Inhibition of glycosaminoglycan modification of perlecan domain I by site-directed mutagenesis changes protease sensitivity and laminin-1 binding activity

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Abstract Glycosaminoglycan attachment to perlecan domain I (173 residues) was completely prevented by site-directed mutagenesis of Ser-65, Ser-71 and Ser-76 as shown by recombinant production in mammalian cells. This did not interfere with the proper folding of the domain's SEA module but enhanced its sensitivity to neutral proteases. Lack of substitution also abolished binding to the two major heparin binding sites of laminin-1.

Key words: Basement membrane; Proteoglycan; Proteolysis; Recombinant protein; Site-directed mutagenesis

1. Introduction

Perlecan is an abundant proteoglycan of basement membranes and other pericellular matrices and is usually modified by three glycosaminoglycan (GAG) chains attached to one end of its elongated core protein [1,2]. These side chains, in particular heparan sulfate, can bind fibroblast growth factor-2 (FGF-2) which is important in the regulation of cytokine action during wound healing and angiogenesis [3–6]. They may also modulate integrin-mediated cell adhesion to perlecan [7,8] and integrate it into basement membrane structures by binding to laminin-1 and collagen IV [9].

The major GAG attachment sites of perlecan were localized to its N-terminal domain I in several recombinant studies [5,10–12]. They were shown to be substituted by heparan and chondroitin/dermatan sulfate chains which were, however, smaller than those of tissue-derived perlecan. A further, probably minor attachment site which exists on the C-terminal perlecan domain V may be important in the regulation of cell adhesion and heparin binding [13]. Three serines within typical SGD consensus sequences of domain I could be identified as GAG-linked attachment sites by biochemical analysis of a recombinant fragment [11] and by site-directed mutagenesis of a truncated domain I segment [12]. The latter lacked the SEA module, which is considered to be associated with regions of extensive GAG- or *O*-linked carbohydrate modification in several proteins [14].

In order to study the structure and function of domain I and its SEA module we have now prepared a recombinant fragment in which GAG modification was completely abolished by alanine mutagenesis of Ser-65, Ser-71 and Ser-76. This fragment I mutant was obtained in high yields and had a SEA module which was properly folded but became distinctly sensitive to cleavage by neutral proteases. The mutant also failed to bind to laminin-1 but retained perlecan-specific immunological epitopes which could be detected in tissues.

2. Materials and methods

2.1. Expression vector and transfection

The introduction of three mutations in the perlecan domain I cDNA followed previously used strategies [15]. The overlapping complementary primers 5′-AGCAGGAGATGGCCTTGGCGCCGGAGATGTGGGCGCCGGGGACTTC and 5′-GGCGCCCACATCTCCGGCGCCAAGGCCATCTCCTGCTGCATCATCT were used to change three Ser to Ala codons (underlined) by PCR amplification with the corresponding 5′ and 3′ terminal primers used before in the production of domain I [11] in order to prepare two subfragments. These were purified and used with the terminal primers to produce a cDNA encoding the entire mutated domain I. After restriction with *Nhe*I and *Xho*I, this was inserted in frame with the BM-40 signal peptide of the episomal expression vector pCEP-Pu [16]. The correct sequence and in-frame insertion were confirmed by cycle sequencing on a 373A DNA sequencer (Applied Biosystems). Transfection of EBNA-293 cells and selection of cells by puromycin in order to obtain serum-free medium and to purify the fragment I mutant followed a previous protocol [16].

2.2. Protein purification and modification

Recombinant GAG-substituted perlecan fragments IA and IB and the fragment I mutant were purified on DEAE cellulose and Superose 12 as previously described [11]. Proteolytic fragment E3 [17] and recombinant fragment α1VI/V [18] corresponded to C- and N-terminal regions of the laminin α1 chain. Digestions with heparitinase and chondroitinase ABC [11] and with *N*-glycosidase F [19] followed previous procedures. Digestions (24 h, 37°C) with endoproteinase Glu-C, trypsin and pancreatic elastase (enzyme:substrate ratio 1:100) were carried out in 0.05 M Tris-HCl, pH 7.4. Digests were then separated by reversed phase HPLC on a C18 column with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid.

2.3. Analytical and immunological methods

Amino acid and hexosamine compositions were determined after hydrolysis with 6 M and 3 M HCl, respectively, on a Biotronik LC 3000 analyzer. Edman degradation on a 473A sequencer followed the manufacturer's instructions (Applied Biosystems). Circular dichroism (CD) spectra were recorded and evaluated as described before [11]. SDS electrophoresis in 5–20% polyacrylamide gels followed established protocols. Immunological assays [11] and indirect immunofluorescence [13] were performed with previously described rabbit antisera [11,13]. Solid phase binding assays followed a standard protocol [20].

3. Results

An episomal expression vector was used to produce a perlecan fragment I mutant in which the three potential GAG attachment sites, Ser-65, Ser-71 and Ser-76, were mutated to Ala. This mutant was readily expressed in human EBNA-293

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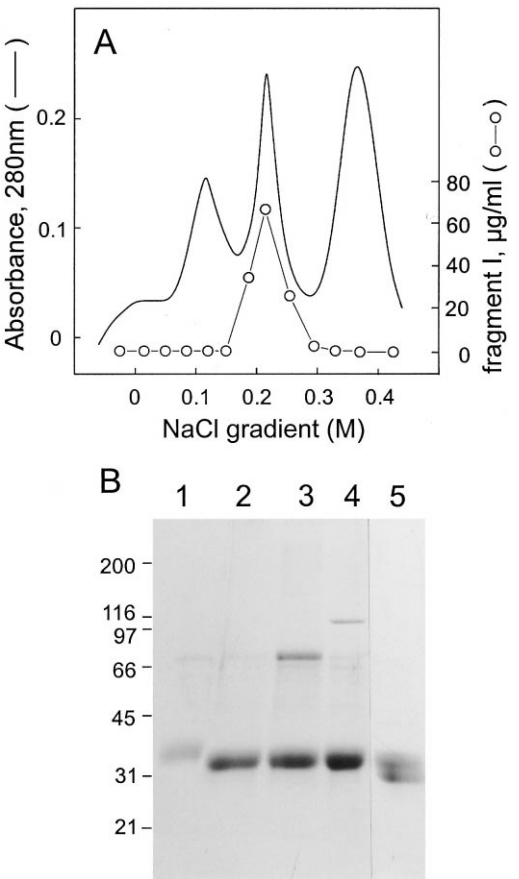

Fig. 1. Chromatography of perlecan fragment I mutant on DEAE cellulose (A) and SDS gel electrophoresis of purified fragments prior to or after enzymatic digestion (B). A: Serum-free conditioned medium (1 l) was passed over a DEAE column (2.5×25 cm) which was eluted with a 500 ml linear gradient of 0–0.5 M NaCl, as indicated at the bottom. The effluent was monitored for absorbance (280 nm) and for fragment I concentration (μg/ml) as determined by radioimmunoinhibition assay. B: Electrophoresis shows fragment IA after heparitinase digestion (lane 1) and purified fragment I mutant prior to (lane 2) and after digestion with heparitinase (lane 3), chondroitinase (lane 4) or *N*-glycosidase F (lane 5). The gel was calibrated with marker proteins (in kDa) shown in the left margin.

cells, as shown by electrophoresis (32 kDa band) of serum-free conditioned medium and radioimmunoassay (production 15 μg/ml/day). The recombinant fragment I mutant eluted from a DEAE cellulose column in a narrow peak at around 0.22 M NaCl (Fig. 1A) and was obtained in purified form after molecular sieve chromatography, as shown by electrophoresis (Fig. 1B, lane 2). Recombinant perlecan fragments IA, which contains heparan sulfate, and IB, which contains heparan/chondroitin sulfate, were previously shown [11] to run as a broad electrophoretic band of 70–100 kDa and to elute from DEAE cellulose at 0.33 M and 0.4 M NaCl, respectively. Neither of these GAG-modified forms could be detected in the culture medium of the fragment I mutant by immunological assays (see Fig. 1A). The purified fragment I mutant showed a single N-terminal sequence APLAVT with APLA being derived from the BM-40 signal peptide [11,16], and contained 4.8 ± 0.5 residues of glucosamine and 4.5 ± 0.4 residues of galactosamine. This is consistent with the modification of one *N*-linked and five *O*-linked oligosaccharide acceptor sites that are present in domain I [11] and the absence of GAG substitutions. In addition, a minor 22 kDa fragment with the starting sequence DDA*A*GDGL (italics denotes first mutation) could be purified from the culture medium, indicating that the mutated fragment is sensitive to endogenous proteolysis.

Heparitinase-treated fragment IA shows a single electrophoretic band of slightly lower mobility than the mutant (Fig. 1B, lane 1), presumably due to some residual GAG segments. Treatment of the mutant with heparitinase or chondroitinase ABC did not change electrophoretic mobility while digestion with *N*-glycosidase F caused partial conversion to a 30 kDa component (Fig. 1B). The CD spectrum of the mutant (not shown) was almost identical to that described for fragment IA [11], indicating 18% α helix and 62% β structure. This is consistent with proper folding of the SEA module, for which similar contents of secondary structure have been predicted from the sequence [14].

Correct folding of the mutant was also indicated from radioimmunoinhibition profiles, which were indistinguishable from those previously shown for fragment IA [11]. Rabbit antibodies which were affinity-purified on a column containing the fragment I mutant were tested for their reaction with tissue forms of perlecan by immunofluorescence. On sections of skeletal muscle, they reacted with endomysium, perimysium, perineurium and small blood vessels (Fig. 2A). The same staining pattern was observed with antibodies against the C-terminal domain V of perlecan (Fig. 2B). In kidney, staining for domain I was observed in glomerular and tubular basement membranes, mesangium and Bowman's capsule and in testis basement membranes around seminiferous tubules were stained (not shown).

The identification of an endogenous fragment of the mutant raised the question whether the GAG chains protect domain I

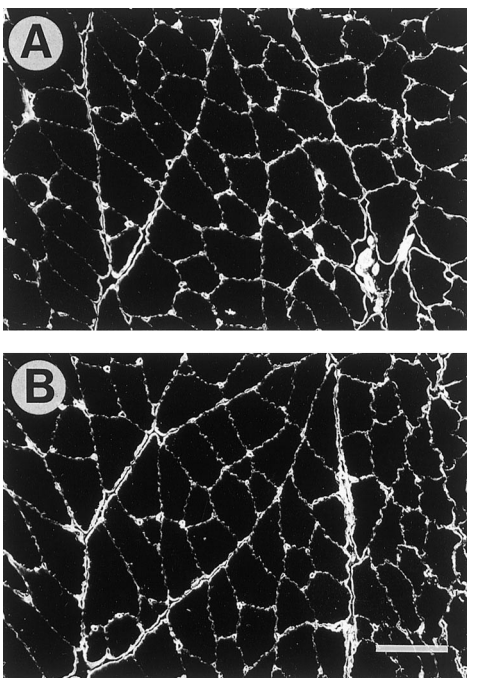

Fig. 2. Indirect immunofluorescence staining of mouse skeletal muscle by affinity-purified antibodies against perlecan domains I (A) and V (B). Bar: 100 μm.

from proteolysis. This was examined by cleavage of fragments IA and IB and of the mutant with trypsin, elastase or endoproteinase Glu-C. Electrophoresis demonstrated complete digestion of the mutant while substantial amounts of the proteoglycan bands of IA and IB still persisted (not shown). Complete digestion of the mutant to small peptides was also demonstrated by reversed phase chromatography, as shown for endoproteinase Glu-C (Fig. 3A). The amounts of small peptides released from fragments IA and IB by the same protease were considerably smaller. Similar but less marked differences were also observed after treatment with trypsin or elastase. Peptides generated by endoproteinase Glu-C were used to locate cleavage sites by Edman degradation (Fig. 3B). This demonstrated relatively little cleavage in the region (positions 22–79) of the mutant preceding the SEA module despite the presence of three Glu residues. Nine major cleavage sites were identified within the SEA module (positions 80–194), including almost every Glu position. From the same digest of fragment IA, only three major cleavage sites could be detected, with lower yields. They included positions near the N- and C-termini of the SEA module and one within its central region (Fig. 3B).

Binding of tissue-derived perlecan to laminin-1, which contains the α1 chain, was previously shown to be mainly mediated by the heparan sulfate chains [9]. This was now examined with the three variants of recombinant domain I using the laminin α1 chain fragments α1VI/V and E3, which are derived from the N- and C-termini, respectively (Fig. 4). A solid phase assay demonstrated a strong binding of fragment IA to E3 and a 10-fold lower reactivity with α1VI/V. For fragment IB, binding was similar but at a slightly lower level, probably due to its lower heparan sulfate content [11]. No substantial reaction could be detected for the fragment I mutant. None of the perlecan fragments bound to fibronectin or vitronectin.

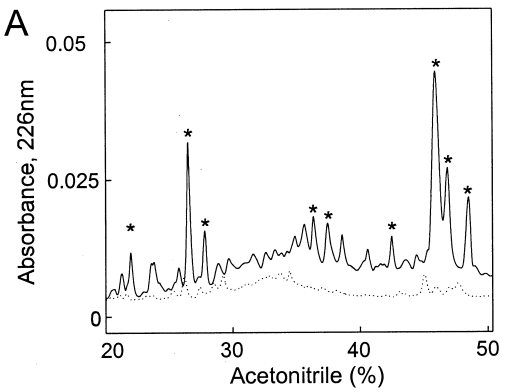

Fig. 3. HPLC separation of peptides obtained from fragment I by endoproteinase Glu-C digestion (A) and identification of peptides by N-terminal sequencing (B). A: The superimposed profiles show the digests of the mutant (solid line) and IB (dotted line) loaded in identical amounts onto the C18 column. Asterisks denote the peaks subjected to sequencing. B: The sequence shows only the SEA module within the position numbers indicated. The underlined sequences represent either full-length peptides (ended by a bar) or a partial sequence (ended by an arrowhead) and were determined from the digest of the mutant. A similar analysis of peptides obtained in low yield from GAG-modified fragment I determined cleavages only at positions 95/96, 139/140 and 163/164.

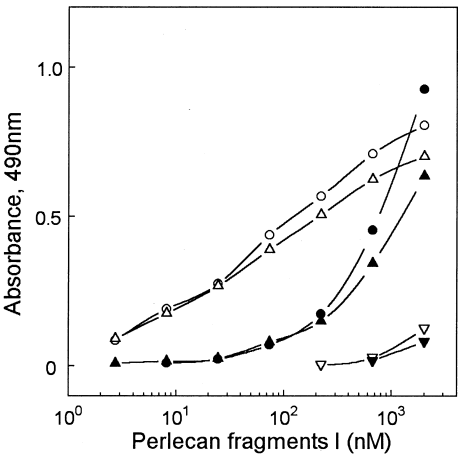

Fig. 4. Solid phase binding assay between perlecan domain I fragments and heparin binding laminin-1 fragments. The laminin fragments E3 (open symbols) and α1VI/V (closed symbols) were used in immobilized form. Soluble recombinant perlecan fragments were IA (○,●) IB (△,▲) and I mutant (▽,▼) at the concentrations indicated.

4. Discussion

The N-terminal domain I of mouse perlecan was predicted to contain a GAG attachment region (positions 22–79) with three SGD acceptor sites [1,2,21] and a SEA module (positions 80–193) folded into α helical and β structures [14]. Recombinant studies provided chemical [11] and mutational [12] evidence for the substitution of Ser-65, Ser-71 and Ser-76 within the SGD sequences by heparan and chondroitin sulfate, but did not exclude a further acceptor site which might be under the control of the SEA module. Our present data, however, do not support this possibility. A further, partial modification by heparan/chondroitin sulfate was also found for perlecan domain V [13]. This GAG substitution was recently localized to a single Ser residue of domain V (M. Friedrich and R. Timpl, unpublished) indicating that mouse perlecan may contain up to four GAG chains.

The data also demonstrated that GAG substitution is not required for the proper folding and secretion of perlecan domain I. The absence of this substitution, however, makes domain I distinctly more vulnerable to attack by several neutral proteases. It has also been shown previously that GAG substitution can protect tissue-derived perlecan from proteolytic cleavage by the isolation of a tryptic 16 kDa core protein fragment carrying most of the GAG side chains [22]. Surprisingly, most of the cleaved peptide bonds could be located to the SEA module despite the prediction of a high content (60%) of secondary structure elements [14] as confirmed here by CD spectroscopy. The SEA module has also been identified in the proteoglycan agrin and several other proteins where it was located adjacent to GAG attachment or mucin-like sites [14]. This could indicate that carbohydrates are required to

stabilize SEA modules and protect them against proteolytic degradation and the extent of substitution may control proteolytic processing. Modification of domain I by GAG does not seem to influence the recognition of perlecan-specific immunological epitopes in the core protein [11]. These epitopes are also exposed in tissue sections, as shown here, which will now allow us to analyze possible proteolytic degradation of domain I during developmental and pathological conditions.

The GAG side chains of perlecan domain I are also involved in binding to FGF-2, which enhances binding to high affinity FGF cell receptors [4,23,24], and in binding to other extracellular ligands such as laminin-1 [9]. The latter interaction could contribute to basement membrane assembly in addition to links between laminin-1 and the perlecan core protein via ternary complexes with nidogen-1 [9]. Here we show that recombinant GAG forms of domain I recognize the C-terminal region of the laminin α1 chain (fragment E3) and its N-terminal counterpart (fragment α1VI/V) with a 10-fold difference in binding activity. A similar difference between the two laminin fragments was observed in their binding to perlecan [18], indicating that the recombinant and tissue-derived perlecan structures contain similar binding epitopes on their heparan sulfate chains despite their substantial difference in size [11]. Yet perlecan shows GAG-mediated binding to fibronectin [9] not found here for recombinant fragment I. The heparan sulfate chains from both sources could therefore be used to distinguish between different GAG binding epitopes in various protein ligands [23,24].

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