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A synthetic peptide corresponding to the 550–585 region of α-dystroglycan binds β-dystroglycan as revealed by NMR spectroscopy

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Abstract We have probed the binding of a synthetic peptide corresponding to the region 550–585 of the α subunit of dystroglycan with a recombinant protein fragment corresponding to the N-terminal extracellular region of β-dystroglycan (654–750), using NMR in solution. In a 30:1 molar ratio, the peptide binds to the recombinant protein fragment in the fast/intermediate exchange regime. By monitoring the peptide intra-residue HN–Hα peak volumes of the 2D TOCSY NMR spectra, both in the absence and in the presence of the recombinant fragment, we determined the differential binding affinities of each amino acid. We found that the residues in the region 550–565 (SWVQFNSNSQLMYGLP) are more influenced by the presence of the protein, whereas the C-terminal portion is marginally involved. These NMR results have been confirmed by solid-phase binding assays.

Key words: Mapping binding site; Nuclear magnetic resonance spectroscopy; Binding epitope; Dystroglycan; Protein–protein interaction

1. Introduction

Dystroglycan (DG) is a membrane receptor that provides a linkage between the cytoskeleton (via dystrophin binding) and the extracellular matrix (via binding to several proteins and proteoglycans) [1,2]. It is a member of the dystrophin–glycoprotein complex (DGC) involved in the maintenance of the structural integrity of sarcolemma in skeletal muscle fibres. In fact, disruption of the DGC is observed in various forms of muscular dystrophy [3,4]. DG was found also to play a crucial role in early development [5]. The receptor is constituted by two subunits, α and β, which arise from post-translational cleavage of a single polypeptide chain [1]. β-DG, which contains the transmembrane domain, interacts with dystrophin and dystrophin-related proteins inside the cytoplasm, whereas α-DG, the highly glycosylated peripheral membrane subunit, binds extracellular matrix proteins containing laminin-type LG domains [6–8]. It is likely that DG also plays a role as a signal transduction protein [9].

Although many biological studies have been devoted to the elucidation of the role of DG in both development and disease [2], very few structural studies have been reported [10]. In fact, the large size of DG (895 amino acids, α-DG = 1–653 and β-DG = 654–895), its high level of glycosylation and its membrane-bound nature make this protein an extremely challenging system using classical structural methods (X-ray and nuclear magnetic resonance (NMR)).

One successful approach has been to dissect this large complex and to study the different subdomains as separate independent entities [11–13]. Using a full series of recombinant fragments and solid-phase binding assays, we found that a region as short as 36 amino acids (550–585) of the α subunit is crucial for interactions with the β subunit (Sciandra, Schneider, Giardina, Baumgartner, Petrucci and Brancaccio, submitted).

In this study, using NMR spectroscopy we have investigated the binding of a synthetic α-DG peptide (550–585) to the recombinant protein fragment corresponding to the entire N-terminal extracellular region of β-DG (654–750) and identified the amino acids responsible for the interaction.

2. Materials and methods

2.1. Recombinant proteins and synthetic peptide

Standard polymerase chain reaction (PCR) protocols were used to amplify the DNA constructs required for the production of the recombinant dystroglycan fragments under analysis. β-DG(654–750) and α-DG(485–651) were prepared as described elsewhere [13]. To prepare the three deleted α-DG fragments, α-DGC(485–585), α-DGC(485–572) and α-DGC(485–550), the same forward primer, 5′-CCC**GTCGAC**AGTGGAGTGCCCCGTGGGGGAGAAC-3′ (*Sal*I restriction site in bold and target sequence underlined) was used together with different 3′ primers, respectively: 5′-CCC**GAATTC**TTAGAGGCCCCCTTTGTCTGTGGCATGC-3′ for α-DGC(485–585), 5′-CCC**GAATTC**TTATTTTCCCACATGGCTGCTGTCAGGC-3′ for α-DGC(485–572), and 5′-CCC**GAATTC**TTACGATTTCTCACCTACTAACTGCTGC-3′ for α-DGC(485–550) (*Eco*RI restriction site in bold and target sequences underlined). The recombi-

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Abbreviations: DG, dystroglycan; DGC, dystrophin–glycoprotein complex; α-DGC, C-terminal region of the α-dystroglycan subunit; β-DG, N-terminus of the β-dystroglycan subunit

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nant proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified as described elsewhere [12,13]. The synthetic peptide α-DGC(550–585) was purchased from Synt:em and further purified using HPLC chromatography. The purified peptide was dissolved in a solution (90% H_2O and 10% D_2O) containing 0.05% acetate buffer and the pH was adjusted to a final value of ∼4.0.

2.2. Solid-phase binding assays

Recombinant β-DG(654–750) was biotinylated as described elsewhere [13]. To assess the binding properties of recombinant α-DG proteins to β-DG(654–750), solid-phase assays were performed as follows: 0.1–0.5 μg/well of each recombinant protein was immobilised on microtitre plates by overnight incubation at 4°C in coating buffer (50 mM $NaHCO_3$ pH 9.6). The plates were washed three times with Tris-buffered saline (TBS), 0.05% Tween-20 (T-TBS), containing 1.25 mM $CaCl_2$ and 1 mM $MgCl_2$ (wash buffer), and then incubated for 1 h in T-TBS containing 3% milk powder for blocking unspecific binding sites. After extensive washing with wash buffer, wells were incubated with biotinylated β-DG(654–750) in T-TBS, 1% bovine serum albumin (BSA), 1% milk powder, 1.25 mM $CaCl_2$ and 1 mM $MgCl_2$ for 3 h at room temperature. The biotinylated β-DG(654–750) bound fraction was detected with the Vectastain AB complex from Vector Laboratories (Burlingame, CA, USA). 5 mg of *p*-nitrophenyl phosphate in 10 ml of 10 mM diethanolamine and 0.5 mM $MgCl_2$ was used as substrate for the alkaline phosphatase and quantitative measurements were carried out at 405 nm. The absorbance values were corrected for the signals obtained incubating biotinylated β-DG(654–750) only with BSA (∼0.5 μg/well). Data were fitted using a hyperbolic model for a single class of binding sites according to the equation: $OD_i = OD_{tot} \times c/(K_d+c)$, where OD_i represents the absorbance measured at increasing concentrations of ligands, K_d is the binding dissociation constant, c is the concentration of biotinylated β-DG(654–750), and OD_{tot} the absorbance at saturation. Data were normalised according to the equation: OD_i/OD_{tot} and are reported as fractional saturation (%).

Table 1
Proton NMR assignments for α-DG(550–585) in H_2O:D_2O (90:10) at pH 4.0 and 25°C

Residue	NH	αH	βH	Others
S550	8.21	4.14		
W551	8.65	4.75	3.27, 3.23	2H 7.22, 4H 7.59, 5H 7.11, 6H 7.21, 7H 7.46, NH 10.10
V552	7.91	3.92	1.84	γCH_3 0.78, 0.74
Q553	8.12	4.08	1.89, 1.83	γCH_2 2.18, 2.14, δNH_2 7.43, 6.84
F554	8.20	4.57	3.08, 2.99	2,6H 7.20, 4H 7.26, 3,5H 7.30
N555	8.32	4.66	2.77, 2.65	γNH_2 7.52, 6.86
S556	8.36	4.37	3.92	
N557	8.44	4.74	2.86, 2.79	γNH_2 7.57, 6.91
S558	8.20	4.32	3.90, 3.85	
Q559	8.29	4.27	2.11, 1.98	γCH_2 2.34, δNH_2 7.47, 6.83
L560	8.01	4.24	1.59	γCH1.48, δCH_3 0.89, 0.82
M561	8.10	4.36	1.92, 188	γCH_2 2.40, 2.33
Y562	8.03	4.54	3.09, 2.90	2,6H 7.09, 3,5H 6.79
G563	8.16	3.87		
L564	7.99	4.60	1.62, 1.58	γCH 1.55, δCH_3 0.93
P565		4.07	2.25, 2.00	γCH_2 1.89, δCH_2 3.63
D566	8.40	4.58		
S567	8.32	4.34	3.95, 3.85	
S568	8.28	4.32	3.83	
H569	8.31	4.71	3.29, 3.13	2H 8.55, 4H 7.25
V570	7.97	4.09	2.07	γCH_3 0.93
G571	8.50	3.92		
K572	8.13	4.27	1.76, 1.67	γCH_2 1.39, 1.32, δCH_2 1.63, εCH_2 2.96
H573	8.56	4.58	3.12, 3.08	2H 8.55, 4H 7.12
E574	8.37	4.23	1.87, 1.81	γCH_2 2.24, 2.18
Y575	8.19	4.46	2.91, 2.83	2,6H 7.02, 3,5H 6.75
F576	8.04	4.55	3.05, 2.92	2,6H 7.17, 4H 7.25, 3,5H 7.29
M577	8.16	4.32	1.95, 1.89	γCH_2 2.44, 2.39
H578	8.37	4.66	3.27, 3.14	2H 8.59, 4H 7.28
A579	8.42	4.35	1.40	
T580	8.17	4.33	4.24	γCH_3 1.19
D581	8.30	4.61	2.80, 2.77	
K582	8.34	4.28	1.89, 1.75	γCH_2 1.46, 1.40, δCH_2 1.67, εCH_2 2.98
G583	8.42	3.94		
G584	8.23	3.94		
L585	8.13	4.30	1.67, 1.59	δCH_3 0.92, 0.86

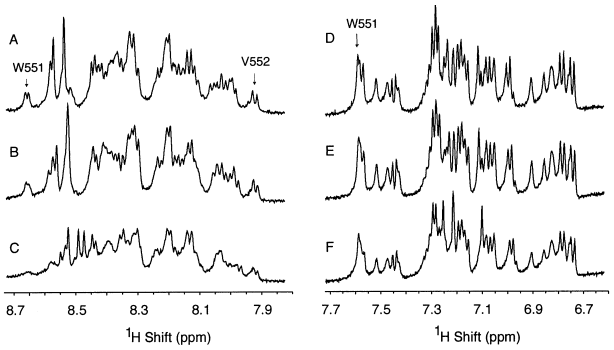

Fig. 1. 1D spectra of 1 mM synthetic α-DGC(550–585) in sodium acetate buffer 0.05%, pH 4.0, at 25°C showing line broadening effects in the amide (A–C) and aromatic (D–F) regions. Peaks referring to Trp^{551} and Val^{552} are indicated by arrows. Different [α-DGC(550–585)]:[β-DG(654–750)] ratios are reported: (A, D) α-DGC(550–585) in the absence of β-DG(654–750), (B, E) 100:1, (C, F) 30:1.

2.3. NMR experiments

All NMR experiments were run on Varian Unity Plus 600 and 800 MHz spectrometers equipped with a triple-resonance probe and a triple-axis gradient unit. The temperature was held constant at 298 K. The proton assignments of the α-DGC(550–585) peptide were obtained with a series of TOCSY and NOESY experiments [14]. The number of scans was 32 for 512 data points in the indirect dimension. The mixing times ranged from 40 to 70 ms for the TOCSY spectra and from 200 to 250 ms for NOESY spectra. The concentration of α-DGC(550–585) peptide was ∼1 mM and a 1:30 [β-DG(654–750)]:[peptide] ratio was used. The spectra were recorded with spectral widths of 7000 Hz and 9000 Hz on the 600 MHz and 800 MHz spectrometers, respectively. Water suppression was achieved using WATERGATE [15] pulse sequence. Data were processed using NMRPIPE [16] and the peak volumes were calculated using a Gaussian fit enclosed in the Sparky software package (http://www.cgl.ucsf.edu/home/sparky/).

3. Results

The primary sequence of the synthetic peptide spanning the amino acid sequence 550–585 of the C-terminal domain of vertebrate α-DG is reported in Table 1 together with the complete resonance assignment, which was accomplished using $^1H/^1H$ NOESY and TOCSY experiments [14]. In the absence of the β-DG(654–750) fragment, the amide region of the peptide displays sharp and poorly dispersed resonances typical of random coil conformation (see Fig. 1A). Further, the $^1H/^1H$ NOESY spectra show only the presence of sequential NOEs in both aliphatic and amide regions (data not shown). From these observations, we conclude that in the absence of the β-DG(654–750) fragment, the peptide is mostly unstructured in aqueous solution. Upon titration with increasing amounts of β-DG(654–750), we observed a general broadening of the peptide resonances (Fig. 1B,C,E,F), indicating that the peptide assumes the correlation time of the β-DG(654–750). From a closer analysis of the 1D ^1H spectra of the peptide amide and aromatic region, it becomes clear that the peptide interacts with the protein at a ratio of 100:1,

showing a high affinity for the extracellular fragment of β-DG(654–750) (see Fig. 1B,E), and that some of the residues are more involved in the binding than others as shown by the differential broadening of the amide and aromatic signals. In Fig. 1C,F, where the molar ratio [peptide]:[protein] is 30:1, one can see that the isolated peak of Trp^{551} is particularly influenced by the presence of the protein, whereas other resonances appear to be very sharp. At [peptide]:[protein] molar ratios lower than 20:1, the peptide resonances become undetectably broad (data not shown). Based on these results, the 30:1 [peptide]:[protein] molar ratio was chosen as the best ratio at which to reveal the differential broadening of the lines and to carry out all our 2D TOCSY experiments. Under these conditions, we can determine which amino acids are responsible for the binding using differential line broadening [17–19]. We found that the presence of β-DG(654–750) significantly influences the peptide TOCSY spectrum in the fingerprint region (Fig. 2A,B). A general broadening of the crosspeaks is observed, but some differential effects are remarkable, indicating that a number of residues belonging to synthetic peptide α-DGC(550–585) interact preferentially with β-DG(654–750). This can be observed in particular for residues Trp^{551}, Val^{552}, Gln^{553}, Asn^{555}, Gln^{559}, Leu^{560}, Met^{561} and Gly^{563}. The differential broadening is illustrated in more detail in Fig. 3 where two selected crosspeaks of the TOCSY spectra and their slices, referring to Gln^{553} and Val^{570}, show very different intensity ratios upon addition of β-DG(654–750), suggesting that Gln^{553} participates more in the binding with β-DG(654–750) than Val^{570}.

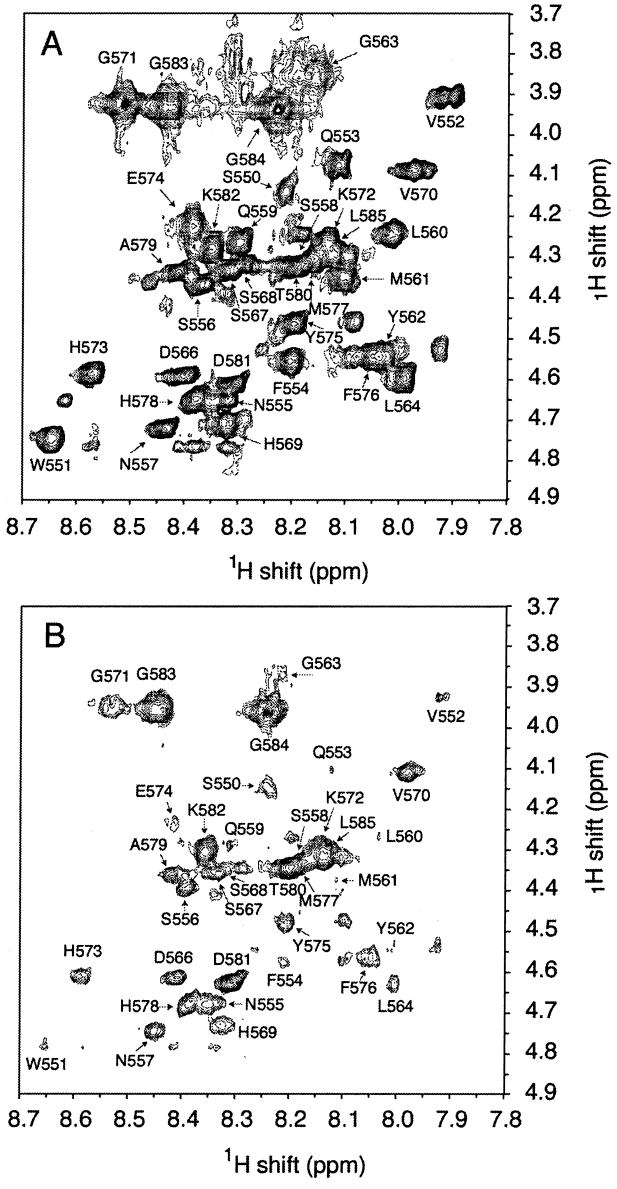

Fig. 2. 2D TOCSY spectra in the fingerprint region of 1 mM synthetic α-DGC(550–585) in sodium acetate buffer 0.05%, pH 4.0, at 25°C in the absence (A) and in the presence (B) of 30 μM β-DG(654–750).

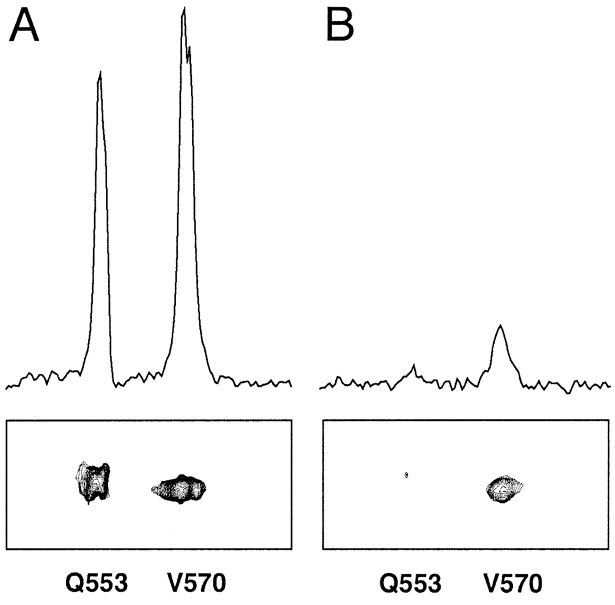

Fig. 3. Selected region of the TOCSY spectra of 1 mM synthetic α-DGC(550–585) in sodium acetate buffer 0.05%, pH 4.0, at 25°C, in the absence (A) and in the presence (B) of β-DG(654–750) 30 μM (bottom). At the top of the figure are shown the slices of the same crosspeaks. A part of the drastic loss of signal to noise ratio from A to B, a significant difference in the relative intensity of the resonances is clearly observed.

In order to determine if changes in peptide backbone conformation are induced by β-DG(654–750), we also performed transfer NOESY experiments (data not shown). Although the interpretation of these experiments was limited by the spectral overlap of several connectivities, more intense transfer NOEs than their corresponding NOEs were observed. It is likely that these effects are due to the increased correlation time associated with the peptide/β-DG(654–750) complex resulting in more efficient cross-relaxation between proton pairs in proximity. However, the small number of new connectivities induced by β-DG(654–750) suggests that the peptide backbone conformation is still substantially unstructured in the bound form.

We evaluated the extent of binding for each amino acid calculating the ratio V_{free}/V_{bound}, where V_{free} is the volume of the intra-residue HN–Hα crosspeak of the 2D TOCSY spectrum in the absence of β-DG(654–750) and V_{bound} is the volume of the same crosspeak in the presence of β-DG(654–750). A plot summarising all the V_{free}/V_{bound} ratios versus residue numbers for all the amino acids is shown in Fig. 4. Although all the resonances are influenced by the interaction with β-DG(654–750), the interaction of the N-terminus of the

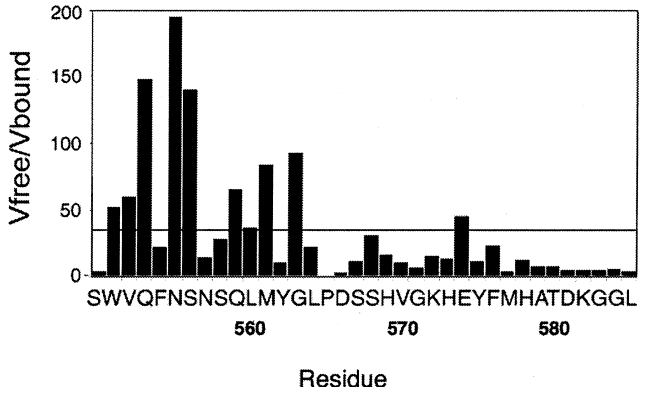

Fig. 4. Plot of the ratios V_{bound}/V_{free} normalised with respect to the maximum values, versus the amino acidic sequence of α-DGC(550–585). The horizontal bar represents the average ratio.

synthetic peptide is more pronounced, while that of the C-terminal portion is only marginally involved.

The V_{free}/V_{bound} ratios measured in a decreasing order are: Asn^{555}, Gln^{553}, Ser^{556}, Gly^{563}, Met^{561}, Gln^{559}, which all belong to the N-terminal portion (550–565, see Fig. 5A). In addition, Val^{552}, Glu^{574} and Trp^{551} also show values over the average (Fig. 4).

In order to validate the results obtained by NMR experiments, we carried out solid-phase binding assays using a new recombinant fragment belonging to the C-terminal region of α-DG, α-DGC(485–572), which includes the N-terminal portion of the 550–585 sequence. α-DGC(485–651), α-DGC(485–585) and α-DGC(485–550) were used as a control (Fig. 5A). Although the K_d values should be considered as apparent, solid-phase binding assays are frequently used to collect qualitative or semi-quantitative reliable estimates on the affinity of interaction between two proteins [20]. We found that α-DGC(485–572) binds to β-DG(654–750) with high affinity ($K_d = 6 \pm 1$ μM) and in a similar fashion as α-DGC(485–651) ($K_d = 6 \pm 2$ μM), which corresponds to the entire α-DG C-terminal domain, and α-DGC(485–585) ($K_d = 12 \pm 2$ μM), whereas α-DGC(485–550) shows no detectable affinity (Fig. 5B).

4. Discussion

DG is a receptor formed by an extracellular subunit, α-DG, and a transmembrane one, β-DG, that interact tightly but non-covalently. In a previous work, we found that the binding epitope for β-DG is located within the C-terminal domain of α-DG and that the binding is independent of glycosylation [13]. Recently, using a progressive deletion recombinant approach, the binding epitope has been further narrowed down to a sequence as short as 36 amino acids (550–585) (Sciandra, Schneider, Giardina, Baumgartner, Petrucci and Brancaccio, submitted). We have used an NMR approach to investigate in more detail the binding between a synthetic peptide corresponding to the 550–585 sequence of vertebrate α-DG and the recombinant fragment β-DG(654–750), which corresponds to the N-terminal extracellular region of β-DG.

It has been shown that the recombinant β-DG(654–750) fragment is mainly unstructured in aqueous solution, with short stretches of secondary structure [21]. Although we cannot rule out the oligomerisation or the formation of aggregates at higher concentrations (> 200 μM [21]), we carried out NMR experiments at a relatively low concentration of β-DG(654–750) (~30 μM), which makes the aforementioned phenomena unlikely. However, it is clear that the plasticity of β-DG(654–750) makes it very difficult to obtain workable NMR data. In spite of these difficulties, NMR methods can be a great tool for obtaining atomic resolution of protein–protein interactions without determining entire protein structures [22]. The binding properties of the molecules can be probed in several ways, depending on the kinetics of the binding. When the time regime of the interaction is fast on the NMR time scale, binding sites can be detected using differences in chemical shifts, while in studies with small ligands bound to macromolecules, the ratios between selective and non-selective relaxation rates can be analysed ([23] and references therein). With fast/intermediate exchange a linewidth broadening is observed. Differential line broadening can be

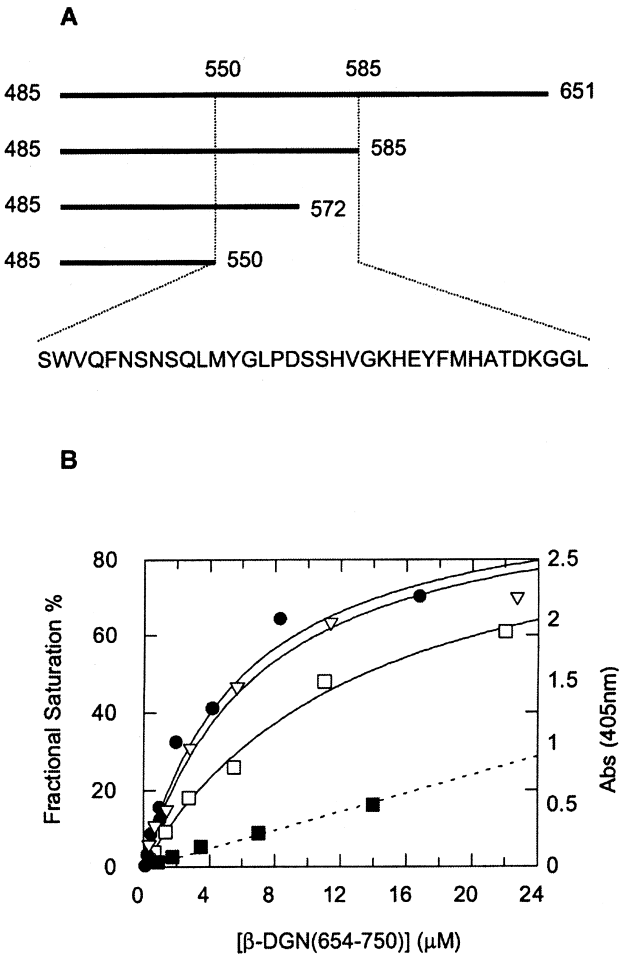

Fig. 5. A: The C-terminal region of α-DGC is extended from amino acid 485 to 653. This region harbours the binding epitope (550–585) for the extracellular domain of β-DG(654–750). The α-DGC(485–651) fragment that corresponds to almost the entire α-DG C-terminal region has been used as a control, together with α-DGC(485–585) and α-DGC(485–550) [13]. B: Solid-phase binding assays, using biotinylated β-DG(654–750) as a ligand, were performed to test the α-DG recombinant fragments' binding properties. The fragments α-DGC(485–651) (▽), α-DGC(485–572) (●) and α-DGC(485–585) (□) show a similar binding activity ($K_d = 6 \pm 2$ μM, $K_d = 6 \pm 1$ μM and $K_d = 12 \pm 2$ μM respectively), whereas fragment α-DGC(485–550) (■) does not bind the extracellular region of β-DG in the concentration range analysed. Data refer to representative experiments.

used to determine the residues directly involved in the binding [17–19]. Thus, the line shapes, or simply the ratios of the peak volumes of peptide in the bound and in the free states, can give a direct estimation of the extent of the involvement of each single amino acid in the binding process.

In this case, assuming that the K_d for the peptide/protein complex is $\sim$6 μM and that the rate of peptide association with β-DG(654–750) is diffusion-limited (10^8 M^{-1} s^{-1}), it can be estimated that the upper limit for the off rate is about 6×10^2 s^{-1}, which is in the range of the intermediate exchange [24,25]. In such a regime of exchange, the broadening of some NH might be due to the averaging of bound resonances which experience large chemical shift changes. A differential line broadening may also result from the different overall correlation time that the peptide assumes when bound to β-DG(654–750).

We were able to demonstrate via 1D and 2D TOCSY experiments (Figs. 1–4) that (i) the synthetic peptide binds to β-DG also in solution, (ii) its N-terminal portion (550–565) is preferentially involved in the binding, as confirmed by solid-phase analysis (Fig. 5), and (iii) residues Asn^{555}, Gln^{553}, Ser^{556}, Gly^{563}, Met^{561} and Gln^{559} are those more likely to interact with β-DG.

By combining NMR methods and solid-phase binding assays, we have identified 'hot spots' within the α/β-DG binding surface. As for other protein–protein interactions [26], we found that the binding is confined to a discrete subset of only 16 residues, ^{550}SWVQFNSNSQLMYGLP^{565}. In fact, the presence of the peptide corresponding to α-DG(485–572) (Fig. 5) is sufficient for the interaction with β-DG(654–750) to take place. Even though Glu^{574} is the only residue within the C-terminal portion of the 550–585 sequence to show some interaction (Fig. 4), its presence is not a requirement for the interaction to take place, as demonstrated by the binding behaviour of α-DGC(485–572) (Fig. 5B).

Our research gives valuable molecular insight into the dimerisation interface of DG and paves the way for further studies employing site-directed mutagenesis, to elucidate the contribution of each single amino acid to the interaction. To investigate details of the local conformation of the bound form of the peptide, we are currently carrying out experiments on a shorter α-DGC peptide and trying to prepare an isotope-labelled β-DG(654–750).

A detailed understanding of the interaction between α- and β-DG at the molecular level would facilitate the rational design of drugs aimed to modulating the function of the DG receptor molecule. For example, a specific inhibitory agent could be used to inactivate this receptor for arenaviruses or *Mycobacterium leprae* infections [27,28] or to strengthen the interactions between the DG subunits in all those severe muscular dystrophies in which the receptor is either absent or reduced within the sarcolemma [2].

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