

Characterization of Human Alpha-Dystrobrevin Isoforms in HL-60 Human Promyelocytic Leukemia Cells Undergoing Granulocytic Differentiation

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The biochemical properties and spatial localization of the protein alpha-dystrobrevin and other isoforms were investigated in cells of the human promyelocytic leukemia line HL-60 granulocytic differentiation as induced by retinoic acid (RA). Alpha-dystrobrevin was detected both in the cytosol and the nuclei of these cells, and a short isoform (gamma-dystrobrevin) was modified by tyrosine phosphorylation soon after the onset of the RA-triggered differentiation. Varying patterns of distribution of alpha-dystrobrevin and its isoforms could be discerned in HL-60 promyelocytes, RA-differentiated mature granulocytes, and human neutrophils. Moreover, the gamma-dystrobrevin isoform was found in association with actin and myosin light chain. The results provide new information about potential involvement of alpha-dystrobrevin and its splice isoforms in signal transduction in myeloid cells during induction of granulocytic differentiation and/or at the commitment stage of differentiation or granulocytic cells.

INTRODUCTION

The large, multisubunit dystrophin glycoprotein complex (DGC) is found in the sarcolemma of striated muscle fibers, and it is essential for maintaining the structural integrity of these fibers during contraction. The complex also constitutes a scaffold for signaling molecules. A specific function of dystrophin complex is to link the extracellular matrix to cortical actin. DGC is made up of the following: the intracellular proteins dystrophin, alpha-dystrobrevin, and syntrophins; the transmembrane proteins beta-dystroglycan, alpha-, beta-, gamma-, and delta-sarcoglycan, and sarcospan; and the extracellularly located alpha-dystroglycan. Dystrobrevin is a dystrophin-related component of the DGC that is situated in the cytoplasm. Dystrophin isolated from the electric organ of *Torpedo californica* has been found to be associated with two proteins, one 58 kDa (homologous with syntrophin) and the other 87 kDa, now known as dystrobrevin (Butler *et al.*, 1992). The mammalian homologue of dystrobrevin is a hydrophilic phosphoprotein that consists of

686 amino acids and has a molecular mass of 77 kDa. Historically, after the first description of dystrobrevin, it was noted that there are multiple splice isoforms expressed from the same gene. In human, Sadoulet-Puccio *et al.* (1996) found six isoforms of dystrobrevin (designated alpha, beta, gamma, delta, epsilon, and zeta) that varied in size from 22 to 80 kDa, and three or possibly four of these (i.e., alpha, beta, gamma, and delta) could be detected by Western blot analysis. Other investigators described the isoforms in parallel giving them numbers 1–4 (Blake *et al.*, 1996). When a separate, homologous gene was discovered, it was called beta-dystrobrevin and the original gene was called alpha-dystrobrevin. Several of the DGC components also exist in two or more isoforms, which are either generated by alternative splicing of a single gene or originate from distinct genes (Peters *et al.*, 1997; Blake *et al.*, 1998). As yet, nothing is known about the roles of the various alpha-dystrobrevin splice isoforms within the DGC.

Most studies of DGC have been focused on organs of mesenchymal origin and the neuromuscular system. Characterization of the expression patterns of the various alpha-dystrobrevin isoforms and their splice variants in skeletal muscle has implied that the individual forms have distinct functions during different stages of differentiation (Blake *et al.*, 1996). Moreover, a number of investigators have shown

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that defects in genes encoding DGC proteins are associated with several types of muscular dystrophy (reviewed by Brown, 1997; Straub and Campbell, 1997; Culligan *et al.*, 1998). In evidence of a signaling capacity of the DGC, it has been reported that neuronal nitric-oxide synthase (nNOS) is associated with the DGC via alpha-dystrobrevin, and there is a loss of nNOS from the sarcolemma in Duchene muscular dystrophy (reviewed by Bredt, 1999). Neither the structure nor the functions of the DGC are completely understood.

Tyrosine kinases are also important factors at the neuromuscular junction (Hoch, 1999; Sanes and Lichtman, 1999). Wagner *et al.* (1993) found that the C terminus of alpha-dystrobrevin is unique and contains tyrosine residues that are phosphorylated *in vivo*. Other investigations (DeChiara *et al.*, 1996; Glass *et al.*, 1996) have shown that agrin regulates the activity of a muscle-specific receptor tyrosine kinase that is essential for proper synapse formation. However, the physiological role of tyrosine phosphorylation of alpha-dystrobrevin in skeletal muscle has not yet been elucidated.

Protein tyrosine phosphorylation provides an essential means of transducing extracellular signals into the nucleus. In general, the goal of our research is to identify the nuclear proteins that are tyrosine-phosphorylated during differentiation of the human promyelocytic leukemia line HL-60. The experimental model is based on differentiation induced by retinoic acid (RA), which causes HL-60 cells to develop into granulocytes (Collins and Gallo, 1977; Breitman *et al.*, 1980).

The results of the present study are the first to show that human alpha-dystrobrevin and its few isoforms are expressed in cells of the promyelocytic HL-60 line. Furthermore, it is found in two cell compartments, the cytosol and the nucleus. Also, we observed a distinct pattern of distribution of alpha-dystrobrevin, including colocalization with actin, in HL-60 promyelocytes, differentiated mature granulocytes, and human neutrophils. Moreover, we discovered that gamma-dystrobrevin isoform undergoes tyrosine phosphorylation soon after the onset of RA-induced differentiation. These findings further support a signaling role for the peripheral dystrophin-associated protein complex (DAPC).

MATERIALS AND METHODS

Cell Culture

Human promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 medium (Invitrogen, Lidingo, Sweden) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) in a 5% CO₂-supplemented incubator at 37°C. Cultures were seeded at a density of 4×10^5 cells/ml (maximum 1.5×10^6 cells/ml) and were subsequently transferred to fresh medium. Cells were not used after passage 60. Granulocytic differentiation was induced by treating 4×10^4 cells/ml with 700 nM RA (Sigma-Aldrich, St. Louis, MO) prepared from a stock solution of 1 mM RA in 96% ethanol (stored at -20°C). The degree of differentiation was measured as the cellular reduction of nitroblue tetrazolium to insoluble blue-black formazan after stimulation with phorbol 12-myristate 13-acetate (Collins and Gallo, 1977). At least 70–75% of the cells were nitroblue tetrazolium-positive in the RA-differentiated population.

Isolation of Neutrophils from Human Blood

To obtain normal neutrophils, heparinized blood (25 ml) from healthy adult volunteers was layered on a 20-ml Polymorphprep

and 50-ml Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient and centrifuged at $600 \times g$ for 45 min at room temperature. The uppermost layer down to the granulocyte band was aspirated, and the band containing granulocytes was transferred to a tube, gently mixed with an equal volume of phosphate-buffered saline (PBS), pH 7.3, and centrifuged at $600 \times g$ for 10 min at room temperature. The cells were resuspended in 9 ml of cold distilled water, 3 ml of 3.4% PBS, and 5 ml of Krebs-Ringer phosphate buffer, pH 7.3, supplemented with 10 mM glucose (without Ca²⁺), and then sedimented at $200 \times g$ for 10 min at 4–8°C. The resulting cell suspension contained at least 95% neutrophils, and eosinophils predominated in the remaining 5%. The neutrophils were routinely pretreated with 5 mM diisopropylfluorophosphate for 15 min on ice to minimize proteolysis and were thereafter kept in a melting ice bath until further analysis.

Isolation and Analysis of Cytosolic and Nuclear Proteins

Nuclei were isolated according to Antalis and Godbolt (1991), with some modifications. Briefly, cells were harvested and washed twice in PBS, pH 7.5, resuspended to 3×10^7 cells/ml in solution A (10 mM NaCl, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 0.05% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 µg/ml each aprotinin, leupeptin, and pepstatin, 5 mM NaF, and 1 mM Na₃VO₄), and then allowed to swell for 15 min at 0°C. Thereafter, the cell suspension was shaken vigorously by hand and immediately mixed 1:1 (vol/vol) with solution B (solution A supplemented with 0.6 M sucrose). The cell homogenates were then centrifuged at $1500 \times g$ for 5 min. Supernatant, corresponding to the cytosolic fraction, was clarified by centrifuging at $15,000 \times g$ for 15 min and then frozen at -76°C. Pelleted nuclei were washed twice by centrifuging at $1500 \times g$ for 5 min, first with a 1:1 mixture of solutions A and B and then three times with solution C (i.e., a 1:1 [vol/vol] mixture of solutions A and B without NP-40). The nuclei were subsequently examined under a light microscope for purity and integrity and used for experiments or frozen at -76°C.

To analyze total nuclear proteins by SDS-PAGE, $\sim 5 \times 10^7$ nuclei/ml were resuspended in an equal amount (vol/vol) of twice-concentrated lysis solution (100 mM Tris, pH 7.4, 5 mM magnesium chloride, 200 mM dithiothreitol [DTT], and 4% SDS), after which three volumes of 1× lysis solution and benzonase (Benzonase Pure Grade; Merck, Darmstadt, Germany) were added to give a final concentration of 2.5 U/ml. The lysates were incubated for 1 h at 0°C and then centrifuged at $15,000 \times g$ for 30 min. For SDS-PAGE, we added 12.5 µl of 0.5 M Tris-HCl, pH 6.8, 30 µl of 10% SDS, 20 µl of 0.5 M DTT, 20 µl of glycerol, and 5 µl of 0.5% bromphenol blue to 100 µl of a cytosolic or nuclear protein fraction and then boiled the samples for 5 min. The supernatants were immediately subjected to electrophoresis or frozen at -76°C.

Total nuclear proteins for two-dimensional electrophoresis (2-DE) analysis were solubilized by mixing $\sim 5 \times 10^7$ nuclei/ml in isoelectric focusing (IEF) sample buffer (8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 1% DTT, 0.8% 2-D pharmalytes, pH 3–10, and 8 mM PMSF), and these samples were incubated and centrifuged as described above. Bromphenol blue (0.01%, wt/vol) was added to the samples before IEF.

Production of Human Anti-Dystrobrevin Antibodies

Polyclonal antibodies against human alpha-dystrobrevin (antibody-Dyst) were generated in rabbits by using the synthetic peptide with a terminal cysteine coupled to keyhole limpet hemocyanin, according to standard methods (Agrisera, Vännäs, Sweden). The synthetic peptide EHEQASQPTPEKAQQ, corresponding to amino acids 439–453 at the COOH-terminal of human gamma-dystrobrevin, was used to produce the antibody-Dyst. The antibody was affinity purified to obtain the total immunoglobulin fraction before use in experiments.

Gel Electrophoresis and Silver Staining

Cytosolic and nuclear proteins were resolved by SDS electrophoresis and two-dimensional electrophoresis (IEF/SDS). For the former technique, we used a 7–15% polyacrylamide gradient gel in Tris-glycine electrophoresis buffer. For 2-DE, we used an Immobilon DryStrip kit at a pH range of 3–10 and an 8–18% gradient of Exel SDS Gel (both from Amersham Biosciences AB, Uppsala, Sweden), according to the instructions of the manufacturer. Total nuclear proteins and proteins coprecipitating with gamma-dystrobrevin were resolved in the 2-DE gels and analyzed either by silver staining (Shevchenko *et al.*, 1996) or by using antibodies of interest.

Immunoblot Analysis

After SDS-PAGE or 2-DE, proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) then blocked by incubating overnight at 4°C with 3% bovine serum albumin (BSA) dissolved in PBS supplemented with 0.18% Tween 20. The membranes were incubated for 1 h at room temperature with antibodies against phosphotyrosine (IgG2bk; Upstate Biotechnology, Lake Placid, NY), dystrobrevin, actin, and myosin light chain (MLC) (both from Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 2 µg/ml in PBS containing 0.18% Tween 20, 0.35 M NaCl, and 1% BSA. The membranes were subsequently washed with PBS-Tween 20 and then incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (DAKO, Copenhagen, Denmark) diluted 1:2000. Thereafter, the filters were washed as described above, and immunoreactive bands were detected by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham Biosciences AB), according to the instructions of the manufacturer. Reprobing of the membranes, if needed, was done according to the standard ECL Western blotting protocols (Amersham Biosciences AB).

Immunoprecipitation

Protein extracts from human neutrophils and from HL-60 cells differentiated for various amounts of time with 700 nM RA were prepared as described above. Immunoprecipitation with a rabbit polyclonal antibody-Dyst was performed as follows. An aliquot of cytosolic protein from $\sim 2.5 \times 10^7$ cells was diluted 1:1 in 2× radioimmunoprecipitation assay (RIPA) buffer (2% NP-40, 2% deoxycholic acid sodium salt, 0.2% SDS, 300 mM NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM EDTA, pH 8.0, 2 mM PMSF, 4 mM Na₃VO₄, 100 mM NaF, and 1 µg/ml each aprotinin, leupeptin, and pepstatin) and then precleared by tumbling for 30 min at 4°C with 20 µl of a 50% slurry of protein A-agarose beads (washed with PBS) according to the manufacturer (Santa Cruz Biotechnology). The beads were removed by centrifugation (10-s pulse in a microcentrifuge), and the precleared extracts were incubated 2–3 h at 4°C with 4 µg of antibody. Thereafter, a 20-µl aliquot of 50% protein A-agarose beads was added, and the samples were tumbled for 1 h at 4°C. The beads were subsequently collected by centrifugation and washed five times with 1 ml of 1× RIPA buffer containing protease inhibitors (as described above).

To perform immunoprecipitation from nuclear fractions, the nuclei from $\sim 2.5 \times 10^7$ cells were solubilized in 1× RIPA buffer containing protease inhibitors and benzonase (Benzonase Pure Grade; Merck) at a final concentration of 2.5 U/ml. The lysates were further incubated for 1 h on ice and then centrifuged at $15,000 \times g$ for 30 min at 4°C. The subsequent immunoprecipitation steps were as described above. For 2-DE analysis, the immunoprecipitates were resuspended in IEF sample buffer, incubated for 30 min at room temperature, centrifuged as described above, and then subjected to IEF. Using preimmune serum instead of antibodies in immunoprecipitation experiments served as a negative control.

In-Gel Tryptic Digestion and Matrix-assisted Laser Desorption Ionization/Time of Flight (MALDI-TOF) Mass Spectrometry

Proteins from proliferating HL-60 cells, RA-treated differentiating HL-60 cells, or human neutrophils were immunoprecipitated, and those that coprecipitated with gamma-dystrobrevin were resolved by 2-DE and then visualized by silver staining (Shevchenko *et al.*, 1996). Gel areas that were deemed to be of interest were cut out and subjected to overnight in-gel tryptic digestion (Shevchenko *et al.*, 1996; Anderson, 1998). Briefly, the gel slices were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini; Eppendorf - 5 Prime, Boulder, CO). The protein spot was rehydrated in 20 µl of 25 mM ammonium bicarbonate, pH 8.3, containing 20 µg/ml modified trypsin (Promega, Madison, WI). Once this solution was fully absorbed by the gel, trypsin-free buffer was added just enough to cover the slice, and the samples were incubated overnight at 37°C. The tryptic peptides were subsequently extracted from the gel slices as follows. Any extraneous solution remaining after the digestion was removed and placed in a fresh tube. The gel slices were first subjected to an aqueous extraction and then to organic extraction with 5% trifluoroacetic acid in 50% acetonitrile, shaking occasionally. The digestion and extract solutions were then combined and evaporated to dryness. For MALDI-TOF analysis, the peptides were redissolved in 3 µl of 30% acetonitrile and 0.01% trifluoroacetic acid and were then prepared with a matrix (α -cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed on a Voyager MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) and externally calibrated using synthetic peptides with known masses. The spectra were obtained in the positive ionization mode at 25 kV. The mass information generated from the composite spectrum was submitted to a search performed with the Protana or EXPASY database, using the MS-Fit algorithm or the PeptIdent search engine.

Immunofluorescence Labeling of Cells

Proliferating HL-60 cells, HL-60 cells differentiated for 24 and 96 h with 700 nM RA, or human neutrophils were washed with PBS, pH 7.6, after which 10 nM formyl-methionyl-leucyl-phenylalanine was added to induce polarized cell morphology. The cells were subsequently incubated for 2–3 min, and then medium was replaced with fresh 4% paraformaldehyde in PBS (pH 7.3). Unstimulated cells were treated in a similar manner, but were not exposed to formyl-methionyl-leucyl-phenylalanine. After the indicated treatment, the cells were fixed for 20 min at room temperature, washed four times with PBS, and then incubated for 1 h at room temperature in blocking buffer (PBS containing 1% BSA, 1 mM EGTA, and 10% goat serum; DAKO, Copenhagen, Denmark). The cells were subsequently incubated for 1 h with antibody-Dyst (10 µg/ml) diluted in blocking buffer and then washed 3 × 5 min with blocking buffer, and incubated for 1 h with secondary Alexa 488-coupled goat anti-rabbit Fab fragments (Molecular Probes, Eugene, OR) at a concentration 15 µg/ml. The F-actin network was labeled by incubating for 30 min with Alexa 594-phalloidin in blocking buffer as suggested by the manufacturer (Molecular Probes). Thereafter, the cells were allowed to adhere on microscope slides coated with poly-L-lysine (Sigma-Aldrich) for 30 min in a wet chamber and then mounted in ProLong mounting medium (Molecular Probes).

Confocal Laser Scanning Microscopy

A Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) was used to localize human dystrobrevin and/or F-actin in both proliferating and differentiated HL-60 cells, as well as in human neutrophils. Fluorescent markers, filters, and thresholds were combined to minimize bleeding between the red and green channels of the microscope. The microscope was

equipped with an Argon ion laser operated at 10–15 mW with 30% efficiency. A wavelength of 488 nm was selected with an interfluorescence filter, the dichroic beam splitter was adjusted to 565 nm (B/S 565), and the second beam splitter was set at 510 nm (DRLP 510). Red and green fluorescinated probes were respectively detected with a 600 EFLP and a 530 DF detector filter. ImageSpace Software (Molecular Dynamics) installed on Silicon Graphics 4D/25 Personal Iris, Iris Indigo, and O2 workstations (Silicon Graphics, Mountain View, CA) was used to run the microscope and to collect data.

RESULTS

Nuclear Protein Patterns in Proliferating and RA-Differentiating HL-60 Cells

To investigate changes in the nuclear protein expression pattern and specific tyrosine phosphorylation in proliferating HL-60 cells and HL-60 cells that had undergone RA-induced differentiation for 30 min, total nuclear proteins were fractionated by 2-DE and then silver stained or transferred to PVDF Immobilon-P membranes. Considering the tyrosine phosphorylation pattern of the transferred proteins (Figure 1, pY), we found the largest variation for proteins with an acidic pI. The total amount of proteins did not change significantly after the brief RA-induced differentiation (Figure 1, silver). However, we did observe a difference between differentiating (RA 30 min) and proliferating HL-60 cells in regard to tyrosine-phosphorylated proteins detected in the nuclei. Some of these proteins appeared in the nucleus early in differentiation (30-min RA treatment) and remained tyrosine-phosphorylated during the precommitment stage of differentiation (up to 24 h; data not shown). Two groups of tyrosine-phosphorylated proteins could be identified: those that were tyrosine phosphorylated in the nuclei of both proliferating and RA-differentiated cells (Figure 1, small arrows); those that were not tyrosine phosphorylated in the nuclei of proliferating cells but were phosphorylated in the nuclei of cells exposed to RA for 30 min (Figure 1B, circles). The 2-DE patterns of silver-stained and tyrosine-phosphorylated nuclear proteins seen in Figure 1 suggest that a majority of the proteins that differed in regard to tyrosine phosphorylation were present in similar amounts in control and differentiating cells. This agrees with the time-dependent modification of tyrosine-phosphorylated proteins we observed in the nuclei of the differentiating HL-60 cells. Thus, 2-DE revealed qualitative and quantitative changes in the tyrosine phosphorylation patterns of proteins that were modified in the nuclei of proliferating and RA-differentiated (30 min) HL-60 cells.

Identification of Proteins Undergoing Tyrosine Phosphorylation Immediately after Onset of RA-induced Differentiation

We found >10 nuclear proteins with acidic pI that were tyrosine phosphorylated after 30 min of exposure to RA (Figure 1B). Of the modified proteins that were translocated into the nucleus after the onset of differentiation, the eight that predominated were as follows (Figure 1B): 35 kDa, pI 4.7; 40 kDa, pI 4.6; 43 kDa, pI 4.8; 45 kDa, pI 4.7; 45 kDa, pI 4.9; 50 kDa, pI 4.6; 56 kDa, pI 4.7; and 60 kDa, pI 4.9. We subjected these acidic proteins to further analysis, because they exhibited particularly extensive ty-

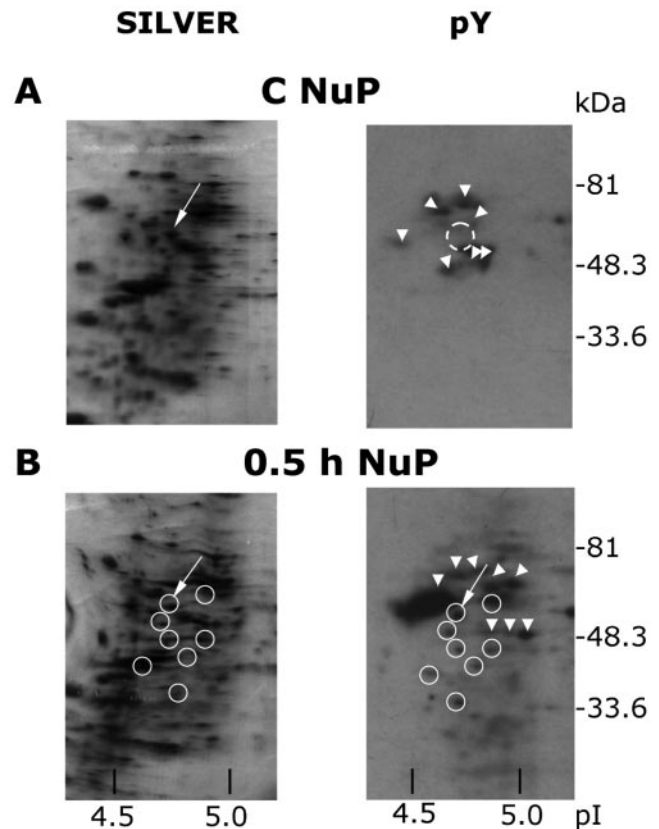


Figure 1. Two-dimensional electrophoretic patterns of total and tyrosine-phosphorylated proteins with acidic pI isolated from the nuclei of differentiating HL-60 cells. Total nuclear proteins were isolated from proliferating control cells (A) and from HL-60 cells induced to differentiate by treatment with retinoic acid (B). The proteins were subjected to 2-DE and then silver stained (silver) or blotted PVDF membranes and checked for tyrosine phosphorylation (pY). (A) Dashed circle in the pY image indicates a protein that was not tyrosine-phosphorylated in the proliferating HL-60 cells but was tyrosine-phosphorylated after 30-min treatment with RA. (B) In both images, the arrow indicates a protein that was tyrosine-phosphorylated in the nuclei after induction of differentiation and was identified as human gamma-dystrobrevin. The arrowheads in pY indicate proteins that were consistently found to be tyrosine-phosphorylated in the nuclei of both proliferating and induced to differentiation cells. The circles in the Silver and pY images show nuclear proteins that were not tyrosine phosphorylated in proliferating cells, but underwent tyrosine phosphorylation after 30-min treatment with RA. Migration of the molecular size marker proteins is indicated to the right (kilodalton values).

rosine phosphorylation. Total nuclear proteins were separated by 2-DE, and proteins of interest were cut out and subjected to tryptic in-gel digestion and mass spectrometric analysis. We subsequently focused our attention on the 56-kDa protein with a pI of 4.7 (arrow in images in Figure 1), which, with the aid of EMBL databases, was identified as human gamma-dystrobrevin, i.e., a short splice isoform of human alpha-dystrobrevin. This protein was one of the first identified and it should be emphasized that this

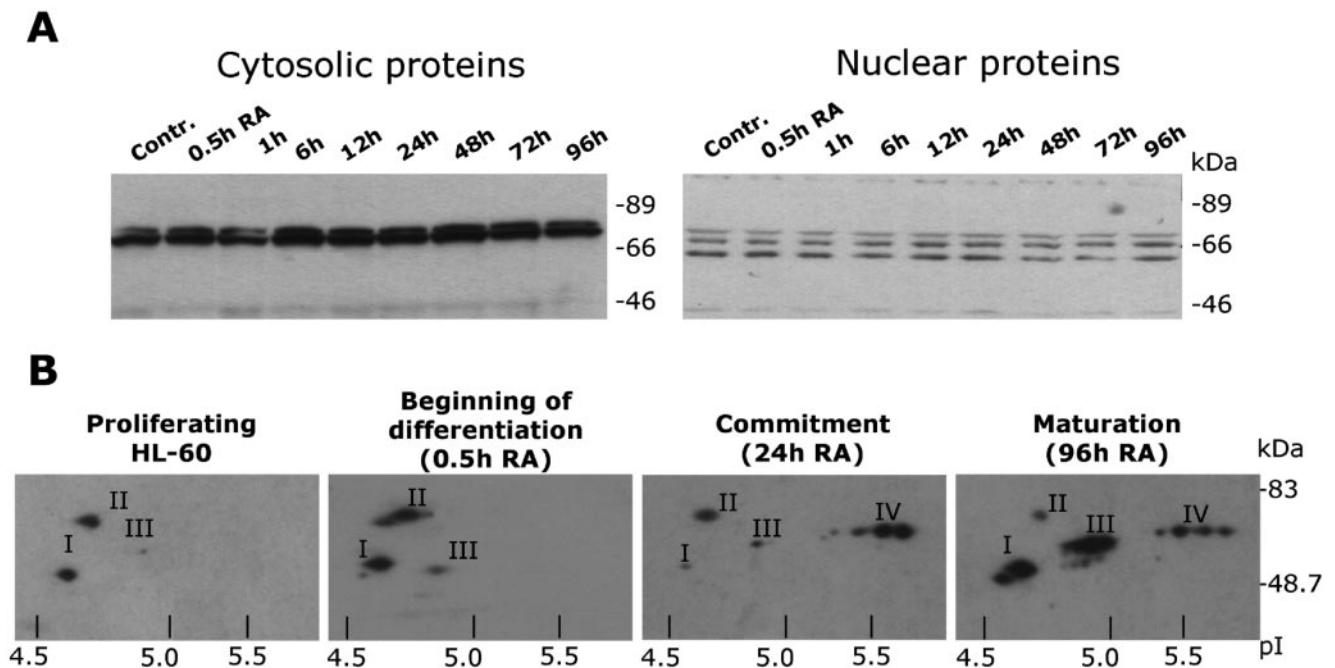


Figure 2. Expression of human alpha-dystrobrevin isoforms in HL-60 cells undergoing granulocytic differentiation. (A) Cytosolic and nuclear proteins were isolated from proliferating and differentiating (0.5–96 h) HL-60 cells as described. The proteins were fractionated by SDS-PAGE on a 7–15% acrylamide gel gradient and transferred on to Immobilon PVDF membrane. The membranes were analyzed with polyclonal antibodies against human alpha-dystrobrevin and developed with an enhanced chemiluminescence detection system. (B) Total nuclear proteins were isolated from proliferating and differentiated (RA for 30 min, 24 h, and 96 h) HL-60 cells. The isolated proteins were fractionated by 2-DE, blotted on PVDF membranes, and analyzed with polyclonal antibodies against human alpha-dystrobrevin-gamma. The numerals I–IV denote different isoforms of human alpha-dystrobrevin detected in the nucleus of the HL-60 cells. Migration of the molecular size marker proteins is indicated to the right (kilodalton values).

particular protein belonged to the group of proteins that were not tyrosine phosphorylated in the proliferating HL-60 cells (dashed circle in Figure 1A, pY) but were tyrosine phosphorylated after only 30 min of treatment with RA (arrow in Figure 1B, pY).

Characterization of Human Alpha-Dystrobrevin Expression during Granulocytic Differentiation of HL-60 Cells

Cytoplasmic and nuclear proteins were isolated from HL-60 cells that were either proliferating (control) or differentiating (RA for 30 min to 96 h). The total expression of cytoplasmic and nuclear alpha-dystrobrevin and its splice isoforms was assessed using the produced rabbit polyclonal antibody-Dyst (Figure 2A). Analyzing the cytosolic samples, the antibody revealed two closely migrating bands with molecular masses of ~75–85 kDa. Moreover, a comparable faint band of ~45 kDa was also detected. In contrast, in the nuclear fraction we identified three to five distinct isoform proteins that varied in size from 55 to 75 kDa. In addition, the antibodies detected two faint bands ~45 and 95 kDa (Figure 2A). The levels of alpha-dystrobrevin in the cytosolic and nuclear fractions were essentially constant during both proliferation and granulocytic differentiation.

To study expression of alpha-dystrobrevin isoforms in the nucleus in greater detail, we analyzed total nuclear proteins by 2-DE of proteins transferred to PVDF membranes. HL-60 cells were examined during proliferation and during the three main stages of granulocytic differentiation, i.e., at 30 min and 24 and 96 h of treatment with RA, respectively, representing the beginning, commitment, and maturation stages. The antibody-Dyst recognized four distinct proteins (I–IV) that varied in size (50–80 kDa) and displayed different pI values (Figure 2B). The relative expression also fluctuated during granulocytic differentiation. Alpha-dystrobrevin isoform I (Figure 2B) was present in the nuclei of both proliferating and differentiated cells, and the largest relative amount of alpha-dystrobrevin isoform I was observed in terminally differentiated granulocytes (Figure 2B, maturation). The relative amount of isoform II was nearly invariable in proliferating and differentiating cells with the exception of higher expression at the beginning of differentiation (Figure 2B, 0.5-h RA). In contrast, the amount of alpha-dystrobrevin isoform III was negligible during proliferation, at the beginning of differentiation, and during the commitment stage, whereas the level increased dramatically during granulocytic maturation. Finally, isoform IV was detected only at the commitment and maturation stages, and its pI values were distinct from isoforms I–III.

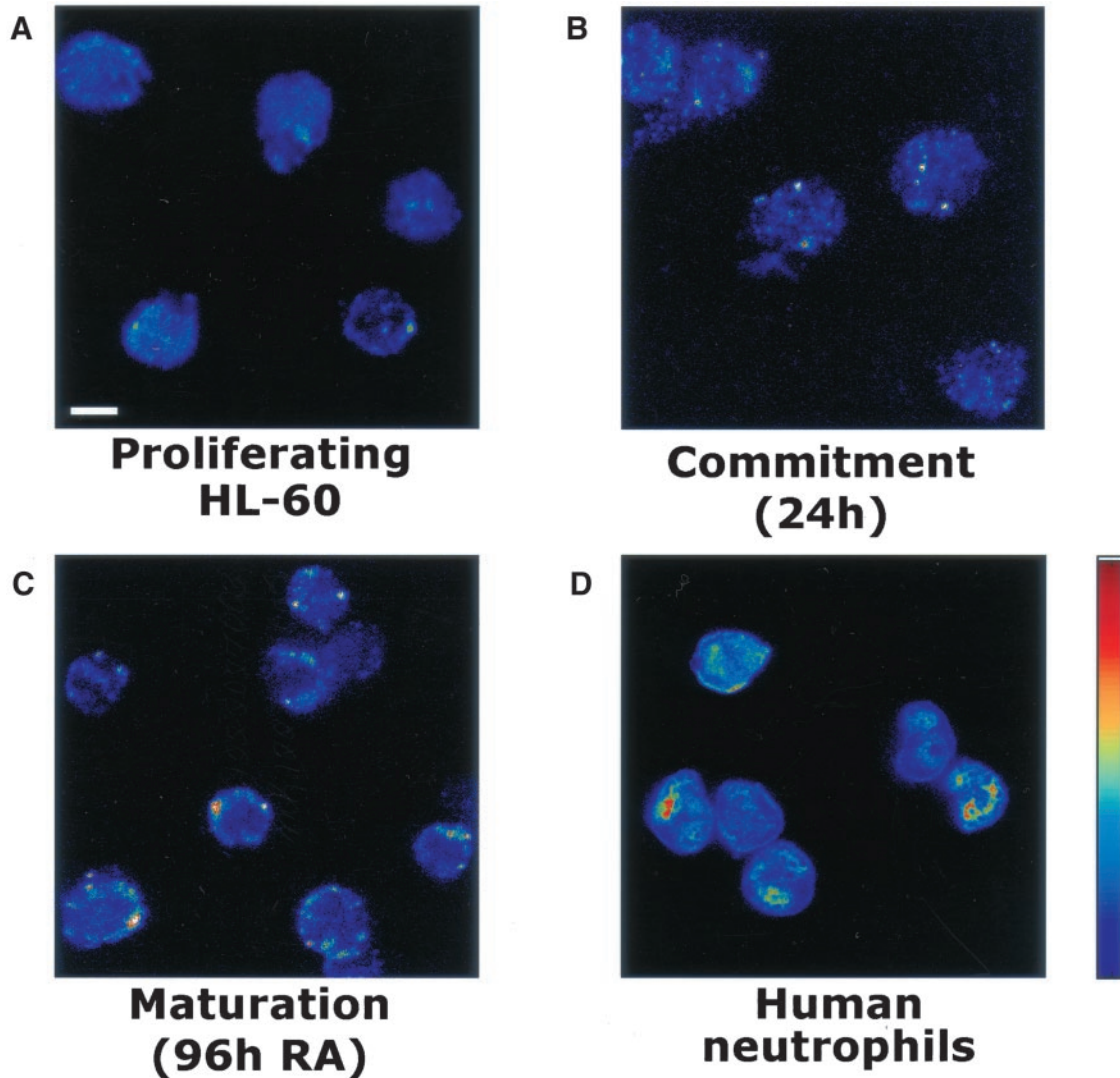


Figure 3. Distribution of human dystrobrevin in HL-60 cells undergoing granulocytic differentiation and in human neutrophils. The confocal images show the localization of human dystrobrevin in proliferating HL-60 cells (A), HL-60 cells at the commitment stage of differentiation (B; after 24-h treatment with RA), mature granulocytes (C; after 96-h RA), and peripheral neutrophils isolated from human blood (D). All cells were fixed and labeled as described in MATERIALS AND METHODS, and the images were obtained under identical conditions. To visualize fluorescence from entire cells, some areas in the images were allowed to saturate. The color scale is presented on the left. Scale bar, 5 μm .

Distribution of Alpha-Dystrobrevin in Differentiating HL-60 Cells and in Human Neutrophils

To determine the localization of human alpha-dystrobrevin during granulocytic differentiation, we labeled HL-60 cells in the three main stages of differentiation and neutrophils from peripheral blood with antibody-Dyst and then examined them in a confocal microscope. We found that the distribution of alpha-dystrobrevin changed in the HL-60 cells during the various stages of differentiation, seen as distinct staining alternating between the plasma membrane, the cytoplasm, and the nucleus (Figure 3, A–C). Particularly in the proliferating cells, the protein

seemed to be dispersed throughout the entire cell volume. During the commitment stage, alpha-dystrobrevin seemed to be more concentrated adjacent to the cell membrane, although the pattern was still diffuse (Figure 3B). However, after 96 h of differentiation (i.e., in granulocyte-like cells), clusters were clearly visible at the cellular and nuclear membranes. Interestingly, we observed obvious dissimilarities in dystrobrevin immunostaining between the isolated normal neutrophils and the HL-60 cells undergoing induced differentiation *in vitro* (Figure 3D): both types of cells exhibited staining close to the cell and nuclear membrane, but only the neutrophils showed especially strong dystrobrevin immunoreactivity in the nuclei.

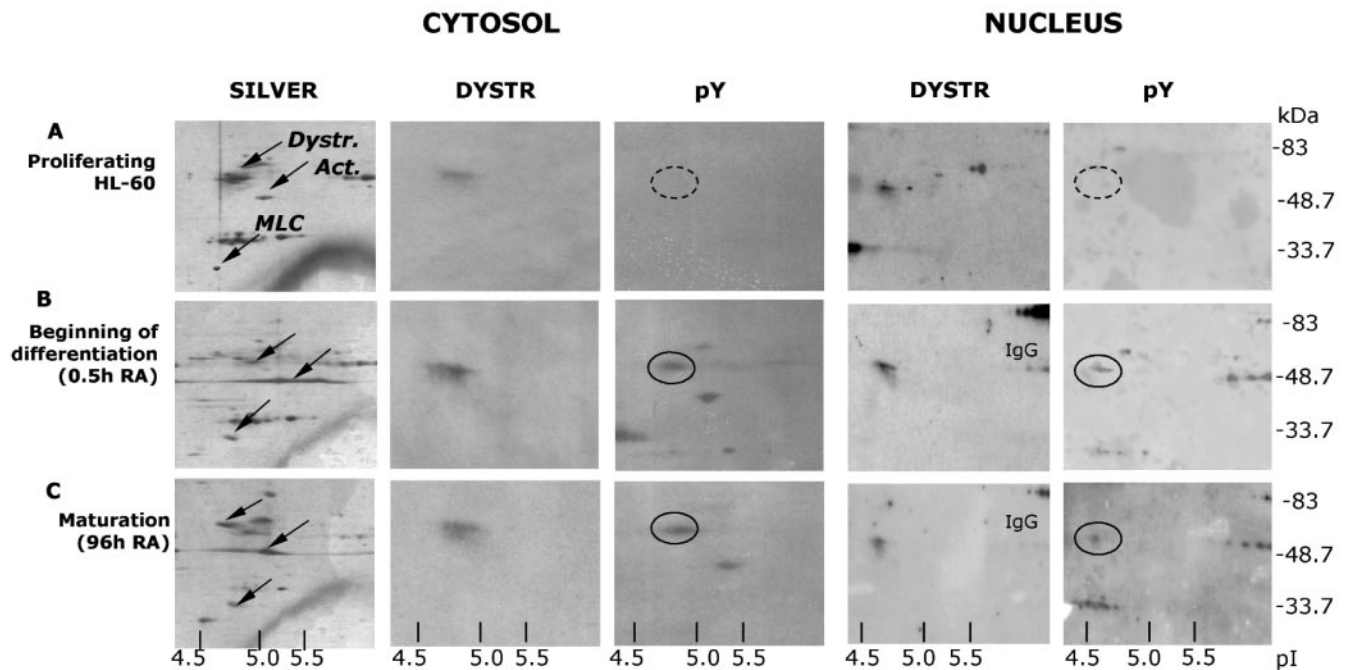


Figure 4. Human alpha-dystrobrevin-associated proteins and tyrosine phosphorylation of alpha-dystrobrevin in proliferating and differentiating HL-60 cells. (Cytosol) Cytosolic proteins were isolated from proliferating and differentiating (0.5, 96 h) HL-60 cells as described and further used for immunoprecipitation of human alpha-dystrobrevin. Coimmunoprecipitated proteins were subjected to 2-DE fractionation and then silver stained (silver) or blotted on PVDF membranes. The proteins on the membranes were detected with antibodies against alpha-dystrobrevin (Dystr) or anti-phosphotyrosine (pY). The dashed circle in A and the circles in B and C (pY) indicate the absence or presence of tyrosine-phosphorylated gamma-dystrobrevin. The membranes were subsequently reprobred with antibodies against actin and MLC. The positions of gamma-dystrobrevin (Dystr), actin (Act), and MLC are designated by arrows in the images to the left (silver). (Nucleus) Total nuclear proteins were extracted from proliferating and differentiated (RA for 30 min and 96 h) HL-60 cells and were used for immunoprecipitation of alpha-dystrobrevin as described. Coimmunoprecipitated proteins were subjected to 2-DE fractionation and then blotted on PVDF membranes. The proteins on the membranes were detected with antibodies against alpha-dystrobrevin (Dystr). The membranes were subsequently reprobred with anti-phosphotyrosine antibodies to ascertain tyrosine phosphorylation (pY). The dashed circle in A and the circles in B and C (pY) indicate the absence or presence of tyrosine-phosphorylated gamma-dystrobrevin. IgG designates human immunoglobulin. Migration of the molecular size marker proteins is indicated to the right (kilodalton values).

Human Alpha-Dystrobrevin Phosphorylations and Protein Associations In Vitro during Differentiation of HL-60 Cells

HL-60 cells cytosolic and nuclear extracts (Figure 4) were prepared and analyzed by immunoprecipitation to delineate phosphorylations and proteins binding patterns. Again, we examined HL-60 cells in the two stages of granulocytic development (at onset of differentiation, after 30 min, and during maturation, 96 h), by using proliferating HL-60 cells as controls. The precipitated complexes were resolved by 2-DE. In SDS-PAGE, at least one 50- to 55-kDa isoform of alpha-dystrobrevin was obscured by the IgG heavy chains in the immunoprecipitated complexes, so we performed 2-DE to improve detection of the dystrobrevin.

The dystrobrevin-binding proteins from cytosol were either visualized by silver staining (Figure 4, cytosol and silver) or transferred to PVDF membranes for Western blotting (Figure 4, cytosol, Dystr, and pY). Silver staining of cytosolic fraction revealed several distinct proteins. Some of the proteins in the immunoprecipitated complexes displayed divergent expression patterns. These were cut out of the 2-DE gel and analyzed by MALDI-TOF. Searches in the

EXPASY database using the PeptIdent search engine resulted in definite identification of two human alpha-dystrobrevin-associated proteins: one was 43 kDa (pI of 5.1) and the other 20 kDa (pI of 4.7), corresponding to human actin and MLC, respectively. After that, we confirmed with Western blotting the presence of actin or MLC (data not shown). The positions of actin and MLC during granulocytic differentiation determined by antibodies are shown in Figure 4 (silver, arrows). Moreover, a number of attempts are being made to identify other proteins visible after IP-experiments on 2-DE silver-stained gels.

Analysis of immunoprecipitates with polyclonal antibody against dystrobrevin showed that the cytosol fraction was enriched with one of the alpha-dystrobrevin isoforms in size corresponding gamma-dystrobrevin (Figure 4, cytosol and pY). Reprobred the same membrane with antiphosphotyrosine antibodies confirmed gamma-dystrobrevin tyrosine phosphorylation (Figure 4, cytosol and pY).

The dystrobrevin-binding proteins from nuclear fraction were also visualized either by silver staining (data not shown) or they were transferred to PVDF membranes for Western blotting (Figure 4, nucleus, Dystr, and pY). In con-

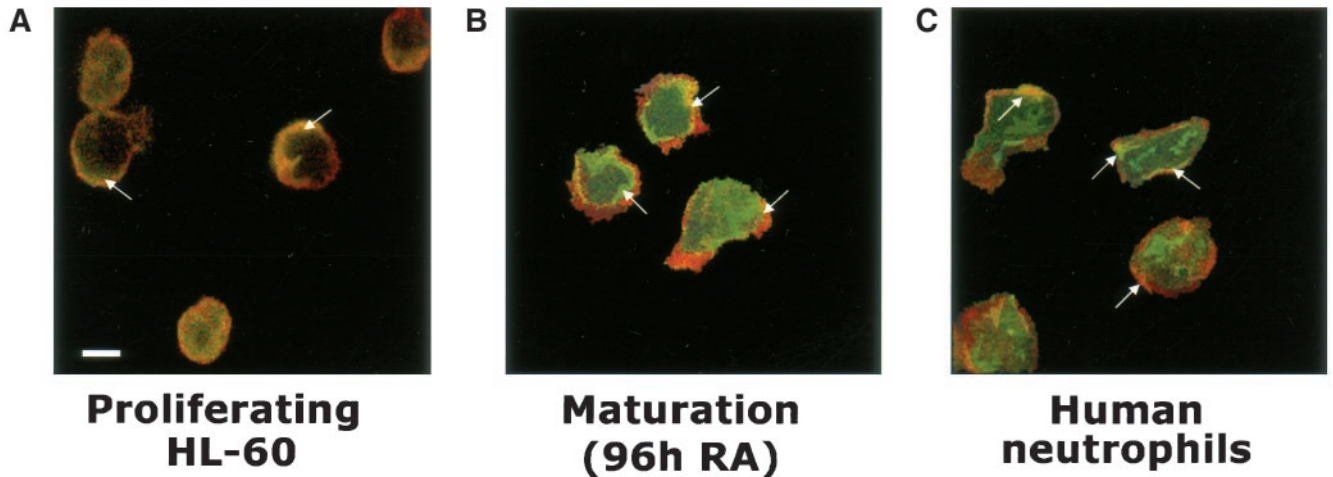


Figure 5. Double-labeling of human dystrobrevin and F-actin in HL-60 cells undergoing granulocytic differentiation and in neutrophils from adult humans. The confocal images show colocalization of human dystrobrevin and F-actin in proliferating HL-60 cells (A), mature granulocytes (B, 96 h with RA), and human neutrophils (C). Cells were treated and visualized as described in the legend of Figure 3. F-actin was stained with Alexa 594-phalloidin. Sections were $\sim 0.6 \mu\text{m}$ in thickness, and the images were obtained under identical conditions. Scale bar, $5 \mu\text{m}$.

trast to immunoprecipitated proteins in cytosol fraction, there were two to three antibody-Dyst reactive spots. A 55-kDa spot (pI of ~ 4.7) was present at all stages of differentiation (Figure 4, nucleus and Dystr). A stretched spot in size ~ 95 kDa (pI of ~ 6.0) was detectable in proliferating cells, at the beginning of HL-60 cell differentiation and at the maturation stage (Figure 4, nucleus A–C and Dystr); and its highest expression level was observed at the beginning of differentiation. Additional spots sizing ~ 70 kDa (pI of 5.7) and 44 kDa (pI of 4.2) were precipitated with antibody-Dyst in the control cells (Figure 4, nucleus A and Dystr). Reprob-ing the same membranes with anti-phosphotyrosine antibodies confirmed tyrosine phosphorylation of gamma-dystrobrevin isoform (as indicated by EMBL database) in HL-60 cells during granulocytic differentiation. More specifically, the tyrosine phosphorylation of the gamma-dystrobrevin isoform (50–60 kDa, pI of ~ 4.6) in differentiating cells (circles in Figure 4, nucleus B and C and pY) was not detected in proliferating control cells (dashed circle in Figure 4, nucleus A and pY). In addition, the 95-kDa spot, reacting with anti-dystrobrevin antibodies, was tyrosine phosphorylated in the beginning of differentiation and in the maturation stage.

Association between Human Alpha-Dystrobrevin Isoforms and F-actin in HL-60 Cells and Neutrophils

To study sites of colocalization of alpha-dystrobrevin and actin, we stained granulocytes differentiated *in vitro* and normal human neutrophils with the antibody-Dyst and the fluorescent anti-rabbit secondary antibody (Figure 5, A–C, green channel). The actin network was labeled with Alexa 594-phalloidin (Figure 5, red channel). However, a high-resolution microscopic analysis of the distribution of cytoskeletal proteins is hampered by the small HL-60 and

neutrophil cell size. Anyway, alpha-dystrobrevin was partly enriched at the outer envelope of the nuclei in both the mature granulocytes and the human neutrophils (Figure 5, B and C). The subcellular distribution of actin and alpha-dystrobrevin was similar and displayed colocalization at the membrane, coupled with immunolabeling of dystrobrevin around the nucleus (arrows in Figure 5). In B and C, there are some regions that show separate green and red fluorescence, suggesting that there are membrane domains where the proteins are close to each other but not colocalizing. Some faint nuclear labeling for dystrobrevin was also observed in proliferating HL-60 cells. Together, these results confirm previous observations and demonstrate that both *in vitro* and *in vivo*, alpha-dystrobrevin interacts with actin.

DISCUSSION

Our results demonstrate that gamma-dystrobrevin, a short isoform of alpha-dystrobrevin, is expressed in human promyelocytic HL-60 cells, and in the nucleus is modified by tyrosine phosphorylation soon after granulocytic differentiation is induced by treatment with RA. By using antibodies raised against human alpha-dystrobrevin, we found this protein in two cell compartments: the cytosol and the nucleus. We also observed distinct dystrobrevin distribution, including colocalization with actin in HL-60 promyelocytes, in HL-60 cells differentiated to mature granulocytes and in human neutrophils. Moreover, we found actin and myosin light chain associated with human alpha-dystrobrevin.

Dystrophin-related and DAPCs are believed to play an important role in the stability and maintenance of the plasma membrane during muscle contraction and relaxation, and they have also been implicated in cell signaling events (Petrof *et al.*, 1993). These proteins are ideally placed to transduce signals from the extracellular matrix to the cytoskeleton and there is evidence of direct phosphorylation of dystrophin and the

DAPC proteins (Luise *et al.*, 1993; Shemanko *et al.*, 1995; Walsh *et al.*, 1995). It has been reported that alpha-dystrobrevin transcripts are abundant in skeletal muscle, heart, and brain, and smaller amounts are present in lung, liver, and pancreas. In addition, Enigk and Maimone (1999) found that alternatively spliced transcripts are differentially expressed and developmentally regulated during muscle cell differentiation. The presence of multiple isoforms with different tissue specificities suggests that dystrobrevin has several functions (Sadoulet-Puccio *et al.*, 1997; Newey *et al.*, 2001). Gieseler *et al.* (1999) have proposed that dystrophin and dystrobrevin participate in the same biological actions, because they found that disruption of these proteins led to identical phenotypes in the nematode *Caenorhabditis elegans*.

Our experiments using the anti-alpha-dystrobrevin antibody in immunoblotting of cytoplasmic and nuclear proteins fractionated by SDS electrophoresis indicated that there are three isoforms of this dystrobrevin in the cytoplasm and three to four in the nucleus. The new antibody was raised against an epitope present in almost all splice isoforms of alpha-dystrobrevin, because these isoforms share an exceptionally high homology. Therefore, in all the experiments using this antibody, a number of isoforms could have been detected. More detailed immunanalysis of the nuclear alpha-dystrobrevin showed that there were the relative amounts of the individual isoforms depended on the stage of differentiation of the cell. This suggests that there are different alpha-dystrobrevin isoforms in the cytoplasm and nucleus of HL-60 cells, which in turn implies that these isoforms possess functional rather than structural properties. Furthermore, the observed differentiation-dependent changes in alpha-dystrobrevin isoforms might be explained in terms of altered splicing events. Thus, the differences between detected isoforms could easily be splice mediated, and phosphorylation may be causing the multiple spots observed for I, II, and IV isoforms during granulocytic differentiation of HL-60 cells.

Additionally, the molecular weights of all the known alpha-dystrobrevin isoforms can be calculated from the published sequences (Blake *et al.*, 1996; Sadoulet-Puccio *et al.*, 1996). Accordingly, the isoform II could be likely alpha-dystrobrevin (dystrobrevin-1), isoform I as gamma-dystrobrevin (dystrobrevin-2), and isoform III and isoform IV as beta-dystrobrevin. Moreover, we are presently working on precise determination of nuclear alpha-dystrobrevin isoforms.

Immunostaining of rat and mouse skeletal muscle with alpha-dystrobrevin-specific antibodies has established that the protein is localized to the sarcolemma and there are higher concentrations at the neuromuscular junctions, which suggests that it has a functional role at both sites (Blake *et al.*, 1996; Grady *et al.*, 2000). Moreover, in our study, alpha-dystrobrevin was associated with the nuclear membrane in HL-60 cells. Judging from the difference in fluorescence between the cytoplasm and the nucleus, dystrobrevin seems to accumulate both in the nucleus and on the cell membrane. Inasmuch as dystrobrevin shows widespread localization along the cell and nuclear membranes and is clustered in differentiating HL-60 cells, this protein may also play an important role in human neutrophils. The increase in alpha-dystrobrevin we observed around/in the nuclei of motile human neutrophils is definitively intriguing.

Little is known about the nuclear dystrophin complex, and it is unclear whether it has structural and/or signaling proper-

ties. Of interest in this context, a beta-dystrobrevin has been found in the nuclei of hippocampal pyramidal neurons (Blake *et al.*, 1999). In addition, González *et al.* (2000) examined the subcellular distribution of the dystrophin Dp71 different splicing isoforms and concluded that it determines the nuclear or cytoplasmic distribution of Dp71. According to Blake *et al.* (1999) there is an association between Dp71 and dystrobrevin, but Dp71 cannot act as a functional substitute for full-length dystrophin. These findings raise the possibility that a DAPC-like complex exists in the nucleus, but the role of DAPC proteins in that location requires further investigation.

Using MALDI, we continued our analysis of proteins that coprecipitated with dystrobrevin, and we discovered two cytoskeletal proteins associated with alpha-dystrobrevin in the granulocytes differentiated *in vitro*. These proteins were identified as human actin and myosin light chain. Again, the DAPC has chiefly two types of functions, that is, it plays a mechanical role and a signaling role. Mechanical models comprise the actin–dystrophin–dystroglycan–laminin axis, which suggests a mechanical link between the cytoskeleton and the extracellular matrix (reviewed by Roberts, 2001). The signaling role is implied by the fact that occurrence of tyrosine phosphorylation of dystrobrevin is specific to the stage of differentiation.

It is now known that there is an indirect interaction between dystrobrevin and F-actin via dystrophin. Therefore, the interaction with MLC, most likely also indirect, seems interesting in a light of the motile capacity of HL-60 derived granulocytes or human neutrophils. The cytoskeleton is a vital factor determining cell shape, it serves as a scaffold for essential cellular enzymes and adhesion molecules, and it provides structural integrity to the cell and regulates many important biochemical events. In neutrophils and other motile cells, polymerization of actin likely drives extension of the lamellae and generates the force underlying this effect by allowing interaction with myosin, polymerization *per se*, and osmotic mechanisms (reviewed by Torres and Coates, 1999). In recent years, it has become clear that the actin cytoskeleton participates in regulation of many other processes, such as receptor affinity, apoptosis, the cell cycle, and signaling to the nucleus. The F-actin specialization may also provide a mechanism for anchorage of signaling molecules, as evidenced by the finding that assembly of this protein is necessary for accumulation of phosphotyrosine (reviewed by Dai *et al.*, 2000). Nonmuscle myosin is an ATPase that forms an enzymatically active complex with actin, and it has been shown that this enzyme provides energy for cell and organelle movement and maintains the structural organization of the cytoskeleton (Korn, 1978). Myosins are capable of either translocating actin filaments or translocating vesicles or other cargo on actin filaments. Apart from the well-characterized role of conventional myosins in muscle contraction, members of the myosin superfamily are involved in a number of cellular functions, including membrane trafficking, cell motility, cytokinesis, organelle transport, and signal transduction (Mermall *et al.*, 1998).

In the present report using confocal laser scanning microscopy, we have been able to demonstrate colocalization of alpha-dystrobrevin and actin in proliferating HL-60 cells, mature granulocytes differentiated *in vitro*, and normal human neutrophils. It should be noted that the apparent fluorescence intensity in a particular region of the cell is not related to the relative amount of fluorophore in the $\sim 0.6\text{-}\mu\text{m}$ -thick confocal section. In conventional fluorescence microscopy, cell thickness

can be a problem when studying colocalization, because different fluorescent proteins can appear to be in the same position even although they are actually separated along the Z axis (reviewed by Torres and Coates, 1999). In conclusion, there is evidence that alpha-dystrobrevin may play a significant morphological and/or physiological role in phagocytic function.

It is now generally accepted that phosphorylations and dephosphorylations of proteins are important factors in regulation of essentially all cellular functions (Graves and Krebs, 1999), because they modulate the intrinsic biological activity, subcellular location, and half-life of individual proteins, and also control docking with other proteins and cellular metabolism in general. The state of phosphorylation of a protein is a flexible mechanism for reversibly altering conformation and function (Cohen, 2000).

The C terminus of dystrobrevin has four potential tyrosine phosphorylation sites, and it is highly similar to the cysteine-rich C-terminal end of dystrophin. Not all splice variants of dystrobrevin contain the phosphotyrosine tail, and differential splicing confers tissue-specific sensitivity to extracellular signals mediated by tyrosine kinases and plays a role in regulating subcellular distribution (Balasubramanian *et al.*, 1998). The cited investigators found that dystrobrevin isoforms that do contain the phosphotyrosine tail seem to be synapse specific. The 62-kDa isoform that lacks the phosphotyrosine tail is widely expressed, whereas the 90- and 97-kDa isoforms are restricted to brain and muscle. Obviously, the presence of phosphotyrosines in the sequence of dystrobrevin makes this protein an excellent candidate for regulation by tyrosine phosphorylation. It is still not known what signaling events are blocked by tyrosine phosphorylation in muscle, although reports have indicated that alpha-dystrobrevin-1 may be phosphorylated by a tyrosine kinase of the src-family (Nawrotzki *et al.*, 1998). Concerning other DGC proteins, Ilsley *et al.* (2001) have shown that beta-dystroglycan is tyrosine-phosphorylated in muscle cells and that such phosphorylation is important for regulation of the interaction between dystrophin and beta-dystroglycan.

Our analysis of proteins coprecipitating with human alpha-dystrobrevin showed that gamma-dystrobrevin isoform (58 kDa, pI of 4.6) was tyrosine-phosphorylated soon after HL-60 cells were treated with RA, and this phosphorylated state was maintained until the cells became mature granulocytes. We therefore suggest that RA-induced tyrosine phosphorylation modulates the affinity of this dystrobrevin isoform for other associated proteins, possibly resulting in different abilities of the proteins to participate in cellular signaling.

Signaling models of dystrobrevin function comprise the predominance of associations with molecules whose main role is in communication. Early biochemical studies demonstrated that alpha-dystrobrevin was associated with dystrophin and syntrophin (Butler *et al.*, 1992; Wagner *et al.*, 1993), and later investigations revealed additional interaction with utrophin (Nawrotzki *et al.*, 1998; Peters *et al.*, 1998) and the sarcoglycan-sarcospan complex (Yoshida *et al.*, 2000). It is assumed that the three-syntrophin isoforms act as modular adapters that recruit signaling proteins to the sarcolemma via their interaction with the dystrophin complex. Froehner *et al.* (1997) and other researchers (Brenman *et al.*, 1996; Peters *et al.*, 1997; Gee *et al.*, 1998) have found that syntrophins bind the following important signaling proteins: nNOS, voltage-activated sodium chan-

nels, aquaporins, stress-activated kinase-3, and microtubule-associated serine-threonine kinase.

Although we now have significant biochemical, cell biological, and genetic information on dystrophins, dystrobrevins, and their "collaborators," the exact functions of these molecules remain unclear (reviewed by Roberts, 2001). More recent studies have implicated the DAPC in signaling events, thus this complex now seems to be more dynamic than previously assumed. In addition, the interaction of dystrobrevin with other dystroglycan complex proteins may facilitate the transduction of signals between the DAPC and extracellular proteins and other signaling pathways. With this in mind, we are currently examining possible interactions of alpha-dystrobrevin with additional dystrophin-complex-related proteins. Our data also give some evidence of the effects of the association of this protein with the DAPC and the potential involvement of alpha-dystrobrevin in signal transduction in myeloid or phagocytic cells.

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