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Research article

THE EFFECT OF THE LIPID-BINDING SITE OF THE ANKYRIN-BINDING DOMAIN OF ERYTHROID β-SPECTRIN ON THE PROPERTIES OF NATURAL MEMBRANES AND SKELETAL STRUCTURES

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Abstract: It was previously shown that the beta-spectrin ankyrin-binding domain binds lipid domains rich in PE in an ankyrin-dependent manner, and that its N-terminal sequence is crucial in interactions with phospholipids. In this study, the effect of the full-length ankyrin-binding domain of β-spectrin on natural erythrocyte and HeLa cell membranes was tested. It was found that, when encapsulated in resealed erythrocyte ghosts, the protein representing the full-length ankyrin-binding domain strongly affected the shape and barrier properties of the erythrocyte membrane, and induced partial spectrin release from the membrane, while truncated mutants had no effect. As found previously (Bok *et al.* Cell Biol. Int. 31 (2007) 1482-94), overexpression of the full-length GFP-tagged ankyrin-binding domain aggregated and induced aggregation of endogenous spectrin, but this was not the case with overexpression of proteins truncated at their N-terminus. Here, we show that the aggregation of spectrin was accompanied by the aggregation of integral membrane proteins that are

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Abbreviations used: DiD – 1,1'dioctadecyl 3,3,3'3'tetramethylindocarbocyanine; PC – phosphatidylcholine; PE – phosphatidylethanolamine; SDS PAGE – SDS polyacrylamide gel electrophoresis

known to be connected to spectrin via ankyrin, i.e. Na^+K^+ATP-ase, IP3 receptor protein and L1 CAM. By contrast, the morphology of the actin cytoskeleton remained unchanged and aggregation of cadherin E or N did not occur upon the overexpression of either full-length or truncated ankyrin-binding domain proteins. The obtained results indicate a substantial role of the lipid-binding part of the β-spectrin ankyrin-binding domain in the determination of the membrane and spectrin-based skeleton functional properties.

Key words: Spectrin-lipid interactions, Ankyrin-binding domain, Resealed ghosts, Membrane skeleton properties, Transmembrane protein aggregation

INTRODUCTION

Spectrin was originally discovered in erythrocytes by Marchesi and Steers [1]. Now, spectrins are recognized as a large class of proteins that are found in all vertebrate and invertebrate cells, and that are differentially expressed during development [2, 3]. In non-erythroid cells, spectrins form an extended protein network just below the plasma membrane bilayer by linking various actin fibers and many integral membrane proteins. They are also present in the cytosol, and can bind other proteins via numerous interaction motifs, such as the SH3 domain [4-6] or calmodulin-binding domain in αII-spectrin. Over 50 protein ligands for spectrins have been found [3].

One of the most studied biological membranes is that of the red blood cell. During its 120-day life span, a red blood cell circulates many times in the human body, often being squeezed through capillaries with an up to four-fold smaller diameter. The remarkable mechanical properties of the erythrocyte originate from the unique architecture of its plasma membrane, which is its main load-bearing component, as there are no stress fibers inside the cell [7-9]. An important element controlling the shape and mechanical properties of red blood cells is the membrane skeleton. This subcortical two-dimensional meshwork is composed mainly of spectrin and actin together with other proteins that regulate spectrin-actin interaction and participate in linking it to the integral membrane proteins through several pathways of protein-protein interaction: spectrin-ankyrin-band 3 (AE1; anion exchanger 1) and a connection involving Rh, RhAG, CD47, protein 4.2 and/or ankyrin or the ternary interaction protein 4.1-p55-glycophorin C [10-15]. Recent data also implies the participation of many other membrane proteins including band 3 protein (AE1) and adducin in the spectrin-actin junction of the erythrocyte membrane skeleton [16, 17]. Erythrocytes with defects in their spectrin-based (membrane) skeleton lose their shape and stability, as seen in hemolytic anemia in humans [18].

There are also many indications that spectrin binds directly to membrane lipids (e.g. [19], for a review, see [20]). Its high affinity lipid-binding sites seem to be confined to particular regions [19, 21, 22]. Previously published studies from our laboratory [21, 23, 24] revealed that an ankyrin-sensitive phospholipid-binding

site is located at the N-terminal part of the erythroid (residues 1768-1805) and non-erythroid (residues 1776-1906) β-spectrin ankyrin-binding domain [25]. Moreover, we found that the N-terminal parts of both β-spectrin ankyrin-binding domains are important for this activity [23, 24].

A previous study by our group [24] showed that overexpression of the full-length ankyrin-binding domain induced aggregation of endogenous spectrin together with GFP-tagged recombinant protein. This was not the case for GFP-tagged proteins truncated at their N-terminus. This drew our attention to the possible role of this part of the spectrin molecule in determining the properties of the membrane and the distribution of the membrane integral proteins, an issue which has yet to be elucidated. As this is a fragment of a large, constitutively-expressed protein, it is rather difficult to construct a model system to study its biological role. Therefore, to at least partially answer this question, two competition models were used.

One of the models was red blood cell ghosts resealed in the presence of expressed polypeptides representing the full-length or truncated ankyrin-binding domain of β-spectrin [26, 27]. This experimental system permitted the observation of the effect of the polypeptides encapsulated in the erythrocyte ghosts on their morphology, barrier properties and simultaneous spectrin release. The other model was HeLa cells, in which it was possible to observe the effect of overexpression of the full-length or truncated ankyrin-binding domain of erythroid and non-erythroid β-spectrins on actin cytoskeleton morphology and the distribution of some integral membrane proteins. The aggregation of spectrin upon transfection with recombinant proteins representing the full-length erythroid or non-erythroid spectrin ankyrin-binding domains (and not their truncated mutants) was accompanied by the aggregation of integral membrane proteins that are known to be connected to spectrin via ankyrin, i.e. Na^+K^+ATP-ase, IP3 receptor protein and L1 CAM. However, the morphology of the actin cytoskeleton remained unchanged, and the aggregation of cadherin E or N did not occur upon overexpression of either the full-length or truncated ankyrin-binding domain proteins.

MATERIALS AND METHODS

Polypeptide expression and purification

Plasmids encoding the full-length ankyrin-binding domain proteins of erythroid β-I spectrin (DWA, residues 1638-1952), N1C (residues 1767-1952) and non-erythroid β-II spectrin (DWAN, residues 1740-1981), and the truncated erythroid fragments Fr 3 and Fr 5 (residues 1776-1952 and 1806-1952, respectively) and a truncated non-erythroid fragment (DWANN2 residues 1857-1981) were generated as described previously (Tab. 1) [23, 24]. They were used for protein expression experiments. Recombinant proteins were expressed in the bacterial BL21(DE3)pLysE expression system using 0.5 M IPTG as an inducer

for 3 h at 37ºC, then extracted with 8 M urea (20 mM Tris-HCl, 100 mM NaCl, pH 8), and purified via immobilized Co^{2+}-affinity chromatography (Clontech).

Tab. 1. The βI and βII-Spectrin fragments used in this study.

Parent molecule	Name	Residue number
βI-Spectrin	DWA	1638-1952
	N1C	1767-1952
	Fr 3	1776-1952
	Fr 5	1806-1952
βII-Spectrin	DWAN	1740-1981
	DWANN2	1857-1981

The His-Tag proteins were analyzed in Coomassie blue-stained SDS-PAGE 10% gels (not shown). Before use in the resealing experiments, hemoglobin was added to the obtained protein solutions to a final concentration of 1 mg/ml, and dialyzed into a resealing buffer without urea.

Preparation of the resealed ghosts and analysis of spectrin release

Fresh blood samples were collected from healthy human volunteers using an anti-coagulant (0.8% citric acid monohydrate, 2.2% trisodium citrate, 2.2% glucose). The red blood cells were washed three times with 10 mM Tris-HCl buffer (pH 7.4) containing 120 mM KCl. Intact cells were lysed in ice-cold lysis buffer containing MgATP (5 mM Tris-HCl, 5 mM KCl, 1 mM $MgCl_2$, 0.6 mM MgATP, pH 7.4) and centrifuged (15000 g, 15', 4ºC). This step was repeated until the ghosts were pale pink. Then they were suspended in the resealing buffer (150 mM KCl, 1.6 mM $MgCl_2$, 1 mM DTT, 0.6 mM MgATP, pH 7.4) and incubated first for 10 min on ice and then for various lengths of time with gentle shaking (0-60 min, at 37ºC, 225 rpm) in the presence or absence of 1 mg/ml hemoglobin-stabilized polypeptides corresponding to the full-length or truncated ankyrin-binding domain at a final concentration of 2 mg/ml. After resealing, the ghosts were centrifuged (10000 g, 5 min, 4ºC), and the pellets and supernatants were collected for further analyses.

The level of endogenous spectrin extracted from the erythrocyte ghosts during resealing with the ankyrin-binding domain and its mutants was analysed via Coomassie Blue-stained SDS-PAGE (7% gels) of the obtained supernatants. The resealed protein content of the ghosts was assessed via SDS-PAGE of the pellet proteins.

The morphology of the resealed erythrocyte ghosts

After resealing in the presence of the various polypeptides, the erythrocyte ghosts were fixed by resuspending them in 4% PFA in the resealing buffer (1:1, 10 min, room temperature) and washed three times at room temperature with PBS (pH 7.4, 7000 g, 5 min, room temperature). The fixed and washed

ghosts were stained with Vybrant® DiD (1 μM/ml in PBS, pH 7.4, Invitrogen) and incubated for 30 min at 37ºC. Next, the ghosts were washed three times with PBS without DiD, centrifuged at 10000 g for 5 min at room temperature, mounted (VectaShield for fluorescence, Vector) on glass microscope slides, and covered with coverslips. The images were acquired with a Zeiss LSM 510 Meta microscope using a 1.4 Plan Apochromat lens, 63x lens objective.

The barrier properties of the resealed ghosts

The Hi-Tech single-mixing stopped-flow system was used to study the behaviour of the erythrocyte ghost membranes resealed in the presence of various polypeptides upon dilution with a hypotonic (water) solution [28]. The erythrocyte ghosts were isolated and resealed for 30 min as described above. After resealing, the suspension was centrifuged (10000 g for 10 min, 4ºC), and the ghost pellet was resuspended in PBS and used for stopped-flow analysis. In this experimental approach, the light scattering at 90º to the incident beam was used to follow the erythrocyte size changes induced by the osmotic pressure over time. The collected results were the kinetic traces of the light scattering changes.

The transient expression of fluorescent recombinant proteins in HeLa cell culture

The ankyrin-binding domain constructs in the pEGFP-C1 vector were obtained as described previously by Bok *et al.* [24]. Purified plasmid DNA was used to transfect cultured HeLa cells and the observations were made 24 h after transfection.

Immunostaining

Before immunostaining, the cells were washed several times with PBS and fixed with formaldehyde (4%) in PBS. Next, they were permeabilized with 0.1% Triton X-100 in PBS, and finally washed a few times with PBS. The cells were blocked for 20 min with 100% FCS at room temperature, and then incubated for 2 h, either with polyclonal antibodies: 2 μg/ml rabbit anti-human α1-Na^+/K^+-ATPase, 2 μg/ml goat anti-human L1-CAM, or 1 μg/ml rabbit anti-human IP3R-I/II/III (all from Santa Cruz Biotechnology); or with mouse monoclonal antibodies: 4 μg/ml anti-human E (Chemicon/Milipore) or N-cadherin IgM (Upstate/Milipore). Then the cells were washed three times in PBS. As a secondary antibody, we used 3.75 μg/ml Cy™5- or TRITC-conjugated goat anti-rabbit, or donkey anti-goat, or rabbit anti-mouse Igs (all from Jackson ImmunoResearch). The preparations were washed three times with PBS and then with distilled water, and were finally mounted by Vectashield with DAPI (Vector Laboratories, Inc.) on glass slides. Changes in the localization of the F-actin filaments were detected using 2.5 μg/ml phalloidin conjugated with rhodamine B (TRITC-Phalloidin B, Fluka). The cells were incubated with phalloidin in PBS for 15 min, washed three times with PBS and distilled water, and mounted by Vectashield without DAPI. The cells were observed via confocal optics using a Zeiss LSM 510 Meta microscope using 1.4 Plan Apochromat lens, 63x lens objective.

RESULTS

The effect of the presence of the ankyrin-binding domain or its mutants on spectrin release from the membrane during resealing and on the morphology of erythrocyte ghosts

The erythrocyte ghosts were resealed in the presence of a protein corresponding to either the full-length or truncated ankyrin-binding domain to test the possibility of endogenous spectrin release through a competitive mechanism. The SDS-PAGE results for the supernatants collected after the resealing incubation of the ghosts in the presence or absence of the studied polypeptides (Fig. 1) indicated that the recombinant full-length ankyrin-binding domain (DWA) and its truncated functional analog N1C, induced a partial (25-30% of the total ghost spectrin) release of endogenous spectrin from the erythrocyte ghosts as quantitated by a densitometric analysis of Coomassie-stained electropherograms (Fig. 1A, B). The appearance of spectrin was observed during the first minutes of incubation with these fragments.

Different results were obtained when the truncated mutants lacking their N-terminal region were used. When Fr 3 or Fr 5 were present during the resealing incubation, no spectrin extraction was observed (Fig. 1C). There were also no traces of spectrin in the supernatant from the control ghosts resealed in the absence of polypeptides and from the ghosts resealed in the presence of hemoglobin. After the above-described experiments, the morphology of the resealed ghosts was examined. Their shape was observed first in Nomarsky optics (not shown) and then under a fluorescence/confocal microscope using a highly lipophilic fluorescent dye, DiD, which stains the lipid phase of biological membranes. The analysis revealed that the largest effect on the morphology of the erythrocyte ghosts, and therefore possibly on the membrane-membrane skeleton interactions, was exerted by the DWA and N1C polypeptides,

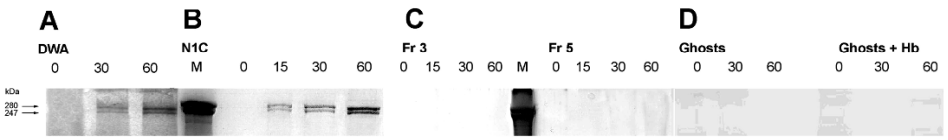


Fig. 1. The release of spectrin during the resealing of the erythrocyte ghosts in the presence of the full-length or truncated ankyrin-binding domain. The results shown are from the SDS-PAGE analysis of supernatants from ghosts incubated for resealing with a "resealing buffer" (see Materials and Methods) containing 2 mg/ml of DWA (panel A) or N1C (panel B) after the indicated incubation time. M – molecular mass marker for the erythrocyte ghost proteins. There is a clearly visible extraction of endogenous spectrin. Panel C shows the supernatants collected from ghosts resealed in the presence of 2 mg/ml Fr 3 and Fr 5 proteins, and panel D shows the supernatants from the control, "empty" ghosts and from those incubated with hemoglobin in the resealing buffer (for further details see Materials and Methods).

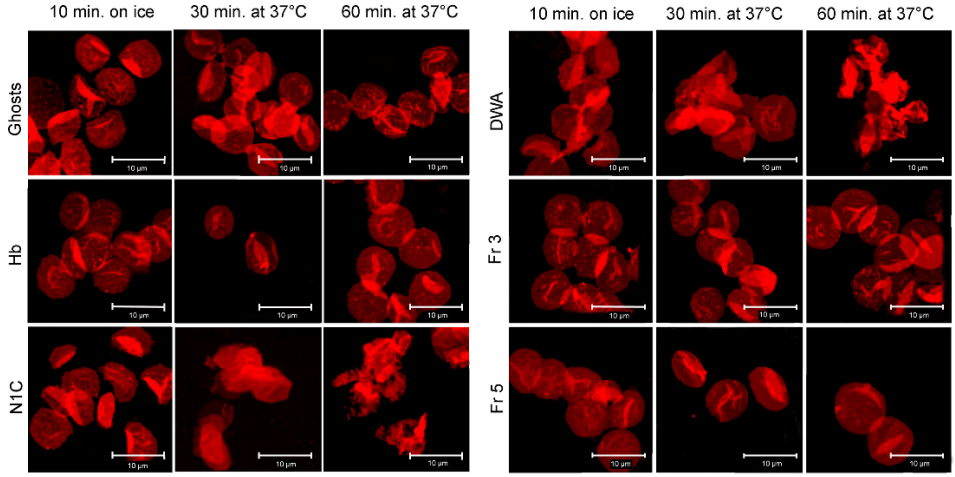


Fig. 2. Erythrocyte shape after resealing in the presence of the studied recombinant polypeptides representing the full-length or truncated ankyrin-binding domain. The images represent ghosts collected during resealing (0, 30 and 60 min respectively in the left, middle and right column of each panel). The ghosts were stained with the fluorescence dye Vybrant® DiD, and the images were acquired with an Olympus Fluoview 500 confocal scanning microscope. A PLAPO 60x oil immersion objective was used. The images in each panel are 2D pictures, the bar is 10 μm. Ghosts – hemoglobin-free resealed ghosts (control), Hb – ghosts resealed in the presence of 2 mg/ml hemoglobin, N1C, DWA, Fr 3, Fr 5 – ghosts resealed in the presence of 2 mg/ml N1C, DWA, Fr 3 or Fr 5, respectively. For further details see Materials and Methods.

which represent the full-length sequence of the studied domain. These polypeptides caused a complete loss of the normal, discoidal shape, aggregation and membrane folding of the erythrocyte ghosts (Fig. 2, left panel, bottom row, and right panel, top row). The morphology of the ghosts resealed in the presence of the fragments Fr 3 and Fr 5 (Fig. 2, right panel, middle and bottom row) was comparable to that of the control ghosts (Fig. 2, left panel, top and middle row). We may conclude that the presence of the N-terminal region in the DWA protein was important to its ability to compete with native spectrin, which most probably affected the shape of the resealed ghosts.

The effect of the resealed full-length and truncated ankyrin-binding domain on the barrier properties of erythrocyte ghosts

Normally, the analysis of hemolysis kinetics in the stopped-flow regime is used to follow the ability of erythrocyte plasma membranes to withstand osmotic stress, with the results giving insight into the mechanical properties of the membrane [28]. In the case of resealed ghosts, the swelling/lysis kinetics are rather difficult to follow, as the membrane barrier properties are not maintained to a high degree, and the initial level of "impermeability" can be different for different preparations studied. However, it appeared that these barrier properties (plateau value) varied between ghosts resealed in the presence or absence of

polypeptides corresponding to the full-length and truncated ankyrin-binding domain. The results presented in Fig. 3 indicate that the light-scattering change values corresponding to the initial barrier properties observed for ghosts resealed in the presence of a functional ankyrin-binding domain (DWA and N1C) were much smaller than those for mutants in which the N-terminal part had been deleted (Fr 3 and Fr 5). The data for Fr 3 and Fr 5 was similar to the control values observed in the presence of the buffer only or in the presence of haemoglobin. It seems that competition of DWA and N1C with native spectrin for the erythrocyte membrane sites (also involving spectrin release) most probably prevented the re-establishment of the membrane barrier, which resulted in smaller volume changes under osmotic stress. The other possibility, that this interaction would affect the mechanical properties of the membrane, is rather unlikely, as only the amplitude, but not the slope of the initial part of the curve is subject to change. Altogether, the observed changes in the resealed erythrocyte ghost membrane properties suggest an important role of the N-terminal part of the β-spectrin ankyrin-binding domain.

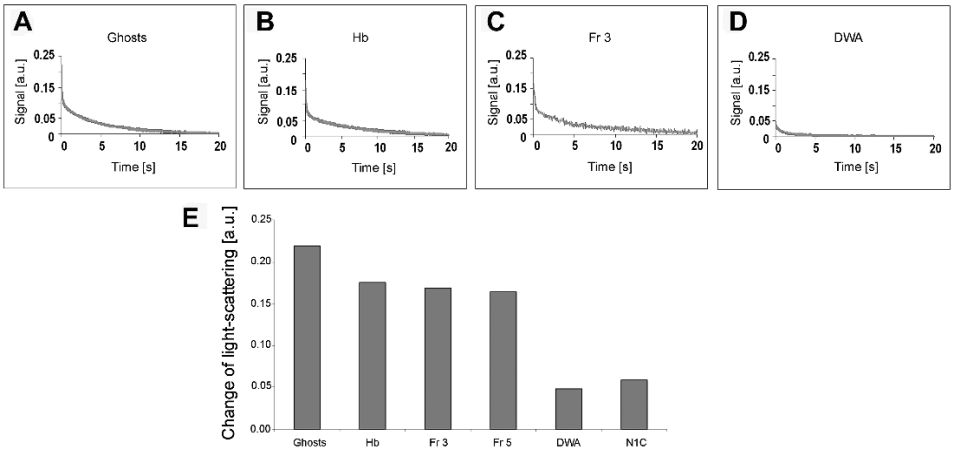


Fig. 3. Changes in the membrane barrier properties upon resealing in the presence or absence of polypeptides corresponding to the full-length or truncated ankyrin-binding domain. Resealed ghosts were obtained as described in the legends to Figs 1 and 2 and in the Materials and Methods section. Upper panel: examples of stopped-flow light-scattering traces collected at 400 nm. Each trace represents the average of 14-17 individual experiments. Lower panel: bar graph representing the average amplitude values obtained for a particular preparation. The numbers correspond to the amplitude values (for further details see Materials and Methods).

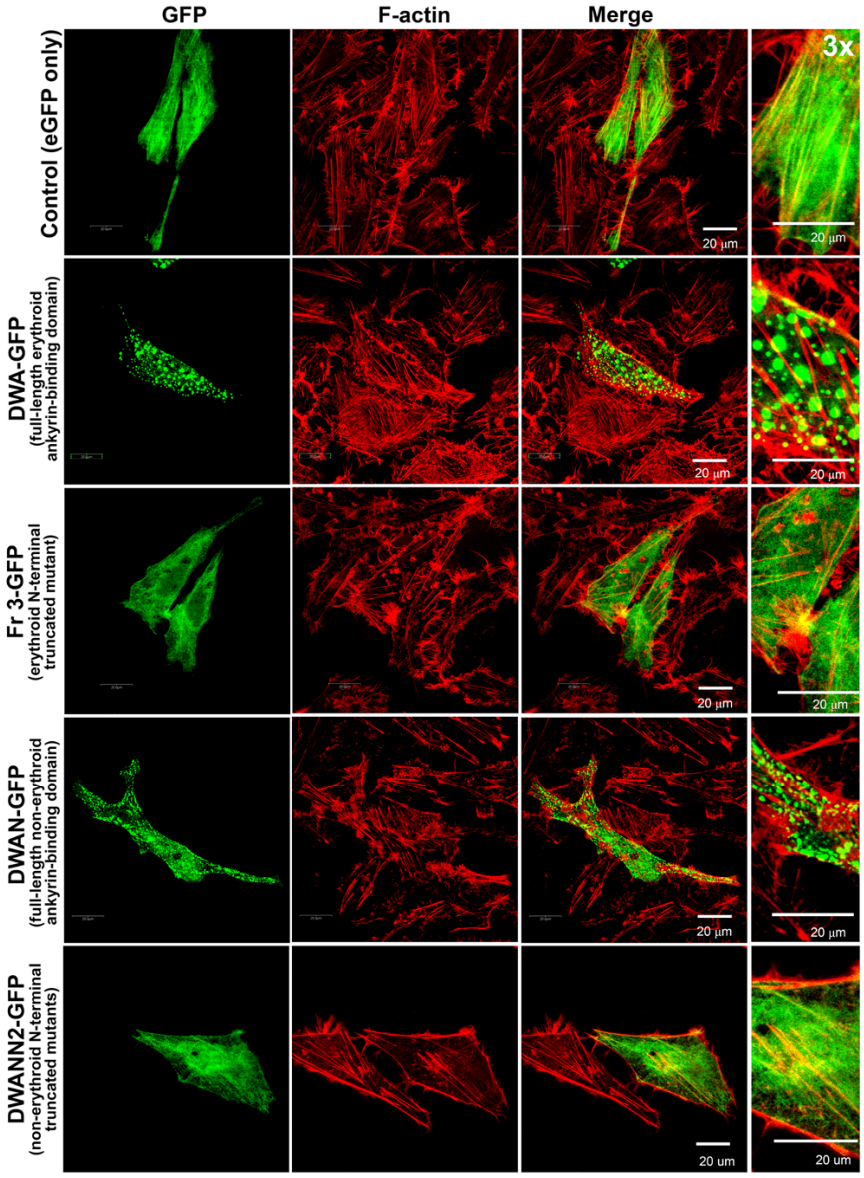


Fig. 4. Transient overexpression of the full-length ankyrin-binding domain induces aggregation of spectrin but does not affect the actin cytoskeleton morphology in HeLa cells. HeLa cells were transiently transfected with pEGFP-C1 vector (top row), or the same vector containing inserts coding for the full-length erythroid spectrin ankyrin-binding domain (DWA-GFP) or its N-terminal deletion mutant (Fr 3-GFP), or the full-length non-erythroid spectrin ankyrin-binding domain (DWAN-GFP) or its N-terminal deletion mutant (DWANN2). Left column: fluorescence of GFP. Middle column: actin filaments stained with TRITC-conjugated Phalloidin. Right column: overlay. Inset: Zoomed (3x) fragment of the right column photograph. Bar = 20 mm. Observations were performed using an LSM510 META Zeiss microscope and a Pln Apo 63x1.4 objective.

The effects of full-length or truncated ankyrin-binding domain overexpression in HeLa cells on the morphology of the actin skeleton and the distribution of several integral membrane proteins: Na^+K^+ATPase, IP3 receptor, L1 CAM and E and N cadherins

Living cells transfected with the GFP-tagged construct of this domain were chosen for experiments to yield more information on the mode of action of recombinant ankyrin-binding protein in the cell. Earlier data [24] showed that overexpression of the full-length ankyrin-binding domain induced aggregation of endogenous spectrin together with GFP-tagged recombinant protein. This was not the case for His-tagged proteins truncated at the N-terminus.

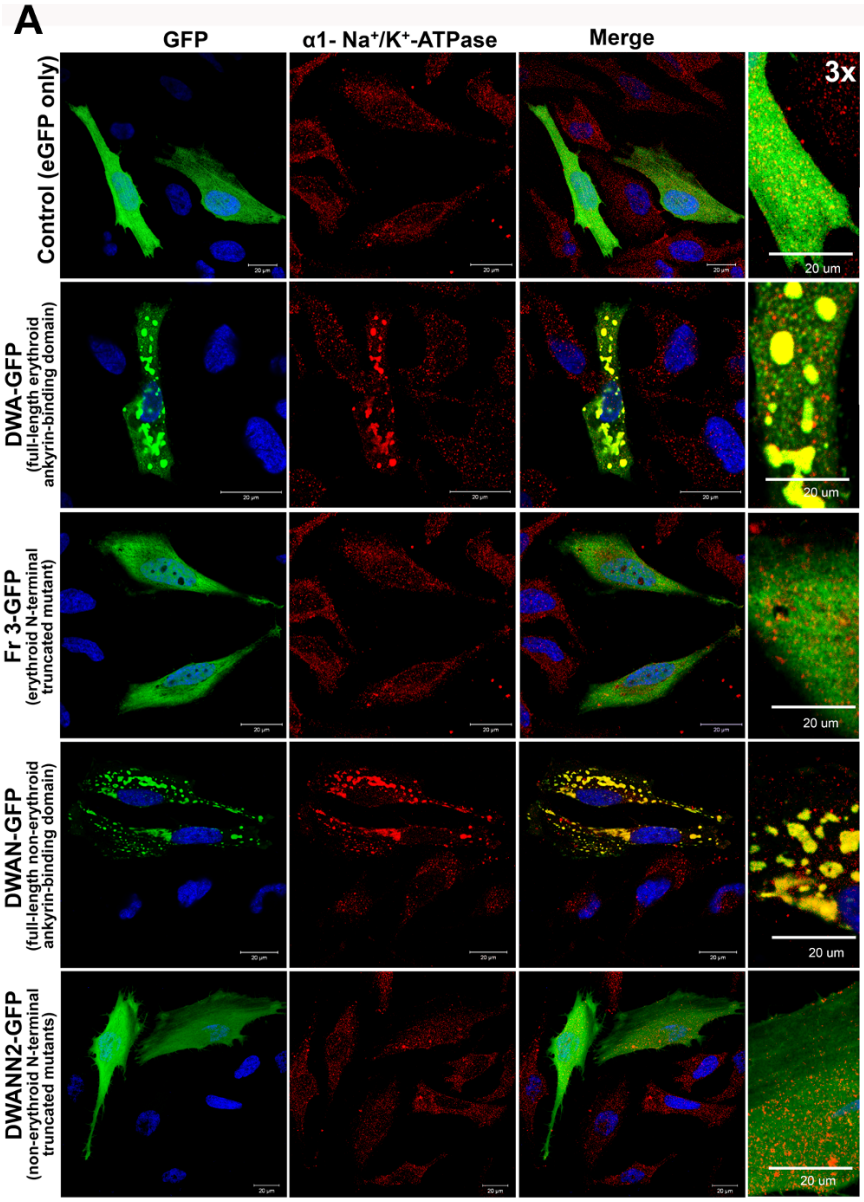
It was interesting to test whether this redistribution of spectrin elements affects the actin cytoskeleton. As shown in Fig. 4, the RITC-Phalloidin staining pattern of the actin cytoskeleton did not undergo significant changes in cells overexpressing any of the ankyrin-binding domain constructs including GFP alone when compared to the untransfected cells.

The aggregation of spectrin was accompanied by the aggregation of certain membrane proteins which are known to be connected to spectrin via ankyrin, i.e. Na^+K^+ATP-ase, IP3 receptor protein and L1 CAM (Fig. 5A and Tab. 2). The aggregation of these proteins was not observed in the case of overexpression of recombinant proteins truncated at their N-terminus, representing either erythroid or non-erythroid spectrin ankyrin-binding domains. On the other hand, as mentioned above and shown in Fig. 4, the morphology of the actin cytoskeleton remained unchanged, and cadherin E and N did not aggregate (Fig. 5 and Tab. 2) upon overexpression of full-length or truncated ankyrin-binding domain proteins.

Tab. 2. The co-distribution of selected transmembrane proteins with spectrin aggregates following the transfection of HeLa cells with a vector coding for the EGFP-conjugated full-length ankyrin-binding domain. For details see the Materials and Methods section and Fig. 5.

Transmembrane protein	Co-distribution
Na^+K^+ ATP-ase	+
IP3R1/I/II/III	+
L1-CAM	+
E-cadherin	-
N-cadherin	-

In summary, all of the above data indicates that the N-terminal part of the ankyrin-binding domain of the beta spectrins, which was also found to have ankyrin-dependent lipid (PE-rich) binding activity, plays an important role in maintaining some of the crucial membrane properties such as the barrier properties and in particular those connected to spectrin function: maintaining the cell shape and the distribution of certain membrane proteins.


A
GFP
α1- Na+/K+-ATPase
Merge
Control (eGFP only)
3x
20 um
DWA-GFP
(full-length erythroid ankyrin-binding domain)
20 um
Fr 3-GFP
(erythroid N-terminal truncated mutant)
20 um
DWAN-GFP
(full-length non-erythroid ankyrin-binding domain)
20 um
DWANN2-GFP
(non-erythroid N-terminal truncated mutants)
20 um

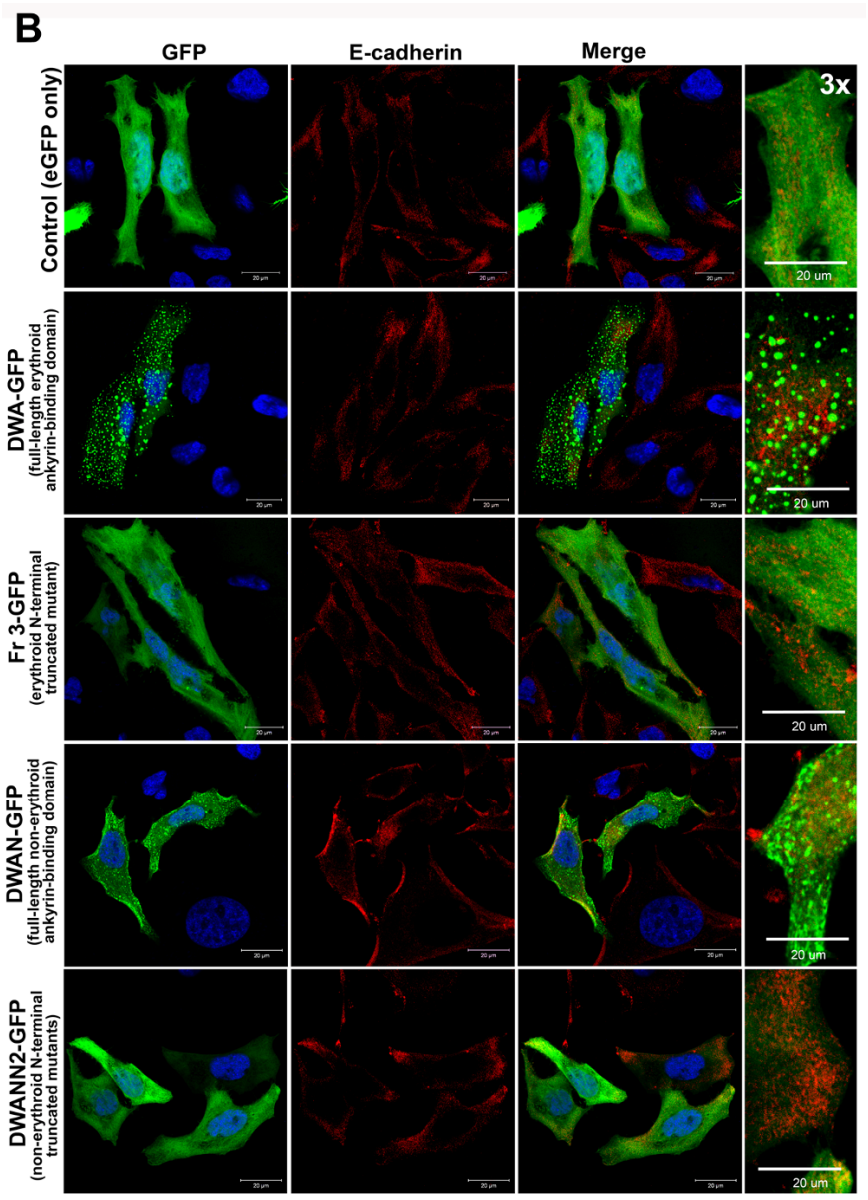


Fig. 5. ATP-ase but not cadherin E epitopes follow spectrin aggregation upon the transient expression of the full-length ankyrin-binding domain. HeLa cells were transiently transfected with pEGFP-C1 vector (top row), or the same vector containing inserts coding for the full-length erythroid spectrin ankyrin-binding domain (DWA-GFP) or its N-terminal deletion mutant (Fr 3-GFP), or the full-length non-erythroid spectrin ankyrin-binding domain (DWAN-GFP) or its N-terminal deletion mutant (DWANN2). The cells were harvested and immunostained with polyclonal rabbit anti-human ATP-ase and probed with TRITC-labelled goat anti-rabbit IgG (A) or with mouse monoclonal anti-human cadherin E probed with Cy^{TM}5 rabbit anti-mouse antibody (B). Other details as in Fig. 4.

DISCUSSION

It was shown previously that spectrins bind membrane phospholipids, in particular PE-rich mono- and bilayers (for a review see [20]). This binding was found to be ankyrin-sensitive, i.e. it was inhibited by purified erythrocyte ankyrin [21]. It was shown that the binding site for PE-rich lipids, which is sensitive to ankyrin inhibition, was located in a 38-residue N-terminal fragment of the β-spectrin ankyrin-binding domain, and that the presence of the first 8 residues was crucial for this activity. An analogous role was shown for the N-terminal part of the non-erythroid β-spectrin ankyrin-binding domain (residues 1776-1906) [23]. Moreover, Bok *et al.* [24] found that when protein constructs corresponding to the full-length domain and containing an "intact" N-terminal sequence were transiently overexpressed in the cells of several lines, specific aggregation of the spectrin-based skeleton was observed. The fact that the aggregates contained endogenous spectrin was confirmed by the observation that anti-spectrin antibodies depleted of their anti-ankyrin-binding domain reactivity still reacted with the aggregates. Here, we addressed the question of how the polypeptides corresponding to the full-length and truncated ankyrin-binding domain would affect some of the properties of the natural membrane. As the transfection of erythroid cells is not a simple task, we decided to load the expressed and purified proteins into the cell interior by taking advantage of the ability of the red blood cell membrane to reseal under appropriate salt and temperature conditions. Such ghosts re-establish some of the native erythrocyte membrane properties such as shape and membrane phospholipid asymmetry, and partially regain the membrane barrier properties [26, 27]. Our assumption was that if the studied recombinant protein was active and in the ghosts at high concentrations, the protein should at least to a certain degree compete with the native spectrin. This appeared to be true in the case of the full-length ankyrin-binding domain and in the case of the mutant that had an intact N-terminal part. More interestingly, these proteins dramatically affected the morphology of the resulting resealed ghosts; entrapping these peptides within the ghosts caused complete loss of the normal discoidal shape, aggregation and membrane folding. Truncated mutants (Fr 3 and Fr 5) of the ankyrin-binding domain truncated by 8 or 38 residues from the N-terminal region did not compete with endogenous spectrin for binding to the ghost membrane; in fact, reconstitution of the membrane with Fr 3 and Fr 5 caused neither spectrin release nor substantial changes in the ghost morphology. Our earlier data [23] indicated that these mutants were still able to bind erythrocyte ankyrin *in vitro*, but that their ability to bind PE-rich mono- and bilayers was markedly reduced. We therefore assume that the removal of the first amino acid residues, cruicial in binding the membrane phospholipids, markedly reduced the polypeptide's ability to compete with erythrocyte spectrin. As a consequence, the endogenous spectrin was still anchored via these sites into the membrane, thus maintaining the correct shape of the erythrocyte ghosts.

The importance of the N-terminal region of this domain might indicate that the lipid-binding activity of this domain plays a significant role in spectrin-membrane interactions in the natural erythrocyte membrane. The presence of full-length (DWA and N1C) ankyrin-binding domain polypeptides during the resealing of the erythrocyte membrane probably induced a decrease in the barrier properties, as shown in the lower light-scattering change values observed under conditions of osmotic stress. A much smaller effect (if any) was observed when the truncated mutants were used. The competitive interaction of the polypeptide corresponding to the ankyrin-binding domain containing an intact N-terminal sequence with the membrane bilayer prevented the re-estblishing of the barrier properties to a much greater extent than the interactions of those truncated at the N-terminus, indicating that not only the mechanical but also the permeability properties had been changed. This may further indicate that the presence of a lipid-binding (PE-rich) region in this domain is crucial in the inhibition of the resealing process, suggesting that lipid-binding activity is important for this process. These conclusions concur with the data of other researchers [29], who found that spectrin extractability from resealed ghost membranes was much less efficient than from regular ghosts, i.e. ghosts with scrambled PS-asymmetry.

To get more detailed insight into the mechanism of changes occurring in the membrane in the presence of an excess of protein corresponding to the ankyrin-binding domain, we used the model of HeLa cells transiently transfected with the GFP construct. The spectrin distribution changes occurring upon the transfection of HeLa cells with a vector containing an insert coding for the full-length ankyrin-binding domain of either erythroid or non-erythroid spectrin was reported earlier by our laboratory [24]. Here, we showed that the expression of the full length ankyrin-binding domain induced aggregation of some transmembrane proteins, such as Na^+K^+ATP-ase, IP3 receptor protein and L1 CAM, which are known to interact with spectrin via ankyrin together with the endogenous spectrin-GFP-ankyrin-binding domain. This event appeared to be dependent on the presence of the N-terminal part of the ankyrin-binding domain, suggesting that the lipid-binding activity of this part may be important in this event. On the other hand, overexpression of the ankyrin-binding domain does not seem to have a substantial effect on the actin cytoskeleton morphology observed after staining with phalloidin under a fluorescent microscope. Also, the appearance of E and N cadherins, whose link to actin filaments is independent of ankyrin, remained unchanged in HeLa cells overexpressing the full-length ankyrin-binding domain. Neither the actin filaments nor the E and N cadherin distribution were affected by the overexpression of truncated mutants of the ankyrin-binding domain or by green fluorescent protein alone (Figs 4 and 5B, and Tab. 2). Similar results with overexpression of the β-spectrin ankyrin-binding domain were reported in [30], but they used only the full-length ankyrin-binding domain without further mutations, i.e. without exploring the role of the lipid-binding part. It is most probable that an excess of ankyrin-binding domain protein does not disrupt ankyrin-spectrin interactions since ankyrin-linked

transmembrane proteins follow the pattern of the spectrin/ankyrin-binding domain protein in the transfected cell.

Previously, we proposed that the N-terminal, lipid-binding part of the ankyrin-binding domain is responsible for anchoring the membrane skeleton to the membrane bilayer in cases when ankyrin is not present in sufficient quantity to provide an attachment site for the spectrin tetramer or when its activity is suppressed, e.g. by phosphorylation [31]. Considering the obtained results, we suggest that this part of the domain is also responsible for maintaining the spectrin tetramers (in particular, their middle parts) and therefore, keeping the ankyrin-transmembrane protein complexes at a proper distance from each other. This may be involved in maintaining the membrane's mechanical and/or barrier properties as well as for the proper lateral distribution of integral proteins linked to spectrin via ankyrin. Part of this event could be a result of partial spectrin release from the membrane. In the case of erythrocyte membranes, 25-30% of endogenous spectrin was released during resealing with proteins representing the full-length ankyrin-binding domain. In the case of transfected cells, the amount of spectrin is much more difficult to assess, as we did not obtain a high transfection efficiency. The fact that a substantial amount of Na^+K^+ATPase or other integral proteins co-aggregate with the spectrin-ankyrin-binding domain-GFP conjugate suggests that this fraction of cellular spectrin is still membrane-bound.

It should be noted here that on the basis of structural studies, the same fragment of the ankyrin-binding domain was recently implied to be a part of an ankyrin binding-site by Ipsaro *et al.* [32]. Actually, it is the same fragment which was found by us [33, 34] to form an amphipathic helix, but they suggest that the hydrophilic side of it is involved in ankyrin-binding; this could explain the competition between lipids and ankyrin for β-spectrin [21]. However, recent structural and above all mutational studies [35] seem to suggest that the neighboring part of the β-spectrin is engaged in ankyrin binding, so the effect on the erythrocyte membrane is rather lipid-binding site dependent.

The data presented here sheds new light on the role of the lipid-binding part of the β-spectrin ankyrin-binding domain. This part of the molecule seems to be involved in preventing spectrin release from the membrane upon resealing, in controlling the regaining of the resealed ghost shape, and in barrier properties. It is also involved in preventing the aggregation of spectrin and integral membrane proteins in non-erythroid membranes. There is a substantial possibility that all these features are connected with the capability of interaction of this part of the molecule with membrane lipids.

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