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Structural and Functional Analysis of the N-Terminal Extracellular Region of β-Dystroglycan

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A protein fragment corresponding to the mouse β-dystroglycan N-terminal extracellular region from position 654 to 750, β-DG(654–750) was recombinantly expressed in BL21(DE3) *Escherichia coli* cells. Secondary structure prediction of the protein fragment reveals about 70% of random coil, as confirmed by circular dichroism analysis. Moreover, fluorescence analysis shows that the tryptophan residue in position 659 lays in a solvent-exposed fashion. These data suggest that the β-DG(654–750) is likely to have a quite flexible structure and to be only partially folded. Interestingly, the protein still retains its biological function since using solid-phase assays we have detected binding of biotinylated β-DG(654–750) both to native α-dystroglycan and to a recombinant fragment which spans the C-terminal region of α-dystroglycan.

Dystroglycan is a highly glycosylated protein receptor for extracellular matrix proteins which consists of two proteins, α and β, encoded by a single gene (1, 2). Dystroglycan is synthesized as a precursor which is cleaved into an extracellular (α) and a transmembrane subunit (β). The highly glycosylated α-dystroglycan was originally identified in rabbit skeletal muscle where it was localized within the external side of sarcolemma (3), while the transmembrane spanning β-dystroglycan was found to bind with its cytosolic C-terminal proline-rich sequence the C-terminal end of dystrophin (4). Dystroglycan, in association with sarcoglycans and sarcospan, forms the monomeric dystrophin glycoprotein complex, which plays an important role as a mechanical anchor between the extracellular matrix and the actin cytoskeleton in muscle and in a wide variety of tissues (5–9). Moreover, dystroglycan has also a crucial function during early morphogenesis as revealed by its gene disruption experiment in mice which showed an impairment of their embryonic development as early as day 6.5 (10, 11).

α-Dystroglycan binds laminin, agrin and perlecan with high affinity in a calcium-dependent fashion (12–15). A number of experimental evidences indicates that glycosylation is strictly required for α-dystroglycan binding activity (5, 16). It is likely that α-dystroglycan carbohydrate moieties protruding out of its central mucin-like region are crucial for the formation of these interactions as also shown by the lack of binding to laminin and agrin observed on the recombinant N-terminal region expressed in bacterial cells (17).

Although α-dystroglycan structure was not solved at the atomic resolution yet, in recent years several structural aspects of the α-dystroglycan molecule have been investigated. By electron microscopy analysis it was shown that α-dystroglycan has a dumbbell-like shape in which two globes are connected by an elongated rod corresponding to the central mucin-like region (18). It was also demonstrated that its N-terminal region expressed in *Escherichia coli* (*E. coli*) cells represents an autonomous folding globular unit and this domain was crystallized in order to solve its structure at the atomic level (17, 19). It was also found that the N-terminal region (approximately between positions 77 and 175) shares a significant degree of identity and similarity with sequences belonging to the immunoglobulin κ family (19).

Besides α-dystroglycan, a great deal of experimental work has been also carried out on the cytoplasmic tail of β-dystroglycan which harbours binding epitopes for a number of cytosolic proteins like dystrophin, its isoform Dp116, the growth receptor binding adaptor protein (Grb2) and rapsyn (4, 20–24), while little or no

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experiments have been collected so far on its extracellular region, between positions 654 and 750 (1, 22). It is likely that the N-terminal extracellular region of β-dystroglycan plays an important role in the formation of the tight, not covalent, interaction with α-dystroglycan. In addition, it might represent a novel protein domain since data base search does not reveal a significant similarity to any other protein.

We expressed the N-terminal region of β-dystroglycan in *E. coli* cells and started to characterize both its structural and functional properties. Our results indicate that: (i) the recombinant protein is prevalently organized in a random coil fashion, with the secondary structure prediction supporting this notion; (ii) the protein still binds native α-dystroglycan even in the absence of glycosylation, thus retaining its biological function; and (iii) it also binds a recombinant protein fragment spanning the C-terminal region of α-dystroglycan.

MATERIALS AND METHODS

DNA manipulations and recombinant fragments purification. The full-length cDNA coding for murine dystroglycan (α + β), cloned into the episomal expression vector pCEP from Invitrogen (San Diego, CA) in collaboration with Dr. Ulrike Mayer (Martinsried, Germany), was used as template to generate by polymerase chain reaction (PCR) the β-DG(654–750) DNA construct. Specific oligonucleotide primers were used to amplify the 654–750 sequence: forward 5′CCC**GGATCC**TCTATCGTGGTGGAATGGACCAACA-3′, and reverse 5′CCC**GAATTC**TTAGTAAACATCGTCCTCACTGCTCTTC-3′ (*Bam*HI and *Eco*RI restriction sites in bold, and target sequences underlined). The DNA product was purified and inserted in a bacterial vector (which also contained a N-terminal His_6-tag and a thrombin cleavage site) for the expression of proteins as thioredoxin fusion products (25). The fusion protein was expressed quantitatively in the *E. coli* BL21(DE3) strain and purified using nickel nitrilotriacetate affinity chromatography. Pure mouse β-dystroglycan, starting with foreign residues *Gly-Ser*, was obtained after thrombin cleavage. The same procedure was used to prepare the C-terminal α-dystroglycan fragment α-DGC(485–651) and also the N-terminal α-dystroglycan fragment $\alpha\text{-DGN}(30–315)^{WT}$ and the single mutant $\alpha\text{-DGN}(30–315)^{R168H}$ (19,26). Tricine SDS/PAGE according to Shägger and von Jagow (27) was used to check purity. Native chick kidney α-dystroglycan was purified as described elsewhere (8).

N-terminal sequencing. The amino acid sequence of the protein fragment, β-DG(654–750), was determined by automated Edman degradation using a Perkin-Elmer (Norwalk, USA) model AB 476A sequencer. Sample (0.5 nmol) was loaded onto a polyvinylidene difluoride membrane (PROBLOTT, Perkin-Elmer) coated with polybrene (2 μl, 20 mg/ml 70% methanol).

Mass spectrometry. Mass spectral analysis of β-DG(654–750) was carried out using an electrospray mass spectrometer, LCQ model Finnigan (San Jose, USA), in positive mode. A protein solution, desalted by RP-HPLC with an acetonitrile gradient (10–50%) in 0.2% trifluoroacetic acid, was directly infused at 5 μl/min. Full scan spectrum was acquired in the range of 150–2000 amu. Deconvolution of the multicharged spectrum was performed by a Finnigan software.

Fluorescence and circular dichroism. Fluorescence emission spectrum (Ex^{λ} = 280 nm) was recorded at 25°C in a 1-cm quartz cell, using a Spex (Edison, USA) FluoroMax spectrofluorimeter, in 0.1 M sodium phosphate, 150 mM NaCl pH 6.5. For protein denaturation experiments the fluorescence emission was monitored at 350 nm for β-DG(654–750) and at 342 nm for α-dystroglycan recombinant fragments (in both cases Ex^{λ} = 280 nm). Guanidinium hydrochloride (Gdn/HCl) was purchased from Fluka (Buchs, Switzerland). Circular dichroism was recorded at 20°C in a 0.1-cm quartz cell, using a Jasco (Easton, USA) Model J-720 spectropolarimeter, in aqueous solution at very low ionic strength. The spectrum was the average of multiple accumulations and was noise-reduced using standard procedures. The signal was expressed as mean molar ellipticity [θ] (deg cm^2 $dmol^{-1}$) equal to $[\theta]_{obs}(10c \cdot n \cdot l)$, where $[\theta]_{obs}$ is the measured ellipticity (mdeg), c is the protein concentration (M), n is the number of amino acid residues/chain, and l is the path-length of the cell (cm).

The concentration of β-DG(654–750) was estimated both using the Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, USA) and spectrophotometrically using an extinction coefficient at 280 nm of ≈8000 M^{-1} cm^{-1}, which was measured using the method of Gill and von Hippel (28).

β-DG(654–750) biotinylation. Recombinant β-DG(654–750) was biotinylated in 50 mM sodium phosphate buffer at pH 7.4, with 0.5 mg/ml sulfosuccinimidobiotin (S-NHS-biotin) from Pierce (Rockford, USA) for 30 min on ice and dialysed overnight against 10 mM Tris/HCl, 150 mM NaCl pH 7.4. The optimal dilution for signal detection was determined by dot blot analysis.

Solid-phase binding experiments. Solid-phase binding assays were carried out as described elsewhere (8), with the following modifications. Briefly, ≈1 μg/well of α-dystroglycan recombinant fragments $\alpha\text{-DGN}(30–315)^{WT}$ and α-DGC(485–651) and 0.1 to 0.5 μg/well (in different experiments) of chick kidney α-dystroglycan were immobilized on microtiter plates by overnight incubation at 4°C in 50 mM sodium bicarbonate, pH 9.6. The remaining binding sites were saturated by 1 h incubation with 10 mM Tris/HCl 150 mM NaCl, pH 7.4 (TBS), containing 1.25 mM $CaCl_2$, 1 mM $MgCl_2$ and 3% milk powder. After blocking and extensive washing with TBS containing 0.1% Tween 20 (T-TBS) wells were incubated with biotinylated β-DG(654–750) in TBS containing 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA, 1% milk powder, 0.05% Tween-20, pH 7.4, for 2 h at room temperature (≈25°C). Wells were washed 4 times with T-TBS containing 1.25 mM $CaCl_2$, 1 mM $MgCl_2$ and the biotinylated β-DG(654–750) bound fraction was detected with the VECTASTAIN ABC immunodetection kit from Vector Laboratories (Burlingame, USA). 0.4 mg/ml o-phenylenediamine dihydrochloride (OPD) in 80 mM citrate/phosphate buffer containing 0.015% H_2O_2, pH 5.3, was used as the horseradish peroxidase substrate, and quantitative measurements were carried out at 490 nm. The absorbance values were corrected for the signals obtained incubating biotinylated β-DG(654–750) only with BSA (1 μg/well). Data were fitted using a single class of equivalent binding sites, using the equation: $OD_{490nm} = (OD_{tot} * c/(K_d + c))$, where OD_{490nm} represents absorbance, K_d is the binding dissociation constant, c is the concentration of biotinylated β-DG(654–750), and OD_{tot} absorbance at saturation.

RESULTS AND DISCUSSION

The N-terminal residue of β-dystroglycan, Ser^{654} has been previously assigned by direct N-terminal sequencing on the mature protein both on the sheep brain (29) and on the *Torpedo californica* electric organ postsynaptic membranes protein (30). The sequence of the murine β-dystroglycan extracellular region, from position 654 to 750, is reported in Fig. 1. Accordingly, we have recombinantly expressed in *E. coli* cells as a fusion protein and, after thrombin cleavage, purified (Fig. 2), a protein fragment, β-DG(654–750), which exactly corresponds to this region aside from the first two initial foreign residues, Gly and Ser. The yield of the recombinant protein was approximately 5 mg per liter of bacterial culture.

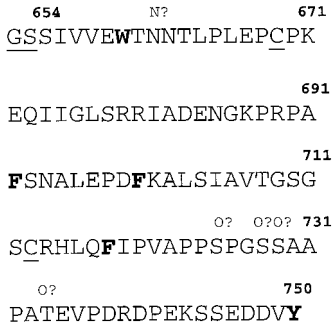

FIG. 1. Primary structure of β-DG(654–750). This protein fragment (99 amino acids) corresponds to the extracellular region of β-dystroglycan. β-Dystroglycan originates from the posttranslational cleavage of the dystroglycan precursor at the Gly-Ser site in position 653–654 and after the Tyr residue in position 750 starts its transmembrane region (1). Foreign and cysteine residues are underlined. Aromatic residues are in bold. Numbers refer to the dystroglycan sequences alignment (1). Putative N-glycosylation sites (N?), based on the NxT/S consensus sequence, and O-glycosylation sites (O?), based on the *NetOGlyc* program analysis (31) available at the *ExPASy* server (Geneva, Switzerland), are also indicated.

The electrophoretic mobility (Fig. 2) of the protein fragment indicates a relative molecular mass of about 14 ÷ 14.5 kDa which is different from its deduced mass (10528.7 Da). In order to check whether this discrepancy was due to an incorrect expression of the fragment, we have carried out both N-terminal sequence analysis (data not shown) which confirmed the N-terminal sequence GSSIVV. . . (see Fig. 1) and mass spectrometry measurement which yielded a mass value of 10526 (data not shown) that nicely corresponds to the expected mass. Thus, the anomalous electrophoretic behaviour is not depending on differences in the primary structure of the recombinant β-DG(654–750) fragment and is likely to mirror the

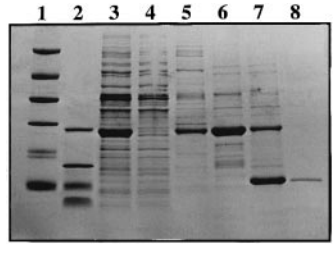

FIG. 2. Purification of β-DG(654–750). SDS-Tricine PAGE was used to analyze the various steps during purification using nickel nitrilotriacetate affinity chromatography. Lane 1, molecular mass markers (94, 67, 43, 30, 20.1 and 14.4 kDa); lane 2, molecular mass markers (26.6, 17, 14.2 and 6.5 kDa); lane 3, total bacterial extract; lane 4, flow-through fraction; lane 5, wash fraction; lane 6, fraction eluted with 1 M imidazole; lane 7, fraction digested with thrombin; lane 8, purified β-DG(654–750). Although the β-DG(654–750) molecular mass as measured by mass spectrometry (10526 Da) is in good agreement with its calculated mass (10528.7 Da), the protein fragment displays an anomalous electrophoretic mobility (14 ÷ 14.5 kDa) (see text).

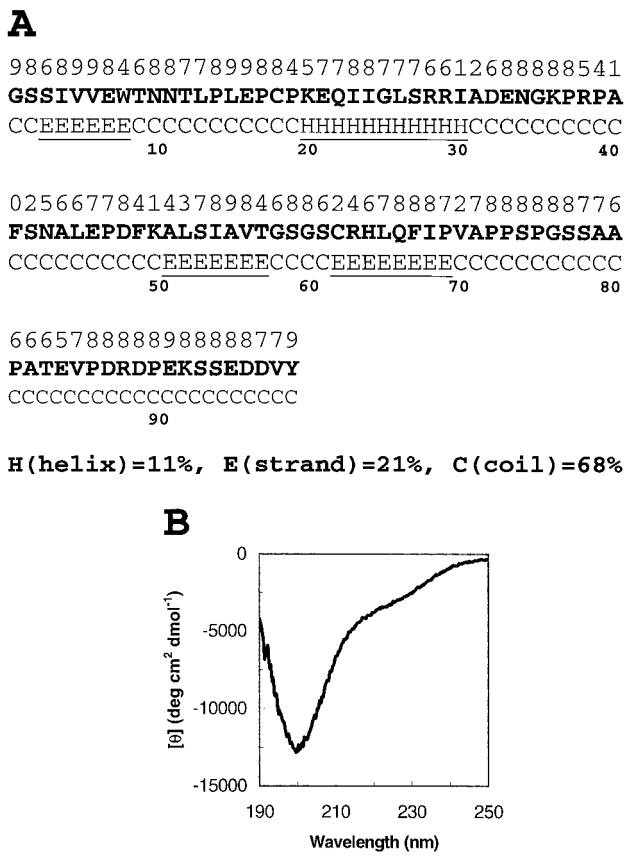

FIG. 3. β-DG(654–750), circular dichroism and secondary structure prediction. (A) Secondary structure prediction was performed using the *PSIpred* protein structure prediction server available at the University of Warwick (32). Numbers from 0 = low to 9 = high refer to the confidence level. The putative elements of secondary structure are underlined and the target sequence is reported in bold. (B) Circular dichroic spectrum of β-DG(654–750) between 190 and 250 nm was carried out at 20°C and normalized as described in the Materials and Methods section.

one of native β-dystroglycan, which runs as a 43 kDa band (1) while its calculated molecular mass is 26.4 kDa. In fact, the highly reduced electrophoretic mobility of native β-dystroglycan is not likely to depend only on the presence of its carbohydrate fraction.

As revealed by far-UV circular dichroism spectrum (minimum at ≈200 nm), the β-DG(654–750) protein fragment is likely to possess only few secondary structure elements (either α-helix or β-strand), confirming the results of the secondary structure prediction (Figs. 3A and 3B). In addition, fluorescence analysis suggests that the single Trp^{659} residue is solvent-exposed, since its emission maximum is at ≈350 nm (Fig. 4A) (33–34). Consequently, no transition curves were measured as a function of guanidinium hydrochloride (Gdn/HCl) concentration monitoring Trp(659) fluorescence (Fig. 4B). These latter results indicate that at least the N-terminal region of the protein, encompassing the Trp^{659} residue (see Fig. 1), is likely to be organized in a

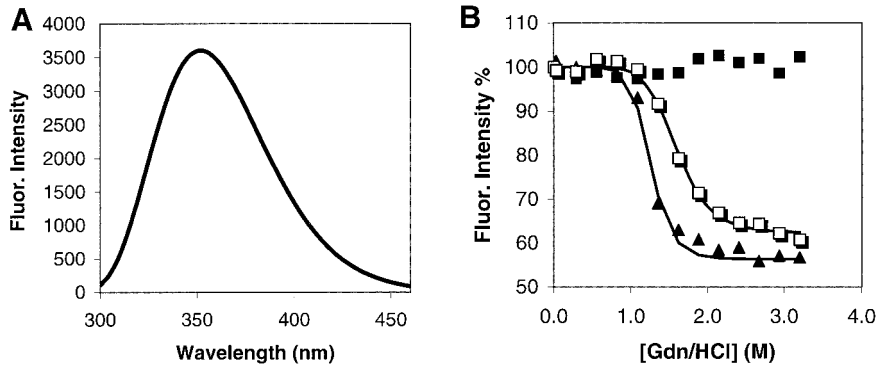

FIG. 4. Fluorescence analysis. (A) Emission spectrum of β-DG(654–750) between 300 and 450 nm (Ex^{λ} = 280 nm). (B) Fluorescence signal as a function of Gdn/HCl concentration. No transition was detected for β-DG(654–750) (■). For comparison, experiments on α-DGN(30–315)^{WT} (▲) and α-DGN(30–315)^{R168H} (□) are also reported.

rather flexible fashion, which might represent a partially folded state.

As a matter of fact, *in vivo* the extracellular region of β-dystroglycan is covalently linked to its transmembrane segment, from residue 751 to 774 (see 1), and this could strongly influence its overall structural organization. Moreover, the lack of glycosylation in the recombinant bacterial protein might also contribute for its increased flexibility and reduce its overall stability (35, 36). The primary structure shows the presence of one N-glycosylation consensus motif (Asn in position 661) and three O-glycosylation putative sites (Ser in positions 725, 728 and 729) (see Fig. 1). However, both Ser^{725} and Ser^{729} are not conserved within other mammalian dystroglycan sequences (1). To clarify the possible role of glycosylation on the structural organization and stability of the protein, the recombinant production of the extracellular region of β-dystroglycan in an eukaryotic expression system would be desirable.

Interestingly, we have measured high affinity binding (K_d is about 10 μM) of the β-DG(654–750) fragment both to native α-dystroglycan and to a recombinant fragment spanning its C-terminal region, α-DGC(485–651) (Fig. 5). This suggests that the protein still retains its crucial biological function even in the absence of glycosylation. It is not possible to rule out that the lack of glycosylation in β-DG(654–750) is affecting the strength of the interaction with α-dystroglycan, eventually reducing it from the nanomolar to the micromolar range. The low amount of secondary and possibly also tertiary structure displayed by β-DG(654–750) might suggest that only a small portion of the protein, represented by a limited number of side chains, is interacting with α-dystroglycan. On the other hand, it is also possible that α-dystroglycan acts as a structure-inducer and influences the overall conformation of soluble β-DG(654–750), drastically changing the level of its secondary and tertiary structures.

Both SDS-PAGE analysis, performed in reducing and nonreducing conditions, and sequence analysis of peptides obtained following tryptic digestion of the non reduced β-DG(654–750) protein fragment (data not shown) suggest that the cysteine residues in positions 669 and 713 are present in reduced form. This is apparently in contrast with previous experiments suggesting the presence of at least one disulphide bridge in the extracellular region of *Torpedo californica* β-dystroglycan, based on the different electrophoretic mobility observed under reducing and nonreducing conditions (30). The absence of the disulphide bridge in our recombinant β-dystroglycan protein fragment could influence its overall structural arrangement and reduce its stability (37), even though its functional properties seem to be not particularly affected (see Fig. 5). It is noteworthy that although recent experiments suggest the presence of a disulphide bridge in murine β-dystroglycan (38), no direct and conclusive data are available confirming its presence in mammalian β-dystroglycans.

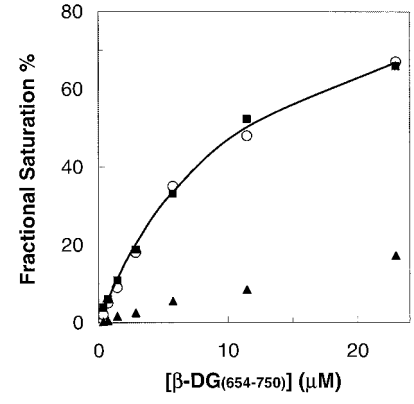

FIG. 5. Solid-phase biotinylated-ligand binding assay. Biotinylated β-DG(654–750) binding to native chick kidney α-dystroglycan (○) and recombinant α-dystroglycan protein fragments, α-DGC(485–651) (■) and α-DGN(30–315)^{WT} (▲). Data were normalized using the OD_{tot} parameter and experimental values were fitted according to the equation described in the Materials and Methods section. Data refer to one representative experiment.

Our results indicate that the C-terminal region of α-dystroglycan is responsible and sufficient for the interaction with β-dystroglycan since we detected only negligible binding to the α-dystroglycan N-terminal fragment α-DGN(30–315)^{WT} (Fig. 5). Based on its dumbbell-like shape, it was previously proposed that the that the N-terminal region of α-dystroglycan is directed towards the extracellular matrix space (18). Intuitively, this implies that its C-terminal region is located next to the cell surface. Although no conclusive evidences have been collected confirming such topology at the plasmalemmal surface, our data seem to corroborate this view. In addition, it can not be ruled out that the extracellular region of β-dystroglycan might also be involved in the formation of interactions with other proteins as, for example, sarcoglycans and sarcospan both belonging to the dystrophin glycoprotein-complex in muscles and other tissues (1, 9, 38).

In conclusion, our results represent the first binding data collected on the interaction between α- and β-dystroglycan using a solid-phase technique. We have also assigned to the C-terminal region of α-dystroglycan the β-dystroglycan binding epitope. Although glycosylation of the extracellular region of β-dystroglycan and its membrane-associated location may modulate the strength of this interaction *in vivo*, our recombinant system seems to be well suited to analyze its molecular mechanism in more detail.

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