Structural basis for the high-affinity interaction of nidogen-1 with immunoglobulin-like domain 3 of perlecan

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Nidogen and perlecan are large multifunctional basement membrane (BM) proteins conserved in all metazoa. Their high-affinity interaction, which is likely to contribute to BM assembly and function, is mediated by the central G2 domain in nidogen and the third immunoglobulin (IG)-like domain in perlecan, IG3. We have solved the crystal structure at 2.0 Å resolution of the mouse nidogen-1 G2–perlecan IG3 complex. Perlecan IG3 belongs to the I-set of the IG superfamily and binds to the wall of the nidogen-1 G2 β -barrel using β -strands C, D and F. Nidogen-1 residues participating in the extensive interface are highly conserved, whereas the corresponding binding site on perlecan is more variable. We hypothesize that a second, as yet unidentified, activity of nidogen overlaps with perlecan binding and accounts for the unusually high degree of surface conservation in the G2 domain.

Keywords: immunoglobulin-like domain/interaction/
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Introduction

Basement membranes (BMs) are thin sheets of specialized extracellular matrix underlying epithelia and surrounding peripheral nerve axons, muscle and fat cells. Basement membranes act as barriers and scaffolds during tissue development and regulate many cellular activities, including growth, differentiation and migration. The basic constituents of BMs are collagen IV, laminin, nidogen/entactin and the major heparan sulfate proteoglycan perlecan (Timpl and Brown, 1996; Erickson and Couchman, 2000). These large mosaic glycoproteins are ancient and have been exquisitely conserved throughout metazoan evolution (Hynes and Zhao, 2000). A multitude of intermolecular interactions have been defined *in vitro* and are believed to determine the ultrastructure and *in vivo* functions of BMs (Timpl and Brown, 1996). For instance, the high-affinity binding of nidogen-1 to the laminin γ 1 chain has been shown to be critical for BM formation and branching epithelial morphogenesis (Ekblom *et al.*, 1994; Mayer *et al.*, 1998). Nidogen-1 also contains binding sites

for collagen IV and perlecan (Fox *et al.*, 1991; Battaglia *et al.*, 1992; Reinhardt *et al.*, 1993), but the physiological relevance of these activities is not fully understood. Mice lacking nidogen-1 have no overt phenotype, presumably because of compensation by the second mammalian nidogen, nidogen-2 (Murshed *et al.*, 2000). Absence of the unique nidogen in *Caenorhabditis elegans* results in aberrant axonal migration in spite of morphologically normal BMs, suggesting a role of nidogen in cell migration rather than BM assembly (Kim and Wadsworth, 2000). A gene knockout of perlecan in the mouse leads to multiple BM and cartilage defects (Arikawa-Hirasawa *et al.*, 1999; Costell *et al.*, 1999). However, because of the multifunctional nature of perlecan, it is not clear which aspects (if any) of the complex phenotype relate to the loss of nidogen binding.

Nidogen-1 is a 150 kDa glycoprotein consisting of three globular regions, G1–3, with the laminin-binding site located within G3 (Fox *et al.*, 1991). Perlecan binds to G2 (Reinhardt *et al.*, 1993; Hopf *et al.*, 2001b) and we have recently shown that a single immunoglobulin (IG)-like domain in perlecan, IG3, is sufficient for high-affinity ($K_d \sim 10$ nM) binding (Figure 1) (Hopf *et al.*, 1999, 2001b). The crystal structure of nidogen-1 G2 revealed a β -barrel with a surprising similarity to green fluorescent protein and a cluster of highly conserved residues on the barrel surface; site-directed mutagenesis showed these nidogen-1 residues to be involved in perlecan binding (Hopf *et al.*, 2001a). To gain further insight into the nidogen–perlecan interaction, we have now determined the crystal structure of a complex of nidogen-1 G2 and perlecan IG3. This structure affords, for the first time, an atomic view of a protein–protein interaction involved in BM assembly (Liddington, 2001).

Results

Mouse nidogen-1 G2 (residues 357–638) and perlecan IG3 (residues 1765–1858) were expressed in 293-EBNA cells. The two protein fragments form a stable 1:1 complex, which was purified by size-exclusion chromatography and crystallized. The structure of the nidogen-1 G2–perlecan IG3 complex was solved by molecular replacement and refined at 2.0 Å resolution to $R_{\text{free}} = 0.245$.

The structure of nidogen-1 G2 in the complex (Figure 2) is very similar to that of the fragment in isolation and consists of an N-terminal epidermal growth factor-like (EG) domain and an 11-stranded β -barrel (strands A–K) with a central irregular α -helix (Hopf *et al.*, 2001a). Notably, segments that were disordered in the original structure (mainly in the EG domain) are clearly defined in the complex, revealing the N-terminal β -hairpin of the EG domain and the disulfide bridge linking the EG domain to the β -barrel. Strong spherical electron density near the

Fig. 1. Domain organization of mouse perlecan (Noonan *et al.*, 1991) and nidogen-1 (Mann *et al.*, 1989). HS, heparan sulfate oligosaccharide chains; SEA, domain found in sea urchin sperm protein, enterokinase, agrin; LA, LDL receptor type A; IG, immunoglobulin-like; L4, laminin domain IV; LE, laminin type epidermal growth factor-like; LG, laminin G-like; EG, epidermal growth factor-like; TY, thyroglobulin-like; G1–3, nidogen globular domains. The double-headed arrow indicates the high-affinity interaction between nidogen-1 G2 (in cyan) with the preceding EG domain in green) and perlecan IG3 (in magenta).

E–F turn indicates the presence of a metal ion. Based on the nature and geometry of the ligands (tetrahedral coordination by Asp511, His513, His515, as well as His1809 from a packing-related perlecan IG3 domain) and an average metal–ligand distance of 2.1 Å, the ion has been assigned as zinc, although cobalt from the chelating chromatography used in the purification of nidogen-1 G2 cannot be ruled out.

Perlecan IG3 belongs to the I-set of the IG superfamily (Harpaz and Chothia, 1994), in which strand A is interrupted by a bulging loop and switches between the two β -sheets, resulting in a sandwich of two four-stranded sheets, ABED and A'GFC (Figure 2). The same arrangement of β -strands is found in the prototype I-set member telokin (Holden *et al.*, 1992), which can be superposed onto perlecan IG3 with an r.m.s. deviation of 1.7 Å (84 C α atoms). Significant differences are the lack of a short β -strand C' in perlecan IG3, a longer D–E hairpin in telokin, and a differently positioned A–A' bulge, resulting in a longer β -strand A' in perlecan IG3. As in almost all members of the IG superfamily, strands B and F of perlecan IG3 are connected by a buried disulfide bridge between Cys1792 and Cys1839.

Fig. 2. The structure of the nidogen-1 G2–perlecan IG3 complex. (A) Two orthogonal views of the complex. The nidogen-1 G2 fragment is composed of an N-terminal EG domain (in green) and an 11-stranded β -barrel domain (in cyan); perlecan IG3 is in magenta. Disulfide bridges are in yellow and a zinc ion is shown as a red sphere. The β -strands in nidogen-1 G2 and perlecan IG3 are labelled A–K and A–G, respectively. (B) Sequence of the perlecan IG3 domain and its secondary structure elements. (A) and Figure 3 were made with BOBSRIPT (Esnouf, 1997) and RASTER3D (Merritt and Bacon, 1997).

Perlecan binds to a relatively flat region on the nidogen-1 G2 β -barrel, with the long axis of the IG3 domain roughly perpendicular to the central helix of the

Fig. 3. Stereoview of the nidogen-1 G2–perlecan IG3 interface. Nidogen-1 and perlecan residues are in cyan and magenta, respectively, and are labelled, as are β -strands contributing to the interface. Hydrogen bonds are indicated by thin black lines. The view direction is similar to the lower panel in Figure 2A.

barrel. Upon complex formation, 2000 Å² of solvent-accessible surface area become buried in an extensive interface. The majority of contacts are made by strands A–C of nidogen-1 G2 and strands C and F of perlecan IG3. Additionally, strands J and K of nidogen-1 G2 interact with strand D of perlecan IG3. Hence, the location of the perlecan-binding site on the β -barrel of nidogen-1 G2 is entirely consistent with our previous mutagenesis data (Hopf *et al.*, 2001a). In the two areas of extensive contact, there is good shape complementarity between nidogen-1 G2 and perlecan IG3, resulting in a general absence of water molecules. We also note that the polypeptide chain termini of both nidogen-1 G2 and perlecan IG3 are distant and pointing away from the interface (see below).

The core of the interface is formed by the packing of strand C of perlecan IG3 and residues preceding it onto strands B and C of nidogen-1 G2 in a way that allows Ala1800, Thr1802 and Val1804 (perlecan) to interdigitate with His429, Tyr431, Tyr440 and Ala442 (nidogen) (Figure 3). This extensive apolar contact is strengthened by a hydrogen bond from Tyr1801 to Glu616 in the J–K loop of nidogen-1 G2 and hydrogen bonds between Ser1842 in strand F of perlecan IG3 and His429 and Arg403 (nidogen). The latter residue also donates a hydrogen bond to the peptide carbonyl group of residue 1843 in the F–G turn of perlecan IG3 (not shown). Arg620 (nidogen) is buried in the interface by the Tyr1801 (perlecan) side chain and donates a hydrogen bond to the peptide carbonyl group of Tyr1801. The guanidinium group of Arg620 also hydrogen bonds to a number of water molecules that fill a small cavity in the interface. The other main contact in the interface is centered on the side chain of Phe1820 (perlecan), which is bound snugly in a hydrophobic pocket created by Tyr440, Phe609, Leu611, Arg620 and Ala622 (nidogen). Two hydrogen bonds involving the preceding residue, Asp1819, complete the interface. The putative zinc ion in nidogen-1 G2 is not directly involved in perlecan binding, but the E–F turn, which provides three metal ligands, is in van der Waals contact with the F–G hairpin of perlecan IG3.

Discussion

The biological function of all extracellular matrices is determined by their composition and ultrastructure, which, in turn, depend on a multitude of often weak interactions between large modular proteins. Recombinant production, site-directed mutagenesis and, less frequently, structural studies of isolated domains have been employed to study protein–protein interactions between matrix proteins. The nidogen-1 G2–perlecan IG3 complex reported here provides the first detailed view of an interaction between two matrix proteins that are common to all metazoan organisms (Hynes and Zhao, 2000).

IG domains, together with the topologically similar fibronectin type III domains, are the most abundant domains found in cell adhesion and receptor molecules (Schultz *et al.*, 2000). The prominent role of IG domains in both homo- and heterophilic protein–protein interactions is reflected by the large number of structures that have been determined, both of individual domains and of macromolecular complexes (Chothia and Jones, 1997; Wiesmann *et al.*, 2000). Interestingly, IG domains are not nearly as widespread in secreted extracellular matrix proteins, and perlecan is unusual in containing a long tandem array of IG domains (Figure 1). The structure of perlecan IG3 bound to the β -barrel of nidogen-1 G2 further illustrates the enormous versatility of the IG domain in mediating protein–protein interactions. Immunoglobulin domains do not contain a unique ligand recognition site, but achieve high-affinity ligand binding in many different ways (Chothia and Jones, 1997; Wiesmann *et al.*, 2000). In this respect we note that the nidogen-binding site of perlecan IG3 is similar to the integrin-binding sites of VCAM-1 and ICAM-2 in that β -strand C' of the typical I-set IG domain is missing, and strands C, D and F provide many of the critical ligand-binding residues (Jones *et al.*, 1995; Casasnovas *et al.*, 1997).

The results of previous mapping and mutagenesis experiments (Hopf *et al.*, 2001a,b) are in good agreement with the nidogen-1 G2–perlecan IG3 structure. First, the

location of polypeptide chain termini in the complex supports the notion that domains flanking G2 in nidogen-1 and IG3 in perlecan are not needed for high-affinity binding. In fibronectin, by contrast, most activities require the cooperation of more than one domain (Leahy *et al.*, 1996; Sharma *et al.*, 1999; Pickford *et al.*, 2001). Moreover, using loop chimeras, perlecan IG3 loops B–C and F–G were identified as crucial for nidogen-1 binding, and mutation of Tyr1801 to alanine reduced nidogen-1 binding ~5-fold (Hopf *et al.*, 2001b). The two loop regions and Tyr1801 indeed participate in the major interface contact (Figure 3). Finally, a series of alanine substitutions within nidogen-1 G2 was used to map the perlecan-binding site (Hopf *et al.*, 2001a). This demonstrated critical roles of His429, Tyr431 and Arg620, in excellent agreement with the nidogen-1 G2–perlecan IG3 structure. Curiously, mutation of Tyr440 in nidogen-1 G2 (Hopf *et al.*, 2001a) and of Asp1819/Phe1820 in perlecan IG3 (Hopf *et al.*, 2001b) affected the nidogen-1–perlecan interaction only modestly, despite the fact that these residues interact intimately in the complex (Figure 3). It is conceivable that the close packing around Phe1820 may have formed adventitiously in the crystal, but a more likely explanation is that the mutations resulted in some structural rearrangement that obscured the important role of Phe1820.

Zinc has been shown to inhibit weakly the binding of perlecan to nidogen-1 (Reinhardt *et al.*, 1993) and a zinc ion is observed bound to nidogen-1 G2 in the complex. The zinc-binding E–F loop is in close proximity to two exposed apolar residues in the F–G turn of perlecan IG3, Met1844 and Phe1845, and thus could affect perlecan binding. However, given that a stable nidogen-1 G2–perlecan IG3 complex was obtained in the presence of a metal ion, a physiological role of zinc in modulating the nidogen–perlecan interaction seems unlikely.

The perlecan-binding residues of mouse nidogen-1 are strikingly conserved in all metazoan nidogens (Hopf *et al.*, 2001a). In fact, all nidogen residues shown in Figure 3 are invariant in mammalian nidogen-1 and nidogen-2, which share ~50% overall sequence identity (Kohfeldt *et al.*, 1998). A domain with 21% sequence identity to mouse nidogen-1 G2 is present in a human secreted protein (DDBJ/EMBL/GenBank accession Nos AJ306906 and AF156100) related to *C.elegans* hemicentin (Vogel and Hedgecock, 2001), but the perlecan-binding site of nidogens is not conserved in this protein. If perlecan binding is indeed a conserved property of all nidogens, it should be possible to identify a similarly stringent local sequence conservation in all metazoan perlecan. We aligned all IG domains of perlecan from *C.elegans* (unc-52; SwissProt Q06561), *Drosophila* (S.Baumgartner, personal communication), mouse (SwissProt Q05793) and human (SwissProt P98160), which differ subtly in their modular organization, using a set of strictly conserved core residues corresponding to Trp1805, Leu1824 and Cys1839 in mouse perlecan IG3 (data not shown). We then looked for conservation of the nidogen-binding residues shown in Figure 3. In human perlecan, IG3 could be clearly identified as the nidogen-binding domain, with all critical residues absolutely conserved. Residues corresponding to Tyr1801, Asp1819 and Phe1820 (mouse perlecan) proved to be highly discriminating, with no

other IG domain in mouse or human perlecan (a total of 35 domains) containing a similar arrangement.

Surprisingly, we were unable to identify a nidogen-binding IG domain in either *C.elegans* or *Drosophila* perlecan, even when we allowed for changes to match the few, mostly conservative substitutions in the corresponding nidogens. Of course, this may simply indicate that the worm and fly perlecan are too distant in evolution from the mouse protein for a nidogen-binding site to be discerned. Alternatively, it is possible that perlecan binding is unique to mammalian (or vertebrate) nidogens and that the conserved residues on the nidogen G2 β -barrel participate in a more ancient function, perhaps related to cell migration. We have recently shown that collagen IV competes with perlecan for binding to the conserved patch on unglycosylated mouse nidogen-1 G2 (Hopf *et al.*, 2001a). It is conceivable that nidogen G2 is complexed differentially in tissues, depending on the availability of its two ligands, perlecan and collagen IV. The constraints of maintaining a functional binding site for two disparate ligands could account for the unusually high degree of surface conservation in nidogen G2. An answer to these questions will have to await the biochemical characterization of invertebrate nidogens and further genetic experiments, such as a nidogen-1/nidogen-2 double knockout in the mouse.

Materials and methods

Non-glycosylated mouse nidogen-1 G2 (residues 357–638) and perlecan IG3 (residues 1765–1858) were expressed in 293-EBNA cells and purified as described [Hopf *et al.*, 2001a,b; the sequence numbering includes the signal peptide in perlecan (Noonan *et al.*, 1991) but not in nidogen-1 (Mann *et al.*, 1989)]. The nidogen-1 G2–perlecan IG3 complex was prepared by adding a 1.5-fold molar excess of perlecan IG3 to 4.5 μ M nidogen-1 G2 in 0.2 M ammonium acetate pH 6.8, followed by 10-fold concentration and purification on a Superdex 75 column (Pharmacia) in the same buffer. The nidogen-1 G2–perlecan IG3 complex (1 mg total) was concentrated to 6 mg/ml in 0.04 M ammonium acetate pH 6.8. A single crystal measuring $0.05 \times 0.1 \times 0.1$ mm was obtained by the hanging drop method (1 μ l each of protein and reservoir solution) using 10% PEG 6000, 5% 2-methyl-2,4-pentanediol (MPD), 0.1 M HEPES pH 7.5 as precipitant. The crystal was flash frozen to 100K in 15% PEG 6000, 5% MPD, 0.1 M HEPES pH 7.5, 30% glycerol and diffraction data were collected with a Quantum4 CCD detector on station 9.6 at the SRS Daresbury ($\lambda = 0.87$ Å). The space group is $P2_12_12_1$ with unit cell dimensions $a = 55.95$ Å, $b = 72.30$ Å, $c = 103.97$ Å. The asymmetric unit contains one 1:1 complex and ~50% solvent. The diffraction data were processed with MOSFLM (Leslie, 1994) and programs of the CCP4 suite (CCP4, 1994) to yield 29 096 unique reflections in the 20–2.0 Å resolution range (completeness = 99.8%; R_{merge} on intensities = 0.089; multiplicity = 3.6). The structure was solved by molecular replacement with AMoRe (Navaza, 1994) using the nidogen-1 G2 structure (Hopf *et al.*, 2001a) as a search model. The perlecan IG3 domain was built with O (Jones *et al.*, 1991) and the complex refined with CNS (Brünger *et al.*, 1998). The final model includes nidogen-1 residues 359–631 and perlecan residues 1769–1857 (a total of 2802 protein atoms), 175 water molecules, a HEPES buffer molecule, and a metal ion bound in a crystal contact which was refined as Zn^{2+} . The final R -factor is 0.213 ($R_{\text{free}} = 0.245$) using all data with $F > 0$ from 20 to 2.0 Å resolution. The r.m.s. deviations from ideality for bond lengths and angles are 0.006 Å and 1.2°, respectively. A total of 90.0% of the residues are in the core regions of the Ramachandran plot, and only one residue is in a disallowed region.

Coordinates

The coordinates of the nidogen-1 G2–perlecan IG3 complex structure have been deposited in the Protein Data Bank (accession code 1gl4).

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