Direct interaction of β -dystroglycan with F-actin

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Dystroglycans are essential transmembrane adhesion receptors for laminin. α -Dystroglycan is a highly glycosylated extracellular protein that interacts with laminin in the extracellular matrix and the transmembrane region of β -dystroglycan. β -Dystroglycan, via its cytoplasmic tail, interacts with dystrophin and utrophin and also with the actin cytoskeleton. As a part of the dystrophin–glycoprotein complex of muscles, dystroglycan is also important in maintaining sarcolemmal integrity. Mutations in dystrophin that lead to Duchenne muscular dystrophy also lead to a loss of dystroglycan from the sarcolemma, and chimaeric mice lacking

muscle dystroglycan exhibit a severe muscular dystrophy phenotype. Using yeast two-hybrid analysis and biochemical and cell biological studies, we show, in the present study, that the cytoplasmic tail of β -dystroglycan interacts directly with F-actin and, furthermore, that it bundles actin filaments and induces an aberrant actin phenotype when overexpressed in cells.

Key words: actin binding, actin bundling, dystroglycan, F-actin, muscular dystrophy, utrophin.

INTRODUCTION

Dystroglycan is a cell adhesion receptor that is an essential part of the link between the actin cytoskeleton and the extracellular matrix (see [1] for a review). Previous work has demonstrated that the spectrin family proteins dystrophin and utrophin mediate the link between the C-terminal cytoplasmic tail of β -dystroglycan and the actin cytoskeleton. The WW and EF-hand domains of utrophin or dystrophin associate with the 20 terminal residues of the cytoplasmic tail of β -dystroglycan [2–4], and the calponin homology domains of dystrophin and utrophin bind directly to F-actin [5,6]. On the extracellular face, the N-terminus of β -dystroglycan associates with α -dystroglycan. α -Dystroglycan, in turn, binds a number of extracellular matrix proteins including laminin [7]. The importance of this physical link is exemplified by mutations in either dystrophin or laminin, which give rise to severe forms of muscular dystrophy [8,9]. Furthermore, dystroglycan-deficient mice fail to develop after embryonic day 6.5, suggesting a more fundamental role for dystroglycan [10], and targeted disruption of dystroglycan in muscle leads to a muscular dystrophy phenotype [11,12]. More recent studies have also suggested that dystroglycan may play a role in cell polarity in non-muscle tissues [13,14]. Whether this is associated with the ability of dystroglycan to associate with the actin cytoskeleton or is part of a further novel function for dystroglycan is yet to be established. Similar to dystroglycan, other cell adhesion receptors associate with the actin cytoskeleton, including cadherins and integrins, via cytoskeletal linking proteins and do not normally interact directly with F-actin. The presence of multiple links between an adhesion receptor and F-actin provides for a high degree of flexibility, fidelity and regulation of the receptor or cytoskeleton, although, for integrins, direct associations between the integrin cytoplasmic tail and actin have been reported (see [15] for a review). In the present study, we report a direct interaction between the cytoplasmic tail of β -dystroglycan and F-actin and demonstrate a novel role for dystroglycan in the organization of the actin cytoskeleton in fibroblast cells that is independent of its normal cytoskeletal linker, utrophin.

MATERIALS AND METHODS

Yeast two-hybrid analyses

The cytoplasmic domain of β -dystroglycan (DGc) from mouse (residues 781–893) was used as a bait in a GAL4-based yeast two-hybrid screen (Matchmaker; Clontech) against a human skeletal-muscle cDNA library (Clontech) according to the manufacturer's instructions. The *Saccharomyces cerevisiae* strain PJ69-4A was initially transformed with the β -dystroglycan bait construct in vector pAS2-1 and expression of the β -dystroglycan protein was confirmed by Western-blot analysis for β -dystroglycan. After co-transfection of the human skeletal-muscle cDNA library in pACT2, an initial round of selection on media lacking histidine was performed on the 900 000 transformants, followed by a further round of selection on adenine. Of the 150 positive colonies selected on histidine or adenine, 78 grew on media lacking both histidine and adenine. After plasmid rescue, the 74 plasmids containing open reading frames were transformed back into PJ69-4A containing β -dystroglycan to confirm activation of the reporter genes, and into PJ69-4A without β -dystroglycan to demonstrate lack of self-activation.

Expression constructs

A full-length mouse dystroglycan cDNA encoding α - and β -dystroglycans (kindly provided by Derek Blake, University of Oxford, Oxford, U.K.) was cloned into the pEGFP vector (Clontech) using *Sma*I and *Sal*I restriction sites engineered by PCR. All subsequent constructs containing deletion and mutations of α/β -DG (full-length dystroglycan) were generated by PCR and cloning into the pEGFP vector using the same sites, and the fidelity of all the constructs was verified by nucleotide sequencing. Residue numbers for the mouse DG–GFP (dystroglycan–green fluorescent protein) constructs were: α/β -DG, 1–893; Δ DGc (deletion of DGc), 1–773; DGc, 774–893; Δ DGe [deletion of DGe (extracellular domain of dystroglycan)], 1–652 + 752–893; and α -dystroglycan, 1–652. The AP–DG (alkaline phosphatase–dystroglycan) construct was provided by Marius Sudol (Mount

Abbreviations used: α/β -DG, full-length dystroglycan; AP, alkaline phosphatase; DG, dystroglycan; DGC, dystrophin–glycoprotein complex; DGc, cytoplasmic domain of β -DG; DGe, extracellular domain of β -DG; GFP, green fluorescent protein; GST, glutathione S-transferase; PAK, p21-activated kinase.

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Sinai School of Medicine, New York, NY, U.S.A.) [4]. Dominant-negative GTPase constructs N19Rho, N17Rac and N17Cdc42 [16,17] were obtained from Anne Ridley (Ludwig Institute, London, U.K.). DGc (residues 775–893) was produced by PCR and cloned into the pET vector pSJW1 ([18], but see [18a]) using *Nde*I and *Sal*I restriction sites. DGc was expressed as a native protein, lacking any fusion tag, in *Escherichia coli* BL21 (DE3). After lysis, DGc was purified using DEAE–Sephacel in 20 mM Tris/HCl (pH 8.0), 1 mM EGTA and 1 mM dithiothreitol, followed by purification through a hydroxyapatite column in 5 mM KH_2PO_4 (pH 7.0), 1 mM EGTA and 100 mM NaCl. DGc was eluted from the DEAE–Sephacel column using a NaCl concentration of 200–300 mM and was collected from the flow-through of the hydroxyapatite column. Despite having a molecular mass of only 12 kDa, DGc runs on SDS/PAGE with an apparent molecular mass of 23 kDa, possibly due to its high proline content.

Cell culture

Tissue culture, transient transfection and immunofluorescence microscopy of REF52 cells were performed as described previously [19]. Cells were deemed to have an actin phenotype if a cell had over 30 small F-actin-containing protrusions around its periphery. For analysis of the effects of dominant-negative small GTPases, only cells staining positive for both dystroglycan (GFP) and the myc epitope were scored as described above. Primary embryonic fibroblasts were prepared from normal and utrophin-deficient mouse embryos [20] and treated as for REF52 cells above. Selection of Swiss 3T3 cells stably transfected with the α/β -DG–GFP construct or GFP empty vector was performed in the presence of 600 $\mu\text{g}/\text{ml}$ G418 (Promega). Clonal populations of cells were isolated by limiting dilution and fluorescent-activated cell sorting using the expressed GFP signal. Active Cdc42 assay was performed on lysates from Swiss 3T3 cells stably expressing either GFP or DG–GFP by pull-down with a GST–PAK construct (where PAK stands for p21-activated kinase) and Western-blot analysis for total and active Cdc42 as described previously [21]. PAK is regulated by binding-activated GTP-bound Cdc42. Therefore GST–fusion proteins to the Cdc42-binding site in PAK can be used to probe specifically for active Cdc42. By comparing the level of total Cdc42 with the amount of Cdc42 that binds to GST–PAK by Western-blot analysis, one can determine the relative amount of active Cdc42 in the cell lysate.

Analysis of actin interactions

Filamentous rabbit muscle actin (5 μM) was incubated in actin-binding buffer [22] in the absence or presence of increasing concentrations of DGc and centrifuged at 360 000 *g* (high speed) or 13 000 *g* (low speed). Equivalent volumes of supernatant and pellet fractions were subjected to SDS/PAGE. Falling-ball viscometry was performed as described previously [6] using 5 μM G-actin and increasing concentrations of DGc. Briefly, glass capillaries (100 μl ; Corning, Corning, NY, U.S.A.) were loaded with G-actin and DGc in the presence of actin-binding buffer and polymerization salts (50 mM KCl/2 mM MgCl_2 /1 mM EGTA) and allowed to polymerize overnight at 4 °C. Capillaries were brought to room temperature (20 °C) and the viscosity of each capillary was determined relative to F-actin alone by the time it took a 0.6 mm stainless-steel ball to drop 10 cm through the capillary.

Figure 1 Biochemical analysis of dystroglycan binding to actin

(A) F-actin was subjected to high- and low-speed sedimentation in the absence (A) or presence of DGc (A + DGc). Supernatant (S) and pellet (P) fractions were separated by SDS/PAGE. Numbers on the right refer to molecular-mass standards (in kDa). Note that despite having a molecular mass of only 12 kDa, DGc runs on SDS/PAGE with an apparent molecular mass of 23 kDa, probably due to its high proline content. The first two lanes in each panel are controls for F-actin alone. At high speed, most of the F-actin is recovered in the pellet fraction, whereas at low speed most of the F-actin is recovered in the supernatant fraction. At high speed, DGc was recovered in the pellet fraction along with F-actin, indicating that it is a bona fide F-actin-binding protein, whereas at low speed, DGc caused a shift of F-actin from the supernatant to the pellet fraction, suggestive of an F-actin-bundling activity. (B) Falling-ball viscometry with 5 μM actin polymerized in the presence of increasing concentrations of DGc. DGc caused a concentration-dependent increase in relative viscosity, indicative of actin bundling.

Electron microscopy analysis

Actin filaments were polymerized as mentioned above and incubated in the absence or presence of 1 μM DGc for 3 h. Filaments were then placed on to carbon Formvar-coated discs, and negatively stained with Nanovan (Universal Biologicals, Cambridge, U.K.) and visualized on a Zeiss 902 electron microscope.

Protein immunoblotting

For direct detection of proteins, 5 μg of the total cell lysate was separated by SDS/PAGE, transferred to PVDF and probed with the appropriate antiserum: β -dystroglycan polyclonal serum (1:10000) [23], β -dystroglycan monoclonal 43DAG/8D5 (1:50) (Novacastra Laboratories, Newcastle upon Tyne, U.K.), GFP (1:5000) (provided by Francis Barr, Max Planck Institute, Munich, Germany) and utrophin (1:10000) [2] as required. Detection was by reaction with a horseradish peroxidase-conjugated secondary antibody and visualization was by ECL[®] according to the manufacturer's instructions (Amersham Biosciences).

Figure 2 Electron microscopy of dystroglycan-induced F-actin bundles

Electron microscopy of negatively stained actin filaments in the absence (A, B) or presence (C, D) of DGc. DGc caused a pronounced bundling of F-actin filaments, as indicated by arrows in (C, D), when compared with F-actin alone. At higher power (D and inset), DGc induced ordered F-actin bundles. Arrowheads indicate individual actin filaments within the bundle, as compared with the single filaments seen with F-actin alone (B). Scale bar, 200 nm.

RESULTS

To identify potential novel interacting partners for dystroglycan, we performed a yeast two-hybrid screen using the mouse DGc as a bait against a human skeletal-muscle cDNA library. From 900 000 transformants, 74 positive interacting clones were identified, of which two independently verified hits were to full-length clones of actin $\alpha 1$. Whereas dystroglycan is part of a complex in muscle and non-muscle cells that interacts with the actin cytoskeleton, via dystrophin or utrophin respectively, dystroglycan itself has not been shown previously to interact directly with the actin cytoskeleton.

Biochemical interaction of dystroglycan with actin

To verify the identification of actin as an interacting protein in the yeast two-hybrid assay, we investigated the interaction between the bacterially expressed DGc and actin. We were unable to demonstrate any direct interaction between purified DGc and purified G-actin by affinity chromatography with immobilized DGc, or between β -dystroglycan and G-actin by immunoprecipitation from REF52 cell lysates (results not shown). However, as shown in Figure 1(A), DGc interacted with F-actin in a high-speed F-actin sedimentation assay. At a high g -value, F-actin was pelleted and any F-actin-binding protein was recovered in

the pellet fraction with F-actin. Most of the F-actin alone was recovered in the pellet fraction as expected. In the presence of DGc, F-actin was also recovered in the pellet fraction along with some of the DGc, suggesting that DGc can interact with F-actin. Surprisingly, when subjected to low-speed sedimentation, DGc was also recovered in the pellet fraction along with a substantial portion of the F-actin, whereas, in the actin-only control, most of the F-actin remained in the supernatant fraction. DGc alone remained in the soluble fraction at either high or low centrifugation speeds (results not shown). These results are suggestive of an actin-bundling activity for DGc, resulting in the formation of higher-order F-actin structures that sediment at low g -value. To confirm further an actin-bundling activity, we performed falling-ball viscometry with increasing concentrations of DGc. As can be seen in Figure 1(B), increasing concentrations of DGc resulted in an increase in relative viscosity, typical of an actin-bundling protein. An actin-bundling activity of DGc was further confirmed by electron microscopy of F-actin in the presence of DGc. As can be seen by electron microscopy, DGc caused a pronounced bundling of F-actin as seen both at low magnification (indicated by arrows in Figure 2C) and high magnification (arrowheads in Figure 2D and inset) when compared with the predominantly single filaments seen with F-actin alone (Figures 2A and 2B).

Figure 3 Dystroglycan overexpression induces alterations in the actin cytoskeleton

An α/β -DG fused to GFP at the C-terminus of β -dystroglycan was transiently expressed in REF52 fibroblasts. (A) Empty GFP vector alone was uniformly distributed through the cytoplasm (green), with no apparent effect on the morphology of the normal actin stress fibre of these cells, as determined by phalloidin staining (red). (B–D) In contrast with the empty GFP vector alone, DG–GFP transfection caused a dramatic rearrangement of the actin cytoskeleton. The DG–GFP is localized at the cell periphery where it stains the plasma membrane and, in particular, the actin-rich protrusions, as indicated by arrows in (B) and inset. With increasing levels of DG–GFP expression, as determined by GFP fluorescence, the effect on the actin cytoskeleton and the number of actin-rich surface protrusions increase (C, D). The actin cytoskeleton becomes more restricted to the periphery, with many small dystroglycan-rich protrusions (arrows in C) and, in extreme cases, the formation of microvilli-like structures on the apical surface of the cell, as indicated by arrowheads in (D). Scale bar, 25 μ m.

Induction of membrane extensions by overexpression of dystroglycan in fibroblast cells

Since DGc was capable of interacting with F-actin *in vitro*, we examined the ability of dystroglycan to interact with the actin cytoskeleton by transfection of an α/β -DG–GFP into the rat embryo fibroblast cell line REF52. Dystroglycan overexpression led to a marked alteration of cell morphology, with the induction of numerous actin-rich surface protrusions around the cell periphery and on the apical surface, and a dramatic reorganization of the actin cytoskeleton (Figure 3). β -DG–GFP seemed to localize to the cell membrane as expected. With increasing levels of β -DG–GFP expression, as determined by the intensity of GFP fluorescence, there was an increase in both the number of actin-rich surface protrusions and in the extent of the remodelling of the actin cytoskeleton (Figures 3B–3D). In the presence of GFP alone, the REF52 cells had their normal complement of stress fibres arranged in a semi-orthogonal pattern (Figure 3A). However, after expression of dystroglycan, these stress fibres were rearranged into a circumferential belt of actin cables with prominent actin-rich filopodial protrusions (Figure 3B and inset). At higher levels of dystroglycan expression, the actin cables became even more dispersed towards the cell periphery (Figure 3C). Therefore the dramatic reorganization of the actin cytoskeleton observed could be due to bundling activity of dystroglycan when overexpressed at

the cell surface. Whereas dystroglycan expression had a dramatic effect on the organization of the actin cytoskeleton, as determined by immunofluorescence microscopy, there was no apparent gross perturbation of either the microtubule or intermediate filament networks (results not shown).

To address the contribution of different domains of dystroglycan to the observed phenotypes, we constructed a series of deletion mutants in α - and β -dystroglycans (Figure 4A). All constructs were expressed efficiently and produced proteins of the expected molecular mass as determined by SDS/PAGE and Western-blot analysis for the GFP moiety (see Figure 5). Constructs were localized to the membrane as determined by GFP fluorescence, with the exception of the intracellular domain of β -dystroglycan (DGc), which was localized predominantly to the nucleus. An actin phenotype as shown in Figure 3 was evident only when DGc was present, i.e. α/β -DG, Δ DGc and AP-DG (Figure 4A). Quantitative results for some of these constructs are presented in Figure 4(B). When DGc is deleted (Δ DGc), the percentage of cells showing an actin phenotype was decreased from 68 to 22%, whereas the DGc alone was not capable of inducing any alteration in the actin cytoskeleton when compared with GFP alone. This is probably because, similar to GFP, DGc was also localized predominantly to the nucleus. The actin phenotype was still evident when a direct fusion was made between α -dystroglycan and the transmembrane region of

Figure 4 Deletion analysis of the regions in dystroglycan required to induce morphogenetic changes

(A) Schematic representation of dystroglycan and the dystroglycan deletion mutants used to study the morphogenetic effects. All constructs are fused to GFP at the C-terminus with the exception of the AP-DG construct, which is fused to AP at the N-terminus. On the right is a qualitative summary of the ability of these constructs to induce an actin phenotype in REF52 cells, based on the quantitative analysis shown in (B). Results are the means $\pm$ S.D. from at least three independent experiments. ss, signal sequence; α -DG, α -dystroglycan; e, DGe; tm, transmembrane region of β -dystroglycan; c, DGc; AP-DG, AP fused to the transmembrane and cytoplasmic domain of β -dystroglycan; GFP, GFP empty vector.

β -dystroglycan to make a chimaeric molecule (Δ DGe), suggesting that DGe was not necessary for the phenotype. Furthermore, α -dystroglycan alone was also not capable of inducing a change in actin. The latter is not surprising given that α -dystroglycan is a peripheral membrane protein, and without its cellular anchor, β -dystroglycan is unlikely to have a direct link to actin. The transmembrane and intracellular domains of β -dystroglycan, which were fused to AP to promote its efficient insertion into the membrane [4], produced as dramatic an actin phenotype as the α/β -DG construct (74 % versus 68 %; Figure 4B). Taken together, these results suggest that DGc is both necessary and sufficient to induce the actin phenotype, but only when correctly targeted to the plasma membrane.

Utrophin is not required for the dystroglycan-induced actin phenotype

Despite the expression of DG-GFP, typically not more than the endogenous levels (Figure 5A, upper panels), the expression

Figure 5 The utrophin-dystroglycan complex and actin rearrangement

(A) Dystroglycan and utrophin protein levels in REF52 cells expressing α/β -DG-GFP. Cell lysates from transient transfections of REF52 cells expressing the empty GFP vector (GFP), α/β -DG-GFP (α/β -DG) or non-transfected cells (—), probed with antisera against GFP (upper panel), β -dystroglycan (middle panel) or utrophin (lower panel). GFP staining indicates the relative levels of GFP and DG-GFP expression compared with the endogenous levels of β -dystroglycan and utrophin, which appear to be unaltered in expressing cells. (B) Quantification of protrusion formation in primary embryo fibroblasts from wild-type (UTRN+) and utrophin knockout (UTRN-) mice and when expressing α/β -DG-GFP (α/β -DG). The number of cells determined to have an actin phenotype was almost identical whether cells were from normal or utrophin-deficient mice. Results are means $\pm$ S.D. (C) Western-blot analysis of the relative levels of dystroglycan and dystroglycan-GFP expression in Swiss 3T3 cells stably expressing either GFP alone (lane 1) or DG-GFP (lane 2). (D) Cdc42 assay of lysates from Swiss 3T3 cells stably expressing either GFP alone (upper panel) or DG-GFP (lower panel). Total Cdc42 levels in stably transfected Swiss 3T3 cells as determined by direct Western-blot analysis of whole-cell lysates (lanes 1); active Cdc42 levels as determined by pull-down with a GST-PAK fusion protein that binds only to active Cdc42, followed by Western-blot analysis for Cdc42 (lanes 2).

of its normal intracellular binding partner utrophin [2,24] was not altered (Figure 5A, lower panel). Furthermore, the actin phenotype appeared to be independent of utrophin, the normal non-muscle cytoskeletal anchor for dystroglycan [2,6], and dystrophin, which is not expressed in mesenchymal cells. The DG-GFP construct was expressed in fibroblasts derived from wild-type and utrophin-null embryos [20]. DG-GFP expression induced the same degree of actin rearrangement in approx. 80 % of cells from either background (Figure 5B). This result strongly suggests that the rearrangement of the actin cytoskeleton induced by β -dystroglycan is a novel and separate function from its known role as an adhesion receptor linking the actin cytoskeleton to the extracellular matrix via utrophin and dystrophin. Effects on the actin cytoskeleton, rearrangement and reduction of F-actin stress fibres and induction of numerous actin-rich protrusions (Figures 3B–3D) have been associated previously with the activation of the Rho family GTPase, Cdc42 [16]. To assess the contribution of Cdc42 activation to the dystroglycan-induced actin rearrangements, we measured the levels of active Cdc42 in Swiss 3T3 cells stably expressing either GFP alone or DG-GFP (Figure 5C). However, the levels of Cdc42 activity as determined by the GST-PAK binding assay [21] were not different for the

Figure 6 Actin morphology in cells overexpressing dystroglycan

Actin morphology, as determined by rhodamine-phalloidin staining in cells expressing either GFP alone (**A, C**) or dystroglycan-GFP (**B, D**). (**A, B**) Utrophin-deficient fibroblasts; asterisks indicate cells transformed with GFP (**A**) or dystroglycan-GFP (**B**). Stable transformants of Swiss 3T3 fibroblasts (**C, D**) expressing either GFP (**C**) or dystroglycan-GFP (**D**). Arrows in (**B, D**) indicate actin-rich surface protrusions. Scale bar, 25 μ m.

GFP- and DG-GFP-expressing cells (Figure 5D). This result suggests that the increase in the number of actin-rich surface protrusions in these cells was not simply due to increased Cdc42 activity, but was probably due to a direct or some other signalling effect of DG. As seen in Figure 6, DG-GFP expression in utrophin-deficient fibroblasts resulted in a circumferential band of actin stress fibres and numerous peripheral F-actin protrusions (Figure 6B) very similar to that seen in REF52 cells expressing DG-GFP (Figure 3B), whereas GFP alone did not affect the normal stress fibre morphology of the utrophin-deficient fibroblasts (Figure 6A). Swiss 3T3 cells stably expressing GFP have some filopodia-like structures as well as prominent F-actin-rich lamellipodial protrusions (Figure 6C). However, DG-GFP expression induces a dramatic increase in the number of filopodia with actin-rich protrusions present all over the cells (Figure 6D).

To assess further the contribution of Cdc42 to the observed filopodia phenotype, we performed co-expression studies with dominant-negative Cdc42 and DG-GFP. The stable Swiss 3T3 cells were not suitable for this experiment, since they turn off dystroglycan expression when deprived of serum, a phenomenon noted in other cell types [25]. Serum deprivation was necessary to visualize the effects of the dominant-negative GTPases. Instead, the assays were performed in REF52 cells. Despite the Cdc42 activation assay showing no difference in overall Cdc42 activity (Figure 5D), somewhat surprisingly dominant-negative Cdc42 inhibited the filopodia phenotype (Figure 7). Dominant-negative Rac and Rho, which are predominantly involved in the

regulation of membrane ruffles and stress fibres respectively, or the empty myc vector had no effect on filopodia formation (Figure 7). Therefore these results suggest that, whereas dystroglycan expression does not affect global Cdc42 activity, the induction of filopodia by dystroglycan still requires Cdc42 activity for this effect.

DISCUSSION

The present study indicates a novel function for dystroglycan in the regulation of the actin cytoskeleton and cell morphology, via a potential direct interaction with the actin cytoskeleton and independent of utrophin. DGc is both necessary and sufficient (providing it is correctly localized) to induce alterations in the actin cytoskeleton. These findings have wide implications for our understanding of the function of dystroglycan in a variety of muscular dystrophies and during *Mycobacterium leprae* infection as well as the fundamental role of dystroglycan in the regulation and organization of the actin cytoskeleton and cellular morphology.

The interaction between dystroglycan and actin was first identified in a yeast two-hybrid screen where the proteins are targeted to the yeast nucleus by the GAL4 fusion [26]. This might suggest that dystroglycan interacted with monomeric G-actin. However, affinity chromatography and immunoprecipitation

Figure 7 Dominant-negative Cdc42 inhibits dystroglycan-induced filopodia

REF52 cells were co-transfected with dystroglycan-GFP and either empty myc vector (myc), dominant-negative Rac (Rac-), dominant-negative Rho (Rho-) or dominant-negative Cdc42 (Cdc42-). Cells staining positive for both dystroglycan (GFP) and the myc epitope were scored for an actin phenotype as described in the Materials and methods section. Empty myc vector, dominant-negative Rac and dominant-negative Rho had no effect on the ability of dystroglycan to induce filopodia-like structures, whereas dominant-negative Cdc42 dramatically decreased the number of peripheral actin-rich structures to levels seen in non-transfected cells. Results are means $\pm$ S.D. from at least three independent experiments.

experiments failed to demonstrate an interaction between G-actin and dystroglycan. Furthermore, if dystroglycan did interact with G-actin to any extent, this would be apparent in the actin interaction assays performed. If dystroglycan acted as a G-actin-sequestering protein, then the amount of F-actin that sedimented at high speed would be decreased in the presence of dystroglycan, resulting in an increase in the amount of actin in the supernatant fraction. By the same token, increasing concentrations of dystroglycan would tend to decrease actin viscosity in falling-ball assays due to the decreased amount of F-actin available for any bundling activity; neither of these is the case (see Figures 1A and 1B). The GAL4 activation domain-actin fusion protein must therefore be capable of polymerizing in the yeast nucleus for the interaction with dystroglycan to take place. Actin fusion proteins can polymerize to form functional filaments, e.g. GFP-actin and F-actin-binding proteins and polymeric actin are normally present in the nucleus of many cells (for recent reviews see [27,28]). Therefore, probably DGc was able to interact with polymeric mammalian actin in the yeast nucleus to activate the reporter genes.

Dystroglycan clearly has both a biochemical actin-bundling activity and a cellular effect on the organization of F-actin. To bundle F-actin, a protein must be multimeric and contain one actin-binding site, or be monomeric with multiple actin-binding sites. Previous work indicates that the cytoplasmic tail of dystroglycan is monomeric ([2]; S. J. Winder, unpublished work) and therefore must contain multiple actin-binding sites. Sequence searches with the cytoplasmic tail of β -dystroglycan reveal no significant overall similarity. However, several short regions of similarity exist between previously delineated actin-binding regions in other actin-binding proteins, including utrophin, gelsolin, tropomodulin and vasodilator-stimulated phosphoprotein (Figure 8). Analysis of the role of these potential actin-binding sites in F-actin bundling by β -dystroglycan is currently in progress.

Deletion mutagenesis of dystroglycan clearly demonstrated a requirement for both DGc and its correct localization to

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781 KLTLEDQATFIKK 803
121 KLTGLLWSIILH 133
    ****      :*:
    utrophin ABS2

804 GVPIIFADELDDSKPPSSSMPLILQ 829
126 GAAAIFTVQLDDYLNGRAVQHR-EVQ 150
    *   **:   ***      :      :*
    gelsolin segment 1

830 EEKAPLPPPEYPSQSVP 846
114 EGGGPPPPPALPTWSVP 130
    *   *   ***   .*:   ***
    VASP

858 EYTPLRDEDPNA 869
169 QYKVPDDEEPS 180
    .* *:   ***:***
    tropomodulin

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Figure 8 Similarity of DGc to other actin-binding proteins

Identification of significant similarities between DGc (upper sequence) and other F-actin-binding proteins (lower sequences). Residue numbers for the segments of individual proteins are indicated. ABS2, actin-binding site 2; VASP, vasodilator-stimulated phosphoprotein. *, identity; ':, highly conserved residues; '.', weakly conserved residues.

the plasma membrane in the induction of surface protrusions and reorganization of actin stress fibres in fibroblast cells. Cell adhesion and organization of the actin cytoskeleton are important determinants in maintaining cell polarity. Recent studies have also indicated an important role for dystroglycan in cell polarity in non-muscle cells [13,14], suggesting that the correct localization of dystroglycan plays an important role in cellular organization, potentially through direct connections to the actin cytoskeleton. The small Rho family GTPase Cdc42 is also an important polarity determinant that has potent effects in regulating the actin cytoskeleton, although evidence presented in this paper suggests that the effects of dystroglycan on the actin cytoskeleton were not through any obvious alteration of the levels of active Cdc42 in the cell. Despite the Cdc42 activation assay showing no difference in Cdc42 activity, somewhat surprisingly dominant-negative Cdc42, but not dominant-negative Rac or Rho, inhibited the filopodia phenotype. This would initially seem paradoxical. However, Cdc42 activity is required for new actin polymerization and filopodia formation [16,29]. Therefore it is not too surprising that inhibition of Cdc42 activity leads to a decrease in filopodia, whether mediated by Arp2/3 and fascin [30], IRSp53 and Mena [31], or as in this case, by dystroglycan. Thus increased dystroglycan expression and localization to the membrane, providing increased actin-bundling potential at the membrane, would appear to be sufficient to induce filopodia under normal levels of new actin polymerization, without affecting overall Cdc42 activity. Nonetheless, some Cdc42 activity is required to maintain dynamic actin polymerization for dystroglycan to induce filopodia, as dominant-negative Cdc42 inhibits dystroglycan-induced filopodia.

The effects of dystroglycan expression on the actin cytoskeleton did not require the presence of utrophin, through which dystroglycan would normally connect to the actin cytoskeleton [6]. Expression of dystroglycan in utrophin-null fibroblasts produced qualitatively similar rearrangements of the actin cytoskeleton as seen in normal fibroblast cells. These results would

tend to suggest a direct role for dystroglycan in regulating the actin cytoskeleton independent of its link to the actin cytoskeleton through utrophin. Utrophin-null mice have a relatively benign phenotype, with only a moderate change in neuromuscular junction architecture [20,32]. It is possible that dystroglycan itself could compensate for the missing actin-binding role of utrophin, despite the fact that, in mice lacking dystrophin and utrophin, this compensation is clearly insufficient to prevent the severe muscular dystrophy seen in these animals [33,34]. The absence of dystrophin alone is evidently enough to disrupt completely the dystrophin–glycoprotein complex (DGC), including dystroglycan, since, in both humans and mice, loss of dystrophin results in all DGC components being lost from the sarcolemma [35,36]. Furthermore, the F-actin side-binding activity of dystrophin [37,38] would also appear to be required for integrity of the subsarcolemmal actin cytoskeleton [39], a role clearly not fulfilled by endogenous dystroglycan alone. Dystrophin, similar to utrophin, can associate with the side of actin filaments [38,40]. As such, dystrophin may serve to stabilize them to a greater extent than is possible by the short (12 kDa) tail of β -dystroglycan. Alternatively, the mode of dystroglycan binding to F-actin, as a bundling protein, may not serve the same function as the side-binding activity of dystrophin or utrophin, and thus cannot compensate for their loss. Identification of β -dystroglycan as an F-actin bundling protein gives a new insight into the role of the DGC in muscular dystrophies and provides new possibilities for therapeutic intervention.

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