Utrophins compensate for Dp71 absence in mdx^{3cv} in adhered platelets

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Platelet adhesion is a critical step due to its hemostatic role in stopping bleeding after vascular damage. Short dystrophins are the most abundant *dmd* gene products in nonmuscle tissues, and in association with cytoskeleton proteins contribute to their intrinsic function; while utrophins are dystrophin-homologous related family proteins with structural and functional similarities. We previously demonstrated the presence of Dp71 isoforms, utrophins, and various dystrophin-associated proteins and their participation in cytoskeleton re-organization, filopodia and lamellipodia extension, and in centralizing cytoplasmic granules during the adhesion process of human platelets. To evaluate the morphologic changes and actin-based structures of mdx^{3cv} platelets during the adhesion process, we compared the topographic distribution of Dp71d/Dp71Δ_{110^m}/utrophins and dystrophin-associated protein in adhered platelets from dystrophic mdx^{3cv} mouse. By confocal microscopy, we showed that absence of Dp71 isoforms in platelets from this animal model disrupted dystrophin-associated protein expression and distribution without modifying the platelet morphology displayed during the glass-adhesion process. By immunoprecipitation assays, we proved that up-regulated utrophins were associated with dystrophin-associated proteins to conform the dystrophin-associated protein complex corresponding to utrophins, which might compensate for Dp71 absence in mdx^{3cv} platelets. *Blood Coagul Fibrinolysis* 19:39–47 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The platelet cytoskeleton is responsible for the maintenance and change of morphology, adhesion, aggregation, and contractility to sustain its hemostatic role. Re-organization of actin into functional networks is regulated by actin-binding proteins [1] that in turn have control of specific signaling pathways [2]. Upon stimulation with the subendothelial matrix exposed by blood vessel damage, platelets change shape, rounding and extruding dynamic filopodia, and the contractile ring appears around the granulomere. After platelets adhere and spread, lamellipodia, cytoplasmic and membrane protein recruitment to the attachment site occurs, which is enhanced via release of granule contents [3].

Duchenne muscular dystrophy is an X-linked genetic disease characterized by progressive muscle degeneration [4]. The *dmd* gene is transcribed from at least seven promoters encoding multiple tissue-specific proteins that differ in their N-terminal region [5]. Dystrophin is the largest 427 kDa protein, and other products are named in reference to their respective molecular weights, including Dp260, Dp140, Dp116, and Dp71 [6]. All dystrophin proteins have been detected in the nervous system [7]; however, Dp71 is the most abundant *dmd* gene product in nonmuscle tissues, in which Dp71 transcripts are alternatively spliced for exons 71–74 and/or 78, generating isoforms of 70–75 kDa and 55 kDa, respectively [8,9]. Dp71d is the isoform in which exon 78 is conserved, while Dp71f is the isoform in which it is absent, affecting the open reading frame with an insert of 31 amino acid residues (founder sequence) [8,10]. Additionally, protein products without exons 71–74 could be alternatively spliced for exon 78 or not, and are denominated Dp71Δ_{110^a} and Dp71Δ_{110^m}, respectively [9].

The utrophin family has very similar properties to the dystrophin family and is also transcribed from several promoters. Variable splicing patterns have been described for both families, these giving rise to several C-terminal protein products that enable both molecules to bind dystrophin-associated protein (DAP) – β-dystroglycan, dystrobrevins, and syntrophins [11] – conforming the respective dystrophin-associated protein complex

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(DAPC). Dystroglycan is now thought to be a structural molecule that participates in cell signaling, in cytoskeleton re-organization, and as a potential tumor suppressor [12]. Through the syntrophin PDZ domain, DAPC anchors some ion channels and signaling proteins in plasma membranes; moreover, it has been reported that neuronal nitric-oxide synthase is also associated with DAPC [13,14].

We recently characterized the expression pattern of Dp71 isoforms, utrophin gene products, and DAP (β-dystroglycan, α-syntrophins, α-dystrobrevins) and determined their association as Dp71d/Dp71Δ_{110^m} ~ DAPC and Up400/Up71~DAPC in platelet structures displayed during the adhesion process [15]. Additionally, we have shown the structural role of Dp71d/Dp71Δ_{110^m} ~ DAPC and the structural and signaling role of Up400/Up71~DAPC in the focal-stress-fiber complex in human platelets [16]. To elucidate feasible roles of proteins, several mouse models have been developed: the mdx^{3cv} strain has a mutation at exon 62 of the *dmd* gene that affects all Duchenne muscular dystrophy gene products; this model has been used to determine the role of Dp71 isoforms in the central nervous system (brain, retina) [17,18] and spermatozoa [19].

In platelets from mdx^{3cv} mice, we found decreased expression of Dp71d/Dp71Δ_{110^m} isoforms and DAP; nonetheless, no morphologic changes were evident during adhesion. Moreover, Up400 expression was up-regulated and was associated with DAP and actin, conforming the Up400~DAPC that might compensate for absence of Dp71 isoforms, aiding in the performance of its hemostatic role.

Materials and methods

All reagents were purchased from Sigma Chemical Co. (St Louis, Missouri, USA) unless otherwise indicated.

Antibodies

Dys2 and β-dystroglycan were purchased from Novocastra (Newcastle-on-Tyne, UK) and α-dystrobrevin was from Transduction Laboratories (Lexington, New York, USA), while α-syntrophin and actin antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). The antiutrophin K7 antibody was previously characterized [20].

Animals

C57BL/6 (Wt) and heterozygote mdx^{3cv} mutant mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and bred at an animal facility as described in [19].

Isolation of platelets from C57BL/6 and mdx^{3cv} mice

Mouse blood was collected by eye puncture from 12-week-old wild-type or mdx^{3cv} mice. Blood was immediately mixed with citrate anticoagulant at pH 6.5 (93 mmol/l sodium citrate, 70 mmol/l citric acid, and 140 mmol/l dextrose) at a ratio of nine parts of blood per one part of anticoagulant. Platelet-rich plasma was obtained as described in [21].

Platelet adhesion and fixation

Platelets were set on glass coverslips for 20 min, with removal of platelets not adhered through washing with Hank's balanced saline solution. For immunofluorescence, cells were fixed and permeabilized with a mixture of 2% *p*-formaldehyde, 0.05% glutaraldehyde, and 0.04% NP-40 in the cytoskeleton-stabilizing solution PHEM (100 mmol/l PIPES, 5.25 mmol/l HEPES, 10 mmol/l EGTA, 20 mmol/l $MgCl_2$) for 20 min.

Immunofluorescence staining

To visualize actin filaments, platelets were incubated with 0.1 μg/ml FITC phalloidin. The antibodies used were diluted in 0.1% bovine serum albumin in phosphate-buffered saline and incubated with platelets for 2 h with gentle agitation. Coverslips were washed with PHEM solution and incubated with secondary goat antirabbit antibodies conjugated to Alexa-Fluor-568 (Molecular Probes, Eugene, Oregon, USA) for 1 h and then washed several times and mounted in Vectashield (Vector Laboratories, Inc., Burlingame, California, USA). Slides were observed in an epifluorescence Leika confocal system TCS-SP2. Five optical sections were scanned at 0.18 μm using the laser confocal microscope dual-channel imaging system. Control samples without first antibody were observed for each immunostaining.

Western blot

Protein extracts of resting platelets containing sodium dodecyl sulfate and β-mercaptoethanol were boiled for 5 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Proteins were transferred to nitrocellulose membranes in a semi-dry system (Owl Separation Systems, Portsmouth, New Hampshire, USA). Protein was quantified using the DC kit (Bio-Rad, Hercules California, USA), and 80 μg protein were loaded onto the gels. Membranes were blocked in Tris-buffered saline (2 mmol/l Tris base, 500 mmol/l NaCl, pH 7.4) and 5% powdered nonfat milk. As controls of protein loading, actin and β-tubulin were detected. Blots were washed with Tris-buffered saline + 0.1% Tween 20 and were incubated overnight at 4°C with primary antibody, washed, and subsequently incubated with the secondary antibody conjugated to horseradish peroxidase for 1 h. Membranes were washed and detected by enhanced chemoluminescence (ECL Western Blot System; Amersham, Little Chalfont, UK) and were documented on X-omat film (Kodak; Rochester, New York, USA). Detected proteins were quantified by comparative scanning and analyzed with MCID imaging research version 7.0 evaluation software (Interfocus Imaging, Linton, UK). In all cases, wild-type and mdx^{3cv} samples were processed for

western blot or immunofluorescence staining simultaneously.

Immunoprecipitation assays

Wild-type and mdx^{3cv} resting platelets were lysed for 15 min at 4°C with an equal volume of 2 × lysis buffer (2 mmol/l EGTA, 100 mmol/l HEPES, 150 mmol/l NaCl, 2% NP-40, pH 7.4) containing a protease inhibitor cocktail. Lysates were incubated for 2 h at 4°C with K7 antibody for utrophin gene product precipitation and were subsequently incubated overnight with Rec Protein G-sepharose (Zymed, San Francisco, California, USA). Immunoprecipitates were recuperated by centrifugation and washed with NP-40-free lysis buffer, then resuspended in 2 × sample buffer (125 mmol/l Tris–HCl, 4% sodium dodecyl sulfate, 20% glycerol, 0.01 mg/ml β-mercaptoethanol, and bromophenol blue, pH 6.8) and boiled for 5 min. Immunoprecipitated proteins were analyzed by western blot as previously described. For identification of each revealed protein, total brain extract was detected simultaneously (data not shown).

Statistical analysis

Statistical analysis was performed using the Rank sum test, and *P* values less than 0.05 were considered significant.

Results

Distribution of actin filaments in mdx^{3cv}-adhered platelets is similar to that of wild-type platelets

Platelet activation induced by adhesion to the surface triggers a change from discoid to extended shape on the substrate and is the result of a rapid polymerization in parallel bundles and in cortical networks. Actin filaments are regulated and maintained by interactions with actin-binding proteins to perform four basic structures that contribute to platelet function: filopodia; lamellipodia; contractile ring, and stress fibers [22].

We recently characterized the expression and distribution pattern of Dp71 isoforms in human platelets, confirming their active participation in actin cytoskeleton re-organization from resting to adhered stage, intervening in shape changes, granule centralization, and aggregation [15]. To determine whether the Dp71d/Dp71Δ_{110m} pattern of expression is modified in platelets of mdx^{3cv} mice, protein extracts were analyzed by western blot and compared with samples from human platelets and wild-type mouse. We observed a different pattern expression of Dp71d between human and mice samples, because the bands observed showed a slight lower relative mass. In Fig. 1A, two bands of 55 and 76 kDa corresponding to Dp71Δ_{110m} and Dp71d were detected with Dys2 monoclonal antibody from wild-type whole-platelet extracts. Faint bands corresponding to the same proteins were observed in platelet extracts from mdx^{3cv} mice (Fig. 1A), confirming the genotype of this dystrophic animal model [23]. Actin detection was included as a loaded control (Fig. 1A′).

To determine whether diminution of Dp71d/Dp71Δ_{110m} isoforms expression could modify actin filament organization, adhered platelets from wild-type and deficient-dystrophin-expression mdx^{3cv} mouse were stained for Dp71d/Dp71Δ_{110m} and actin filaments and were analyzed by confocal microscopy.

Our results showed that actin filaments were concentrated at the filopodia, cytoplasm, and in some cases as dispersed aggregates in filopodial platelets from wild-type mice and from mdx^{3cv} mice. Moreover, in full-extended platelets, actin was concentrated at the lamellipodia zone, the contractile ring (arrow), and as discrete punctuate pattern at the plasma membrane of wild-type mouse (Fig. 1Ba, arrowhead).

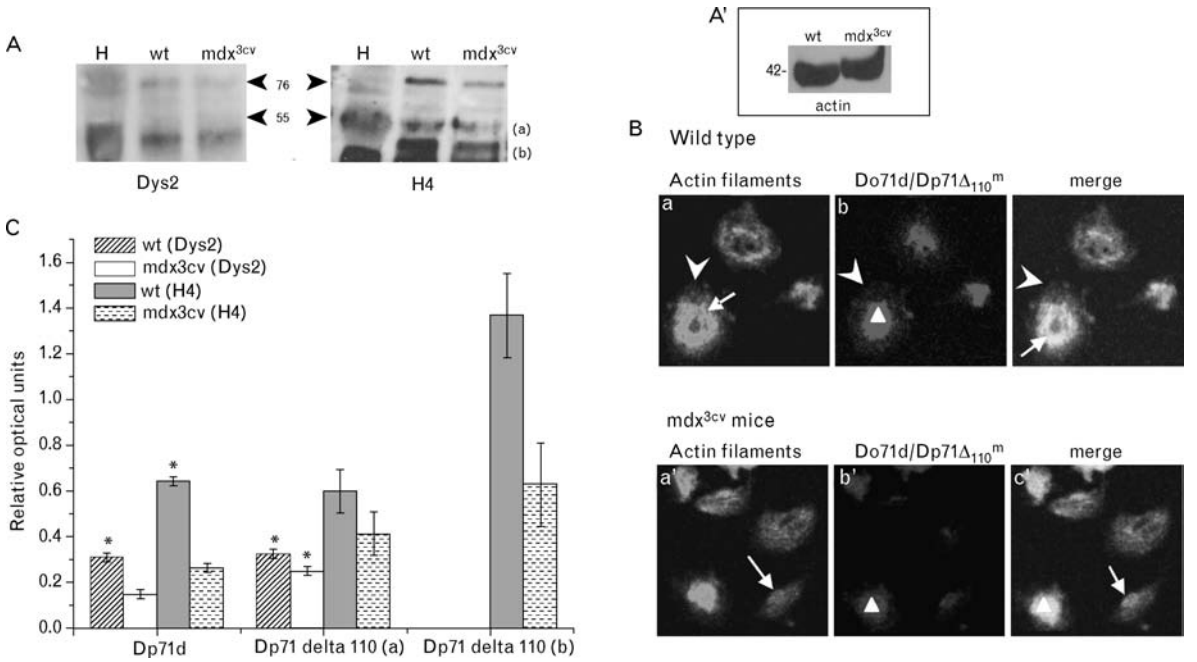
Dp71d/Dp71Δ_{110m} staining in wild-strain platelets was found concentrated at the granulomere zone (triangle), at the filopodia, and at a lower intensity at the cytoplasm and decorating the plasma membrane (Fig. 1Bb, arrowhead). In contrast, a dramatic decrease of immunofluorescence for Dp71d/Dp71Δ_{110m} was observed in platelets from mdx^{3cv} (Fig. 1Bb′), which enables a description of its distribution; however, residual staining was visualized at the granulomere (triangle).

Merging of the images corresponding to Dp71d/Dp71Δ_{110m} and filamentous actin of wild-type mice showed that co-localization occurred in the filopodia and in the granulomere zone of filopodial platelets, while in full-spread platelets co-localization was more concentrated at the contractile ring (arrow) and the cytoplasm, and some patches were observed at the plasma membrane (arrowhead) (Fig. 1Bc). In contrast, in mdx^{3cv} platelets the co-distribution of Dp71d/Dp71Δ_{110m} and actin filaments was scarce and was only observed at the granulomere (triangle) and contractile ring (arrow; Fig. 1Bc′). The levels of Dp71 isoforms determined with both antibodies (Dys2 and H4) in mdx^{3cv} platelets was quantified and graphed. Analysis corroborated diminution of Dp71 isoforms in extracts from mdx^{3cv} platelets (Fig. 1C).

Dystrophin-associated protein expression is altered in absence of Dp71

Previously, we corroborated the association of DAP (β-dystroglycan, α-syntrophin, and α-dystrobrevins) with Dp71d/Dp71Δ_{110m} and Up400/Up71, which conform Dp71d/Dp71Δ_{110m} ~ DAPC and Up400/Up71~DAPC, respectively, participating in adhesion, aggregation, and cytoplasmic granule centralization [15]. To determine whether DAP expression was modified in mdx^{3cv} platelets, β-dystroglycan, α-syntrophin, and α-dystrobrevin-1 and α-dystrobrevin-2 were determined by western blot assays in platelets from wild-type and mdx^{3cv} mice

Fig. 1



Comparative topographic distribution of actin cytoskeleton between adhered platelets from wild-type and dystrophic mice. (A) Presence of Dp71d/Dp71Δ_{110^m} in whole human platelet extracts from wild-type and mdx^{3cv} mice were determined by western blot. Protein bands of 76 and 56 kDa corresponding to Dp71d and Dp71Δ_{110^m} were observed in wild-type (wt) samples; remnants of the same proteins appeared as faint bands in mdx^{3cv} mice. The relative optical density of actin was compared to equilibrate each sample. (A′) Actin-loaded control. (B) wt and mdx^{3cv} mouse-adhered platelets were processed for double-labeling using FITC-phalloidin/a (a′) and Dys2 antibody followed by a secondary antibody, TRITC, to detect Dp71d/Dp71Δ_{110^m}, and were analyzed by confocal microscopy. Merging of the respective staining images is shown in (c, c′), respectively. Granulomere area (triangle), contractile ring (arrow), and plasma membrane patches (arrowhead). Scale bar = 2 μm. (C) Relative optical units obtained with MCID software for each Dp71disoform from wt (lined histograms) and mdx^{3cv} total extract platelets (white histograms). Results are the mean ± SD; $n = 3$. $^*P < 0.05$.

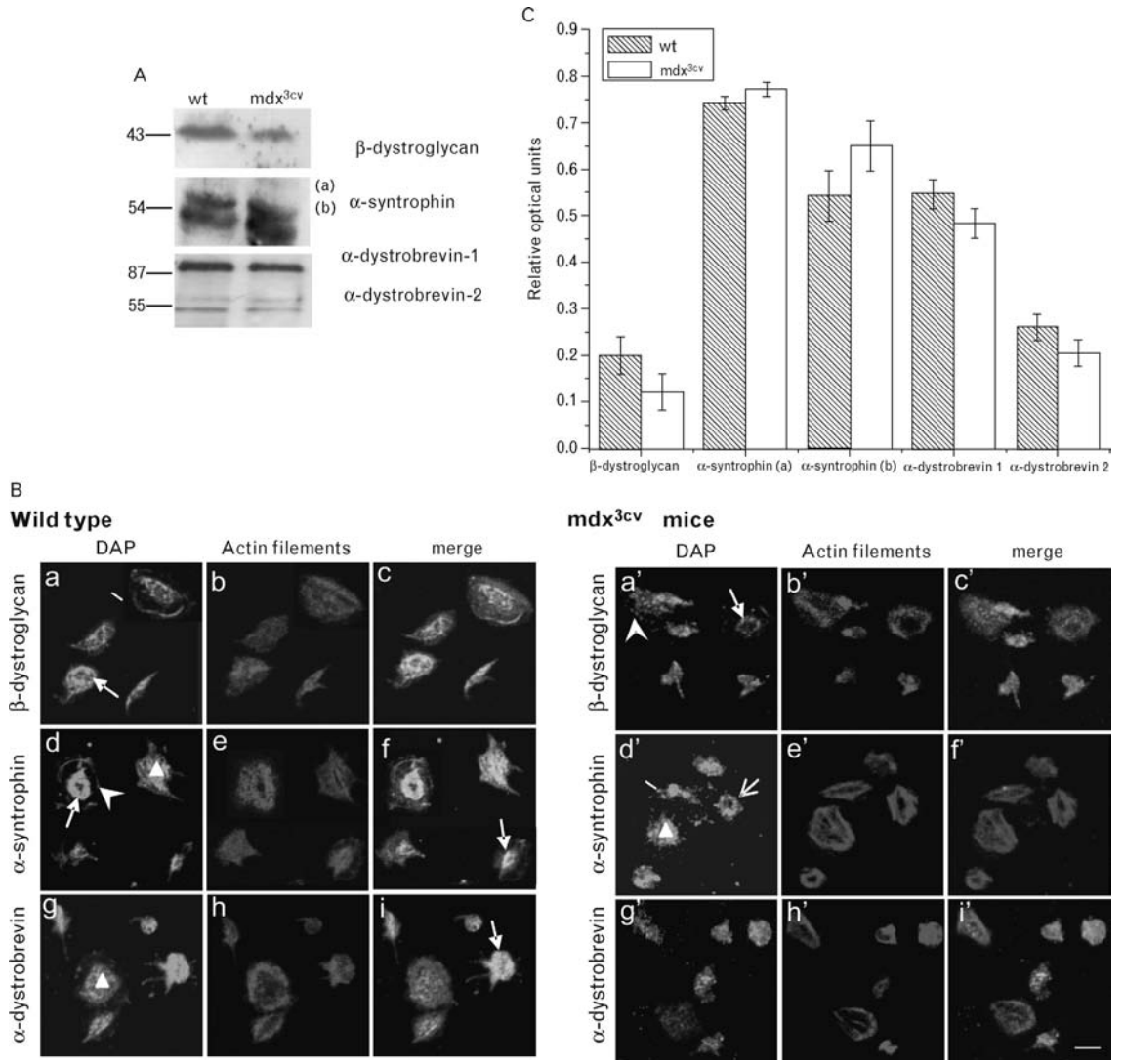
(Fig. 2A). Proteins were detected with specific antibodies and identified by their relative mass: β-dystroglycan, 43 kDa; α-syntrophin, 54 kDa; and α-dystrobrevin-1 and α-dystrobrevin-2, 84, and 55 kDa, respectively. Corresponding bands to these proteins were less intense in platelet extracts from mdx^{3cv} mouse than in extracts from wild-type mouse (Fig. 2A). The difference in DAP presence was variable, but the relative optical units showed the diminution of the expression of β-dystroglycan and α-dystrobrevin-1 and α-dystrobrevin-2, as well as the increase of the bands corresponding to α-syntrophin (Fig. 2C).

To investigate whether absence of Dp71 isoforms modifies the spatial distribution of DAP (β-dystroglycan, α-syntrophin, and α-dystrobrevins) in relation to actin filaments, confocal microscopy analysis was performed in adhered platelets from mdx^{3cv} platelets and compared with wild-type-mice platelets (Fig. 2B).

β-Dystroglycan in platelets from wild-type and mdx^{3cv} mice showed a similar distribution (Fig. 2Ba, Ba′). In filopodial stages from both strains, β-dystroglycan was observed as homogeneously dispersed staining at the cytoplasm and filopodia. In full-spread wild-type platelets, β-dystroglycan appeared outlining the plasma membrane (arrowhead), the contractile ring (arrow), and filopodia (Fig. 2Ba), while staining corresponding to platelets from mdx^{3cv} mice was diminished with a discontinuous pattern around the plasma membrane (arrowhead), contractile ring (arrow), and filopodia (Fig. 2Ba′). The actin filament distribution pattern from wild-type and mdx^{3cv} mice was similar to that described in Fig. 1 and will not be described further.

Merging of β-dystroglycan and filamentous actin in wild-type platelets showed scarce co-localization evidenced at the filopodia, contractile ring (arrow), and plasma membrane (Fig. 2Bc). In comparison, β-dystroglycan and filamentous actin co-localization was confined only to filopodia and to the contractile ring (arrow) of some platelets from mdx^{3cv} mice (Fig. 2Bc′). In wild-type and mdx^{3cv} filopodial platelets, α-syntrophin (Fig. 2Bd, Bd′) was dispersed as discrete aggregates at the filopodia and at the cytoplasm, whereas in well adhered platelets the label was observed at the contractile ring (arrow), granulomere (triangle), and as patches at the plasma membrane (arrowhead). The presence of α-syntrophin

Fig. 2



Comparative topographic distribution and expression pattern of dystrophin-associated proteins (DAP) in adhered platelets from wild-type and dystrophic mice. (A) Whole platelet extracts from wild-type and mdx^{3cv} mice were processed by western blot. Protein bands corresponding to β-dystroglycan (43 kDa), α-syntrophins (56 kDa), and α-dystrobrevin-1 and α-dystrobrevin-2 (84 and 55 kDa, respectively) were revealed. (B) Platelets from wild-type (wt) and mdx^{3cv} strains were double-stained with antibodies against β-dystroglycan (a, a′), α-syntrophins (d, d′), and α-dystrobrevins (g, g′) and revealed with a secondary antibody, FITC, and actin filaments using TRITC-phalloidin. Samples were analyzed by confocal microscope. Granulomere area (triangle), contractile ring (arrow), and plasma membrane patches (arrowhead). Scale bar = 2 μm. Results are the mean ± SD; $n = 3$. $^*P < 0.05$. (C) Relative optical units of DAP by western blot assays obtained with MCID software for each isoform from wt (lined histograms) and mdx^{3cv} total extract platelets (white histograms). Results are the mean ± SD; $n = 3$. $^*P < 0.05$.

was increased in mdx^{3cv} platelets and was localized in the granulomere (triangle) and contractile ring (arrow) of full-spread platelets. α-Syntrophin and filamentous actin merging showed co-localization at the contractile ring (arrow), and the filopodia in wild-type platelets (Fig. 2Bf).

α-Dystrobrevins were homogeneously distributed at the filopodia, granulomere, and as granular staining in wild-type platelet cytoplasm (Fig. 2Bg); however, low α-dystrobrevin staining distributed in the cytoplasm was observed in mdx^{3cv} platelets (Fig. 2Bg′). Scarce α-dystrobrevins and actin filament co-localization was observed at the corresponding images (Fig. 2Bi, Bi′) for wild-type and mdx^{3cv} samples. In wild-type platelets, co-localization of these proteins was more evident in filopodial stages. In platelets from mdx^{3cv} mouse, no overlapping was detected (Fig. 2Bi′). The corresponding quantitative analysis of these images corroborated DAP data observed by western blot detection, which is the down-regulation of β-dystroglycan and α-dystrobrevin-1 and α-dystrobrevin-2 and the up-regulation of α-syntrophin (Fig. 2C).

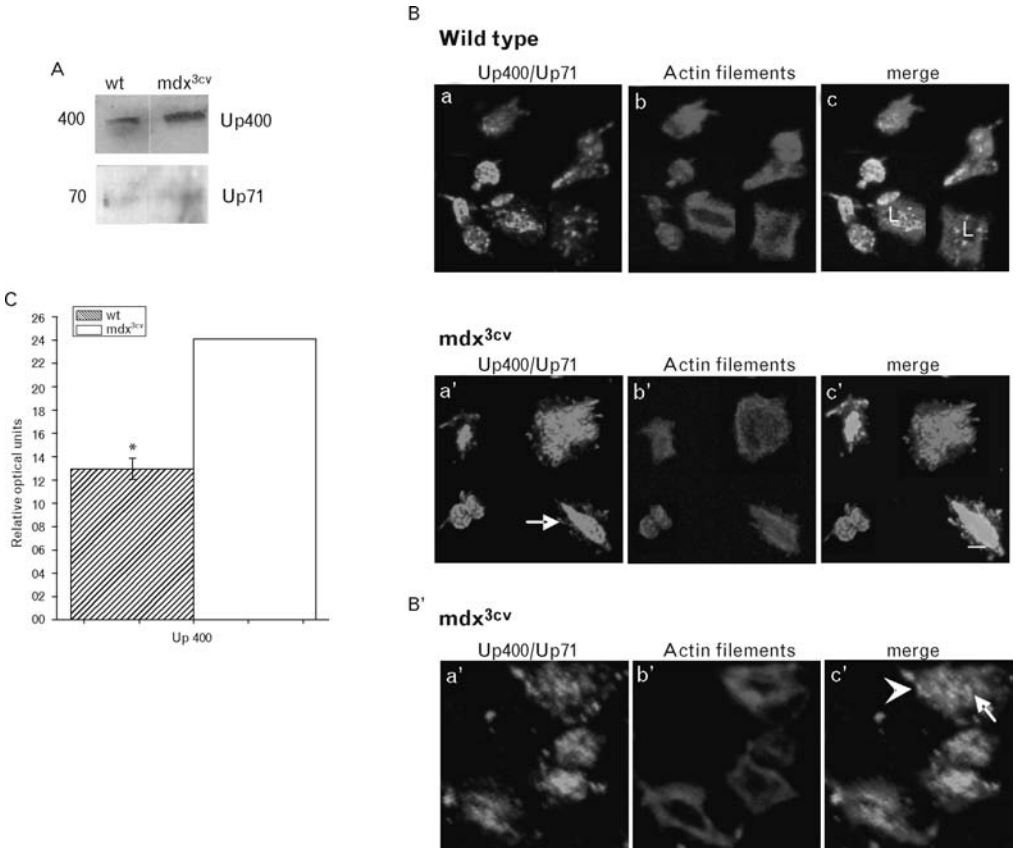
Up400/Up71 expression is increased in mdx^{3cv} platelets and their distribution is favored toward the plasma membrane

Previous studies have demonstrated that, in Dp71-deficient mdx^{3cv} brain, utrophin isoforms were up-regulated [24]; in order to determine whether utrophins were overexpressed in the absence of Dp71, we compared the Up400/Up71 presence and distribution in relation to actin filaments in platelets isolated from mdx^{3cv} mice.

In Fig. 3A, expression of Up400/Up71 by western blot analysis in wild-type and in mdx^{3cv} platelet extracts is shown. It is clear that Up400 was up-regulated in the absence of Dp71 isoforms, and quantitative analyses corroborated this observation (Fig. 3C).

The immunofluorescence staining distribution of Up400/Up71 and actin is observed in Fig. 3B. In filopodial wild-type platelets, Up400/Up71 were found in the filopodia and in the plasma membrane with some submembrane aggregates (Fig. 3Ba), while in full-spread platelets a punctuate pattern was present at the cytoplasm (Fig. 3Ba). In filopodial platelets, actin filaments and Up400/Up71 coincided at the plasma membrane and filopodia; nevertheless, no co-distribution was observed in lamellipodial platelets (Fig. 3Bc). It was notorious that a very intense label of Up400/Up71 was present at the filopodia, cytoplasm, and plasma membrane of filopodial and full-spread platelets from the mdx^{3cv} mouse (Fig. 3Ba′). It is likely that Up400/Up71 over-staining prevented feasible co-localization with actin filaments (Fig. 3Bc′). We therefore modified the staining conditions (the primary antibody was diluted twice) (Fig. 3B′). Co-distribution of Up400/Up71 and actin filaments was evident in mdx^{3cv} platelets in the plasma membrane (triangle), filopodia, and contractile ring

Fig. 3



Utrophins are overexpressed in mdx^{3cv}-adhered platelets. (A) Utrophin pattern expression in wild-type (wt) and mdx^{3cv} platelets was evaluated by western blot assays. Protein bands corresponding to Up400 (400 kDa) and Up71 (76 kDa) were comparatively revealed using K7 antibody in wt and mdx^{3cv} samples. (B) wt and mdx^{3cv} mice-adhered platelets were comparatively co-stained for Up400/Up71 using the K7 antibody and a secondary antibody, FITC, and TRITC-phalloidin. The respective images were analyzed by confocal microscope. Filopodia (arrow), and plasma membrane patches (arrowhead). Scale bar = 2 μm. (B′) wt and mdx^{3cv} mice-adhered-platelets were co-stained for Up400/Up71 using the twice-diluted K7 antibody and a secondary antibody, FITC (Up400/Up71) and TRITC-phalloidin. The respective images were analyzed by confocal microscope. L, filopodial platelets; plasma membrane patches (arrowhead). Scale bar = 2 μm. (C) Relative optical units of Up400 by western blot assays were obtained with MCID software for each isoform from wt (lined histograms) and mdx^{3cv} total extract platelets (white histograms). Results are the mean ± SD; $n = 3$. $^*P < 0.05$.

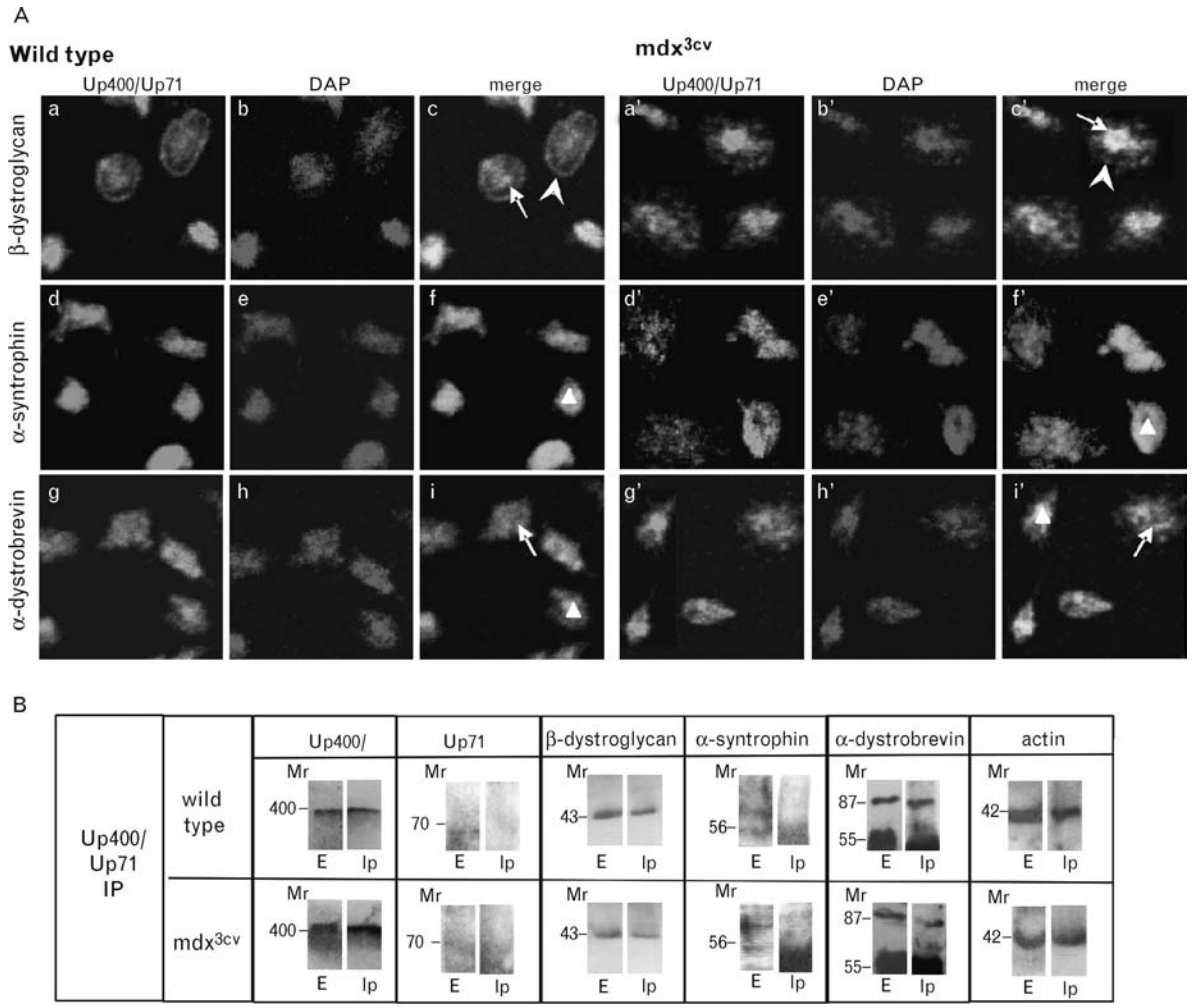
(arrow); this pattern was similar to that observed for human platelets [15].

Up400/Up71 is associated with dystrophin-associated protein in the absence of Dp71 isoforms

To investigate the feasible compensatory role of Up400/Up71 in the absence of Dp71 isoforms, we performed double immunofluorescence assays using FITC secondary antibody-labeled K7 polyclonal antibody and TRITC secondary antibody, detecting each of the specific anti-DAP antibodies. Since the utrophin and DAP distribution have been previously analyzed (Fig. 3), we describe only the corresponding merging herein. β-Dystroglycan and utrophins were co-distributed at the granulomere, contractile ring (arrow), and plasma membrane patches (arrowhead) in both samples (Fig. 4Ac, Ac′); this was more evident for mdx^{3cv} platelets (Fig. 4Ac′). α-Syntrophin and Up400/Up71 were similarly co-localized in mdx^{3cv} and wild-type platelets, and were concentrated at the granulomere (triangle) and plasma membrane (Fig. 4Af, Af′). Co-staining of α-dystrobrevins and Up400/Up71 was more evident for mdx^{3cv} than for wild-type platelets, and was observed in the granulomere (triangle), plasma membrane, and filopodia (Fig. 4Ai′).

To characterize Up400/Up71∼DAPC, immunoprecipitation assays were performed with K7 polyclonal antibody against Up400/Up71 (Fig. 4B) in platelets from mdx^{3cv} compared with the wild-type strain. Total extracts and co-immunoprecipitated proteins were analyzed by

Fig. 4



Co-distribution and co-precipitation of Up400/Up71, dystrophin-associated proteins (DAP), and actin in mdx^{3cv} platelets. (A) mdx^{3cv}-adhered and wild-type (wt)-adhered platelets were co-stained for Up400/Up71 using the K7 antibody revealed with FITC-conjugated secondary antibody and various specific DAP antibodies (identified on the left side of each row of images) revealed with TRITC-conjugated secondary antibody. The respective images were analyzed by confocal microscope. Contractile ring (arrow), granulomere (triangle), and plasma membrane patches (arrowhead). Scale bar = 2 μm. (B) Immunoprecipitations were performed with K7 polyclonal antibody (Ip) in extracts of mdx^{3cv} and wt platelets. Proteins from total extracts (E) and immunoprecipitates (Ip) were analyzed by immunoblot using antibodies against Up400/Up71, β-dystroglycan, α-syntrophins, α-dystrobrevins, and actin. Proteins were identified as in Fig. 2B by their respective relative mass (Mr).

immunoblots using antibodies against Up400/Up71, β-dystroglycan, α-syntrophin, α-dystrobrevins, and actin.

Up400 pulled down with β-dystroglycan, α-dystrobrevin-1 and α-dystrobrevin-2, and actin in precipitates from mdx^{3cv} and wild-type platelets; however, α-syntrophin could not be well visualized due to the interference of IgG present. It was notorious that Up400 was more concentrated in mdx^{3cv} platelets in relation to wild-type samples, confirming its over-expression. Our results showed that these DAP remnants in mdx^{3cv} platelets were associated with Up400/Up71 in dystrophic platelets, and that this DAPC was associated with actin.

Discussion

We previously demonstrated the presence of Dp71 isoforms (Dp71d, $Dp71\Delta_{110^m}$, $Dp71\Delta_{110^a}$, and Dp71f) and utrophin gene products (Up400, Up71) in human platelets, which were associated with DAP to form Dp71d/$Dp71\Delta_{110^m}$ ~ DAPC and Up400/Up71~DAPC during the adhesion process [15]. To elucidate the feasible role of these complexes, we compared the DAPC component distribution in adhered platelets from mdx^{3cv} mice with that of wild-type mice. In this study, we showed that the Dp71d absence in mdx^{3cv} mouse was manifested by a decrease in the level of β-dystroglycan and, to a lesser degree, α-dystrobrevins; an increase in the level of α-syntrophin; overexpression and re-distribution of Up400; and an association of Up400/Up71 with DAP conforming the Up400~DAPC.

The adhesion process involves several steps including platelet receptor activation, which triggers signaling into the cell, recruiting cytoplasm and membrane proteins to the attachment site, and actin filament re-organization into higher-order networks. Actin filaments and other cytoskeletal proteins are arranged in characteristic patterns, localizing at specific platelet structures such as the filopodia, lamellipodia, contractile ring, and stress fibers, and mediating spreading on the substrate, granule secretion, and platelet contraction [22].

Western blot and immunofluorescence data showed decreased expression and suggest a differential distribution pattern of Dp71d/$Dp71\Delta_{110^m}$, β-dystroglycan, and α-dystrobrevins, as well as increased expression of α-syntrophins in platelets from mdx^{3cv} mice in accordance with previous reports demonstrating that some DAPC members were altered by an absence of Dp71 in non-muscle cells [17–19]; thus, the DAP expression pattern in the mdx^{3cv} animal model is cell specific [19]. It has been reported that Dp71 and β-dystroglycan link actin and are needed for actin–cytoskeleton anchorage to the plasma membrane through the DAPC [15,25,26]. Platelets are heterogeneous fragment cells that adhere asynchronously, so morphologic differences are not easy to evaluate. Our results, however, suggested that neither mutation of Dp71 nor decreased β-dystroglycan expression modified the actin-based structures triggered during the glass-adhered platelet [22]. As actin filaments, Dp71 isoforms, and the DAP distribution in wild-type mouse-adhered platelets were similar to those observed in human-adhered platelets, we inferred that these were associated with Dp71d/$Dp71\Delta_{110^m}$ conforming Dp71d/$Dp71\Delta_{110^m}$ ~ DAPC.

Owing to the fact that utrophin is a dystrophin-related protein with broad tissue distribution that performs similar cellular functions in association with DAP [7] and that it is up-regulated during absence of dystrophin [18,27,28], we have proposed a dual role (structural and signaling) for Up400/Up71~DAPC [16]. Their overexpression and re-distribution in mdx^{3cv} platelets therefore strongly suggested that they might compensate for Dp71 absence, as observed in other cellular systems [19,29]. It is notorious that up-regulation of utrophin preserved the platelet hemostatic function.

Taken together, our results indicate that, notwithstanding Dp71 mutation and decreased DAP expression, the adhered-platelet morphology in mdx^{3cv} mice was not altered. Up400 is clearly overexpressed, and therefore it might be a good candidate to replace Dp71d/$Dp71\Delta_{110^m}$ ~ DAPC disruption. In the dystrophic forebrain, β-dystroglycan expression is not drastically affected, possibly due to the up-regulation of utrophin isoforms that partially compensate for the deficiency in brain dystrophins [24]. Furthermore, co-localization and immunoprecipitation data confirmed that utrophins formed actin cytoskeleton-bound Up400/Up71~DAPC, which might possess an anchorage and structural role during the adhesion process, contributing to normal clot formation.

The bleeding tendency in patients with Duchenne muscular dystrophy during invasive surgical procedures [30,31] has not been attributed to blood coagulation abnormalities or deficiencies; nonetheless, these have been associated with impaired vessel reactivity [32]. In conclusion, our data reinforce the proposal of utrophin replacement for Duchenne muscular dystrophy therapy [33].

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