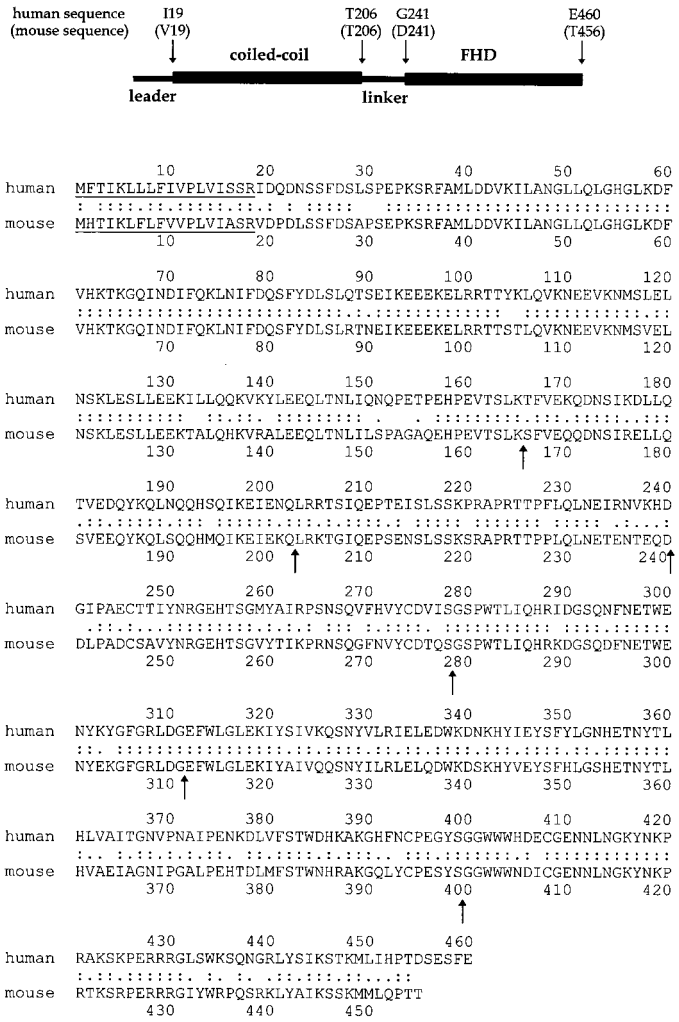

FIG. 1. (**Top**) Domain structure of ANGPTL3, with human and mouse protein numberings indicated. (**Bottom**) Pairwise alignment between human and murine ANGPTL3. Conserved residues are indicated by colons; conservative substitutions are indicated by periods. The approximate locations of intron donor sites in murine ANGPTL3 are indicated by arrows. The predicted secretory signal peptide is underlined.

The boundary between the linker peptide and the FHD was determined by visual inspection of the crystal structure of the fibrinogen γ chain portion of fibrinogen fragment D (Spraggon *et al.*, 1997; Yee *et al.*, 1997). We place the N-terminus of the FHD in that structure roughly at Ile174 (Ile148 in the crystal structure numbering). The corresponding residue in ANGPTL3, as determined by a pairwise alignment, is Gly241. We place the linker peptide between Thr206 (directly following a basic dipeptide) and Asp240. The choice of Thr206 was guided by the predicted coiled-coil potential of the N-terminal polypeptide, which drops off sharply around Thr206.

The FHD boundaries for other angiopoietins were determined by alignment with the FHD from ANGPTL3. Figure 2 shows a multiple alignment of the FHDs from several related genes: ANGPT1 (Davis *et al.*, 1996), ANGPT2 (Maisonpierre *et al.*, 1997), ARP-1 (Kim *et al.*, 1999a), ARP-2 (Kim *et al.*, 1999b), ANGPT4 (Valenzuela *et al.*, 1999), CDT6 (Peek *et al.*, 1998), and FIBG (fibrinogen γ: Lottspeich and Henschen, 1977). As determined from an intrafamily comparison, the FHD segments have approximately equal percentage identities, ranging from about 36 to 40%. The calcium binding site for fibrinogen γ is underlined in Fig. 2. It is apparent from the multiple alignment that ANGPTL3, unlike the other family members, does not contain the motif of acidic residues determining a calcium binding site. All sequences have a common motif of four cysteines. ANGPT1, ANGPT2, and ANGPT4 have, in addition, two extra cysteines in the area of the calcium binding site (Valenzuela *et al.,* 1999). According to the fibrinogen γ chain structure, these two cysteines reside on the turn of an exposed loop. Whether these are involved in disulfide linking of angiopoietin monomers is not known. Absent from all angiopoietins, but present in the fibrinogen γ chain, is a short C-terminal segment that contains the Factor XIII cross-linking sites.

The N-terminal coiled-coil domains of all angiopoietins have some similarity to myosins, prototypical coiled-coil forming proteins. The N-terminal domain of ANGPTL3 is predicted to contain amphipathic helices

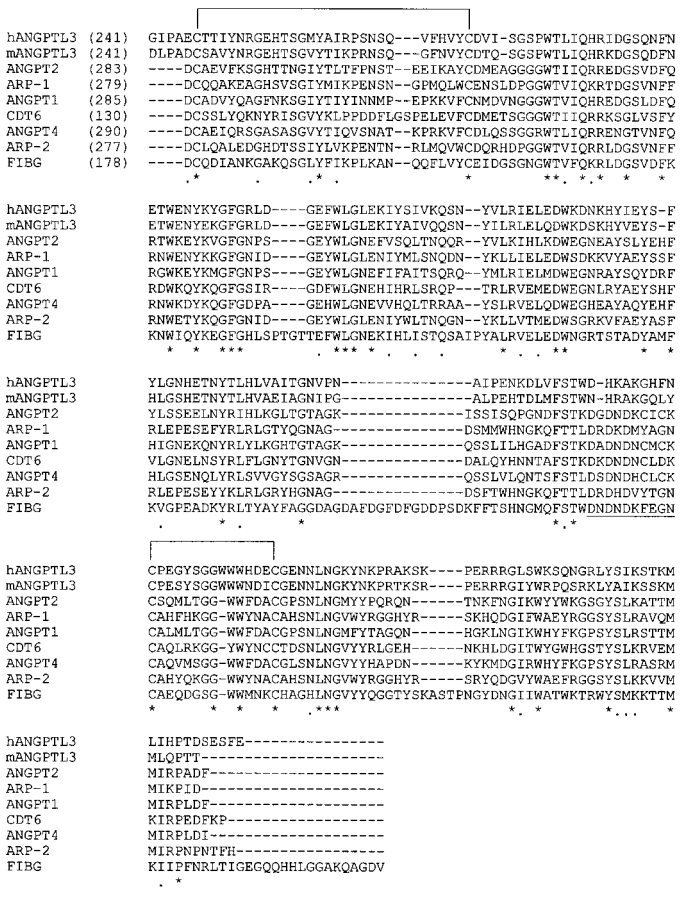

FIG. 2. A multiple alignment of the fibrinogen homology domains for fibrinogen γ and the angiopoietins. Conserved residues are indicated by asterisks, while conservative substitutions are indicated by dots. Two disulfide bonds, as inferred by analogy with fibrinogen γ, are indicated. The area of the calcium binding site in fibrinogen gamma is underlined.

TABLE 1

Intron Donor and Acceptor Sites in Murine ANGPTL3

Intron No.	Donor site		Intron length (bp)	Acceptor site	
	Exon	Intron		Intron	Exon
1	ATCACTCAAA	gtaagtagaa	585	ctgtgttcag	AGTTTTGTAG
2	AGAAAAGCAG	gtaagtagcc	1054	cttttatcag	CTCAGAAAGA
3	GAACAAGATG	gtaagataat	1077	tcaattctag	ACCTTCCTGC
4	ACCCAATCAG	gtaaacccat	269	cactctccag	GCAGTCCATG
5	AGGCTCGATG	gtaaaatgat	2445	ttttctttag	GAGAATTTTG
6	AGTTACTCAG	gtatctcatt	195	tctttttag	GTGGCTGGTG

Note. Ten nucleotides flanking each site are shown. The total length of each intron is indicated.

necessary for coiled-coil formation, with predicted trimer forming potential. Though the N-terminal domains of ANGPT1, ANGPT2, and ANGPT4 have substantial similarity to one another, at 40 to 60% identity, the N-terminal domains of ANGPTL3, ARP-1, ARP-2, CDT6, and FIBG have a lower level of similarity, around 20 to 25% identity, to ANGPT1, ANGPT2, or ANGPT4. The extended helical domain of ANGPTL3 was predicted to contain two N-linked glycosylation sites, at Asn23 and Asn115. We have confirmed that Asn115 is glycosylated, while Asn23 is not (data not shown). The glycosylated state of Asn115 is perfectly consistent with the coiled-coil predictions, as Asn115 is predicted to lie in the "f" or solvent-exposed location of the helical heptad coiled-coil motif (Lupas *et al.,* 1991).

Gene Structure of Murine ANGPTL3

Using the isolation and sequencing procedure outlined under Materials and Methods, a contiguous genomic segment of 11287 bp containing the murine ANGPTL3 locus was produced. Inspection of this sequence and comparison with the ANGPTL3 cDNA revealed that ANGPTL3 is encoded by seven exons. Table 1 lists the intron donor and acceptor sites for these exons. The approximate locations of the intron junctions on the murine ANGPTL3 polypeptide are indicated in Fig. 1. Interestingly, the predicted boundary between the linker peptide and the FHD lies at an intron junction, and the predicted N-terminus of the linker peptide also lies close to an intron junction. This indicates that the linker peptide resides predominately on its own exon. The N-terminal coiled-coil domain of ANPGT5 is encoded by two exons, while the FHD is encoded by four exons. Inspection of the ANGPTL3 gene sequence upstream of the predicted transcription initiation site revealed the sequence TATATAAA at nucleotide −31, which represents a likely binding site for the transcription factor TFIID.

Northern Blot Analysis

Four transcript sizes were observed on human MTNI blots in liver at approximately 4.5, 3.0, 2.8, and 1.7 kb, and a faint signal was observed at approximately 1.7 kb in kidney (Fig. 3a). No other signals were observed on MTNI (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas), MTNII (spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes), or MTNIII (stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, and bone marrow) blots. The 1.7-kb band corresponds

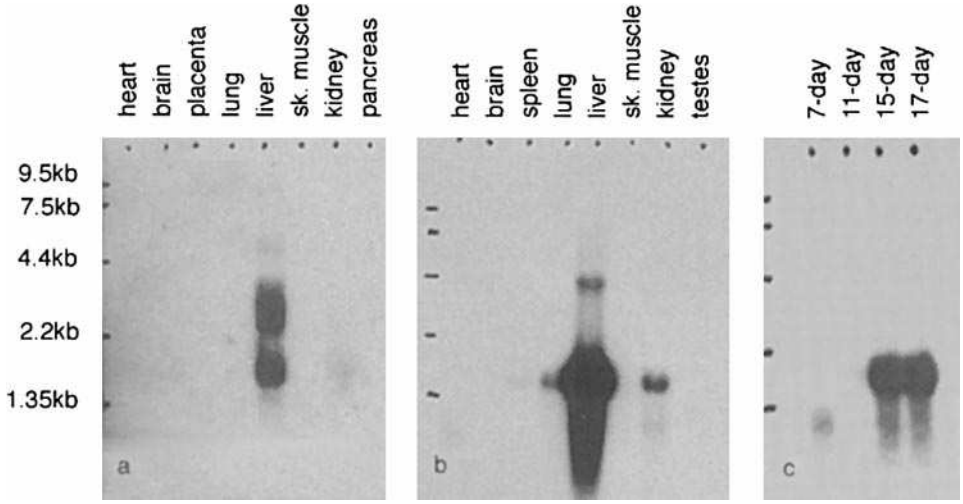

FIG. 3. Northern blot analysis of ANGPTL3. (**a**) Human MTNI; (**b**) mouse MTN; (**c**) mouse embryo.

to the 1.5 kb of sequence obtained from EST319951, not including the poly(A) tail. Given the multiexon structure of the ANGPTL3 gene, it is possible that the larger transcripts represent incompletely spliced RNA. The Master Dot blot showed signals in liver and fetal liver and a faint signal in kidney, with no signal in any other tissue (data not shown). Two transcript sizes were observed on the Mouse Multiple Tissue Northern blot in liver at approximately 4.4 and 1.7 kb, and a faint signal was observed at approximately 1.7 kb in kidney. One transcript size was observed on the Mouse Embryo blot at approximately 1.7 kb in 15- and 17-day embryo. No signal was detected in 7- or 11-day embryo.

Chromosomal Mapping

Human ANGPTL3 was mapped to chromosome 1 on both the lower resolution GeneBridge 4 (GB4)and the medium-resolution Stanford G3 RH panels. The results showed further that ANGPTL3 maps 2.74 cR_{3000} distal from the GB4 framework marker D1S230 and 25.37 $cR_{10,000}$ distal from the Stanford G3 framework marker AFM225zg7 (D1S246). D1S246 is also contained in the D1S203 (92.5 cM)–D1S2865 (123.9 cM) interval in the NCBI/International RH Mapping Consortium GeneMap '98 chromosome 1 RH map (http://www.ncbi.nlm.nih.gov/genemap98/). On the GB4 RH map, the framework markers proximal and distal to ANGPTL3 were D1S230 and WI-9515 (D1S2423), respectively. The use of surrounding cytogenetically mapped genes places ANGPTL3 in the 1p31 region of chromosome 1. Additionally, the use of the RH positioned markers flanking ANGPTL3 positions the gene in the 1p31.1–p22.3 region on the integrated LDB chromosome 1 map (The Genetic Location Database, University of Southampton, WWW server: http://cedar.genetics.soton.ac.uk/public_html/).

Murine ANGPTL3 mapped to mouse chromosome 4 on the T31 RH panel with linkage to D4Mit166 and D4Mit219 at 44.5 and 49.6 cM, respectively. This region of mouse chromosome 4 is syntenic to the human 1p31 chromosomal region where the human version of ANGPTL3 maps.

DISCUSSION

By searching EST databases for signal sequences and amphipathic helices, we have identified a new angiopoietin-like gene, ANGPTL3. ANGPTL3 is a 460-amino-acid polypeptide with the characteristic structure of angiopoietins: a signal peptide, an extended helical domain, a short linker peptide, and a globular FHD.

The ANGPTL3 polypeptide exhibits two features that distinguish it from other angiopoietins. First, while it contains the four conserved cysteines implicated in the intramolecular disulfide bonds of the FHD, it does not contain the two other cysteines that are found within the FHD of ANGPT1, ANGPT2, and ANGPT4. In fact, ANGPTL3 contains no other cysteines and consequently is predicted to form noncovalent rather than disulfide-linked oligomers. Second, ANGPTL3 does not contain the characteristic calcium binding motif found in the other angiopoietins. The lack of this motif is unusual in a FHD (Doolittle *et al.*, 1997).

Multiple tissue Northern blots show that ANGPTL3 is expressed principally in the liver. Murine embryo Northern blots show the presence of ANGPTL3 transcript as early as day 15. It appears that ANGPTL3 is expressed early during liver development and that expression is maintained in adult liver.

While the name "angiopoietin" implies activity in angiogenesis, other members of this family may not be involved in angiogenesis, but rather in mesenchymal–endothelial interactions in other complex tissues such as the eye (CDT6) or the liver (ANGPTL3). CDT6 is expressed specifically by corneal stromal cells (Peek *et al.*, 1998). *Scabrous* is involved in eye development in *Drosophila* (Lee *et al.*, 1996). Other FHD-containing proteins such as tenascin are also implicated in mesenchymal–epithelial interactions and morphogenesis (Erickson, 1993). It is unknown whether the paradigm of agonist–antagonist pairs will hold true for the newly identified angiopoietin-like genes such as ANGPTL3.

Valenzuela *et al.* (1999) have suggested that the designation *angiopoietin* be reserved for factors known to bind to the Tie2 receptor. In this respect, ANGPTL3 may not be a true angiopoietin. ANGPTL3 may represent a ligand for a different receptor, perhaps a homologue of the Tie1 or Tie2 receptor, involved in liver biology. We speculate that new angiopoietin-like genes, specific to other tissue types, remain to be discovered.

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