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Characterization of an Importin in α/β-Recognized Nuclear Localization Signal in β-Dystroglycan

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ABSTRACT

β-dystroglycan (β-DG) is a widely expressed transmembrane protein that plays important roles in connecting the extracellular matrix to the cytoskeleton, and thereby contributing to plasma membrane integrity and signal transduction. We previously observed nuclear localization of β-DG in cultured cell lines, implying the existence of a nuclear targeting mechanism that directs it to the nucleus instead of the plasma membrane. In this study, we delineate the nuclear import pathway of β-DG, characterizing a functional nuclear localization signal (NLS) in the β-DG cytoplasmic domain, within amino acids 776–782. The NLS either alone or in the context of the whole β-DG protein was able to target the heterologous GFP protein to the nucleus, with site-directed mutagenesis indicating that amino acids R^{779} and K^{780} are critical for NLS functionality. The nuclear transport molecules Importin (Imp)α and Impβ bound with high affinity to the NLS of β-DG and were found to be essential for NLS-dependent nuclear import in an in vitro reconstituted nuclear transport assay; cotransfection experiments confirmed the dependence on Ran for nuclear accumulation. Intriguingly, experiments suggested that tyrosine phosphorylation of β-DG may result in cytoplasmic retention, with Y^{892} playing a key role. β-DG thus follows a conventional Impα/β-dependent nuclear import pathway, with important implications for its potential function in the nucleus. J. Cell. Biochem. 110: 706–717, 2010.

KEY WORDS: β-DYSTROGLYCAN; NLS; IMPORTIN α/β; NUCLEAR IMPORT; GFP

Dystroglycan (DG), best known as a central component of the dystrophin-associated protein complex (DAPC) in muscle, is an important cell adhesion receptor that links the intracellular skeleton and the extracellular matrix [Ervasti and Campbell, 1993]. DG is encoded by a single gene (DAG1) and cleaved into two proteins post-translationally, resulting in an extracellular peripheral membrane glycoprotein, α-DG, and a transmembrane glycoprotein, β-DG, which remain non-covalently linked at the cell membrane [Henry and Campbell, 1996; Di Stasio et al., 1999; Winder, 2001]. In skeletal muscle, α-DG links laminin-2 and agrin in the basal lamina with β-DG in the sarcolemma [Ervasti and Campbell, 1993; Fallon and Hall, 1994; Gee et al., 1994; Ekblom, 1996]. On the cytoplasmic side of the sarcolemma, β-DG interacts with dystrophin or utrophin which are linked in turn to F-actin [Jung et al., 1995; Winder, 2001].

Bárbara Lara-Chacón and Mario Bermúdez de León contributed equally to this study.

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Disruption of the DAPC complex underlies the molecular pathogenesis of a variety of forms of muscular dystrophy, revealing that the extracellular matrix–cytoskeleton linkage is critical for maintaining the structural integrity of the sarcolemma [Ervasti et al., 1990; Ervasti and Campbell, 1993; Campbell, 1995].

In addition to the crucial structural role of β-DG at the plasma membrane, it has recently been shown that this protein also functions as a dynamic signal transducing molecule. β-DG is subject to adhesion-dependent tyrosine phosphorylation by Src family kinases [James et al., 2000; Ilsley et al., 2001; Sotgia et al., 2001], acting as a scaffold for the ERK-MAP kinase cascade [Spence et al., 2004b], interacting with ezrin to mediate cytoskeleton remodeling and induce peripheral filopodia and microvilli [Spence et al., 2004a; Batchelor et al., 2007], and has also been implicated in survival signaling through Akt [Rando, 2001; Langenbach and Rando, 2002]. Furthermore, β-DG interacts with the adaptor molecule Grb2 [Yang et al., 1995; Russo et al., 2000], and rapsyn, a peripheral protein required for acetylcholine receptor clustering [Cartaud et al., 1998; Bartoli et al., 2001; Jacobson et al., 2001].

So far, all cell functions of β-DG relate to its localization at the plasma membrane and interaction with cytoskeletal and cytoplasmic proteins. Therefore, it was surprising to find in our previous studies the presence of β-DG in the nucleus of epithelial HeLa and muscular C2C12 cells [Fuentes-Mera et al., 2006; Gonzalez-Ramirez et al., 2008]. In HeLa cells, β-DG colocalizes with some dystrophin-associated proteins (DAPs), including dystrophin Dp71, α- and β-dystrobrevin and nNOS, to form a DAPC-like complex in the nucleus [Fuentes-Mera et al., 2006]. Moreover, β-DG was recovered in nuclear matrix preparations from these cells and found to be associated with the nuclear matrix proteins lamin B1 and actin, suggesting that binding of β-DG to the nuclear matrix occurs through its interaction with those proteins. In C2C12 muscle cells, β-DG was recovered together with emerin (an inner nuclear membrane protein) and lamin A/C (a protein of the nuclear lamina) in nuclear envelope extracts [Gonzalez-Ramirez et al., 2008], implying that β-DG can associate with the nuclear envelope.

Proteins generally gain access to the nucleus through the action of a nuclear localization signal (NLS) that confers recognition by members of Importin (Imp) superfamily, which mediate translocation through the nuclear pore complexes (NPCs) [Jans et al., 2000; Pemberton and Paschal, 2005]. The existence of β-DG in the nucleus opens the novel possibility of additional nuclear roles for this multifunctional protein. In the present study, we characterize the nuclear import pathway of β-DG, delineating a functional NLS in the sequence of β-DG located within amino acids 776–782. Furthermore, using direct binding assays, we show that Impα and Impβ as well as the Impα/β heterodimer bind with high affinity to the NLS of β-DG and that the latter can target a heterologous protein such as green fluorescent protein (GFP) to the nucleus in a reconstituted in vitro nuclear transport system. Transfection experiments confirm dependence of β-DG nuclear import on Ran; that β-DG appears to possess a conventional NLS-/Imp-dependent nuclear import pathway raises the intriguing possibility of previously unrecognized nuclear functions for β-DG.

MATERIALS AND METHODS

ANTIBODIES

The following antibodies were used: JAF, a rabbit polyclonal antibody that recognizes the last seven amino acids of the C-terminal of β-dystroglycan (β-DG) [Rivier et al., 1999]; C-20, a goat polyclonal antibody raised against a peptide mapping at the C-terminus of β-DG (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal antibodies H-70, H-110, and FL against calnexin, lamin A/C, and GFP respectively, and a monoclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology). A mouse monoclonal antibody that recognizes the central domain of actin [Garcia-Tovar et al., 2001] was kindly provided by Dr. Manuel Hernandez from CINVESTAV-IPN, Mexico.

CELL CULTURE AND TRANSFECTION

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) neonatal calf serum, 25 U/ml penicillin and 25 μg/ml streptomycin. Where indicated, HeLa cells, grown on coverslips at 80% confluence were treated for 6 h at 37°C with 20 μg/ml *Agaricus bisporus* lectin (Sigma, St. Louis, MO), prediluted in DMEM. C2C12 cells were grown in DMEM supplemented with 10% fetal bovine serum, 25 U/ml penicillin, 25 μg/ml streptomycin, and 1 mM sodium pyruvate. In some experiments, C2C12 cells were treated with 2 mM peroxyvanadate (Sigma) in serum-free DMEM medium for 3 h at 37°C to evidence phosphorylation of β-DG. Hepa-1 cells were grown in 90% of DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. A549 cells were grown in Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. HTC cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, L-glutamine. For in vitro transport assay experiments, HTC cells were trypsinized and seeded onto glass coverslips 2 days prior to use to achieve a confluence of 70% at the time of experimentation. All cell cultures were maintained in a humidified 37°C incubator with 95% air and 5% CO_2.

For transfection, cells were seeded onto glass coverslips and grown overnight to ~70% confluence. On the following day, cells were transfected with 5 μg of DNA premixed with 5 μl of Plus reagent and 8 μl of lipofectamine (Invitrogen, Carlsbad, CA) in serum free medium. The cells were washed with culture medium and maintained in the appropriate growth medium supplemented with serum for 24 h prior to analysis.

PLASMID CONSTRUCTS

To construct vector pGFP-β-DG, a human β-DG cDNA was amplified from HeLa cells by PCR using a M-MLV reverse transcriptase coupled to a high fidelity polymerase (*Pfu* turbo; Stratagene, La Jolla, CA) and primers DIS5-F 5′-TTGCGGCCGC-AAGGGCCCTCCATCGTGGTGGAATGGAC-3′ and DIS3-R 5′-TTGC-GGCCGCAAGGGCCC**TTATTA**AGGTGGGACATAGGGAG-3′, containing *Apa*I restriction sites (bold letters denote stop codons). PCR product was digested with *Apa*I and cloned into the *Apa*I-digested pQBI25-

fC1 vector (Quantum Biotechnologies, Inc., Montreal, Canada). For construction of vector pGFP-β-DGΔNLS, the NLS of β-DG was deleted on the pGFP-β-DG vector using the QuickChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. The forward and reverse primers were 5′-GCCATGATCTGCTACCTTACCCTTGAGGACCAGG-CC-3′ and 5′-GTCCTCAAGGGTAAGGTAGCAGATCATGGCAATGC-A-3′; respectively. To generate the plasmid encoding GFP-β-DG-Cyto, a β-DG cDNA fragment corresponding to the cytoplasmic domain (amino acids 774–895) was amplified by PCR using pGFP-β-DG as the template and cloned into *Bgl*II–*Eco*RI digested pEGFP-C1 vector (Promega). The β-DG-CytoY892F encoding derivative was generated via PCR mutagenesis using oligonucleotides 5′-TATA-GATCTTGCTACCGCAAGAAGCGGAAGGGC-3′ (forward) and 5′-A-GAATTCAAGGTGGGACAAAGGGAGGAGGTG-3′(reverse) (bold letters denote the mutated codon). For construction of plasmid pTetra-GFP-NLS-β-DG and NLS mutant derivatives thereof, double-stranded oligonucleotides containing terminal phosphorylated overhangs for *Bgl*II and encoding the NLS of β-DG (^{776}RKKRKGK^{782}) or designed mutants, were inserted individually into the pTetra-GFP [Beetz et al., 2004], previously digested with *Bgl*II. The forward and reverse primers for the wild-type NLS were 5′-GATCC CGC AAG AAG CGG AAG GGC AAG A-3′ and 5′-GATCT CTT GCC CTT CCG CTT CTT GCG G-3′, respectively. The forward and reverse primers, respectively, for mutA-E (bold letters denote mutated codons) were 5′-GATCC **GCC** AAG AAG CGG AAG GGC **GCG** A-3′ and 5′-GATCT **CGC** GCC CTT CCG CTT CTT **GGC** G-3′ (mutA); 5′-GATCC CGC **GCG GCG** CGG AAG GGC AAG A-3′ and 5′-GATCT CTT GCC CTT CCG **CGC CGC** GCG G-3′ (mutB); 5′-GATCC CGC AAG AAG **GCG GCG** GGC AAG A-3′ and 5′-GATCT CTT GCC **CGC CGC** CTT CTT GCG G-3′ (mutC); 5′-GATCC CGC AAG AAG CGG **GCG** GGC AAG A-3′ and 5′-GATCT CTT GCC **CGC** CCG CTT CTT GCG G-3′ (mutD); and 5′-GATCC CGC AAG AAG **GCG** AAG GGC AAG A-3′ and 5′-GATCT CTT GCC CTT **CGC** CTT CTT GCG G-3′ (mutE). The plasmid construct for bacterial expression of GFP fused to the NLS or the cytoplasmic domain of β-DG was generated using the Gateway™ system (Invitrogen), whereby a β-DG cDNA fragment encoding the putative NLS (amino acids 774–789) or the cytoplasmic domain (amino acids 774–895) was PCR amplified using primers containing attB1 and attB2 recombination sites (forward 5′-GGGGACAAGTTTGTA-CAAAAAAGCAGGCTGCTACCGCAAGAAGCGGAAGG-3′ and reverse 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGCCTGGTCCT-CAAGGG-3′). The PCR fragment was recombined into the pDONOR207 vector via the BP recombination reaction and the resulting plasmid, pDONOR207-β-DG, was then used to perform an LR recombination reaction with the prokaryotic vector pDEST-Rfb-GFP to generate the pDEST-Rfb-GFP-β-DG-NLS or the pDEST-Rfb-GFP-β-DG-Cyto vector. The integrity of all constructs was confirmed by DNA sequencing.

Expression vectors encoding GFP alone or fused to the SV40 large T-antigen NLS (GFP-Tag-NLS) as well as glutathione *S*-transferase (GST) alone or fused to mouse Importins (Imps) have been previously described [Hubner et al., 1997; Baliga et al., 2003]. The plasmid encoding DsRED-RanQ69L (DsRED-dominant negative mutant of Ran) was kindly provided by Michael Green [Heilman et al., 2006].

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cells plated on coverslips were washed in PBS, fixed with 4% formaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.2% Triton X-100 for 2 min, followed by blocking for 20 min with 1% gelatin/1.5% FBS in PBS, and incubation overnight at 4°C with the specific anti-β-DG antibody JAF. Cells were then washed with PBS and incubated for 1 h at 4°C with a fluorescein-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA). To stain nuclei, cells were incubated for 10 min at room temperature with 500 μg/ml propidium iodide (PI, Sigma). For GFP-fluorescence analysis, cells were fixed 24 h post-transfection and stained with PI, as described above. After washing, cell preparations were mounted on microscope slides with VectaShield (Vector Laboratories, Inc., Burlingame, CA) and visualized on a confocal laser scanning microscope (TCP-SP2, Leica, Heidelberg, Germany) using 63× and 100× oil-immersion plan apochromat objectives (NA 1.32 and 1.4, respectively). Image analysis of digitized confocal microscopic files using the Image J 1.62 software enabled determination of the nuclear-cytoplasmic ratio ($F_{n/c}$). The $F_{n/c}$ was calculated using the equation $F_{n/c} = (F_n - F_b)/(F_c - F_b)$, where F_n, F_b, and F_c represent the nuclear, background, and cytoplasmic fluorescence values, respectively; the results were plotted using the SigmaPlot software [Hu and Jans, 1999; Forwood et al., 2001].

SUBCELLULAR FRACTIONATION

For total cell extracts, 500 μl of lysis buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM PMSF, 1% NP-40, and 1X complete (Roche Applied Science, Indianapolis, IN)] were added to a cell monolayer and incubated in ice for 20 min. Cells were scraped and centrifuged at 12,000*g* for 5 min. Protein concentrations were determined by the Bradford protein dye binding method (Bio-Rad, Hercules, CA), using bovine serum albumin as standard. To isolate cytosolic and nuclear extracts, cells were washed twice with cold PBS, scraped, and centrifuged at 200*g* for 5 min at 4°C. The cell pellet was resuspended in 4.2 ml of cold TM-2 buffer (10 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 0.5 mM PMSF and 1X complete) and incubated for 10 min on ice; 1.8 ml of Triton X-100 5% (volume/volume) was then added and the homogenate incubated on ice for 10 min. Nuclei were separated from cytosol by ten passes through a 22 gauge needle and centrifugation at 2,000*g* for 5 min at 4°C. The supernatant was saved as the cytosolic fraction and the pellet resuspended in 3 ml of buffer A (0.25 M sucrose, 50 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 1X Complete, and 0.5 mM PMSF) at a density of 17×10^6 nuclei/ml, prior to the addition of 6 ml of buffer B [2.3 M sucrose, 50 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 1X Complete (Roche Applied Science), and 0.5 mM PMSF] and mixing by inversion. The mixture was then underlaid with 2 ml of buffer B and centrifuged at 141,000*g* for 1 h at 4°C. The nuclear pellet was suspended in lysis buffer, sonicated, and centrifuged at 15,000*g* for 2 min at 4°C, with the supernatant retained, and protein concentration measured using the Bradford assay.

IMMUNOPRECIPITATION AND WESTERN BLOTTING

Immunoprecipitation assays were carried out on C2C12 cytoplasmic and nuclear extracts using an anti-phosphotyrosine antibody, as

described [Calderilla-Barbosa et al., 2006]. Immunoprecipitated proteins were detected by Western analysis, Protein samples (80 μg) were electrophoresed on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose (Hybond-N+, Amersham Pharmacia, GE Healthcare, Buckinghamshire, UK) using a Transblot apparatus (Bio-Rad). Membranes were blocked for 1–3 h in TBS-T [100 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween-20] with 6–15% (w/v) low-fat dried milk and then incubated overnight at 4°C with the appropriate primary antibody. Following three washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) and developed using the ECL Western blotting analysis system (Amersham Pharmacia, GE Healthcare).

PROTEIN EXPRESSION AND PURIFICATION

Fusion proteins GFP-Tag-NLS, GFP-β-DG-NLS (aa 774–789), GFP-β-DG-Cyto (aa 774–895) and GFP were purified from bacteria as His6-tagged proteins using nickel affinity chromatography under denaturing conditions [Ghildyal et al., 2005]. Briefly, a single colony of BL21 (DE3) transformed bacteria was used to inoculate 800 ml of LB medium plus 100 μg/ml ampicillin and 25 μg/ml kanamicine and the bacterial culture was grown at 28°C to an OD_{600nm} of 0.6 and then induced with 1 mM IPTG for 2 h. Bacterial cells were harvested by centrifugation at 5,000*g* for 10 min and the pellet was resuspended in His-lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, and 1 M NaCl) and lysed with 3 mg/ml lysozyme on ice for 30 min in the presence of 1 U/ml DNase and Complete EDTA-free protease inhibitors (Roche Applied Science). The lysate was centrifuged at 11,000*g* for 45 min at 4°C and the soluble fraction incubated with 4 ml of preequilibrated Ni-NTA beads slurry (Qiagen) for 1 h at 4°C. Beads were washed and protein eluted in His-lysis buffer containing 200 mM imidazole. Imidazole was removed by dialysis against PBS. GST-tagged mouse Imp proteins were expressed and purified as previously [Hubner et al., 1997]. All proteins were concentrated using VivaSpin™ concentrators (Millipore) and the protein concentration estimated by Bradford Assay. Predimerization of Impα and Impβ was performed at 13.6 μM for 15 min at room temperature.

IN VITRO NUCLEAR TRANSPORT ASSAY

Nuclear import kinetics were measured using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy as previously [Jans et al., 1996]. NLS-/Imp-dependent nuclear protein import can be reconstituted in this system in the presence of reticulocyte lysate (Promega), an ATP regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine phosphate, and 2 mM ATP), 70 kDa Texas Red-conjugated dextran (to assess nuclear integrity), 5 μM GFP fusion protein and IB buffer (110 mM KCl, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $CaCl_2$, 20 mM HEPES and 1 mM dithiothreitol, pH 7.4) in a final volume of 5 μl. The involvement of individual Imps in β-DG nuclear import was determined by preincubating reticulocyte lysate for 15 min at room temperature with monoclonal antibodies to Impα2 (Rch1) or β1 (BD Biosciences, San Jose, CA); this antibody has been used previously to block nuclear transport of various different Impα-recognized NLS-containing proteins [Forwood et al., 2001; Ghildyal et al., 2005; Hearps and Jans, 2006]. The requirement for ATP was tested by apyrase pretreatment of both the reticulocyte lysate (800 U/ml for 15 min at room temperature) and unperforated HTC cells (0.2 U/ml for 15 min at 37°C) to break down ATP, and omitting ATP regenerator from the sample as previously [Jans et al., 1996; Forwood et al., 2001; Ghildyal et al., 2005]. In some experiments, reticulocyte lysate was preincubated with 300 μM GTPγS (non-hydrolysable GTP analogue) for 10 min at room temperature to inhibit Ran and thereby block Imp-cargo complexation. Image analysis for the $F_{n/c}$ ratio was performed as previously, with curve fitting performed using the Kaleidagraph 2.1 software [Hu and Jans, 1999; Forwood et al., 2001].

GEL MOBILITY SHIFT ASSAY

The interaction of the NLS or cytoplasmic domain of β-DG with Imps was analyzed by native gel electrophoresis as previously [Forwood et al., 2001; Wagstaff and Jans, 2006]. Ten micromolars of different Imps and/or the Impα/β heterodimer was incubated with 2 μM of GFP-β-DG-NLS or GFP alone (as control) in PBS for 15 min at room temperature. Sucrose was added to the reaction, and the mixture was loaded into a preequilibrated 4–20% gradient polyacylamide gel. Complexes were visualized on an AlphaImager® HP (AlphaInnotech).

ALPHAScreen ASSAY

The interaction between GFP-fusion proteins and Imps was assessed using an established ALPHAScreen assay (Perkin Elmer, Wellesley, MA) [Wagstaff and Jans, 2006], a 384-well white opaque plate (Perkin Elmer) was coated with 30 mM of His6-tagged GFP-β-DG-NLS or GFP-β-DG-Cyto fusion protein per well and incubated for 30 min with increasing concentrations of GST-Imp subunits (0–60 nM). One microliter of 1:10 dilution of the Nickel chelate acceptor beads and 1 μl of BSA 2.5% were subsequently added and the samples incubated at room temperature for 90 min. One microliter of 1:10 dilution of the streptavidin donor beads was then added and the samples incubated at room temperature for a further 2 h. Binding was subsequently detected using a FusionAlpha plate reader (Perkin Elmer).

RESULTS

ENDOGENOUS β-DYSTROGLYCAN LOCALIZES IN THE NUCLEUS

We previously observed β-DG in the nucleus of cells of the HeLa human cervical cancer line [Fuentes-Mera et al., 2006], raising the question of whether β-DG may be generally present in the nucleus in various different cell types. To address this, the subcellular localization of β-DG was analyzed in the A549 human lung, Hepa-1 mouse hepatic and C2C12 mouse muscle cell lines, and compared to results for HeLa cells. Figure 1A shows the results from immunofluorescence and confocal microscopy analysis using the β-DG-specific antibody JAF [Rivier et al., 1999]. In all cell lines, β-DG was detected predominantly in the cytoplasm, but also to differing extents in the nucleus: in the cytoplasm, β-DG localized to the cell periphery, decorating the filopodia-like surface protrusions, and towards the perinuclear region in a rim-like structure. Inside the nucleus, β-DG localization varied between the different lines: in

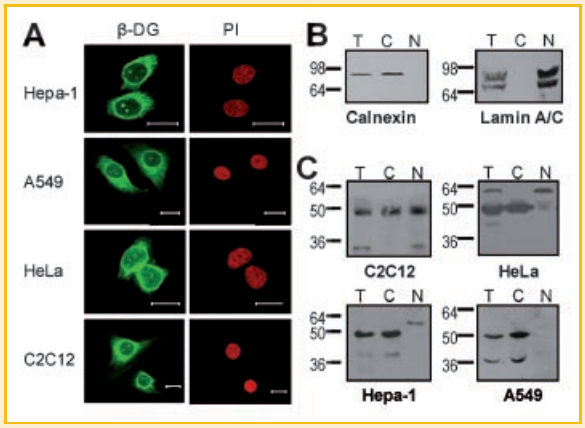

Fig. 1. Subcellular distribution of β-dystroglycan in various cell lines. Panel A: Cells cultured on glass cover slips were fixed and stained with the anti-β-dystroglycan antibody JAF, followed by a fluorescein-conjugated secondary antibody (green color) with nuclei stained with propidium iodide (PI; red color). Samples were imaged using a confocal microscope with a typical single optical Z-section shown. Bar = 20 μm. Panels B and C: Cell cultures were fractionated into total (T), cytoplasmic (C) and nuclear (N) protein extracts, resolved by SDS–PAGE and subjected to Western analysis for β-DG using the antibody JAF (panel C). Nuclear (lamin A/C) and cytoplasmic (calnexin) proteins were analyzed in parallel as controls (panel B), with the position of running of protein markers shown on the left. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Hepa-1 cells, it displayed a discontinuous punctuate staining with some intense-labeled foci, while in the remaining cell lines (A549, HeLa, and C2C12), it showed a homogenous pattern of distribution with the presence of comparatively less intense fluorescent foci. To corroborate the nuclear localization of β-DG, total cytoplasmic and nuclear protein extracts were obtained from each cell line and subjected to immunoblotting analysis, using the anti-β-DG antibody JAF (Fig. 1B). The fractionation procedure was validated by probing C2C12 cell extracts for calnexin (a cytoplasmic protein) and lamin A/C (a nuclear protein). As expected, calnexin (90 kDa) was absent from the nuclear fraction but present in the cytosolic extracts, whereas lamin A/C (69/62 kDa) was found solely in the nuclear fraction. Identical results were obtained with extracts of the remaining three cell lines (data not shown). In C2C12 cells, the anti-β-DG antibody revealed two immunoreactive bands: the first one, positioned just below the 50 kDa protein marker, was observed in cytoplasm and nucleus, while the second one, migrating below the 36 kDa marker, was found solely in the nuclear fraction, In the HeLa and Hepa-1 cell lines, the β-DG protein band that migrates below the 50 kDa marker was predominant in the cytosol but barely detected (HeLa cells) or even absent (Hepa-1 cells) from the nucleus. Instead, a slower-migrating band, situated above the 50 kDa protein marker, was present exclusively in the nuclear fraction of both cell types. Finally, in the A549 cells the β-DG band below the 50 kDa was prominent in the cytoplasm but virtually absent from the nucleus, while a second band located between the 50 and 36 kDa markers was restricted to the cytosolic fraction. It has been observed that phosphorylation of β-DG causes an upward mobility shift of the protein [James et al., 2000]; therefore, the immunoreactive bands migrating above the expected molecular weight of β-DG (43 kDa) might correspond to phosphorylated variants (see below). Overall, the immunofluorescence and Western blot analyses confirmed the ability of endogenously expressed β-DG to localize in the nucleus.

CHARACTERIZATION OF THE NLS OF β-DYSTROGLYCAN

To identify the molecular signal responsible for the nuclear localization of β-DG, its primary amino acid sequence was analyzed using the protein domain prediction program PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html). This analysis unveiled a putative monopartite NLS located in the cytoplasmic domain of β-DG within residues 776–782 (Supplemental Fig. 1A). Interestingly, the amino acid sequence of the putative NLS of β-DG is totally conserved among the orthologous proteins of different species (Supplemental Fig. 1B). To assess the functional significance of the NLS motif, the cDNA of β-DG was fused to the 3′-end of the GFP cDNA to generate the pGFP-β-DG expression vector, and site-directed mutagenesis performed to delete the NLS and generate the pGFP-β-DGΔNLS vector (Fig. 2A). To confirm the expression of GFP-β-DG and GFP-β-DGΔNLS fusion proteins, total extracts from transiently transfected C2C12 cells were subjected to Western blotting analysis using the anti-β-DG antibody JAF (Fig. 2B).

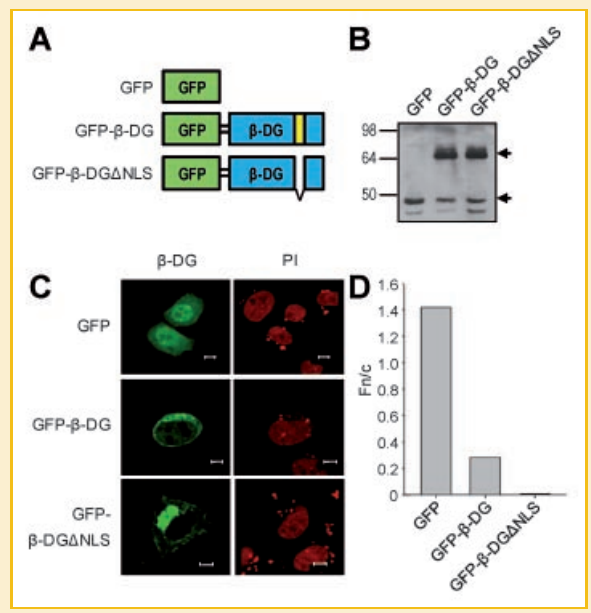

Fig. 2. Nuclear localization of β-dystroglycan requires the NLS within amino acids 776–782. Panel A: Schematic illustration of the expression vectors used to evaluate the functionality of the NLS of β-dystroglycan (β-DG), where NLS is denoted by a yellow square. Panel B: HeLa cell lysates expressing GFP, GFP-β-DG and GFP-β-DGΔNLS fusion proteins were analyzed by Western blotting using the anti-β-DG antibody JAF. The running position of protein markers is shown on the left. Arrows at the right denote the migration of the endogenous β-DG and the GFP-β-DG protein fusions. Panel C: HeLa cells cultured on glass cover slips were transiently transfected to express GFP, GFP-β-DG and GFP-β-DGΔNLS and cells were fixed 48 h post-transfection and stained with propidium iodide (PI) to enable the nuclei to be visualized. Cells were imaged by confocal microscopy, with typical single optical Z-sections shown. Scale bars represent 8 μm. Panel D: A quantitative analysis of the levels of nuclear accumulation ($F_{n/c}$ ratio) of GFP, GFP-β-DG, and GFP-β-DGΔNLS was estimated, as described in the Materials and Methods Section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

A protein band of 72 kDa corresponding to the GFP-β-DG fusion was detected in the lysates of cells expressing GFP-β-DG or GFP-β-DGΔNLS fusion proteins but not in the lysate of cells producing GFP alone. Additionally, a protein band that migrates below the 50 kDa marker that must correspond to the endogenous β-DG protein was revealed in all of the cell extracts. Subcellular localization was also evaluated by confocal fluorescence microscopy (Fig. 2C). GFP alone was found equally distributed between cytoplasm and nucleus ($F_{n/c}$ of 1.2) in keeping with the fact that it is small enough (molecular weight of 27 kDa) to enter the nucleus by passive diffusion through the NPC (Fig. 2D). GFP-β-DG displayed a dotted labeling pattern distributed mainly in the cytoplasm with only a faint signal in the nucleus ($F_{n/c}$ of 0.2) (Fig. 2D). Since GFP-β-DG is greater than the upper limit (45 kDa) for free diffusion through the NPC, this implies that its nuclear accumulation is the result of an active transport. Remarkably, GFP-β-DGΔNLS was totally excluded from the nucleus, with striking accumulation in the perinuclear region resembling the endoplasmic reticulum ($F_{n/c}$ of 0.008) (Fig. 2D). These results demonstrate that the amino acid sequence ^{776}RKKRKGK^{782} represents a functional NLS for β-DG sufficient to target a heterologous protein (GFP) to the nucleus, and necessary for nuclear entry of β-DG.

SITE-DIRECTED MUTAGENIC ANALYSIS OF THE β-DYSTROGLYCAN NLS

β-DG exhibits diverse interactions with cytoskeletal and cytoplasmic proteins that may interfere with its nuclear transport signaling. In order to test the efficacy of the NLS out of the context of full-length β-DG, the sequence encoding the NLS motif was cloned into the Tetra-GFP reporter system, which is based on four in-frame fused copies of EGFP [Beetz et al., 2004]. Additionally, a series of mutants, each of which contained one or two basic residues within the NLS substituted by alanine were also engineered in the Tetra-GFP vector. Expression of the reporter proteins bearing the NLS of β-DG or its mutated variants was evaluated in transfected C2C12 cells by Western analysis. A band of the expected 110 kDa was detected exclusively in the lysates of transfected cells (Supplemental Fig. 2), confirming the integrity of the protein constructs. Then, subcellular localization of the reporter proteins was analyzed by confocal fluorescence microscopy and quantitative imaging analysis was performed to determine their nuclear to cytoplasmic ratio ($F_{n/c}$—see the Materials and Methods Section). As expected, the Tetra-GFP alone (110 kDa) showed exclusive cytoplasmic distribution; however, the inclusion of the NLS of β-DG or that of the SV40 large T-antigen (NLS-SV40, used as a positive control) resulted in predominantly nuclear localization (Fig. 3A). Image analysis revealed that the NLS of β-DG is highly efficient in transporting Tetra-GFP to the nucleus, compared with the NLS-SV40 ($F_{n/c}$ of 1.7 and 2.0, respectively) (Fig. 3B). The mutA β-DG derivative displayed only a moderate reduction in nuclear accumulation ($F_{n/c}$ of 0.4), compared to the wild-type construct (Fig. 3A,C), suggesting that the central basic residues of the NLS are critical to NLS. To explore this in more detail, the central core residues, K^{777} and K^{778} were mutated in mutB, whereas R^{779} and K^{780} were substituted in mutC. Nuclear accumulation of mutB was

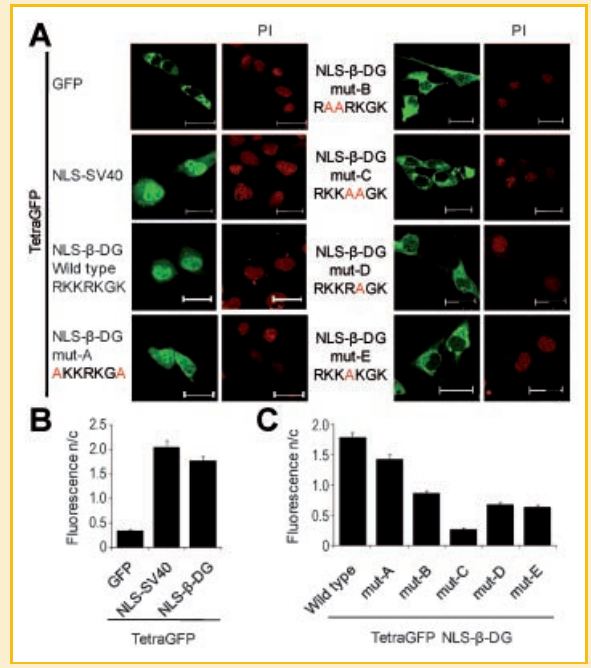

Fig. 3. In vivo nuclear import efficiency of the β-dystroglycan NLS and derivative mutants. Panel A: C2C12 cells expressing Tetra-GFP alone or fused to the NLS of SV40 large T-antigen (NLS-SV40, positive control) or the NLS of β-dystroglycan (NLS-β-DG) or its derivative NLS mutants (mutA-mutE) were grown on glass cover slips, fixed 24 h post-transfection, stained with propidium iodide (PI, red color) to visualize nuclei and then, subjected to confocal microscopy analysis. The NLSs are shown in single-letter amino acid code with mutated residues denoted in red. Typical single optical Z-sections are shown. Bar = 30 μm. Panel B: Confocal images shown in panel A were analyzed using the Image J software and a quantitative analysis of the levels of nuclear accumulation ($F_{n/c}$ ratio) of Tetra-GFP, NLS-SV40, and NLS-β-DG was estimated, as described in the Materials and Methods Section. Results represent the mean ± SEM from a series of three separate experiments where each data point for the $F_{n/c}$ represents the mean of >100 separate measurements. Panel C: Quantitative analysis of the levels of nuclear accumulation of β-DG NLS mutants was performed as described above and compared with that of the wild-type NLS. Results represent the mean ± SEM of three separate experiments (n > 100).

diminished approximately 50% ($F_{n/c}$ of 0.8) whereas that of mutC displayed a more drastic reduction ($F_{n/c}$ of 0.2), compared with the wild-type NLS (Fig. 3A,C). These results suggest that R^{779} and K^{780} play a pivotal role in the nuclear uptake of β-DG. Mutants D (R^{779}/A) and E (K^{780}/A) were then generated to evaluate the individual contribution of each residue. MutD as well as mutE caused a significant reduction in the nuclear accumulation of the Tetra-GFP reporter protein ($F_{n/c}$ of 0.6), suggesting that both residues are equally important to the β-DG NLS functionality (Fig. 3A,C). Since β-DG exerts diverse interactions with cytoskeletal and cytoplasmic proteins that may modulate its nuclear transport, the importance of R^{779} and K^{780} was confirmed in the context of the full-length β-DG. Supplemental Figure 3 shows that mutation of the two basic residues provoked a drastic reduction in the nuclear localization of full-length β-DG (GFP-β-DG vs. GFP-β-DG-mutC).

β-DYSTROGLYCAN NUCLEAR IMPORT IS DEPENDENT ON RAN, AND MEDIATED BY IMPORTINS α AND β

NLS-dependent nuclear protein import generally involves the recognition and binding of the NLS by Imps and the subsequent translocation of the protein cargo into the nucleus through the NPC. The NPC is made of protein building blocks—nucleoporins—some of which contain the covalently attached sugar moiety *N-acetyl* glucosamine, which binds the edible mushroom *Agaricus bisporus* lectin (ABL) with high affinity. Blockage of nuclear uptake by ABL is diagnostic for nuclear entry via the NPC [Yu et al., 1999]. Therefore, we examined the effect of ABL on the nuclear translocation of endogenous β-DG in HeLa cells by immunofluorescence analysis. Figure 4 shows that ABL-treatment impeded nuclear entry of β-DG, resulting in an exclusively cytoplasmic localization pattern for this protein, consistent with the idea that β-DG enters the nucleus through the NPC.

Imp-dependent nuclear protein import is integrally dependent on the guanine nucleotide binding protein Ran. To test whether nuclear accumulation of β-DG may be dependent on Ran, a negative-dominant mutant of Ran deficient in GTP hydrolysis (RanQ69L) was used. As previously observed, Tetra-GFP fused to the NLS of β-DG exhibited almost complete nuclear localization in the CC12 cells ($F_{n/c}$ of 2.3); however, ds-Red-RANQ69L coexpression markedly inhibited nuclear accumulation (Fig. 5A), resulting in an $F_{n/c}$ of 1 (Fig. 5B). This inhibition of nuclear accumulation was comparable to the effect of ds-Red-RANQ69L overexpression on nuclear accumulation of Tetra-GFP fused to the NLS of SV40 large T-antigen, whose nuclear import is strongly dependent on Ran, and mediated by the Impα/β heterodimer (Fig. 5A,B). The results clearly indicate Ran dependence of nuclear accumulation mediated by the β-DG NLS, implying Imp dependence of the nuclear import process.

To assess Imp involvement in the nuclear import of β-DG, constructs were generated to express two different fragments of β-DG (aa 774–789 and 774–895), both containing the NLS, fused to GFP (yielding GFP-β-DG-NLS and GFP-β-DG-Cyto, respectively); these were expressed in bacteria and purified. The ability of GFP-β-DG-NLS and GFP-β-DG-Cyto to interact with Imps, expressed as GST fusion proteins, was assessed using a gel shift mobility assay followed by fluorimaging (Fig. 6A). Preincubation with Impα or Impβ alone, or the Impα/β heterodimer, resulted in altered mobility of both GFP-β-DG-NLS and GFP-β-DG-Cyto. As previously [Wagstaff and Jans, 2006], the NLS of SV40 large T antigen within GFP-Tag-NLS was used as a positive control recognized by Impα as well as the Impα/β heterodimer but not by Impβ alone, confirming the reliability of this assay. The binding affinity of the respective Imps for the NLS of β-DG was measured in an ALPHAScreen assay, as previously [Hearps and Jans, 2006; Wagstaff and Jans, 2006]. Figure 6B shows that GFP-β-DG-NLS as well as GFP-β-DG-Cyto bound with high affinity to both Impα and Impβ alone, but the affinity was even higher (<1 nM) for the Impα/β heterodimer; clearly, the β-DG NLS is accessible to bind Imps when in the context of the β-DG cytoplasmic domain. No binding to Imps was observed with GST alone (negative control).

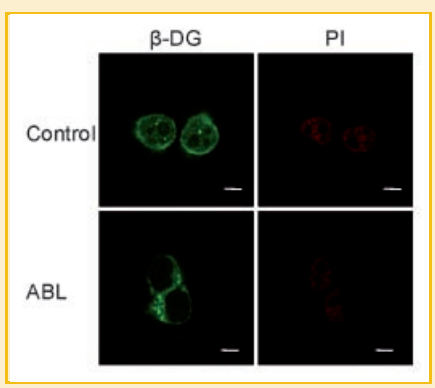

Fig. 4. The *Agaricus bisporus* lectin inhibits nuclear import of β-dystroglycan. HeLa cells, seeded onto coverslips were pretreated with 20 μg/ml *Agaricus bisporus* lectin (ABL) for 6 h at 37°C. Cells were then fixed and permeabilized to analyze β-dystroglycan (β-DG) distribution, using the primary anti-β-DG antibody JAF and a fluorescein-conjugated secondary antibody (green color). Nuclei were stained with propidium iodide (PI, red color). After double labeling, cell preparations were imaged by CLSM with typical single optical Z-sections shown. Control: cells with no treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

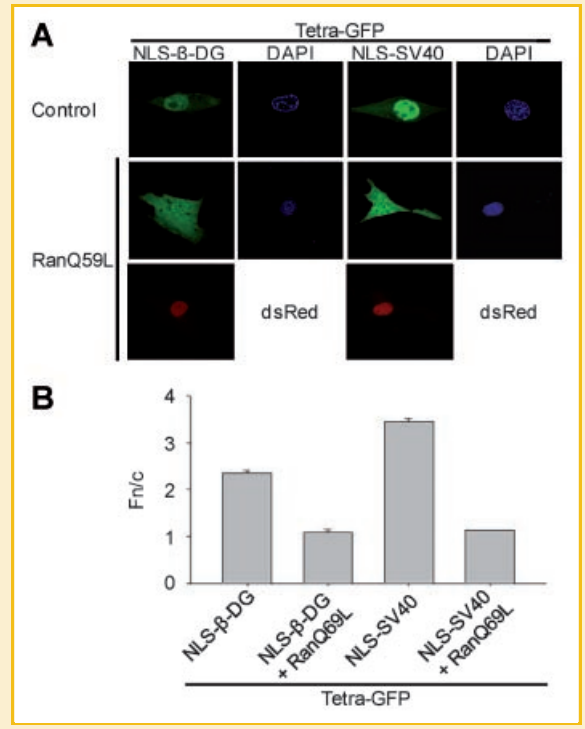

Fig. 5. Nuclear accumulation of the β-DG NLS is reduced by the Ran negative-dominant mutant RanQ69L. Panel A: C2C12 cells were cotransfected with plasmids expressing Tetra-GFP (green) fused to the NLS of either β-dystroglycan (NLS-β-DG) or SV40 large T-antigen (NLS-SV40, positive control) together without or with plasmid encoding the dominant negative Ran derivative dsRed-RanQ69L (red). Cells were fixed 24 h post-transfection, stained with DAPI (blue) to visualize nuclei and then, subjected to confocal microscopic analysis. Typical single optical Z-sections are shown. Panel B: Confocal images shown in panel A were analyzed with the Image J software and a quantitative analysis of the levels of nuclear accumulation ($F_{n/c}$ ratio) performed as described in the legend to Figure 3.

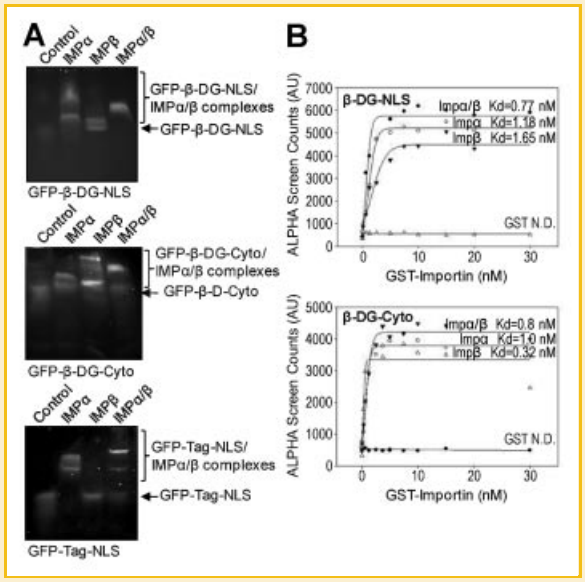

Fig. 6. The β-DG NLS is recognized with high affinity by the importin α/β heterodimer. Panel A: Mobility gel shift assays were performed by incubating bacterially expressed GFP-β-DG-NLS (upper) GFP-β-DG-Cyto (middle) or GFP-Tag-NLS (lower) proteins with 10 μM of GST-tagged mouse Importin (Imp)α or Impβ, or predimerized Impα/β1 for 15 min at room temperature prior to native PAGE and fluorimaging. The running position of GFP-tagged proteins, and complexes thereof with Imps are indicated. Panel B: To determine the affinity of binding, GFP-tagged proteins were incubated with increasing concentrations (0–60 nM) of GST-tagged mouse Impα, Impβ or predimerized Impα/β1, or GST itself as a control, and an ALPHAScreen assay performed as described in the Materials and Methods Section. Sigmoidal curves were fitted using the SigmaPlot software to determine the apparent dissociation constants (K_d) as indicated. Each data point represents the average of three measurements from a single typical experiment from a series of three separate experiments. ND, not determined.

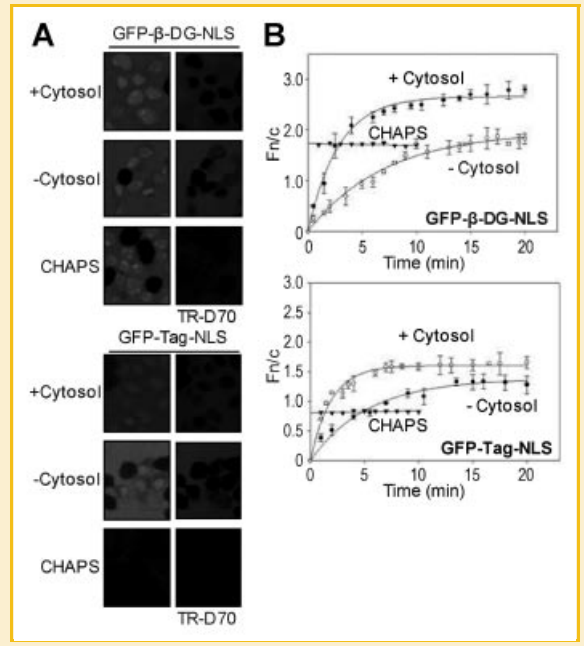

Fig. 7. Nuclear import of β-dystroglycan is dependent on cytosolic factors. Nuclear import of GFP-β-DG-NLS was reconstituted in vitro in mechanically perforated HTC cells in the absence or the presence of exogenous cytosol and an ATP regenerating system, as described in the Materials and Methods Section. Panel A: Confocal microscopic images were acquired at the indicated times to visualize the accumulation of GFP-β-DG-NLS or the control protein GFP-Tag-NLS into intact nuclei (monitored by exclusion of 70 kDa Texas Red-labeled dextran, TR-D70). Nuclear accumulation of the reporter proteins was also analyzed in the absence of an intact nuclear membrane (indicated by the lack of exclusion of TR-D70), induced by the addition of 0.025% CHAPS. Panel B: Image analysis was performed on the CLSM images using ImageJ (NIH) software. All values were contained within the linear fluorescence range. The nuclear to cytoplasmic fluorescence ratio ($F_{n/c}$) was calculated as described in the Materials and Methods Section. Results represent the mean ± SEM from a series of five separate experiments where each data point for the $F_{n/c}$ represents the mean of >15 separate measurements. Note that since in the absence of an intact nuclear membrane, steady state is reached within minutes, the data for CHAPS-treated samples are only presented for the first 10 min.

To confirm the role of Imps in β-DG nuclear import, an established in vitro reconstituted system, revolving around the use of mechanically perforated rat hepatoma (HTC) cells, was used [Hu and Jans, 1999; Jans et al., 1996; Forwood et al., 2001; Harley et al., 2003; Hearps and Jans, 2006]. The system is essentially identical to the digitonin-permeabilized cell system [Adam et al., 1990, 1991], in that NLS-dependent nuclear protein import can be reconstituted through the addition of exogenous cytosol and an ATP-regenerating system. Significantly, GFP-β-DG-NLS accumulated to a greater extent than GFP-Tag-NLS in this system (maximal $F_{n/c}$ of ca. 2.7 compared to 1.6), indicating functionality of the β-DG NLS (Fig. 7A,B). To investigate the dependence of GFP-β-DG-NLS nuclear import on cytosolic factors, the assay was carried out in the absence of exogenous cytosol. GFP-β-DG-NLS accumulated to a lower extent ($F_{n/c}$ of ca. 1.9 compared to 2.6), indicating that cytosolic components are required for efficient β-DG nuclear import (Fig. 7A,B). To test the ability of GFP-β-DG-NLS to bind to nuclear components, the nuclear envelope was permeabilized with CHAPS and the degree of nuclear accumulation assessed. As previously [Ghildyal et al., 2005], GFP-Tag-NLS does not bind to nuclear components and equilibrates between the nucleus and cytoplasm in the absence of a barrier to diffusion between the two compartments yielding an $F_{n/c}$ value close to 1.0. In contrast, GFP-β-DG-NLS was found to accumulate in the nucleus of CHAPS-treated cells to an $F_{n/c}$ value of 1.7, presumably due to the binding of β-DG NLS to nuclear components (Fig. 7A,B).

To identify cytosolic factor(s) required for β-DG import, antibodies capable of binding and inhibiting the action of specific Imps were added to the reaction. Figure 8A,B shows that antibodies to both Impα and Impβ markedly decreased the levels of GFP-β-DG-NLS nuclear accumulation. Nuclear accumulation of the control molecule GFP-Tag-NLS was inhibited by both anti-Impα and -Impβ antibodies (Fig. 8A,C), demonstrating their functionality and that the effects on β-DG NLS were specific. The results indicate that β-DG nuclear import depends on both Impα and Impβ, consistent with the results obtained in the IMP binding assays.

Further analysis was performed by omitting the ATP regenerating system from the assay, as well as treating both cells and the exogenous cytosol with apyrase to remove all traces of ATP [Jans

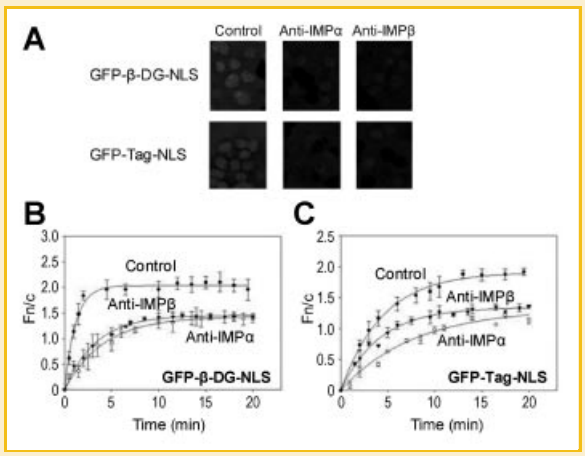

Fig. 8. Nuclear import of β-dystroglycan involves Impα and Impβ. Nuclear import of GFP-β-DG-NLS and GFP-Tag-NLS was reconstituted in vitro as per Figure 7 in the presence of antibodies to Impα or Impβ at 20 and 45 μg/ml, respectively. Panel A: Representative CLSM images of GFP-β-DG-NLS and GFP-Tag-NLS in the absence (Control) or presence of antibodies to Impα or Impβ as indicated. Panels B and C: Nuclear import kinetics of GFP-β-DG-NLS (panel B) and GFP-Tag-NLS (panel C) in the absence or presence of antibodies to Impα or Impβ were determined as described in the legend to Figure 7. Data represent the mean ± SEM from two separate experiments, where each point for the $F_{n/c}$ represents the mean of >10 separate measurements.

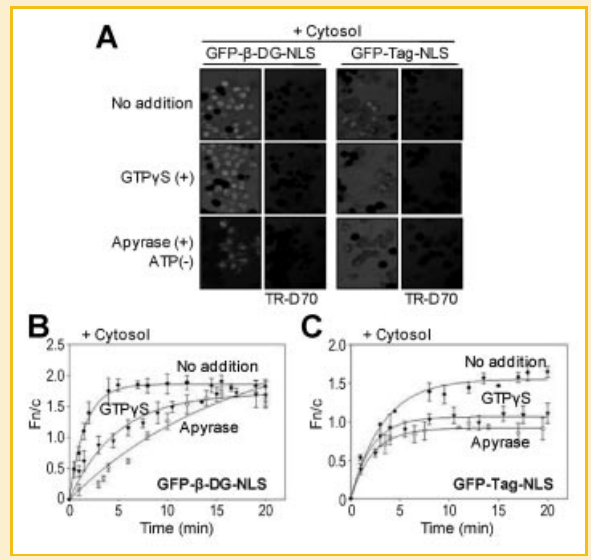

Fig. 9. Nuclear import of β-dystroglycan requires Ran GTP and ATP. Nuclear import of GFP-β-DG-NLS and GFP-Tag-NLS was reconstituted in vitro as described in the legend to Figure 7, following either pretreatment of both the exogenous cytosol and unperforated cells with apyrase (800 and 0.2 U/ml, respectively) to remove ATP from the system or preincubation of exogenous cytosol with 5 μM GTPγS [Jans et al., 1996; Forwood et al., 2001; Ghildyal et al., 2005]. Cells without any treatment were used for comparison. Panel A: Representative confocal microscopic images of the accumulation of GFP-β-DG-NLS and GFP-Tag-NLS in untreated cells or following treatments with Ran GTPγS or apyrase as indicated. Panels B and C: Nuclear import kinetics of GFP-β-DG-NLS (B) and GFP-Tag-NLS (C) were determined in untreated cells or following treatment with Ran GTPγS or apyrase, as described in the legend to Figure 7. Results represent the mean ± SEM from a series of three separate experiments where each point for the $F_{n/c}$ represents the mean of >12 separate measurements.

et al., 1996; Adam et al., 1990, 1991; Forwood et al., 2001; Ghildyal et al., 2005]; import was also analyzed in the presence of the non-hydrolysable GTP analogue GTPγS, which locks Ran in the GTP bound form that binds Impβ, and thus inhibits IMP interaction with import cargo. Figure 9A,B shows that although GFP-β-DG-NLS protein accumulated in the nucleus in the absence of ATP, the nuclear import rate was reduced sevenfold, whereby half-maximal nuclear accumulation was attained at 1.1 min in the presence of ATP compared to 7.8 min in its absence. Similarly, GTPγS reduced the nuclear import rate almost threefold ($t_{1/2}$ of 3.2 min compared to 1.1 min in the absence of GTPγS). As previously [Hearps and Jans, 2006], nuclear import of GFP-NLS-SV40 was inhibited by both cell treatments (Fig. 8A,C). Overall, the results support the idea that the β-DG NLS mediates nuclear import through an active mechanism that requires Imps and Ran, consistent with the results in Figure 5.

PHOSPHORYLATION AT Tyr^{892} MODULATES β-DG NUCLEAR IMPORT

Phosphorylation is one of the main mechanisms controlling the nuclear transport of proteins [Jans et al., 2000; Poon and Jans, 2005; Wagstaff and Jans, 2009]. As a preliminary step towards determining whether phosphorylation at Tyr^{892} may modulate β-DG subcellular distribution, C2C12 cells were treated with the phosphatase inhibitor peroxyvanadate (PV) for 3 h to increase the levels of phosphorylation in the cell, and then cytosolic and nuclear extracts prepared, prior to immunoprecipitation using a phosphotyrosine-specific antibody and Western analysis for β-DG (Fig. 10A). Immunoprecipitated phospho-Tyr-β-DG clearly migrated more slowly in the nuclear/cytosolic extracts, relative to total extracts (T in Fig. 10A), consistent with β-DG being Tyr-phosphorylated. Supporting this idea, immunoprecipitated β-DG from whole cell extracts from peroxyvanadate-treated using the anti-β-DG antibody followed by Western analysis using an anti-phosphotyrosine antibody indicated a Tyr-phosphorylated β-DG form with essentially identical electrophoretic mobility (Supplemental Fig. 4). Strikingly, PV treatment resulted in a marked increase in nuclear levels of phospho-Tyr-β-DG, implying that phosphorylation of β-DG may facilitate its nuclear import (Fig. 10A).

To assess directly the effect of phosphorylation of Tyr^{892} on β-DG nuclear import, the subcellular localization of a GFP-tagged constructs containing the cytoplasmic tail of β-DG with Tyr^{892} (wild-type; GFP-β-DG-Cyto) or a non-phosphorylatable Phe^{892}-substituted derivative (β-DGY892F) was analyzed by confocal microscopy in transfected C2C12 cells. Figure 10B shows that whilst GFP-β-DG-Cyto was distributed equally between the cytoplasm and the nucleus ($F_{n/c}$ of 1.2), GFP-β-DGY892F accumulated more strongly in the nucleus ($F_{n/c}$ of 1.64). Overall, these results are consistent with the idea that phosphorylation influences β-DG nuclear import.

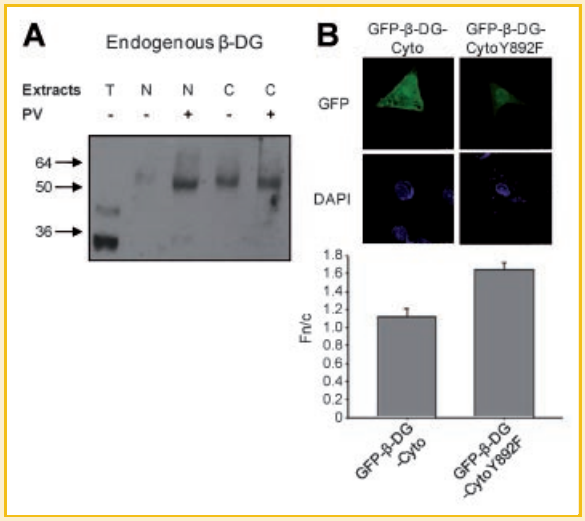

Fig. 10. Tyrosine phosphorylation of β-DG modulates negatively its nuclear localization. Panel A: Cytoplasmic (C) and nuclear (N) extracts obtained from non-transfected C2C12 cells cultured in the presence or absence of peroxyvanadate (PV) as indicated, were immunoprecipitated with an anti-phosphotyrosine antibody resolved by SDS-PAGE and subjected to Western analysis for β-DG using the anti-β-DG antibody JAF. As a control, immunodetection of β-DG was carried out in total cell extracts (T) without immunoprecipitation. Migration of protein markers is shown on the left. Panel B: C2C12 cells expressing GFP fused to the cytoplasmic tail of β-dystroglycan containing the tyrosine 892 intact (GFP-β-DG-Cyto) or mutated to phenylalanine (β-DG-CytoY892F) were grown on glass cover slips, fixed 24 h post-transfection, stained with DAPI (blue color) to visualize nuclei and then, subjected to confocal microscopic analysis. Typical single optical Z-sections are shown; a number of images were analyzed using the Image J software to give a quantitative analysis of the levels of nuclear accumulation ($F_{n/c}$ ratio), as described in the legend to Figure 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In this study, we delineate the nuclear import pathway of the transmembrane DAPC component β-DG. To be imported into the nucleus, proteins conventionally need to be actively transported through the nuclear pore via an NLS-/Imp-dependent process [Jans et al., 2000; Pemberton and Paschal, 2005]. In line with this, we show here that β-DG possesses a basic NLS (^{776}RKKRKGK^{782}) within its cytoplasmic domain, which mediates active nuclear transport of β-DG, in a process dependent on Impα/β and Ran. That the NLS is necessary for β-DG nuclear localization is shown by the fact that deletion of the sequence from full-length β-DG abolishes nuclear localization of a GFP-β-DG fusion protein, whilst the NLS alone is sufficient to target a Tetra-GFP reporter protein to the nucleus, as well as GFP in an in vitro reconstituted system. Mutagenic analysis revealed that residues R^{779} and K^{780} are essential for NLS functionality in the context of both full-length β-DG and a Tetra-GFP fusion. Oppizzi et al. [2008] reported that two lysines (^{793}KK^{794}) 10 amino acids downstream of the NLS may also play a role in nuclear accumulation, but our work here with full-length β-DG and the large Tetra-GFP reporter protein show that ^{776}RKKRKGK^{782} is both necessary and sufficient for β-DG nuclear targeting.

Through transfection experiments, direct binding studies and an in vitro reconstituted nuclear transport assay, we show that the NLS mediates nuclear import actively through the nuclear pore via a conventional pathway dependent on Impα and β, and Ran. The high binding affinity of the Impα/β heterodimer for the β-DG NLS, even in the context of the cytoplasmic domain of β-DG (<1 nM) is consistent with the idea that Impα/β is likely to be responsible for β-DG nuclear import in vivo, although interaction with nuclear components, mediated by the β-DG NLS, may also contribute to β-DG nuclear accumulation.

Exactly how β-DG may traffic from its conventional plasma membrane location is unclear; further, β-DG associates with the actin-based cytoskeleton [Chen et al., 2003] and a number of cytoplasmic proteins [Yang et al., 1995; Cartaud et al., 1998; Russo et al., 2000; Bartoli et al., 2001; Rando, 2001; Langenbach and Rando, 2002; Spence et al., 2004b; Batchelor et al., 2007] that could anchor the molecule to the cytoplasmic compartment and restraining it from transport to the nucleus. For instance, β-DG binds to dystrophin to form the DAPC, involved in plasma membrane stability and cell signaling [Jung et al., 1995]. In concordance, our results show that the NLS of β-DG alone is more efficient at targeting GFP to the nucleus than the β-DG cytoplasmic domain or full-length β-DG in transfected cells; clearly, other sequences within β-DG modulate NLS accessibility as well as β-DG interaction with other proteins. Importantly, the β-DG NLS overlaps with the protein domain of β-DG that interacts with ezrin to modulate cytoskeletal remodeling and filopodia formation [Spence et al., 2004a]. Hence, it is conceivable that competition between Imps and ezrin for binding to the NLS of β-DG may well regulate β-DG nuclear import.

Since phosphorylation of β-DG is known to affect its subcellular localization [Sotgia et al., 2003], we decided to ascertain whether this modification may affect β-DG nuclear import. Our analysis indicates increased accumulation of tyrosine-phosphorylated β-DG exclusively in the nucleus of peroxyvanadate-treated cells, indicating that its nuclear localization can be regulated. Interestingly, the cytoplasmic tail of β-DG containing substitution of Tyr^{892} with a non-phosphorylatable phenylalanine residue is imported to the nucleus more efficiently than wild-type variant. Since tyrosine 892 phosphorylation of β-DG promotes its association with SH2-domain containing proteins such as c-Src. Fyn, Csk, NCK, and SHC [Sotgia et al., 2001], the Y892F mutation would weaken all of these cytoplasmic interactions, and thereby facilitate interaction with Impα/β nuclear import. Therefore, it seems that specific tyrosine phosphorylation may result in cytoplasmic retention of β-DG, thereby inhibiting its nuclear import; since inhibition of dephosphorylation (Fig. 10A) seems to enhance nuclear accumulation, the clear implication is that β-DG may have phosphorylation sites that can either enhance or inhibit its nuclear import. Elucidating this potential regulatory mechanism is a focus of future work in this laboratory.

β-DG is largely known as dystrophin-associated transmembrane protein involved in plasma membrane stability, adhesion, cytoskeletal remodeling, and cell signaling [Yang et al., 1995; Cartaud et al., 1998; James et al., 2000; Russo et al., 2000; Sotgia et al., 2000, 2001; Bartoli et al., 2001; Ilsley et al., 2001; Rando, 2001; Langenbach and

Rando, 2002; Spence et al., 2004a,b; Batchelor et al., 2007]. Accordingly, its nuclear localization as described here and elsewhere [Fuentes-Mera et al., 2006; Gonzalez-Ramirez et al., 2008; Oppizzi et al., 2008] opens up the possibility of additional functions within the nucleus for this multi-functional protein, in processes such as gene transcription, RNA processing or mRNA transport [Bartova and Kozubek, 2006; Schneider and Grosschedl, 2007]. Interestingly, in our previous study [Gonzalez-Ramirez et al., 2008] we found enriched protein levels of β-DG in the nuclear envelope fraction of C2C12 muscle cells, similar to those detected for the nuclear envelope markers emerin and lamin A/C. Furthermore, immunostaining of β-DG was evident in the nuclear periphery of the cell lines analyzed herein, which is consistent with the labeling pattern of inner nuclear membrane proteins. On the other hand, we have also reported the recovery of β-DG and some dystrophin-associated proteins, including α1-dystrobrevin, β-sarcoglycan and nNOS, in the nuclear matrix of HeLa cells [Fuentes-Mera et al., 2006]. Overall, these results suggest that, additional to its role at the plasma membrane, β-DG may be a nuclear envelope component, possibly within the inner nuclear membrane, which might play a role in nuclear architecture. The nuclear envelope is involved in the maintenance of nuclear shape and integrity, organization of chromatin, splicing and modulation of gene expression [Hetzer et al., 2005; Bartova and Kozubek, 2006; Schneider and Grosschedl, 2007]. In fact, the crucial role of nuclear envelope in cell/tissue function has been highlighted by the identification of a variety of human diseases, ranging from cancers to some forms of muscular dystrophies, linked to mutations in proteins of the nuclear pore, the inner nuclear membrane and the nuclear lamina [Crisp and Burke, 2008; Parnaik, 2008]. Therefore, it is tempting to propose that β-DG might participate in the modulation of nuclear envelope-associated nuclear processes, although evaluation of this hypothesis will require future studies.

In conclusion, we have established that β-DG has a functional NLS mediating active targeting to the nucleus through Impα and Impβ and Ran. The presence of β-DG within the nucleus suggests a potential role for this protein in nuclear processes.

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