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 Dp71Δ110) in the membrane cytoskeleton of human platelets. Both Dp71Δ110 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, Dp71Δ110 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 Dp71Δ110 was localized to the submembranous cytoskeleton. In addition to Dp71Δ110, platelets contained several components of the dystrophin-associated protein complex, including β-dystroglycan and syntrophin. To better understand the potential function of Dp71Δ110, collagen adhesion assays were performed on platelets isolated from wild-type or Dp71-deficient (mdxnev) mice. Adhesion to collagen in response to thrombin was significantly decreased in platelets isolated from mdxnev mice, compared with wild-type platelets. Collectively, our results provide evidence that Dp71Δ110 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) 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 cytoplasmic 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 ~38 kDa (designated Dp71Δ110) in total protein lysates from adult human brain. The observation that Dp71Δ110 fails to react with a monoclonal antibody (MANEX374A) directed against epitopes within exons 73 and 74 provides further evidence that Dp71Δ110 is derived from Dp71 transcripts lacking exons 71–74.

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-
Dp71 Isoforms in the Platelet Membrane Cytoskeleton

**Table 1** Antibodies used in this study and their epitope location

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location of epitope</th>
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<tr>
<td>1461&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C-terminal 17 amino acids of muscle dystrophin and Dp71</td>
</tr>
<tr>
<td>462B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Alternative C-terminal 31 amino acids of muscle dystrophin and Dp71</td>
</tr>
<tr>
<td>MANDYS&lt;sup&gt;1m&lt;/sup&gt;</td>
<td>Central rod domain of muscle dystrophin</td>
</tr>
<tr>
<td>MANCH&lt;sup&gt;3m&lt;/sup&gt;</td>
<td>Central domain of utrophin</td>
</tr>
<tr>
<td>MANEXT7374&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Amino acids encoding exons 73 and 74 of dystrophin</td>
</tr>
<tr>
<td>NME&lt;sup&gt;1&lt;/sup&gt;-&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N-terminal 7 amino acids of Dp71</td>
</tr>
<tr>
<td>Dystrobrevin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Amino acids 249–403 of mouse α-dystrobrevin-1</td>
</tr>
<tr>
<td>γ-Sarcoglycan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Amino acids 167–178 of rabbit γ-sarcoglycan</td>
</tr>
<tr>
<td>β-Dystroglycan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C-terminal 15 amino acids of human β-dystroglycan</td>
</tr>
<tr>
<td>Syntrophin&lt;sup&gt;m&lt;/sup&gt;</td>
<td>Torpedo syntrophin</td>
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</tbody>
</table>

<sup>a</sup> Affinity-purified polyclonal antibody (p) and monoclonal antibody (m).

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 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-terminus/β-dystroglycan coding exons 73 and 74 of dystrophin (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 Coulter 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<sup>9</sup> platelets/ml in Tyrode buffer (138 mM NaCl, 1.85 mM KCl, 12 mM Na<sub>2</sub>CO<sub>3</sub>, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM calcium chloride, 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 µM benamidine, 2 µM 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 Transciptase-PCR Analysis—Total RNA extracted from fresh human platelets using the TRIzol reagent procedure (Invitrogen) was stored at −70 °C in diethyl pycrocarbonate-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′-TGCACTAGCT-GAAAAGCTGCC-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′-GCTTCCTGATGATCTCAGAGG-3′; position 11205–11223) and 2296 (5′-TCTGAAATCTATTCCT-GAAAAGCTGCC-3′; position 11259–11276). Random hexameric-primed first-strand cDNA served as template for PCR analysis using primers GAP1 (5′-CCACCATGGCGAATCCAGTTGCA-3′; sense) and GAP2 (5′-TCTAGCGGAGGTTCATGCG-3′; antisense) for glyceraldehyde-3-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<sub>2</sub>, 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 profile of 1 cycle 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 85 °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). 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. Gold particles were allowed to bind to the gold coverslips (Thermofax, Invitrogen) for 30 min at room temperature. After washing several times with PBS, followed by PBS containing 0.5% bovine serum albumin (BSA) and 0.1% glycine to quench residual aldehyde, coverslips were rinsed with PBS containing 0.5% BSA, and then incubated with the dytrophin-specific C-terminal antibody 1461 for 1 h at room temperature. 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...
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, 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 Δ110 containing the C-terminal sequence of muscle dystrophin. Dp71 Δ110 containing the alternative 31-amino acid C-terminal sequence due to the splicing of exon 78, kDa, molecular mass markers.

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, 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 Δ110 containing the C-terminal sequence of muscle dystrophin. Dp71 Δ110 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 (MANDSY1) 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. 1D). Consistent with our previous findings (27), utrophin was identified in platelets using a monoclonal antibody (MANCHO3) against the C-terminal domain of utrophin (Fig. 1E). 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 Δ110 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 Δ110 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-
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 Dp71 is alternatively spliced for exon 78.

**Identification of Dp71Δ110, Dystrophin, and/or Utrophin in the Megakaryocytic Dami Cell Line**—Because platelets are derived from megakaryocytes, we studied the expression of Dp71Δ110, 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. 3A, Dp71Δ110 containing the C-terminal sequence of muscle dystrophin was found in platelets and Dami cells. However, compared with platelets, Dp71Δ110 containing the alternative 31-amino acid C-terminal sequence was not detected (Fig. 3B). These findings suggest that, in Dami cells, Dp71Δ110 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 Dp71Δ110 transcripts containing exon 78 (data not shown). As shown previously in Fig. 1, there was no evidence of Dp71Δ110 in skeletal muscle (Fig. 3, A and B). Dami cells, like platelets, do not contain full-length dystrophin (Fig. 3C) but express utrophin (Fig. 3D).

**Association of Dp71Δ110 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 Dp71Δ110 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. 4A, 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). Dp71Δ110-containing the C-terminal sequence of muscle dystrophin was recovered exclusively in the detergent-insoluble fraction (Fig. 4A, lane 3). In contrast, equivalent amounts of Dp71Δ110 containing the alternative 31-amino acid C-terminal sequence were recovered in both the detergent-insoluble and -soluble fractions (Fig. 4B, 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. 2](image-url)  
**Alternative splicing of exon 78 in Dp71Δ110 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, 4X174 DNA marker.

![Fig. 3](image-url)  
**Detection of Dp71Δ110 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 Dp71Δ110 (A and B), dystrophin (C), or utrophin (D).

![Fig. 4](image-url)  
**Colocalization of Dp71Δ110 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 Dp71Δ110 (A and B) or utrophin (C).
The blots were immunostained for Dp71, utrophin, vinculin, or spectrin, as indicated. Pellets were solubilized in SDS-PAGE sample buffer, separated on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were immunostained for Dp71, utrophin, vinculin, or spectrin, as indicated.

**Fig. 5.** Redistribuition of Dp71 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 (1×10⁷ 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 Dp71, utrophin, vinculin, or spectrin, as indicated.

Redistribution of Dp71 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 α₅β₃ 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, Dp71 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 was also observed with utrophin. In addition, the rate at which Dp71 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 Dp71 and utrophin are part of the membrane cytoskeleton that is incorporated into integrin-rich cytoskeletal complexes upon thrombin-induced platelet activation.

**Fig. 6.** Intracellular localization of Dp71 in human platelets. Scanning electron micrographs of Dp71 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 IgG conjugated goat anti-rabbit IgG (A) or antibody 1461 (B), followed by goat anti-rabbit IgG conjugated 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 Dp71 in normal human platelets. Platelets were immunoperoxidase-stained with either control rabbit IgG (C) or IgG antibody 1461 (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 Dp71 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.

Intracellular Localization of Dp71 in Platelets—Scanning immunoelectron microscopy, performed on semi-permeabilized intact human platelets, revealed Dp71 close to the plasma membrane, with the majority localized to pseudopodia (Fig. 6B, arrowheads). No labeling of the plasma membrane was observed in the negative control (Fig. 6A). Transmission immunoelectron microscopy also revealed Dp71 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. 6E, arrow). In contrast, there was no significant immunoperoxidase staining of the plasma membrane in negative controls (Fig. 6C).

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, Dp71, and syntrophin, the redistribution of syntrophin was examined in activated platelets. As observed for utrophin and Dp71, syntrophin sedimented at low g-forces when normal platelets were activated.
Dp71 Isoforms in the Platelet Membrane Cytoskeleton

**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 x 10⁶ 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. 8A). 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, GPIIIb–IIIa, and pp60src (27, 44).

Platelets from patients with Glanzmann’s thrombasthenia are deficient in αIIbβ3 and do not undergo αIIbβ3-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. 8B), a result consistent for utrophin, GPIIIb–IIIa, and pp60src (27, 44). These findings indicate that syntrophin, like Dp71Δ110 and utrophin, is part of the platelet membrane cytoskeleton that is incorporated in integrin-rich cytoskeletal complexes as a result of integrin-mediated transmembrane signaling.

**Thrombin-stimulated Platelet Adhesion Is Decreased in mdx°cv Mice**—mdx°cv 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 Dp71Δ110 affects platelet function, platelet adhesion (measured as RFU/10⁷ platelets) to collagen-coated wells was compared in platelets from age-matched wild-type or mdx°cv mice. As shown in Fig. 9, no difference in adherence was observed between platelets from wild-type or mdx°cv mice when platelet inhibitors (PGE1, tephravine, and EDTA) were added (unstimulated, 255 ± 61 versus 256 ± 49 RFU/10⁷ platelets, respectively). Although platelets from wild-type or mdx°cv mice exhibited increased adherence to collagen-coated wells in the absence of platelet inhibitors, no significant difference was observed between wild-type or mdx°cv platelets (collagen, 127 ± 145 versus 1460 ± 114 RFU/10⁷ 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°cv mice (thrombin + collagen, 3494 ± 326 versus 2052 ± 162 RFU/10⁷ platelets, respectively). These findings indicate that platelet adhesion to collagen in response to thrombin is decreased in mdx°cv mice, raising the possibility that Dp71Δ110 plays a role in thrombin-stimulated platelet adhesion.

**DISCUSSION**

In the present study, we have identified and characterized 55- to 60-kDa isoforms of Dp71 (designated Dp71Δ110) 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 Dp71Δ110 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°cv mice, as compared with platelets from age-matched wild-type mice, suggests a role for Dp71Δ110 in thrombin-stimulated platelet adhesion.

Several lines of evidence indicate that Dp71Δ110 is a component of the platelet membrane cytoskeleton. First, Dp71Δ110...
was recovered in the high speed fraction from detergent-lysed platelets. Second, like the membrane cytoskeletal proteins vinculin and spectrin, Dp71Δ110 redistributed from the high speed detergent-insoluble fraction to the low speed detergent-insoluble fraction upon platelet stimulation by thrombin. Third, immunoelectron microscopy localized Dp71Δ110 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 Dp71Δ110 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, Dp71Δ110 would not be expected to act as a membrane stabilizer. However, the identification of Dp71Δ110 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 αvβ1, the fibronectin receptor on platelets, transiently associates with dystrophin in developing cultured myotubes (51). Dp71Δ110 may also play a role in signal transduction by localizing signaling molecules such as phosphatidylinositol 3'-kinase and p21ras 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 α1β3, 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, Dp71Δ110 could potentially interact with Rap1b or similar platelet proteins, thereby linking platelet signaling with cytoskeletal reorganization.

Like platelets, human brain also contains Dp71Δ110 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- and β1-syntrophin (46, 53, 54). Therefore, it is likely that the syntrophins identified in platelets do not associate with Dp71Δ110 but with utrophin. However, Dp71Δ110 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 Dp71Δ110.

The observation that Dp71Δ110 containing the C-terminal sequence of muscle dystrophin is found exclusively in the high speed detergent-insoluble fraction, whereas equivalent amounts of Dp71Δ110 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 C2C12 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 Dp71Δ110. 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 p34cdc2 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 pp60src and pp62−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 Dp71Δ110 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 mdx3cv mice supports the concept that DMD is associated with a platelet function disorder and raises the possibility that Dp71Δ110 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 Dp71Δ110 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 Dp71Δ110 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 Dp71Δ110 and if loss of Dp71Δ110 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 mdx3cv mice could possibly explain the increased perioperative bleeding observed in some DMD patients. However, because mdx3cv platelets are still responsive to collagen, it is likely that the deficiency of Dp71Δ110 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 Dp71Δ110 and its role in platelet function and hemostasis.

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