alpha-Dystrobrevin distribution and association with other proteins in human promyelocytic NB4 cells treated for granulocytic differentiation

Veronika Borutinskaité, Karl-Eric Magnusson and R Navakauskiene

Veronika Borutinskaité, Karl-Eric Magnusson and R Navakauskiene, alpha-Dystrobrevin distribution and association with other proteins in human promyelocytic NB4 cells treated for granulocytic differentiation, 2011, MOLECULAR BIOLOGY REPORTS, (38), 5, 3001-3011.
http://dx.doi.org/10.1007/s11033-010-9965-9

Alpha-Dystrobrevin distribution and association with other proteins in human promyelocytic NB4 cells treated for granulocytic differentiation.

V.V. Borutinskaite[a,b]*, R. Navakauskiene[a], K.-E. Magnusson[b]

[a] Department of Developmental Biology, Institute of Biochemistry, LT-08662 Vilnius, Lithuania;

[b] Division of Medical Microbiology, Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden.

Footnotes

*Corresponding author: V.V. Borutinskaite, Department of Developmental Biology, Institute of Biochemistry, LT-08662 Vilnius, Lithuania. Voice: +37052729327, fax: +37052729196, e-mail: verbu@imk.liu.se

Keywords:

Alpha-dystrobrevin, Histone deacetylase inhibitor, BML-210, retinoic acid, granulocytic differentiation.

Abbreviations used:

α-DB, alpha-Dystrobrevin; ATRA, all-trans retinoic acid; HDACI, histone deacetylase inhibitor.

Abstract

Dystrobrevins bind directly to dystrophin and are prominent components of the dystrophin-associated protein complex that links the cytoskeleton to the extracellular matrix. They are involved in brain development, synapse formation and plasticity, as well as water and ion homeostasis. However, the role of dystrobrevins in non-muscular cells is not clear. In this study, we show that different alpha-dystrobrevin isoforms are present in promyelocytic leukemia (NB4) cells. Only the biggest alpha-dystrobrevin isoform (DB-alpha), which can be important for its function, was expressed in the membrane fraction of NB4 cells; the other α-DB isoforms were found in the hydrophilic cell fractions. Employing the immunoprecipitation and mass spectrometry, we identified novel α-DB-interacting proteins involved in cytoskeleton reorganization (actin, tropomyosin, gelsolin, tubulin) and signal transduction process (stathmin, prohibitin, RIBA) during proliferation and differentiation of NB4 cells.

Our results suggest that α-DB isoforms play a central role in cytoskeleton reorganization via their multiple interactions with actin and actin-associating proteins and may participate in signal transduction process during NB4 cell granulocytic differentiation via directly and non directly associated proteins.

Introduction

The dystrophin-associated protein complex (DAPC) is expressed in muscular and non-muscular tissues. It is considered both as a mechanical component of cells linking the cortical actin cytoskeleton to the extracellular matrix, and a highly dynamic multifunctional structure that can serve as a scaffold for the interaction of signaling proteins [1]. Moreover, mutations in genes encoding any of the ten DAPC components or DGC-related genes lead to muscular dystrophies in humans or animal models. These include α-, β-, γ-, and δ-sarcoglycan, α-dystrobrevin (α-DB), laminin α2, and four enzymes that selectively glycosylate dystroglycan [2].

The key part of the signaling function of the dystrophin complex seems to be mediated by dystrobrevins (DB). The DBs form a family of dystrophin-associated proteins which are localized at the inner surface of cell membranes and are products of two genes: α- and β-DB. Each gene produces multiple transcripts that localize distinctly in the cell and may thus have potentially different physiological roles through interactions with other proteins.

The full role of DB in cell signaling remains to be elucidated. Targeted disruption of the α-DB gene in mice results, however, in muscular pathology but without the membrane fragility characteristic of dystrophin-deficient muscular dystrophy [3]. These findings suggest that α-DB is important for muscle function, perhaps in a non-structural way [4].

There are at least eight different α-DB isoforms generated by alternative splicing. α-DB has no enzymatic activity of its own, and its involvement in the signaling pathway is dependent on the interaction with other proteins. α-DB has several functional domains, i.e. zink-binding, syntrophin-binding and coiled-coil. Moreover, it can bind one or two syntrophin molecules that recruit nNOS, protein kinases and membrane protein receptors to the DAPC, and indeed mice lacking α-DB

display defects in NOS-mediated signaling. Through the coiled-coil domain, α-DB can interact with dystrophin and F-actin, and through its N-terminal region with the sarcoglycan complex [5]. Using immunoprecipitation and the yeast two-hybrid methodology, it has been demonstrated that α-DB can interact with desmuslin via the ROD domain, with dysbindin and syncoilin via the coiled-coil domain [6,7]. Since syncoilin can associate with desmin, this could be important for maintaining muscle fiber integrity and for linking the DAPC to the cytoskeleton [8]. It was shown that α-DB can bind to the inward rectifier potassium channel (Kir2.x) and to the kinesin heavy chain Kif5A via the ZZ domain, a role for α-DB as a motor protein receptor has been suggested [9].

It was displayed that α-DBs are expressed not only in muscular cells, but also in human epithelial cervical cancer cells (HeLa) and human promyelocytic leukemia cells (HL-60), but the functions in non muscle cells are still not clear [10,11]. Since the phosphorylation state of DB-gamma changed during the differentiation process after HL-60 cell treatment with all-trans retinoic acid (ATRA), it was suggested that this modulates the association of α-DB with other proteins, possibly resulting in a different ability of the proteins to participate in cellular signaling [11].

ATRA has been applied successfully in the treatment of acute promyelocytic leukemia (APL), and 13-cis-retinoic acid is effective against juvenile chronic myelogenous leukemia and mycosis fungoides, a cutaneous T-cell lymphoma. Combinations of ATRA with specific histone deacetylase inhibitors (HDACIs) have displayed synergistic activity in studies in vitro [12]. HDACI can affect cancer cell division and induce differentiation or apoptosis. Phenylbutyrate is used clinically in humans, and the availability of novel HDAC inhibitors warrants the pursuit of preclinical and clinical studies, particularly in combination with retinoids [13,14,15].

In the present study, we have examined α-DB expression and its possible binding partners in human promyelocytic leukemia (NB4) cells induced to granulocytic differentiation with ATRA alone and in combination with HDACI BML-210. BML-210 is a novel HDACI that was used in our previous studies. We showed that ATRA together with BML-210 increased differentiation level at earlier time pint of treatment than ATRA alone [12]. HDACI BML-210 alone after 96 h of treatment induce apoptosis in NB4 cells, but no differentiation [12]. To identify novel α-DB-interacting proteins in contral and treated NB4 cells, we used immunoprecipitation and mass spectrometry. Indeed, we identified new proteins that could be divided into several groups depending on their function. β-Actin, α/β-tubulin, tropomyosin, gelsolin, desmin and stathmin are cytoskeletal proteins involved in cell growth and/or maintenance processes. Another group (prohibitin, STAG1, RIBA and annexinA4) is responsible for signal transduction and cell communication. We have also identified HSP90, HSP70, chaperonin-60 and BiP which are engaged in protein metabolism, signal transduction and gene transcription regulation [16]. We have furthermore displayed that the α-DB sub-cellular localization changed in NB4 cells undergoing differentiation or apoptosis after treatment with ATRA and the novel HDACI BML-210. Also we determined co-localization of α-DB with F-actin and HSP90 in untreated NB4 cells. To summarize, our study has revealed that α-DBs are important for cytoskeleton reorganization via interactions with cytoskeleton proteins like actin and tropomyosin, and for cell signaling through

association with proteins being involved in signal transduction, such as RIBA, prohibitin and others.

Materials and methods

Cell culture

The human APL cell line NB4 was cultured in RPMI medium (Gibco Invitrogen, Lidingö, Sweden) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 20 mM glutamine at 37 ^{0}C in humidified air and 5% CO_2. Cultures were seeded at a density of 4×10^5 cells/ml and were (at maximum 1.5×10^6 cells/ml) subsequently transferred to a fresh medium.

Chemicals and antibodies

Retinoic acid was purchased from Sigma Chem. Co (St.Louis, MO) and added to the medium from solution of 500 µM to a final concentration of 1 µM or 10 µM. ATRA was dissolved in ethanol. BML-210 (Biomol, Plymouth Meeting, PA) was dissolved in DMSO (stock solution 10 mM) and added to the medium to a final concentration of 5 µM, 10 µM and 30 µM. Polyclonal antibodies against alpha-dystrobrevin (against unique peptide EHEQASQPTPEKAQQ corresponding to amino acids 439-453) were generated according to standard methods by Agsera (Vannas, Sweden). Horseradish peroxidase-conjugated secondary antibodies (DAKO Denmark A/S, Glostrup, Denmark) were used.

Hydrophilic and hydrophobic protein fraction preparation

Hydrophilic and hydrophobic protein fractions were isolated with the MEM-PER Eukaryotic Membrane Protein Extraction Reagent Kit according to the manufacturer's instructions (Pierce Biotech., Rockford, IL). The hydrophobic fraction contains the integral membrane and membrane-associated proteins. The hydrophilic fraction contains the soluble cell proteins.

Preparation of cytosolic and nuclear proteins

For isolation of cytosolic and intact nuclei the cells (5×10^6 to 10^7) were harvested by centrifugation at 500×g for 6 min, washed twice in ice-cold PBS. Cytosolic and nuclear protein fractions were isolated with Nuclei Isolation Kit buffer according to the manufacturer's instructions (Sigma, St. Louis, MO)

Also isolated nuclei were used for separation of hydrophilic and hydrophobic nuclear proteins by using MEM-PER Eukaryotic Membrane Protein Extraction Reagent Kit according to the manufacturer's instructions (Pierce Biotech.).

Isolation of neutrophils from human blood

The neutrophils were isolated essentially according to Boyum [17] as described by Kulyte et al. [11].

Immunoprecipitation

Immunoprecipitation with rabbit polyclonal antibodies against alpha-Dystrobrevin was performed as follows. Fifty µl of prepared Protein A/G slurry (Santa Cruz Biotechnology, Santa Cruz, CA) washed with PBS was added to the cell fraction and incubated with rotation to remove endogenous IgG at 4 ^{0}C for 30 min. Beads were pulled down by centrifugation at

10,000×g for 10 min at 4 ^{0}C, and the supernatant was transferred to a fresh Eppendorf tube. 12.5 µg of primary antibodies (anti-dystrobrevin, or irrelevant IgG) was added to the Eppendorf tube containing the cold precleared lysate and incubated at 4 ^{0}C for 4 h. One hundred µl of Protein A/G was added to the lysate and rotated at 4 ^{0}C overnight. Beads were pulled down by centrifugation at 10,000×g for 10 min at 4 ^{0}C, the supernatant was carefully removed, and beads were washed 3−5 times with 500 µl of 1× RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, pH 8.0, 0.1%SDS, 1% NP-40, 1% DOC).

2-D electrophoresis

For 2DE, the immunoprecipitated protein complexes were actively solubilised in 80 µL of IEF buffer (8M urea, 2M thiourea, 4% CHAPS, 1% DTT (dithiothreitol), 0.002% Bromphenol blue, 0.8% Pharmalyte 3-10) and in 120 µL of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 0.2% DTT, 0.002% Bromphenol blue, 0.5% Pharmalyte, pH range 4−7), and then Immobiline DryStrips strips, pH 4−7 (Amersham Biosciences, Sweden), were hydrated with the rehydration solution in a strip holder for 16 h at room temperatute. The first dimension of isoelectric focusing was performed with a 2DE Multiphor unit (Amersham Biosciences) by steps of increased voltage up to ~30 kVh.

Focusing Immobiline DryStrips were incubated for 15 min at room temperature in electrophoresis buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT, followed by 15 min in electrophoresis buffer with 2% (w/v) iodoacetamide.

The second dimension was performed in a gradient gel (8–18% ExcelGel SDS polyacrylamide). 2-DE gels were silver-stained according to Shevchenko et al. [18]. Spots were selected by visual inspection and gel slices were excised by scalpel.

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight) analysis

The gel areas of interest were cut out and subjected to in-gel tryptic digestion according to Shevchenko et al. [18,19]. For MALDI-TOF-MS analysis, samples were desalted and purified using C18zipTips (Millipore, Salna, Sweden) following the manufacturer's instructions (Millipore). After that, 1 µl of each sample was mixed with 1 µl α-cyano-4-hydroxicinnamic acid matrix (in 70% acetonitrile with 0.3% (v/v) trifluoroacetic acid) and spotted on the target plate. Samples were analyzed with a MALDI–TOF MS using a Voyager-DE™ Pro (Applied Biosystems, Framingham, MA). Positive ionization, acceleration voltage 20 kV, grid voltage 75%, guide wire 0.02 and the extraction delay time 200 ns were used to collect spectra in the mass range of 700−4000 Da. Reflector mass spectra were acquired and calibrated either externally or internally, using trypsin autolysis peptides (*m/z* 842.5200, 1045.5642, 2211.1046). Data processing of the spectra was performed with Data Explorer™ Version 4.0 (Applied Biosistems). Mass spectrometry data were searched against the human protein database (a subset of proteins from the NCBI non-redundant protein database) using the MASCOT software search algorithms (Matrix Science Ltd, London, UK). Restrictions were passed on mass tolerance (±50 ppm), maximum missed cleaveges by trypsin (up to 1), and cysteine modification by carbamidomethylation.

Immunofluorescence labeling of cells

NB4 cells were treated with varying concentrations of ATRA and BML-210 as described above. After 24−96 h treatment, cells were rinsed in ice-cold phosphate saline buffer (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 150 mM NaCl, pH 7.6).

Cover-slips with the captured cells were rinsed three times in phosphate buffer (PBS, pH 7.6) and fixed for 15 min in phosphate buffer supplemented with 3.3% (w/v) paraformaldehyde. Then cells were rinsed three times in PBS, pH 7.6, and permeabilized with 3.3% Triton X-100 for 15 min. The cells were blocked with phosphate buffer containing 5% (v/v) goat serum (DAKO) for 60 min at room temperature. Then, the cover-slips were rinsed and incubated with the indicated primary antibodies for 90 min at 37 ^{0}C and tree times rinsed with PBS, pH 7.6. Finally, the cover-slips were incubated with secondary antibodies, i.e. Alexa 564-coupled goat anti-rabbit or Alexa 488-coupled goat anti-rabbit Fab fragments (Molecular Probes, Eugene, OR) at a concentration 15 μg/ml for α-DB visualization, or Alexa 488-coupled goat anti-mouse Fab fragments for visualization of HSP90. Also, Alexa 488-phalloidin was used for F-actin visualization. Slides were subsequently examined by confocal laser scanning microscopy.

Confocal laser scanning microscopy (CLSM)

A Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) was used for the visualization of human α-DB in both proliferating and differentiated NB4 cells. Fluorescent markers, filters, and thresholds were combined to minimize the bleeding between the red and the 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 barrier filter was set at 510 nm (EFLP 510). Red and green fluorescinated probes were respectively detected with a 600 EFLP and a 545 DF 30 detector filter. ImageSpace Software (Molecular Dynamics) installed on Iris Indigo and O2 workstations (Silicon Graphics, Mountain View, CA) were used to run the microscope and to collect data.

For confocal imaging, we also used a Bio-Rad Radiance 2100 and Radiance 2000MP (Carl Zeiss, Jena, Germany) to assess dual labeling of both filamentous actin (F-actin) and α-Dystrobrevin (α-DB), or heat-shock protein 90 (HSP90) and α-DB. Images were taken in sequence after inserting the signal enhancing lenses by activating channel 1 (blue); not used: Mai-Tai laser (815 nm), with dicroic beam-splitter 500DCLPXR, blocking filter BGG22 and emission filter D488/10; channel 2 (green): Argon laser (488 nm), no blocking filter and emission filter HQ545/40; and channel 3 (red): Argon laser (488 and 514 nm), no blocking filter and the emission filter E600 LP. The microscope was a Nikon Eclipse TE2000U (Tokyo, Japan), equipped with PlanApo DicH x60 oil immersion objective (NA 1.40).

Results

Distribution of human dystrobrevin in different compartments of proliferating NB4 cells

According to UniProtKB/Swiss-prot α-DB protein (Q9Y4J8) analysis, 8 named isoforms produced by alternative splicing: DTN-1 represented by isoform with the molecular weights of 84 kDa (Q9Y4J8-1), DB-alpha by 77,6 kDa (Q9Y4J8-2), DTN-2 by 65,15 kDa (Q9Y4J8-3), DB-beta by 64,7 kDa (Q9Y4J8-4), DB-gamma by 59 kDa (Q9Y4J8-5), DB-epsilon by 43,6 kDa (Q9Y4J8-6), DTN-3 by 42,4 kDa (Q9Y4J8-7), and DB-zeta by 22 kDa (Q9Y4J8-8) proteins, respectively [20,21].

To characterize the distribution of α-DB isoforms in different cell fractions, hydrophilic and hydrophobic proteins, also cytosolic and nuclear proteins were isolated from proliferating NB4 cells. Proteins were further fractionated with SDS-PAGE, transferred to PVDF membrane, and analyzed with a rabbit polyclonal antibody against α-DB.

In the hydrophobic protein fraction of whole NB4 cell lysates, which contains all membrane fractions, the antibody revealed two closely migrating bands with the molecular weight of approximately 75 and 80 kDa, representing the DB-alpha splice isoform (Fig. 1, B). DB-alpha, DB-beta, DB-gamma and DB-zeta isoforms were present both in the cytosolic (Fig. 1, D) and the hydrophilic fractions (Fig. 1, A), albeit in different proportions. Only the DB-alpha isoform were present in the hydrophobic nuclear fraction (Fig. 1, C), whereas the hydrophilic nuclear fraction contained the DB-alpha and DB-beta isoforms (Fig. 1, E). The band with 70 kDa molecular weigh in hydrophilic fractions can be beta-DB gene product (DTN-B1).

In this study we treated NB4 cells with 1 μM ATRA alone or in combination with 5 μM BML-210 (novel HDACI) to induce cell differentiation into granulocyte-like cells. As we showed previously [12], 1 μM ATRA alone and in combination with 5 μM BML-210 can induce differentiation up to 80 % after 96 h. To induce apoptosis we used 10 μM of BML-210. The levels of α-DB isoforms were essentially constant in all fractions during both proliferation and granulocytic differentiation of NB4 cells, i.e. after treatment with ATRA alone or in combination with the novel HDACI BML-210 for 96 h (data not shown).

Identification of proteins associated with α-DB in proliferating and differentiating NB4 cells

NB4 cells were treated with ATRA or BML-210 alone or in combination. Hydrophilic proteins were isolated from proliferating NB4 cells and those treated with the chemical agents as described in Material and Methods, and further co-immunoprecipitated with antibodies against α-DB. Immunoprecipitates were then separated with 2-DE and visualized by silver staining [18,19]. Approximately 100−120 protein spots were seen on each gel (Fig. 2). By quantitative and statistical analyses, about 45% of these were differently expressed in the untreated (control) cells and the treated cells. Some differently expressed spots were excised and subjected to in-gel tryptic digestion and identification by mass spectrometry analysis (MALDI-TOF-MS) and human protein database searching. The positions of all proteins identified on 2-D gels were within the expected range of their theoretical isoelectric points and molecular sizes. These analyses resulted in the identification of both known and novel α-DB -interacting proteins. All proteins with a MASCOT total protein score > 75 were considered as real and as high confidence proteins within a dystrobrevin-associated protein complex. The identified

proteins are presented in Table 1. Some of them are important for cell growth and/or homeostasis, such as α/β-tubulin, β-actin, desmin, tropomyosin 3, stathmin and gelsolin. Others are involved in protein metabolism (disulphide isomerase (ER-60), BRCA1-associated protein) and the heat shock proteins 90Ad, HSP 70-8 and HSP70-9β. Yet another group (STAG1, Annexin A4, prohibitin and RIBA) comprised proteins responsible for signal transduction and cell communication processes.

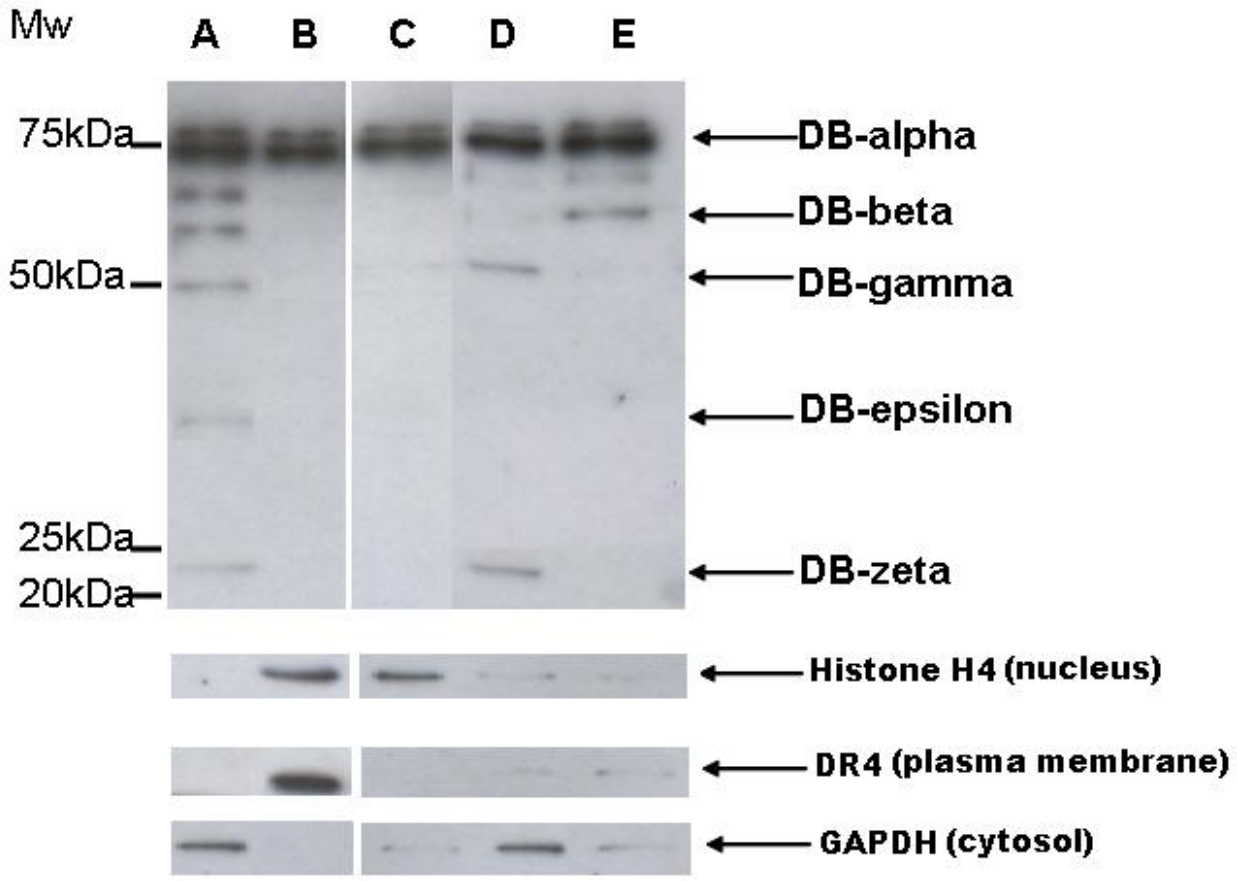

Fig. 1. Expression of α-DB isoforms in NB4 cells.
Hydrophobic and hydrophilic proteins from the NB4 cell line were solubilised and extracted using the Mem-PER Kit. The total hydrophilic (soluble proteins) NB4 cell fraction (A), the total hydrophobic (membrane proteins) fraction (B), the nuclear hydrophobic (C) fraction, the nuclear hydrophilic (E) and the cytosolic (D) fractions were fractionated in 8-18% SDS-PAGE gel and visualized by Western blot with polyclonal antibodies against α-DB. Proteins regarded as markers for different cell compartments were detected using protein-specific antibodies and HRP-conjugated secondary antibodies with chemiluminescent detection. Migration of the molecular size marker proteins is indicated to the left (kDa values).

Figure 3 describes a putative network of α-DB-associated proteins, which was performed using Ingenuity pathway analysis finder (www. Ingenuity.com) or Human Protein Reference Database by providing the Unigene/Swiss-Prot accession number of the proteins whose were identified from gels (Fig. 2) and listed in Table 1.

Distribution of α-dystrobrevin in proliferating and differentiating NB4 cells

By confocal microscopy, we assessed the localization of α-DB in the proliferating NB4 cells, in NB4 cells induced for granulocytic differentiation with ATRA and in human polymorphonuclear leukocytes (neutrophils) isolated from peripheral blood. In proliferating (control) NB4 cells, α-DB was found to be dispersed throughout the entire cytoplasm and enriched in

the plasma membrane (Fig. 4). After a 96-h treatment of NB4 cells with BML-210 was no differentiation detected and no changes in localization of α-DB were observed (data not shown). After a 96-h treatment of NB4 cells with ATRA, when about 80% of differentiated cells in cell culture were counted, α-DB localized both to the cytoplasm and to the nucleus (Fig. 4). Interestingly, we observed obvious similarities in the α-DB immunostaining for isolated mature neutrophils and for NB4 cells undergoing ATRA-induced differentiation *in vitro* (Fig. 4): both types of cells showed α-DB immunoreactivity in the nucleus, which was especially strong in the neutrophils.

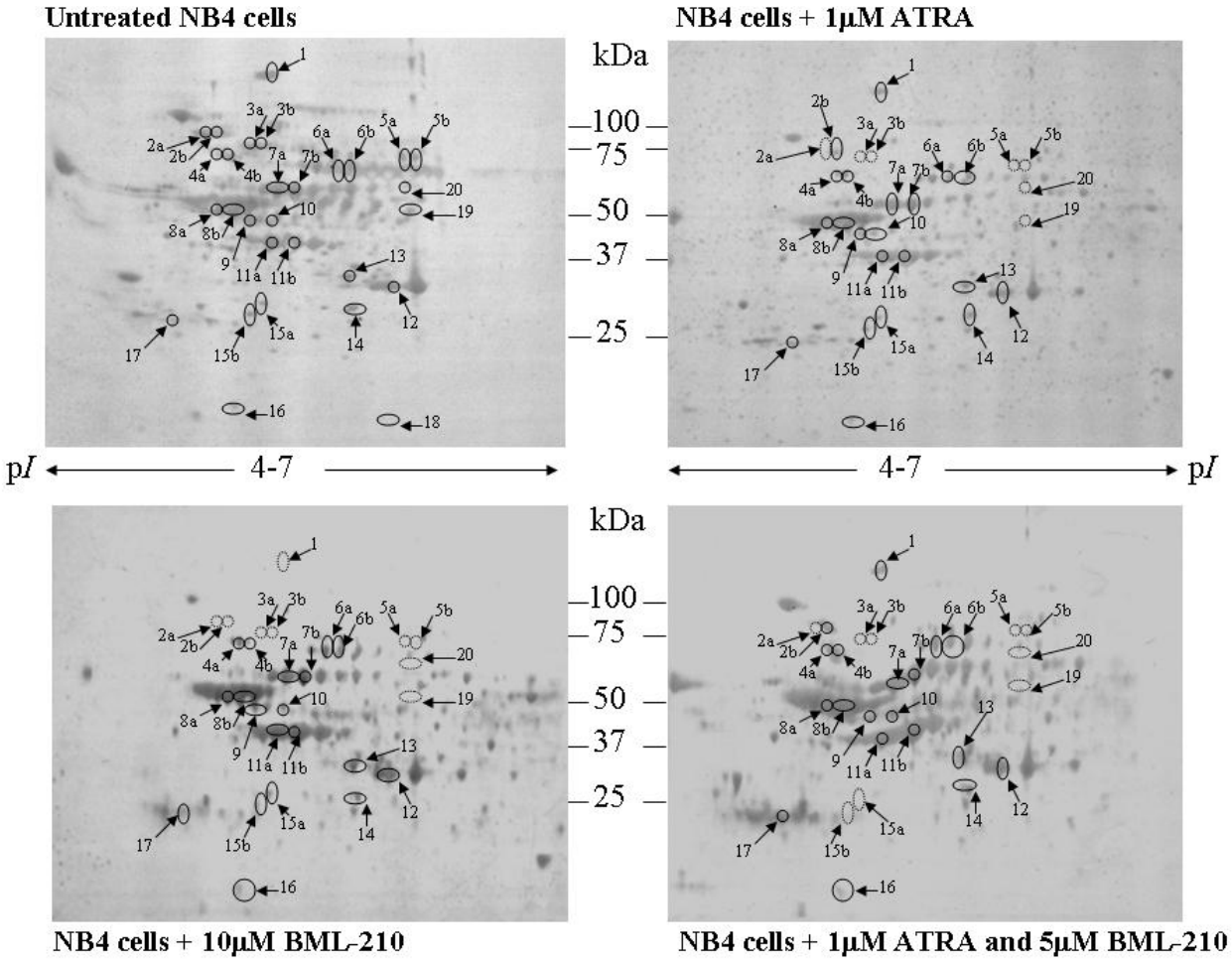

Fig.2. Human α-DB-associated proteins in proliferating and differentiating NB4 cells.
Hydrophilic proteins from untreated NB4 cells and cells treated with ATRA or BML-210 alone or in combination were isolated using the Mem-PER Kit, proteins were immunoprecipitated with anti-α-DB, fractionated in 2-DE system and visualized by silver staining. Numbers refer to identified proteins, which are listed in Table 1. Circles represent proteins that expression level increased or remained at the same level in untreated and treated NB4 cells. Dotted circles represent proteins that expression level decreased after treatment of NB4 cells. Migration of the molecular size marker proteins is indicated on the center (kDa).

In conclusion, we observed an altered localization of α-DB after treatments with ATRA in NB4 cells. This may be indicative of an important role of α-DB in cell proliferation and granulocytic differentiation.

Co-localization of human α-dystrobrevin isoforms with F-actin and HSP90 in NB4 cells

Through the mass spectrometry experiments, we identified HSP90 as a putative α-DB-interacting protein. We therefore examined whether α-DB and HSP90 co-localized in NB4 cells (Fig. 5, B). HSP90 was present both in the

cytoplasm and in the nucleus of NB4 (Fig. 5, B). α-DB was also found in the cytoplasm and the nuclei, but also in the proximity of the plasma membrane. Incidentally, the sub-cellular distribution of HSP90 and α-DB was very similar and displayed a clear co-localization in the cytoplasm.

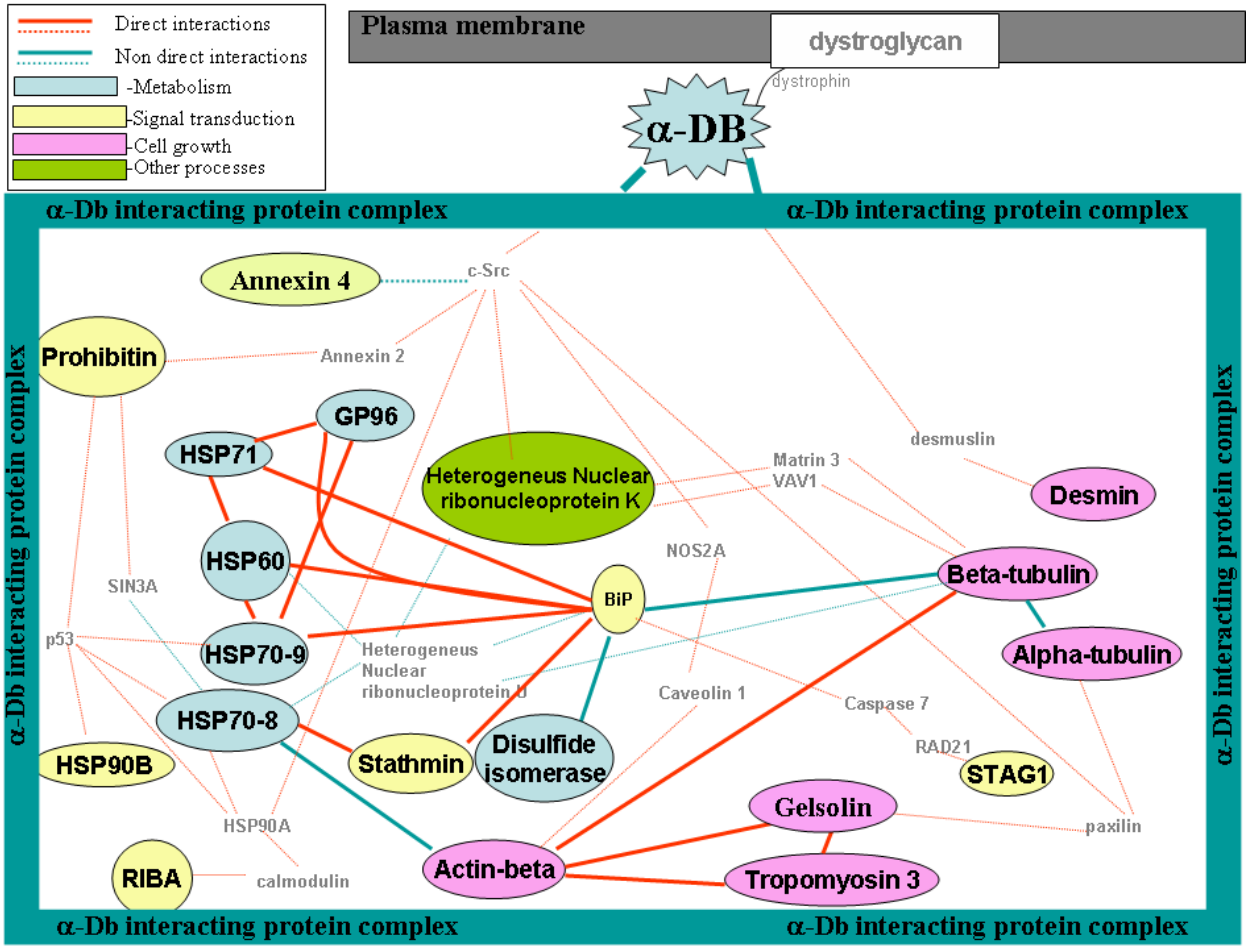

Fig.3. The network of α-dystrobrevin-associated proteins.
These- group of biologically related proteins, generated on the basis of an experimentally derived dataset. The graphic layout and network display were performed using the Ingenuity pathway analysis finder (www. Ingenuity.com) or Human Protein Reference Database by providing the Unigene/Swiss-Prot accession number of the proteins those were identified from gels (Fig. 3) and are listed in Table 1.

The co-localization of α-DB with F-actin, was assessed using the Alexa 563-fluorescent anti-rabbit secondary antibody for α-DB and the Alexa 488-phalloidin for F-actin network labeling. Generally, the distribution of F-actin and α-DB were similar in proliferating NB4 cells; they co-localized at the plasma membrane, in the cytoplasm and around the nucleus (Fig. 5, A).

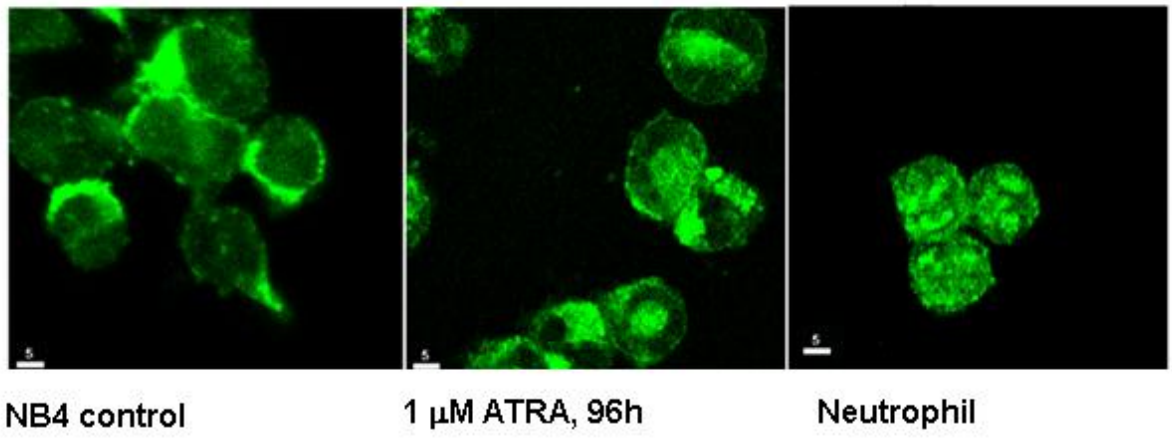

Fig. 4. Distribution of human α-DB in NB4 cells.
Confocal images demonstrating the localization of human α-DB in proliferating (control), induced to granulocytic differentiation with 1 μM ATRA NB4 cells, and in human neutrophils. Bar: 5 μm.

Discussion

The two classes of DB, α and β, bind directly to dystrophin and are prominent components of the DAPC which links the cytoskeleton to the extracellular matrix. Furthermore, the DAPC has been recognized to be molecularly heterogeneous. It is present in numerous tissues and is involved in brain development, synapse formation and plasticity, and in water and ion homeostasis [22]. There is still little data concerning the roles of DB in other cell types.

Alternative splicing of the primary α-DB transcript can produce a number of specific isoforms and post-transcriptional modifications. Such multiple α-DB isoforms are known to localize differently in cardiac and skeletal muscles [20] and have potentially different physiological roles through their interactions with different proteins within and outside of the DAPC [23]. We have shown that α-DB isoforms are expressed in NB4 cells, and that the level of α-DB isoforms expression differs in various cell fractions, such as cytosol, nucleus and the hydrophobic cell compartments. Thus three to five DB isoforms were present in the hydrophilic fraction of the whole cell lysates, in the cytoplasm and the nucleus. However, the hydrophobic fraction contains only the biggest protein isoform (DB-alpha) that confirm their association with membrane structures (Fig.1, B).

It has been shown by Yoshida M. et al. [5] that the N-terminal tail of DB participates in the interaction with the sarcoglycan–sarcospan complex. Fuentes-Mera et al. [10] have found that DAPC is present in the nuclei of HeLa cells and can associate with the nuclear matrix. Our results support earlier findings that only biggest α-DB isoform shows a tight interaction with DAPC at the plasma membrane and at the nuclear membrane of NB4 cells.

Recently, several DB–interacting proteins have been identified, by biochemical methods and employing the yeast two-hybrid system. It was, for instance, that α-DB can interact with syncoilin, desmuslin, actin, dysbindin and Kif5 [4,6,7]. All these data suggest that α-DB plays the key role in cytoskeleton reorganization or acts as a motor protein receptor [9,24]. To identify proteins interacting with α-DB in NB4 cells, we immunoprecipitated hydrophilic cell proteins with polyclonal

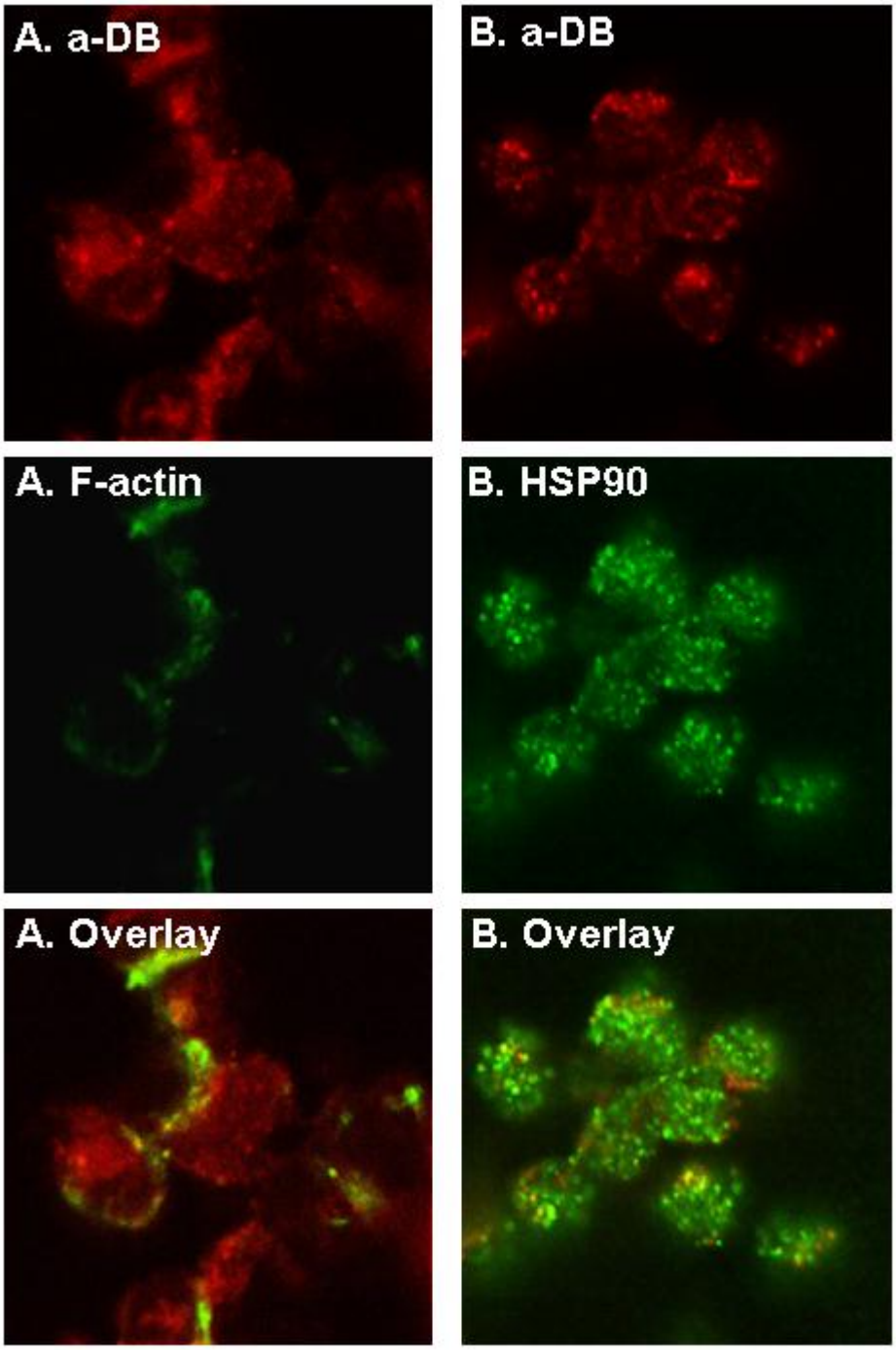

Fig. 5. **Dual immunofluorescence labeling of human α-DB and actin filaments (F-actin) (A) or α-DB and HSP90 proteins (B) in proliferating NB4 cells.**
Cells were fixed and incubated with Alexa 488-phalloidin or anti-mouse HSP90 antibody (visualized with Alexa 488-conjugated goat anti-mouse antibodies), and then incubated with anti-rabbit α-DB antibody (visualized with Alexa 563-conjugated goat anti-rabbit antibodies). The cells display clear co-localization between α-DB and F-actin (A) or α-DB and HSP90 (B) proteins at cytoplasm. Bar: 5 μm.

anti α-DB antibodies. In Table 1 we list the proteins identified by MALDI-TOF-MS analysis as being associated with α-DB. These proteins can be grouped depending on their function: the first contains β-Actin, α/β-tubulin, tropomyosin, gelsolin, desmin and stathmin, which are cytoskeleton proteins and involved in cell growth and/or maintenance processes; the second group comprises proteins responsible for signal transduction and cell communication processes (prohibitin, STAG1, RIBA and AnnexinA4); finally, the third group includes HSP90, HSP70, chaperonin-60 and BiP which can be

Table 1. α-DB associated proteins identified by mass spectrometry.

Spot No.	calculated		theoretical		Spot No - Coverage (%)	Accession Number (GI)	Protein name	Domains	Biological activity
	Mw (kDa)	pI	Mw (kDa)	pI					
1	~130	~5.4	140	5.3	1-5 %	40352781	Cohesin subunit SA-1 (STAG1)	Coiled-coil	Component of cohesin complex, a complex required for the cohesion of sister chromatids after DNA replication; may also play a role in spindle pole assembly during mitosis.
2a; 2b	~90	~4.7	90	4.7	2a-18%; 2b-20%	15010550	Heat shock protein Gp96 precursor	HATPase Ciled-coil	Molecular chaperone that functions in the processing and transport of secreted proteins.
3a; 3b	~80	~5.2	83	5.0	3a-38%; 3b-40%	17865718	Hsp90-1,beta	Coiled-coil	Molecular chaperone. It has ATPase activity, interacts with TP53/p53.
4a; 4b	~70	~4,8	72	5,1	4a-46%; 4b-48%	14916999	78 kDa glucose-regulated protein precursor (BiP) (GRP78)	Coiled-coil Signal peptide	It plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
5a; 5b	~74	~6.0	73	5.9	5a-18%; 5b-20%	24234688	Heat shock 70kDa protein 9B (GRP75)	Coiled-coil	Implicated in the control of cell proliferation and cellular aging. May also act as a chaperone.
6a; 6b	~71	~5,5	70	5,4	6a-20%; 6b-22%	123648	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	Coiled-coil	Chaperone.
7a; 7b	~60	~5,3	61	5,7	7a-30 %; 7b-20%	306890	Chaperonin (HSP60)	Coiled-coil	Implicated in mitochondrial protein import and macromolecular assembly. May facilitate the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.
8a	~52	~4,8	50	4,7	8a-25%	18088719	Beta-tubulin	TUBULIN Coiled-coil	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain.
8b	~52	~5,0	50	4,9	8b-20%	2843123	Alpha-tubulin	TUBULIN Coiled-coil	Tubulin is the major constituent of microtubules. It binds

									two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain.
9	~48	~5,0	47	5,0	9-32%	61104905	Heat shock protein 90Ad	HATPase	ATP binding.
10	~48	~5,1	53	5.2	10-33%	21358854	Desmin	Coiled-coil	Desmin are class-III intermediate filaments found in muscle cells. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures.
11a; 11b	~40	~5,3	41	5,5	11a-36%; 11b-38%	14250401	Beta-actin	ACTIN Coiled-coil	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
12	~32	~5,7	33	5,6	12-24%	39645467	Annexin A4	ANX	Calcium/phospholipid-binding protein, which promotes membrane fusion and is involved in exocytosis.
13	~33	~5,5	34	5.5	13-25%	55958543	heterogeneous nuclear ribonucleoprotein K	Nuclear localization signal, RNA binding	One of the major pre-mRNA-binding proteins. Binds tenaciously to poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences. Can also bind poly(C) single-stranded DNA.
14a; 14b	~28	~5,5	29	5,5	14a-38%; 14b-41%	66267315	prohibitin	PHB Coiled-coil	Prohibitin inhibits DNA synthesis. It has a role in regulating proliferation. As yet it is unclear if the protein or the mRNA exhibits this effect. May play a role in regulating mitochondrial respiration activity and in aging.
15a;	~29	~5,0	29	4,7	15a-23%;	19072649	TPMsk3	Coiled-coil	Binds to actin filaments in muscle and non-muscle cells.
15b	~28	~5,0	26	4,8	15b-38%	55665778	tropomyosin 3	Coiled-coil	Binds to actin filaments in muscle and non-muscle cells. Plays a central role, in association with the

									troponin complex, in the calcium dependent regulation of vertebrate striated muscle contraction. Smooth muscle contraction is regulated by interaction with caldesmon. In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments.
16	~15	~4,8	17	4,8	16-28%	2286103	RIBA	RAS	Binds and exchanges GTP and GDP.
17	~27	~4,5	22	4,6	17-17%	55960303	gelsolin	Signal peptide GEL	Calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping). It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed.
18	~18	~5,6	17	5,7	18-34%	5031851	Stathmin		Involved in the regulation of the microtubule (MT) filament system by destabilizing microtubules. Prevents assembly and promotes disassembly of microtubules. Phosphorylation at Ser-16 may be required for axon formation during neurogenesis.
19	~55	~5,6	57	5,8	19-15%	2245365	ER-60 (disulfide isomerase)	Signal peptide	Catalyzes the rearrangement of -S-S- bonds in proteins.
20	~62	~5,6	68	5,6	20-10%	10800417	BRCA1 associated protein		Deubiquitinating enzyme which may be involved in BRCA1 signal transduction pathway.

involved in protein metabolism, signal transduction and gene transcription regulation [16].

These DB-associated proteins can bind directly or indirectly to α-DB via accessory proteins, that are recruited to dystrobrevin as part of multiprotein complexes (Fig. 3). All the thirteen identified α-DB-associated proteins (STAG1, Gp96, HSP90-β, BiP, HSP70-9, HSP70-8, chaperonin 60, α/β-tubulin, desmin, β-actin, prohibitin and tropomyosin) contain the coiled-coil (CC) domain. We predict that these proteins directly interact with α-DB via the coiled-coil domain. Thus, the proteins with CC domain may act as a bridge linking α-DB with the other α-DB -interacting proteins.

Moreover, we found changes in the expression of proteins, such as gelsolin, Gp96, HSP90-1β, STAG1, HSP70-9B, tropomyosin and prohibitin, during treatment with ATRA or BML-210 or in combination (Fig. 2). Gelsolin expression was increased after combined treatment with combination of ATRA and BML-210 (Fig. 2). It is known that gelsolin is an actin-binding protein implicated in remodeling the actin cytoskeleton in numerous cellular processes and has been suggested to play a role in apoptotic resistance [25]. In this study we observed that prohibitin expression levels were decreased after NB4 cell treatments (Fig. 2). It was shown by Mishra and co-authors that prohibitin may act as a tumor suppressor protein and being involved in Ras/Raf signaling pathway [26]. Both HSP70-8 and HSP90-1β were decreased during the treatments (Fig. 2). Generally, HSP90α and HSP90β are found in the cytosol, where they are supposedly required for the stability and functional maturation of certain signaling proteins such as steroid receptors, Raf serine kinases, the cyclin-dependent kinase 4 (cdk4) and some receptor tyrosine kinases [27,28]. It has furthermore been shown that HSP90 inhibitors cause growth arrest followed by differentiation and apoptosis [29].

Roberts has reported the DAPC to perform two distinct functions, i.e. it plays both a mechanical and a signaling role. The mechanical models comprise the actin-dystrophin-dystroglycan-laminin axis, suggesting a mechanical link between the cytoskeleton and the extracellular matrix [30].

Immunostaining of NB4 cells with α-DB -specific antibodies has shown that it is primarily localized close to the plasma membrane of proliferating cells (Fig. 4). After treatment of NB4 cells with the differentiating agent ATRA, α-DB was also detected in the nucleus. Thus, our data clearly show that α-DB changed its sub-cellular localization during the granulocytic differentiation of NB4 cells.

To sum it up, we have identified a novel ensemble of α-DB interacting proteins in promyelocytic leukemia cells, proteins which are important in cytoskeleton reorganization and signal transduction. We have also found that α-DB changes sub-cellular compartment after treatment with chemical agents (ATRA alone and together with BML-210). Altogether, our data suggest that α-DB may function as an important structural and signaling protein during proliferation and differentiation processes in human promyelocytic cells.

Acknowledgements

This research was supported by the Swedish Institute (Visby Program No. 00879/2009), the Swedish Research Council, Lithuanian State Science and Studies Foundation (No.V-12/2009) and EU Marie Curie Programme Fellowship for V.V. Borutinskaite.

References

1. Rando TA (2001) The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle Nerve 24: 1575-1594

2. Dalkilic I, Kunkel LM (2003) Muscular dystrophies: genes to pathogenesis. Curr Opin Genet Dev 13:231–238

3. Grady RM, Grange RW, Lau KS, Maimone MM, Nichol MC, Stull JT, Sanes JR (1999) Role for α-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. Nat Cell Biol 1: 215-220

4. Albrecht DE, Froehner SC (2004) DAMAGE, a Novel α-Dystrobrevin-associated MAGE Protein in Dystrophin Complexes. J Biol Chem 279(8): 7014-7023

5. Yoshida M, Hama H, Ishikawa-Sakurai M, Imamura M, Mizuno Y, Araishi K, Wakabayashi-Takai E, Noguchi S, Sasaoka T, Ozawa E (2000) Biochemical evidence for association of dystrobrevin with the sarcoglycan–sarcospan complex as a basis for understanding sarcoglycanopathy. Hum Mol Genet 9: 1033-1040

6. Mizuno Y, Thompson TG, Guyon RJ, Lidov HGW, Brosius M, Imamura M, Ozawa E, Watkins SC, Kunkel LM (2001) Desmuslin, an intermediate filament protein that interacts with α-dystrobrevin and desmin. PNAS 98(11): 6156-6161

7. Newey SE, Howman EV, Ponting PCh, Benson AM, Nawrotzki R, Loh NY, Davies KE, Blake DJ (2001) Syncoilin, a Novel Member of the Intermediate Filament Superfamily that Interacts with a-Dystrobrevin in Skeletal Muscle. J Biol Chem 276: 6645-6655

8. Costa ML, Escaleira R, Cataldo A, Oliveira F, Mermelstein CS (2004) Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. Braz J Med Biol Res 37(12): 1819-1830

9. Ceccarini M, Torreri P, Lombardi DG, Macchia G, Macioce P, Petrucci TC (2005) Molecular basis of Dystrobrevin interactionwith kinesin heavy chain: structural determinants of their binding. J Mol Biol 354: 872-882.

10. Fuentes-Mera L, Rodríguez-Muñoz R, González-Ramírez R, García-Sierra F, González E, Mornet D, Cisneros B (2006) Characterization of a novel Dp71 dystrophin-associated protein complex (DAPC) present in the nucleus of HeLa cells: members of the nuclear DAPC associate with the nuclear matrix. ExpCell Res 312: 3023-35

11. Kulyte A, Navakauskiene R, Treigyte G, Gineitis A, Bergman T, Magnusson KE (2002) Characterization of human alpha-dystrobrevin isoforms in HL-60 human promyelocytic leukemia cells undergoing granulocytic differentiation. Mol Biol Cell 13(12): 4195-4205

12. Savickiene J, Borutinskaite VV, Treigyte G, Magnusson KE, Navakauskiene R (2006) The novel histone deacetylase inhibitor BML-210 exerts growth inhibitory, proapoptotic and differentiation stimulating effects on the human leukemia cell lines. Eur J Pharmacol 549(1-3): 9-18

13. Chen Z, Wang W, Pan J, Chen L, Xue Y (1999) Combination of all-trans-retinoic acid with butyric acid and its prodrugs markedly enhancing differentiation of human acute promyelocytic leukemia NB4 cells. Chin Med J Engl 112: 352–355

14. Coffey DC, Kutko MC, Glick RD, Butler LM, Heller G, Rifkind RA, Marks PA, Richon VM, La Quaglia MP (2001) The histone deacetylase inhibitor, CBHA, inhibits growth of human neuroblastoma xenografts in vivo, alone and synergistically with all-trans-retinoic acid. Cancer Res 61: 3591–3594

15. Demary K, Wong L, Spanjard RA (2001) Effects of retinoic acid and sodium butyrate on gene expression, histone acetylation and inhibition of proliferation of melanoma cells. Cancer Lett 163**:** 103–107

16. Waleryck D, Kudla G, Gutkowska M, Wawrzynow B, Muller L, King FW, Helwak A, Boros J, Zylicz A, Zylicz M (2004) Hsp90 chaperones wild-type p53 tumor suppressor protein. J Biol Chem 279(47): 48836-48845

17. Boyum A (1968) A one-stage procedure for isolation of granulocytes and lymphocytes from human blood. General sedimentation properties of white blood cells in a 1 g gravity field. Scand J Clin Lab Invest Suppl 97: 51-76

18. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68: 850-858

19. Shevchenko A, Chernushevic I, Wilm M, Mann M (2002) 'De novo' sequencing of peptides recovered from in-gel digested proteins by nanoelectrospray tandem mass spectrometry. Mol Biotechnol 20: 107–118

20. Nawrotzki R, Loh NY, Ruegg MA, Davies KE, Blake DJ (1998) Characterisation of alpha-dystrobrevin in muscle. J Cell Sci 111: 2595-2605

21. Sadoulet-Puccio HM, Khurana TS, Cohen JB, Kunkel LM (1996) Cloning and characterization of the human homologue of a dystrophin related phosphoprotein found at the Torpedo electric organ post-synaptic membrane. Hum Mol Genet 5:489-496

22. Haenggi T, Fritschy JM (2006) Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. Cell Mol Life Sci 63: 1614-31

23. Lien CF, Hazai D, Yeung D, Tan J, Fuchtbauer EM, Jancsik V, Gorecki DC (2006) Expression of α-dystrobrevin in blood-tissue barriers: sub-cellular localisation and molecular characterisation in normal and dystrophic mice. Cell Tissue Res 327(1): 67-82

24. Grady RM, Akaaboune M, Cohen AL, Maimore MM, Lichtman JW, Sanes JR (2003) Tyrosine- phosphorylated and nonphosphorylated isoforms of α-dystrobrevin: roles in sceletal muscle and its neuromuscular and myotendious junction. J Cell Biol 160(5): 741-752

25. Ahn JS, Jang IS, Rhim JH, Kim K, Yeo EJ, Park SC (2003) Gelsolin for senescence associated resistance to apoptosis. Ann N Y Acad Sci 1010: 493-495

26. Mishra S, Murphy LC, Murphy LJ (2006) The Prohibitins: emerging roles in diverse functions. J Cell Mol Med 10(2): 353-363

27. Pratt WB (1997) The role of the Hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signalling via MAP kineses. Ann Rev Pharmacol Toxicol 37: 297-326

28. Schulte TW, Blagosklonny MV, Romanova L, Mushinski JF, Monia BP, Johnston JF, Nguyen P, Trepel J, Neckers LM (1996) Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf- 1-MEK-mitogen-activated protein kinase signaling pathway. Mol Cell Biol 16: 5839-5845

29. Srethapakdi M, Liu F, Tavorath R, Rosen N (2000) Inhibition of Hsp90 Function by Ansamycins Causes Retinoblastoma Gene Product-dependent G_1 Arrest. Cancer Res 60: 3940-3946

30. Roberts RG (2001) Dystrophins and dystrobrevins. Genome Biol 2(4). REVIEWS3006.