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# β-Dystrobrevin, a Kinesin-Binding Receptor, Interacts With the Extracellular Matrix Components Pancortins

**Caterina Veroni, Margherita Grasso, Gianfranco Macchia, Carlo Ramoni, Marina Ceccarini, Tamara C. Petrucci, and Pompeo Macioce***

Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Roma, Italy

The dystrobrevins (α and β) are components of the dystrophin-associated protein complex (DPC), which links the cytoskeleton to the extracellular matrix and serves as a scaffold for signaling proteins. The precise functions of the β-dystrobrevin isoform, which is expressed in nonmuscle tissues, have not yet been determined. To gain further insights into the role of β-dystrobrevin in brain, we performed a yeast two-hybrid screen and identified pancortin-2 as a novel β-dystrobrevin-binding partner. Pancortins-1–4 are neuron-specific olfactomedin-related glycoproteins, highly expressed during brain development and widely distributed in the mature cerebral cortex of the mouse. Pancortins are important constituents of the extracellular matrix and are thought to play an essential role in neuronal differentiation. We characterized the interaction between pancortin-2 and β-dystrobrevin by in vitro and in vivo association assays and mapped the binding site of pancortin-2 on β-dystrobrevin to amino acids 202–236 of the β-dystrobrevin molecule. We also found that the domain of interaction for β-dystrobrevin is contained in the B part of pancortin-2, a central region that is common to all four pancortins. Our results indicate that β-dystrobrevin could interact with all members of the pancortin family, implying that β-dystrobrevin may be involved in brain development. We suggest that dystrobrevin, a motor protein receptor that binds kinesin heavy chain, might play a role in intracellular transport of pancortin to specific sites in the cell. 

**Key words:** dystrophin; neuronal differentiation; brain development; molecular motors

Dystrobrevins are dystrophin-related and -associated proteins encoded by two separate genes, α and β (Ambrose et al., 1997; Loh et al., 1998). They are cytoplasmic components of the dystrophin-associated protein complex (DPC), which, by linking the actin cytoskeleton to the extracellular matrix, is critical for the integrity of muscle fibers, and which has more recently been recognized as a scaffold for signaling proteins (Rando, 2001; Blake et al., 2002). Mutations in some DPC members result in various forms of muscular dystrophy, the most common of which are Duchenne (DMD) and Becker (BMD) muscular dystrophies (Straub and Campbell, 1997). The finding that many DMD patients suffer from a cognitive impairment that is the result of gene mutation and not the psychological consequence of the physical handicap, has been amply confirmed (for reviews see Blake and Kroger, 2000; Anderson et al., 2002). The brain dysfunction often associated with muscular dystrophies is thought to be a nonprogressive disorder that occurs early, probably during embryogenesis, whereas the muscle disease arises later and progressively (Moizard et al., 1998). Muscular dystrophies are indeed multisystem disorders affecting not only skeletal, smooth, and cardiac muscles but also the endocrine system and the central nervous system (CNS). Several DPC proteins are present in central neurons, where they appear to interact in a manner similar to that seen in muscle and form DPC-like complexes. Despite growing evidence that these complexes are important for cerebral development and function, their role remains unclear.

α-Dystrobrevin is expressed predominantly in skeletal muscle, heart, lung, and brain (Blake et al., 1996; Sadoulet-Puccio et al., 1996), whereas β-dystrobrevin is restricted to nonmuscle tissues and is abundantly expressed in brain, lung, kidney, and liver (Peters et al., 1997; Blake et al., 1998). The precise role of dystrobrevins is still elusive, although their cellular functions are beginning to be elucidated through the study of their associated proteins. Several dystrobrevin-associated proteins have recently been described, including syncoilin (Newey et al., 2001), desmuslin (Mizuno et al., 2001), DAMAGE (Albrecht and Froehner 2004), and dysbindin (Benson et al., 2001). Among these, dysbindin, a coiled-coil protein involved in the biogenesis of lysosome-related organelles (Li et al., 2003) and in

Margherita Grasso's current address is Department of Histology and Medical Embryology, University of Rome La Sapienza, 00161 Roma, Italy

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*Correspondence to: Dr. Pompeo Macioce, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy. E-mail: macioce@iss.it

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neuronal transmission and vulnerability (Numakawa et al., 2004), has also been found to be a major schizophrenia-susceptibility factor (Straub et al., 2002).

We have reported that dystrobrevin binds to the cargo-binding domain of kinesin heavy chain and have suggested a role for dystrobrevin as a motor protein receptor (Macioce et al., 2003). We have also recently described a difference in the binding properties of dystrobrevin isoforms for kinesin and found that in vitro phosphorylated β-dystrobrevin showed reduced binding capacity toward kinesin (Ceccarini et al., 2005). We suggested that the association of dystrobrevin with kinesin could be affected by structural changes in β-dystrobrevin induced by interacting proteins and/or posttranslational modifications (Ceccarini et al., 2005), implying an emerging role of dystrobrevin as a multifunctional scaffold protein involved in intracellular transport and cell signaling. Recently, Grady and coworkers (2006) observed abnormal motor behavior and cerebellar synaptic defects in double knockout mice lacking both α- and β-dystrobrevin and suggested that motor deficits in muscular dystrophy patients may reflect not only peripheral derangements but also CNS defects.

In the present study, we characterized the association of β-dystrobrevin, the dystrobrevin isoform expressed in nonmuscle tissues, with a novel binding partner, pancortin. Pancortins are neuron-specific olfactomedin-related glycoproteins that are components of the extracellular matrix and have been proposed to support neuronal differentiation and survival (Nagano et al., 2000).

## MATERIALS AND METHODS

### Plasmid Constructs

Construction of pGEX-6P/β-DB, pGBKT7/β-DB, and pCMV-HA/β-DB is described in detail elsewhere (Macioce et al., 2003), as is construction of pGEX-4T/α-DB1 (Ceccarini et al., 2005). β-DB amino-terminal- and carboxyl-terminal-deleted mutants were obtained by PCR with pGEX-6P/β-DB as a template and were subcloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA) as reported previously (Ceccarini et al., 2005). After EcoRI digestion, amino-terminal-deleted β-DB fragments were inserted into the EcoRI site of pGADT7 vector (Clontech, Logan, UT). βDBΔ6, a naturally occurring β-dystrobrevin splice variant lacking exon 6, isolated during the full-length β-dystrobrevin cloning described by Ceccarini et al. (2005) and subcloned into pCRII-TOPO, was utilized as template to obtain $\beta DB\Delta_{202-236}$. To restore the correct open reading frame, two PCR rounds were run in order to insert the last nucleotide of exon 6 ($G_{835}$). In the first reaction, two different fragments were amplified with the following pairs of primers: βDBF 5′ GGG AAG CGA GCC AGG ATG ATT 3′/βDBΔ6R 5′ CAC AGG ATG GAA GAC CTG CTG CGG AAA ACA 3′ and βDBΔ6F 5′ TGT TTT CCG CAG CAG GTC TTC CAT CCT GTG 3′/βDBR 5′ CAG GAC ACA GGA GTA ACC TCT 3′. The amplification products were subsequently joined together and allowed to extend. The resulting filaments were amplified by a second PCR utilizing βDBF and βDBR primers. Pancortin DNA insert coding for amino acids 18–153 of pancortin-2 and the upstream HA epitope tag was amplified by PCR from pACT2/pan template and subcloned into pCRII-TOPO to create pCRII-TOPO/pan. The pancortin-2 fragment was then EcoRI-restricted and subcloned into the EcoRI site of pCMV-Myc (Clontech) to generate pCMV-Myc/pan. pGEX-6P/pan was obtained by EcoRI digestion of pCMV-Myc/pan and insertion of the pancortin-2 fragment into the EcoRI site of pGEX-6P-2 vector (Amersham Pharmacia Biotech, Arlington Heights, IL). Pancortin B part was obtained by PCR with pACT2/pan as a template and subcloned into pCRII-TOPO vector to create pCRII-TOPO/$PAN_{B\ part}$. The correct orientation of cDNA inserts was verified by restriction enzyme and/or PCR analysis. Sequence analysis was used to check that they were in-frame.

### Yeast Two-Hybrid Screen

Two-hybrid screening was carried out by yeast mating, with the Matchmaker Gal4 Two-Hybrid System 3 (Clontech), as reported elsewhere (Macioce et al., 2003). Briefly, the Gal4 DNA binding domain construct pGBKT7/β-DB was used to transform *Saccharomyces cerevisiae* MATa yeast strain AH109. AH109[pGBKT7/β-DB] was then used as a bait strain to screen a mouse brain cDNA library (Clontech), which was cloned into the activation domain (AD) vector pACT2, and pretransformed in the *S. cerevisiae* MATα yeast strain Y187. The yeast mating screening was performed according to the manufacturer's instructions. Unique inserts were sequenced, and DNA and protein sequence analyses were performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). After isolation of the pACT2 plasmids encoding the library clones, these plasmids were tested for autoactivation of the reporter gene in yeast by both cotransformation, using either the empty bait plasmid pGBKT7 or the same plasmid encoding an unrelated negative control (lamin C), and yeast mating, using Y187 [pGBKT7] as the empty bait strain, or Y187[pGBKT7-lamin C] as a negative control. Isolates growing in SD/-TLHA/X-α-Gal, minimal synthetic dropout medium (SD) lacking Trp, Leu, His, and Ade (-TLHA) and including 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal), and resulting in blue staining without the presence of the β-DB bait, were excluded from further investigation. Activation of the reporter genes in the positive colonies was confirmed in the same experiments.

### In Vitro Transcription and Translation

TNT SP6 or T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) in the presence of PROMIX (70% L-[$^{35}$S]methionine; 30% L-[$^{35}$S]cysteine; Amersham Pharmacia Biotech) was used to transcribe and translate in vitro pCRII-TOPO/pan and pCRII-TOPO/$PAN_{B\ part}$ to obtain $^{35}$S-labeled pancortin-2 (aa 18–153) and pancortin B part, respectively. The same procedure was followed for pCRII-TOPO/β-DB, pCRII-TOPO/$\beta\text{-}DB_{357-615}$, pGADT7/$\beta\text{-}DB_{399-615}$, pGADT7/$\beta\text{-}DB_{399-511}$, pGADT7/$\beta\text{-}DB_{469-615}$, pGADT7/$\beta\text{-}DB_{500-615}$, pCRII-TOPO/$\beta\text{-}DB_{1-436}$, pCRII-TOPO/$\beta\text{-}DB_{1-352}$, pCRII-TOPO/$\beta\text{-}DB_{1-284}$, and pCRII-TOPO/$\beta\text{-}DB_{1-236}$, as well as for pCRII-TOPO/$\beta\text{-}DB\Delta_{202-236}$, to obtain the corresponding polypeptides. Newly synthesized proteins were separated by SDS-PAGE and analyzed with an Instant-Imager (Packard).

### In Vitro Interaction Between β-Dystrobrevin and Pancortin

pGEX-6P/β-DB, pGEX-6P/pan, and pGEX-6P were used to obtain recombinant glutathione S-transferase (GST)-β-DB, GST-pan, and GST, basically as previously described (Macioce et al., 2003). GST-β-DB, GST-pan, and GST bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech) were equilibrated in binding buffer (20 mM Hepes, pH 7.4, 0.15 M NaCl, 1 mM dithiothreitol, 0.2% Triton X-100, 5 μM $MgSO_4$, and protease inhibitors). For binding assays, 5–20 μl of in vitro-translated reaction products was incubated with 5–20 μl of 50% slurry bead-bound GST-fusion proteins overnight at 4°C on a rotator. After extensive washes with binding buffer, bound radioactive proteins were resuspended in Laemmli's loading buffer and separated by SDS-PAGE. Gels were dried, and radiolabeled proteins were detected by autoradiography.

### Antibodies

A dystrobrevin mAb (clone 23; BD Transduction Laboratories, Mountain View, CA) or a polyclonal antibody raised against β-dystrobrevin (Macioce et al., 2003) was used to detect dystrobrevins. Additional antibodies used were mouse monoclonal anti-PDI (Stressgen, Vancouver, British Columbia, Canada), antigolgin-97 mouse monoclonal CDF4 (Molecular Probes, Eugene, OR), c-Myc-tag mAb (Clontech), and HA-tag polyclonal antibody (Clontech).

### Pull-Down Experiments From Rat Brain Lysates

For the GST pull-down assays, 5 g freshly prepared rat brain was homogenized in 15 ml lysis buffer (20 mM Hepes, pH 7.4, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 1 mM dithiothreitol, and protease inhibitors) and the lysate cleared by centrifugation at 20,000*g* for 30 min at 4°C. To minimize nonspecific binding, 1 mg of proteins from the lysate was preincubated in binding buffer with 5 μl of 50% slurry bead-bound GST protein, overnight at 4°C on a rotator. The precleared supernatant was then incubated for 2 hr with 5 μl of 50% slurry bead-bound GST-pancortin-2, and the beads were washed six times with 0.5 ml binding buffer. Bead-bound GST protein was used as control. After washing, the samples were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the β-dystrobrevin polyclonal antibody. Bound antibodies were visualized by using the ECL system (Pierce, Rockford, IL).

### Immunofluorescence of Transfected Cells

Cos-7 cells grown in DMEM/10% fetal bovine serum were seeded on sterile, untreated glass coverslips and transfected with Fugene 6 (Roche) according to the manufacturer's instructions. Twenty-four to thirty-six hours after transfection, the cells were washed with TBS, fixed with 3.7% formaldehyde in TBS for 15 min at room temperature, and permeabilized for 5 min in TBS containing 0.5% Triton X-100. After washing, cells were blocked for 30 min in TBS containing 3% normal goat serum and incubated for 1–3 hr with primary antibodies diluted in TBS containing 1% normal goat serum. Cells were then incubated with the appropriate secondary antibodies for 1 hr and mounted with VectaShield (Vector Laboratories). Alexa Fluor 488 and Alexa Fluor 568 $F(ab')_2$ fragment of goat anti-rabbit or anti-mouse immunoglobulin G (Molecular Probes) secondary antibodies were used. The coverslips were examined under a Leica TCS 4D confocal microscope. Image acquisition and processing were performed in SCANware and Multicolor Analysis (Leica Lasertechnik Gmbh) and Adobe Photoshop software.

## RESULTS

### Interaction of Pancortin-2 and β-Dystrobrevin in the Yeast Two-Hybrid System

In a previous paper, we described the yeast two-hybrid screen performed on a mouse brain cDNA library with β-dystrobrevin as bait (Macioce et al., 2003). End sequencing and BLAST searching of positive clones revealed that one of them contained the near-entire coding sequence (amino acids 18–153) of pancortin-2 (GenBank accession No. BAA28766). We confirmed the pancortin-2/β-dystrobrevin interaction in the yeast system by mating MATa strain AH109 transformed with pACT2/Pan with MATα strain Y187 transformed with pGBKT7/β-DB (Fig. 1A). Pancortin-2 interacts with β-dystrobrevin but not with the unrelated protein lamin C, nor does it transactivate reporter gene expression (Fig. 1A). The same results were obtained by β-galactosidase assay after cotransformation of bait and prey plasmids into the yeast strain Y187 (data not shown).

### Association With Pancortin-2 Involves β-Dystrobrevin Domain Spanning Amino Acids 202–236

The direct interaction between pancortin-2 and β-dystrobrevin was confirmed by GST pull-down experiments performed with either GST-β-dystrobrevin and in vitro-transcribed/translated pancortin-2 (Fig. 1B) or GST-pancortin-2 and in vitro-transcribed/translated β-dystrobrevin (Fig. 2B, first lanes). Interestingly, the association of dystrobrevin with pancortin seems to be limited to β-dystrobrevin; we found that GST-pancortin-2 failed to interact with in vitro-transcribed/translated α-dystrobrevin (not shown).

To characterize further the structural requirements of the interaction between pancortin-2 and β-dystrobrevin, we used a set of progressively smaller $^{35}S$-labeled amino-terminal- and carboxyl-terminal-deleted β-dystrobrevin mutants (Fig. 2A) and tested them for interaction with recombinant GST-pancortin-2. β-Dystrobrevin carboxyl-terminal-deleted mutants were pulled down by GST-pancortin-2 (Fig. 2C, right), whereas all amino-terminal-deleted mutants except for $\beta\text{-DB}_{469–615}$ were not (Fig. 2B, right). The strong interaction of the amino-terminal fragment $\beta DB_{1–236}$, together with the rather feeble interaction of $\beta DB\Delta_{202–236}$, a deletion mutant devoid of amino acids 202–236 (Fig. 3), clearly indicated that a major site of interaction for pancortin-2 is comprised within amino acid residues 202 and 236 of the β-dystrobrevin molecule. In addition, a clear band given by the coprecipitation of

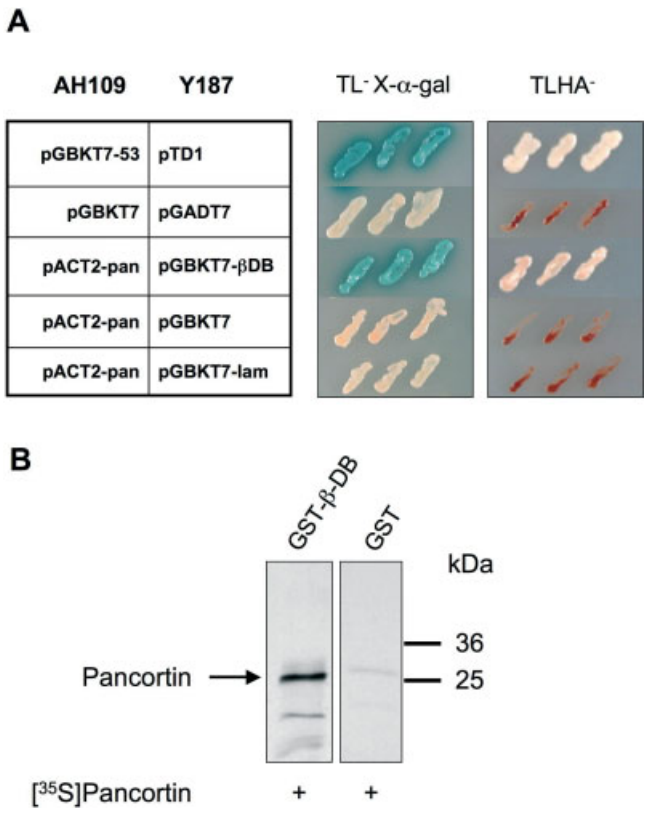


Fig. 1. Direct interaction of β-dystrobrevin and pancortin-2 in vivo and in vitro. **A:** Yeast mating experiments: diploids containing interacting clones of pancortin-2 (pan) and β-dystrobrevin (βDB) demonstrated α-galactosidase activity with the production of the blue coloration on double dropout X-α-gal medium ($TL^-$ X-α-gal) and grew vigorously on quadruple dropout medium ($TLHA^-$). A diploid strain derived by mating AH109 [pGBKT7-53] and Y187[pTD1-1] was used as a positive control; Y187[pGBKT7-lamin C (lam)] provided a negative control. **B:** Binding of $^{35}$S-labeled pancortin to immobilized GST-β-dystrobrevin (GST-β-DB). Proteins pulled down by GST-βDB, or GST, immobilized on glutathione-Sepharose beads, were analyzed by SDS-PAGE and detected by autoradiography. Molecular mass markers are indicated at right (kDa).

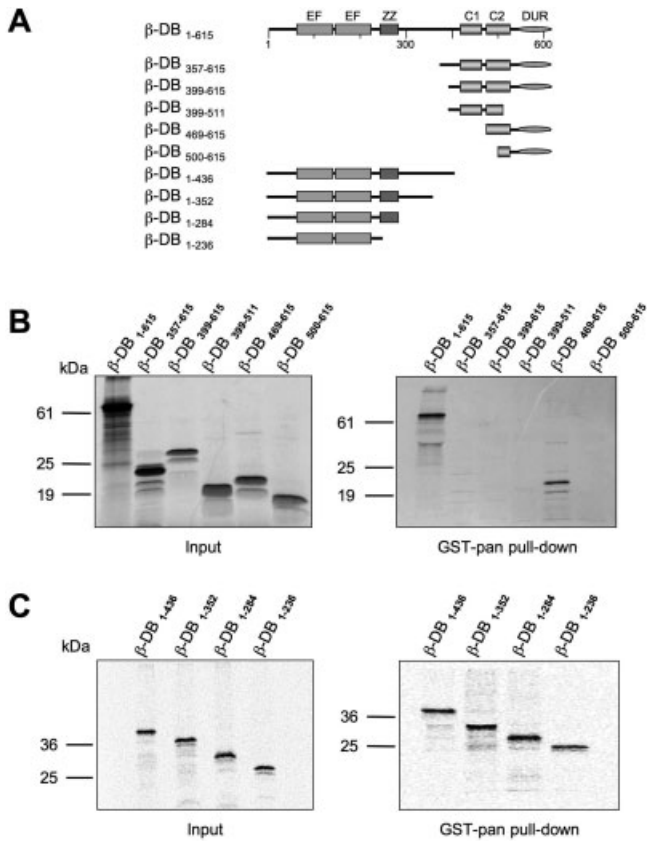


Fig. 2. Analysis of β-dystrobrevin regions involved in the interaction with pancortin-2. **A:** Schematic representation of full-length β-dystrobrevin and β-dystrobrevin-deleted proteins used in the pull-down assay. The positions of EF-hand (EF) motifs, the ZZ domain (ZZ), and the two coiled-coil regions (C1, C2) are shown. **B:** Binding of $^{35}$S-labeled β-dystrobrevin and its amino-terminal-deleted mutants to immobilized pancortin-2. **C:** Binding of $^{35}$S-labeled β-dystrobrevin carboxyl-terminal-deleted mutants to immobilized pancortin-2. Aliquots of in-vitro-transcribed and translated proteins (input) and the proteins bound to pancortin-2, immobilized on glutathione-Sepharose beads (GST-pan pull-down), were analyzed by SDS-PAGE and detected by autoradiography (B) or by an Instant Imager (C). Molecular mass markers are indicated (kDa).

β-$DB_{469–615}$ and GST-pancortin-2 raises the possibility of an additional, albeit minor, binding site for pancortin-2 within the β-dystrobrevin region comprising the coiled-coil C2 and the extreme carboxyl-terminus of the molecule (Fig. 2B, right).

## Association With β-Dystrobrevin Involves Pancortin B Part

Pancortin isoforms include four splice variants, pancortins-1–4, related to one another by their shared B part and by two pairs of alternative N-terminal (A1 or A2 part) and C-terminal (C1 or C2 part) regions (Fig. 4A; Nagano et al., 1998). The region of interaction of β-dystrobrevin on pancortin-2 was determined by using recombinant full-length GST-β-dystrobrevin as well as amino-terminal- and carboxyl-terminal-deleted β-dystrobrevin mutants to precipitate $^{35}$S-labeled B part of pancortin. Our results showed that pancortin B part was efficiently pulled down by full-length GST-β-dystrobrevin and the GST-β-dystrobrevin carboxyl-terminal-deleted mutant β-$DB_{1–352}$ but not by the GST-amino-terminal-deleted mutant β-$DB_{399–615}$ or by GST protein alone (Fig. 4B). These data indicate that the site of interaction with β-dystrobrevin is contained within the central region of pancortin-2, i.e., within the B part that is common to all four pancortin isoforms.

## Immunolocalization of Pancortin-2 and β-Dystrobrevin in Transfected COS Cells

The in vivo association of β-dystrobrevin with pancortin-2 was studied by overexpressing both proteins in COS-7 cells. Prior to these studies, we determined the dis-

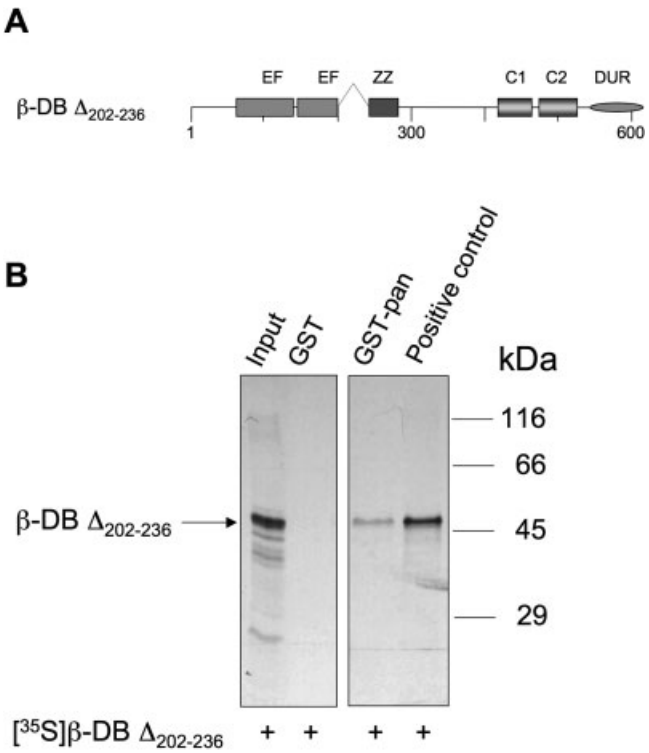


Fig. 3. Lack of binding of the $^{35}$S-labeled β-dystrobrevin mutant devoid of amino acids 202–236 (β-DB $\Delta_{202-236}$), depicted in **A**, with GST-pancortin-2 (GST-pan; **B**). Proteins pulled down by GST, GST-pan, or GST-PKA used as positive control, immobilized on glutathione-Sepharose beads, were analyzed by SDS-PAGE and detected by autoradiography. Molecular mass markers are indicated (kDa).

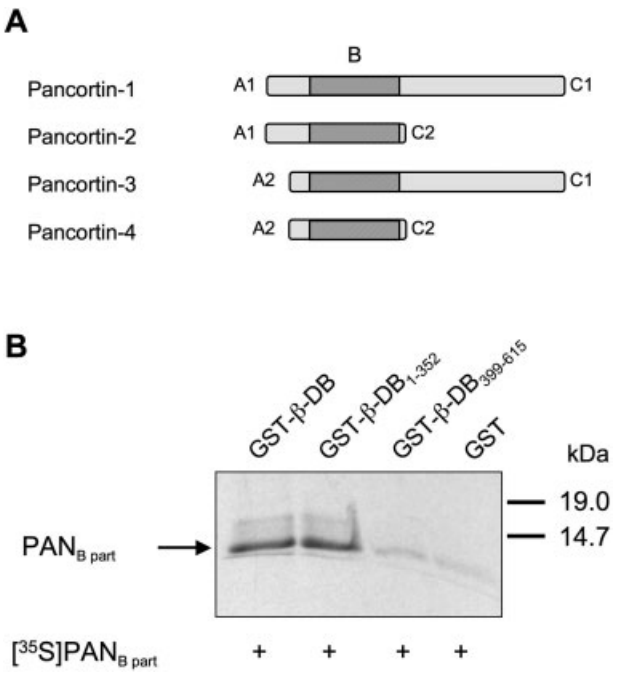


Fig. 4. Analysis of pancortin regions involved in the interaction with β-dystrobrevin. **A:** Schematic representation of pancortin-1–4 structure: pancortins are encoded by a single gene that produces four different proteins sharing a common portion, the B part, in the middle of their structure, with two variations at the N-terminal (A1 or A2 part) and C-terminal (C1 or C2 part) sides. **B:** Binding of $^{35}$S-labeled B part of pancortin ($PAN_{B\ part}$) to immobilized β-dystrobrevin full-length and truncated mutants. Molecular mass markers are indicated (kDa).

tributions of β-dystrobrevin and of pancortin-2 by single transfection of pCMV-HA/β-DB and of pCMV-Myc/pancortin-2. Immunofluorescence labeling of cells transiently transfected with pancortin-2 and visualized by the suitable anti-tag antibody showed that pancortin-2 immunoreactivity often appears concentrated in vesicular-tubular structures next to the nucleus (Fig. 5a). Single staining with the monoclonal anti-PDI and antigolgin 97 antibodies, which reveal the endoplasmic reticulum and the Golgi apparatus, respectively, suggested that pancortin-like immunoreactivity localizes mainly in the endoplasmic reticulum of transfected COS cells. The transfected β-dystrobrevin, visualized either by a polyclonal β-dystrobrevin antibody (Fig. 5b) or by the suitable anti-tag antibody (not shown), displayed an intense punctate staining pattern distributed throughout the cell (Fig. 5b), as previously described (Macioce et al., 2003). When the constructs encoding pancortin-2 and β-dystrobrevin were cotransfected, immunofluorescence microscopy of the two coexpressed proteins indicated that part of the intensely stained β-dystrobrevin punctae were changed by pancortin-2 overexpression (Fig. 5c,f,i) into tubular-vesicular structures that also seemed to be localized in the endoplasmic reticulum (Fig. 5d,g,j). In these structures, β-dystrobrevin and pancortin-2 showed a degree of colocalization ranging from weak to virtually complete (Fig. 5e,h,k), thus strengthening the hypothesis of their association in a living system.

### In Vivo Association of Pancortin-2 and β-Dystrobrevin

To characterize further the interaction of β-dystrobrevin and pancortin-2 in vivo, we performed coprecipitation experiments with rat brain extracts. By using GST pull-down to coprecipitate pancortin-2 and their associated proteins, we found that pancortin-2 and β-dystrobrevin coprecipitated in the same complex (Fig. 6). Western blot with the polyclonal β-dystrobrevin antibody, which has previously been shown to recognize all dystrobrevin isoforms (Macioce et al., 2003), also indicated that α-dystrobrevin did not coprecipitate with the GST-pancortin-2 complex (Fig. 6), confirming that in rat brain the interaction of pancortin-2 with β-dystrobrevin is specific.

## DISCUSSION

In this study, we have identified pancortin-2 as a new β-dystrobrevin-associated protein, and characterized the interaction of pancortin-2 and β-dystrobrevin. Pancortin-2 is a member of the pancortins, a family of extracellular matrix glycoproteins comprising four different isoforms,

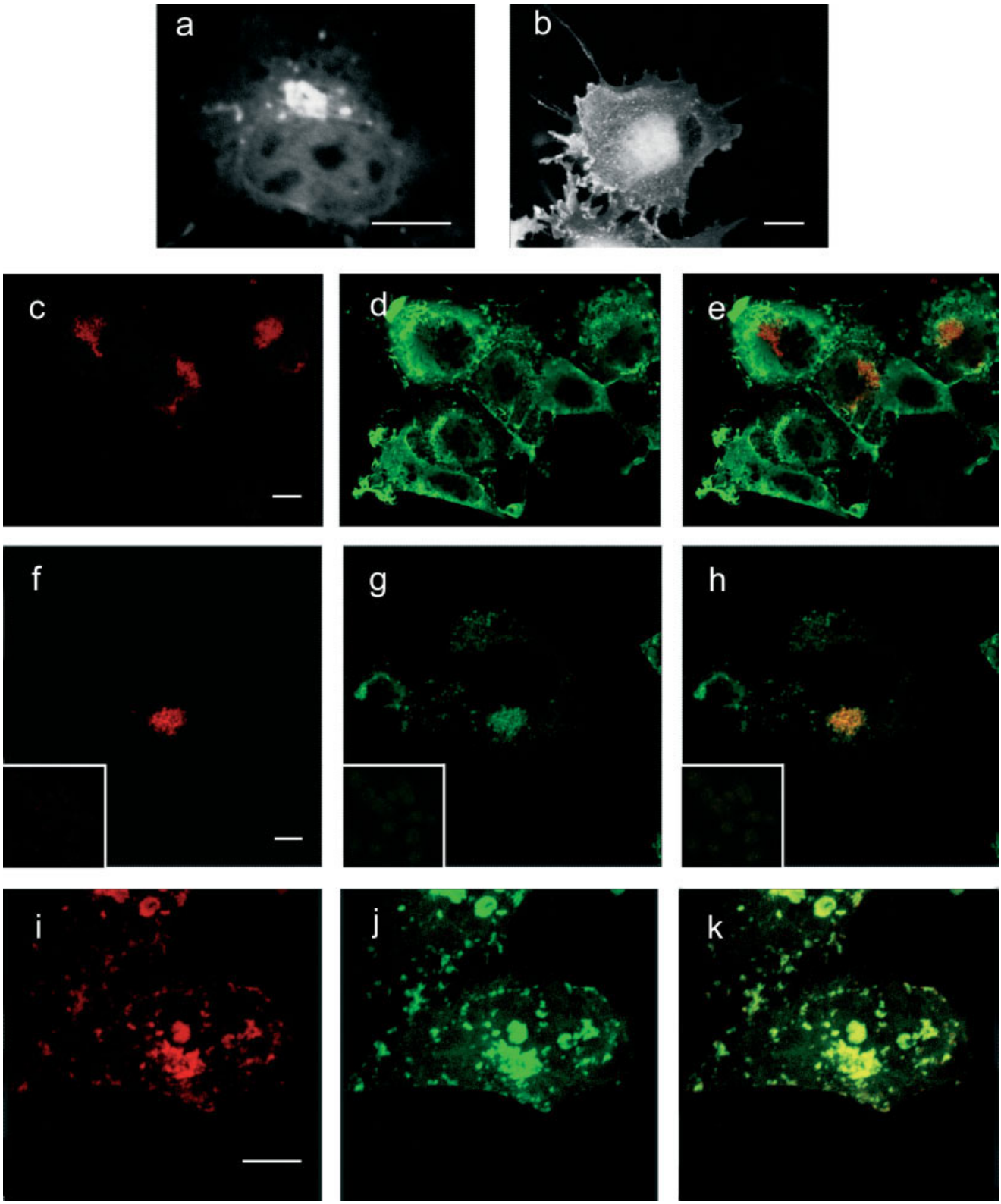


Fig. 5. Colocalization of β-dystrobrevin and pancortin-2 in transfected COS-7 cells. The subcellular distribution of pancortin and β-dystrobrevin was determined by transfecting COS-7 cells with pCMV-Myc/pan (**a**) and pCMV-HA/βDB (**b**), respectively. Pancortin-2 immunoreactivity often appears concentrated in vesicular-tubular structures next to the nucleus (a), whereas β-dystrobrevin is located in intensely stained punctae distributed throughout the cell (b). Cotransfection of pancortin-2 and β-dystrobrevin resulted in a change of the organization mostly of β-dystrobrevin (**d,g,j**), whereas pancortin-2 (**c,f,i**) remained concentrated in vesicular-tubular structures. The merged images (**e,h,k**) show different degrees of colocalization of the two proteins. Pancortin-2 was detected by using the monoclonal anti-c-Myc antibody, followed by Alexa Fluor 568 goat anti-mouse IgG secondary antibody (a,c,i). β-Dystrobrevin was detected by using the polyclonal anti-β-dystrobrevin antibody, followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (b,d,j). In the merged images (e,h,k), colocalization areas are rendered in yellow. **Insets** in f–h represent untransfected controls. Images a,b and i,l,m are the sums of the entire stacks of the cell monolayer. Images c–e and f–h are central sections of the cell. Scale bars = 10 μm in a,b,c (applies to c–e), f (applies to f–h), i (applies to i–k).

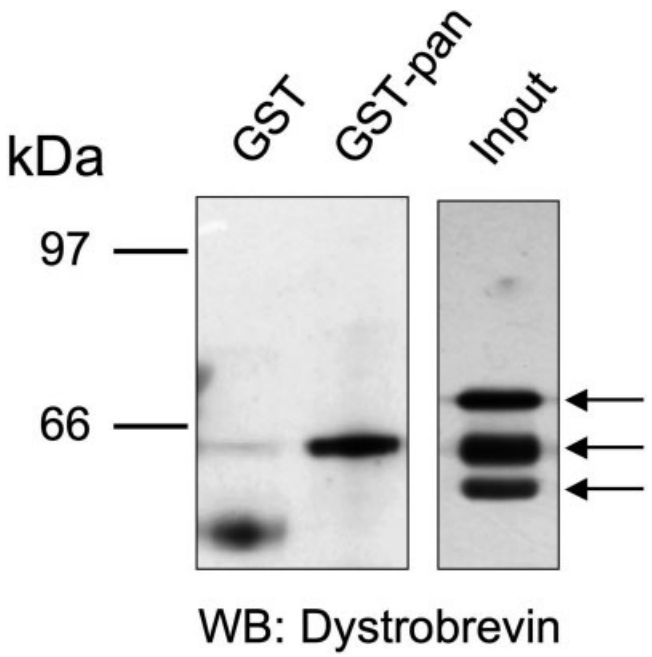


Fig. 6. Direct interaction of β-dystrobrevin and pancortin-2. Precleared rat brain lysate was incubated with GST-pancortin-2 (GST-pan) or GST protein (GST) prebound to glutathione-Sepharose beads. After extensive washing, bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with polyclonal anti-β-dystrobrevin antibody, and revealed by ECL. Bands corresponding to major dystrobrevin isoforms (α-dystrobrevin 1, 78 kDa; β-dystrobrevin, 59 kDa; α-dystrobrevin 2, 55 kDa) are indicated by arrows at right. Molecular masses of size markers are indicated at left (kDa). WB, Western blot.

formed by differential promoter usage and alternative splicing of a single gene (Danielson et al., 1994). Some of these proteins are expressed predominantly in the cortex of the mouse (Nagano et al., 1998, 2000) and rat (Danielson et al., 1994) brain. In the mouse, pancortins-1–4 share a common portion, the B part in the middle of their structure, with two splice variations at the amino-terminal (A1 or A2 part) and carboxyl-terminal regions (C1 or C2 part; Nagano et al., 1998). Each part of pancortin is differently named in the rat and the mouse (Danielson et al., 1994), whereas, in *Xenopus* and chick, pancortin orthologues are kown as *noelins* (Moreno and Bronner-Fraser, 2001). Noelin-1 enhances neural crest generation in chick (Barembaum et al., 2000) and promotes neurogenesis in *Xenopus* (Moreno and Bronner-Fraser, 2001). An important role in neuronal differentiation in mice has also been suggested for pancortins (Nagano et al., 2000; Ando et al., 2005). Consistently, in the middle of embryogenesis, pancortin mRNAs are widely distributed in the brain; as development progresses, their expression becomes confined to certain brain areas, suggesting that the extracellular matrix is adapted to the physiological specialties of specific cell populations. Recently, a transient but robust expression of A1- and A2-pancortin mRNAs and their translated molecules has been demonstrated in the developing brain, especially in the thalamus, although almost no pancortin mRNAs were detected in the adult thalamus. The expression of A2-pancortin (the pancortin isoforms for which the amino-terminus is composed of the A2 part; pancortin-3 and pancortin-4) mRNAs is more dominant than that of A1-pancortin (the pancortin isoforms for which the amino-terminus is composed of the A1 part; pancortin-1 and pancortin-2) mRNAs in the prenatal mouse cerebral cortex, despite the fact that levels of the former are very low in the adult (Nagano et al., 1998). Pancortin-3 (one of the A2-pancortins) seems to be secreted more efficiently than pancortin-1 (one of the A1-pancortins) from COS-7 cells, suggesting that the secretion of these two varieties of pancortin is regulated differently (Nagano et al., 2000). There is evidence that all four pancortins multimerize, and that the two conserved cysteine residues in the common B region are necessary for multimerization and secretion of pancortin-3 (Ando et al., 2005). These results and the finding that in *Xenopus* pancortin isoforms can bind to and antagonize one another suggest that interacting pancortin isoforms may play a role in regulating the timing of differentiation (Moreno and Bronner-Fraser, 2005).

Here we present evidence that β-dystrobrevin interacts directly with pancortins. The interaction meets several criteria of specificity, including interaction by yeast two-hybrid, coprecipitation in vitro and from rat brain extracts, and colocalization in transfected cells. In addition, we found that α-dystrobrevin, a closely related homologue of β-dystrobrevin, does not interact with pancortin-2.

Interacting proteins are likely to be involved in the same biological functions, and their interaction may link biological functions together into larger cellular processes. Our characterization of β-dystrobrevin association with pancortin therefore suggests the possibility that β-dystrobrevin might play a role in neuronal differentiation and brain development.

We have previously reported differences in the expression of some DPC components during retinoic acid (RA)-induced neuronal differentiation of P19 cells. We showed that Dp427 and Dp71 dystrophin isoforms and α-dystrobrevin 2 expression correlate temporally with neuronal differentiation of P19 cells, whereas dystroglycan, α-dystrobrevin 1, and β-dystrobrevin seemed to be constitutively expressed (Ceccarini et al., 2002). However, during mouse embryogenesis, an interesting correlation between α-dystrobrevin 1 expression and the induction of various differentiation processes has been described for the developing nervous system (Lien et al., 2004). Similarly, although there are no comparable reports on β-dystrobrevin, we cannot exclude the possibility that β-dystrobrevin expression might increase during brain development.

Here we have mapped the major region of β-dystrobrevin that binds pancortin-2 to a short segment contained between the EF hand-like and ZZ domains and that comprises amino acids 202–236. However, our data also suggest the presence of a secondary, weaker binding site for pancortin-2 in the β-dystrobrevin carboxyl-terminal region, although the possibility of a nonspecific coiled-coil interaction between β-dystrobrevin C2 region and pancortin B

part cannot be excluded. We also localized the β-dystrobrevin binding site on pancortin-2 and found that it lies in the B part of the molecule, the central region that is shared by all members of the pancortin family. Our data imply that β-dystrobrevin may associate with any one of the pancortins, and this is consistent with the finding that the B part is necessary for multimerization and secretion of pancortins (Ando et al., 2005; Moreno and Bronner-Fraser, 2005).

Although its spatial and temporal expressions during development have yet to be analyzed, β-dystrobrevin is expressed in adult brain; it has been reported to be most abundant in the hippocampus, cerebellum, and cortex (Blake et al., 1998, 1999). Indeed, a robust expression of pancortin has also been found to be localized throughout the mature cerebral cortex and in the hippocampus (Nagano et al., 1998, 2000). In rat, the pancortin orthologue BMZ was found primarily in the Golgi apparatus of podocytes and neurons (Kondo et al., 2000); by using an antibody against the B part, pancortin immunoreactivity was localized mainly in the endoplasmic reticulum (ER) of cortical neurons (Nagano et al., 1998). Our immunofluorescence microscopy results suggest that, in cotransfected COS-7 cells, pancortin-2 and β-dystrobrevin colocalize at the ER.

Because of its nature as a scaffolding protein that can assemble large protein–protein complexes at the plasma membrane, β-dystrobrevin might possibly anchor pancortin to the DPC. Still, the functional significance of this anchoring activity remains elusive.

An alternative hypothesis stems from the fact that pancortins are extracellular matrix constituents. They are N-glycosylated and secreted (Ando et al., 2005; Moreno and Bronner-Fraser, 2005), after which they have to be transported from the ER to the outside of the cell. Intracellular transport usually occurs through the action of molecular motors of the kinesin and dynein superfamilies, which actively move along microtubules carrying various cargoes (Hirokawa, 1998). We have previously shown that dystrobrevin binds the cargo-binding domain of the kinesin heavy chain and suggested a role for dystrobrevin as a motor protein receptor (Macioce et al., 2003). Recently, we reported that dystrobrevin and kinesin interact with a high binding affinity, which is consistent with microtubule-mediated transport, and suggested that their interaction may be regulated by phosphorylation (Ceccarini et al., 2005). In neurons, motor proteins such as kinesin are involved in the targeting and localization of specific proteins within distinct molecular and functional domains. The interaction between the adaptor/scaffolding protein dystrobrevin and the motor protein kinesin may play a role in the transport and targeting of pancortin to specific sites in the cell, with the differences in the binding properties of dystrobrevin isoforms reflecting their functional diversity within the same cell type. Phosphorylation events could have a regulatory role in this context. Taken together, our results reveal an emerging role of dystrobrevin as a multifunctional scaffold protein, with exciting new functions in brain development as well as intracellular transport and cell signaling.

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