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Research article

Function, expression and localization of annexin A7 in platelets and red blood cells: Insights derived from an annexin A7 mutant mouse

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Abstract

Background: Annexin A7 is a Ca^{2+}- and phospholipid-binding protein expressed as a 47 and 51 kDa isoform, which is thought to be involved in membrane fusion processes. Recently the 47 kDa isoform has been identified in erythrocytes where it was proposed to be a key component in the process of the Ca^{2+}-dependent vesicle release, a process with which red blood cells might protect themselves against an attack by for example complement components.

Results: The role of annexin A7 in red blood cells was addressed in erythrocytes from *anxA7*$^{-/-}$ mice. Interestingly, the Ca^{2+}-mediated vesiculation process was not impaired. Also, the membrane organization appeared not to be disturbed as assessed using gradient fractionation studies. Instead, lack of annexin A7 led to an altered cell shape and increased osmotic resistance of red blood cells. Annexin A7 was also identified in platelets. In these cells its loss led to a slightly slower aggregation velocity which seems to be compensated by an increased number of platelets. The results appear to rule out an important role of annexin A7 in membrane fusion processes occurring in red blood cells. Instead the protein might be involved in the organization of the membrane cytoskeleton. Red blood cells may represent an appropriate model to study the role of annexin A7 in cellular processes.

Conclusion: We have demonstrated the presence of both annexin A7 isoforms in red blood cells and the presence of the small isoform in platelets. In both cell types the loss of annexin A7 impairs cellular functions. The defects observed are however not compatible with a crucial role for annexin A7 in membrane fusion processes in these cell types.

Background

Annexin A7 is a Ca^{2+}- and phospholipid-binding protein, which was isolated as the agent that mediated aggregation of chromaffin granules and fusion of phospholipid membranes in the presence of Ca^{2+}. This activity led to the proposal of annexin A7's involvement in the exocytotic secretion of catecholamines [1]. The protein belongs to a family of evolutionarily conserved proteins of a bipartite structure with a variable N-terminal and a conserved C-terminal domain. The C-terminal domain is responsible for the Ca^{2+}- and phospholipid-binding, the N-terminal domain appears to confer functional diversity [2-4]. The binding to negatively charged phospholipids is thought to be mediated by Ca^{2+} ions.

Annexin A7 is unique in that it carries an extraordinarily long and hydrophobic amino terminus with more than 100 amino acids. Alternative splicing gives rise to two isoforms of 47 and 51 kDa. Both isoforms differ by an additional cassette exon located in the first third of the N-terminal domain. Most tissues harbor only the 47 kDa isoform, both forms are found in brain and heart, while the large isoform is exclusively expressed in mature skeletal muscle.

At the cellular level annexin A7 can be detected in the cytosol, at the plasma membrane, around the nucleus, at vesicular structures including adrenal chromaffin granules, and at the t-tubule system [5,6]. Annexin A7 translocates to membranes in a Ca^{2+}-dependent fashion and, when intracellular Ca^{2+} levels rise, sequentially redistributes to the plasma and the nuclear membrane as well as to intracellular vesicles. Furthermore, annexin A7 associates with lipid rafts [7]. Lipid rafts play a key role in membrane budding and in vesiculation processes such as endo- and exocytosis [8-10].

Two binding partners of annexin A7 have been identified, sorcin and galectin-3 [11-13]. Sorcin is a Ca^{2+}-binding protein and belongs to the penta EF-hand protein family [14]. Like annexin A7 it binds to membranes in a Ca^{2+}-dependent manner. Sorcin also has been described as interaction partner of the ryanodine receptor, and appears to modulate its function [15]. The influence of the sorcin/annexin A7 interaction on the ryanodine receptor is unknown. Sorcin and annexin A7 are coexpressed in all tissues examined so far [11]. The binding of annexin A7 and sorcin is Ca^{2+}-dependent and occurs at micromolar Ca^{2+} concentrations. The binding sites have been localised to the amino terminal GGYY and GYGG motifs in sorcin and to the GYPP motif in the amino terminus of annexin A7. The proteins bind to each other with a stoichiometry of two sorcin molecules per annexin A7 molecule [12].

Galectin-3 is a multifunctional oncogenic protein with an anti-apoptotic activity found in the extracellular space, in the nucleus and cytoplasm and in mitochondria. Cytoplasmic galectin-3 correlates with tumor progression and protects mitochondrial integrity. Down regulation of annexin A7 prevents galectin-3 translocation to the perinuclear membrane and increases galectin-3 secretion. For annexin A7 a role for galectin-3 trafficking, apoptosis regulation, and mitochondrial integrity was proposed [13].

The cellular role of annexin A7 is not well understood. It is thought to regulate and stabilize membrane domains and to have a role in Ca^{2+} homeostasis and Ca^{2+}-dependent signaling pathways. These proposals are supported by data obtained from analysis of annexin A7 deficient mouse mutants. Two annexin A7 129Sv null mice strains were generated independently using a different strategy. The one of Srivastava et al. [16] is lethal. Heterozygous mice exhibit an insulin secretion defect and tumor phenotypes. The null strain reported by Herr et al. [17] is viable, healthy, and shows no insulin secretion defect or other obvious defects. However, in isolated cardiomyocytes the frequency-induced shortening is disturbed. Here we have focused on the analysis of red blood cells and platelets of the *anxA7*$^{-/-}$ mutant. Generally, red blood cells and platelets were thought not to contain annexin A7 and only recently the 47 kDa isoform has been reported as component of red blood cells [7].

Red blood cells undergo various biochemical or morphological changes that appear to be Ca^{2+}-dependent processes [18,19]. One of them is the release of hemoglobin-containing exovesicles occurring in vivo as well as in vitro [7,20]. This process is thought to represent a protective method of the red blood cell against an attack by for example complement components. Red blood cells which lack the ability to vesiculate cause a disease with red blood cell destruction and haemoglobinuria [21]. There exist two types of vesicles, micro- and nanovesicles with a size of 180 nm and 60 nm, respectively. They are enriched in cholesterol and sphingolipid rich lipid raft domains that are associated with proteins like acetylcholinesterase, cell surface proteins including a complement receptor, and the lipid raft proteins stomatin and flotillin, but they lack any cytoskeletal protein [7]. Furthermore, they contain sorcin and annexin A7 attached to the lipid rafts. Both proteins are more abundant in nanovesicles. The vesicle formation goes along with several other changes in the red blood cell like cytoskeletal rearrangements and changes in the phospholipid orientation in the cellular membrane.

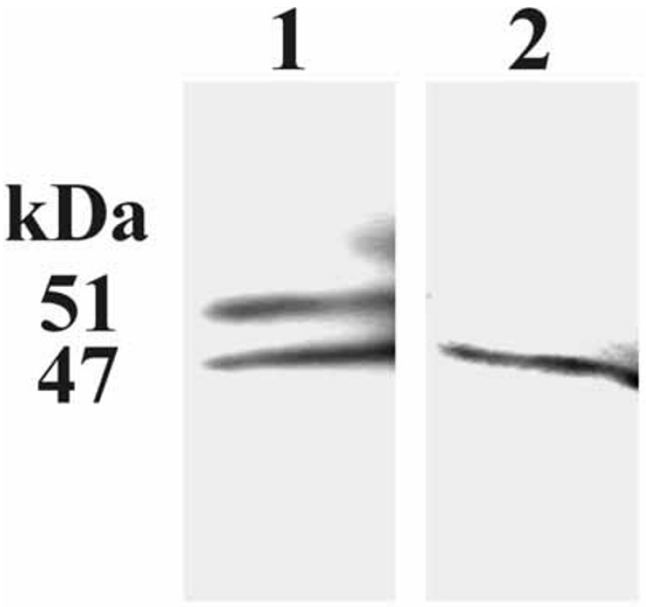

Figure 1
Expression of annexin A7 in human red blood cells (1) and human platelets (2). Protein homogenates were separated by SDS-PAGE (12 % acrylamide). The resulting western blot was probed with mAb 203–217, visualization was by a secondary peroxidase coupled antibody followed by ECL. Both isoforms are detected in red blood cells, in platelets only the small isoform is present.

Results

Annexin A7 expression in red blood cells, red blood cell derived exovesicles and in platelets

Salzer et al. recently reported the presence of the 47 kDa isoform of annexin A7 and its partner sorcin in micro- and nanovesicles derived from red blood cells where they are located in the lumen and are also enriched in membrane rafts [7]. We have now extended these findings and show here that the 51 kDa isoform is present as well. In western blots of human red blood cells the 47 and 51 kDa isoforms were detected using mAb 203–217 (Fig. 1). The 47 kDa isoform was detected in silver stained gels and its identity with annexin A7 confirmed by peptide mass fingerprinting (data not shown). The 51 kDa isoform was only detected in western blots.

Its presence in vesicles led to the suggestion, that, along with raft domains, annexin A7 plays a role in membrane organization and the vesiculation process. To examine this, we analysed the ability of red blood cells derived from the annexin A7 knock out mice (*anxA7*$^{-/-}$) to form exovesicles. Because of better accessibility and larger available amounts that led to clear results, we included our data obtained with human blood. Independent of the presence of annexin A7 both types of exovesicles, micro- and nanovesicles, were released after Ca^{2+}/ionophore treatment as determined by acetylcholine esterase activity and microscopic examination. Annexin A7 is present in both vesicle types where it is more enriched in nanovesicles (Fig. 2). The 51 kDa isoform is only detectable in nanovesicles. The quantity of both vesicle types did not differ between wild type and knock out red blood cell vesicles as determined by the AChE-values (A_{405} nanovesicles: ~0.18; A_{405} microvesicles: ~0.9; both, for wt and ko). It appears that annexin A7, although it has been described to fuse membranes, is not a key component in the process of the formation of red blood cell exovesicles. When we probed for the presence of sorcin in wild type and mutant vesicles we found that the sorcin levels were reduced in the mutant nanovesicles (Fig. 2).

To study the distribution of annexin A7 in red blood cells we used self forming iodixanol density gradients. Both isoforms were present in the soluble fraction as well as in the gradient fractions where they are assumed to be associated with membranes. These membranes are exclusively plasma membranes as red blood cells are free of any organelles. The distribution was however not homogeneous throughout the gradient. This could reflect the binding of annexin A7 to membrane subdomains that have different lipid or lipid/protein composition as discussed by Salzer et al. [7]. Likewise, sorcin does not exhibit a homogeneous distribution in the gradient. Moreover, it segregates into vesicles which are not associated with annexin A7 (Fig. 3). We also tested platelets for annexin A7 expression and detected the 47 kDa isoform (Fig. 1).

The lack of annexin A7 changes red blood cell morphology and osmotic resistance

As annexin A7 is a component of red blood cell vesicles we studied the consequences of the loss of the protein in the *anxA7*$^{-/-}$mouse for red blood cell morphology and osmotic resistance. In a standard 'blood smear' we did not note a deformation of the cells, however dark field microscopy revealed changes in shape and diameter of the *anxA7*$^{-/-}$red blood cells. They had a statistically significant larger cell diameter of 6.0 μm compared to wild type with 5.7 μm ($p = 0.015$, $n = 40$), a remarkably lower emphasized central impression and a more flat shape (Fig. 4). No significant change in the mean corpuscular volume (MCV) was measured with the ADVIA 120 cell counter.

Shape and MCV of a red blood cell essentially influence its function and capillary passage. The typical biconcave form depends on various influences including the membrane lipid composition and the submembranous cytoskeleton [32] where annexin A7 might play a role. The

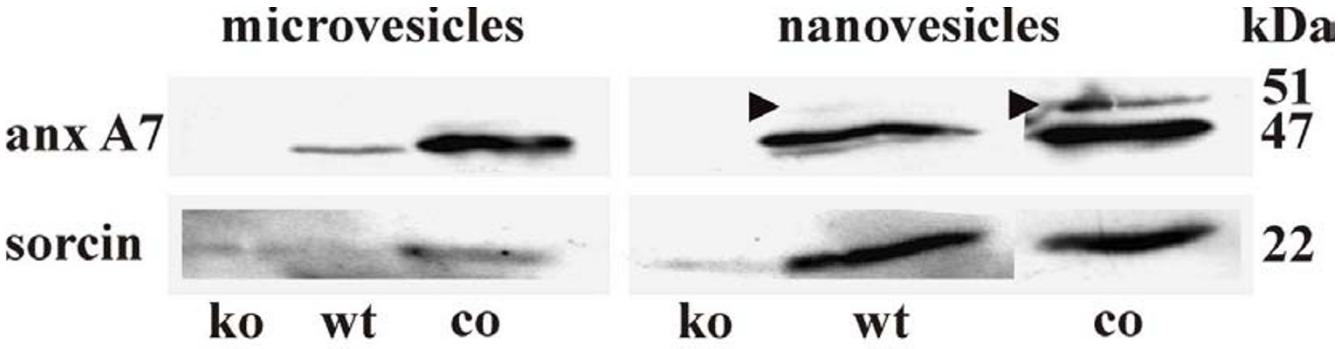

Figure 2
Enrichment of annexin A7 and sorcin in exovesicles derived from red blood cells. Vesicles from wild type (wt) and *anxA7*$^{-/-}$ mutant (ko) were generated with Ca^{2+}/ionophore treatment, isolated by differential centrifugation and analysed by immunoblotting with mAb 203–217 and a sorcin polyclonal antibody. In general, annexin A7 and sorcin are more abundant in nanovesicles. The 51 kDa isoform of annexin A7 (arrows) is only observed in nanovesicles. Samples of both vesicles types were normalized according to their acetylcholine esterase activity. Human red blood cells (co) were used for control and normalized independently.

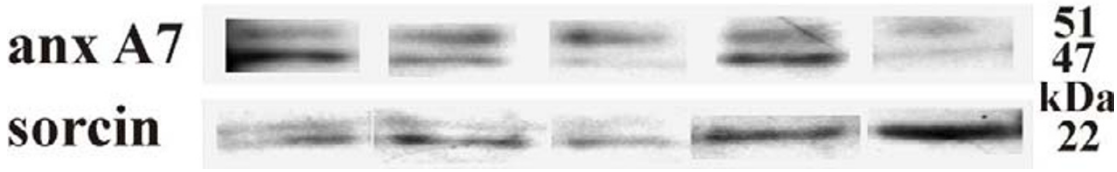

Figure 3
Association of annexin A7 and sorcin with distinct red blood cell plasma membrane fractions. Lysed human red blood cells were added to a self forming iodixanol density gradient. The density of the gradient increases from left (1.06 g/ml) to right (1.20 g/ml). Fractions were analysed by immunoblotting using mAb 203–217 and the sorcin polyclonal antibody.

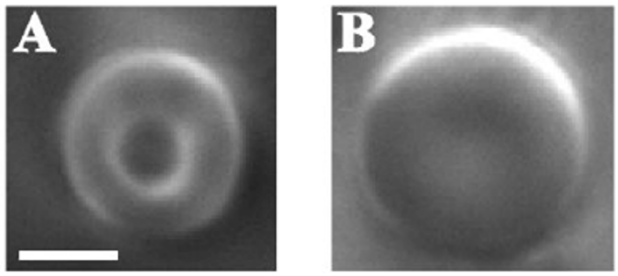

Figure 4
Dark field microscopy of red blood cells from *anxA7*$^{-/-}$ mutant (B) and wild type mice (A). The mean cellular diameters are 6.0 μm and 5.7 μm, respectively. The size differences are statistically significant ($p = 0.015$; $n = 40$). Bar, 3 μm.

osmotic resistance, which is the resistance towards changes in the extracellular ionic strength, is a convenient assay for analysis of the red blood cell integrity. It is measured as the sodium chloride concentration at which cellular lysis starts (minimal resistance) up to a complete lysis (maximal resistance) of the cells [33]. Osmotic resistance increases with higher MCV, larger surface area and a higher degree of cellular metabolism stabilizing the intracellular ion levels. Aged red blood cells show a lowered MCV and lower osmotic resistance [33]. Similar properties can be observed in red blood cells with lowered membrane permeability. The osmotic resistance of annexin A7 deficient red blood cells is significantly increased compared to wild type cells. The minimal resistance value of knock out cells is observed in a 0.60 % NaCl solution versus 0.65 % for wild type cells. 50 % haemolysis is achieved at 0.516 % NaCl solution for knock out versus 0.564 %

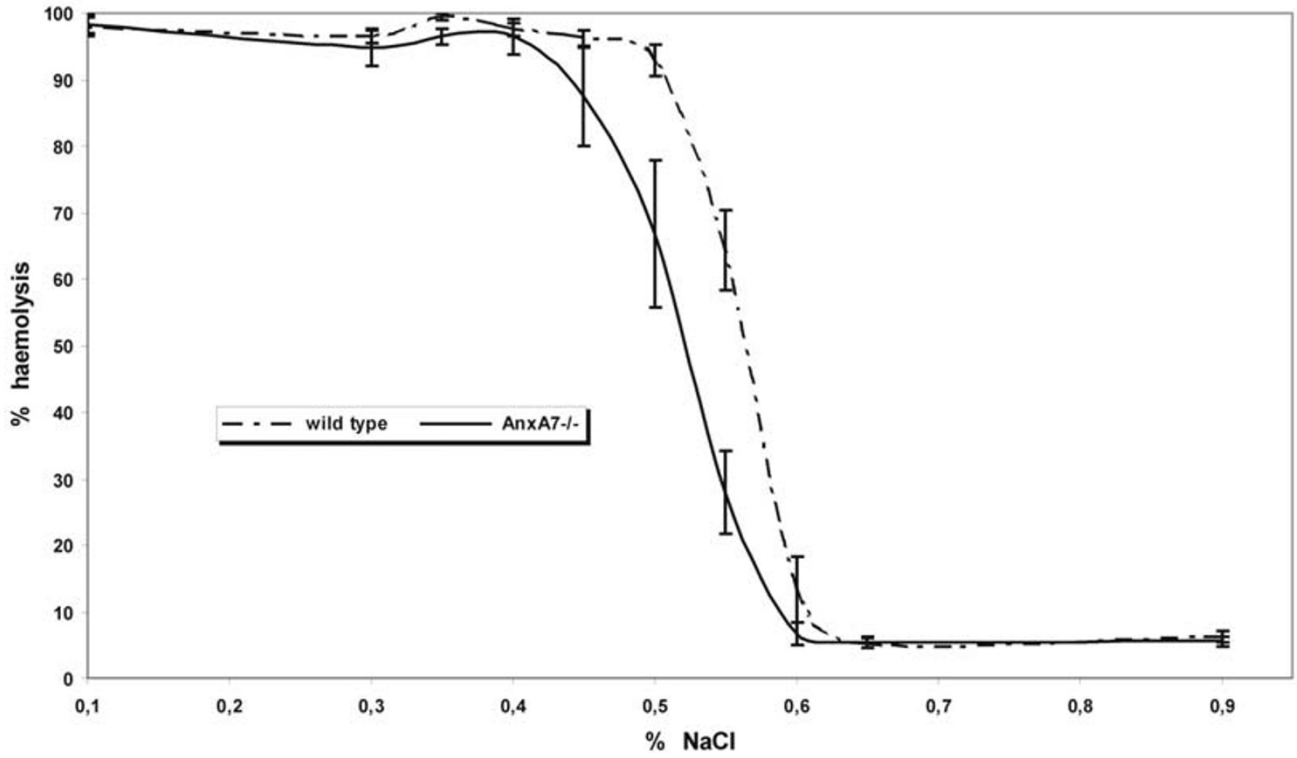

Figure 5
Osmotic resistance curves of red blood cells from wild type and *anxA7*$^{-/-}$ mutant mice. The osmotic resistance towards changes in the extracellular ionic strength is a convenient assay for analysis of the red blood cell integrity. It is measured as a sodium chloride concentration in which cellular lysis starts (minimal resistance) up to a complete lysis (maximal resistance) of a red blood cell. The osmotic resistance of annexin A7 deficient red blood cells is significantly increased compared to the one of wild type ($p = 0.00066$; $n = 8$).

NaCl for wild type cells (mean values, $p = 0.00066$, $n = 8$; Fig. 5). The resistance width of the knock out cells is slightly lower (0.20 % instead of 0.25 % NaCl solution). We also performed direct measurements of the red blood cell membrane deformability, to further characterize a possible role of annexin A7 on the membrane stability. With micropipette experiments we tried to correlate mechanical characteristics of the red blood cells with their morphology. We could not observe any statistically significant difference of values describing membrane rigidity and lysis force in these experiments (M. Heil, B. Hoffmann and R. Merkel, unpublished results). However, the distinction observed in the osmotic resistance experiments was highly significant and reflects a mean value over a high number of different red blood cells.

These data suggest that annexin A7 contributes to the shape of red blood cells and the osmotic resistance. As we have not observed a binding of annexin A7 to F-actin (data not shown) it could do so either by alteration of the membrane rigidity and/or by affecting the ion homeostasis.

The lack of annexin A7 affects primary haemostasis ex vivo
The use of an advanced electronic cell counter/flow cytometer (ADVIA 120) allowed us to screen other parameters as well despite of the limited murine blood volume. We analysed leukocytes, red blood cells, haemoglobin content, haematocrit, mean cellular volume, mean cellular haemoglobin, mean cellular haemoglobin concentration, platelets, neutrophiles, lymphocytes, monocytes, eosinophiles and basophiles. In these tests of whole blood we found only a change in the platelet numbers, whereas all other haematological parameters were not affected. The mean platelet counts from wild type and *anxA7*$^{-/-}$ mutant mice are determined as $674 \times 10^3/\mu l$ and $774 \times 10^3/\mu l$, respectively. The platelet counts in knock out mice are significantly higher ($p = 0.0275$; $n_{\text{knock out}} =$

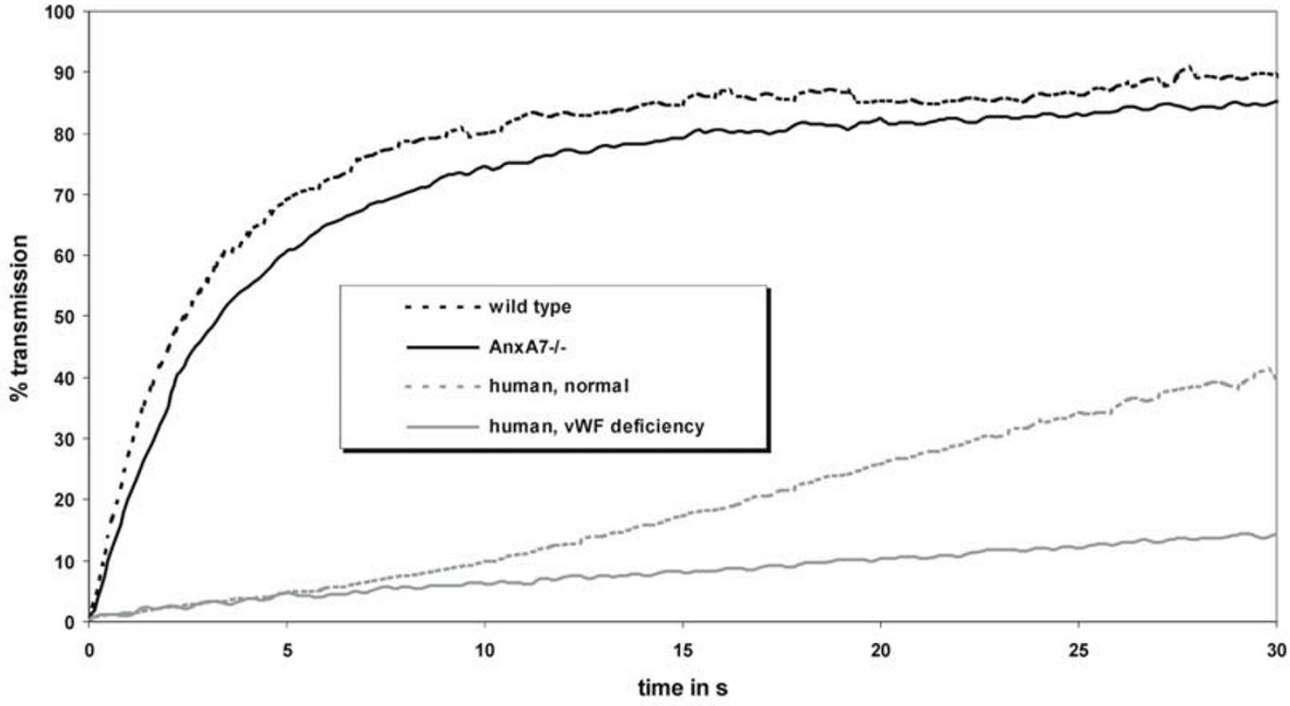

Figure 6
Platelet aggregation curves from platelet rich plasma of annexin A7 knock out and wild type mice. The transmission values were measured with an APACT photometer and aggregation was initiated by adding ristocetin. The first thirty seconds are given. Constant platelet counts were used throughout all experiments. The aggregation curve data were analysed for slope values, the maximal aggregation amplitude of every single curve was set to 100 %. Both murine curves differ significantly at a time point of seven seconds after platelet initiation, the mutant shows a slightly lower aggregation velocity ($p = 0.0287$, $n_{knock\ out} = 16$, $n_{wild\ type} = 15$, mean $_{knock\ out} = 67.7$ % transmission, mean $_{wild\ type} = 77.1$ % transmission, standard error $_{knock\ out} = 8.3$ % transmission, standard error $_{wild\ type} = 13.8$ % transmission). Murine platelets react immediately to the initiating chemical and show no lag phase like the normal human platelets. For comparison of the range of the platelet impairment slightly affected human platelets are shown as well (von Willebrand syndrome).

11, $n_{wild\ type} = 14$). An increase in platelet counts is a rather uncommon disorder. In humans, platelet counts normally increase only transiently as in reactive thrombocytosis and under postoperative conditions or are largely increased in neoplastic diseases. By contrast, the *anxA7*$^{-/-}$ mice are healthy and viable. Therefore we tested the platelet function and performed aggregometry measurements with platelet rich plasma. Platelet activation is observed as a morphological change from the resting discoid state to activated spherical cells with pseudopods. The morphological changes are due to cytoskeletal rearrangements. In vitro the activated cells form aggregates recruiting additional cells in the solution thereby reducing its cloudiness. Analysis of the transmission values of aggregation curves after platelet activation by ristocetin addition (von Willebrand cofactor) showed that both curves differed significantly at the time point of seven seconds after platelet initiation ($p = 0.0287$, $n_{knock\ out} = 16$, $n_{wild\ type} = 15$; Fig. 6). Annexin A7 deficient platelets showed a slightly lowered aggregation velocity. When we compared the aggregation curves of human platelets from a healthy donor with the ones obtained from an individual with a von Willebrand factor type 1 defect, we found that the difference in the curves was much more pronounced as observed in our studies of healthy mouse platelets and *anxA7*$^{-/-}$ platelets. Furthermore we found that murine platelets responded immediately to the initiating chemical whereas human platelets have a lag phase [23].

Discussion

Annexin A7 is thought to have a role in vesicle fusion and in regulating and stabilizing membrane domains [1]. The protein has been localized to the plasma membrane including raft subdomains, furthermore it is present in the

cytosol where it is found on subcellular vesicle-like structures. This distribution appears to be independent of the tissue or cell type analysed. In specialized or terminally differentiated cells, expression levels increase and frequently the larger of the two isoforms is being expressed [6,34,35].

Red blood cells, which were thought to be devoid of annexin A7, were recently shown to harbor the 47 kDa isoform together with its binding partner sorcin [7]. We showed the presence of the 51 kDa isoform as well, adding red blood cells to the list of cells that harbor this isoform. Salzer et al. [7] not only demonstrated the presence of annexin A7 in red blood cells, they also showed that annexin A7 and sorcin were enriched in membrane raft domains of nanovesicles formed from red blood cells in vitro. Raft domains in general are considered key players in membrane organization and in mediating the vesiculation process. They result from the clustering of sphingolipids and cholesterol in the outer leaflet of the membrane connected to phospholipids and cholesterol in the inner leaflet and are enriched in special proteins. Biochemically rafts are defined as membranes that are resistant to extraction by cold Triton X-100 and can be floated to low densities in sucrose gradient centrifugation [36].

The findings of Caohuy et al. [37] that annexin A7 mediates membrane aggregation also suggested that annexin A7 is an essential component during vesiculation. In addition they reported a further activation of the vesiculation process by GTP and proposed that annexin A7 acts as a GTPase. Similarly, the presence of GTP enhanced the secretion of surfactant by annexin A7. These results were thought to support a direct role for annexin A7 in surfactant secretion, but in these studies annexin A7 mediated membrane fusion was separated from a second GTP-dependent mechanism [38].

Having an annexin A7 knockout mouse we tested the involvement of annexin A7 in budding of red blood cell vesicles. Our data suggest that the efficiency of micro- and nanovesicle formation is not significantly different in wild type and mutant red blood cells. Likewise, *Dictyostelium discoideum* cells, in which the single annexin gene was inactivated, were not impaired in processes requiring membrane fusion [[39]; and data unpublished]. It might well be that the observed fusion of phospholipid layers by annexin A7 in the presence of high Ca^{2+} concentrations may turn out to be an in vitro effect. Similarly, the high abundance of annexin A7 in micro- and nanovesicles might be fortuitous as a significant percentage of the red blood cell membrane, with which annexin A7 associates when the Ca^{2+} levels rise, appears to be involved in the formation of exovesicles. Furthermore, the distribution of both annexin A7 isoforms in gradients of red blood cells indicates a function which differs from organizing subcellular membranes or membrane pathways. Whereas annexin A2 is tightly associated with endosomes and may have functions in membrane fusion and the endosomal pathway, annexin A7 distributes with plasma membrane raft domains of different types and densities. Interestingly, in vesicles of red blood cells lacking annexin A7 the amount of sorcin is lowered, indicating that its localisation is affected by the loss of annexin A7 or its expression during red blood cell development was reduced.

Having excluded a direct role in vesicle formation and membrane fusion, annexin A7 might act by its property as Ca^{2+}-binding protein [17]. Previous results in which annexin A7 was shown to play a role in membrane aggregation might be explained by a supportive function of annexin A7 in that it interferes with the local Ca^{2+} homeostasis thus influencing membrane organization. As the annexins belong to a wide spread and evolutionarily conserved protein family, redundant, but not identical functions are expected. However, so far no other members of the annexin family, which may substitute for annexin A7, have been described in red blood cells.

The structural basis for the elasticy of red blood cells in circulation are long range molecular functions of the plasma membrane and the membrane associated part of the cytoskeleton [40-43]. Major constituents of the membrane skeleton are spectrin tetramers linked together by short actin filaments and several other proteins covering the entire cytoplasmic surface of the red blood cell membrane [44]. Membrane cholesterol diminishes red blood cell haemolysis by the complement complex, whereas depletion of raft cholesterol abrogates association of all raft proteins with no significant effect on areas in the rest of the cell membrane and deformability [45,46].

Annexin A7 knock out mouse red blood cells show a more flat shape and have a higher osmotic resistance. The presence of annexin A7 may alter the membrane flexibility in that it supports the typical biconcave membrane shape and leads to a lower minimal osmotic resistance. Annexin A7 does not appear to have an influence on plasma membrane integrity itself as the maximal osmotic resistance value stays constant. Ca^{2+} and membrane skeletal proteins are known to play key roles on the red blood cell membrane shape and stability [47]. Annexins have been reported to bind directly to F-actin, we could however exclude such an activity for annexin A7. Nevertheless it might interact with other components and have a role in organizing the membrane cytoskeleton, controlling raft protein associations or influencing the ionic strength of

red blood cells on its own or by interfering with other signaling pathways.

As discussed above annexin A7 could be involved in the cytoplasmic Ca^{2+} homeostasis. It has been demonstrated that micromolar changes of the intracellular Ca^{2+} concentration exert a profound effect on the membrane properties that regulate red blood cell deformability [48,49]. Furthermore, the intracellular Ca^{2+} was shown to regulate the membrane stability through modulation of cytoskeletal protein interactions [50]. Ca^{2+} also induces a transbilayer phospholipid redistribution inducing a shape change of red blood cells [51]. In addition, Ca^{2+} ions can drive spiculation of the membrane bilayer without involving the cytoskeleton [52].

We have also observed a defect in platelet function in the annexin A7 mutant. In the platelet aggregation experiments the initial aggregation velocity was slightly, but significantly lower in platelets lacking annexin A7. These data are not reflected by a change in the in vivo bleeding time. However the reaction speed of murine platelets is much higher than those of human and due to the technical setup we might have missed larger changes in the mouse samples. The immediate reaction of mouse platelets and the absence of a lag phase after triggering may be caused by an immediately initiated thrombin generation [22]. As known from the frequent human vWF-syndrome or other haemostatic diseases, small defects often are clinically silent and do not result in physiological changes under normal conditions. Only in situations with severe physical injury, distinct environmental influences or additional platelet attributes like the platelet antigen polymorphism [53] differences might become apparent.

Conclusions

In this paper we report the presence of both annexin A7 isoforms in red blood cells where they are abundant in nanovesicles that form upon Ca^{2+} addition. The proteins are however not required for the vesiculation process. They are also not essential for the formation of specific membrane domains such as lipid rafts, as exogenously added recombinant annexin A7 redistributed to similar positions in a density gradient containing membranes derived from red blood cells of *anxA7*$^{-/-}$mice. The observed cell shape change of erythrocytes lacking annexin A7 might be due to alterations in the erythrocyte cytoskeleton. As a direct interaction with F-actin was ruled out, this effect might be mediated by annexin's Ca^{2+}-binding activity.

Methods

Acquisition of murine and human blood samples

Blood from 129Sv wild type and annexin A7 knock out mice (*anxA7*$^{-/-}$) kept under pathogen-free conditions and fed with regular food, was collected in 1 ml syringes with a 22 gauge needle punctating the right cardiac ventricle immediately after they had been killed by cervical dislocation. Coagulation generally was inhibited with 1/10 volume of 10 mM NaEDTA, pH 7.5, already present in the syringe. The mice were killed by cervical dislocation in order to avoid any effect of anaesthetic chemicals on blood parameters. The wild type and mutant mice were matched regarding their age, weight and sex. The daytime of blood collection, sampling site and collection method were kept constant during all experiments. A typical murine blood sample volume consisted of 550 µl, with variations ranging from 200 µl to 900 µl. Human blood samples from the authors were collected by vein puncture using common EDTA-monovettes (Sarstedt).

We performed our studies using well standardized sampling conditions, as differences in the blood sampling site, for example between tail and heart, show large changes in all cell type counts. First and second sample draw affect blood measurements, too [22]. Also the genetic background in common laboratory and transgenic mice affects the phenotype. A change from one strain to another may protect mice from effects of the primarily induced genetic defect. For example, BALB/c mice have a significantly increased velocity of platelet aggregation as compared to 129Sv mice, which were used throughout this study [23].

Self forming iodixanol density gradients of human red blood cells

The subcellular distribution of annexin A7 was addressed according to [24]. Red blood cells were centrifuged and washed in isotonic buffered salt solution, collected in ice cold lysis buffer (20 mM Hepes, pH 7.4, 200 µM $CaCl_2$, 0.25 M sucrose, and proteinase inhibitors (Roche)), homogenized by a loosely fitting dounce homogenizer and additional passages through a 22 gauge needle. A 50 % iodixanol solution was established by mixing Optiprep solution (60 % iodixanol; Sigma) with buffer (120 mM Hepes, pH 7.4, 1.2 mM $CaCl_2$, 0.25 M sucrose). Seven parts of this 50 % iodixanol solution were mixed with thirteen parts of the red blood cell solution, generating a gradient solution containing 17.5 % iodixanol. The mixture was filled into a centrifuge tube underlayed by cushions of 30 % and 35 % iodixanol and centrifuged for 3 hours at 270,000 × g in a SW41 Ti swing out rotor (Beckman) to achieve a nearly linear density gradient (approximately 1.06 g/ml to 1.20 g/ml) [24]. Fractions of 0.6 ml were collected from the top of the gradient by a 1 ml syringe without using a needle and immediately frozen in liquid nitrogen for further use.

Exovesicle generation from human and murine red blood cells

Red blood cells naturally can form hemoglobin containing exovesicles. In vitro these vesicle formation is Ca^{2+} induced. Freshly collected lithium-heparin anticoagulated blood samples were centrifuged for 5 minutes at 400 × g and 4°C. The erythrocytes were washed five times with 20 mM Hepes, pH 7.5, and 150 mM NaCl (washing buffer). A volume of 500 µl washed red blood cells were combined with 3 ml of vesiculation buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ adjusted to 5 µM Ca^{2+} ionophore A23187) and incubated for 30 minutes at 37°C. After incubation EDTA was added to the solution to a final concentration of 5 mM and centrifuged for 5 minutes at 400 × g and 4°C. The supernatant was collected and centrifuged for 20 minutes at 15,000 × g and 4°C. The resulting pellet was resuspended in washing buffer and centrifuged at 400 × g again to collect a supernatant enriched with red blood cell microvesicles. The supernatant of the 15,000 × g step was further centrifuged for 60 minutes at 100,000 × g and 4°C. The final pellet of this step was resuspended in washing buffer and centrifuged at 15,000 × g again to purify a supernatant enriched in nanovesicles according to [7,20]. The vesicles were assayed for acetylcholine esterase activity and used for further analysis.

Acetylcholine esterase assay

A 30 µl sample of red blood cell vesicles was mixed with an equal volume of buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 0.5 % Triton X-100), vortexed and incubated for 5 minutes at 37°C. Subsequently 640 µl sodium phosphate buffer (100 mM Na_2PO_4, pH 7.6), 50 µl DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), 10 mM in sodium phosphate buffer), and 50 µl acetylthiocholine chloride (12,5 mM in H_2O) were added. The reaction took place at room temperature and was measured photometrically at a wavelength of 405 nm according to the Ellmann' method [25].

Determination of the osmotic resistance of red blood cells

A Na_2HPO_4/NaH_2PO_4 buffered NaCl gradient (pH 7.4) comprised of the following NaCl steps was used for this assay: 0.90%, 0.65%, 0.60%, 0.55%, 0.50%, 0.45%, 0.40%, 0.35%, 0.30%, 0.10%. For each step 5 ml of the gradient step solution and 50 µl blood sample were carefully inverted four times and incubated for 30 minutes in the dark. Samples were carefully inverted again and centrifuged at 1,500 × g for 10 minutes. From 1 ml supernatant of each step the extinction at 546 nm wavelength was measured. The experiment was performed at room temperature.

Platelet aggregation assay

Approximately 500 µl of murine blood were centrifuged at 200 × g for 15 minutes. The resulting supernatants were transferred to new tubes and used as platelet rich plasma (PRP). The platelet aggregation measurements were done according to the method developed by Born and Cross [26] using an APACT photometer (Labor, Ahrendsburg, FRG) and the APACT software (APACT 1.4). Subsequently samples of the PRP were adjusted with buffer (5 mM Hepes, 150 mM NaCl, pH 7.3) to 250,000 platelets/µl. A constant platelet count was used throughout allowing comparisons between wild type and knock out murine and human samples. 250 µl adjusted platelets were prewarmed to 37°C and used in the assay, and the aggregation process was started by adding 25 µl of a prewarmed 16.5 mg/ml ristocetin solution (DiaMed Diagnostica, Bensheim, FRG; concentration in the assay: 1.5 mg/ml), a reagent which worked reliably in our hands with mouse and human platelets. Each murine assay represents a different animal. Analyses of human blood samples were performed as described in the manufacturer's protocol. Several repeated measurements were done per human individual to control the reproducibility. The acquired aggregation curve data, measured as ascending degree of transmission, were analysed for maximal slope values, while the maximal aggregation amplitude of every single curve was set to 100 %. In general, all platelet procedures were done at 22°C within four hours after collecting the blood sample.

Miscellaneous methods

For standard haematopoetic measurements and cell counts on murine and human blood samples about 300 µl of blood were analysed with an ADVIA 120 electronic cell counter (Bayer). With this equipment red blood cells and platelets are "transformed" into spherical bodies without changing their volume. Therefore number and volume of red blood cells and platelets are not calculated values, but directly measured values. The following data were statistically analysed: leucocytes, red blood cells, haemoglobin, haematocrit, mean cellular volume, mean cellular haemoglobin, mean cellular haemoglobin concentration, platelet number, neutrophiles, lymphocytes, monocytes, eosinophiles, basophiles.

Peptide mass fingerprinting was performed on a Bruker Reflex IV MALDI-TOF mass spectrometer. Recombinant annexin A7 was purified according to [27], F-actin-binding of recombinant annexin A7 was done as described [28]. SDS-PAGE and western blotting were done as described [29,30]. Detection in immunoblots was with enhanced chemiluminescence [17]. Antibodies employed were mab 203–217 directed against mouse annexin A7 core domain [5] and a polyclonal rabbit anti-sorcin antibody [31].

For statistical analyses the following tests were performed: David et al. range test to ensure samples are from a normal distribution, F-test looking for a samples' homoscedascity, Student's T-test to judge if samples of different groups have a different mean value and are parts from different normal distributions. The exact probability values and the significance of an analysis are indicated when the experiments are described.

Authors' contributions

CH and CSC planned and carried out the experiments with the red blood cells and platelets and the fractionation studies and drafted the manuscript, GL was responsible for platelets, RK gave support on the ADVIA 120 cell counter, evaluated and discussed the results, SMP and BSG gave support on the APACT system, platelet aggregation experiments, and evaluated the results, CZ was responsible for the sorcin studies, MS studied the cytoskeletal activities of the protein, AAN conceived of the studies and participated in its design. All authors read and approved the manuscript.

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References

1. Creutz CE, Pazoles CJ, Pollard HB: **Identification and purification of an adrenal medullary protein (synexin) that causes calcium dependent aggregation of isolated chromaffin granules.** *J Biol Chem* 1978, **253:**2858-2866.
2. Raynal P, Pollard HB: **Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins.** *BBA* 1994, **119:**63-93.
3. Gerke V, Moss SE: **Annexins and membrane dynamics.** *BBA* 1997, **1357:**129-154.
4. Gerke V, Moss SE: **Annexins: from structure to function.** *Physiol Rev* 2002, **82:**331-371.
5. Selbert S, Fischer P, Pongratz D, Stewart M, Noegel AA: **Expression and localization of annexin VII (synexin) in muscle cells.** *J Cell Sci* 1995, **108:**85-95.
6. Clemen CS, Herr C, Lie AA, Noegel AA, Schröder R: **Annexin VII: an astroglial protein exhibiting a Ca2+-dependent subcellular distribution.** *NeuroReport* 2001, **12:**1139-1144.
7. Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R: **Calcium-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin.** *Blood* 2002, **99:**2569-2577.
8. Huttner WB, Zimmerberg J: **Implications of lipid microdomains for membrane curvature, budding and fission.** *Curr Opin Cell Biol* 2001, **13:**478-484.
9. Ikonen E: **Roles of lipid rafts in membrane transport.** *Curr Opin Cell Biol* 2001, **13:**470-477.
10. Schmidt K, Schrader M, Kern HF, Kleene R: **Regulated apical secretion of zymogens in rat pancreas. Involvement of the lycosylphosphatidylinositol-anchored glycoprotein GP-2, the lectin ZG16p, and cholesterol-glycosphingolipid-enriched microdomains.** *J Biol Chem* 2001, **276:**14315-14323.
11. Brownawell AM, Creutz CE: **Calcium-dependent binding of sorcin to the N-terminal domain of synexin (Annexin VII).** *J Biol Chem* 1997, **272:**22182-22190.
12. Verzili D, Zamparelli C, Mattei B, Noegel AA, Chiancone E: **The sorcin-annexin VII calcium-dependent interaction requires the sorcin N-terminal domain.** *FEBS Lett* 2000, **471:**197-200.
13. Yu F, Finley RLJ, Raz A, Kim HR: **Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation.** *J Biol Chem* 2002, **277:**15819-15827.
14. Maki M, Narayana SV, Hitomi K: **A growing family of the Ca2+-binding proteins with five EF-hand motifs.** *Biochem J* 1997, **328:**718-720.
15. Meyers MB, Pickel VM, Sheu SS, Sharma VK, Scotto KW, Fishman GI: **Association of sorcin with the cardiac ryanodine receptor.** *J Biol Chem* 1995, **270:**26411-26418.
16. Srivastava M, Atwater I, Glasman M: **Defects in inositol 1,4,5-trisphosphate receptor expression, Ca(2+) signaling, and insulin secretion in the anx7(+/-) knockout mouse.** *Proc Natl Acad Sci* 1999, **96:**13783-13788.
17. Herr C, Smyth N, Ullrich S, Yun F, Sasse P, Hescheler J, Fleischmann B, Lasek K, Brixius K, Schwinger RH, Fassler R, Schroder R, Noegel AA: **Loss of annexin A7 leads to alterations in frequency-induced shortening of isolated murine cardiomyocytes.** *Mol Cell Biol* 2001, **21:**4119-4128.
18. Li Q, Jungmann V, Kiyatkin A, Low PS: **Prostaglandin E2 stimulates a Ca2+-dependent K+ channel in human erythrocytes and alters cell volume and filterability.** *J Biol Chem* 1996, **271:**18651-18656.
19. Yang L, Andrews DA, Low P: **Lysophosphatidic acid opens a Ca(++) channel in human erythrocytes.** *Blood* 2000, **95:**2420-2425.
20. Allan D, Thomas P, Limbrick AR: **The isolation and characterization of 60 nm vesicles ('nanovesicles') produced during ionophore A23187-induced budding of human erythrocytes.** *Biochem J* 1980, **188:**881-887.
21. Whitlow M, Iida K, Marshall P, Silber R, Nussenzweig V: **Cells lacking glycan phosphatidylinositol-linked proteins have impaired ability to vesiculate.** *Blood* 1993, **81:**510-516.
22. Nemzek JA, Bolgos GL, Remick W, Remick DG: **Differences in normal values for murine white blood cell counts and other haematological parameters based on sampling site.** *Inflamm res* 2001, **50:**523-527.
23. Zumbach A, Marbet GA, Tsakiris DA: **Influence of the genetic background on platelet function, microparticle and thrombin generation in the common laboratory mouse.** *Platelets* 2001, **12:**496-502.
24. Graham J, Ford T, Rickwood D: **The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol.** *Anal Biochem* 1994, **220:**367-373.
25. Steck TL, Kant JA: **Preparation of impermeable ghost and inside-out vesicles from human erythrocyte membranes.** *Methods Ezymol* 1974, **31:**172-180.
26. Born GVR, Cross MJ: **The aggregation of blood platelets.** *J Physiol* 1963, **168:**178-195.
27. Liemann S, Bringemeier I, Benz J, Gottig P, Hofmann A, Huber R, Noegel AA, Jacob U: **Crystal structure of the C-terminal tetrad repeat from synexin (annexin VII) of Dictyostelium discoideum.** *J Mol Biol* 1997, **270:**79-88.
28. Khurana B, Khurana T, Khaire N, Noegel AA: **Functions of LIM proteins in cell polarity and chemotactic motility.** *Embo J* 2002, **21:**5331-5342.
29. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriphage T4.** *Nature* 1970, **227:**680-685.
30. Towbin H, Staehelin T, Gordon J: **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** *Proc Nat Acad Sci USA* 1979, **76:**4350-4354.
31. Zamparelli C, Ilari A, Verzili D, Giangiacomo L, Colotti G, Pascarella S, Chiancone E: **Structure-function relationships in sorcin, a member of the penta EF-hand family. Interaction of sorcin fragments with the ryanodine receptor and an Escherichia coli model system.** *Biochemistry* 2000, **39:**658-666.
32. Harvey JW: **Erythrocyte Metabolism.** *Clinical Biochemistry of Domestic Animals* 4th edition. *Academic Press. San Diego, California*; 1989:185-234.
33. Jain NC: **Essentials of veterinary hematology.** *Lea & Febiger, Philadelphia, Pennsylvania* 1993:139.

34. Magendzo K, Shirvan A, Cultraro C, Srivastava M, Pollard HB, Burns AL: **Alternative splicing of human synexin mRNA in brain, cardiac, and skeletal muscle alters the unique N-terminal domain.** *J Biol Chem* 1991, **266:**3228-3232.
35. Clemen CS, Hofmann A, Zamparelli C, Noegel AA: **Expression and localisation of annexin VII (synexin) isoforms in differentiating myoblasts.** *J Muscle Res Cell Motil* 1999, **20:**669-679.
36. Simons K, Ehehalt R: **Cholesterol, lipid rafts, and disease.** *J Clin Invest* 2002, **110:**597-603.
37. Caohuy H, Srivastava M, Pollard HB: **Membrane fusion protein synexin (annexin VII) as a Ca2+/GTP sensor in exocytotic secretion.** *Proc Natl Acad Sci USA* 1996, **93:**10797-10802.
38. Chander A, Sen N, Spitzer AR: **Synexin and GTP increase surfactant secretion in permeabilized alveolar type II cells.** *Am J Physiol Lung Cell Mol Physiol* 2001, **280:**L991-L998.
39. Doring V, Veretout F, Albrecht R: **The in vivo role of annexin VII (synexin): characterization of an annexin VII-deficient Dictyostelium mutant indicates an involvement in Ca(2+)-regulated processes.** *J Cell Sci* 1995, **108:**2065-2076.
40. Byers TJ, Branton D: **Visualization of the protein associations in the erythrocyte membrane skeleton.** *Proc Natl Acad Sci USA* 1985, **82:**6153-6157.
41. Saxton MJ: **The membrane skeleton of erythrocytes.** *A percolation model* 1990, **57:**1167-1177.
42. Berk DA, Hochmuth RM: **Lateral mobility of integral proteins in red blood cell tethers.** *Biophys J* 1992, **61:**9-18.
43. Mohandas N, Chasis JA: **Red blood cell deformability, membrane material properties and shape: regulation by transmembrane, skeletal and cytosolic proteins and lipids.** *Semin Hematol* 1993, **30:**171-192.
44. Gilligan DM, Bennet V: **The junctional complex of the membrane skeleton.** *Semin Hematol* 1993, **30:**74-83.
45. Ilangumaran S, Hoessli DC: **Effects of cholesterol depletion by cyclodextrin on the spingolipid microdomains of the plasma membrane.** *Biochem J* 1998, **335:**433-440.
46. Samuel BU, Mohandas N, Harrison T: **The Role of cholesterol and glycosylphosphatidylinsoitol-anchored proteins of erythrocyte rafts in regulating raft protein content and malaria infection.** *J Biol Chem* 2001, **276:**29319-29329.
47. Kumar J, Gupta CM: **Membrane skeletal protein structure and interactions in human erythrocytes after their treatment with diamide and calcium.** *Indian J Biochem Biophys* 1992, **29:**123-127.
48. Palek J, Liu SC: **Dependence of spectrin organization in red blood cell membranes on cell metabolism: implications for control of red cell shape, deformability, and surface area.** *Semin Hematol* 1979, **16:**75-93.
49. Oonishi T, Sakashita K, Uyesaka N: **Regulation of red blood cell filterability by Ca2+ influx and cAMP-mediated signaling pathways.** *Am J Physiol* 1997, **273:**C1828-C1834.
50. Takakuwa Y, Mohandas N: **Modulation of erythrocyte membrane material properties by Ca2+ and calmodulin. Implications for their role in regulation of skeletal protein interactions.** *J Clin Invest* 1988, **82:**394-400.
51. Lin S, Yang E, Huestis WH: **Relationship of phospholipid distribution to shape change in Ca(2+)-crenated and recovered human erythrocytes.** *Biochemistry* 1994, **33:**7337-7344.
52. Lange Y, Gough A, Steck TL: **Role of the bilayer in the shape of the isolated erythrocyte membrane.** *J Membr Biol* 1982, **69:**113-123.
53. Weiss EJ, Bray PF, Tayback M: **A polymorphism of a platelet glycoprotein IIIa as an inherited risk factor for coronary thrombosis.** *New Engl J* 1996, **334:**1090-1094.