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Experimental Hematology

Experimental Hematology 2010;38:618–628

Distribution of dystrophin- and utrophin-associated protein complexes during activation of human neutrophils

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(Received 23 October 2009; revised 12 March 2010; accepted 22 April 2010)

Objective. **Dystrophins, utrophins, and their associated proteins are involved in structural and signaling roles in nonmuscle tissues; however, description of these proteins in neutrophils remained unexplored. Therefore we characterize the pattern expression, and the cellular distribution of dystrophin and utrophin gene products and dystrophin-associated proteins (i.e., β-dystroglycan, α-syntrophin, and α-dystrobrevins) in relation to actin filaments in resting and activated with formyl-methionyl-leucyl-phenylalanine human neutrophils.**

Materials and Methods. **Resting and fMLP-activated human neutrophils were analyzed by immunoblot and by confocal microscopy analysis. Immunoprecipitation assays were performed to corroborate the presence of protein complexes.**

Results. **Immunoprecipitation assays and confocal analysis demonstrated the presence of two dystrophin-associated protein complexes in resting and activated neutrophils: the former formed by Dp71d/Dp71Δ_{110}^{m} and dystrophin-associated proteins (β-dystroglycan, α-syntrophin, α-dystrobrevin-1, and -2), while the latter contains Up400, instead of Dp71d/Dp71Δ_{110}^{m}, as a central component of the dystrophin-associated protein complexes (DAPC). Confocal analysis also showed the subcellular redistribution of Dp71d/Dp71Δ_{110}^{m} ~ DAPC and Up400 ~ DAPC in F-actin − based structures displayed during activation process with fMLP.**

Conclusions. **Our study showed the existence of two protein complexes formed by Dp71d/Dp71Δ_{110}^{m} or Up400 associated with DAPs in resting and fMLP-treated human polymorphonuclears. The interaction of these complexes with the actin cytoskeleton is indicative of their dynamic participation in the chemotaxis process.** **© 2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Polymorphonuclear neutrophilic granulocytes (also known as PMNs or neutrophils) are generated continuously from hematopoietic stem cells in the bone marrow. The differentiation of promyelocytes via myelocytes and metamyelocytes into bone marrow neutrophils is directed by a transcriptional program that promotes cell-cycle exit and acquisition of highly specific functions that are crucial for the immune response [1]. Neutrophils kill and degrade microorganisms via the production and finely tuned release of granule proteins synthesized during neutrophil differentiation and stored in mobilizable organelles dispersed throughout the cytoplasm in unstimulated neutrophils; but upon activation, the granules fuse with the plasma membrane, thereby discharging their content from the cell [2–4]. Activated neutrophils also synthesize chemokines and cytokines, which recruit and regulate the inflammatory response of other effector cells including macrophages, T cells, and neutrophils themselves [5].

The microfilamentous cytoskeleton is a highly dynamic network made of actin and numerous actin-associated proteins, which participates in the regulation of the processes previously mentioned. Neutrophils double their F-actin content within seconds after stimulation with chemotaxis agonists, and a striking reorganization of the actin cytoskeleton has been observed with areas of intensive actin turnover, localized in the lamellipodia during migration [6,7].

Dystrophin is a cytoskeleton protein essential for skeletal function, as indicated by the lethal pathophysiology associated with its deficiency, namely, Duchenne muscular dystrophy

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Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exphem.2010.04.010.

0301-472X/$ - see front matter.
doi: 10.1016/j.exphem.2010.04.010

(DMD). Dystrophin confers resistance on the sarcolemma against the stress of contraction-relaxation cycles by interacting with cytoskeletal and membrane partners [8–10]. Alternative usage of internal promoters of the DMD gene produces different dystrophin isoforms; full-length dystrophin isoforms (Dp427) are under the control of three independently regulated promoters referred to as brain, muscle, and Purkinje, while four additional promoters give rise to truncated C-terminal isoforms, namely Dp260 (260 kDa), Dp140 (140 kDa), Dp116 (116 kDa), and Dp71 (71 kDa) [11–14].

A homologue protein family of dystrophin encoded by an alternative gene at chromosome 6 comprises the utrophin family, which is composed of full-size utrophin (Up400), Up140 (140 kDa), and Up71 (70 kDa) [15,16] share similar binding domains for dystrophin-associated glycoproteins and actin filaments [17–19].

Dystrophin can be associated with the plasma membrane of cardiac and skeletal muscle interacting with the integral membrane protein dystroglycan, which in turn binds to laminin on the extracellular face. The dystrophin−dystroglycan complex interacts with integral (sarcoglycans) and peripheral (syntrophin and dystrobrevin) membrane proteins to form the dystrophin glycoprotein complex [20]. This protein complex interacts with F-actin to establish a link between the extracellular matrix and the internal cytoskeletal network [21–23]. Utrophins share with dystrophin the protein-binding domains for dystrophin-associated glycoproteins and actin filaments [17–19].

Dp71 is the major dystrophin expressed in nonmuscle cells, such as neurons [11], glia [24], spermatozoa [25], and platelets [26,27]. Dp71 transcripts are alternatively spliced in exons 71−74 and 78 to produce multiple products of 70−78 kDa. For instance, the Dp71d isoform messenger RNA preserves exon 78, while those of $Dp71\Delta_{110}{}^{a}$ and $Dp71\Delta_{110}{}^{m}$ suffer alternative splicing at exons 71–74 and 78 or at exons 71–74, respectively [27,28].

Presence of the dystrophin-associated protein complexes (DAPC) has been described in different tissues, participating in cell adhesion, signal transduction, and plasma membrane stability. Taking into account the diverse functionality of the DAPC, we have begun a long-term study aimed at defining the function of the DAPC in blood tissue. Previously, we revealed the presence of DAPC corresponding to Dp71 isoforms (Dp71/$Dp71\Delta^{110}{}_{m}$∼DAPC) and utrophin gene products (Up400/Up71∼DAPC) in platelets, and demonstrated its central participation in the remodeling of cytoskeleton during the adhesion process [29]. Hence, in the present study, these dystrophin-associated complexes have been investigated in resting and activated neutrophils.

Materials and methods

Antibodies

The monoclonal and polyclonal antibodies used in this study are listed in Table 1.

Neutrophil isolation

Neutrophils were isolated from peripheral blood anticoagulated with ethylenediamine tetraacetic acid (final concentration, 4 mM) obtained from voluntary blood donors. A density separation medium was prepared by careful layering of one part of Lymphoprep (Griner Bio-One, Monroe, NC, USA) over four parts of Polymorphprep (Griner Bio-One, Monroe, NC, USA). Whole blood was layered on an equal volume of separation liquid and the mixture was centrifuged in a swing-out rotor for 40 minutes (480*g*, room temperature), yielding an upper band containing mononuclear cells, a middle band containing neutrophils, and a pellet of red blood cells. Neutrophils were harvested and the separation liquid was removed by dilution in phosphate-buffered saline (PBS) and another centrifugation for 10 minutes at 480*g* [30]. A brief (10-second) hypotonic lysis of contaminating erythrocytes was performed by suspending the cells in 3 mL of 1 mM PBS. Isotonicity was then restored by adding 7 mL 1.3% NaCl to 1 mM PBS. After an additional washing in PBS containing 0.2% bovine serum albumin, the isolated neutrophils were resuspended in the same buffer at the desired concentration.

Table 1. Characteristics of the different antibodies used in this study

Antibody	Position of antigen	Specificity	Reference/resource
H4 pAb	C-T last 11 aa	Dp427, Dp260, Dp140, Dp116, Dp71	[50]
Dys2 mAb	C-T last 17 aa	Dp427, Dp260, Dp140, Dp116, Dp71	Novocastra, Newcastle, UK
Utrophin 3B6 mAb	Peptide 261-371 human utrophin	Up400	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
K7 pAb	C-T last 15 aa	Up400, Up140,Up71	[50]
β-Dystroglycan L-20 pAb	Peptide C-T	β-Dystroglycan	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
α-Syntrophin pAb	Peptide C-T	α-Syntrophin	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
α-Dystrobrevin pAb	C-T last 19 aa	α-Dystrobrevin	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
α-Actin mAb	C-T aa human origin	Actin	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

mAb = monoclonal antibody; pAb = polyclonal antibody.

The resulting cell population contained 95% to 97% neutrophils, as verified by fluorescence-activated cell sorter analysis (data not shown).

Coating of coverslips

Glass coverslips were washed with methanol and overlaid with 0.25 mL poly-L-lysine (0.2 mg/mL in water). After evaporation of fluid at 50° to 65°C, the poly-L-lysine–coated coverslips were washed twice with distilled water.

Cell activation and immunofluorescence staining

Neutrophils were seeded on poly-L-lysine–coated coverslips and after 20 minutes were stimulated with 100 nM formyl-methionyl-leucyl-phenylalanine for 10 minutes and fixed for 20 minutes with 2% paraformaldehyde/PBS and 0.04% NP40 in the cytoskeleton stabilizing solution PHEM (100 mM PIPES, 5.25 mM HEPES, 10 mM ethylene glycol tetraacetic acid, 20 mM $MgCl_2$, pH 6.9; Sigma Chemical Co., St Louis, MO, USA). Resting and activated neutrophils were first incubated for 1 hour with 0.1 μg/mL fluorescein isothiocyanate citrate (FITC) phalloidin (Sigma Chemical Co.) to label actin filaments, and then incubated for 2 hours with the appropriate primary antibodies (Table 1) diluted in 0.1% fetal bovine serum in PBS. Cells were washed with PBS and incubated for 1 hour with the secondary anti-mouse, anti-rabbit, or anti-goat antibodies conjugated to Alexa-Fluor-568 or Alexa-Fluor-488 (Molecular Probes, Eugene, OR, USA). For counterstaining, nuclei were dyed with 4′,6-diamidino-2-phenylindole (Sigma Chemical Co.), washed, and mounted in Vectashield media (Vector Laboratories, Inc., Burlingame, CA, USA). Slides were observed using a Leica confocal instrument model TCS-SP5 Mo, and images were taken at 63× zoom 3× at 1024 × 1024 pixels with an HCX PL APO 63×/1.40–0.60 DIL CS oil immersion. Optical sections [z] were captured at 0.18 μm. Negative controls included cells incubated with an irrelevant polyclonal antibody or cells just exposed to the fluorescent-secondary antibodies. None of these controls showed any conspicuous signal.

Western blotting analysis

Resting and fMLP-activated PMN were resuspended in 50 mM Tris-HCl (pH 8) containing complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Homogenates were sonicated and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). A portion (50 μg) of protein extract was mixed with an equal volume of 2× sample buffer (50 mM Tris–HCl [pH 6.8], 15% [w/v] sodium dodecyl sulfate, 5% [v/v], β-mercaptoethanol, 20% [v/v] glycerol, 0.1% [w/v] bromophenol blue) and boiled for 3 minutes. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% [w/v]) and electrotransferred onto nitrocellulose membrane, using a semi-dry system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Membranes were incubated with the appropriate primary antibodies, and then with horseradish peroxidase–conjugated secondary antibodies, visualized using an enhanced chemoluminescence Western blotting analysis system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and documented using X-omat film (Kodak, Rochester, NY, USA). Negative controls consisted of transferred strips incubated only with horseradish peroxidase–conjugated secondary antibodies.

Immunoprecipitation assays

Resting and activated PMN were resuspended in 50 mM Tris-HCl (pH 8) containing complete Protease Inhibitor Cocktail (Roche). Homogenates were sonicated with an equal volume of 2× RIPA buffer (10 mM Tris-HCl [pH 7.4], 1 mM ethylene glycol tetraacetic acid, 158 mM NaCl, 10 mM Na_3MoO_4, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine tetraacetic acid, 2 mMNa_3VO_4). After determination of protein concentration by the Bradford method, cell lysates were added to an equal volume of ice-cold RIPA buffer containing 1% (w/v) Triton X-100, and 0.1% (w/v) sodium dodecyl sulfate. Lysates were incubated for 2 hours at 4°C with the appropriate immunoprecipitating antibodies (Table 1) and subsequently incubated overnight with Protein G plus Sepharose (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were separated by centrifugation, washed with lysis buffer, resuspended in 2× sample buffer, and then boiled for 5 minutes. Immunoprecipitated proteins were analyzed by Western blot, as described previously.

Results

Dp71d and Dp71Δ_{110}^{m} short dystrophin isoforms are part of the human neutrophil cytoskeleton and codistribute with the actin cytoskeleton upon activation

To identify the expression pattern of Dp71 isoforms in neutrophils or PMN under resting and activated conditions, we essayed Western blot reactions using the anti-dystrophin antibody H4 directed against epitopes localized in the dystrophin C-terminal region (Table 1). Because brain expresses all members of the dystrophin family, human brain extract was used as positive control.

The anti-dystrophin antibody H4 revealed the presence of two bands comigrating to those found in the brain extract, the first with a relative molecular weight of 55 kDa that may correspond with Dp71Δ_{110}^{m}, a Dp71 isoform generated by alternative splicing of exons 71–74, and the second, with a predicted molecular weight of 76 kDa that must correspond with Dp71d. It is important to note that these results showed the presence of Dp71d and Dp71Δ_{110}^{m} in activated and resting PMN as the main dystrophin gene products. The Dp140 was evidently observed for brain samples but faint for PMN (Fig. 1A).

To evaluate the subcellular distribution of Dp71 isoforms, we analyzed double immunofluorescence reactions of resting and activated PMN by confocal microscope. Because Dys2 antibody recognizes the C-terminal region of dystrophin, the Dp71Δ_{110}^{m} and Dp71d distribution observed are referred to as Dp71Δ_{110}^{m}/Dp71d (Fig. 1B). In resting PMN, Dp71Δ_{110}^{m}/Dp71d was found at the plasma membrane and at the cytoplasm surrounding the nuclei. F-actin was observed around the plasma membrane and beneath it, freeing the space corresponding to the nuclei of resting PMN (Fig. 1B). The Dp71Δ_{110}^{m}/Dp71d distribution pattern was similar to that of actin, and a clear colocalization was observed at the plasma membrane and scarcely around the nuclei.

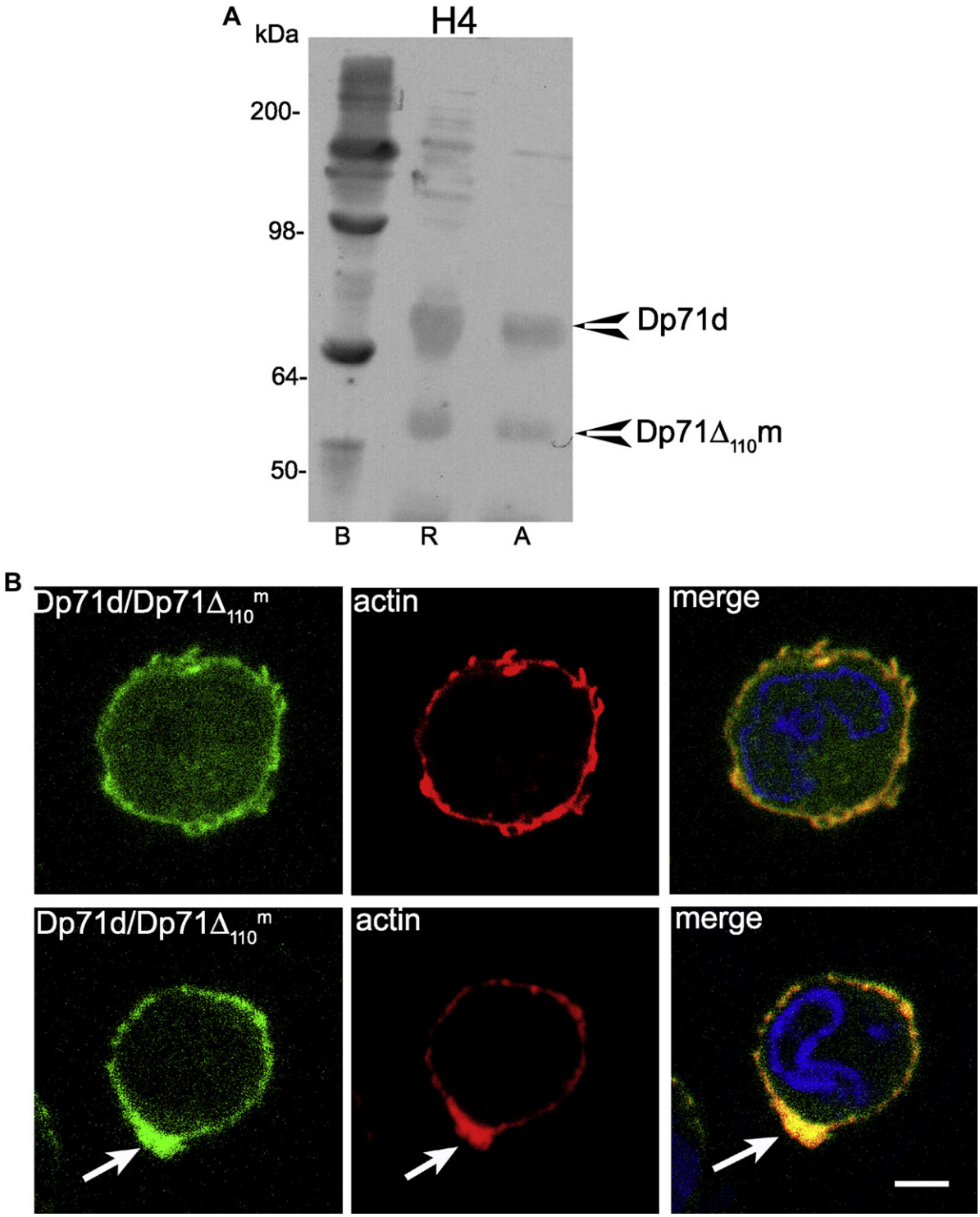


Figure 1. Detection and topographical distribution of short dystrophins in human neutrophils. Expression and distribution of short dystrophin isoforms in human neutrophils. (**A**) Western blots of whole protein lysates, obtained from human brain (B) and human resting (R) and activated (A) neutrophils were analyzed, using the H4 anti-dystrophin antibody. Proteins detected with H4 were identified as Dp71 (76 kDa) and Dp71$\Delta_{110}{}^{m}$ (55 kDa) isoforms (arrows). Migration of protein markers is shown on the left. (**B**) Resting and formyl-methionyl-leucyl-phenylalanine-activated neutrophils were seeded on glass cover slips and immunostained with the primary anti-dystrophin antibody H4 and the specific signal for Dp71d/Dp71$\Delta_{110}{}^{m}$ was developed using a secondary fluorescein-conjugated antibody (green color). Cells were counterstained with 4′,6-diamidino-2-phenylindole (blue color) and rhodamine-conjugated phalloidin (red color) to visualize nuclei and cytoplasmic actin, respectively. After triple labeling, cell preparations were subjected to confocal microscopy analysis. Merged images are shown on the right panels. Arrows denote plasma membrane protrusions of activated cells. Scale bar = 5 μm.

In activated PMN, Dp71Δ_{110}^{m}/Dp71d was redistributed in fine aggregates at the cytoplasm and around the nuclei these clumps were also observed at actin-based protrusions (arrow). Quiescent neutrophils polarize as a consequence of exposure to a uniform concentration of fMLP, and a drastic reorganization of actin filaments throughout the entire body of the cell was observed, and synthesized actin polymers protrude in the forward direction (Fig. 1B, arrow) [31]. Colocalization of Dp71Δ_{110}^{m}/Dp71d with F-actin was evident in these cellular regions (arrow in merge image). Negative controls were performed using only the FITC secondary antibody (Supplementary Figure E1, online only, available at www.exphem.org). Because brain extract was used as positive control for Western blot assays, we included PC12 cells as positive controls for localization of dystrophin family proteins (Supplementary Figure E2, online only, available at www.exphem.org).

Utrophins are present in PMN

To determine the presence of utrophin family proteins, resting and activated PMN and brain extracts were analyzed by immunoblot with K7 antibody directed to the C-terminal last 15 aa of Up400. This antibody revealed the presence of Up400 with bands of approximately 395 kDa showed in resting and activated PMN. Up71 was observed with bands of 76 kDa, which was more evident for activated than for resting PMN and scarce for brain extract (Fig. 2A).

Additionally, a 160-kDa protein band was observed in activated PMN extracts, but was faintly detected in brain extracts; this band might correspond to Up140, an utrophin isoform identified in brain [16] or could be a degradation product of Up400 (Fig. 2A).

Cellular distribution of utrophins was performed, using an anti-utrophin K7 antibody (Table 1). In resting PMN, Up400/Up71 was confined as thick aggregates at the plasma membrane and beneath it; colocalizing with F-actin mainly at the plasma membrane (Fig. 2B). Activated ones conserved plasma membrane clumps of Up400 and exhibited a cytoplasmic homogeneous distribution of this protein, locating at plasma membrane extensions (arrows) and colocalizing with F-actin (Fig. 2B, arrows). Negative controls were performed using only the FITC secondary antibody (Supplementary Figure E1).

Dystrophin-associated proteins are present in resting and activated PMN

To ascertain the presence of dystrophin-associated proteins (DAPs) in PMN, specific antibodies directed to each of the DAP analyzed (i.e., β-dystroglycan, α-syntrophin, and α-dystrobrevin) were employed for immunoblotting and immunofluorescence assays.

An immunoreactive band corresponding to β-dystroglycan (43 kDa) was found in brain, as well as in resting and activated PMN extracts. An additional band of 65 kDa was observed in PMN extracts, being apparently a little stronger in activated extracts. A band of 56 kDa that must correspond to α-syntrophin was revealed in the different lysates. Two bands of 84 kDa and 55 kDa that must correspond to α-dystrobrevin-1 and α-dystrobrevin-2, respectively, were found in resting and activated PMN extracts, as well as in the brain lysate (Fig. 3A).

Distribution of DAPs was analyzed by immunofluorescence assays. In resting PMN, β-dystroglycan, being a transmembranal protein, was observed surrounding and beneath the plasma membrane as discrete aggregates, colocalizing with actin at these zones (Fig. 3B).

It is noteworthy that in activated PMN, β-dystroglycan distribution changed to a punctuate pattern with a homogenous distribution in the cytoplasm and scarce presence in the nuclei. Codistribution with actin was observed at the plasma membrane, cytoplasm, and lamellipodium (Fig. 3C, arrow). α-Syntrophin showed a discontinuous distribution that formed cytoplasmic patches of labeling and scarce colocalization with F-actin at the plasma membrane (Fig. 3B). In contrast, activated PMN exhibited dotted staining of α-syntrophin distributed at the cytoplasm showing some zones of more intense labeling beneath the plasma membrane (asterisks), including the lamellipodium and the nuclear area. Such a pattern of distribution slightly colocalized with that of F-actin (Fig. 3C). Finally, the α-dystrobrevin antibody revealed polarized distribution in the cytoplasm of resting PMN (Fig. 3B). However, in activated PMN, α-dystrobrevins encompassed extended regions of the cytoplasm, nuclei, and cell periphery including actin-based structures; colocalization with actin filaments was observed specially at the regions of active polymerization (Fig. 3C, arrows). Negative controls were performed using only the FITC secondary antibody (Supplementary Figure E1).

Dystrophins and utrophins colocalize in resting PMN and redistribute in activated PMN

The presence of dystrophin and utrophin isoforms in resting and activated PMN together with the different DAPs makes the formation of a DAPC containing Dp71Δ_{110}^{m}/Dp71d and/or Up400/Up71 feasible. To test this idea, the potential colocalization of Dp71Δ_{110}^{m}/Dp71d or Up400/Up71 with the DAPs (β-dystroglycan, α-syntrophin, and α-dystrobrevin) was analyzed by confocal microscopy assays.

Figure 4 shows that Dp71Δ_{110}^{m}/Dp71d colocalized with the three DAPs assayed in both resting and activated PMN. In resting PMN, codistribution was located in the cell periphery, beneath the plasma membrane and surrounding the nuclei of the cells (Fig. 4A), while in activated cells the label was redistributed to the cytoplasm and to the actin-based protrusions (Fig. 4B, arrows).

Merging confocal microscopy images of Up400/Up71 and DAPs showed colocalization in resting and activated PMN. In resting PMN, the proteins were found beneath the cytoplasmic membrane and cytoplasm (Fig. 5A), while

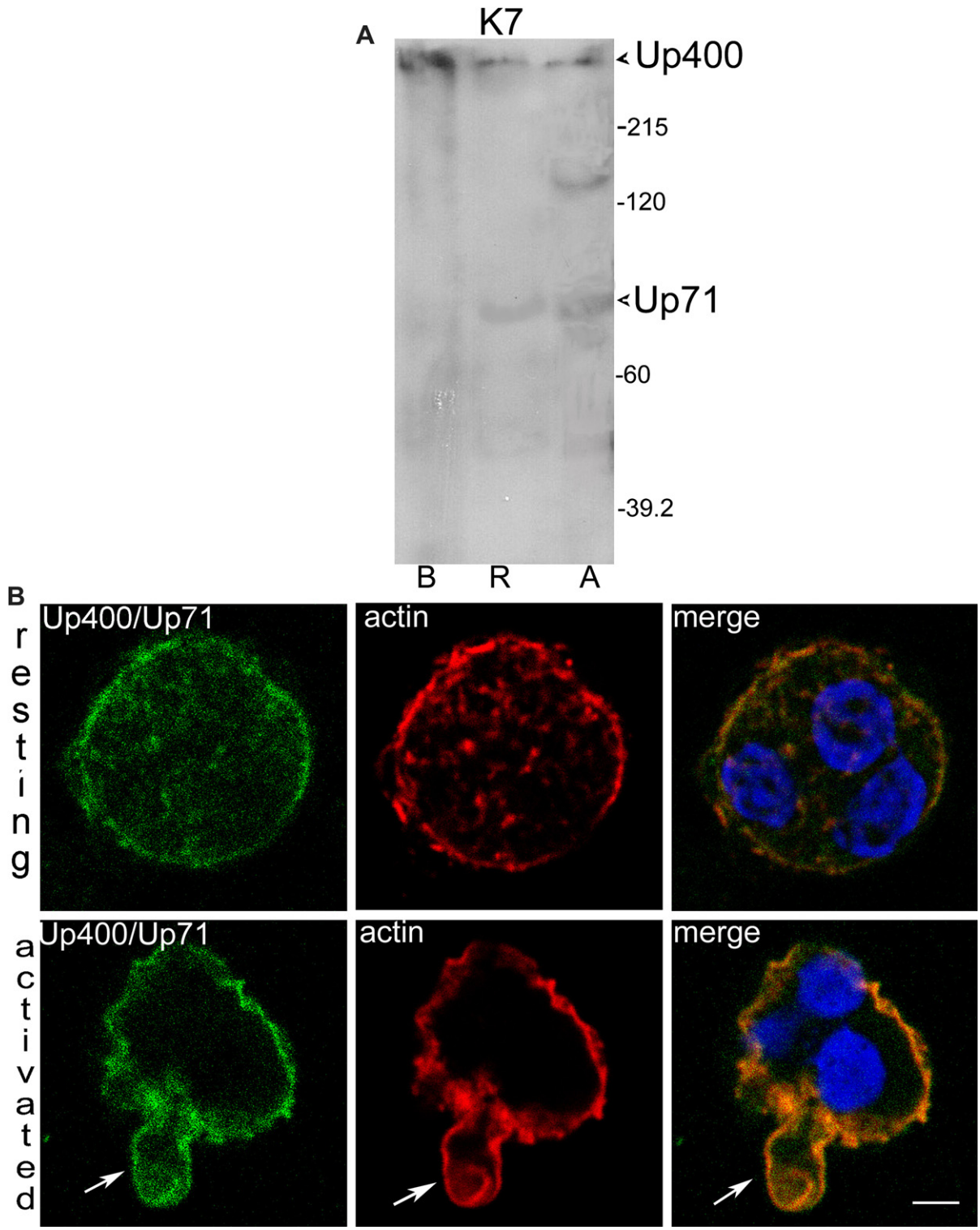


Figure 2. Expression and distribution of utrophin gene products in human neutrophils. (**A**) To identify utrophin isoforms expressed in neutrophils, Western blotting analysis of whole protein lysates from human brain (B) and human resting (R) and activated (A) neutrophils were carried out. Up400 (395 kDa) and Up71 (76 kDa) was revealed with the K7 antibody (arrows). Migration of protein markers is shown on the left. (**B**) Resting and formyl-methionyl-leucyl-phenylalanine-activated neutrophils were analyzed by immunofluorescence and confocal microscopy using the primary anti-utrophin K7 and the specific signal for Up400/Up71 were developed with a secondary fluorescein-conjugated antibody (green color). Cells were counterstained with 4′,6-diamidino-2-phenylindole (blue color) and rhodamine-conjugated phalloidin (red color) to visualize nuclei and actin filaments, respectively. Merged images are shown on the right panels. Arrows denote plasma membrane protrusions of activated cells. Scale bar = 5 μm.

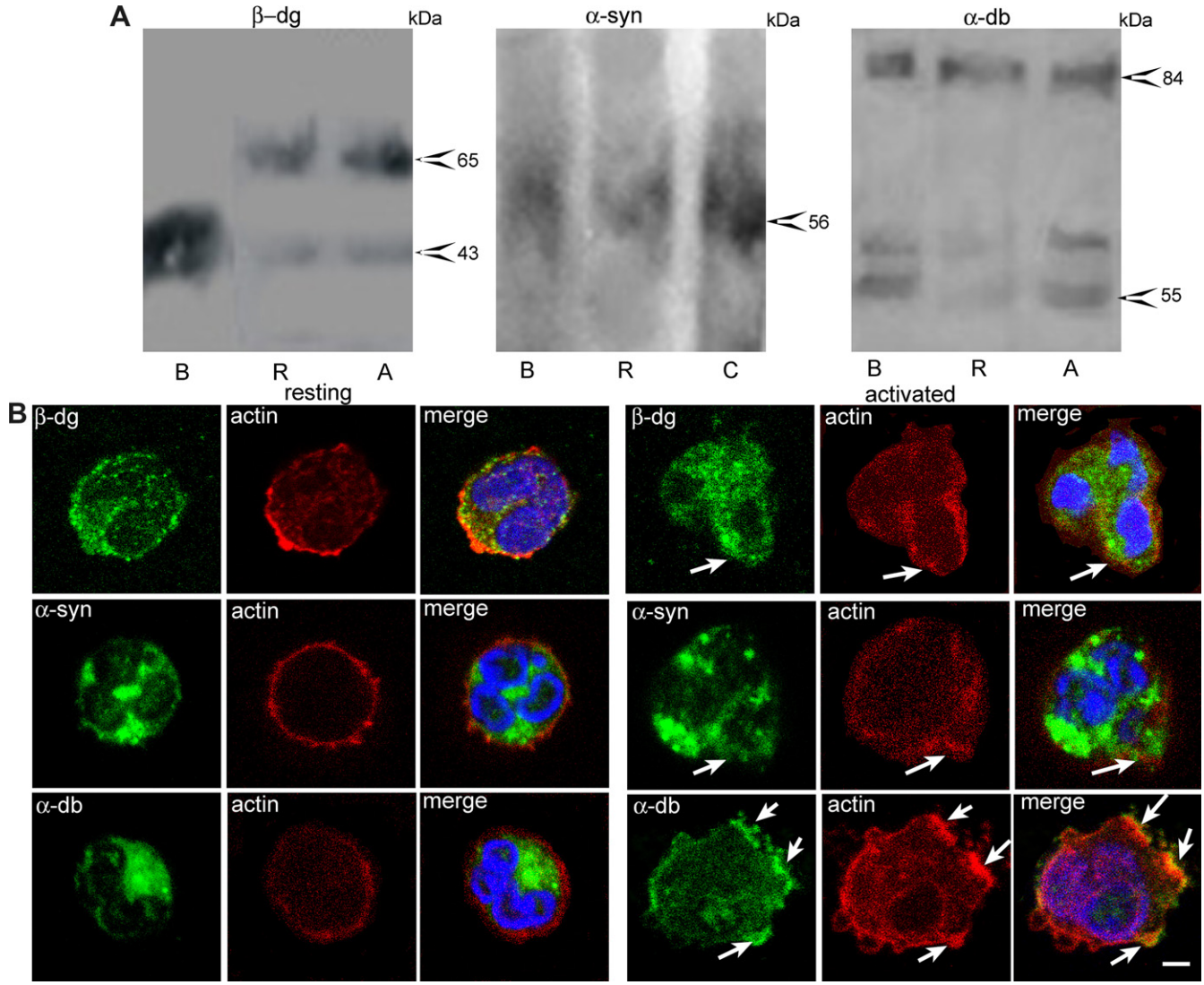


Figure 3. Identification and distribution of dystrophin-associated proteins and actin during human neutrophils activation. (**A**) Whole protein lysates from human brain (B) and human resting (R) and activated (A) neutrophils were comparatively analyzed by immunoblotting. Blots were immunostained with antibodies against β-dystroglycan (β-dg), α-syntrophin (α-syn), and α-dystrobrevins (α-db). Arrows on the right denote the specific protein bands for β-dystroglycan (43 and 65 kDa), α-syntrophin (54 kDa) and α-Db-1 and α-Db-2 dystrobrevin (84 and 55 kDa, respectively). (**B**) Confocal microscopy analysis of resting and activated human neutrophils were carried out with primary antibodies direct to β-dystroglycan, α-syntrophin and α-dystrobrevins and the specific signal for each protein was developed using a secondary fluorescein-conjugated antibody (green color). Counterstaining with 4′,6-diamidino-2-phenylindole (blue color) and rhodamine-conjugated phalloidin (red color) were carried out to decorate nuclei and actin filaments, respectively. Merging images are shown on the right panels. Plasma membrane actin-based structures (arrows). Scale bar = 5 μm.

in activated PMN, Up400/Up71 and DAPs distributed homogeneously colocalizing in the cytoplasm and membrane extensions (Fig. 5B, arrows). Negative controls were performed using only the tetrarhodamine isothiocyanate secondary antibody (Supplementary Figure E3, online only, available at www.exphem.org).

Altogether, these results suggest the distribution of a DAPC in resting and activated PMN that contains $Dp71\Delta_{110}{}^{m}$/Dp71d or Up400/Up71 as central component undergoes reorganization according to the functional stage of the cell participating in the morphological changes of the cell in order to perform their function.

Short dystrophins and utrophin gene products form complexes in resting and activated PMN

To confirm the existence of protein associations between $Dp71\Delta_{110}{}^{m}$/Dp71d and/or Up400 with DAPs, immunoprecipitation assays were carried out in resting and activated PMN, utilizing antibodies specific to dystrophin (Dys2) and utrophin (3B6). In addition, interaction of DAPCs with the actin-based cytoskeleton was analyzed with an immunoprecipitating antibody directed to actin. Coimmunoprecipitated proteins were analyzed by immunoblotting, employing antibodies against actin, DAPs, dystrophin, and utrophin.

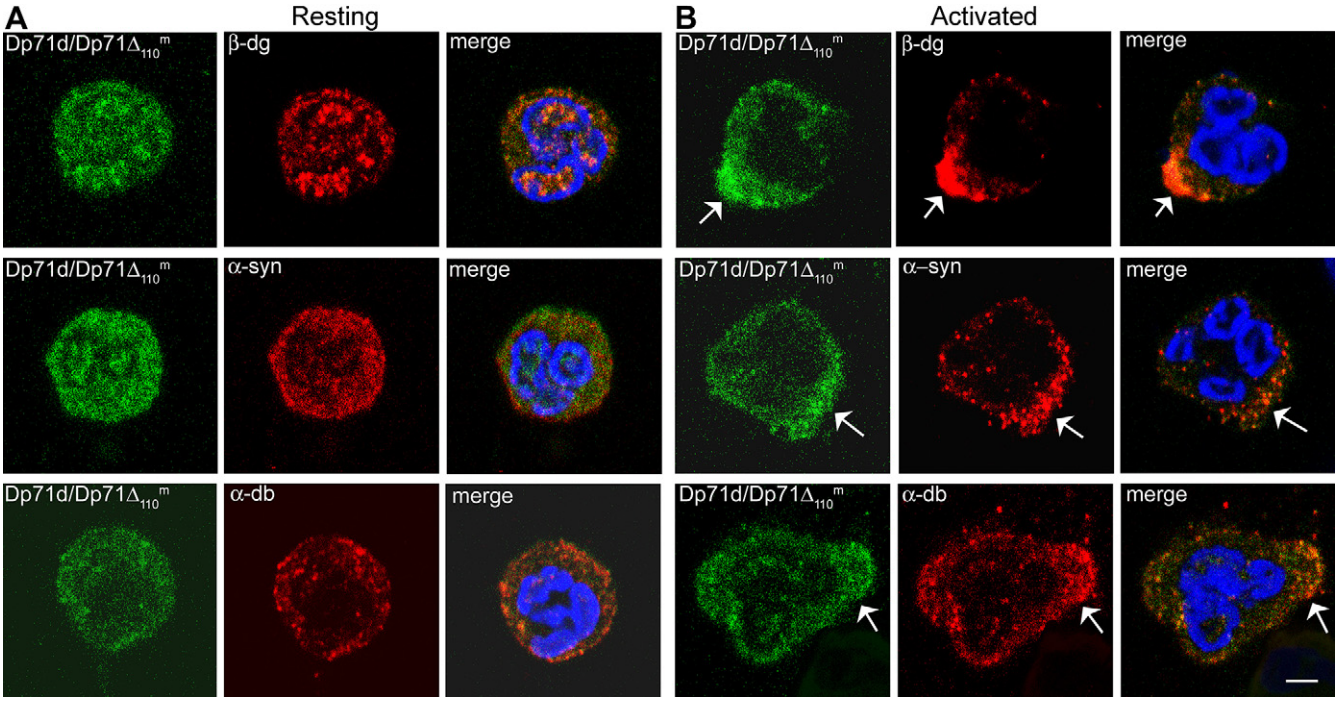


Figure 4. Colocalization of Dp71 with dystrophin-associated proteins (DAPs) in human resting and activated human neutrophils. (**A**) Resting and (**B**) activated neutrophils were stained for Dp71d/Dp71Δ^{m}_{110} using the anti-dystrophin antibody Dys2 and costained either anti−β-dystroglycan (β-dg), anti-−α-syntrophin (α-syn), or α-dystrobrevin (α-db) antibody. Developing of Dp71d/Dp71Δ^{m}_{110} was carried out with a secondary fluorescein-conjugated antibody (green color), while developing of each DAP was performed with a secondary rhodamine-conjugated antibody (red color). 4′,6-Diamidino-2-phenylindole (blue color) was used to stain the nuclei. Plasma membrane protrusions (arrows). Scale bar = 5 μm.

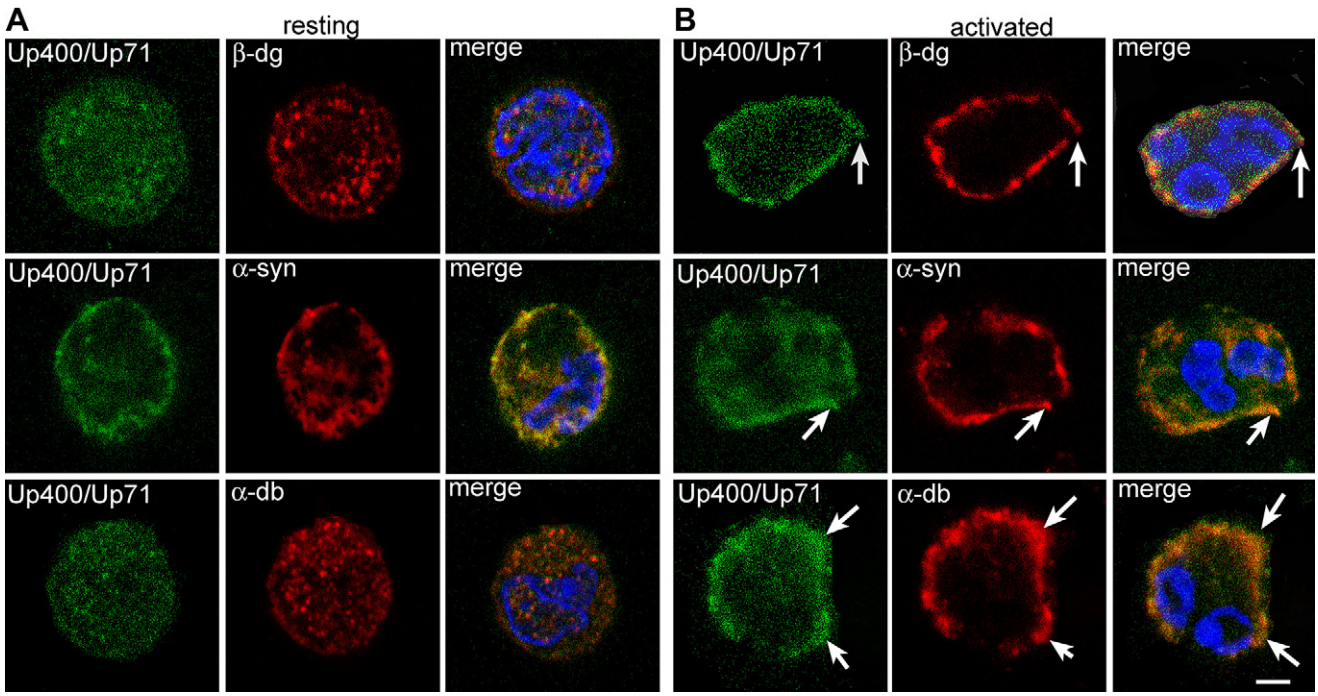


Figure 5. Colocalization of utrophin and dystrophin-associated proteins (DAPs) during activation of human neutrophils. (**A**) Resting and (**B**) activated neutrophils were double immunolabeling with the anti-utrophin antibody K7 and either anti−β-dystroglycan (β-dg), anti−α-syntrophin (α-syn), or α-dystrobrevin (α-db) antibody. Developing of Up400/Up71 staining was carried out with a secondary fluorescein-conjugated antibody (green color) while developing of each DAP staining was performed with a secondary rhodamine-conjugated antibody (red color), while nuclei were identified with 4′,6-diamidino-2-phenylindole (blue color). Scale bar = 5 μm.

Actin immunoprecipitation from resting and activated PMN pulled down Dp71Δ_{110}^{m}, Dp71d, Up400, β-dystroglycan 43 kDa), α-syntrophin, α-dystrobrevin-1, and -2. The 65-kDa protein band of β-dystroglycan was barely detected in the immunoprecipitate of resting PMN and undetected in that of activated PMN (Fig. 6).

Immunoprecipitation of Dp71d/Dp71Δ_{110}^{m} recovered with different efficiency the immunoreactive bands of 43 kDa and 65 kDa corresponding to β-dystroglycan, while the 43-kDa band was barely detected in resting and activated PMN, the 65-kDa band was clearly observed under both conditions. Interestingly, the 65-kDa band was apparently more abundant in activated than in resting PMN. In addition, α-syntrophin, α-dystrobrevin-1, -2, and actin proteins were coimmunoprecipitated from resting and activated PMN, and α-dystrobrevin-1 -2 was faintly detected in fMLP-treated PMN (Fig. 6).

The anti-utrophin antibody 3B6 immunoprecipitated Up400 together with β-dystroglycan, α-syntrophin, α-dystrobrevin-1 and -2, and actin in resting and activated PMN (Fig. 6, Up400, immunoprecipitates). Regarding β-dystroglycan isoforms, the 65-kDa protein band was recovered more efficiently than that of the 43-kDa protein under both conditions.

Altogether, these results show the existence of two protein complexes in resting and fMLP-treated PMN: the former formed by Dp71d/Dp71Δ_{110}^{m} and several DAPs (i.e., β-dystroglycan, α-syntrophin, α-dystrobrevin-1, and -2), while the latter contains Up400, instead of Dp71d/Dp71Δ_{110}^{m}, as a central component of the DAPC, which also suggest a stepwise interaction of these complexes with the actin cytoskeleton.

Discussion

In the present study, we described, to our knowledge for the first time the pattern of expression and subcellular localization of dystrophin (Dp71d and Dp71Δ_{110}^{m}) and utrophin (Up400 and Up71) isoforms, as well as those of the DAPs (β-dystroglycan, α-syntrophin, and α-dystrobrevin-1 and -2) in human resting and activated neutrophils (PMN) using immunofluorescence and immunoblotting analyses. Furthermore, we demonstrated by immunoprecipitation assays the existence of alternative DAPCs in both resting and activated the PMN, which are composed of Dp71d/Dp71Δ_{110}^{m} or Up400 and β-dystroglycan, α-syntrophin, and α-dystrobrevin-1 and -2. We also revealed that DAPCs are associated with the actin cytoskeleton, likely via the interaction of actin with the actin-binding motifs present in utrophin, Dp71, and/or β-dystroglycan [19,32]. Two different β-dystroglycan isoforms (43 and 65 kDa) were found in the DAPCs; however, while the 43 kDa variant associated with

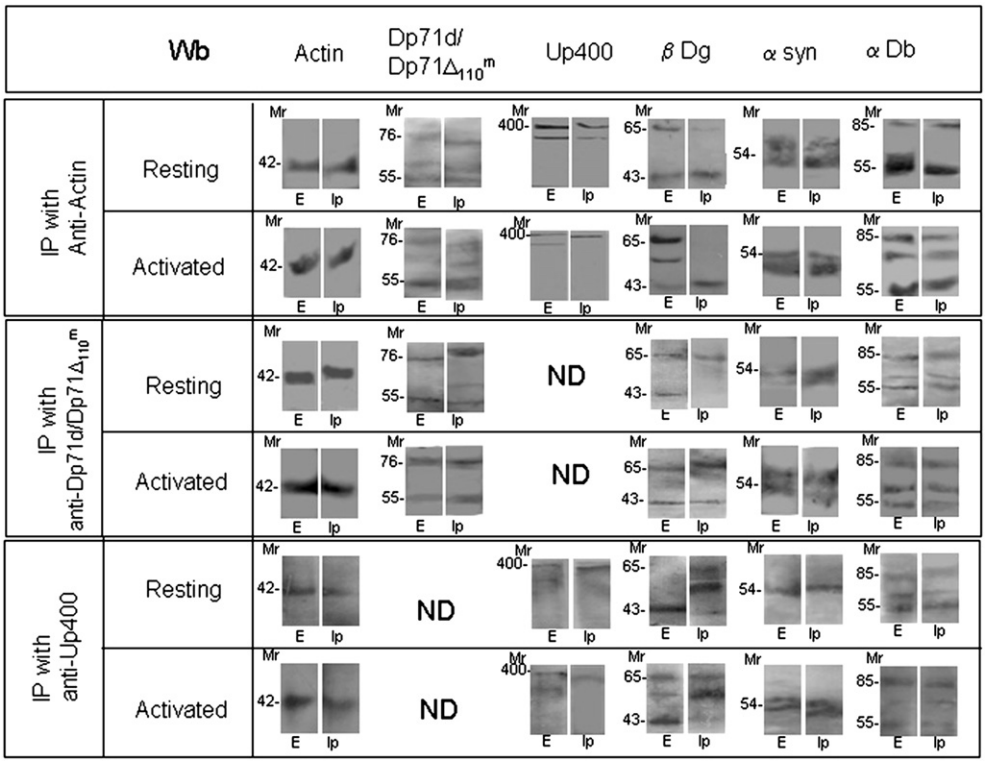


Figure 6. Dp71d/Dp71Δ_{110}^{m} and utrophin proteins form protein complexes with dystrophin-associated proteins (DAPs) and actin in resting and activated neutrophils. Protein extracts from resting (R) or activated (A) human neutrophils were immunoprecipitated with anti-actin, anti-Dp71d/Dp71Δ_{110}^{m} or anti-utrophin antibodies. Proteins from total extracts (E) and immunoprecipitates (IP) were analyzed by Western blotting (Wb) using antibodies against actin, β-dystroglycan (β-dg), α-syntrophin (α-syn), α-dystrobrevins (α-db), Dp71d/Dp71Δ_{110}^{m}, and Up400/Up71. Migration of protein markers is shown on the left.

actin in activated neutrophils, the 65 kDa variant did not, suggesting that these isoforms possess different affinity properties between each other, as described previously [33]. Interestingly, Up71 protein band was revealed with more intensity in activated than resting neutrophils, suggesting that Up71 levels increase during this process. Further studies are necessary to determine whether this regulation occurred at transcriptional or translational level. In neutrophils, the actin-based cytoskeleton is reorganized upon activation, manifesting in the formation of lamellipodia and pseudopodia for migration and phagocytosis [34]. It is noteworthy that the subcellular distribution of Dp71d/Dp71Δ_{110}^{m}, Up400/Up71, β-dystroglycan, α-syntrophin, and α-dystrobrevin-1 and -2 underwent a drastic reorganization in fMLP-activated neutrophils, concentrating together with actin filaments in the specialized cytoskeletal structures mentioned previously (Figs. 1–3). These data suggest that Dp71d/Dp71Δ_{110}^{m} and the DAPs might play an important role in PMN cytoskeleton remodeling. In this respect, it has been demonstrated that β-dystroglycan can induce filopodia formation in both a Cdc42- and an ezrin-dependent manner [35,36] and podosome formation via its Src-mediated phosphorylation and further interaction with Tks5 [37]. Therefore, it is plausible that β-dystroglycan participates in the lamellipodium formation of PMN, a process that has been shown to require Gβγ-dependent activation of Cdc42 [38]. Lamellipodia and filopodia are stabilized by adhesion to the extracellular matrix by integrin receptors that are also linked with the actin cytoskeleton; integrin-based adhesions are organized into short-lived focal complexes and their mature derivatives, focal adhesions [39]. Interestingly, we have previously shown that β-dystroglycan is a component of the integrin-adhesion complex in platelets, functioning as a bridge to communicate actin and microtubules [40]. Therefore, it is tempting to speculate that β-dystroglycan has a role in the adhesion, and ultimately in the migration process, of neutrophils. Leukocyte migration and trafficking are regulated through the dynamic reorganization of actin microfilaments and microtubules, which suppress polarity and enhance directional migration [41,42]. In this regard, it has been reported that β-dystroglycan is required to polarize the epithelial cells and the oocyte in *Drosophila* [43], which supports the hypothesis that β-dystroglycan participates in neutrophil migration. We must bear in mind the possibility that the presence of the proteins described herein could be part of the membrane granules.

It is noteworthy that Dp71 and different DAPs localized to the nuclei of neutrophils. Recently, Dp71 and several DAPs were found to be associated with the nuclear envelope of C2C12 muscle cells, suggesting that these proteins are involved in nuclear structure, and thereby in the modulation of nuclear processes [44]. Study of the nuclear function of Dp71 and DAPs in neutrophils is currently in process at our laboratory.

DMD is the most common and severe neuromuscular disease, characterized by respiratory failure as the primary cause of death and two thirds of these deaths occurred during acute infection [45,46]. The neutrophil is the principal phagocyte delivered to inflammatory sites extending pseudopodia to engulf microorganisms [47]. The phagosome formation and subsequent maturation involve cytoskeletal rearrangements, as well as the involvement of the endocytic pathway [48] in which β-dystroglycan has shown a relevant role [49]. Therefore, based on our data, we strongly suggest that key functions of PMN, such as chemotaxis and phagocytosis, might be affected in DMD patients and might be related to the infection-mediated death in these patients. Future studies based on knockdown experiments will undoubtedly demonstrate the role of DAPC members in neutrophils function.

Finally, the present study has revealed the existence of two protein complexes formed by Dp71d/Dp71Δ_{110}^{m} or Up400 associated with DAPs in resting and fMLP-treated human polymorphonuclears, indicating their dynamic participation in the chemotaxis process.

Acknowledgments

We thank Dr. Mornet for the generous gift of H4 and K7 antibodies. This work was supported by research grants Consejo Nacional para la Ciencia y la Tecnología (CONACyT) (México, D.F.) CB2007-79700 and Secretaría de Investigación y Posgrado (SIP) (México, D.F.) SIP0298 to D.C.

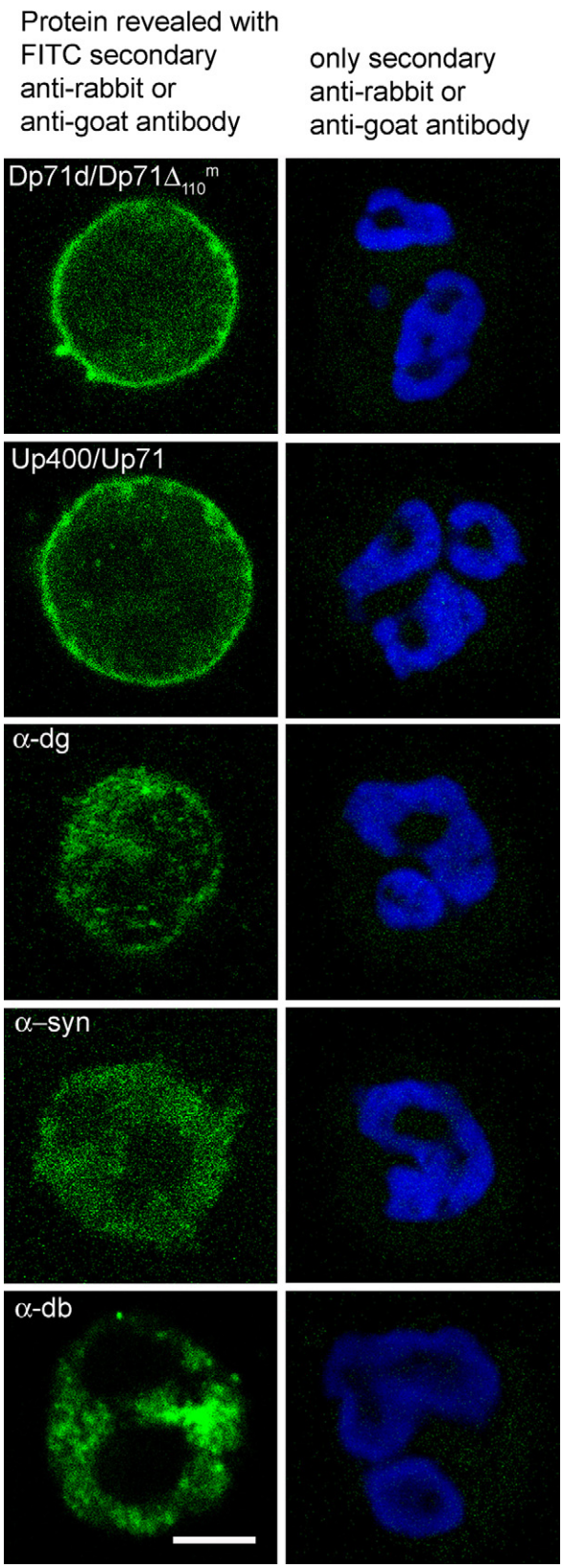
Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

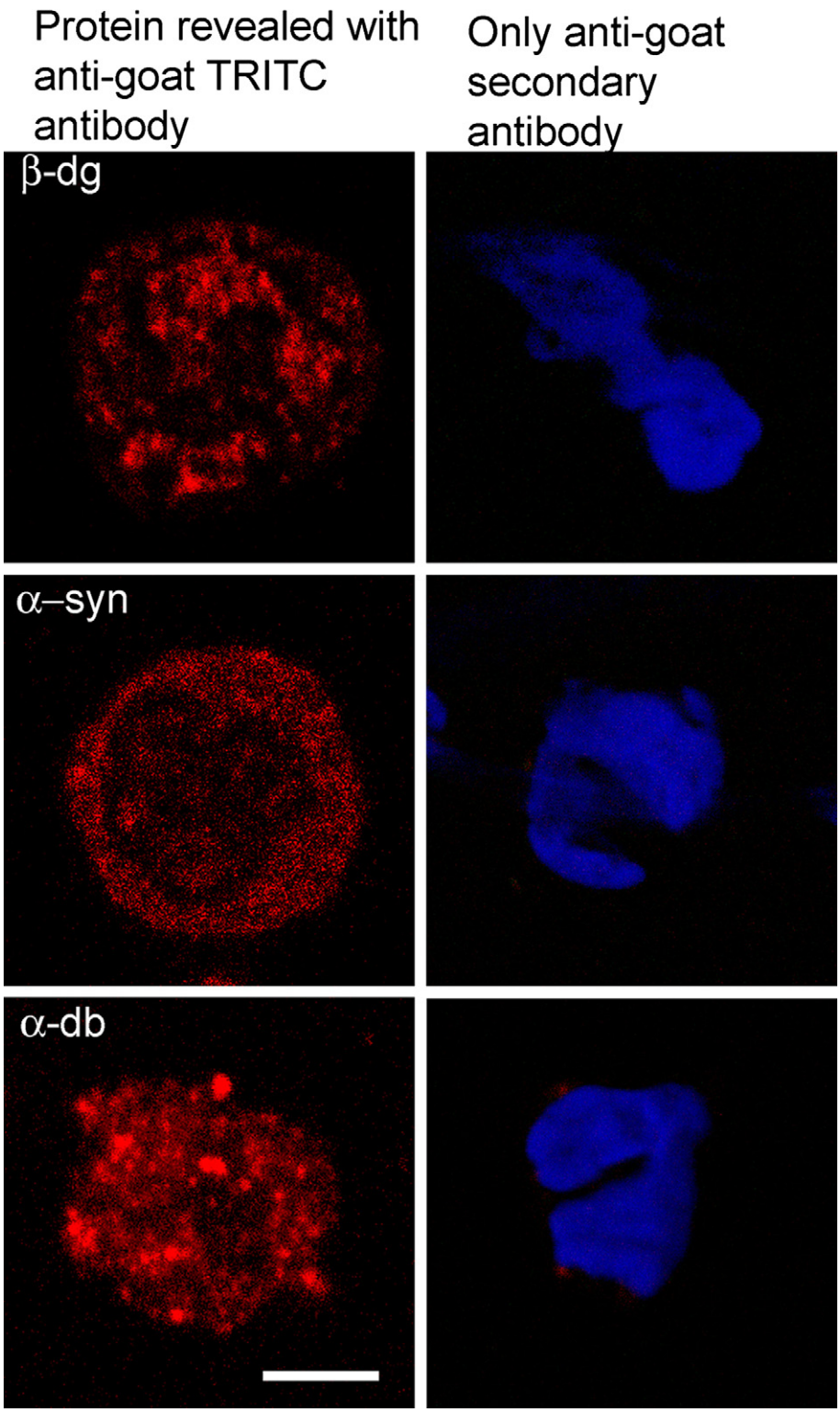
References

1. Akashi K, He X, Chen J, et al. Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood. 2003;101:383–389.
2. Borregaard N, Miller LJ, Springer TA. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science. 1987;237:1204–1206.
3. Segal AW. How neutrophils kill microbes. Annu Rev Immunol. 2005; 23:197–223.
4. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect. 2003;5:1317–1327.
5. Theilgaard-Monch K, Knudsen S, Follin P, Borregaard N. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. J Immunol. 2004; 172:7684–7693.
6. Hartwig JH. Actin-binding proteins 1: spectrin superfamily. Protein Profile. 1994;1:706–778.
7. Carlier MF. Control of actin dynamics. Curr Opin Cell Biol. 1998;10: 45–51.
8. Coffey AJ, Roberts RG, Green ED, et al. Construction of a 2.6-Mb contig in yeast artificial chromosomes spanning the human dystrophin gene using an STS-based approach. Genomics. 1992;12:474–484.
9. Roberts RG, Coffey AJ, Bobrow M, Bentley DR. Exon structure of the human dystrophin gene. Genomics. 1993;16:536–538.

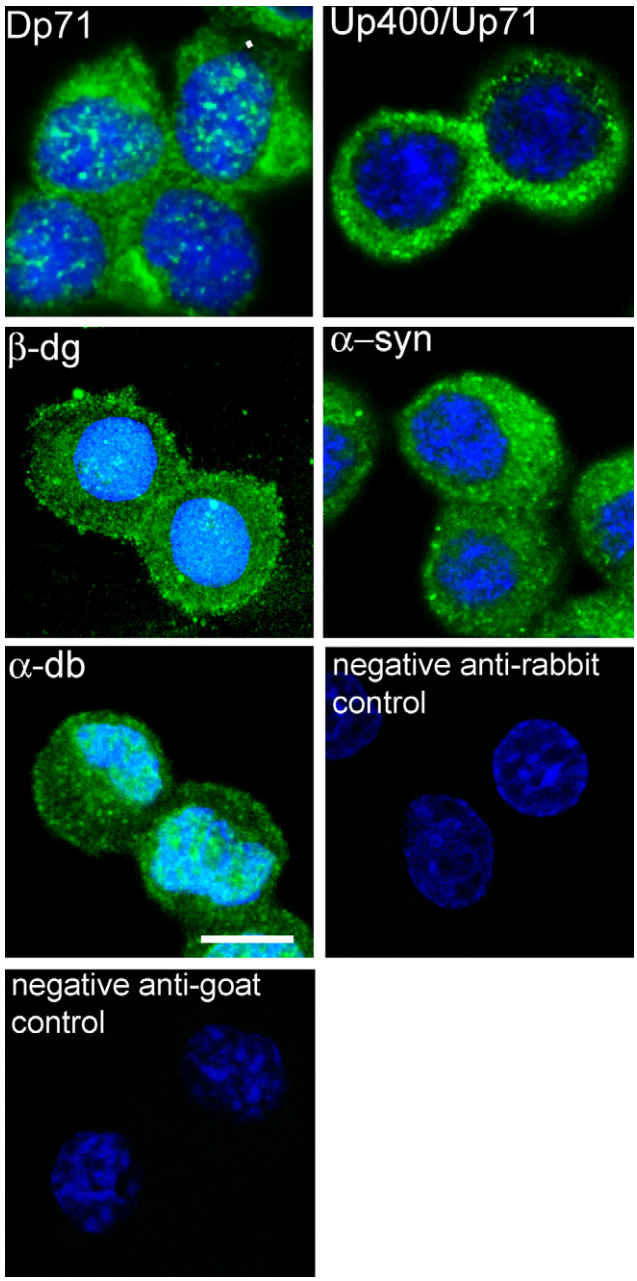
10. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell. 1987;50:509–517.
11. Bar S, Barnea E, Levy Z, Neuman S, Yaffe D, Nudel U. A novel product of the Duchenne muscular dystrophy gene which greatly differs from the known isoforms in its structure and tissue distribution. Biochem J. 1990;272:557–560.
12. Rapaport D, Greenberg DS, Tal M, Yaffe D, Nudel U. Dp71, the nonmuscle product of the Duchenne muscular dystrophy gene is associated with the cell membrane. FEBS Lett. 1993;328:197–202.
13. Gorecki DC, Monaco AP, Derry JM, Walker AP, Barnard EA, Barnard PJ. Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. Hum Mol Genet. 1992;1: 505–510.
14. Byers TJ, Lidov HG, Kunkel LM. An alternative dystrophin transcript specific to peripheral nerve. Nat Genet. 1993;4:77–81.
15. Khurana TS, Hoffman EP, Kunkel LM. Identification of a chromosome 6-encoded dystrophin-related protein. J Biol Chem. 1990;265:16717–16720.
16. Wilson J, Putt W, Jimenez C, Edwards YH. Up71 and up140, two novel transcripts of utrophin that are homologues of short forms of dystrophin. Hum Mol Genet. 1999;8:1271–1278.
17. James M, Nguyen TM, Wise CJ, Jones GE, Morris GE. Utrophin-dystroglycan complex in membranes of adherent cultured cells. Cell Motil Cytoskeleton. 1996;33:163–174.
18. Moores CA, Kendrick-Jones J. Biochemical characterisation of the actin-binding properties of utrophin. Cell Motil Cytoskeleton. 2000; 46:116–128.
19. Winder SJ, Hemmings L, Maciver SK, et al. Utrophin actin binding domain: analysis of actin binding and cellular targeting. J Cell Sci. 1995;108(Pt 1):63–71.
20. Winder SJ, Gibson TJ, Kendrick-Jones J. Dystrophin and utrophin: the missing links!. FEBS Lett. 1995;369:27–33.
21. Rando TA. The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle Nerve. 2001;24:1575–1594.
22. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature. 1992;355:696–702.
23. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol. 1993;122:809–823.
24. Claudepierre T, Mornet D, Pannicke T, et al. Expression of Dp71 in Muller glial cells: a comparison with utrophin- and dystrophin-associated proteins. Invest Ophthalmol Vis Sci. 2000;41:294–304.
25. Hernandez-Gonzalez EO, Martinez-Rojas D, Mornet D, Rendon A, Mujica A. Comparative distribution of short dystrophin superfamily products in various guinea pig spermatozoa domains. Eur J Cell Biol. 2001;80:792–798.
26. Earnest JP, Santos GF, Zuerbig S, Fox JE. Dystrophin-related protein in the platelet membrane skeleton. Integrin-induced change in detergent-insolubility and cleavage by calpain in aggregating platelets. J Biol Chem. 1995;270:27259–27265.
27. Austin RC, Fox JE, Werstuck GH, et al. Identification of Dp71 isoforms in the platelet membrane cytoskeleton. Potential role in thrombin-mediated platelet adhesion. J Biol Chem. 2002;277:47106–47113.
28. Austin RC, Howard PL, D'Souza VN, Klamut HJ, Ray PN. Cloning and characterization of alternatively spliced isoforms of Dp71. Hum Mol Genet. 1995;4:1475–1483.
29. Cerecedo D, Martinez-Rojas D, Chavez O, et al. Platelet adhesion: structural and functional diversity of short dystrophin and utrophins in the formation of dystrophin-associated-protein complexes related to actin dynamics. Thromb Haemost. 2005;94:1203–1212.
30. Ferrante A, Thong YH. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. J Immunol Methods. 1980;36: 109–117.
31. Cassimeris L, McNeill H, Zigmond SH. Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. J Cell Biol. 1990;110:1067–1075.
32. Chen YJ, Spence HJ, Cameron JM, Jess T, Ilsley JL, Winder SJ. Direct interaction of beta-dystroglycan with F-actin. Biochem J. 2003;375: 329–337.
33. Royuela M, Chazalette D, Hugon G, et al. Formation of multiple complexes between beta-dystroglycan and dystrophin family products. J Muscle Res Cell Motil. 2003;24:387–397.
34. Niggli V. Signaling to migration in neutrophils: importance of localized pathways. Int J Biochem Cell Biol. 2003;35:1619–1638.
35. Batchelor CL, Higginson JR, Chen YJ, Vanni C, Eva A, Winder SJ. Recruitment of Dbl by ezrin and dystroglycan drives membrane proximal Cdc42 activation and filopodia formation. Cell Cycle. 2007;6: 353–363.
36. Spence HJ, Chen YJ, Batchelor CL, et al. Ezrin-dependent regulation of the actin cytoskeleton by beta-dystroglycan. Hum Mol Genet. 2004; 13:1657–1668.
37. Thompson O, Kleino I, Crimaldi L, Gimona M, Saksela K, Winder SJ. Dystroglycan, Tks5 and Src mediated assembly of podosomes in myoblasts. PLoS One. 2008;3:e3638.
38. Li Z, Hannigan M, Mo Z, et al. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell. 2003;114:215–227.
39. Verkhovsky AB, Chaga OY, Schaub S, Svitkina TM, Meister JJ, Borisy GG. Orientational order of the lamellipodial actin network as demonstrated in living motile cells. Mol Biol Cell. 2003;14:4667–4675.
40. Cerecedo D, Cisneros B, Suarez-Sanchez R, Hernandez-Gonzalez E, Galvan I. beta-Dystroglycan modulates the interplay between actin and microtubules in human-adhered platelets. Br J Haematol. 2008; 141:517–528.
41. Jones GE. Cellular signaling in macrophage migration and chemotaxis. J Leukoc Biol. 2000;68:593–602.
42. Worthylake RA, Burridge K. Leukocyte transendothelial migration: orchestrating the underlying molecular machinery. Curr Opin Cell Biol. 2001;13:569–577.
43. Deng WM, Schneider M, Frock R, et al. Dystroglycan is required for polarizing the epithelial cells and the oocyte in Drosophila. Development. 2003;130:173–184.
44. Gonzalez-Ramirez R, Morales-Lazaro SL, Tapia-Ramirez V, Mornet D, Cisneros B. Nuclear and nuclear envelope localization of dystrophin Dp71 and dystrophin-associated proteins (DAPs) in the C2C12 muscle cells: DAPs nuclear localization is modulated during myogenesis. J Cell Biochem. 2008;105:735–745.
45. Walton JN. Muscular dystrophies and their management. Br Med J. 1969;3:639–642.
46. Calvert LD, McKeever TM, Kinnear WJ, Britton JR. Trends in survival from muscular dystrophy in England and Wales and impact on respiratory services. Respir Med. 2006;100:1058–1063.
47. Malech HL, Gallin JI. Current concepts: immunology. Neutrophils in human diseases. N Engl J Med. 1987;317:687–694.
48. Vieira OV, Botelho RJ, Grinstein S. Phagosome maturation: aging gracefully. Biochem J. 2002;366:689–704.
49. Zhan Y, Tremblay MR, Melian N, Carbonetto S. Evidence that dystroglycan is associated with dynamin and regulates endocytosis. J Biol Chem. 2005;280:18015–18024.
50. Dalloz C, Sarig R, Fort P, et al. Targeted inactivation of dystrophin gene product Dp71: phenotypic impact in mouse retina. Hum Mol Genet. 2003;12:1543–1554.



Supplementary Figure E1. Fluorescein isothiocyanate−negative immunofluorescence neutrophils control. Resting neutrophils were immunolabeled with the anti-dystrophin antibody H4, anti-utrophin antibody K7 and either anti−β-dystroglycan (β-dg), anti−α-syntrophin (α-syn), or α-dystrobrevin (α-db) antibody. The primary antibody staining was carried out with a secondary fluorescein-conjugated antibody (green color) while nuclei were identified with 4′,6-diamidino-2-phenylindole (blue color). Negative controls were performed adding only the fluorescein-conjugated antibody. Scale bar = 5 μm.



Supplementary Figure E2. Tetrarhodamine isothiocyanate–negative immunofluorescence neutrophils control. Resting neutrophils were immunolabeling with anti–β-dystroglycan (β-dg), anti–α-syntrophin (α-syn), or α-dystrobrevin (α-db) antibodies. The primary antibody staining was carried out with a secondary rhodamine-conjugated antibody (red color), while nuclei were identified with 4′,6-diamidino-2-phenylindole (blue color). Negative controls were performed adding only the rhodamine-conjugated antibody. Scale bar = 5 μm.



Supplementary Figure E3. Distribution of dystrophin-, utrophin-, and dystrophyn-associated proteins in PC12 cells. Cells, plated onto laminin-coated coverslips were processed for immunofluorescence single staining by overnight incubation at 4°C with the following primary antibodies: anti-H4, anti-K7, anti−β-dystroglycan, anti−α-syntrophin and anti−α-dystrobrevins and the specific signal for each protein was developed using a secondary fluorescein-conjugated antibody (green color). Counterstaining with 4′,6-diamidino-2-phenylindole (blue color) was carried out to decorate nuclei. Negative controls were performed adding only the fluorescein-conjugated antibody. Scale bar = 2 μm.