Binding of von Willebrand factor to the small proteoglycan decorin

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Abstract The small proteoglycan decorin plays an important role in the organisation of the extracellular matrix by binding to several components, including collagen and fibronectin. In this work, we report the dose-dependent and saturable interaction of decorin with the adhesive glycoprotein, von Willebrand factor (VWF). This interaction was mediated by the glycosaminoglycan side chain of decorin and was critically regulated by the degree of sulfation, but not by the amount of iduronic acid. Both chondroitin sulfate and dermatan sulfate, in addition to heparin, were found to bind VWF equally well. Although soluble decorin prevented VWF binding to heparin, purified VWF-A1 domain failed to interact with the proteoglycan. These results identify VWF as a new partner for the small proteoglycan, decorin, in the structural organisation of the extracellular matrix.

Keywords: von Willebrand Factor; Decorin; Glycosaminoglycan; Collagen; Heparin

1. Introduction

von Willebrand factor (VWF) is a multimeric adhesive protein, which plays a crucial role in platelet adhesion to the subendothelial matrix. At the site of vessel wall injury, binding of VWF to the GPIb-IX-V receptor on circulating platelets promotes cell rolling and tethering, eventually leading to firm platelet adhesion and thrombus formation [1,2]. The mature VWF molecule contains four repeated A, B, C, and D domains [3]. The A1 domain contains the platelet-binding site, and interacts with the leucine-rich repeats (LRRs) and flanking sequences in the GPIbα subunit of the GPIb-IX-V complex [4,5].

Subendothelial VWF interacts with different components of the extracellular matrix, including collagen and the glycosaminoglycan (GAG), heparin [6,7]. VWF binds to heparin through the A1 domain and through a sequence in the N-terminus of the protein [7,8]. Some studies have suggested that this interaction may influence platelet adhesion [9,10]. VWF interaction with collagen is mediated by both the A1 and A3 domains [11]. Recent findings have suggested that incorporation of VWF into fibrillar collagen enhances platelet adhesion [12].

Decorin is a member of the small leucine-rich proteoglycan family comprising a 40 kDa protein core with 10 LRRs bound to a single chondroitin/dermatan sulfate (CS/DS) side chain [13]. Decorin from different tissues shows great heterogeneity in terms of both CS/DS ratio and degree of sulfation of the GAG chain [14]. Decorin regulates matrix assembly by binding to several proteins including thrombospondin, fibronectin and collagen. In particular, decorin binding to collagen negatively modulates the lateral association of collagen molecules, resulting in a reduction of fibril diameter [15]. Decorin also interacts with growth factors and growth factors receptors to regulate cellular responses [16,17]. We have previously shown that decorin also supports both platelet adhesion and activation by binding to the integrin $\alpha_2\beta_1$ [18].

In this work, we report that decorin directly binds VWF. This interaction is supported by the CS/DS side chain of the proteoglycan and does not involve the A1 domain of VWF. These results identify decorin as a new physiological partner for VWF, and provide evidence for a more complex interplay among collagen, VWF, and decorin in the organisation of the subendothelial matrix.

2. Materials and methods

2.1. Materials

Heparin, CS, DS, chondro-4-sulfatase, chondro-6-sulfatase, *o*-phenylenediamine dihydrochloride (OPD) tablets, and anti-collagen type I antibody were from Sigma (Milan, Italy). Chondroitinase ABC and ACII were from Seikagaku (Tokyo, Japan). Purified human VWF and isolated VWF A1 domain were prepared as described [19,20]. Microtiter plates were from Nunc International (Roskilde, Denmark). Collagen type I was purchased from Collagen Corporation (Freemont, CA, USA). Anti-VWF antibody, C20, was from Santa Cruz Biotechnology (Heidelberg, Germany). Peroxidase-conjugated anti-VWF antibody was from Dako (Lostrup, Denmark). The anti-A1 domain monoclonal antibodies have been previously characterised [21]. Peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Milan, Italy). Chemiluminescence reaction reagents were from Pierce.

2.2. Decorin purification and characterisation

Decorin was purified from bovine tendon or aorta by extraction with 4 M guanidine hydrochloride, ultracentrifugation on a CsCl gradient, and ion-exchange chromatography essentially as previously described [22,23]. Each decorin preparation was characterised by SDS–PAGE, N-terminal sequencing, and circular dichroism spectroscopy, as described [18,24]. Moreover, the presence of possible contaminating

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Abbreviations: VWF, von Willebrand factor; GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; LRRs, leucine-rich repeats

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collagen was investigated by a quantitative hydroxyproline assay and immunochemical detection. No hydroxyproline was detected and the amount of proline evaluated by amino acid analysis was consistent with that expected for purified decorin. No traces of collagen were detected using specific antibodies (data not shown).

2.3. Solid phase binding assay

Wells of microtiter plates were coated in triplicate with 4 μg of BSA, collagen, decorin, or decorin protein core (in 50 μl of PBS), or with 8 μg of heparin, purified decorin GAG chain, CS, or DS (in 50 μl of $NaHCO_3$ buffer, pH 9.6) for 16 h at 4 °C. The efficiency of coating with GAGs was verified by uronic acid assay on the adsorbed materials and found to be comparable for all the GAGs used in this study (data not shown). Upon coating, wells were washed four times with 500 μl of washing buffer (0.15 M NaCl, 0.05% Tween 20) and incubated with 100 μl of 1% BSA in PBS for 1 h at room temperature. After three washes, plates were incubated with 2 μg of purified VWF in 50 μl of PBS (unless otherwise stated) for 2 h at room temperature. Bound VWF was detected by incubation of the wells with peroxidase-conjugated anti-VWF antibody (1:5000 dilution in PBS containing 1% BSA and 0.05% Tween 20) for 2 h at room temperature. After three washes, the colorimetric reaction was initiated by addition of 50 μl of substrate solution (0.04% OPD, 0.04% H_2O_2, 514 mM Na_2HPO_4, and 24.3 mM citric acid, pH 5), and stopped, after 10 min, with 50 μl of 3 N HCl. Absorbance was measured at 490 nm using an ELISA microplate reader. In some experiments, the purified A1 domain (0.5 μg in 50 μl of PBS) instead of intact VWF was added to decorin, heparin, or collagen-coated wells. In these cases, bound protein was detected using a mixture of three different monoclonal antibodies against the A1 domain (6G2, CRI, and 5D2, 0.5 μg in PBS containing 1% BSA and 0.05% Tween 20) followed by a peroxidase-conjugated secondary anti-mouse antibody (1:5000 in PBS containing 1% BSA and 0.05% Tween 20). The colorimetric reaction was performed using OPD substrate, as described above.

2.4. Overlay experiments

Ten μg of decorin purified from aorta or tendon, BSA, or collagen were spotted onto nitrocellulose membranes using a Bio-Dot microfiltration apparatus (Bio-Rad). Membranes were blocked with 5% BSA in TBS for 1 h at room temperature and then incubated with purified VWF (40 μg/ml solution in PBS) for 16 h at 4 °C. Nitrocellulose membranes were then washed 4 times with 10 ml of washing buffer (0.05 mM Tris, 0.2 NaCl, 0.1 % polyethylene glycol 20 000, 0.1% BSA, and 0.05% Tween 20, pH 7.4) and then incubated with anti-VWF antibodies (1:200 dilution in washing buffer) for 2 h at room temperature. Upon extensive washing, membranes were incubated with peroxidase-conjugated secondary antibody and reactive protein visualised by enhanced chemiluminescence reaction.

2.5. Enzymatic digestions of decorin and analysis of disaccharide composition

Enzymatic digestions of decorin were performed to prepare the decorin protein core and to allow further characterisation of the GAG chains by HPLC analysis. Decorin was digested with chondroitinase ABC, chondroitinase ACII, chondro-4-sulfatase, chondro-6-sulfatase, or papain essentially as described [25,26]. After digestion with ABCase and ACII, the resultant disaccharides were analysed by HPLC using a Supelcosil column (TM-SAX 1, Supelco) equilibrated with 5 mM KH_2PO_4, pH 4.5. Elution was performed using a linear gradient (from 5 to 400 mM KH_2PO_4) at a flow rate of 1 ml/min, with detection at 232 nm. The percentage content of iduronic acid in the samples was calculated as described [27].

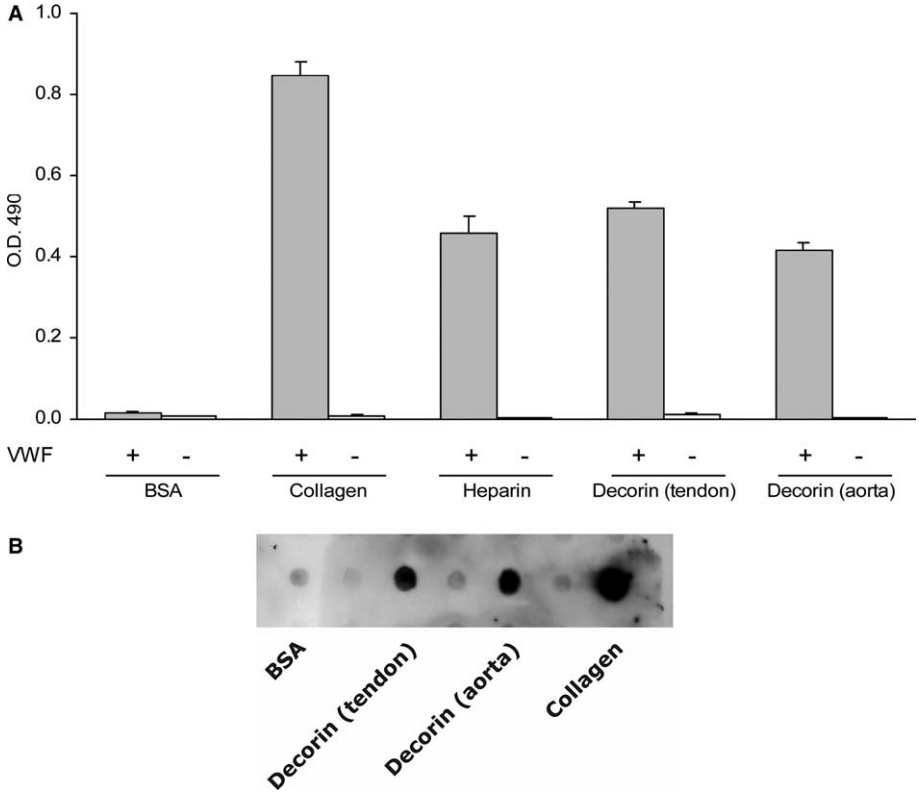

Fig. 1. Binding of VWF to decorin. (A) Polystyrene plates were coated with BSA, collagen, heparin, decorin from tendon and decorin from aorta, and then incubated with (+) or without (−) 2 μg of purified VWF for 2 h. Bound protein was revealed by a colorimetric reaction upon incubation with a peroxidase-conjugated anti-VWF antibody. Results are means ± S.D. of three different experiments. (B) BSA, decorin from tendon and decorin from aorta were spotted onto nitrocellulose and incubated with purified VWF. Bound protein was revealed by a chemiluminescent reaction after incubation with an antibody against VWF and a peroxidase-conjugated anti-mouse antibody. The figure is representative of three different experiments.

3. Results

A potential interaction between VWF and decorin was investigated by a solid-phase binding assay using immobilised ligands and soluble purified VWF. Fig. 1A shows that purified VWF failed to bind immobilised albumin, but interacted with both heparin and collagen, as expected. Moreover, VWF was found to also bind decorin purified from both tendon and aorta. The interaction between VWF and decorin was confirmed by dot-blot assay. Fig. 1B shows that decorin, purified from either tendon or aorta and spotted onto nitrocellulose, supported binding of VWF with similar efficiency. In the light of these results, subsequent experiments were performed with decorin obtained from tendon, a much richer and easier to obtain source of the proteoglycan.

A time course of VWF binding to immobilised decorin is reported in Fig. 2A. The amount of bound protein increased progressively and reached the maximum after 2 h of incubation. The specificity of VWF binding to decorin was then investigated by incubating increasing amounts of purified VWF with immobilised decorin or BSA. Fig. 2B shows that VWF binding to decorin was dose-dependent and saturable. A modified Scatchard-type analysis was performed according to a previously described approach [7,24,28,29] and revealed a single binding site for VWF, with an apparent Kd of about 20 nM.

Binding of VWF to decorin was drastically reduced by increasing the ionic strength, but was not affected by high concentrations of Triton X-100, suggesting an ionic rather than hydrophobic interaction (Fig. 3). In order to identify the region of decorin involved in VWF binding, solid-phase binding assays were performed with isolated protein core or GAG chain, obtained upon decorin digestion with chondroitinase ABC, or papain, respectively. Fig. 4 shows that decorin GAG chain was able to bind VWF in a very similar manner to the intact proteoglycan. By contrast, VWF only minimally interacted with the isolated decorin protein core. These results indicate that binding of VWF to decorin occurs mainly through interaction with the CS/DS chain.

The composition of the GAG side chain of decorin isolated from different tissues is rather variable in terms of disaccharide composition and sulfation level [14]. Therefore, in

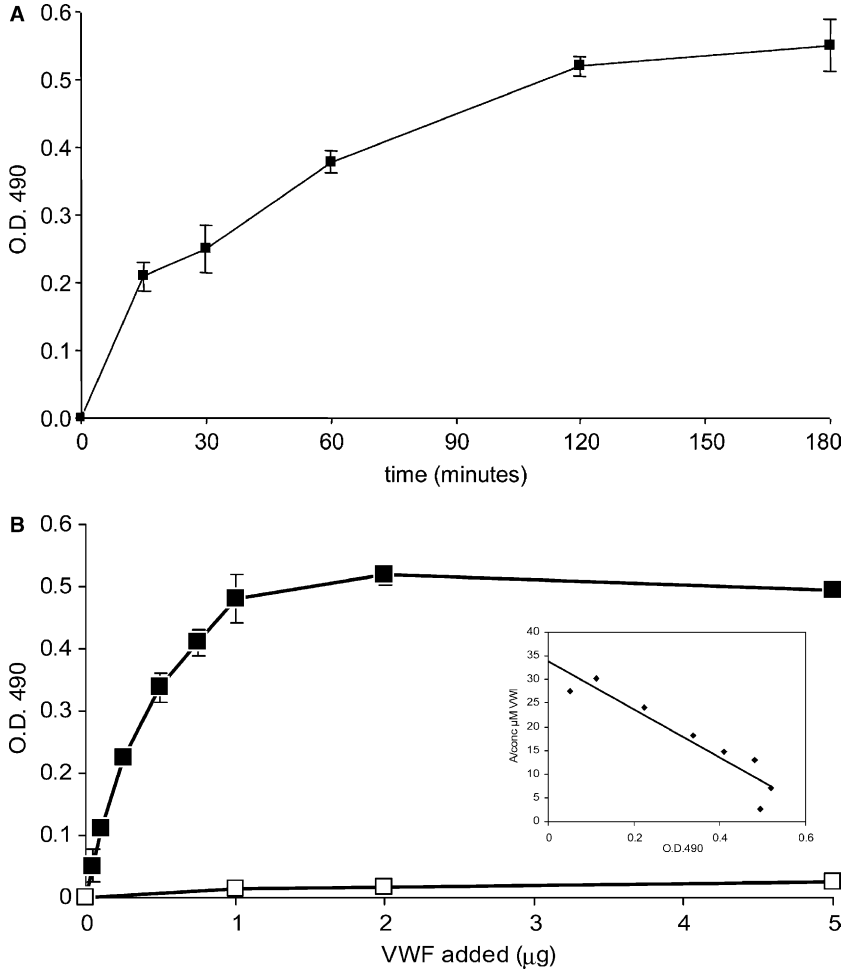

Fig. 2. Kinetics and saturation binding of VWF to decorin. (A) Immobilized decorin from tendon was incubated with 2 μg of purified VWF for increasing times, and the amount of bound protein was revealed by incubation with a peroxidase-conjugated anti-VWF antibody. Results are means ± S.D. of three different experiments. (B) Immobilised decorin (black squares) or BSA (open squares) was incubated with increasing amounts of purified VWF for 2 h, and bound protein revealed using a peroxidase-conjugated anti-VWF antibody followed by a colorimetric reaction. Data are means ± S.D. of three different experiments. The inset shows a Scatchard-type plot of the experimental data for VWF binding to decorin. The K_d value was calculated per VWF subunit, assuming a molecular mass of 275 kDa.

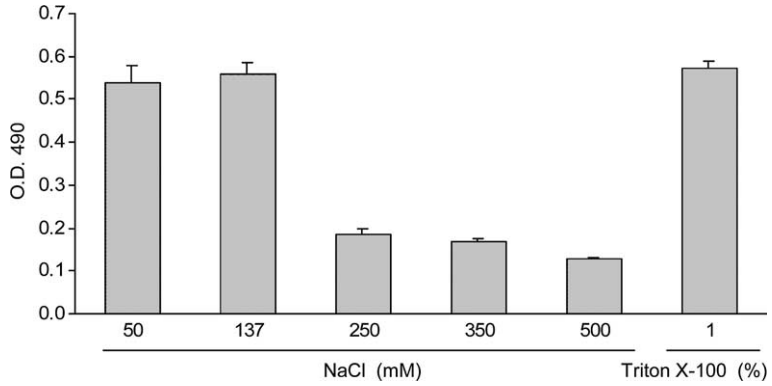

Fig. 3. Effect of increased ionic strength on VWF binding to decorin. Immobilized decorin was incubated with 2 μg of purified WVF in the presence of increased concentrations of NaCl, or 1% Triton X-100, as indicated. Bound protein was revealed upon incubation a peroxidase-conjugated anti-VWF antibody. Results are means ± S.D. of three different experiments.

order to get further insight into the structural requirements for VWF binding, we analysed the composition of the GAG chains of the decorin preparations from tendon and aorta. The results are summarised in Table 1. The GAG chain of decorin from tendon was primarily composed of CS, containing about 80% glucuronic acid-galactosamine disaccharide. By contrast, decorin from aorta was mainly composed of DS, as it contained about 80% iduronic acid-galactosamine. In both cases, however, the degree of sulfation of the GAG chain was very similar and greater than 90%. Fig. 4 clearly shows that removal of sulfate groups from the GAG chain completely abolished the binding of VWF to decorin. A comparable inhibition of VWF binding was observed upon digestion of decorin with either chondro-4-sulfatase, or chondro-6-sulfatase, or with the two enzymes added together (Fig. 4), suggesting that both 4- and 6-sulfates are important for protein interaction. Analysis of decorin GAG chain revealed that non-sulfated disaccharides increased from 8.86% to 78.25% upon treatment with chondro-4- and 6-sulfatases, confirming the efficiency of the digestion. Moreover, we found that, in addition to heparin, CS and DS were also able to bind VWF with similar efficiency (Fig. 4). As reported in Table 1, the CS used in these studies did not contain any detectable iduronic acid, while glucuronic acid in DS was about 2% of the total level of uronic acid. These results indicate that the different ratio of CS/DS in the GAG chain of decorin does not drastically affect VWF binding, as long as the negative charges of the sulfate groups are maintained.

We next investigated whether interaction with decorin could affect the ability of VWF to bind immobilised heparin. Fig. 5A shows that soluble decorin inhibited, in a dose-dependent manner, the interaction of VWF not only with immobilised decorin, but also with heparin. These results suggest that decorin and heparin may share common binding sites on the VWF molecule. Since a major heparin binding

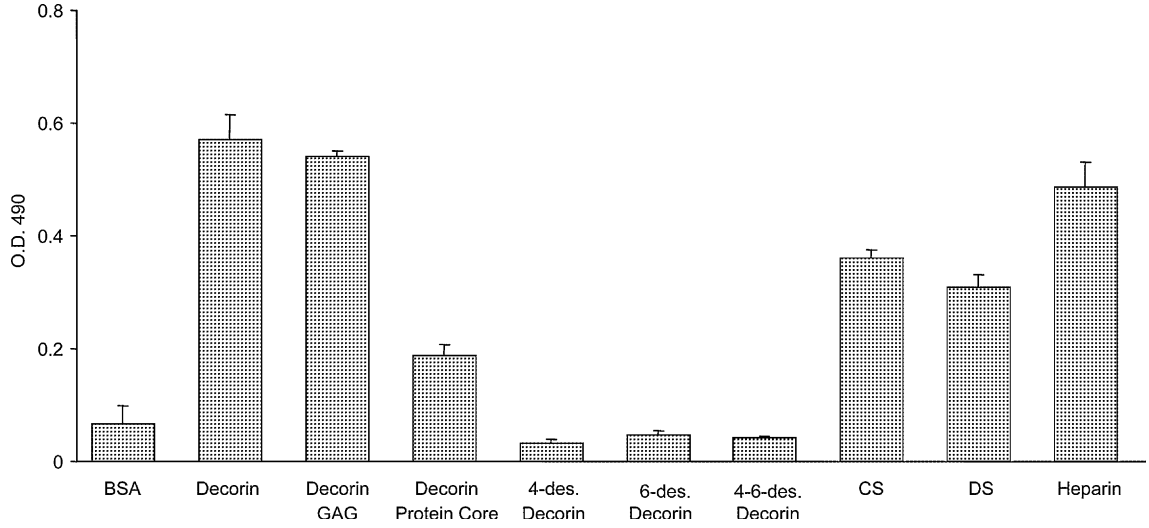

Fig. 4. VWF binding to GAGs. Wells of microtiter plates were coated with BSA, decorin, decorin GAG chain, decorin protein core, desulfated decorin obtained by digestion with chondro-4-sulfatase and chondro-6- sulfatase, alone and in combination (4-des. decorin, 6-des. decorin, and 4-6-des. decorin, respectively), chondroitin sulfate (CS), dermatan sulfate (DS) or heparin, as indicated. VWF binding to immobilised ligands was then measured as described above. Results are means ± S.D. of three different experiments.

Table 1
Analysis of GAGs composition

	GlcUA (%)	IduUA (%)	SO_4^{2-}/100 res
Decorin tendon	78.8	21.2	92.63
Decorin aorta	21.6	78.4	91.18
CS	100	0	96.59
DS	2.15	97.85	91.82

The GAG chains of decorin from tendon and from aorta, as well as commercially available CS and DS, were characterised in terms of percentage of glucuronic acid (GlcUA) and iduronic acid (IduUA), and sulfate content, as described in Section 2.

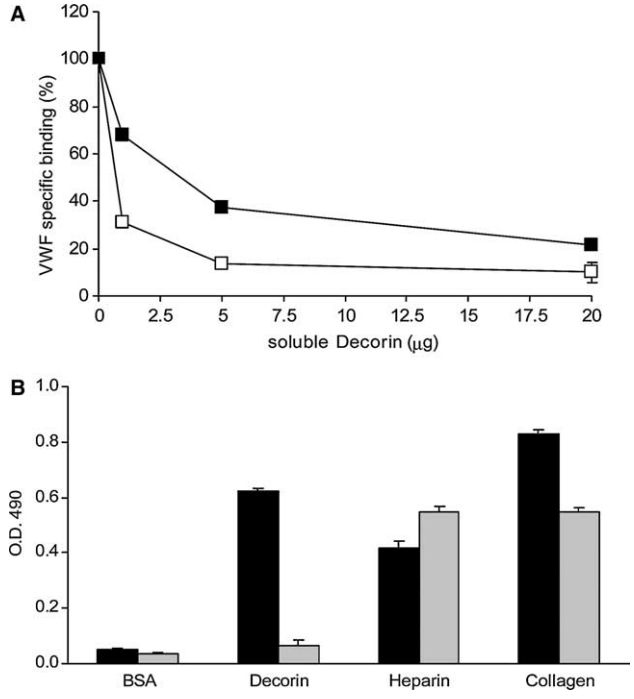

Fig. 5. Analysis of the decorin binding domain on VWF. (A) Purified VWF (2 μg) was incubated with immobilised decorin (black squares) or heparin (open squares) in the presence of increasing amounts of soluble decorin. Bound VWF was then revealed by reaction with a peroxidase-conjugated anti-VWF antibody. Results are expressed as % of specific binding, calculated upon subtraction of the binding to BSA coated wells. The binding of VWF in the absence of added soluble decorin has been considered as 100%. Results are means ± S.D. of three different experiments. When not evident, standard deviations are within the open or black squares. (B) Purified VWF (black bars) or isolated VWF A1 domain (dotted bars) was incubated with immobilised BSA, decorin, heparin and collagen for 2 h. The binding to the immobilised molecules was revealed by immunoreaction with anti-VWF antibody or with a mixture of three different monoclonal antibodies against VWF A1. Results are means ± S.D. of three different experiments.

site is located in the A1 domain of VWF [8,9], we then ascertained whether this domain was involved in the interaction with decorin. Fig. 5B shows that in contrast to intact VWF, the isolated A1 domain was unable to bind immobilised decorin, but efficiently recognised both heparin and collagen, as expected. Moreover, we found that three different monoclonal antibodies against the A1 domain did not alter the interaction of VWF with decorin (data not shown). Therefore, these results argue against a role for the VWF A1 domain in decorin binding.

4. Discussion

In this work, we have identified the small proteoglycan decorin as a new interactor of VWF. Direct binding between decorin and VWF was demonstrated by both solid phase and overlay assays, and found to be specific, dose-dependent, saturable, and mainly mediated by the GAG chain of the proteoglycan. Although the decorin protein core contains 10 LRRs similar to those present in the GPIbα subunit of the platelet receptor for VWF, it does not seem to play a major role in VWF binding. On the other hand, the finding that the decorin GAG chain fully supports interaction with VWF is in line with the reported ability of VWF to interact with heparin [7,8]. This interaction, whose biological significance is largely unclear, has been generally considered a circumstantial evidence for the ability of VWF to interact with proteoglycans containing sulfated disaccharides, although this has never been experimentally confirmed. Therefore, our results represent the first direct evidence for the ability of VWF to bind oligosaccharide chains different from heparin and physiologically present in proteoglycans of the extracellular matrix. The GAG chain of decorin is a mixture of CS and DS, whose relative abundance is greatly different, depending on the tissue type and age of the molecule [14]. In particular, we found that the iduronic acid content of decorin from bovine tendon was only about 20% of the total uronic acid content, but reached almost 80% in decorin from aorta. Despite this, both decorin preparations were equally able to bind VWF. Recently, decorin has been shown to also bind tenascin-X through the GAG chain, but this interaction has been found to be mediated by DS, but not CS, stretches [30]. Therefore, although the content of iduronic acid has been generally considered critical for the ability of GAG chains to display biological effects, our results indicate that this parameter is not significantly relevant for the ability of the GAG chain of decorin to bind VWF. This is also supported by the finding that purified CS chains, which did not contain iduronic acid, interacted with VWF with the same efficiency as DS chains containing almost exclusively iduronic acid. By contrast, we found that the degree of sulfation of galactosamine residues is critical for VWF binding. In fact, enzymatic removal of the sulfate groups completely abolished the ability of decorin to bind VWF. Therefore, interaction between decorin and VWF in different tissues may be modulated by the degree of decorin sulfation, rather than by a different degree of glucuronic acid conversion into iduronic acid.

Although decorin prevented VWF binding to heparin, our results with antibodies against the A1 domain, as well as with isolated A1 domain, which represents, together with a region in the N-terminus of the protein, a known heparin binding region, excluded a direct involvement of this domain in decorin binding. This finding may have physiological consequences. The A1 domain mediates many biological activities of VWF, including its interaction with collagen and platelets [4,6]. Therefore, it is likely that interaction with decorin does not directly compete with the ability of VWF to bind collagen or to stimulate platelets. Moreover, it is known that decorin binds to collagen through the protein core and is incorporated into

newly formed collagen fibres [15]. Moreover, collagen fibres can associate with multimeric VWF in the subendothelium, through direct binding to the A1 and A3 domains [11]. The results presented in this work indicate that an additional direct interaction may occur within this macromolecular complex between VWF and the GAG chain of decorin. Therefore, our results are indicative of a more complex organisation of the extracellular matrix, and suggest that a trimeric complex including collagen fibres, decorin and VWF is the real molecular entity of the subendothelial matrix that is presented to circulating platelets at the site of vessel wall injury. It is noteworthy that all three proteins can bind and activate platelets. Moreover, in contrast to the interaction with VWF, decorin binding to platelets involves the protein core rather than the GAG chain [18]. Whether interaction between decorin and VWF and their incorporation into collagen fibrils may result in an additive or synergistic effect on platelet activation is currently under investigation.

In conclusion, our results report a new direct interaction between two important elements of the extracellular matrix, the multimeric adhesive protein VWF and the small proteoglycan decorin. This finding provides new insights in the complexity of the architecture of the subendothelial matrix, and may have relevant physiological implications for the ability of these components to stimulate platelet adhesion and activation at the site of vascular injury.

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