Adhesion-dependent tyrosine phosphorylation of β-dystroglycan regulates its interaction with utrophin

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Accepted 9 March; published on WWW 18 April 2000

SUMMARY

Many cell adhesion-dependent processes are regulated by tyrosine phosphorylation. In order to investigate the role of tyrosine phosphorylation of the utrophin-dystroglycan complex we treated suspended or adherent cultures of HeLa cells with peroxynitrate and immunoprecipitated β-dystroglycan and utrophin from cell extracts. Western blotting of β-dystroglycan and utrophin revealed adhesion- and peroxynitrate-dependent mobility shifts which were recognised by anti-phospho-tyrosine antibodies. Using maltose binding protein fusion constructs to the carboxy-terminal domains of utrophin we were able to demonstrate specific interactions between the WW, EF and ZZ domains of utrophin and β-dystroglycan by co-immunoprecipitation with endogenous β-dystroglycan. In extracts from cells treated with peroxynitrate, where endogenous β-dystroglycan was tyrosine phosphorylated, β-dystroglycan was no longer co-immunoprecipitated with utrophin fusion constructs. Peptide ‘SPOTs’ assays confirmed that tyrosine phosphorylation of β-dystroglycan regulated the binding of utrophin. The phosphorylated tyrosine was identified as Y892 in the β-dystroglycan WW domain binding motif PPxY thus demonstrating the physiological regulation of the β-dystroglycan/utrophin interaction by adhesion-dependent tyrosine phosphorylation.

Key words: β-Dystroglycan, Adhesion, Tyrosine phosphorylation, WW domain

INTRODUCTION

Urophin is a ubiquitous cytoskeletal protein forming a link between the actin cytoskeleton and the extracellular protein laminin via a membrane glycoprotein complex which includes α- and β-dystroglycans (Ervasi and Campbell, 1993; Matsumura et al., 1992; Tinsley et al., 1992; Winder et al., 1995b). In normal skeletal muscle, where utrophin is restricted to the myotendinous and neuromuscular junctions, utrophin binds to a multimeric protein complex comprising dystroglycans and sarcoglycans. This complex is indistinguishable from the dystrophin glycoprotein complex found in the rest of the sarcolemma (Matsumura et al., 1992), though there is differential distribution of the syntrophins between these complexes. In non-muscle tissues however, sarcoglycans, with the possible exception of β-sarcoglycan (Bonnemann et al., 1995; Lim et al., 1995), and dystrophin (except in neuronal tissues) are not expressed and utrophin associates specifically with α- and β-dystroglycan at the cell membrane (James et al., 1996; Matsumura et al., 1997). In non-muscle cells in culture, utrophin and dystroglycans are associated with cell-cell and cell-matrix adhesion structures (Belkin and Burridge, 1995a,b; Belkin and Smallheiser, 1996; James et al., 1996; Khurana et al., 1995) and may therefore play a role in the organisation of focal adhesions or adherens junctions and may exist as distinct cell adhesion complexes in their own right. The assembly and disassembly of many adhesion structures are known to be regulated by tyrosine phosphorylation (Burridge and Chrzanowska-Wodnicka, 1996). Both utrophin and dystroglycans have been demonstrated to play a key role in adhesion-mediated events. The dystroglycan gene encodes both α- and β-dystroglycan as a single transcript which is post-translationally cleaved (Ibraghimov-Beskrovnaya et al., 1992). Disruption of dystroglycan function, either by antibodies (Durbeej et al., 1995) or gene knockout (Henry and Campbell, 1998; Williamson et al., 1997) has a profound effect on developmental processes that rely on cell adhesion. Antibodies which block α-dystroglycan binding to laminin in epithelial cells inhibit branching morphogenesis (Durbeej et al., 1995), whilst transgenic disruption of the dystroglycan gene (DAG1) in mice leads to embryonic lethality.
at around day 6.5 due to the failure of Reichert’s membrane to form (Williamson et al., 1997). Embryonic stem cells deficient for α- and β-dystroglycan are unable to assemble basement membrane (Henry and Campbell, 1998). A significant reduction of α- and β-dystroglycan in the sarcolemma is also symptomatic of mutations in the dystrophin gene which lead to Duchenne and Becker muscular dystrophies (Ervasti et al., 1994; Ponting et al., 1996). WW domains are 30 amino acid motifs involved in protein-protein interactions through proline-rich motifs in their cognate ligands. EF hands are highly conserved motifs in their cognate ligands. EF hands and may play a regulatory or a structural role. The ZZ domain is a putative zinc finger motif found in various functionally distinct proteins that is believed to mediate protein-protein interactions. It has been shown previously that the cysteine-rich region of dystrophin is required for the binding of dystrophin to β-dystroglycan and the C-terminal domain further increases the binding affinity (Jung et al., 1995; Rosa et al., 1996; Suzuki et al., 1994). Most recently it has been documented that the WW domain in concert with the EF hands binds to the C-terminal PPxY motif of β-dystroglycan and that this binding is enhanced by the presence of the ZZ domain (Rentschler et al., 1999).

In this current study we investigated the binding of the utrophin carboxy-terminal domains to β-dystroglycan using utrophin fusion proteins as probes to identify β-dystroglycan binding sites in utrophin. In addition we have examined the role of adhesion-dependent tyrosine phosphorylation of β-dystroglycan in regulating the interaction between β-dystroglycan and the carboxy terminus of utrophin. Using a phosphorylation state-specific monoclonal antibody we have mapped the site of tyrosine phosphorylation to the essential tyrosine in the β-dystroglycan WW domain binding motif PPxY and demonstrated that phosphorylation of this tyrosine regulates the β-dystroglycan-utrophin interaction.

MATERIALS AND METHODS

Cell culture
HeLa cells were maintained in RPMI 1640 media (Gibco BRL) supplemented with 5% foetal calf serum (Gibco BRL). In order to inhibit the activity of tyrosine phosphatases and effectively block the cells in a tyrosine phosphorylated state, peroxovanadate treatment was carried out on confluent cultures of HeLa cells. Adherent or suspended cells were washed with serum-free RPMI and then incubated in peroxvanadate (2 mM H2O2, 1 mM sodium orthovanadate in serum-free RPMI media) or 100 nM calycin A (Calbiochem) for various times at 37°C. Cells were washed once in cold phosphate buffered saline (50 mM sodium phosphate, pH 7.2, 150 mM NaCl) before being harvested in ice-cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylene glycol-bis-(β-aminoethul ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 100 μM leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 μM N-tosyl-L-phenylalnine chloromethyl ketone (TPCK)) for 30 minutes on ice. Cells which were not treated with phosphatase inhibitors were incubated for an equivalent time in serum-free media before being harvested in RIPA as above. After harvesting, cells were briefly sonicated to shear the DNA and stored at −20°C until assay. For studies with cells in suspension, HeLa cells were first removed from the culture vessel by PBS/EDTA treatment and resuspended in serum free RPMI 1640, with 2% bovine serum albumin to prevent non-specific adhesion, and peroxvanadate as above. Cells were maintained at 37°C in a 50 μl tube on a tube roller for 1 hour. Cells that were still in suspension after one hour were decanted to a fresh tube, recovered by centrifugation and extracted in RIPA buffer as above, adapted from Renshaw et al. (1997).

Antisera
The anti-β-dystroglycan antibody 43DAG1/8D5 was a gift from L. Anderson (University of Newcastle) and MANDAG2 (Helliwell et al., 1994) was kindly provided by G. E. Morris (NE Wales Institute, Wrexham). Polyclonal antisera against bacterially expressed maltose binding protein (RAB4) and the utrophin carboxy-terminal coiled coil domain (RAB5), residues 3204-3433 of human utrophin (Winder and Kendrick-Jones, 1995) were raised in rabbits using standard techniques. RAB4 and RAB5 antisera were effective on western blots at dilutions of 1:5,000 and 1:10,000, respectively, and the utrophin antisemum did not recognise dystrophin in western blots of whole rat skeletal muscle extracts or in mouse C2C12 mouse myotubes (data not shown). Anti-phospho-tyrosine monoclonal (PY20) was from Transduction Laboratories.

Generation of utrophin fusion-proteins
The limits of the utrophin cysteine rich domains WW, EF and ZZ were identified by sequence alignment (Schultz et al., 1998; Sudol, 1996a; Thompson et al., 1997) and secondary structure prediction (Rost et al., 1994; Schultz et al., 1998). Specific utrophin cysteine-rich domains were amplified by polymerase chain reaction (PCR) using domain specific primers to the residues shown in Fig. 3A. 5′ primers contained BamHI and NdeI restriction sites and 3′ primers contained a stop codon and SacII restriction site. PCR products were cloned into the unique BamHI and SacII restriction enzyme sites of pMAL-C2 (New England BioLabs). Cultures of transformed E. coli (BL21 (DE3)) were grown at 37°C until the OD600 reached 0.5, and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2 hours. Following harvesting by centrifugation at 4,000 g for 20 minutes the cell pellet was re-suspended in 50 ml column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 100 μM leupeptin, 1 mM PMSF, 100 μM TPCK) and stored at −20°C overnight. After thawing, the cell suspension was sonicated in short 15 second pulses for a total time of 2 minutes before being centrifuged at 9,000 g for 30 minutes MBP fusions were purified by a single passage over an amylase resin (New England Biolabs) column. Bound fusion proteins were eluted using column buffer containing 10 mM maltose. Fusion containing fractions identified on Coomassie-stained SDS gels were pooled, dialysed against RIPA buffer overnight and then stored at −20°C until use. A human utrophin construct encompassing the WW, EF and ZZ regions (residues 2783-3199) fused to GST was generated by cloning a BamHI and EcoRI digested PCR-generated fragment into the BamHI and EcoRI sites of pGEX-2TK.
In vitro binding and immunoprecipitation assays

For immunoprecipitation of β-dystroglycan/MBP-fusion complexes, fusion proteins were used at a final concentration of 10 μg/ml. Cell extracts in RIPA were initially cleared by incubation with Protein A-Sepharose for 60 minutes at 4°C. The required volume of each extract was added to 100 μl aliquots of cleared cells, the total volume of each was made up to 175 μl using RIPA and 50 μl RIPA containing 1% BSA was added to each tube. After 2 hours incubation at 4°C 10 μl of 43DAG1/8DS and/or MANDAG2 was added to each tube and the cells were incubated for a further hour at 4°C. In order to precipitate the antibody-protein complex, 50 μl of a 20% slurry of Protein A-Sepharose was added per sample, and the cells were incubated for a further hour at 4°C. The samples were centrifuged briefly to pellet the Sepharose beads, and the beads were washed 4 times with RIPA. The final pellet was re-suspended in SDS-PAGE loading buffer. Immunoprecipitation of β-dystroglycan, phosphorylated dystroglycan containing proteins and utrophin were carried out with the relevant antisera at dilutions of 1:20, 1:200 and 1:20 specifically and recovered with Protein A-Sepharose as above.

SDS-PAGE and Western blotting

Samples from immunoprecipitation experiments were run on 3-15% acrylamide SDS-polyacrylamide gels with a 3% stacking gel under reducing conditions (Laemmli, 1970) and blotted onto polyvinylidene difluoride (PVDF) membranes in 25 mM Tris, 198 mM glycine, 10% methanol. Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 30 minutes and incubated overnight at 4°C in appropriately diluted primary antibody in 5% skimmed milk in TBS and washed 3 times in TBS with 0.5% Tween-20 before addition of the alkaline-phosphatase conjugated secondary antibody in 1% skimmed milk in TBS. Membranes were incubated for 2 hours in secondary antibody at room temperature. After washing in TBS/Tween, blots were developed using 0.4 mM nitroblue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM MgCl2, 100 mM Tris-HCl, pH 9.5. Enhanced chemifluorescence detection of utrophin maltose binding protein fusion proteins was performed using a Vistra ECF western blotting kit (Amersham Life Sciences) according to the manufacturer’s instructions. Images were captured on a Storm 460 Phosphorimager in fluorescence mode and data quantified using ImageQuant software.

Two-dimensional gel electrophoresis

β-Dystroglycan was immunoprecipitated from equal amounts of protein (as determined by BCA assay; Pierce) from suspended or adherent and peroxynitrate treated or untreated cell extracts, prepared as above. Equal amounts of immunoprecipitated complexes were subjected to first dimension isoelectric focusing on 180 mm, pH 3-10 Immobiline Drystrips (Amersham Pharmacia Biotech) on an Amersham Pharmacia Biotech Multiphor System according to the manufacturer’s instructions. Equilibrated drystrips were then separated in the second dimension on reducing 3-20% SDS-polyacrylamide gels. All 4 gels were electroblotted onto PVDF and immunoblotted with a cocktail of MANDAG2 and 43DAG8/DS (1:500 and 1:50, respectively). The 4 immunoblots were developed simultaneously by ECL (Amersham Pharmacia Biotech) onto the same piece of x-ray film to avoid differences in exposure time. Blots were stripped (65°C for 45 minutes in 62.5 mM Tris-HCl, pH 6.75, 2% SDS, 100 mM mercaptoethanol) blocked as above and reprobed with anti-phospho tyrosine antisera and detection by ECL as described above. Epitope mapping of β-dystroglycan monoclonal antibody MANDAG2 on ‘SPOTs’ membranes (see below) was performed as a normal western blot detection as described above. Following incubation with MANDAG2 antibody and washing, blots were developed by ECL.

‘SPOTs’ membrane synthesis

The ‘SPOTs’ technique of peptide synthesis on derivatised cellulose membrane was performed as described (Blankenmeyer-Menge et al., 1980; Frank and Doring, 1988; Kramer et al., 1993). All reagents and equipment, including amino acids, derivatised membranes, incubation trays and software (‘SPOTs’: release 1.0), were purchased from Cambridge research Biochemicals and Genosys Biotechnologies, Inc.

Hybridisation of ‘SPOTs’ membranes with 32p-labeled utrophin GST fusion protein

A utrophin WW-EF-ZZ-GST fusion protein was purified from bacterial lysates on glutathione beads and phosphorylated in situ using the catalytic subunit of cAMP-dependent protein kinase as described previously (Rentschler et al., 1999). SPOTs membranes were blocked as above and then probed with the 32P-labeled utrophin GST-fusion protein.

RESULTS

Treatment of tissue culture cells with phosphatase inhibitors such as peroxynitrate or okadaic acid results in an apparent increase in phosphorylation levels, enabling the visualisation of transient phosphorylation events. Western blotting of HeLa cell extracts treated with peroxynitrate, with antibodies to phosphorylated dystroglycan, showed a dramatic increase in the levels of tyrosine phosphorylation up to one hour with a slight decline in phospho-tyrosine levels up to six hours. With prolonged exposure to peroxynitrate cells began to round up and detach from the substratum (data not shown). Western blotting of HeLa cell extracts with antibodies against β-dystroglycan

![Fig. 1. Tyrosine phosphorylation of β-dystroglycan and utrophin in HeLa cells. (A) Western blot of: untreated confluent HeLa cells lane 1; peroxynitrate treated HeLa cells lane 2; calyculin A treated HeLa cells lane 3; detected with β-dystroglycan antibody MANDAG2. (B,C) Western blots of RIPA extracts of HeLa cells with (+) peroxynitrate (PV) treatment, immunoprecipitated (IP) and detected (Blot) with the indicated antisera; β-DG, MANDAG2 against β-dystroglycan; pTyr, PY20 against phospho tyrosine; Utr, RAB5 against utrophin. Numbers represent approximate molecular mass in kDa.](image-url)
revealed a pronounced retardation in electrophoretic mobility in extracts treated with peroxyvanadate (Fig. 1A; lane 1 and 2) consistent with β-dystroglycan being phosphorylated. Calyculin A, an inhibitor of type 1 and 2A serine/threonine phosphatases (Ishihara et al., 1989), added to HeLa cells over the same period however, did not result in any marked difference in electrophoretic mobility (Fig. 1A, lane 3). To confirm that the change in electrophoretic mobility was indeed due to tyrosine phosphorylation and not simply the activation of a serine/threonine kinase by a tyrosine kinase, we immunoprecipitated with antisera against β-dystroglycan or phospho-tyrosine and western blotted with β-dystroglycan antisera. As shown in Fig. 1B, β-dystroglycan antisera immunoprecipitated the authentic 43 kDa β-dystroglycan band and the electrophoretically retarded band (Fig. 1B, lanes 1, 2). Furthermore the phospho-tyrosine immunoprecipitate was recognised by β-dystroglycan antisera in the peroxyvanadate treated cell extracts (Fig. 1B, lane 3). Conversely western blotting of β-dystroglycan immunoprecipitates with anti-phospho-tyrosine antibodies also detected β-dystroglycan (see Fig. 2B). Taken together, these data demonstrate unequivocally that the electrophoretically retarded β-dystroglycan is tyrosine phosphorylated. Similar results were obtained when utrophin immunoprecipitates were western blotted with antisera against either utrophin or phospho-tyrosine (Fig. 1C). In the presence of peroxyvanadate the electrophoretic mobility of utrophin was retarded and the electrophoretically retarded band was specifically recognised by antibodies to phospho-tyrosine. This demonstrates, that like β-dystroglycan, treatment of HeLa cells with peroxyvanadate leads to the phosphorylation of utrophin on tyrosine residues. Direct treatment of RIPA cell extracts with peroxyvanadate did not alter the electrophoretic mobility of β-dystroglycan or utrophin, indicating that the observed mobility shifts were not due to direct modification of thiols in β-dystroglycan and utrophin (Mikalsen and Kaalhus, 1998), data not shown. Similar results for both β-dystroglycan and utrophin were obtained with a number of cultured cell types including: fibroblast, REF52; myoblast, C2C12; epithelial, COS-7; and primary umbilical vein endothelial cells (data not shown) indicating that this is a widespread regulatory event.

Peroxyvanadate treatment of cells can cause artefactual phosphorylation of proteins due to inappropriate or uncontrolled activation of signalling cascades. Peroxyvanadate, however, has proven immensely useful for ‘trapping’ transient tyrosine phosphorylation events. To determine if the tyrosine phosphorylation of β-dystroglycan that we had observed was as a result of a physiological stimulus and not merely an artefact of the pharmacological dose of peroxyvanadate, we examined the effect of peroxyvanadate treatment on adherent and suspended HeLa cells. Fig. 2A shows a distinct electrophoretic mobility shift in response to cell adhesion and suspended HeLa cells. Adhesion alone in the absence of peroxyvanadate caused a slight electrophoretic mobility shift by comparison with suspended cells (Fig. 2A, lanes 1, 2), addition of peroxyvanadate caused a further electrophoretic mobility shift in both suspended and adherent cells (Fig. 2A, lanes 3, 4). Notably the ability of the monoclonal antibody MANDAG2 to recognise β-dystroglycan in western blots was reduced with the increase in tyrosine phosphorylation, suggesting that the phosphorylated tyrosine is within the last 20 amino acids of β-dystroglycan against which the antibody was raised (compare also lanes 1 and 2 in Fig. 1B). Two-dimensional gel and western blotting analysis of β-dystroglycan immunoprecipitated from adherent or suspended HeLa cells in the presence or absence of peroxyvanadate, are shown in Fig. 2B. Western blot detection with anti-phospho tyrosine antisera reveals an increasing number of charged species as a result of both cell adhesion and addition of peroxyvanadate, suggesting a possible hierarchy of tyrosine phosphorylation leading to the adhesion-dependent phosphorylation of two tyrosine residues (Fig. 2B, upper panel). There is a complete absence of a phospho-tyrosine signal in the suspended peroxyvanadate treated cells. One clear spot of tyrosine phosphorylated β-dystroglycan is present in suspended cells treated with peroxyvanadate and in untreated adherent cells, whereas in adherent cells treated with peroxyvanadate we see at least two incompletely resolved spots giving the appearance of an ellipse. The pattern of β-dystroglycan spots in the lower panel recapitulates those of the upper panel (where present) and shows that approximately...
equal amounts of β-dystroglycan were present in the original samples subjected to two-dimensional electrophoresis. These data therefore support the notion that β-dystroglycan is phosphorylated on tyrosine in response to cell adhesion.

In order to identify the β-dystroglycan binding site on utrophin, MBP-fusion proteins to various regions of the cysteine rich region of utrophin (Fig. 3A, upper) were used as affinity probes for β-dystroglycan in extracts of HeLa cells. Following immunoprecipitation with antibodies MANDAG2 and 43DAG1/8D5 against β-dystroglycan, MBP-utrophin fusion proteins co-immunoprecipitated with β-dystroglycan were detected by western blotting with anti-MBP antisera (Fig. 3B, middle). All MBP-fusion constructs were co-immunoprecipitated with β-dystroglycan with the exception of MBP-WW. Densitometric quantification of the MBP-fusions detected, revealed that almost twice as much MBP-WWEFZZ was bound as any other fusion, indicating that the complete cysteine-rich region of utrophin is the most effective in binding.
domains needed for utrophin binding to residues. We therefore repeated the experiments to identify the phosphorylation of not calyculin A, resulted in the adhesion-dependent binding to calcium binding, and this is doubtful (Winder et al., 1995a, that if the EF hands in utrophin are functional in terms of Mg²⁺ (presence of mM EGTA and EDTA). This would suggest that if the EF hands in utrophin are functional in terms of calcium binding, and this is doubtful (Winder et al., 1995a, 1997), then the presence of divalent cations is not required for binding to β-dystroglycan under these conditions.

Treatment of tissue culture cells with peroxysyvanadate, but not calyculin A, resulted in the adhesion-dependent phosphorylation of β-dystroglycan and utrophin on tyrosine residues. We therefore repeated the experiments to identify the domains needed for utrophin binding to β-dystroglycan in extracts from HeLa cells that had previously been treated with peroxysyvanadate. As can be seen in Fig. 3B (lower panel) the prior phosphorylation of β-dystroglycan on tyrosine almost completely abolished the ability of utrophin fusions to be co-immunoprecipitated with β-dystroglycan, with the exception of MBP-ZZ. As shown in Fig. 3D, the binding of the complete cysteine-rich region, domains WW, EF and ZZ, was completely inhibited by tyrosine phosphorylation of β-dystroglycan following treatment with peroxysyvanadate as compared to MBP alone. There was no evidence from western blots of MBP-fusions undergoing mobility shifts, to suggest that any of the MBP-fusions themselves became phosphorylated during the course of the experiments, suggesting that the effect of peroxysyvanadate was directly on β-dystroglycan within cells. Furthermore, despite the apparent inability of the β-dystroglycan monoclonal MANDAG2 to recognise tyrosine phosphorylated β-dystroglycan on western blots it was still able to recognise tyrosine phosphorylated β-dystroglycan in solution. This is apparent from the ability of MANDAG2 to immunoprecipitate tyrosine phosphorylated β-dystroglycan which was detected on two dimensional gels with anti-phospho-tyrosine antisera (Fig. 2B) and to immunoprecipitate the ZZ domain fusion with tyrosine phosphorylated β-dystroglycan (Fig. 3B, lower panel).

As shown in Fig. 1B (lanes 1 and 2) and Fig. 2A, the monoclonal antibody MANDAG2 was less efficient at recognising tyrosine phosphorylated β-dystroglycan on western blots, though this did not appear to affect its ability to immunoprecipitate β-dystroglycan from cell extracts as indicated above. We therefore epitope mapped the MANDAG2 antibody using a series of ‘SPOTs’ membranes covering the entire cytoplasmic domain of β-dystroglycan (Rentschler et al., 1999). As shown in Fig. 4, MANDAG2 recognised a single peptide, spot 31, corresponding to the ultimate 12 amino acids of β-dystroglycan (TPYRSPPPYYVPP). MANDAG2 was originally raised against a peptide corresponding to the ultimate 15 amino acids of β-dystroglycan (Helliwell et al., 1994). More detailed analysis of the epitope by systematic mutation of the peptide recognised in Fig. 4B revealed that the essential amino acids in the epitope were PxYVP in the ultimate 5 amino acids of β-dystroglycan (Fig. 5, Table 1). Furthermore as suggested by the relative inability of MANDAG2 to recognise tyrosine phosphorylated β-dystroglycan (Figs 1, 2), MANDAG2 was unable to recognise any peptide containing a phospho-tyrosine at the second last position (Y), but was unaffected by phosphorylation of tyrosine, serine or threonine alone in any of the other positions in the last 15 amino acids,
MANDAG2 is sensitive to phosphorylation of tyrosine in the epitope PxYVP, which in part overlaps the WW domain binding motif in β-dystroglycan PPyV (Rentschler et al., 1999) and is therefore an effective reporter for tyrosine phosphorylation of the β-dystroglycan WW domain binding motif. From these data and those in Figs 1 and 2, it is possible to deduce that adhesion-dependent tyrosine phosphorylation of β-dystroglycan on its ultimate tyrosine within the WW domain binding motif is able to regulate the binding to utrophin. In order to test this more directly we probed the same SPOTs membranes used in Figs 4 and 6 with a α3P-labeled GST-fusion protein comprising the WWEFZZ region of utrophin. As demonstrated previously for the dystrophin WWEFZZ region (Jung et al., 1995; Rentschler et al., 1999) utrophin also bound almost exclusively to a peptide corresponding to the last 12 amino acids of β-dystroglycan (Fig. 7A). On longer exposure of the SPOTs membrane, weaker interactions with other peptides are apparent, e.g. peptides 2,3,4,6,7,29,30 and binding of utrophin to peptides occurred equally well in the presence or absence of phosphorylation.

### Table 1. Single amino acid substitutions within the β-dystroglycan carboxy-terminal peptide that significantly reduce binding of MANDAG2

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Summary of the results shown in Fig. 5. Each amino acid of the wild-type peptide was sequentially replaced with the other 19 amino acids, and binding of the β-dystroglycan monoclonal MANDAG2 was compared to that of the wild-type peptide. The uppermost row represents the amino acid positions of the wild-type β-dystroglycan peptide. The leftmost column represents all the replacement amino acids. The intersections correspond to the spot numbers where substitutions occurred. Substitutions which significantly reduced binding are indicated by shading. ‘x’ denotes the amino acid of the wild-type peptide. Spot 192 and 288 are control spots where no peptide was synthesised.

Fig. 6. Effect of phosphorylation on the binding of MANDAG2 to a C-terminal β-dystroglycan peptide. (A) A peptide equivalent to that described in Fig. 5A was synthesised containing the phospho-amino acids pY, pT and pS at each possible position in the peptide, either singly or in multiples. The membrane was probed with monoclonal MANDAG2 as described above. Only peptides containing a pY at Y892 were not recognised by MANDAG2. (B) Actual sequence of the corresponding peptide spots in A.

Fig. 7. Identification of β-dystroglycan peptides which interact with GST-utrophin WWEFZZ and the effect of β-dystroglycan phosphorylation. (A) SPOTs membrane as described in Fig. 4 was probed with 32P-labelled GST-WWEFZZ. This utrophin WWEFZZ fusion protein bound almost exclusively to peptide corresponding to the last 12 amino acids of β-dystroglycan (Fig. 7A). On longer exposure of the SPOTs membrane, weaker interactions with other peptides are apparent, e.g. peptides 2,3,4,6,7,29,30 and binding of utrophin to peptides occurred equally well in the presence or absence of phosphorylation.
absence of calcium (data not shown). Furthermore phosphorylation of the tyrosine residue in the WW domain binding motif PPxY was able to completely inhibit the binding of utrophin (Fig. 7B). Phosphorylation of the serine in the n-1 position adjacent to the PPxY motif was also able to regulate binding, as has been demonstrated for other WW domains binding to their cognate ligands (A. Korosi, A. Chang and M. Sudol, unpublished observations). This confirms the hypothesis that adhesion-dependent tyrosine phosphorylation of β-dystroglycan within the WW domain binding motif is able to regulate the WW domain-mediated interaction between utrophin and β-dystroglycan. This is the first demonstration of a physiologically relevant tyrosine phosphorylation of a WW domain ligand and has parallels with the tyrosine phosphorylation of SH3 domain ligands regulating SH3-mediated interactions.

DISCUSSION

Numerous studies have described serine/threonine phosphorylation of dystrophin in vivo, in vitro or by endogenous co-purifying kinases, reviewed in (Michalak et al., 1996; Winder et al., 1997), although few studies have identified functional consequences of these phosphorylation events. Dystrophin has been shown to be tyrosine phosphorylated in the postsynaptic membrane of Torpedo electric organ (Wagner and Huganir, 1994). Two-dimensional gel electrophoretic analysis of the dystrophin glycoprotein complex suggests phosphorylation of dystrophin, β-dystroglycan and possibly other components (Yamamoto et al., 1993). More recently, Yoshida and colleagues (1998) demonstrated adhesion-dependent tyrosine phosphorylation of α- and γ-sarcoglycan but not β-sarcoglycan or β-dystroglycan in L6 myoblasts. Tyrosine phosphorylation of utrophin and β-dystroglycan has not previously been described.

The role of β-dystroglycan in the extracellular matrix and regulation of utrophin binding to β-dystroglycan by tyrosine phosphorylation may be involved in cellular processes that require modulating of cell-substratum interactions and cytoskeletal connections associated with them. β-dystroglycan and utrophin are located at sites of cell-cell and cell substrate contact (Belkin and Burridge, 1995a,b; Belkin and Smallheiser, 1996; James et al., 1996; Khurana et al., 1995). The adhesion-dependent tyrosine phosphorylation of β-dystroglycan occurred whether HeLa cells were plated onto fibronectin or laminin coated substrates, suggesting that the kinase responsible for phosphorylating β-dystroglycan was not specifically activated by the binding of α-dystroglycan to laminin (M. James and S. J. Winder, unpublished observations), but was a more general adhesion signal potentially involving integrin engagement. The phosphorylation of β-dystroglycan on tyrosine did still occur in focal adhesion kinase null cells (a generous gift from Dr D. Illic, San Francisco) indicating that this kinase was not required for β-dystroglycan phosphorylation (S. J. Winder, unpublished observation). The adhesion-dependent phosphorylation of β-dystroglycan by yet unidentified tyrosine kinases and in response to cell adhesion, leads to its release from utrophin and the underlying cytoskeleton. This may be required for processes such as cell adhesion, migration, proliferation and differentiation. Furthermore the identification of tyrosine phosphorylation on β-dystroglycan from C2C12 myotubes (J. L. Ilsley and S. J. Winder, unpublished observations) suggests a role for tyrosine phosphorylation in regulating β-dystroglycan-dystrophin interactions. Such regulation may have implications in the pathogenesis and treatment of Duchenne muscular dystrophy. The recent identification of α-dystroglycan as the cellular receptor for Mycobacterium leprae (Rambukkana et al., 1998) and for arenaviruses such as Lassa fever virus (Cao et al., 1998), also raises the possibility that infection by these organisms may exert part of their pathogenic effect by disruption of signalling events associated with β-dystroglycan phosphorylation.

The modular protein domains of the dystrophin and utrophin cytoine-rich region show an extremely high degree of sequence identity, see Winder et al. (1997) for review. Studies with in vitro translated β-dystroglycan and dystrophin (Jung et al., 1995), showed that high affinity binding of dystrophin carbox terminus GST-fusion proteins to β-dystroglycan required the presence of the complete cytoine-rich domain (shown later to comprise WW, EF and ZZ domains) and that fusions comprising WW-EF bound weakly and WW alone not at all. However, no other dystrophin GST-fusion constructs; EF, ZZ, or EF-ZZ were found to bind under the conditions of these in vitro assays. These apparently conflicting results may reflect differences in the precise limits of the fusion proteins used in this study, the use of MBP as tags in recombinant proteins, and also that we used tissue cell extracts as a source of β-dystroglycan rather than in vitro translated protein. Additionally, more recent studies with dystrophin GST-fusion constructs containing well demarcated modular domains suggest that there is weak interaction between the EF hand region and also a contribution from the ZZ domain of dystrophin to β-dystroglycan binding (Rentschler et al., 1999). β-Dystroglycan from cellular sources may also be complexed with other proteins, such as the adapter protein Grb2 (Yang et al., 1995), or other as yet unidentified proteins, that facilitate binding of EF and ZZ domains to β-dystroglycan (Rentschler et al., 1999). The possibility remains that there are fundamental differences between the carboxy-terminal domains of utrophin and dystrophin with respect to the interaction with β-dystroglycan. This seems unlikely, however, as utrophin has been shown to effectively replace dystrophin in mouse models of muscular dystrophy (Tinsley et al., 1996).

Tyrosine phosphorylation of β-dystroglycan prevented the binding of all utrophin MBP-fusions with the exception of MBP-ZZ, suggesting that ZZ is involved in an interaction somehow different to the WW and EF domains, possibly with a different region of β-dystroglycan, or even with another protein associated with β-dystroglycan such as Grb2 (Yang et al., 1995). It is largely assumed that dystrophin and utrophin WW domains associate with β-dystroglycan via the WW domain binding motif PPxY (Chen and Sudol, 1995) located in the extreme carboxy terminus of β-dystroglycan (see Fig. 8). A recent thorough detailed analysis of the precise requirements for the dystrophin WW domain binding to β-dystroglycan has revealed that this is indeed the case (Rentschler et al., 1999). As shown here, the presence of both the WW domain and ZZ domain are required for the high affinity interaction between utrophin and β-dystroglycan, though the precise target sequence for the ZZ domain on β-
Tyrosine phosphorylation regulates dystroglycan

Table 2. Role of phosphorylation in regulation of SH and WW domain interactions

<table>
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<tr>
<th>Domain</th>
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<th>Regulated by (de)phosphorylation</th>
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<td>pS/Tp</td>
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A brief list of consensus motifs for interaction with the indicated SH or WW domain. Domain, represents the domain class, SH2, SH3 or WW domain types I, II and III. Ligand, represents the defined consensus sequence for binding to the respective ligand. Phosphorylation obligatory, defines whether phosphorylation of a core ligand residue is required for the interaction with the respective domain. Regulated by (de)phosphorylation, indicates where dephosphorylation (shown in parentheses) may act as a regulatory step or whether phosphorylation of the core residue itself leads directly to regulation of binding. *, when the tyrosine is phosphorylated. ‡, this represents the common residues identified between Pin1 and Cdc25 (Lu et al., 1999) and does not represent a true experimentally defined consensus.

REFERENCES


