Disruption of the dystroglycan gene in humans and mice leads to muscular dystrophies and nervous system defects including malformation of the brain and defective synaptic transmission. To identify proteins that interact with dystroglycan in the brain we have used immunoaffinity purification followed by mass spectrometry (LC/MS-MS) and found that the GTPase dynamin 1 is a novel dystroglycan-associated protein. The α-dystroglycan-dynamin 1 complex also included α-dystroglycan and Grb2. Overlay assays indicated that dynamin interacts directly with dystroglycan, and immunodepletion showed that only a pool of dynamin is associated with dystroglycan. Dystroglycan was associated and co-localized immunohistochemically with dynamin 1 in the central nervous system in the outer plexiform layer of retina where photoreceptor terminals are found. Endocytosis in neurons is both constitutive, as in non-neural cells, and regulated by neural activity. To assess the function of dystroglycan in the former, we have assayed transferrin uptake in fibroblastic cells differentiated from embryonic stem cells null for both dystroglycan alleles. In wild-type cells, dystroglycan formed a complex with dynamin and codistributed with cortactin at membrane ruffles, which are organelles implicated in endocytosis. Dystroglycan-null cells had a significantly greater transferrin uptake, a process well known to require dynamin. Expression of dystroglycan in null cells by infection with an adenovirus containing dystroglycan reduced transferrin uptake to levels seen in wild-type embryonic stem cells. These data suggest that dystroglycan regulates endocytosis possibly as a result of its interaction with dynamin.

Dystroglycan (DG) is encoded by a single gene (dag1), and is synthesized as a precursor protein that is cleaved into α and β subunits. α-DG is a peripheral membrane protein that binds, via its carbohydrate side chains (1, 2), to globular motifs in the α chain of several laminins (3–5), agrin (6–9), perlecain (10, 11), and α-neurexin (12). β-DG is a transmembrane protein bound non-covalently to α-DG that interacts intracellularly with dystrophin, utrophin, rapsyn, caveolin-3, and growth factor receptor-bound protein 2 (Grb2) (13). In skeletal muscle α- and β-DG form the functional core of a larger complex that links the extracellular matrix to the cytoskeleton and serves to maintain the integrity of myofibers faced with the stress of repeated contractions (14). Hence, mice with skeletal muscle deficient in DG develop a severe muscular dystrophy (15–16).

DG is expressed in the nervous system and in many tissues other than skeletal muscle (17). Mice null for dag1 die at E6.5–7.0, long before muscle differentiation (18). DG has been implicated in the formation of several (2, 11, 19), but not all, basement membranes (15–16, 20–21, and in Drosophila, DG is necessary for epithelial polarization (22). In the nervous system, DG is localized at glial endfeet in contact with basement membranes around blood vessels (23) and beneath the meningeal basement membranes (2). These mice also have defects in neuronal migration in the cerebral cortex and cerebellum (2). In humans, similar defects, called lissencephalies, are associated with profound mental retardation and are manifested in Walker Warburg Syndrome, Muscle Eye Brain Disease, and Fukuyama Muscular Dystrophy. All of these are congenital muscular dystrophies that result from mutations in genes known or suspected to regulate glycosylation of α-DG (32). Interestingly, mice with a targeted deletion of dag1 in the brain have a similar phenotype (19), suggesting that many functions of DG are mediated by carbohydrate side chains, which are responsible for most ligand binding (1, 2, 33). In addition, mice lacking DG in the brain have defects in long term potentiation in the hippocampus (19), and myd mice have an abnormal electroretinogram with no signs of disrupted neuronal migration in the retina (20). DG is also necessary for stabilization of the postynaptic density of acetylcholine receptors on skeletal myotubes in culture (11, 34) and at nerve-muscle synapses in vivo (11, 15).

Emerging evidence indicates that the proteins that interact with DG in non-muscle tissues differ from those in muscle. For example, α-neurexin is complexed with α-DG in the central nervous system (CNS), whereas perixxin is associated with DG in Schwann cells (35). To identify novel DG interactors we have used immunoaffinity chromatography to purify DG and its associated proteins from rat brains. We report that β-DG...
forms a complex with dynamin 1, a GTPase essential for several modes of endocytosis including regulated endocytosis during synaptic vesicle release and constitutive endocytosis, which occurs in neuronal and non-neuronal cells alike (36–38). The β-DG-dynamin complex in the CNS also contains α-DG and the SH2 (Src homology 2)/SH3 (Src homology 3) adapter protein Grb2 and has the potential to anchor dynamin at discrete sites within the cell. In an overlay assay, β-DG interacts directly with brain dynamin. In the retina, DG is associated and immunohistochemically colocalized with dynamin at the outer plexiform layer where previous ultrastructural studies (27, 39) have found DG to be distributed presynaptically in synapses formed by photoreceptors onto bipolar and horizontal cells. Finally, in fibroblasts differentiated from embryonic stem (ES) cells, DG associates with dynamin and codistributes with cortactin at membrane ruffles, which are organelles implicated in endocytosis. Cells null for DG have an increased uptake of transferrin, and re-introduction of DG into these null cells reduces this increase to normal levels.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antisera to β-DG and monoclonal antibody (mAb) 1B7 to α-DG were characterized previously (11, 40). mAbs to the following proteins were used for Western blotting: β-DG (1:250) (Novocastra Laboratories Ltd.); α-DG (IIH6) (1:1000) (Upstate Biotechnology); Grb2 (1:500); GST (1:1000) (Santa Cruz Biotechnology). mAb Hudy-1 (Upstate Biotechnology), which recognizes dynamin 1 found in the brain as well as the more ubiquitous dynamin 2 (41), was used at 1:40,000 dilution for Western blotting and 1:200 dilution for immunocytochemistry. mAbs to cortactin (Upstate Biotechnology) were used at 1:250. Anti-α-synuclein (874) was a gift from Peter McPherson (McGill). Horseradish peroxidase-conjugated goat anti-mouse (1:2000) and goat anti-rabbit antisera (1:4000) (Jackson ImmunoResearch Laboratories) were used as secondary antibodies in Western blots. Rhodamine-conjugated donkey anti-rabbit (1:2000) and fluorescent (FITC)-conjugated donkey anti-mouse antisera (1:200) (Jackson ImmunoResearch Laboratories) were used for immunofluorescence staining.

**Tissue Extraction**—Rat brains and hippocampi were dissected from adult rats euthanized according to the Guidelines of McGill University Health Center Animal Facility. Tissues were homogenized on ice four times (10 s each) with a Polytron tissue homogenizer in phosphated buffered saline (PBS, pH 9.0) containing 1% sodium deoxycholate, 0.5 mM CaCl$_2$, and a protease inhibitor mixture (Roche Applied Science). The homogenates were incubated at 37°C for 30 min followed by addition of Triton X-100 to a final concentration of 0.5% in a total volume of 6 ml. After 2 h of incubation at 4°C with occasional vortexing, the homogenates were centrifuged at 37,000 g for 30 min. The supernatant was collected and subjected to SDS-PAGE, and protein concentrations were determined (DC Protein Assay Kit, Bio-Rad).

Synaptosomes were prepared from rat brains as described by Sugita et al. (12) with minor modifications. Briefly, rat brains were removed and homogenized with a Teflon glass homogenizer in a buffer containing 20 mM Tris-HCl (pH 7.2), 0.32 M sucrose, 1 mM CaCl$_2$, and protease inhibitor mixture (Roche Applied Science). The homogenates were incubated at 37°C for 30 min followed by addition of Triton X-100 to a final concentration of 0.5% in a total volume of 6 ml. After 2 h of incubation at 4°C with occasional vortexing, the homogenates were centrifuged at 37,000 × g for 30 min. The supernatant was collected, and protein concentrations were determined (DC Protein Assay Kit, Bio-Rad).

**Immunoprecipitation**—Crude synaptosomes or retinas were solubilized in PBS (pH 9.0) containing 1% sodium deoxycholate, 0.5% Triton X-100, 1 mM CaCl$_2$, and protease inhibitor mixture (buffer B) and centrifuged for 37,000 g for 1 h. The resulting pellets were treated with 20 μl of protein G-agarose (Invitrogen) for 1 h. After preclearing, 20 μl of protein G beads conjugated to relevant antibodies or preimmune sera were added to the preclearced supernatants and incubated overnight at 4°C with constant shaking. The agarose beads were then pelleted by centrifugation at 4,000 × g for 2 min. The supernatants were collected, and the beads were washed twice with 1 ml of buffer B, followed by 0.5 ml of 0.5 M NaCl in buffer B, and finally with 0.5 ml of buffer B containing 5 mM EDTA. The washed beads were then boiled in sample buffer for 5 min and subjected to SDS-PAGE and Western blotting.

**Assay for β-DG**—G Thyroplastic tail of β-DG was amplified by PCR from a plasmid containing the full-length DG cDNA sequence using sense 5′-GAAATCTTCTACTGCAAGAAGG-3′, and antisense primers 5′-GCTCTAGGATAGGGGAACTACCG-3′. The amplified fragment (digested with NcoI and XhoI) was used to set up an immunoaffinity assay with GST-DG (IIH6) (1:1000) (Upstate Biotechnology); Grb2 and has the potential to anchor dynamin at discrete sites within the cell. In an overlay assay, β-DG interacts directly with brain dynamin. In the retina, DG is associated and immunohistochemically colocalized with dynamin at the outer plexiform layer where previous ultrastructural studies (27, 39) have found DG to be distributed presynaptically in synapses formed by photoreceptors onto bipolar and horizontal cells. Finally, in fibroblasts differentiated from embryonic stem (ES) cells, DG associates with dynamin and codistributes with cortact in membrane ruffles, which are organelles implicated in endocytosis. Cells null for DG have an increased uptake of transferrin, and re-introduction of DG into these null cells reduces this increase to normal levels.

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**Immunofluorescence Chromatography**—Antisera to β-DG (11), bovine serum albumin (control), or rabbit immunoglobulin (control) were covalently coupled to Affi-Gel 10 following the manufacturer’s instructions (Bio-Rad). Five milligrams of hippocampal extract (2–3 ml) were incubated overnight with 1 ml of each Affi-Gel-protein conjugate. The slurry was packed into a column, washed first with 10 ml of PBS (pH 9.0) containing 1% sodium deoxycholate, 0.5% Triton X-100, and 0.15 M sodium chloride (buffer A), followed by a wash with 10 ml of 0.5 M NaCl in buffer A, and finally 10 ml of 5 mM EDTA in buffer A. Proteins still bound to the column were eluted at low pH with 0.1 M glycine (pH 2.5) and neutralized with 0.5 M of 1 M Tris-HCl (pH 8.0). The glycine and triethylamine eluates were pooled, dialyzed against PBS overnight, and then concentrated to equal volumes (Millipore Centricon filters), and equal aliquots of the eluates were subjected to SDS-PAGE. SDS-PAGE and Western blotting were carried out as described previously (9).
tirified at 16,000 × g for 20 min, and supernatants were used for immunochemical studies.

**Adenoviral Constructs and Infection**—To express full-length DG in DG-null fibroblastic cells, we generated a replication-defective adenovirus using the AdEasy system (Qbiogene) as described by the manufacturer. In brief, DG cDNA was subcloned into the multiple cloning site of pAdTrack-CMV(GFP) (GFP and DG are under separate CMV promoters producing two separate proteins), linearized using PmeI, and cotransfected with pAdEasy-1 into BJ5183, an electrocompetent E. coli strain. Recombinants (AdDG/GFP) were screened by colony size and confirmed by restriction digests. They were then linearized using PacI, purified, and transfected into HEK293 cells using Lipofectamine reagent (Invitrogen). Adenovirus production was observed by plaque formation and by GFP fluorescence. Viral particles were isolated from cell lysates and amplified through several rounds of infections in HEK293 cells.Titers were determined by TCID_{50} infection test as described by the manufacturer (Qbiogene). For DG expression, ES cells were grown at low confluence, infected with 10^{5} plaque forming units/ml (multiplicity of infection, 5) of control Ad-GFP or of AdDG/GFP for 18 h in growth medium. Infection efficiency was monitored by GFP reporter gene expression and was maximal 2 days postinfection. At this point, transferrin uptake was assayed.

**Immunohistochemistry**—Three-month-old male mice (strain C57BL/6) were anesthetized using xylazine (2 mg/ml, Bayer Inc.) and ketamine hydrochloride (15 mg/ml, Ayerst Veterinary Laboratory). After the anesthesia, mice were perfused intraventricularly with PBS followed by 4% paraformaldehyde. Whole eyes were dissected from the animals and postfixed overnight in 4% paraformaldehyde followed by cryopreservation in 30% sucrose. Eyes were then frozen in Tissue-Tek OCT compound (Sakura Finetek) on crushed dried ice and stored at −80°. S-μm horizontal sections were cut using a Leica CM3050S cryostat and stored at −20°. Sections were thawed to room temperature, washed with PBS, and blocked with 10% horse serum and 0.3% Triton X-100 in PBS for 1 h prior to immunostaining. The retinal sections were incubated 1.5 h in blocking buffer containing both Hudy-1 and β-DG antiserum. Incubation with secondary antibodies was done in blocking buffer containing both TRITC-conjugated donkey anti-rabbit antiserum and FITC-conjugated donkey anti-mouse immunoglobulins. Sections were washed three times with PBS after both primary and secondary antibody incubations, mounted in Immuno-fluor mounting medium and visualized using a Leica TCSNTSP confocal microscope.

**RESULTS**

**Identification of Dynamin 1 as a β-DG-associated Protein in Brain**—Hippocampal homogenates were extracted directly with 1% sodium deoxycholate (pH 9.0) and 0.5% Triton X-100. This effectively solubilizes DG as well as most pre- and postsynaptic proteins (44). Immunofluorinity columns conjugated with antisera to β-DG, non-immune rabbit IgGs (control), or BSA (control) were equilibrated with the extracts, washed extensively, and eluted first at low and then at high pH (see “Experimental Procedures”). Following SDS-PAGE analysis of the eluates and Coomassie Blue staining, several protein bands were evident at 43 and ~100 kDa (Fig. 1A) that bound specifically to β-DG antibody columns. The 43-kDa band was confirmed to contain β-DG in Western blots (Fig. 1B). The ~100-kDa band was cut out, digested with trypsin, and subjected to LC/MS-MS analysis (Montreal Proteomics Network). This yielded 21 tryptic peptides whose sequences matched that of rat dynamin 1 (gi: 1188966; 97 kDa) with the peptides covering 25% of the sequence of dynamin 1. The 55-kDa band yielded peptides matching to the IgG heavy chain. Peptides from the ~66-kDa band matched to Hsc70 (not shown). Consistent with the MS results, Western blotting indicated that dynamin 1 was enriched along with β-DG in brain extracts subjected to immunofluorinity chromatography (Fig. 1B). Because dynamin is a relatively abundant cytosolic protein and β-DG is a transmembrane protein, we sought to determine the amount of dynamin 1 bound to β-DG. β-DG could be immunodepleted from brain extracts, although a significant amount of dynamin 1 remained in the supernatant (Fig. 2, A and B). No immunoprecipitation of either protein with control IgG (Fig. 2B) was observed. These results suggest that there is a pool of dynamin 1 tightly associated with β-DG, and a pool of unbound dynamin 1.

**Detection of other DG-associated Proteins in Brain**—To further characterize the β-DG-dynamin complex in the CNS we used antibodies to several DG-associated proteins in muscle. Immunofluorinity-purified fractions of β-DG from brain were Western-blotted with two monoclonal antibodies (mAb IIH6 and 1B7), which detect differentially glycosylated forms of α-DG (25, 40). mAb IIH6, which recognizes larger and more heavily glycosylated forms, detected a band of ~97 kDa by LC/MS-MS. B, column eluates (5%) from 5 mg of hippocampal homogenates were collected, electrophoresed, and Western-blotted with antibodies to dynamin and β-DG to confirm the data in A. Equivalent amounts of eluate from the NRG1G and BSA columns served as controls. The antibodies used as probes are noted on the left and molecular mass markers (kDa) are on the right. Image A or B is from a single gel.

![Image](http://www.jbc.org/)

**FIG. 1.** Immunoaffinity purification of dystroglycan-associated proteins from brain. A, equivalent volumes of eluate (see "Experimental Procedures") from anti-β-DG antibody affinity column (β-DG), nonimmune rabbit IgG column (NRG1G), BSA column (BSA), and hippocampal homogenates (H.H., 2 μg) were loaded, subjected to SDS-PAGE, and stained with Coomassie Blue. Two sets of molecular mass markers (36 kDa–175 kDa) are shown. The arrowhead points to a ~100-kDa band seen only in eluates from the β-DG antibody affinity column. Band 1 is β-DG (43 kDa). The band at 55 kDa is the IgG heavy chain, and the band at ~70 kDa is Hsc70 (data not shown). The band at ~100 kDa was identified as dynamin 1 (97 kDa) by LC/MS-MS. B, column eluates (5%) from 5 mg of hippocampal homogenates were collected, electrophoresed, and Western-blotted with antibodies to dynamin and β-DG. Equivalent amounts of eluate from the NRG1G and BSA columns served as controls. The antibodies used as probes are noted on the left and molecular mass markers (kDa) are on the right. Image A or B is from a single gel.
against β-DG (Fig. 3B) and in the immunoaffinity-purified fractions from brain (data not shown). Taken together, these data indicate that the brain contains a complex of α-β-DG with dynamin 1 and Grb2.

Although Grb2 has the potential to mediate binding to dynamin, other data indicate that SH2-SH3 domain-containing proteins such as amphiphysin (49, 50) and endophilin (51, 52) preferentially bind to proline-rich domains of dynamin (53). This suggested that dynamin might bind directly to β-DG. To test this, brain dynamin was immunoprecipitated with mAb Hudy-1, fractionated electrophoretically, and transferred to PVDF membranes. The PVDF membranes were overlaid with a recombinant protein encompassing the entire cytoplasmic domain of β-DG fused to GST (GST-βDGcyto). We found that GST-βDGcyto but not GST alone bound to bands containing dynamin (Fig. 4A). Thus there appears to be a direct interaction of dynamin with the intracellular region of DG. Interestingly, β-DG immunoaffinity purification (Fig. 3, lanes 6 and 7) revealed no detectable association of DG with amphiphysin suggesting that binding of Grb2 directly or via its SH3 domain occludes binding of amphiphysin in dynamin associated with DG.

Association and Colocalization of DG and Dynamin in the Outer Plexiform Layer of the Retina—DG has been localized to synaptic regions in the brain (40, 54, 55) as well as at GABA-ergic synapses in culture by immunofluorescence microscopy (29, 30). It is challenging to localize antigens within most regions of the CNS so as to ascertain whether they are synaptically localized without resorting to EM. In the retina, however, rod and cone photoreceptors synapse on bipolar and horizontal cells and form so-called “ribbon” synapses where the presynaptic ribbon is readily detectable by light microscopy as plaques (cones) or large puncta (rods) running through the outer plexiform layer (54). Both α- and β-DG have been shown to be expressed in the outer plexiform layer of retina (27, 56, 57), where they are thought to form a complex with dystrophin and β-dystroglycan (58). To extend these studies, we determined whether β-DG is associated with dynamin in the retina. As in the brain, both dynamin and β-DG were coprecipitated (Fig. 5A) with antisera to β-DG. Conversely immunoprecipitation with an antibody to dynamin (Fig. 5B) precipitated β-DG and Grb2 together with dynamin. The amount of β-DG precipitated was less than that in Fig. 5A, consistent with previous data (Fig. 2A) that there is a pool of dynamin unbound to β-DG. Immunohistochemically β-DG immunoreactivity (Fig. 5C, arrowheads) was found in puncta and bands in the outer plexiform layer of retina characteristic of rod and cone photorecep-
diffuse than that of β-DG. Double labeling immunofluorescence showed, however, that essentially all photoreceptor synapses labeled with antisera to β-DG were also positive for dynamin (Fig. 5C, merge). Grb2 was expressed diffusely in virtually all cells of the retina, and, while it overlapped with the distribution of β-DG and dynamin, it is not obvious that this is meaningful (data not shown). Taken together, these data suggest that in the retina, β-DG, dynamin, and possibly Grb2 form a complex in the outer plexiform layer, and this complex is presynaptically localized.

**DG in Endocytosis**—Previously, we have studied DG function in differentiated ES cells wherein both dag1 alleles had been disrupted by homologous recombination (11, 15, 21). Here we first generated mixed cultures of differentiated cells to monitor by immunofluorescence the effect of deletion of dag1 on dynamin-mediated endocytosis. Differentiated ES cells yield a variety of cell types, including fibroblastic cells that quickly migrate out of the embryoid body and are easily visualized as well spread cells on the periphery of the cell mass. In addition, we have established lines of these fibroblastic cells that permit biochemical analysis. Unfortunately, we have not been able to isolate neural stem cells for similar studies nor do we have mice null for dag1 in the CNS from which we could make primary neuronal cultures (19). Nevertheless, constitutive endocytosis of transferrin is essentially the same in neuronal and non-neuronal cells and shares many features with regulated vesicular endocytosis in nerve terminals, including a requirement for dynamin (37).

Fibroblastic cells differentiated from wild-type ES cells express both α-DG (data not shown) and β-DG (Fig. 6, panels C and G) on the surface of the plasma membrane, preferentially in regions called membrane ruffles (Fig. 6, panel A). Ruffles also contain dynamin, β-DG, and cortactin (Fig. 6). Thus DG in these cells might be involved in endocytosis, which frequently occurs at membrane ruffles, and/or could regulate the actin cytoskeleton, a function also attributed to dynamin (59). To assess this further, wild-type or DG-null cells were differentiated from embryoid bodies, and incubated with TRITC-labeled transferrin. In mixed cultures both wild-type and DG-null fibroblastic cells take up transferrin, as demonstrated by the presence of many small, fluorescent puncta visible throughout the thickness of the cells (Fig. 7). Wild-type cells showed both submembranous and cytoplasmic localization of labeled transferrin, indicative of uptake into early and late endosomes (Fig. 7, panels A and C). Few cells had perinuclear staining. In contrast, in DG-null cells transferrin labeling was less frequently concentrated in the region of plasma membrane and most, if not all cells had intense labeling in the cytoplasm especially around the nucleus (Fig. 7, panel D). We quantified the area occupied by transferrin labeling in wild-type and DG-null cells (see “Experimental Procedures”). DG-null cells show a significant, 2.25-fold increase (p < 0.05), in transferrin uptake (Fig. 7, panel E) when compared with wild-type cells. To confirm that DG is associated with dynamin we isolated and expanded fibroblastic cells that appeared essentially identical to those which migrate from embryoid bodies (Figs. 6 and 7). Transferrin uptake by these cell lines was the same as that of fibroblastic cells in mixed cultures (Fig. 8, panels A and B).

Furthermore, immunoprecipitation from wild-type cells showed that a portion of dynamin is associated with β-DG (Fig. 8, panel C, lane wt of upper panel under IP). There was no DG detectable in DG-null cells (Fig. 8, panel C, Pre-IP), and no dynamin was precipitated from these cells (Fig. 8, panel C, IP). Taken together, these observations indicate that β-DG interacts with dynamin at membrane ruffles and suggests a role for DG in regulating endocytosis of transferrin.
To confirm that the difference in endocytosis between wild-type and DG-null cells was caused by DG, we re-introduced DG into the DG-null fibroblastic cells by infection with an adenovirus containing DG/GFP. In these experiments, GFP expression driven by a separate CMV promoter served as a reporter of successful infection. In DG-null cells infected with the DG-GFP virus DG is expressed in the plasma membrane and in ruffles (arrowhead, Fig. 9A, panel c’) similar to the pattern in wild-type cells (Fig. 6). There was no DG expression seen in cells infected with the control adenovirus (GFP) (Fig. 9A, panel b’).

To determine whether transferrin uptake was restored to wild-type levels in infected cells, we incubated wild-type and DG-null cells with rhodamine-conjugated transferrin for 15 min, 24 h after adenovirus infection (Fig. 9B). In uninfected cells transferrin uptake was significantly greater in DG-null cells than in wild-type cells, as noted previously (Figs. 7 and 8). In DG-null cells infected with DG/GFP, there was a decrease in transferrin uptake when compared with DG-null cells infected with GFP alone. The decrease was equivalent to that seen in wild-type cells uninfected or infected with GFP. DG-null cells infected with virus containing DG/GFP (DG-null:DG/GFP) had about a 2-fold reduction in transferrin uptake compared with DG-null cells or null cells infected with GFP (p < 0.05), but no difference compared with wild-type cells or wild-type cells infected with GFP (p > 0.05). Wild-type cells infected with GFP alone have the same transferrin uptake as non-infected wild-type cells (p > 0.05).

**DISCUSSION**

Our results reveal that a complex of β-DG, α-DG, dynamin 1, and Grb2, is found in presynaptic terminals of CNS neurons. This complex is also present in non-neuronal cells where it appears to regulate endocytosis. Several lines of evidence support this claim. First, immunopurification of β-DG-associated proteins followed by SDS-PAGE yielded a 97-kDa band, whose sequence by LC/MS-MS resulted in 21 peptides that covered 25% of the sequence of rat dynamin 1. Sixteen of these peptides were unique for dynamin 1, and five were shared with dynamin 2 but not dynamin 3, both of which are also expressed in the CNS (60). This suggests that dynamin 1 is the major dynamin isoform associated with DG. Second, the association of dynamin was confirmed by immunoprecipitation and extended to show interaction of β-DG with α-DG and Grb2. Other immunoprecipitation data indicate that only a subset of dynamin is associated with β-DG in brain and retina. In an overlay assay, dynamin purified from brain bound directly to the intracellular portion of recombinant β-DG. Third, β-DG is associated and immunohistochemically colocalized with dynamin at ribbon synapses within the outer plexiform layer of the retina. Fourth, in fibroblast-like cells in culture DG is associated with dynamin at cortactin-rich membrane ruffles that are sites of endocytosis (61, 62). Finally, ES cells null for DG had a greater uptake of transferrin, a dynamin-mediated process, than that of wild type cells. Expression of full-length DG in DG-null ES cells restored transferrin endocytosis to levels seen in wild-type cells.

**Dystroglycan Is Found in Nerve Terminals in the CNS—DG** is concentrated at some central (28, 29) and peripheral synapses (8, 25, 26). At neuromuscular junctions DG binds to perlecan (10, 11) and contributes to the assembly of acetylcholinesterase (11) in the synaptic basement membrane. In addition, DG participates in the aggregation of acetylcholine receptors (11, 34) into stable plaques within the myotube membrane. In the hippocampus DG is involved in LTP (19) where it has been reported to function postsynaptically. In our studies immunoaffinity chromatography identified dynamin 1, a neuron-specific form of dynamin (60), as the dominant dynamin isoform associated with DG. Dynamin 2 is also expressed in the CNS and is recognized by mAb Hudyl-1 (41). As a result immunoprecipitations with this mAb from brain homogenates may...
contain DG that is associated with dynamin 2. However, in synaptosomes DG is likely bound to dynamin 1, the dominant isofrom in neurons. Kroger and co-workers (27, 39) have localized DG in the vicinity of synaptic ribbons in the nerve terminals of photoreceptors in the outer plexiform layer of the retina (27, 39), and we have found that DG and dynamin colocalize in ribbon synapses in the outer plexiform layer of the retina (Fig. 5) that are readily detectable by light microscopy (27, 56). We conclude that DG is localized presynaptically where it is associated with dynamin 1.

**Interactions of DG-Dynamin in the CNS—**Blank et al. (58) have suggested that β-dystrobrevin and dystrophin are bound to β-DG based on their localization in the retina. Our biochemical and other data indicate that Grb2 and dynamin also interact with the cytoplasmic tail of β-DG. Thus in the CNS α and β-DG appear to be complexed with conventional components found in muscle, such as dystrobrevin, as well as a novel one, dynamin. Grb2 is an SH2-SH3 domain protein that binds to β-DG via either of its SH3 domains (47, 63) but with different affinities (48). Grb2 also binds to dynamin 1 in brain, again via SH3 domain binding to proline-rich motifs (45, 46). The N-terminal SH3 domain of Grb2 is essential for binding to dynamin (64). So it is reasonable to think that Grb2 may bind to dynamin with its N-terminal SH3 domain and to DG with C-terminal SH3 domain, to form DG-Grb2-dynamin complex. Other studies indicated that amphiphsis and endophilin rather than Grb2 are the major SH3-domain containing protein that interacts with dynamin at nerve terminals (49, 65). We have not, however, detected amphiphsis in immunoprecipitates of DG that contain dynamin (Fig. 3A). Moreover, our overlay data (Fig. 4) indicated that DG can interact directly with dynamin and may not require the intercession of Grb2 in situ. Taken together it appears that DG, Grb2 and dynamin form a complex distinct from dynamin and amphiphsis.

In addition to its intracellular interactions, β-DG also binds to α-DG conferring on the complex the ability to interact with its ligands in the extracellular matrix (32) as well as on other cells (12). For many of its ligands of α-DG binds via its carbohydrate side chain(s) (1) to laminin-like globular motifs (3, 66). Recent data show that aberrant glycosylation of DG in several muscular dystrophies and in the myd mouse leads to greatly reduced ligand binding and electoretinograms with altered b-waves indicative of defective synaptic transmission in the outer plexiform layer (20, 31). Agrin, which contains several G domains and binds to DG (6–9), is found within the synaptic cleft in the outer plexiform layer of the retina (67). Moreover, the ability of α-DG to bind ligand appears necessary for normal synaptic function (2, 31). Thus linkage of dynamin to a transmembrane receptor complex and possibly to the matrix or cell...
adhesion molecules (3, 8, 10, 12, 68) may be important in vesicle recycling during synaptic transmission where spatially regulated subdomains of endocytosis and exocytosis are thought to be critical for evoked release of neurotransmitter (69, 70).

**Dystroglycan in Endocytosis—**To assess the biochemical interactions of DG in cells we have resorted to cultured cells that are null for DG. Unfortunately, we do not have lines of neuronal precursors null for DG, but we do have fibroblastic and epitheliod cells. As a result, we have assayed in fibroblasts

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**Fig. 9. Expression of DG in DG-null cells restores normal levels of transferrin uptake.** A, fibroblasts derived from both wt (panels a’–a”) and DG-null (panels b’–b” and c’–c”) ES cells were infected with an adenovirus containing GFP (panels a’–a”, b’–b”) or DG/GFP (panels c’–c”), and stained with antiserum to DG followed by TRITC-labeled secondary antibody (panels a’, b’, c’). DG was observed at the cell surface and ruffles in DG-null cells infected with DG/GFP (arrowhead, panel c’), similar to labeling in wt cells (see legend to Fig. 6) or wt cells infected with GFP (panel a’). B, rhodamine-conjugated transferrin (Tfn) was incubated with fibroblastic cells for 15 min, 24 h after infection with an adenovirus containing GFP or DG/GFP. DG-null cells infected with virus containing DG/GFP (DG-null:DG/GFP) had decreased Tfn uptake compared with non-infected (DG-null) cells or cells infected with GFP alone (DG-null:GFP). The level of uptake was essentially the same as in wt cells infected with GFP (wt:GFP) or wt cells without viral infection (wt). Fluorescence was quantified (see “Experimental Procedures”) in cells 25–35 μm in diameter to control for variation in cell size. Transferrin fluorescence is often concentrated in a portion of the cell so the full size of the cell was determined from GFP fluorescence in infected cells (A) or by phase imaging of uninfected cells. More than 20 cells were quantified for each treatment and fluorescence intensity was normalized to that in wild-type cells. Bar in A is 50 μm.
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...retardation associated with Duchenne development, these observations may contribute to our understanding of the mental retardation associated with Duchenne muscular dystrophy. In view of recent work implicating DG in synapse formation/function and in neural...
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