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Identification of Dp71 Isoforms in the Platelet Membrane Cytoskeleton

POTENTIAL ROLE IN THROMBIN-MEDIATED PLATELET ADHESION*

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Utrophin is a component of the platelet membrane cytoskeleton and participates in cytoskeletal reorganization (Earnest, J. P., Santos, G. F., Zuerbig, S., and Fox, J. E. B. (1995) *J. Biol. Chem.* 270, 27259–27265). Although platelets do not contain dystrophin, the identification of smaller C-terminal isoforms of dystrophin, including Dp71, which are expressed in a wide range of nonmuscle tissues and cell lines, has not been investigated. In this report, we have identified Dp71 protein variants of 55–60 kDa (designated Dp71Δ_{110}) in the membrane cytoskeleton of human platelets. Both Dp71Δ_{110} and utrophin sediment from lysed platelets along with the high speed detergent-insoluble pellet, which contains components of the membrane cytoskeleton. Like the membrane cytoskeletal proteins vinculin and spectrin, Dp71Δ_{110} and utrophin redistributed from the high speed detergent-insoluble pellet to the integrin-rich low speed pellet of thrombin-stimulated platelets. Immunoelectron microscopy provided further evidence that Dp71Δ_{110} was localized to the submembranous cytoskeleton. In addition to Dp71Δ_{110}, platelets contained several components of the dystrophin-associated protein complex, including β-dystroglycan and syntrophin. To better understand the potential function of Dp71Δ_{110}, collagen adhesion assays were performed on platelets isolated from wild-type or Dp71-deficient (mdx^{3cv}) mice. Adhesion to collagen in response to thrombin was significantly decreased in platelets isolated from mdx^{3cv} mice, compared with wild-type platelets. Collectively, our results provide evidence that Dp71Δ_{110} is a component of the platelet membrane cytoskeleton, is involved in cytoskeletal reorganization and/or signaling, and plays a role in thrombin-mediated platelet adhesion.

Duchenne muscular dystrophy (DMD)[1] is an X-linked recessive disease characterized by progressive degeneration of muscle resulting in early death from respiratory or cardiac failure (1, 2). DMD is caused by mutations in the dystrophin gene, leading to the loss of dystrophin, a 427-kDa membrane-associated cytoskeletal protein. Analysis of dystrophin expression has led to the identification of several tissue-specific and/or developmentally regulated C-terminal dystrophin isoforms generated through differential promoter usage and/or alternative splicing at the 3′-end of the gene (3–7). Dp71, a dystrophin protein of 70–75 kDa, is the major dystrophin gene product found in a wide range of nonmuscle tissues (8–13). The Dp71 transcript, which is regulated by a promoter situated between exons 62 and 63 of the dystrophin gene, encodes the cysteine-rich and C-terminal domains of dystrophin. Because Dp71 transcripts are alternatively spliced for exons 71 and/or 78 in a wide range of nonmuscle tissues, multiple Dp71 protein products of 70–75 kDa are generated, all of which can be identified using dystrophin-specific antibodies directed against the C terminus of the protein (8). In addition to these Dp71 isoforms, we have recently identified a subpopulation of Dp71 transcripts in human brain that do not contain exons 71–74 and are alternatively spliced for exon 78 (14). Consistent with the expected translation of these Dp71 transcripts, immunoblot analysis using dystrophin-specific C-terminal antibodies detected an immunoreactive protein of ~58 kDa (designated Dp71Δ_{110}) in total protein lysates from adult human brain. The observation that Dp71Δ_{110} fails to react with a monoclonal antibody (MANEX7374A) directed against epitopes within exons 73 and 74 provides further evidence that Dp71Δ_{110} is derived from Dp71 transcripts lacking exons 71–74.

Other C-terminal isoforms of dystrophin have been identified in the retina (Dp260) (15) and in the peripheral (Dp116) (16) and central nervous systems (Dp140) (17). In addition, the autosomal homologue of dystrophin (18), utrophin, is expressed in a wide range of nonmuscle tissues and cell lines (19, 20).

Although there is considerable information regarding the tissue distribution and structure of these C-terminal dystrophin isoforms, relatively little is known about their function. Dp71 associates with components of the dystrophin-associated protein complex (DAPC) (21, 22) and localizes to the plasma membrane (23), raising the possibility that Dp71 plays a role in the formation and/or stabilization of the membrane cytoskele-

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[1] The abbreviations used are: DMD, Duchenne muscular dystrophy; DAPC, dystrophin-associated protein complex; Dp71Δ_{110}, 55- to 60-kDa variant of Dp71; Dp71$\Delta_{110}{}^{m}$, Dp71Δ_{110} containing the C-terminal sequences of muscle dystrophin; Dp71$\Delta_{110}{}^{a}$, Dp71Δ_{110} containing the alternative 31-amino acid C-terminal sequence due to the splicing of exon 78; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; BSA, bovine serum albumin; RFU, relative fluorescence units.

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TABLE I
Antibodies used in this study and their epitope location

Antibody[a]	Location of epitope
1461[p]	C-terminal 17 amino acids of muscle dystrophin and Dp71
462B[p]	Alternative C-terminal 31 amino acids of muscle dystrophin and Dp71
MANDYS1[m]	Central rod domain of muscle dystrophin
MANCHO3[m]	C-terminal domain of utrophin
MANEX7374A[m]	Amino acids encoding exons 73 and 74 of dystrophin
NME-1[p]	N-terminal 7 amino acids of Dp71
Dystrobrevin[m]	Amino acids 249–403 of mouse α-dystrobrevin-1
γ-Sarcoglycan[m]	Amino acids 167–178 of rabbit γ-sarcoglycan
β-Dystroglycan[m]	C-terminal 15 amino acids of human β-dystroglycan
Syntrophin[m]	*Torpedo* syntrophin

[a] Affinity-purified polyclonal antibody (*p*) and monoclonal antibody (*m*).

ton. This concept is further supported by studies showing that mice deficient in Dp71 have reduced levels of the DAPC in their brain (24). However, direct evidence that Dp71 is a component of the membrane cytoskeleton or that it is involved in cytoskeletal reorganization and/or transmembrane signaling is lacking.

It is well established that platelets contain a complex membrane cytoskeleton that resembles, at least in part, the cytoskeleton found in muscle (25, 26). Our previous studies have identified utrophin as a component of the platelet membrane cytoskeleton and indicate that it participates in cytoskeletal reorganization during platelet activation (27). Although platelets do not contain full-length dystrophin, the presence of C-terminal dystrophin isoforms was not examined. In the present study, we provide evidence that Dp71Δ_{110} is expressed in human platelets, is a component of the platelet membrane cytoskeleton, and redistributes in integrin-induced reorganization of the cytoskeleton during thrombin-induced platelet activation. In terms of platelet function, adhesion to collagen in response to thrombin was significantly reduced in platelets from Dp71-deficient (mdx^{3cv}) mice, compared with platelets from age-matched wild-type mice. These results suggest that Dp71Δ_{110} plays a potential role in thrombin-stimulated platelet adhesion, possibly by mediating cytoskeletal reorganization and/or signaling.

MATERIALS AND METHODS

Dami Cell Cultures—Dami cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2-95% air.

Antibody Production and Characterization—Table I lists the antibodies used in this study and identifies the epitope location against which they are directed. The production, purification, and characterization of the dystrophin-specific C-terminal antibody 1461 have been described previously (28). The affinity-purified polyclonal antibody 462B, which is directed against the alternative 31-amino acid C terminus of dystrophin, has been described previously (8). MANEX7374A is a monoclonal antibody directed against epitopes within exons 73 and 74 of dystrophin and Dp71 (14, 29). MANDYS1 is a monoclonal antibody directed against the central rod domain of dystrophin that does not cross-react with utrophin (30). MANCHO3 is a monoclonal antibody directed against utrophin that does not cross-react with dystrophin (31). Monoclonal antibodies directed against β-dystroglycan (NCL-43DAG) (32) or γ-sarcoglycan (NCL-g-SARC) were purchased from Novocastra Laboratories (Newcastle, UK). Monoclonal antibodies against dystrobrevin (D62320) were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies against vinculin (V4505) or spectrin (S1390) were purchased from Sigma (St. Louis, MO). Syntrophin antibodies (SYN 1351) were raised against *Torpedo* syntrophin, as described previously (33). The production, purification, and characterization of the Dp71-specific N-terminal antibody, NME-1, has been described (34).

Preparation of Human Platelets—Venous blood was collected from healthy donors into 0.15 volume of 85 mM trisodium citrate, 65 mM citric acid, 2% dextrose (ACD). The blood was centrifuged at 160 × *g* for 15 min at 22 °C, and the platelet-rich plasma was harvested and subjected to an additional centrifugation step. Platelets isolated in this fashion were determined to be >99.9% pure using a Cobas Argos automated cell counter (Roche Molecular Biochemicals, Mississauga, Ontario, Canada). For platelet activation studies, purified platelets were pelleted by centrifugation at 2000 × *g* for 15 min at 22 °C, washed twice in PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4) containing 0.15 volume of ACD and resuspended at a concentration of 1×10^9 platelets/ml in Tyrode's buffer (138 mM NaCl, 2.9 mM KCl, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM calcium chloride, and 0.4 mM magnesium chloride, pH 7.4). Platelets were activated by addition of 1 unit/ml α-thrombin (kindly provided by Dr. John Fenton II, New York Department of Health, Albany, NY).

Isolation and Analysis of Platelet Cytoskeletons—Human platelet lysates were produced by the addition of an equal volume of Triton X-100 lysis buffer (2% Triton X-100, 10 mM EGTA, 100 mM Tris-HCl, 2 mg/ml leupeptin, 100 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, pH 7.4) followed by immediate centrifugation at 15,600 × *g* at 4 °C for 4 min to pellet cytoplasmic actin filaments. The supernatant was subsequently centrifuged at 100,000 × *g* for 2.5 h at 4 °C to pellet components of the membrane cytoskeleton (35). Cytoskeletal components were subjected to immunoblot analysis, as described below.

Reverse Transcriptase-PCR Analysis—Total RNA extracted from fresh human platelets using the TRIzol reagent procedure (Invitrogen) was stored at −70 °C in diethyl pyrocarbonate-treated water. One microgram of platelet RNA was used to synthesize cDNA, using either a primer complimentary to a sequence in the 3′-untranslated region of the human dystrophin/Dp71 cDNA sequence (513, 5′-TGCATAGACGT-GAAAACCTGCC-3′) or random hexamers, as described previously (8). The PCR primers used to amplify DNA segments flanking exon 78 of the dystrophin cDNA were 837 (5′-CCTTCCCTAGTAGTTCAAGAGG-3′; position 11205–11223) and 2296 (5′-TCTAGAATTCTTATTCT-GCTCCTTCTTC-3′; position 11352–11335). Random hexanucleotide-primed first-strand cDNA served as template for PCR analysis using primers GAP1 (5′-CCACCCATGGCAAATTCCATGGCA-3′, sense) and GAP2 (5′-TCTAGACGGAGGTCAGGTCCACC-3′, antisense) for glyceraldehyde-6-phosphate dehydrogenase. All primers were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario, Canada). PCR amplification took place in a final volume of 50 μl containing 2 μl of the RT reaction, 100 ng of primer, 2.5 units of *Taq* polymerase (PerkinElmer Life Sciences) in a buffer consisting of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), and 0.5 mM of each dNTP. All samples were subjected to amplification in a PerkinElmer Life Sciences thermal cycler (model 480) with a step program of 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. Amplified products were separated on a 1.5% agarose-TBE gel containing ethidium bromide.

Immunoblot Analysis—Total protein lysates from human platelets, muscle, or Dami cells were solubilized in SDS-PAGE sample buffer, heated to 95 °C for 2 min, and separated on SDS-polyacrylamide gels under both reducing and nonreducing conditions (36). Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada) and immunostained as previously described (8). After incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies, membranes were developed using the Renaissance chemiluminescence reagent kit (PerkinElmer Life Sciences, Mississauga, Ontario, Canada). Molecular mass markers were purchased from Invitrogen (Burlington, Ontario, Canada) or Bio-Rad.

Immunoelectron Microscopy—Human platelets were fixed and partially permeabilized by incubation for 3 h at 4 °C in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.001% Triton X-100. For immunogold labeling, platelets were allowed to adhere to plastic coverslips (Thermanox, Invitrogen) for 30 min at room temperature. After washing several times with PBS, followed by PBS containing 0.5% bovine serum albumin (BSA) and 0.15% glycine to quench residual aldehyde, coverslips were rinsed with PBS containing 0.5% BSA, and then incubated with the dystrophin-specific C-terminal antibody 1461 (dilution 1:100) for 1 h at room temperature. As a control, coverslips were incubated with normal rabbit IgG in place of rabbit anti-dystrophin IgG. After washing with PBS containing 0.5% BSA, coverslips were incubated with a goat anti-rabbit IgG coupled to 30-nm colloidal gold particles (Amersham Biosciences, Oakville, Ontario, Canada) for

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2 h at room temperature. Coverslips were then washed twice with PBS containing 0.5% BSA and once with PBS before being fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After a series of ethanol dehydration steps, samples were critical-point dried, rendered conductive with a thin layer of evaporated carbon, and then examined in a Jeol JSM 820 electron microscope using mixed backscatter/secondary imaging.

For transmission electron microscopy, platelet samples were incubated with the 1461 antibody as described above, washed with PBS, and then incubated with a HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature. After several washes with PBS, the samples were incubated for 15 min in a fresh solution of 2.8 mM diaminobenzidine in 50 mM TBS containing 0.02% hydrogen peroxide. Immunoperoxidase-labeled platelets were post-fixed in 1% OsO_4/0.1 M sodium cacodylate, dehydrated in an ascending series of ethanol solutions, and embedded in Epon 812 with propylene oxide. Ultrathin sections cut with a diamond knife were then examined in a Jeol 1200EX electron microscope.

Strains of Mice—Wild-type C57BL/6 mice were obtained from Charles River (Montreal, Quebec, Canada). mdx^{3cv} mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Preparation of Mouse Platelets—Mouse blood was collected from the inferior vena cava of anesthetized 10-week-old wild-type or mdx^{3cv} mice into 0.15 volume of ACD and diluted 1:1 in Tyrode's buffer containing 5 μM PGE_1 (Sigma). Platelet-rich plasma was prepared by centrifugation of the blood at 275 × *g* for 15 min at 22 °C through a discontinuous 55%/67% Percoll gradient. After an additional Percoll gradient centrifugation step, platelet-rich plasma was resuspended in Tyrode's buffer containing 5 μM PGE_1. Purified platelets were pelleted by centrifugation at 2000 × *g* for 10 min and resuspended at 1×10^8 platelets/ml aliquots in Tyrode's buffer without PGE_1 (collagen-stimulated platelets), Tyrode's buffer containing 1 mM theophylline, 5 mM EDTA, and 5 μM PGE_1 (unstimulated platelets) or Tyrode's buffer containing 1 unit/ml α-thrombin (thrombin-stimulated platelets).

Collagen Adhesion Assay—Calf skin collagen, Type 1 (Sigma), resuspended in 0.1 mM acetic acid, was added to the wells of 96-well microtiter plates (Nunc) at a concentration of 5 μg/well. After drying overnight in a fume hood, plates were washed three times with PBS. Platelets (1×10^7/well) were added and incubated for 1 h at 37 °C. After gentle washing with PBS containing 5 mM glucose to remove nonadherent platelets, the remaining adherent platelets were fluorescently labeled for 10 min with 20 μg/ml fluorescein diacetate (Sigma) in PBS containing 5.5 mM glucose. The extent of platelet adherence was determined using a Fluorescence Plate Reader (Molecular Devices) by measuring the fluorescence emitted for each well at 530 nm after excitation at 485 nm. The results are presented as relative fluorescence units (RFU) per 1×10^7 platelets and represent the mean ± S.E. of quadruplicate measurements using platelets isolated from eight wild-type or six mdx^{3cv} mice.

Statistical Analysis—Results are presented as the mean ± S.E. Significance of differences between wild-type and mdx^{3cv} platelets were determined by one-way analysis of variance. If significant differences were detected, unpaired Student's *t* tests were performed. For all analyses, p values of <0.05 were considered significant.

RESULTS

Identification of Dp71 Isoforms in Human Platelets—To determine whether human platelets contain dystrophin, C-terminal isoforms of dystrophin and/or utrophin, total platelet lysates were separated on SDS-polyacrylamide gels and subjected to immunoblot analysis. When immunostained with a dystrophin-specific antibody (1461) directed against the C-terminal 17 amino acids of muscle dystrophin, full-length dystrophin was observed in normal muscle but not in DMD muscle or platelets (Fig. 1*A*). However, platelets contained an immunoreactive protein that migrated at an apparent molecular mass of 55–60 kDa (designated $Dp71\Delta_{110}{}^{m}$) under both reducing (Fig. 1*A*) and nonreducing conditions (data not shown). Platelet proteins of similar molecular mass (designated $Dp71\Delta_{110}{}^{a}$) were also identified using an antibody (462B) directed against the alternative 31-amino acid C terminus of dystrophin that results from alternative splicing of exon 78 (Fig. 1*B*) or an antibody (NME-1) specific for the unique seven amino acid N terminus of Dp71 (Fig. 1*C*). The observation that a monoclonal antibody (MANEX7374A) directed against

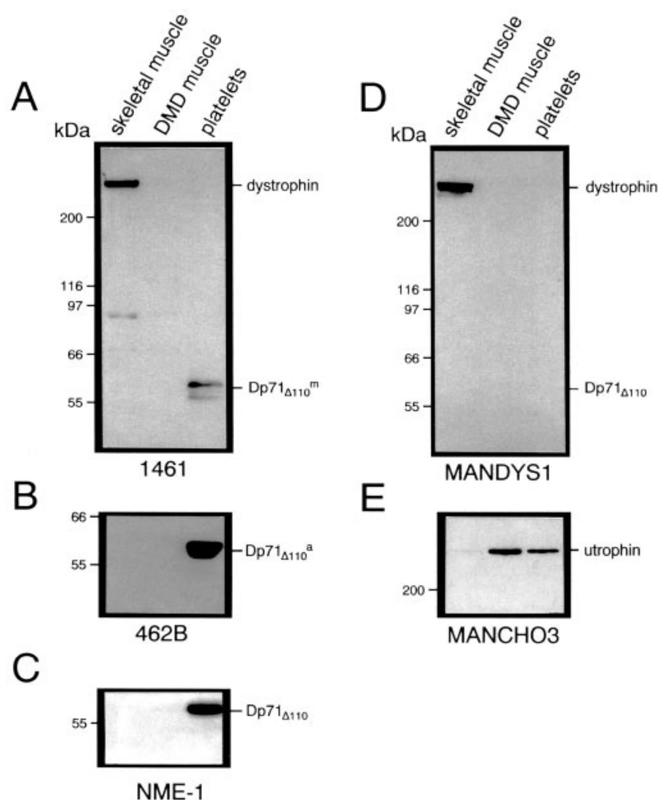

FIG. 1. **Detection of Dp71 protein isoforms in human platelets.** Total protein lysates (40 μg) from adult human skeletal muscle, DMD muscle, and platelets were separated on 7.5% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Blots were immunostained with antibodies directed to either the 17-amino acid C terminus of skeletal muscle dystrophin (*1461*, *A*), the alternative 31-amino acid C terminus of dystrophin due to the splicing of exon 78 (*462B*, *B*), the N terminus of Dp71 (*NME-1*, *C*); the spectrin-repeat rod domain of dystrophin (*MANDYS1*, *D*) or utrophin (*MANCHO3*, *E*). $Dp71\Delta_{110}{}^{m}$, $Dp71\Delta_{110}$ containing the C-terminal sequence of muscle dystrophin. $Dp71\Delta_{110}{}^{a}$, $Dp71\Delta_{110}$ containing the alternative 31-amino acid C-terminal sequence due to the splicing of exon 78. *kDa*, molecular mass markers.

epitopes within exons 73 and 74 failed to detect these proteins (data not shown) suggests that they are derived from Dp71 transcripts deleted for exons 71–74. As expected, a monoclonal antibody (MANDYS1) directed against the spectrin-like repeat domain of dystrophin did not react with these platelet proteins but did react strongly with full-length muscle dystrophin (Fig. 1*D*). Consistent with our previous findings (27), utrophin was identified in platelets using a monoclonal antibody (MANCHO3) against the C-terminal domain of utrophin (Fig. 1*E*). The reactivity and molecular mass of utrophin observed in platelets were comparable to that of utrophin found in DMD muscle (compare *lanes 2* and *3*). The observation that utrophin levels in DMD muscle are increased, compared with normal skeletal muscle, is consistent with previous reports (37, 38).

As described previously for $Dp71\Delta_{110}$ in adult human brain (14), reverse transcriptase-PCR amplification of the open reading frame of Dp71 in adult human platelets resulted in the expected 1.6-kb PCR product (data not shown). To ensure that platelet proteins detected by the dystrophin-specific C-terminal antibodies 1461 and 462B were derived from $Dp71\Delta_{110}$ transcripts alternatively spliced for exon 78, platelet RNA was analyzed by reverse transcriptase-PCR using primers flanking exon 78 (Fig. 2). As expected, a 160-bp fragment was generated from Dp71 cDNA containing an intact exon 78 (Fig. 2, *lane 2*). When platelet cDNA was used as the template, PCR products of 160 and 128 bp were generated (Fig. 2, *lane 3*). Double-

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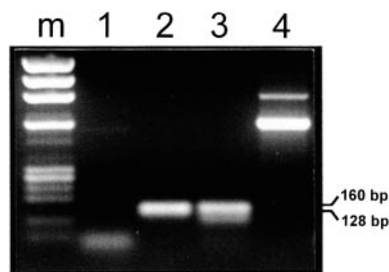

FIG. 2. **Alternative splicing of exon 78 in Dp71Δ_{110} transcripts.** First-strand cDNA was synthesized by using total RNA from human platelets and a primer complimentary to a sequence in the 3′-untranslated region of the Dp71 mRNA (designated primer 513). The cDNA was amplified by PCR with primers specific for the 3′-end of Dp71 (designated primers 837 and 2296) that flank exon 78. PCR products were separated on a 1.5% agarose-TBE gel containing ethidium bromide. *Lane 1*, control PCR products in the absence of platelet cDNA. *Lane 2*, PCR products in the presence of Dp71 cDNA containing an intact exon 78. *Lane 3*, PCR products in the presence of platelet cDNA. *Lane 4*, PCR products, using glyceraldehyde-6-phosphate dehydrogenase primers, in the presence of platelet cDNA primed with random hexamers. The 160-bp band represents PCR products containing an intact exon 78, whereas the 128-bp band represents PCR products spliced for exon 78. *m*, ΦX174 DNA marker.

stranded DNA sequencing of both strands revealed that the 160-bp fragment contained an intact exon 78, whereas the 128-bp fragment was absent for exon 78 (data not shown). As a negative control, omission of platelet cDNA in the reaction mixture resulted in a loss of these two PCR products (Fig. 2, *lane 1*). Thus, the 160- and 128-bp PCR products generated from platelet cDNA result from alternative splicing of the 32-bp exon 78 and provides further evidence that platelet Dp71Δ_{110} is alternatively spliced for exon 78.

Identification of Dp71Δ_{110}, Dystrophin, and/or Utrophin in the Megakaryocytic Dami Cell Line—Because platelets are derived from megakaryocytes, we studied the expression of Dp71Δ_{110}, dystrophin, and utrophin in Dami cells, a megakaryocytic cell line that exhibits many of the morphological and biochemical characteristics of bone marrow megakaryocytes (39). Total protein lysates from skeletal muscle, platelets, and Dami cells were subjected to immunoblot analysis using antibodies specific for the C terminus of dystrophin or utrophin (Fig. 3). As shown in Fig. 3*A*, Dp71$\Delta_{110}{}^{m}$ containing the C-terminal sequence of muscle dystrophin was found in platelets and Dami cells. However, compared with platelets, Dp71$\Delta_{110}{}^{a}$ containing the alternative 31-amino acid C-terminal sequence was not detected (Fig. 3*B*). These findings suggest that, in Dami cells, Dp71Δ_{110} is derived from Dp71 transcripts that retain exon 78. In support of this, RT-PCR analysis of Dami cell RNA revealed the presence of only Dp71Δ_{110} transcripts containing exon 78 (data not shown). As shown previously in Fig. 1, there was no evidence of Dp71Δ_{110} in skeletal muscle (Fig. 3, *A* and *B*). Dami cells, like platelets, do not contain full-length dystrophin (Fig. 3*C*) but express utrophin (Fig. 3*D*).

Association of Dp71Δ_{110} with the Membrane Cytoskeleton in Platelets—Previous studies have shown that dystrophin and its C-terminal isoforms are associated with the membrane cytoskeleton in muscle and nonmuscle tissues (21, 22, 40–42). To determine whether Dp71Δ_{110} associates with the platelet membrane cytoskeleton, platelets were lysed with a Triton X-100-containing buffer, and the cytoskeletal components were isolated by differential centrifugation (Fig. 4). Compared with total platelet lysates (Fig. 4*A*, *lane 1*), the cytoplasmic components of the platelet cytoskeleton, including actin filaments, were sedimented by centrifugation at 15,600 × *g* (low speed pellet, *lane 2*), while the detergent-insoluble membrane cytoskeleton was sedimented by centrifugation of the 15,600 × *g* supernatant at 100,000 × *g* (high speed pellet, *lane 3*). Dp71$\Delta_{110}{}^{m}$ containing the C-terminal sequence of muscle dystrophin was recovered exclusively in the detergent-insoluble fraction (Fig. 4*A*, *lane 3*). In contrast, equivalent amounts of Dp71$\Delta_{110}{}^{a}$ containing the alternative 31-amino acid C-terminal sequence were recovered in both the detergent-insoluble and -soluble fractions (Fig. 4*B*, *lanes 3* and *4*). Consistent with our previous findings (27), utrophin was recovered in the detergent-insoluble fraction, with a minor amount in the detergent-

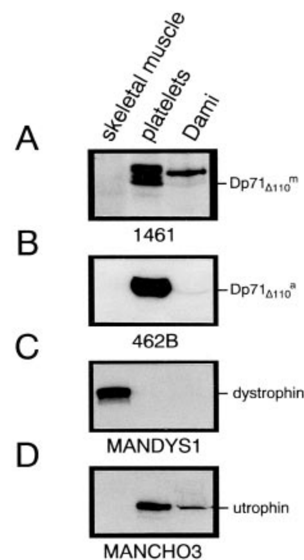

FIG. 3. **Detection of Dp71Δ_{110} in a human megakaryocytic cell line.** Total protein lysates (40 μg) from adult human muscle, platelets, or the megakaryocytic Dami cell line were separated on 7.5% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. Blots were immunostained for Dp71Δ_{110} (*A* and *B*), dystrophin (*C*), or utrophin (*D*).

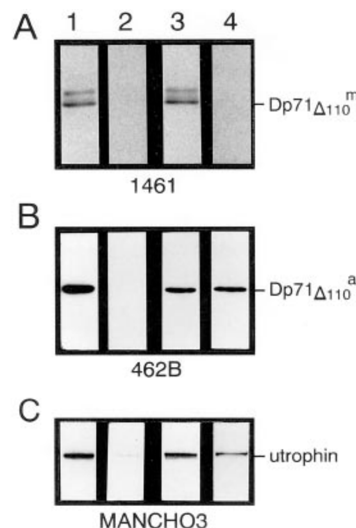

FIG. 4. **Colocalization of Dp71Δ_{110} and utrophin with the platelet cytoskeleton.** Unstimulated adult human platelets (1×10^9 platelets/ml) were resuspended in SDS-PAGE sample buffer (*lane 1*) or lysed by the addition of Triton X-100 lysis buffer (*lanes 2–4*). Platelet lysates were centrifuged for 4 min at 15,000 × *g* and the resulting Triton X-100 insoluble low speed pellet was resuspended in SDS-PAGE sample buffer (*lane 2*). The Triton X-100 supernatant was centrifuged for an additional 2.5 h at 100,000 × *g*, and the resulting Triton X-100 insoluble high speed pellet (*lane 3*) and the high speed supernatant (*lane 4*) were resuspended in SDS-PAGE sample buffer. All samples were electrophoresed through 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were immunostained for either Dp71Δ_{110} (*A* and *B*) or utrophin (*C*).

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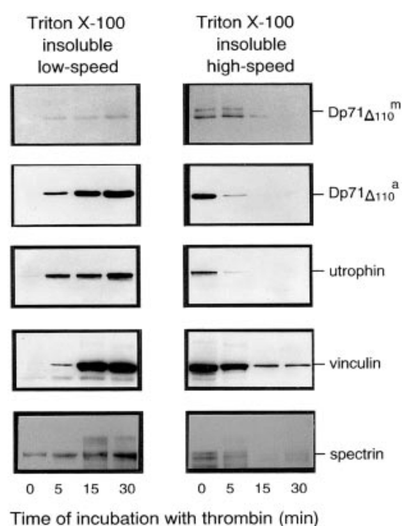

FIG. 5. **Redistribution of Dp71Δ_{110} and utrophin, along with the membrane skeletal proteins vinculin and spectrin, from the high speed detergent-insoluble fraction to the low speed detergent-insoluble fraction of aggregating platelets.** Adult human platelets (1×10^9 platelets/ml) were incubated with thrombin for the indicated times and terminated by the addition of Triton X-100 lysis buffer. Lysates were centrifuged for 4 min at 15,600 × *g* to obtain the low speed detergent-insoluble pellet. Triton X-100 supernatants were centrifuged for a further 2.5 h at 100,000 × *g* to obtain the high speed detergent-insoluble pellets. Pellets were solubilized in SDS-PAGE sample buffer, separated on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were immunostained for Dp71Δ_{110}, utrophin, vinculin, or spectrin, as indicated.

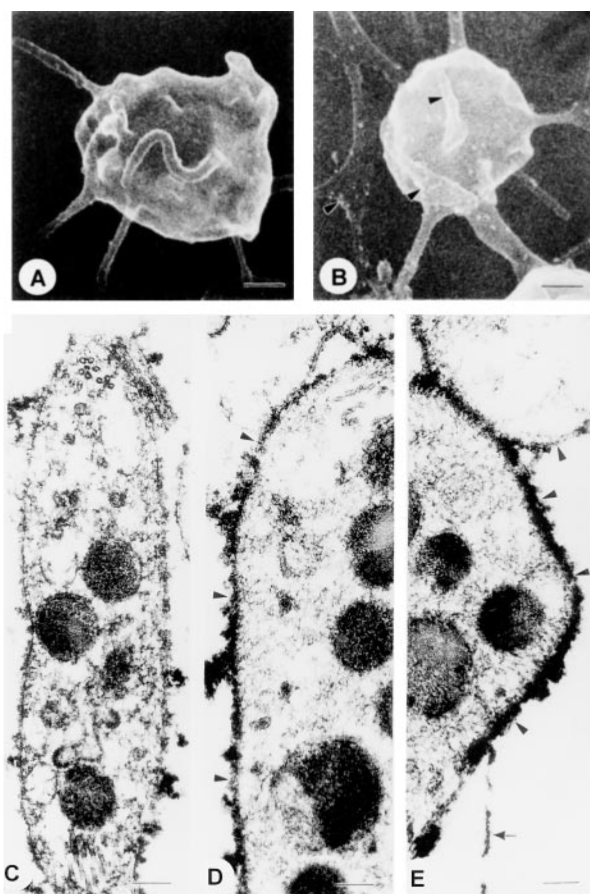

FIG. 6. **Intracellular localization of Dp71Δ_{110} in human platelets.** Scanning electron micrographs of Dp71Δ_{110} in partially permeabilized normal human platelets (*A* and *B*). Platelets were immunostained with either control rabbit IgG (*A*) or antibody 1461 (*B*), followed by goat anti-rabbit gold-30. In *A*, gold particles were absent in control platelets. In *B*, gold particles were positioned close to the plasma membrane with the majority of particles localized to the pseudopodia extending from the body of the platelet (*arrowheads*). The *bar* in *A* and *B* represents 1 μm. *C–E*, transmission electron micrographs of Dp71Δ_{110} in normal human platelets. Platelets were immunoperoxidase-stained with either control rabbit IgG (*C*) or 1461 antibody (*D* and *E*) followed by HRP-conjugated goat anti-rabbit IgG. In *C*, there was no dense reaction product observed in control platelets. In *D* and *E*, the electron-dense reaction product specific for Dp71Δ_{110} was positioned close to the plasma membrane (*arrowheads*). Even where the membrane was dissociated from the body of the platelet the reaction product is present (*arrow*). The *bar* in *C–E* represents 0.25 μm.

soluble fraction (Fig. 4*C*). These findings suggest that Dp71Δ_{110}, like utrophin, is a component of the membrane cytoskeleton.

Redistribution of Dp71Δ_{110} from the High Speed Detergent-insoluble Fraction to the Low Speed Fraction in Thrombin-activated Platelets—Previous studies have shown that the platelet cytoskeleton undergoes reorganization when platelets are stimulated such that actin, myosin, and other cytoskeletal proteins sediment at low *g*-forces from detergent-lysed platelets (43, 44). In addition, fibrinogen is secreted from intracellular granules and binds $\alpha_{IIb}\beta_3$ on adjacent platelets resulting in platelet aggregation. Consequently, a second reorganization of the cytoskeleton takes place such that membrane cytoskeletal proteins (*i.e.* spectrin and vinculin) no longer require high *g*-forces to be sedimented from detergent-lysed platelets and can be sedimented at low *g*-forces. As shown in Fig. 5, Dp71Δ_{110} containing either the C-terminal sequence of dystrophin or the alternative 31-amino acid C-terminal sequence sediment at low *g*-forces when platelets were stimulated with thrombin. This redistribution also was observed with utrophin. In addition, the rate at which Dp71Δ_{110} and utrophin redistributed to the low speed pellet correlated with that of vinculin and spectrin, known platelet membrane cytoskeletal proteins. These findings indicate that both Dp71Δ_{110} and utrophin are part of the membrane cytoskeleton that is incorporated into integrin-rich cytoskeletal complexes upon thrombin-induced platelet activation.

Intracellular Localization of Dp71Δ_{110} in Platelets—Scanning immunoelectron microscopy, performed on semi-permeabilized intact human platelets, revealed Dp71Δ_{110} close to the plasma membrane, with the majority localized to pseudopodia (Fig. 6*B*, *arrowheads*). No labeling of the plasma membrane was observed in the negative control (Fig. 6*A*). Transmission immunoelectron microscopy also revealed Dp71Δ_{110} localized to the plasma membrane of resting platelets (Fig. 6, *D* and *E*, *arrowheads*), even at sites where the membrane had dissociated from the cytoplasm (Fig. 6*E*, *arrow*). In contrast, there was no significant immunoperoxidase staining of the plasma membrane in negative controls (Fig. 6*C*).

Identification of Components of the Dystrophin-associated Protein Complex in Human Platelets—To determine whether human platelets contain components of the DAPC (45), total platelet proteins were subjected to immunoblot analysis using antibodies specific to members of the sarcoglycan, dystroglycan, or cytoplasmic complex (Fig. 7). In contrast to human muscle, platelets did not contain γ-sarcoglycan or the muscle isoforms of α-dystrobrevin, namely α-dystrobrevin-1, -2, and -3. However, platelets were shown to contain both β-dystroglycan (Fig. 7) and syntrophin (Fig. 8). Previous studies have demonstrated that syntrophins associate with dystrophin, utrophin, and multiple forms of Dp71 in a wide range of tissues (46, 47). Given that platelets contain utrophin, Dp71Δ_{110}, and syntrophin, the redistribution of syntrophin was examined in activated platelets. As observed for utrophin and Dp71Δ_{110}, syntrophin sedimented at low *g*-forces when normal platelets were

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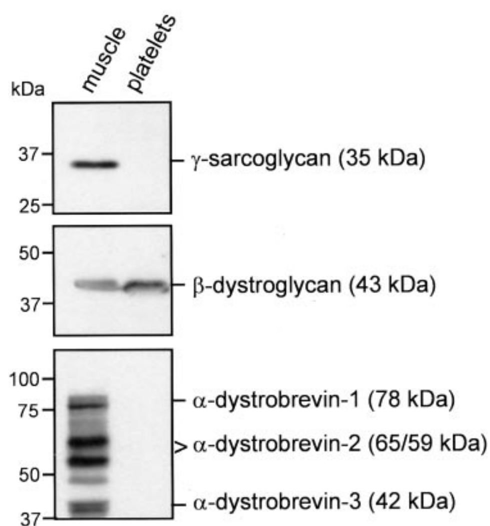

FIG. 7. **Identification of components of the DAPC in human platelets.** Total protein lysates (40 μg) from human muscle or platelets were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunostained with monoclonal antibodies to β-dystroglycan, dystrobrevin, or γ-sarcoglycan, as indicated. *kDa*, molecular mass markers.

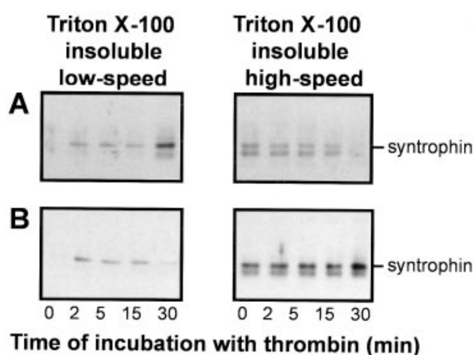

FIG. 8. **Redistribution of syntrophin from the high speed detergent-insoluble fraction to the low speed detergent-insoluble fraction of aggregating platelets.** Adult normal (*A*) or thrombasthenic (*B*) human platelets (1×10^9 platelets/ml) were incubated with thrombin for the indicated times and terminated by the addition of Triton X-100 lysis buffer. Lysates were prepared as described in Fig. 5 and immunostained for syntrophin.

stimulated with thrombin (Fig. 8*A*). Furthermore, sedimentation of syntrophin was accelerated in stirred platelet suspensions in which cell contact and aggregation were maximized (data not shown), a result consistent with utrophin, GPIIb–IIIa, and pp60$^{c\text{-}src}$ (27, 44).

Platelets from patients with Glanzmann's thrombasthenia are deficient in $\alpha_{IIb}\beta_3$ and do not undergo $\alpha_{IIb}\beta_3$-mediated transmembrane signaling (44). In contrast to normal platelets, syntrophin failed to redistribute to the low speed detergent-insoluble pellet of thrombasthenic platelet that were activated in the same way (Fig. 8*B*), a result consistent for utrophin, GPIIb–IIIa, and pp60$^{c\text{-}src}$ (27, 44). These findings indicate that syntrophin, like Dp71Δ_{110} and utrophin, is part of the platelet membrane cytoskeleton that is incorporated into integrin-rich cytoskeletal complexes as a result of integrin-mediated transmembrane signaling.

Thrombin-stimulated Platelet Adhesion Is Decreased in mdx^{3cv} Mice—mdx^{3cv} mice are an established animal model of DMD that are deficient in all forms of dystrophin and Dp71 (48). To determine if the loss of Dp71Δ_{110} affects platelet function, platelet adhesion (measured as RFU/10^7 platelets) to collagen-coated wells was compared in platelets from age-matched wild-type or mdx^{3cv} mice. As shown in Fig. 9, no difference in adherence was observed between platelets from wild-type or mdx^{3cv} mice when platelet inhibitors (PGE$_1$, theophylline, and EDTA) were added (*unstimulated*, 259 ± 61 *versus* 256 ± 49 RFU/10^7 platelets, respectively). Although platelets from wild-type or mdx^{3cv} mice exhibited increased adherence to collagen-coated wells in the absence of platelet inhibitors, no significant difference was observed between wild-type or mdx^{3cv} platelets (*collagen*, 1274 ± 145 *versus* 1460 ± 114 RFU/10^7 platelets, respectively). In contrast, thrombin-stimulated platelets from wild-type mice exhibited significantly higher ($p < 0.05$) adhesion to collagen-coated wells than did thrombin-stimulated platelets from mdx^{3cv} mice (*thrombin + collagen*, 3494 ± 326 *versus* 2052 ± 162 RFU/10^7 platelets, respectively). These findings indicate that platelet adhesion to collagen in response to thrombin is decreased in mdx^{3cv} mice, raising the possibility that Dp71Δ_{110} plays a role in thrombin-stimulated platelet adhesion.

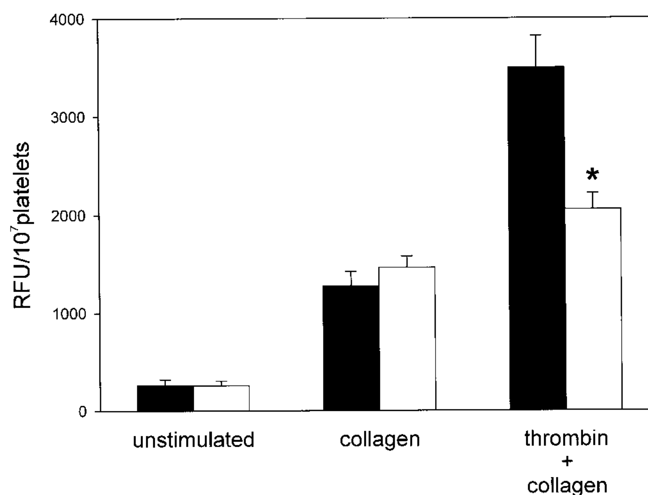

FIG. 9. **Platelets from mdx^{3cv} mice demonstrate decreased adhesion to collagen in response to thrombin.** Freshly isolated platelets from wild-type (*closed bars*) or mdx^{3cv} (*open bars*) mice were allowed to adhere to microtiter plates coated with Type 1 acid-soluble calf skin collagen for 60 min at 37 °C. After removal of nonadherent platelets, adherent platelets were fluorescently labeled with fluorescein diacetate. Fluorescence associated with adherent platelets was measured as relative fluorescence units (RFU) per 1×10^7 platelets. Values represent the mean ± S.E. of quadruplicate measurements from 10-week-old wild-type ($n = 8$) or mdx^{3cv} mice ($n = 6$). *, $p < 0.05$ *versus* thrombin-stimulated wild-type platelets.

DISCUSSION

In the present study, we have identified and characterized 55- to 60-kDa isoforms of Dp71 (designated Dp71Δ_{110}) in human platelets. These isoforms are likely identical to those recently observed in human brain (14), because they are recognized by antibodies specific for the (i) C-terminal sequence of muscle dystrophin, (ii) alternative 31-amino acid C-terminal sequence due to the splicing of exon 78, and (iii) unique 7-amino acid N terminus of Dp71. The presence of Dp71Δ_{110} in the megakaryocytic Dami cell line suggests that these platelet proteins are endogenously produced by megakaryocytes. Although platelets do not appear to contain full-length dystrophin or Dp71, they contain utrophin, the autosomal homologue of dystrophin. The observation that adhesion to collagen in response to thrombin was significantly decreased in platelets from mdx^{3cv}mice, compared with platelets from age-matched wild-type mice, suggests a role for Dp71Δ_{110} in thrombin-stimulated platelet adhesion.

Several lines of evidence indicate that Dp71Δ_{110} is a component of the platelet membrane cytoskeleton. First, Dp71Δ_{110}

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was recovered in the high speed fraction from detergent-lysed platelets. Second, like the membrane cytoskeletal proteins vinculin and spectrin, Dp71Δ_{110} redistributed from the high speed detergent-insoluble fraction to the low speed detergent-insoluble fraction upon platelet stimulation by thrombin. Third, immunoelectron microscopy localized Dp71Δ_{110} in close proximity to the plasma membrane of resting platelets. The observation that Dp71 associates with membrane glycoproteins (21, 22) and localizes to the plasma membrane (23) provides circumstantial evidence that Dp71 is a component of a submembranous cytoskeleton. Our findings provide direct evidence that Dp71Δ_{110} is part of the membrane cytoskeleton of intact platelets and that it reorganizes with utrophin, vinculin, spectrin, and other components of the membrane cytoskeleton upon platelet stimulation by thrombin.

Unlike full-length dystrophin or utrophin, Dp71Δ_{110} would not be expected to act as a membrane stabilizer. However, the identification of Dp71Δ_{110} as a component of the platelet membrane cytoskeleton raises the possibility that it plays a role in binding and/or mediating the distribution of membrane glycoproteins on the platelet surface. This concept is supported by previous studies showing that (i) expression of Dp71 in the muscle of *mdx* transgenic mice restores normal levels of dystrophin-associated glycoproteins (21, 22), (ii) talin, a known component of the platelet membrane cytoskeleton, can interact with dystrophin, presumably through the C-terminal domains (49, 50), and (iii) the integrin $\alpha_5\beta_1$, the fibronectin receptor on platelets, transiently associates with dystrophin in developing cultured myotubes (51). Dp71Δ_{110} may also play a role in signal transduction by localizing signaling molecules such as phosphatidylinositol 3′-kinase and p21^{ras} GTPase-activating proteins at appropriate submembranous locations or by mediating integrin-induced signaling (43, 44). Recent studies have now established that Rap1b, a small GTPase known to promote integrin-dependent adhesion of cells, can augment agonist-induced ligand binding to $\alpha_{IIb}\beta_3$, possibly through its effects on the actin cytoskeleton (52). Given that Rap1b is highly expressed in platelets, is rapidly activated in response to agonists such as thrombin, and co-sediments with the actin cytoskeleton upon platelet activation, Dp71Δ_{110} could potentially interact with Rap1b or similar platelet proteins, thereby linking platelet signaling with cytoskeletal reorganization.

Like platelets, human brain also contains Dp71Δ_{110} transcripts that are alternatively spliced for exons 71–74 and 78 (14). Previous studies have shown that alternative splicing of exons 71–74 in either dystrophin or Dp71 abolishes the binding of α1-, β1-, and β2-syntrophin (46, 53, 54). Therefore, it is likely that the syntrophins identified in platelets do not associate with Dp71Δ_{110} but with utrophin. However, Dp71Δ_{110} could potentially interact with β-dystroglycan, utrophin, other unidentified members of the DAPC, or additional platelet proteins. Clearly, additional studies are required to better define components of the platelet membrane cytoskeleton that associate with Dp71Δ_{110}.

The observation that Dp71Δ_{110} containing the C-terminal sequence of muscle dystrophin is found exclusively in the high speed detergent-insoluble fraction, whereas equivalent amounts of Dp71Δ_{110} containing the alternative 31-amino acid C terminus are found in the high speed detergent-insoluble and high speed detergent-soluble fractions of resting platelets, suggests that alternative splicing of exon 78 may mediate differential protein interactions during thrombin-induced platelet activation. In support of this concept, we have shown that Dp71 isoforms encoding the alternative 31-amino acid C terminus (due to splicing of exon 78) associate with actin bundles in primary myogenic and C_2C_{12} cells (34). Whether this association with actin occurs in platelets and contributes to cytoskeletal reorganization remains to be determined. Alternative splicing may also indirectly affect differential protein interactions by mediating the phosphorylation of Dp71Δ_{110}. Previous studies have shown that the C-terminal domain of full-length dystrophin, as well as Dp71, can be phosphorylated by endogenous protein kinases (21, 55–57). Milner *et al.* (56) showed that two consensus sites for p34^{cdc2} protein kinase lie within the C-terminal region of dystrophin, with one being located in exon 78. In addition, several members of the Src family of tyrosine kinases are found in platelets, with pp60$^{c\text{-}src}$ and pp62$^{c\text{-}src}$ being associated with the membrane cytoskeleton (44). It has been suggested that Src kinases act by phosphorylating key signaling molecules, which in turn regulate signal transduction mechanisms in the platelet. Presently, it is not known which protein kinases, if any, phosphorylate Dp71Δ_{110} *in vivo*.

Reduced platelet adhesion and aggregation have been reported in some DMD patients (58, 59), which may explain the excessive blood loss during spinal surgery observed for some of these patients (60–62). The reduction in collagen adhesion in response to thrombin in platelets from mdx^{3cv} mice supports the concept that DMD is associated with a platelet function disorder and raises the possibility that Dp71Δ_{110} mediates thrombin-stimulated platelet adhesion. It is well established that thrombin induces platelet cytoskeletal reorganization, thereby leading to platelet adhesion and aggregation (25, 26, 43, 44). Given that Dp71Δ_{110} is a component of the platelet membrane cytoskeleton and is incorporated into integrin-rich cytoskeletal complexes following thrombin-induced platelet activation, it is possible that a loss of Dp71Δ_{110} disrupts its association with membrane components important for platelet cytoskeletal reorganization and/or adhesion in response to thrombin. In support of this concept, a marked reduction in the expression of the collagen binding receptor, GPIV (63, 64), has been observed in platelets from DMD patients (58). Additional studies will be necessary to determine whether GPIV or other platelet membrane components associate with Dp71Δ_{110} and if loss of Dp71Δ_{110} leads to a decrease in the level of these membrane components, as was observed for full-length muscle dystrophin (65).

Despite the excessive blood loss during spinal surgery, there is no obvious clinically defined bleeding tendency in DMD. Our findings that platelet adhesion to collagen in response to thrombin is reduced in mdx^{3cv} mice could possibly explain the increased perioperative bleeding observed in some DMD patients. However, because mdx^{3cv} platelets are still responsive to collagen, it is likely that the deficiency of Dp71Δ_{110} does not significantly compromise hemostasis under normal conditions. Analysis of platelets from DMD patients with various mutations, including C-terminal mutations (predicted to disrupt both dystrophin and Dp71 expression), may provide a better understanding of the normal function of Dp71Δ_{110} and its role in platelet function and hemostasis.

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