The NTR module: Domains of netrins, secreted frizzled related proteins, and type I procollagen C-proteinase enhancer protein are homologous with tissue inhibitors of metalloproteases

LÁSZLÓ BÁNYAI AND LÁSZLÓ PATTHY

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Karolina út 29, Budapest H-1113, Hungary

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Abstract

Using homology search, structure prediction, and structural characterization methods we show that the C-terminal domains of (1) netrins, (2) complement proteins C3, C4, C5, (3) secreted frizzled-related proteins, and (4) type I procollagen C-proteinase enhancer proteins (PCOLCEs) are homologous with the N-terminal domains of (5) tissue inhibitors of metalloproteinases (TIMPs). The proteins harboring this netrin module (NTR module) fulfill diverse biological roles ranging from axon guidance, regulation of Wnt signaling, to the control of the activity of metalloproteases. With the exception of TIMPs, it is not known at present what role the NTR modules play in these processes. In view of the fact that the NTR modules of TIMPs are involved in the inhibition of matrixin-type metalloproteases and that the NTR module of PCOLCEs is involved in the control of the activity of the astacin-type metalloprotease BMP1, it seems possible that interaction with metzincins could be a shared property of NTR modules and could be critical for the biological roles of the host proteins.

Keywords: netrins; procollagen C-proteinase enhancer proteins (PCOLCEs); secreted frizzled-related protein (SFRPs); tissue inhibitors of metalloproteinases (TIMPs)

In metazoa most of the extracellular proteins are composed of multiple modules, i.e., independent building blocks that are found in functionally diverse proteins (for reviews, see Patthy, 1985, 1991; Doolittle, 1995). The spread of these protein modules was greatly facilitated by exon shuffling (Patthy, 1987a, 1994).

Prediction of the biological function of the individual modules of multidomain proteins on the basis of homology is not as trivial as in the case of orthologous proteins, which are likely to fulfill very similar roles in different species. In fact, paralogous modules of multidomain proteins (modules of paralogous genes or paralogous modules of orthologous genes) have frequently acquired very different biological roles. Nevertheless, because changes in the function of paralogs by point mutations usually occurs gradually through a continuum of functions, we may expect that some of the functional features of the common ancestor could be retained in all descendants. Accordingly, we may be able to predict the function of novel members of a module family through the analysis of the evolution of functions within that family.

There are several cases where the function of distantly related paralogous modules could be predicted on the basis of homology.

For example, the homologous finger modules of fibronectin and tissue plasminogen activator both proved to be involved in the fibrin affinity of these molecules (Bányai et al., 1983); the homologous type II modules of fibronectin, PDC-109, and gelatinases were shown to be involved in the collagen affinity of these molecules (Bányai et al., 1990; Bányai & Patthy, 1991; Collier et al., 1992). Accordingly, deducing the modular architecture of multidomain proteins not only sheds light on their structural features, but may also provide important clues about the function of its individual modules.

In the present work we show that domains of netrins, complement proteins C3, C4, C5, secreted frizzled-related proteins (SFRPs), and type I ProCOLLAGEN C-proteinase Enhancer proteins (PCOLCEs) are homologous with the N-terminal domains of tissue inhibitors of metalloproteinases (TIMPs). The implications of this homology for the biological roles of the host proteins is discussed.

Results and discussion

Netrins are laminin-related modular proteins (Fig. 1) consisting of a domain homologous with the N-terminal domain VI of laminin B and of three EGF-like repeats related to domain V of laminin B (Ishii et al., 1992; Serafini et al., 1994). The C-terminal third of

Reprint requests to: László Patthy, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Karolina út 29, Budapest H-1113, Hungary; e-mail: patthy@enzim.hu.

Fig. 1. Modular architecture of proteins containing the NTR module. The boxes representing domains of these modular proteins are drawn to scale.

netrins encodes a unique basic domain, hereafter referred to as the NTR domain.

Recent studies (Leyns et al., 1997) have revealed that a family of SFRPs consist of a domain related to the C-terminal domains of netrins and a cysteine-rich domain related to the Wnt-binding domain of frizzled receptors (Fig. 1). In an attempt to find clues for the function of these domains of netrins and SFRPs we have carried out homology searches to identify additional distantly related members of this module family. In earlier studies (Ishii et al., 1992) it has been noted that the C-terminal part of the *Caenorhabditis elegans* netrin, UNC-6, shows modest sequence similarity of uncertain significance with the carboxyterminal part of complement proteins C3, C4, and C5 (Fig. 1). The fact that this domain is absent from the α_2 -macroglobulin, a close relative of complement proteins C3, C4, and C5, did lend support to the notion that this domain corresponds to a mobile module. The biological significance of this weak sequence similarity is supported by an analysis using the consensus sequence method (Patthy, 1987b, 1996). As shown in Figure 2, alignment of the NTR domains of netrins and SFRPs has defined the features typical of this module family. A pattern of six conserved cysteines (C1, C2, C3, C4, C5a, and C6 in Fig. 2) and several conserved blocks of hydrophobic residues (including an YLLLG-like motif) are characteristic of this domain family. It is noteworthy that four of these conserved cysteines (C1,

C3, C4, and C6) are also present in complement components C3, C4, and C5, and these proteins also contain the conserved blocks and the YLLLG-like motif typical of NTR domains.

Homology search with a consensus sequence for NTR domains of netrins (conNTR in Fig. 2) has also identified type I procollagen C-proteinase enhancer proteins (PCOLCEs) as conforming to the consensus rules of this module (Fig. 2), human PCOLCE giving lower E() score (0.087) with this netrin-consensus sequence than most of the SFRP family members. Mouse, rat, and human PCOLCEs are also modular proteins (Fig. 1) that, in addition to the NTR domain identified in this study, contain two tandem CUB domains (Takahara et al., 1994; Ogata et al., 1997). Note that the YLLLG-like motif characteristic of NTR domains of complement C3, C4, and C5, netrins and SFRPs is also conserved in PCOLCEs. Also note that the six cysteines of human PCOLCE align with the conserved cysteines of netrins. Interestingly, rat and mouse PCOLCEs lack two of these cysteines (C2 and C5a in Fig. 2), suggesting that this missing pair of cysteines forms a C2–C5a disulfide bond in the NTR domain of human PCOLCE. On the basis of homology, the four cysteines conserved in all PCOLCEs, netrins, SFRPs are likely to form disulfide bonds in a pattern similar to that determined for complement protein C3 (Dolmer & Sottrup-Jensen, 1993). Accordingly, on the basis of homology we may predict the following disulfide-bond pattern for the NTR mod-

ules of netrins, SFRPs, and human PCOLCE: C1–C4, C2–C5a, and C3–C6. The two cysteines unique to complement proteins C3, C4, and C5 (C* in Fig. 2) form a disulfide bond in a short surface loop of these proteins (Dolmer & Sottrup-Jensen, 1993).

According to the principle of homology walking, the multiple alignment comprising NTR domains of netrins, complement proteins C3, C4, C5, SFRPs, and PCOLCEs was used to deduce characteristic features of the NTR module family to search for additional distantly related members. A search of the database with the derived consensus has identified the N-terminal domains of various members of the family of tissue inhibitors of metalloproteinases (TIMPs) as being significantly similar (Fig. 2). In the output files of these homology searches in the E() score range of $2.2\text{e-}06$ to 0.1, TIMPs are interspersed with PCOLCEs, SFRPs, complement proteins, TIMP1s giving the lowest E() scores ($1.8\text{e-}05$). The significance of the sequence similarity of TIMPs and NTR domains is underlined by the fact that the cysteines shared by complement C3, C4, C5, netrins, and SFRPs are also conserved in the N-terminal domain of TIMPs; furthermore, these cysteines of TIMPs form disulfide bonds (Williamson et al., 1990) in the same pattern as in complement C3: C1–C4, C2–C5b, and C3–C6.

To further test the significance of this sequence similarity (and disulfide-bond pattern similarity), we have predicted the secondary structure of the C-terminal domain of netrins according to a described method (Rost & Sander, 1994; Rost, 1995, 1996). As shown in Figure 2 (top line), the prediction program identified six β -strands and two terminal α -helices. It is noteworthy that the conserved YLLLG-like motif shared by these modules corresponds to a β -strand predicted with high confidence. Significantly, the positions and types of the predicted secondary structure elements of the netrin domain (top line of Fig. 2) are in excellent agreement with those in the known structure of TIMP-2 (bottom line of Fig. 2).

The N-terminal domain of TIMP-2 consists of a five-stranded β -barrel and two terminal α -helices packed side by side against the face of this β -barrel (Williamson et al., 1994; Gomis-Rüth et al., 1997; Muskett et al., 1998). On the basis of homology we may thus predict that the NTR module has a similar fold. Note that the YLLMG-like motif corresponds to strand D of TIMP-2 and that the other conserved blocks of the NTR module also coincide with secondary structural elements of the TIMP-fold (Fig. 2). It is also noteworthy that gaps in the multiple alignment of Figure 2 correspond to surface loops of the TIMP-2 fold. Interestingly, the RGD motifs (recognition sequences for members of the integrin family of adhesion/signaling receptors) that are present in various netrins (Serafini et al., 1994) are found in one of these surface loops (cf. Fig. 2). This fact may provide support for the notion that these RGD motifs may be functional recognition sites for integrins.

To further test the reliability of these structure predictions we have expressed the NTR domain of human PCOLCE and subjected it to structural characterization by circular dichroism (CD) spectroscopy. Analysis of the CD spectra of recombinant NTR domain of human PCOLCE (Fig. 3) predicts 35% β -sheet and 29% α -helix, consistent with the results of our structure predictions.

The homology of domains of netrins, SFRPs, PCOLCEs, and TIMPs may shed some light on the function and structure–function relationships of these molecules.

Vertebrate TIMPs are known to be composed of two domains (Fig. 1), the N-terminal (NTR-related) domain being primarily responsible for the metalloproteinase inhibitor activity of the protein (Murphy et al., 1991; Langton et al., 1998). The short C-terminal domains perform other specific functions, such as providing ad-

Fig. 3. Far-UV CD spectra of recombinant NTR-module of human type I procollagen C-proteinase enhancer protein. Spectra were obtained in 10 mM Tris-HCl, pH 8.0 at 25 °C using 0.1 mg/mL of protein.

ditional binding sites for the prometalloproteinases (Murphy et al., 1991; DeClerck et al., 1993; Willenbrock et al., 1993) or for binding TIMPs to the cell surface (Ko et al., 1997) or to the extracellular matrix (Langton et al., 1998). Isolated N-terminal domains of TIMPs retain their inhibitory activity but lose the binding functions associated with the C-terminal domain (Murphy et al., 1991; DeClerck et al., 1993; Willenbrock et al., 1993; Ko et al., 1997; Langton et al., 1998). Interestingly, two putative *C. elegans* proteins consist of only an NTR domain (Fig. 1).

As for the function of PCOLCEs: they enhance procollagen C-proteinase activity of bone morphogenetic protein-1 (BMP-1), probably by binding to the COOH-terminal propeptide of type I procollagen (Takahara et al., 1994). This activity has been assigned to the two CUB domains because natural processing of the enhancer protein removes the C-terminal NTR-domain, without eliminating enhancer activity (Kessler & Adar, 1989; Hulmes et al., 1997). In fact, there is evidence that removal of the NTR domain leads to a dramatic increase in enhancing activity of PCOLCEs (Kessler & Adar, 1989). There is evidence that the C-proteinase, under certain circumstances, may bind the enhancer protein itself (Takahara et al., 1994; Kessler et al., 1996). In view of the homology of PCOLCE and TIMPs, in view of the fact that PCOLCE interacts with BMP1 (a member of the astacin family of the metzincin superfamily), whereas TIMPs interact with members of the matrixin family of the metzincin superfamily, it is tempting to speculate that the NTR domains are descendants of some ancestral metalloprotease inhibitors. It is noteworthy in this respect that, whereas TIMPs are inhibitors of matrixins, no natural specific

protein inhibitor of astacin-type metalloproteinases has been identified so far (Bond & Beynon, 1995).

There is practically no information on the role of the NTR domains of netrins. Netrins are diffusible proteins that promote axon outgrowth and pioneer axon migrations in nematodes, arthropods, and vertebrates (Ishii et al., 1992; Serafini et al., 1994; Mitchell et al., 1996). They are bifunctional guidance molecules, playing roles as chemoattractants for axons extending toward the ventral midline and chemorepellents for axons growing away from it (Goodman, 1996). Genetic data suggest that both UNC-5 and UNC-40 (members of the immunoglobulin superfamily) are components of the pathway that responds to the UNC-6 protein. Mutations in a member of the TGF- β precursor family, UNC-129, also cause defects in dorsally oriented trajectories of motoraxons that resemble those present in UNC-5, UNC-6, and UNC-40 mutants (Hedgecock et al., 1990; Colavita & Culotti, 1998; Colavita et al., 1998). Although the role of the individual domains of UNC-6 in these processes has not yet been clarified, any model of its functions must take into account its genetic interactions with the TGF- β related UNC-129 pathway (Colavita et al., 1998). It is intriguing to note that metalloproteinases of the astacin family (BMP1/tolloid-type metalloproteases) are intimately involved in the control of the activity of members of the TGF- β family by counteracting the action of chordin (Sarras, 1996; Cho & Blitz, 1998; Wozney et al., 1988).

Nothing is known about the role of the NTR domains of SFRPs. The biological activity of SFRPs as antagonists of Wnt signaling, their role in skeletal morphogenesis, dorsoventral and anteroposterior patterning, and apoptosis is usually assigned to the cysteine-rich domain related to the Wnt-binding domain of frizzled receptors (Hoang et al., 1996; Finch et al., 1997; Leyns et al., 1997; Melkonyan et al., 1997; Rattner et al., 1997; Wolf et al., 1997). In view of the fact that SFRPs also contain a NTR domain related to metalloproteinase inhibitors, the significance of this domain should also be investigated. Because the Spemann organizer that produces chordin and TGF- β family members also produces SFRPs (Leyns et al., 1997), it is tempting to speculate that the TIMP-related part of these molecules may be involved in the regulation of astacin-like metalloproteases controlling the activity of TGF- β s.

Materials and methods

Sequence analysis

Detection of distant homologies was carried out with the consensus sequence method (Patthy, 1987b, 1996). The principle of this procedure is that in the case of distant homologues scores come primarily from positions that are characteristically conserved in the family. Accordingly, in this procedure the evolutionary information characterizing the given family is first condensed into consensus sequences in which low-scoring positions are suppressed thereby sharpening key features of the family. When searching databases with such consensus sequences "bad hits" with variable regions are eliminated thereby increasing the discriminating power of the consensus sequence.

In the present work multiple alignments for the three known subgroups of the NTR module family (netrins, complement proteins, SFRPs) were constructed using Clustal W (Thompson et al., 1994), and consensus sequences were defined for each group according to the consensus sequence procedure (Patthy, 1987b). Unified multiple alignments of NTR modules were constructed using

the consensus sequence-based progressive iterative alignment procedure, and consensus sequences were also derived from these multiple alignments. Database searches were performed and E() scores were calculated to evaluate the significance of the similarities with the FastA programme of the GCG Wisconsin Package Version 9.1 using blosum40, blosum50, blosum62, pam120, and pam250 scoring matrices.

Secondary structure prediction

Secondary structure prediction based on multiple alignments of NTR modules of netrins was carried out with described procedures (Rost & Sander, 1994; Rost, 1995, 1996) using the PHD mail server. The PHD (Profile network prediction HeiDelberg) server predicts secondary structural elements by evaluating the relative probabilities that a given segment can be assigned to helix, strand, or loop. The estimated accuracy of this multiple alignment-based method for the correct prediction of secondary elements is about 72%.

Structural characterization of recombinant NTR module of human PCOLCE

PCR primers (sense: GCGAATTCATATGATATCTCCTGATGCA CCCACCTG, antisense: GGCGAAGCTTGGATCCCTACACAG GTTGAGAGGGGCA) were designed to amplify the segment that corresponds to residues 313–442 of human PCOLCE. The PCR product was cleaved with EcoRI/HindIII and cloned into M13mp18 vector for sequence verification. The insert was excised with EcoRV/HindIII and cloned into PvuII/HindIII digested expression vector pmed 23. This construct (pmed23NTR) encodes a fusion protein (β galNTR) in which the N-terminal 35 residues of β -galactosidase is fused to the recombinant protein. Restriction enzymes were purchased from Biolabs (Beverly, Massachusetts). The M13 sequencing kit used for dideoxy sequencing of cloned DNA fragments and the human liver cDNA library were obtained from Clontech (Palo Alto, California), the expression plasmid pmed23 (Lukachovich et al., 1987) was from Dr. P. Venetianer (Biological Research Center, Szeged, Hungary). *Escherichia coli* strain JM-109 was used to propagate and amplify expression plasmids.

Escherichia coli cells carrying recombinant pmed23NTR plasmids were grown, expression of β -galactosidase fusion proteins was induced with 100 μ mol IPTG, and inclusion bodies containing recombinant protein were isolated essentially as described previously (Bányai & Patthy, 1991). The inclusion bodies were dissolved in 0.1 M Tris-HCl, 8 M urea, 10 mM EDTA, 0.1 M dithiothreitol, pH 8.0, and the mixture was incubated at 25 °C for 60 min with constant stirring. Insoluble cellular debris were removed by centrifugation, and the solubilized proteins were chromatographed on a Sephacryl S-300 column equilibrated with 100 mM Tris-HCl, 8 M urea, 10 mM EDTA, 0.1% 2-mercaptoethanol. Fractions containing pure fusion proteins were identified by SDS-PAGE, pooled and dialysed against 0.1 M Tris-HCl, 10 mM EDTA, pH 8.0 at 25 °C. Precipitated protein was removed by centrifugation, and the supernatant was rechromatographed on Sephadex G-75 column equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, the fractions containing pure recombinant protein were pooled and lyophilized. The β -galactosidase moiety of the fusion protein was removed by limited tryptic digestion according to a described method (Bányai et al., 1994): β galNTR fusion protein (1 mg/mL) was dissolved in 0.1 M ammonium bicarbonate,

pH 8.0 buffer, and was incubated with 0.001 mg/mL trypsin at 25 °C for 60 min. Reaction was arrested with 2 mM phenylmethylsulfonyl fluoride and the digest was applied to a Sephadex G-75 column. TPCK-treated trypsin (Sigma, St. Louis, Missouri) and phenylmethylsulfonyl fluoride (Serva, Heidelberg, Germany) were commercial products.

SDS-PAGE analysis was used to identify fractions containing pure truncated recombinant protein (DEL β galNTR) that corresponds to the NTR domain of human PCOLCE. The concentration of DEL β galNTR protein was determined using the extinction coefficient $9.89 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficients were determined according to a described method (Mach et al., 1992). The composition of protein samples was analyzed by SDS-PAGE using 6–16% and 11–22% linear polyacrylamide gradient slab gels under both reducing and nonreducing conditions (Laemmli, 1970).

Circular dichroism spectroscopy

Far-UV (190–250 nm) CD spectra of recombinant DEL β galNTR were measured using a JASCO J-720 spectropolarimeter thermostatted with a Neslab RT-100 water bath. The CD spectropolarimeter and the optical cells were calibrated with recrystallized *d*-10-camphorsulfonic acid. The measurements were carried out in 0.1 mm pathlength cells and protein solutions of approximately 0.5–1.0 mg/mL. All spectra were measured at 25 °C with a 16 s time constant and a scan rate of 10 nm/min. The spectral slit width was 1.0 nm. All measurements represent the computer average of five scans. Secondary structure of recombinant proteins was estimated from their CD spectra with the J-700 program for Windows Secondary Structure Estimation Ver.1.10.00, JASCO.3.

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