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The vWFA2 domain of type VII collagen is responsible for collagen binding

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ABSTRACT

Type VII collagen (Col7) is the major component of anchoring fibrils and very important for skin integrity. This is emphasized by the Col7 related skin blistering diseases dystrophic epidermolysis bullosa and epidermolysis bullosa acquisita. Structural data that provides insights into the interaction network of Col7 and thus providing a basis for a better understanding of the pathogenesis of the diseases is missing.

We proved that the von-Willebrand-factor A like domain 2 (vWFA2) of Col7 is responsible for type I collagen binding. The interaction has a K_D value of 90 μM as determined by SPR and is enthalpy driven as derived from the van't Hoff equation. Furthermore, a hitherto unknown interaction of this domain with type IV collagen was identified. The interaction of vWFA2 with type I collagen is sensitive to the presence of magnesium ions, however, vWFA2 does not contain a magnesium binding site thus magnesium must bind to type I collagen.

A lysine residue has been identified to be crucial for type I collagen binding. This allowed localization of the binding site. Mutational analysis suggests different interaction mechanisms in different species and that these interactions might be of covalent nature.

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1. Introduction

Type VII collagen (Col7) is the major component of anchoring fibrils and responsible for connecting different skin layers together [1]. The importance of Col7 is highlighted by the skin blistering diseases dystrophic epidermolysis bullosa (DEB) and epidermolysis bullosa acquisita (EBA). DEB is caused by mutations in the type VII collagen gene [2] and EBA is characterized by a loss of tolerance against mainly the N-terminal non-collagenous domain [3,4]. The subdomains of the NC-1 domain have homology to proteins with adhesion functions and mediate interactions with other extracellular matrix proteins [5]. Quantitative data is available for the interaction with type I collagen, type IV collagen or laminin 5 [6].

We recently published an NMR study of the von-Willebrand-factor A like domain 2 (vWFA2) of murine Col7 and identified an interaction with the preceding subdomain [7]. This vWFA2 domain is recognized by autoantibodies in about 80% of EBA patients [3]. Additionally, it has been suggested that vWFA2 is responsible for type I collagen binding [8]. However, the importance of this interaction is subject of controversial discussions [6,8].

In this study we show that murine vWFA2 binds not only type I collagen, but also interacts with type III collagen and type IV collagen. This implies that vWFA2 recognizes the collagen triple helix. Thermodynamic data shows that the binding is enthalpy driven. The interaction is not metal dependent, but actually inhibited by the presence of magnesium ions. Chemical modification and mutations of vWFA2 lead to abrogation of the interaction with type I collagen thus allowing the localization of the binding interface for collagens at vWFA2. Our results have direct implications on the interaction network found at the dermal-epidermal junction and thus on skin stability.

2. Materials and methods

2.1. Expression and purification of vWFA2 wild type and mutant proteins

Expression of labeled and unlabelled proteins has been performed as described in [7]. Typical yields of labeled proteins have been 15 mg/l minimal medium. Mutations of the two lysine residues of vWFA2 have been introduced by the QuickChange Kit (Stratagene) according to the manufactures protocol. R73 and K74 have been changed to glutamine and arginine, respectively in the first mutant protein and K140 to asparagine in the second mutant protein. These mutated sequences represent the amino acid sequence in the human vWFA2 domain. It was anticipated to retain binding of vWFA2 mutants to murine type I collagen and to allow selective biotinylation of the other lysine residue for identification of the type I collagen binding site.

2.2. Biotinylation of vWFA2

Biotinylation was done according to the manufactures protocol. In brief, the buffer was changed to 0.1 M sodium carbonate pH 9.5.

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Protein concentration was above 0.25 mM. NHS-biotin (Sigma) was added from a freshly prepared stock solution (22 mg/ml in DMSO) and incubated for 12 h at room temperature. Final concentration of NHS-biotin was 6.4 mM (2.2 mg/ml NHS-biotin, 10% (v/v) DMSO). DMSO and remaining NHS-biotin have been removed by ultrafiltration (MWCO 10 kD) and the buffer exchanged to 10 mM sodium phosphate pH 7.4. The biotinylation reaction was performed with ^{15}N labeled proteins. ^{1}H,^{15}N-HSQC NMR experiments were used to control that the reaction was quantitative and that the proteins remain folded.

2.3. SPR measurements

SPR measurements have been performed with a BIAcore 3000. Binding of vWFA2 to murine type I collagen (isolated from skin by pepsin digestion; 1066, Chondrex, Inc), human type III collagen (isolated from placenta by pepsin digestion; 889, Yo Proteins), recombinant human type III collagen (345255, BD Biosciences), and murine type IV collagen (isolated from Engelbreth-Hol-Swarm lathrytic mouse tumor; 354233, BD Biosciences) was investigated. Collagen solutions have been prepared according to the suppliers protocols. Collagens dissolved in 10 mM sodium acetate pH 4 were immobilized on a CM5 chip according to the manufacturer protocol resulting in approx. 4000–7000 resonance units (RU). This corresponds to $RU_{max} \approx 500$ for monovalent binding of vWFA2. Experiments have been done at 25 °C in triplicate. Running buffer was 10 mM sodium phosphate buffer pH 7.4. For determination of the standard binding enthalpy ($\Delta H°$) and entropy ($\Delta S°$) a series of binding experiments between 5 °C and 30 °C has been performed. $\Delta H°$ and $\Delta S°$ have been calculated by the van't Hoff equation.

To investigate the influence of magnesium ions on the type I collagen vWFA2 interaction, 5 mM magnesium sulfate was present in the running buffer.

2.4. NMR measurements

A Bruker Avance 500 NMR spectrometer equipped with a TCI cryo probe was used for the experiments. Measurements have been performed at 298 K system temperature. ^{1}H,^{15}N-HSQC have been recorded to verify correct folding of mutant proteins and biotinylated samples. All samples were prepared in 10 mM sodium phosphate buffer pH 7.4 (pH meter reading), 10% (v/v) D_2O. Protein concentrations ranged from 0.1 to 2 mM of protein. Referencing was done via the TSP-d_4 main signal and in the indirect dimension by the Ξ scale (factor for ^{15}N: 0.101329118) or via the signal of G175 (^{1}H: 10.53 ppm, ^{15}N: 113.24 ppm). Data analysis has been done with Topspin 3.1 (Bruker) and CCPN [9].

Manganese(II) sulfate or magnesium sulfate has been added to 0.1 mM vWFA2 to identify potential metal-ion binding sites at vWFA2. Concentrations of manganese(II) sulfate were 50 μM and 150 μM, respectively and 5 mM for magnesium sulfate.

3. Results

3.1. The vWFA2 domain binds collagens

SPR measurements of immobilized type I, III and IV collagen showed an interaction with murine vWFA2 (Fig. 1, Supplemental Fig. A1). The interaction of vWFA2 with murine type I collagen (from skin) and human type III collagen (recombinant and from placenta) shows a K_D of 90 μM at 25 °C. The strength of the interaction with murine type IV collagen is slightly weaker with a K_D of 200 μM. Although data has been fitted to a 1:1 binding stoichiometry, the maximal measured response clearly shows that there must be multiple binding sites.

Measuring the binding affinity at different temperatures allowed determination of the standard binding enthalpy and entropy (Fig. 1). Evaluation of the van't Hoff plot shows that the interaction of vWFA2 with the collagens is enthalpy driven (Table 1). The free energies calculated either from the measured K_D values or from the thermodynamic parameters obtained from the van't Hoff plot are identical.

3.2. Identification of the collagen binding site of the vWFA2 domain

Domains with the von-Willebrand fold can bind collagens either in a metal dependent or independent mode and the binding sites are at different areas. Murine vWFA2 contains two lysine residues with Lys74 present at one potential binding site. This led to the assumption that modifying the lysine side chains influences the binding affinity. To test this, vWFA2 was treated with NHS-biotin, thus attaching biotin to free amino groups i.e. the two lysine side chains and the amino terminus. The protein remains folded and surprisingly, this modification causes abrogation of the vWFA2 type I collagen interaction (data not shown).

To narrow the type I collagen binding at vWFA2 down, two mutant proteins have been generated. In the first mutant protein the amino acids R73 and K74 have been mutated to Q and R, respectively (vWFA2-QR-mutant). These are the amino acids in the human protein and changing these amino acids yields a humanized binding interface as seen in the homology model (Fig. 3). The second lysine, K140, is changed to N, again the corresponding amino acid in the human protein (vWFA2-N-mutant). Both mutants behaved as the wild type protein during expression and purification. ^{1}H,^{15}N-HSQC spectra indicate proper folding with changes in resonances related to the mutations. Both mutant proteins remained folded after biotinylation of the remaining lysine and the N-terminal amino group as shown by NMR spectroscopy.

The vWFA2-N-mutant still bound to type I collagen with slightly lower affinity (200 μM) than the wild type protein. No interaction was detected with biotinylated vWFA2-N-mutant. Surprisingly, the vWFA2-QR-mutant was unable to bind immobilized type I collagen in the SPR experiments.

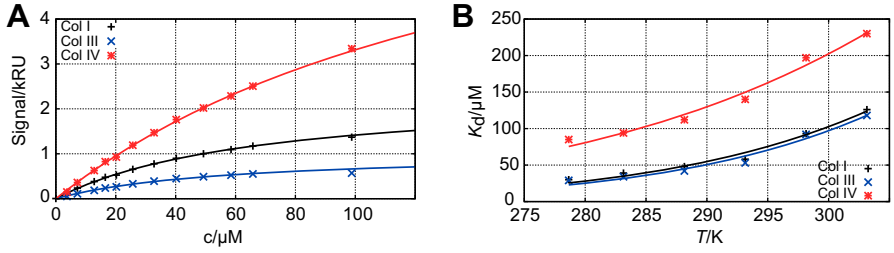

Fig. 1. (A) K_D determination of the vWFA2 with types I, III, and IV collagen by SPR. The measured K_D values in this plot are 68 ± 3 μM (Col I), 57 ± 4 μM (Col III), and 163 ± 13 μM (Col IV), respectively. The sensorgrams are given in Supplemental Fig. A1. (B) van't Hoff Plot for determination of standard binding enthalpy ($\Delta H°$) and entropy ($\Delta S°$). The determined values are given in Table 1.

Table 1
Affinity constants and thermodynamic parameters for the vWFA2 collagen interaction.

Collagen	K_D (25 °C) in μM	$\Delta G°$ (25 °C) in kJ/mol		$\Delta H°$ in kJ/mol	$\Delta S°$ in J/K
I, murine (skin)	93 ± 26 [a]	−23.0 ± 0.6 [b]	−23.0 ± 7.3 [c]	−45.3 ± 3.7	−74.7 ± 12.2
III, human (placenta)	92 ± 38 [a]	−23.0 ± 0.9 [b]	−23.2 ± 9.3 [c]	−47.1 ± 4.7	−80.2 ± 15.4
IV, murine (lathrytic)[d]	197 ± 31 [a]	−21.2 ± 0.4 [b]	−21.3 ± 5 [c]	−32.1 ± 2.5	−36.3 ± 8.4
III, human (recombinant)	63 ± 3	−24.0 ± 0.1 [b]	n. d.	n. d.	n. d.

[a] Mean K_D values are measured in triplicate.
[b] The free enthalpy $\Delta G°$ was calculated from the measured K_D values according $\Delta G° = RT\ln K_D$.
[c] $\Delta H°$ and $\Delta S°$ derived from the van't Hoff Plot were used to calculate the free enthalpy $\Delta G°$.
[d] isolated from Engelbreth-Hol-Swarm lathrytic mouse tumor.

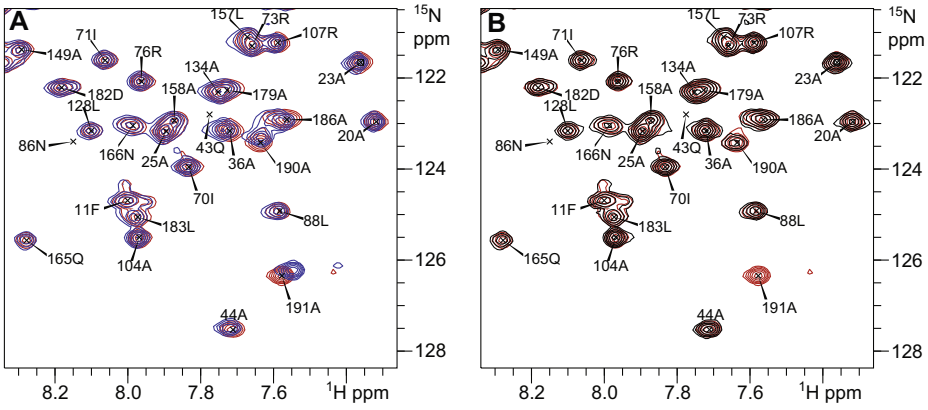

Fig. 2. 500 MHz ^1H,^{15}N-HSQC spectra of 0.1 mM ^{15}N-labeled vWFA2 (red) in presence of 5 mM $MgSO_4$ (A, blue) and 150 μM $MnSO_4$ (B, black). Only peaks that belong to or that are nearby to the N- and C-terminus are shifted in presence of $MgSO_4$. In presence of $MnSO_4$ the signals of residues A191 and G5 disappear due to paramagnetic relaxation. Therefore a natural occurring metal binding site in vWFA2 can be excluded. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

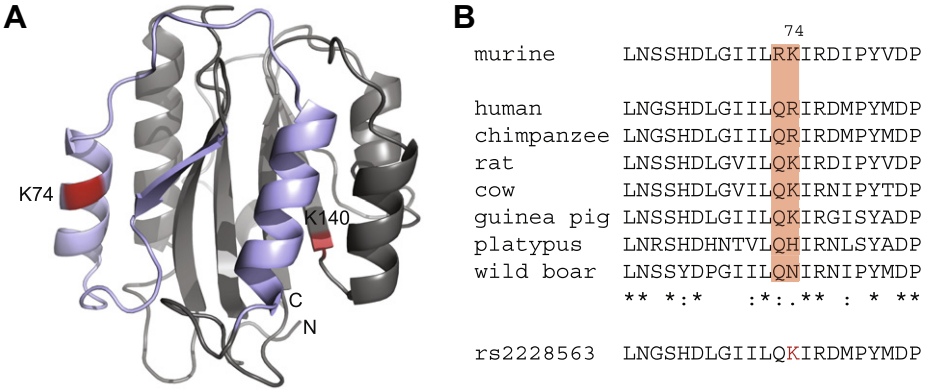

Fig. 3. Binding site of type I collagen to vWFA2 (A). Modification or mutation of K74 abrogates binding of type I collagen. Lysine residues that allowed identification of the binding site are highlighted in red. Binding of collagen to von-Willebrand domains involves either residues on the top in this model or in the front. Because K74 is important for type I collagen binding the binding site must be in front of this model (highlighted in lightblue). Multiple sequence alignment of the vWFA2 domain in different species (B, generated with Clustal Omega). The important residues R73 and K74 are highlighted by a red bar. rs2228563 is SNP in human with a prevalence of about 2 %. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Effects of Mg^{2+} and Mn^{2+} on the vWFA2 domain

As some von-Willebrand domains can bind collagens in a metal dependent way, the influence of magnesium on the interaction was investigated. No interaction of vWFA2 with type I collagen can be detected in SPR measurement with 5 mM $MgSO_4$ in the buffer (Supplemental Fig. A2). NMR measurements have been performed to investigate whether vWFA2 does contain an Mg^{2+} binding site. ^1H,^{15}N-HSQC of ^{15}N labeled vWFA2 in presence of 5 mM $MgSO_4$ showed a distinct shift of resonances of amino acids at the C- and N-terminus, e.g. residues A191, Q189 and G5 (Fig. 2). The cross peaks of G5 and A191 show reduced intensity at 50 μM $MnSO_4$ and disappear completely at 150 μM (Fig. 2).

4. Discussion

Anchoring fibrils consists of Col7 and link different skin layers together. Col7 related diseases result in severe skin blistering. Despite the vast clinical data available for Col7 related diseases, there is a basic knowledge gap on detailed structural data on the interaction network of Col7 with other extracellular matrix components.

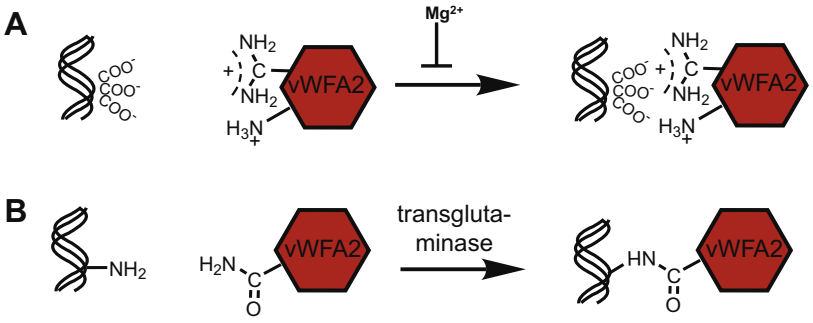

Fig. 4. Possible mechanisms of the vWFA2 type I collagen interaction. The interaction in mouse is based on electrostatic interactions and can be modulated by the presence of magnesium (A). Species that have a glutamine residue in the vWFA2 could form covalent cross links by the formation of isopeptide bonds (B). This requires the action of transglutaminase, an enzyme that is present in skin.

4.1. vWFA2 binds type I collagen

Col7 binds type I collagen but this interaction has been suggested to be of minor importance [6]. Later, it was implied that it is the vWFA2 subdomain of Col7 that interacts with type I collagen and that this interaction is of relevance [8]. We performed SPR measurements with the murine vWFA2 subdomain and murine type I collagen. The K_D of this interaction is of lower magnitude than the K_D for the human proteins determined with the complete NC1 domain by Brittingham and colleagues [6]. However, this study [6] used NC1 that formed dimers and even trimers. This can cause multivalency effects resulting in a higher observed affinity and could also explain the observed interaction of human proteins. Taken this into account and the work of Villone et al. [8] it is very likely that vWFA2 is the sole type I collagen binding site in the NC1 domain.

4.2. vWFA2 also recognizes type IV collagen

Type III collagen and type IV collagen are also recognized with similar affinities than type I collagen. In a previous study, the interaction of NC1 with type IV collagen was found to be of high affinity with a K_D of 2 nM [6]. The major binding site comprises the fibronectin III like domains 7–9 [10]. This region comprises also the binding site for laminin 5. Our data shows that type IV collagen has a second binding site for Col7 that is the vWFA2 subdomain. Because vWFA2 is binding types I and III collagen it is very likely the collagenous domain of type IV collagen that is recognized by vWFA2. Although the binding affinity is of even slightly lower affinity than the vWFA2 type I collagen interaction, in skin this interaction might be more relevant. Since type IV collagen and laminin 5 share a common binding site at the fibronectin III like domains 7–9 in the NC1 domain, this second binding site could enable binding of type IV collagen even if laminin 5 is bound.

4.3. The collagen binding site of vWFA2 is near K74

Combining mutagenesis data and modification of lysine residues, the collagen binding site could be located near K74 and must be similar as for the blood von-Willebrand-factor A3 domain [11]. The binding site does not imply a contribution of a metal ion dependent binding site (MIDAS-motif) as it has been found for integrin α2β1 [12]. However, presence of magnesium ions results in loss of type I collagen binding by vWFA2. Chemical shift mapping identified three resonances that are influenced upon addition of magnesium. These residues are located at the termini and represent an artefact since in native Col7 the vWFA2 subdomain does not have free termini. Because no other resonances are influenced upon addition of metal ions we conclude that vWFA2 does not bind metal ions and that the magnesium must bind to type I collagen. Mg^{2+} binding sites in globular proteins can be identified by bioinformatical approaches [13,14] but these methods have not yet been applied to collagens. Since collagens bind magnesium [15], identification of magnesium binding sites on type I collagen would narrow the motifs that are recognized by vWFA2 readily down. In addition, the presence of several vWFA2 binding sites on type I collagen implies a redundancy for this interaction, i.e. a mutation in type I collagen that abrogates the interaction at one binding site can be compensated.

4.4. Mechanisms of the vWFA2 collagen interaction

Inhibition of type I collagen binding by vWFA2 in presence of magnesium and the presence of two positively charged residues (arginine and lysine) in consecutive positions indicates that electrostatic interactions are important (Fig. 4). Biotinylation of K74 as well as mutation of R73K74 to QR abolishes binding of vWFA2 to type I collagen showing that this interaction is specific.

Electrostatic interactions are common in the extracellular matrix (e.g. proteoglycan-collagen interactions [16]). Surprisingly, a multiple sequence alignment of Col7 vWFA2 domains shows that only the murine protein has two charged residues at this position (Fig. 3). A glutamine seems more important due to its occurrence in all other species. We investigated a vWFA2 mutant with Q73R74 representing the human sequence. This protein showed no interaction with type I collagen. There are several explanations for that observation. Type I collagen could contain only a few binding sites for the humanized mutant thus resulting in much lower signal responses. However, the experimental conditions in [8] i.e. treatment with denaturating agents point toward a covalent interaction between vWFA2 and type I collagen. There are different cross-links imaginable with a lysine residue at type I collagen as a prerequisite. The side chains of lysine residues in collagens can be oxidized thus forming allysines. Allysines mediate cross linking of the collagens [17,18] by formation of an azomethines (Schiff base). However, binding of vWFA2 to recombinant type III collagen renders this mechanism unlikely. Recombinant type III collagen does not contain aldehyde groups. In addition, allysine residues are only found in the telopeptides [17] that are principally removed during the extraction procedure of types I and III collagens used in this study.

The presence of glutamine in the humanized protein and a lysine residue in the murine protein indicates a covalent linkage based on the formation of an isopeptide bond catalyzed by the tissue transglutaminase [19]. The isopeptide bond is formed between a glutamine side chain and the ε-amino group of a lysine residue. Col7 is a target for tissue transglutaminase [20]. In addition, *Col7a*

knockout mice show severe skin blistering that can be rescued by expression of the human *Col7a* gene [21].

Very interestingly, there is single nucleotide polymorphism in human (variant rs2228563) that is present in about 1–2% of the population (www.ensembl.org.) This polymorphism results in a change of arginine to lysine. If our hypothesis is correct, these individuals could show both types of cross linkages.

The present study shows for the first time, that the vWFA2 domain is the collagen binding site in Col7. This interaction is not restricted to type I collagen, also type III and IV collagen are recognized. In mouse the interaction relies on electrostatic interactions and is enthalpy driven. Presence of magnesium ions causes loss of the interaction that would allow modulation of the interaction in vivo. In a humanized protein, no interaction can be found. However, as the amino acid residues at the binding site are highly conserved between species this supports the hypothesis that in human the interaction is of covalent nature and indicates an isopeptide link formation catalyzed by tissue transglutaminase. The detailed structural insights into the interaction network of Col7 will lead to an improved understanding of the skin structure and the pathogenesis of skin blistering diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.119.

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