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Identification of Novel Binding Partners (Annexins) for the Cell Death Signal Phosphatidylserine and Definition of Their Recognition Motif*[S]

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Sabrina Rosenbaum[‡1], Sandra Kreft[‡1], Julia Etich[‡], Christian Frie[‡], Jacek Stermann[‡], Ivan Grskovic[‡], Benjamin Frey[§], Dirk Mielenz[¶], Ernst Pöschl[‖], Udo Gaipl[§], Mats Paulsson[‡‡‡], and Bent Brachvogel[‡2]**

*From the [‡]Center for Biochemistry, Medical Faculty, University of Cologne, 50931 Cologne, Germany, the [§]Department of Radiation Oncology, University Hospital Erlangen, 91054 Erlangen, Germany, the [¶]Division of Molecular Immunology, Nikolaus Fiebiger Centre, University of Erlangen-Nürnberg, 91054 Erlangen, Germany, [‖]School of Biological Sciences, University of East Anglia, Norwich, Norwich NR4 7TJ, United Kingdom, the **Center for Molecular Medicine Cologne (CMMC), and the [‡‡]Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD)*

Identification and clearance of apoptotic cells prevents the release of harmful cell contents thereby suppressing inflammation and autoimmune reactions. Highly conserved annexins may modulate the phagocytic cell removal by acting as bridging molecules to phosphatidylserine, a characteristic phagocytosis signal of dying cells. In this study five members of the structurally and functionally related annexin family were characterized for their capacity to interact with phosphatidylserine and dying cells. The results showed that AnxA3, AnxA4, AnxA13, and the already described interaction partner AnxA5 can bind to phosphatidylserine and apoptotic cells, whereas AnxA8 lacks this ability. Sequence alignment experiments located the essential amino residues for the recognition of surface exposed phosphatidylserine within the calcium binding motifs common to all annexins. These amino acid residues were missing in the evolutionary young AnxA8 and when they were reintroduced by site directed mutagenesis AnxA8 gains the capability to interact with phosphatidylserine containing liposomes and apoptotic cells. By defining the evolutionary conserved amino acid residues mediating phosphatidylserine binding of annexins we show that the recognition of dying cells represent a common feature of most annexins. Hence, the individual annexin repertoire bound to the cell surface of dying cells may fulfil opsonin-like function in cell death recognition.

Discrimination of viable from dying cells is a prerequisite for the efficient clearance of dying and dead cells and to suppress uncontrolled cell lysis and the release of potentially harmful cellular compounds to the local environment. During development and under normal physiological conditions, cells are removed through the process of apoptosis and undergo a strictly defined series of morphological and biochemical changes, before being engulfed by professional phagocytes such as macrophages or even by neighboring cells. A signature of specific "eat me" signals are exposed at the cell surface of apoptotic cells, which enable direct or indirect interactions with phagocytes and dying cells to promote the efficient clearance of apoptotic cells. This reduces the risk of accumulation of secondarily necrotic cells and the release of cellular content to the microenvironment. Exposure of apoptotic-cell associated molecular patterns together with recognition and interpretation of these by phagocytes are crucial steps in the appropriate response of phagocytes toward the engulfed cells (1).

A hallmark of early apoptotic cells is the loss of membrane asymmetry and the exposure of anionic phosphatidylserine (PS)[3] on the outer lipid layer of the cells. In most cells PS predominantly resides in the inner leaflet of the plasma membrane, regulating membrane charge and protein localization (2). This membrane asymmetry is actively maintained by an inward transporting aminophospholipid translocase (3). Upon induction of apotosis, rising cytoplasmic Ca^{2+} levels cause a loss of translocase activity and an activation of bi-directional transporting proteins of the scramblase family to expose PS at the cell surface (4). This phospholipid represents the major recognition cue for the engulfment of dying cells by professional and nonprofessional phagocytes (5). A number of interaction partners for the "eat me" signal PS have been described, including phagocyte cell surface receptors like members of the scavenger receptor family (6) or TIM1 and TIM4 (7), which can directly bind to PS on dying cells. Several PS-binding serum proteins like protein S (8), milk fat globule protein (MFG-E8) (9) and β2-glycoprotein-1 (10) can act as bridging molecules that bind to the signature of the apoptotic cells. The interpretation of the PS signature in the context of bridging molecules and receptors by phagocytes then modulates the response toward apoptotic cells.

Annexin A5 (Anxa5-gene, AnxA5-protein) was identified as a specific interaction partner for PS and is extensively used to detect cell death *in vitro* and *in vivo* (11–13). This protein

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[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany. Tel.: 49-221-478-6996; Fax: 49-221-478-6977; E-mail: bent.brachvogel@uni-koeln.de.

[3] The abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, propidium iodide.

can also inhibit the phagocytosis of dying cells (14, 15) and modulates the cytokine response of phagocytes toward apoptotic and necrotic cells (16). *In vivo*, AnxA5-deficient mice displayed an increased uptake of necrotic cells and a reduced allogenic immune reaction against these (17, 18). Besides AnxA5, two other annexins may play a role in cell death recognition and removal. AnxA1 is externalized upon cell death and can be detected in PS-rich membrane plaques of dying cells (19) where it promotes the removal of dying cells and prevents proinflammatory cytokine production (20). Both AnxA1 and AnxA2 are present at the cell surface of macrophages and may adjust the phagocyte response toward dying cells (21). Most members of the evolutionary conserved, over 1 billion year old annexin family may be involved in the identification and removal of dying cells by phagocytes, but little is yet known about their individual roles as PS-binding immunomodulatory proteins and the underlying molecular mechanisms of PS recognition.

In mammals, the protein family of annexins includes 12 members, with AnxA13 being the phylogenetically oldest annexin (22). All annexins carry four similar domains defined by five α-helices and each domain contains a characteristic type 2 calcium-binding motif (GXGTD-$[X]_{37}$-(D/E)). The four domains are arranged in a disc-like structure with antipodal convex and concave surfaces. Upon binding to calcium the convex side of an annexin specifically interacts with anionic phospholipids, as shown for AnxA5 (23). Because of the structural and functional similarities within the annexin family we speculated that multiple annexins can bind to PS exposed at the apoptotic cell surface. In this study, we produced and purified five annexins and determined their binding toward PS in solid and liquid phase interaction experiments. In addition we characterized their binding to cell surface exposed PS in flow cytometry experiments. Our results show that most annexins interact with PS whereas AnxA8 lacks this ability. Clustal W alignment identified the conserved amino acids mediating the PS recognition by annexins and when introducing these amino residues into the AnxA8 protein using site directed mutagenesis the protein gains the ability to bind to PS. These results indicate that we have identified the essential amino acids for binding of annexins toward PS and that multiple annexins may act as opsonins to influence the recognition of PS at the cell surface of dying cells.

EXPERIMENTAL PROCEDURES

Recombinant Production and Purification of Annexins—DNA fragments encoding mouse full-length Anxa3 (NM_013470.1), Anxa4 (NM_013471.1), Anxa5 (NM_009673.1), Anxa8 (NM_013473.2), and Anxa13 (NM_027211.1) were PCR-amplified from full open reading frame clones (RZPD, Berlin) and fused to att-recombination sites containing a Factor Xa cleavage site at the 5′-end. The corresponding sequences of the individual oligonucleotides are available from the authors. Site-specific recombination (Gateway System, Invitrogen) was used to clone the PCR fragments first into the entry vector pDONR221 and then into the pDEST15 vector (Invitrogen) containing a glutathione *S*-transferase (GST)-tag for purification. Constructs were transfected into *Escherichia coli* (BL21) and fusion proteins were purified by affinity chromatography on glutathione-Sepharose 4B (GE Healthcare). The GST tag was removed by cleavage with Factor Xa according to the manufacturer's protocol (Merck). Purified proteins were separated by SDS-PAGE and visualized by Coomassie staining. The corresponding protein bands were cut out and the identity of purified annexins was confirmed by mass spectrometry peptide mass fingerprinting (Center for Molecular Medicine, University of Cologne). Circular dichroism (CD) spectroscopy measurements were performed on a spectropolarimeter (Jasco J-715). All annexins were dialyzed against 5 mM Tris/HCl (pH 7.4) prior to recording spectra of a concentration of 200 μg/ml. Annexins were labeled with the fluorescent dye Dye490 (DYNOMICS) according to the manufacturer's protocol.

Preparation of Liposomes—Liposomes were prepared as described previously (37). Briefly, 1 mg of liposomes generated from a lipid mixture of phosphatidylcholine (PC):phosphatidylethanolamine (PE) or phosphatidylcholine (PC):phosphatidylethanolamine (PE):phosphatidylserine (PS) were dried under nitrogen, rehydrated with 150 μl of 0.3 M sucrose and incubated for 1 h at room temperature followed by the adjustment of the final volume to 1 ml using H_20. The liposomes were centrifuged for 1 h at 17,900 $\times$ *g*, 4 °C and then resuspended in 500 μl of 20 mM HEPES (pH 7.4). The solution was passed 15 times through an extruder using a 100-nm membrane and then used for experiments.

Protein-Lipid Overlay Assay—Protein-lipid overlay assays were performed as described (38). Lipids (PC, PE, PS) were dissolved in methanol and chloroform mixture to a concentration of 1 mM. Stocks were diluted in a 2:1:0.8 mixture of methanol:chloroform:water to a final concentration of 1, 10, 100, 200, 300, 400, and 500 pmol/μl. 1 μl of each dilution was spotted onto a nitrocellulose membrane (Schleicher& Schuell). After blocking in 1× Tris-buffered saline (TBS), 0.1% Tween-20, 2 mg/ml fatty acid-free bovine serum albumin (BSA) for 1 h at room temperature, membranes were incubated with 10 nM of each annexin for 14 h at 4 °C in blocking solution supplemented with 1 mM $CaCl_2$. Membranes were washed five times with 1× TBS/0.1% Tween-20/1 mM $CaCl_2$ and bound annexins were detected by adding a rabbit antiserum against mouse AnxA3 diluted 1 to 5000 in blocking solution containing 1 mM $CaCl_2$ for 1 h at room temperature. Previous Western blot studies showed that this antiserum can also detect AnxA4, AnxA5, AnxA8, and AnxA13 (supplemental Fig. S1*A*). The primary antibody was detected with a swine anti-rabbit IgG labeled with horseradish peroxidase (DAKO) and visualized by chemoluminescence using standard procedures.

Surface Plasmon Resonance-based Binding Assay—Liposomes were coupled to a BIAsensor chip L1 (BIAcore AB) at a flow rate of 5 μl/min until saturation was reached (~10,000 response units). For all subsequent measurements a flow rate of 10 μl/min was used. The surface of the chip was flushed with 20 mM CHAPS for 1 min. The saturation of binding was confirmed by injecting 25 μl of 0.1 mg/ml BSA in 20 mM HEPES, 250 mM NaCl (pH 7.4). Two independent measure-

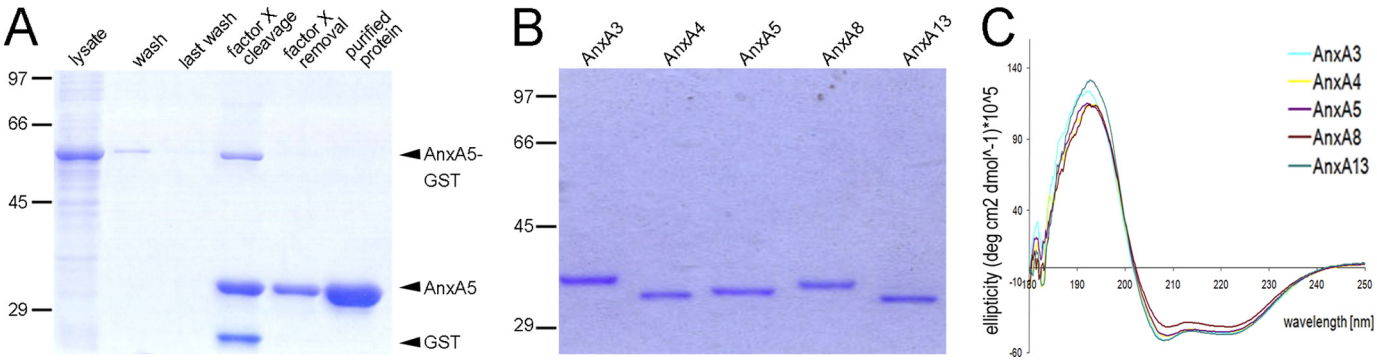

FIGURE 1. **Expression and purification of annexins.** *A*, Coomassie stained SDS-PAGE illustrating different purification steps of AnxA5. After expression in bacteria the protein was purified via its GST tag which was later removed using Factor Xa protease. *B*, all annexins were purified accordingly, migrate at the expected size and show a purity up to 99% as determined by SDS-PAGE and Coomassie staining. Sequence identities were confirmed by peptide mass fingerprinting (not shown). *C*, circular dichroism spectra for recombinant annexins show a high content of α-helical structures.

ments of the binding of individual annexins to liposomes were carried out in the 20 mM HEPES, 250 mM NaCl (pH 7.4) supplemented with 1 mM $CaCl_2$. 30 μl of each annexin dilution was injected and the association was monitored for 180 s. Dissociation was followed for 300 s and the results were analyzed with the BIAevaluation software 4.1 according to the Langmuir model. All binding assays were performed using a BIAcore 2000 (BIAcore AB).

Liposome Binding Assay—Purified annexins were diluted to a final amount of 5 μg of protein in 80 μl of a buffer (20 mM HEPES pH 7.4, 250 mM NaCl) containing either 1 mM or 2.5 mM $CaCl_2$. 20 μl of the liposome suspensions PC/PE (8:2, w/w) or PC/PE/PS (7:2:1, w/w) were added, samples were mixed, incubated for 15 min at room temperature and centrifuged for 30 min at 4 °C and 71,700 × *g*. The supernatants were collected and the pellets were resuspended in the same buffer volume of 20 mM HEPES, pH 7.4, 250 mM NaCl. 30 μl of each sample were separated by SDS-PAGE, and the proteins were visualized by Coomassie staining.

Apoptosis Assay—NIH3T3 cells were plated on 24-well plates (1×10^5 cells/well) in DMEM/F-12 with GlutaMAX™ I (10% FCS, 100 units penicillin/ml, 100 μg/ml streptomycin). After 1 day cell death was induced by addition of 2.5 μM staurosporine (Cayman Chemicals) for 6 h. Cells were isolated by trypsinization and subsequently stained with different fluorescently labeled annexins and propidium iodide and analyzed by flow cytometry (FACSCanto2, Becton Dickinson).

RESULTS

Production of Recombinant Annexins—The open reading frames of AnxA5 and several annexins with unknown roles in cell death recognition were cloned into expression vectors, recombinantly produced in bacteria and purified. The steps of purification in the GST tag-based procedure are shown for AnxA5 (Fig. 1*A*). In bacterial lysates an AnxA5-GST fusion protein of the expected size was detected and after binding of the fusion protein to glutathione-Sepharose, the AnxA5 protein was released by Factor Xa protease cleavage. After removal of Factor Xa only AnxA5 was detected in the supernatant. AnxA3, AnxA4, AnxA8, and AnxA13 were purified according to the same protocol (Fig. 1*B*). Analysis of protein secondary structure by circular dichroism spectroscopy of the purified annexins showed a maximum at 192 nm and minima at 207 and 222 nm typical for the expected high content of α-helical structures in annexins (Fig. 1*C*). The identity of each protein was further confirmed by mass spectrometry peptide mass fingerprinting (not shown).

Annexins Interact with Phosphatidylserine—To test the interactions of individual annexins with specific phospholipids, increasing amounts (1–500 pmol) of phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) were spotted onto nitrocellulose membranes and incubated with purified AnxA3, AnxA4, AnxA5, AnxA8, and AnxA13 (10 nM) in the presence of 1 mM $CaCl_2$. Binding to phospholipids was detected using a cross-reacting antibody that interacts with all annexins (Fig. 2*A*). The tested annexins interact strongly with PS, whereas no binding to PC or PE was found.

The affinities of annexins for PS were further studied by surface plasmon resonance measurements. Liposomes containing PC/PE/PS in a ratio of 7:2:1 (w/w) were immobilized on a BIAsensor chip L1 and the association and dissociation of the different annexins was determined (Fig. 2*B*). AnxA3, AnxA4, AnxA5, and AnxA13 showed a fast, dose-dependent association to liposomes in the presence of 1 mM $CaCl_2$, but a slow dissociation. In contrast, for AnxA8 a faster dissociation from PS-containing liposomes was detected, although a similar dose-dependent binding to liposomes was seen. This is reflected in the calculated K_D values with AnxA8 showing a lower affinity to liposomes (K_D = 26,5 nM) compared with AnxA3, AnxA4, AnxA5, and AnxA13 K_D values. No significant binding was observed for any of the annexins to liposomes containing PC/PE in a ratio of 8:2 (w/w) only (supplemental Fig. S1*B*).

The interactions of annexins with PS-containing liposomes were also studied in liquid phase binding assays (Fig. 2*C*). Liposomes containing PC/PE/PS in a ratio of 7:2:1 (w/w) were incubated with 5 μg of each annexin in the presence of 1 mM $CaCl_2$ for 15 min at room temperature, bound and free protein was separated by ultracentrifugation and analyzed by SDS-PAGE. About 80% of the AnxA4 and AnxA13 and about 50% of the AnxA3 and AnxA5 was recovered in the pellet

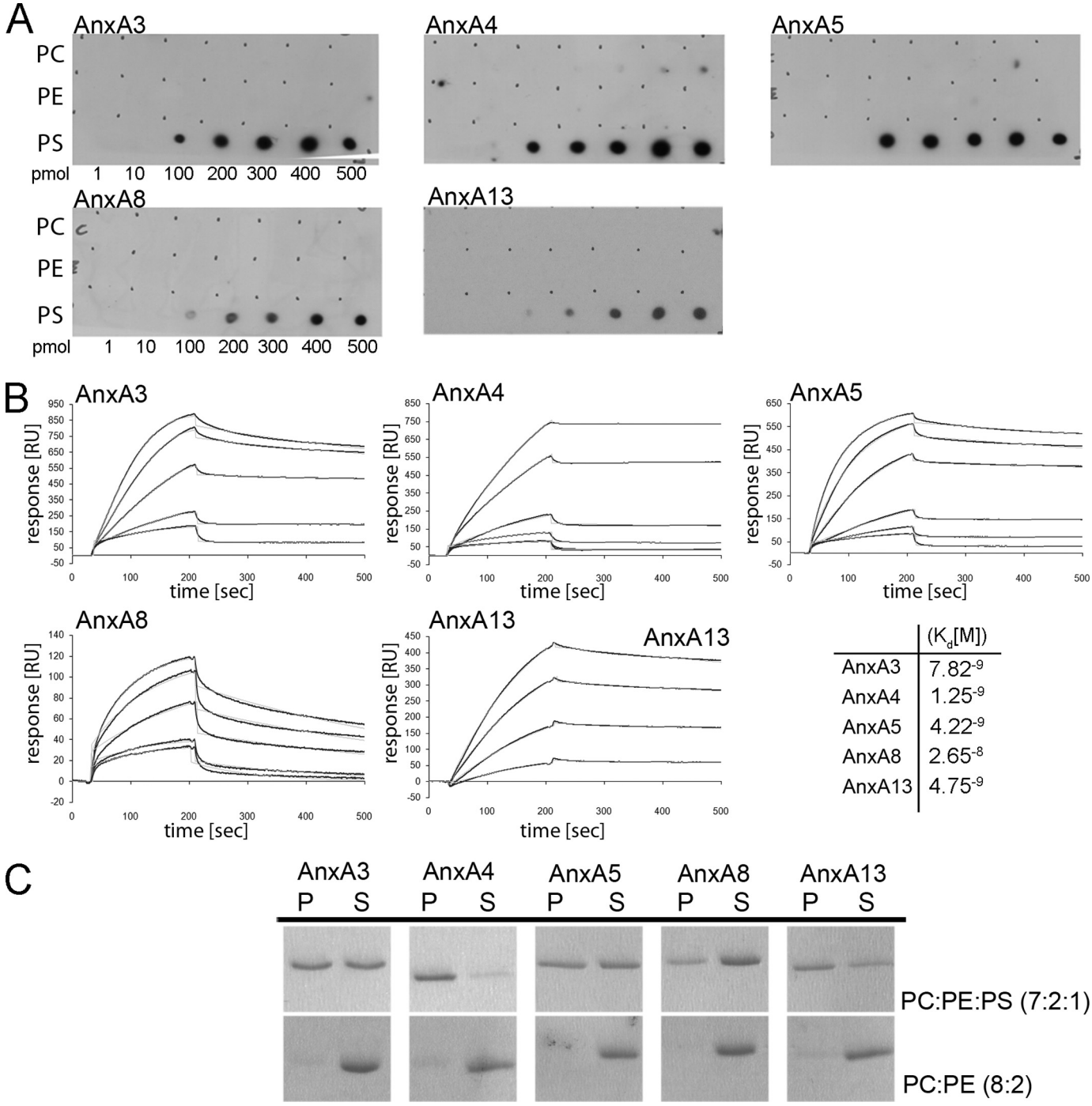

FIGURE 2. **Binding of annexins to phospholipids.** *A*, PC, PE, and PS were spotted onto a nitrocellulose membrane at 1–500 pmol and incubated with AnxA3, AnxA4, AnxA5, AnxA8, and AnxA13 in the presence of 1 mM $CaCl_2$. Annexins bound to phospholipids were detected with a broad-specificity annexin antibody. *B*, surface plasmon resonance measurements were used to study the interactions of soluble annexins with immobilized liposomes containing PC/PE/PS (7:2:1, w/w) mimicking the phospholipid composition of apoptotic cells. Curves in ascending order corresponding to increased concentrations of annexins (100, 80, 50, 25, 12, and 6 nM) are shown. Calculated K_D values for specific interactions are given for individual annexins. *C*, interactions of annexins with liposomes either lacking (PC/PE (8:2, w/w)) or containing PS (PC/PE/PS (7:2:1, w/w)) were analyzed by a liposome binding assay. Bound proteins (pellet fraction, *P*) and unbound annexins (supernatant, *S*) were detected in Coomassie-stained SDS-PAGE gel. No interaction of annexins was detected to liposomes lacking PS.

fraction, while only 20% of AnxA8 was bound to PS containing liposomes. No binding to PC/PE containing liposomes in a ratio of 8:2 (w/w) was observed. Hence, all tested annexins interact specifically with PS containing liposomes at physiologically relevant calcium concentrations and may represent potential binding partners for apoptotic cells.

Annexins Bind to the Cell Surface of Dying Cell—To test the capacity of annexins to interact with the cell surface exposed PS-signature on dying cells the binding was analyzed in flow cytometry experiments. Therefore, all annexin preparations were fluorescently labeled with the chromophore Dye490. Apoptosis was induced in subcon-

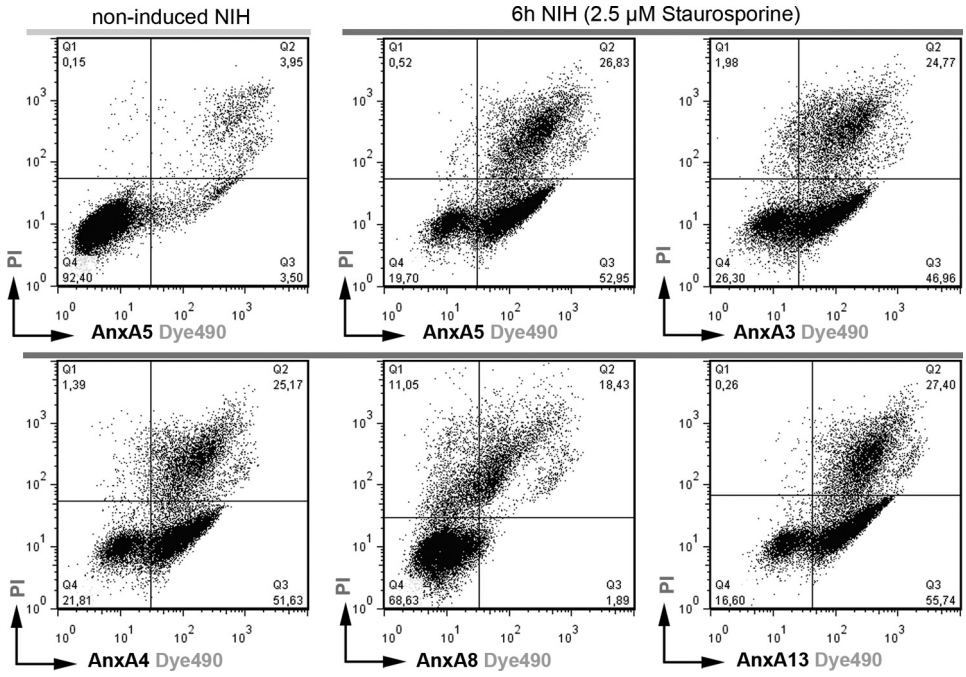

FIGURE 3. **Interaction of annexins with dying cells.** AnxA3, AnxA4, AnxA5, AnxA8, and AnxA13 were conjugated with a fluorescent chromophore (Dye490) and tested for their binding to untreated and dying cells by flow cytometry. Costaining with PI indicated necrotic/dead cells. Apoptotic NIH3T3 cells (addition of 2.5 μM staurosporine for 6 h) were stained with the different annexins as well as propidium iodide.

fluent cultures of NIH3T3 cells for 6 h in the presence of 2.5 μM staurosporine and single cell suspensions were stained with labeled annexins and the DNA stain propidium iodide (PI) prior to analysis by flow cytometry. First binding of AnxA5-Dye490 to dying cells was characterized. Non-induced NIH3T3 cells show only a weak signal for AnxA5-Dye490$^{(+)}$/PI$^{(-)}$ early apoptotic and AnxA5-Dye490$^{(+)}$/PI$^{(+)}$ secondary necrotic cells (Fig. 3, non-induced NIH3T3, Q3 and Q2) and 92% of the cells were negative for both markers (Fig. 3, non-induced NIH3T3, Q4). After induction of apoptosis 53% of the cells correspond to AnxA5-Dye490$^{(+)}$/PI$^{(-)}$ early apoptotic cells and 27% AnxA5-Dye490$^{(+)}$/PI$^{(+)}$ secondary necrotic cells (Fig. 3, *Q3* and *Q2*). Only about 20% of the cells represent AnxA5-Dye490$^{(-)}$/PI$^{(-)}$ viable cells with an unaltered cell surface lipid composition (Fig. 3, *Q4*). Strikingly, binding of labeled AnxA3, AnxA4, and AnxA13 showed a very similar pattern of binding to PS-presenting cells with up to 55% and about 27% of cells representing early apoptotic or secondary necrotic cells, respectively. In contrast, no binding of AnxA8 to apoptotic cells was seen (Fig. 3, AnxA8-Dye490, *Q3*).

Annexins Contain Conserved Amino Acid Residues Mediating Phosphatidylserine Recognition—As AnxA8 represents an evolutionary younger member of the annexin family, it may have lost the capacity to bind to PS due to mutational changes in the highly conserved calcium-binding motifs. To define these variations, protein sequences of AnxA3, AnxA4, AnxA5, AnxA8, and AnxA13 were compared using the ClustalW alignment algorithm (24). Significant differences between AnxA8 and the other annexins were identified within the four conserved GXGT-[X]$_{38}$-(D/E) regions of the calcium type 2-binding motifs of individual annexin repeats. In repeat one, two, and four the negatively charged aspartic acid residues (D) found in AnxA3, AnxA4, AnxA5, and AnxA13 are replaced by an uncharged polar (repeat 1: N40) or positively charged (repeat 2: Lys-112, repeat 4: Arg-272) residue, respectively (Fig. 4, *red boxes*). Additionally, the tryptophan residue in repeat three, known to be involved in AnxA5-phospholipid interactions (25), is altered to methionine in AnxA8. All changes are associated with the GXGT-[*X*]$_{38}$-(D/E) motif and located in calcium binding loops exposed to the cell surface.

The lack of aspartic acid and tryptophan residues in the context of the exposed calcium-binding loops may interfere with the ability of AnxA8 to bind calcium and interact with phospholipids on dying cells. To test for this hypothesis, residues corresponding to functional calcium-binding sites were introduced to AnxA8 by site-specific mutagenesis. The positively charged amino acids in AnxA8 were reverted to aspartic acid in single (A8(K112D); A8(R272D)) or double (A8(K112D, R272D)) mutant proteins. In addition, all four sites were reverted to the corresponding sequences found in apoptotic cell binding annexins A8(N40D, K112D, M194W, R272D). Recombinant proteins were expressed, purified, and their size and purity determined by SDS-PAGE. All AnxA8 mutants showed circular dichroism spectra characterized by a maximum absorbance at 192 nm and minima at 207 nm and 222 nm typical for a high α-helical content. The analysis of thermal stability further indicated correct folding of the mutants as melting temperatures were very similar to wild-type AnxA8 protein (Fig. 5*A*). Hence, reintroducing conserved amino acids into AnxA8 does not affect α-helical folding or decrease the thermal stability.

The binding of the different mutant forms of AnxA8 to PS-containing liposomes was studied in the presence of 1 or 2.5 mM $CaCl_2$ (Fig. 5*B*). At 1 mM $CaCl_2$, about 80% of wild-type AnxA8 or mutants A8(K112D) and A8(R272D) were de-

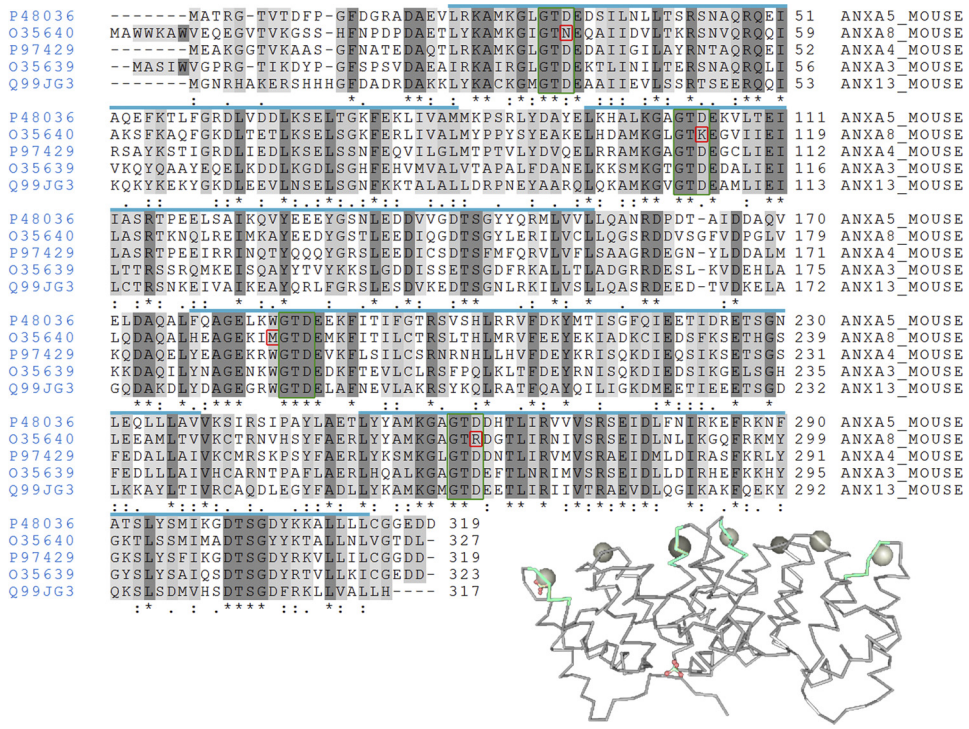

FIGURE 4. **Identification of the PS binding motif.** The amino acid sequences of AnxA3, AnxA4, AnxA5, AnxA8, and Anx13 were compared using the ClustalW multiple sequence alignment program to determine identities, similarities and differences between the annexins. *Top*, four repeats of each annexin are highlighted (*blue line*) and conserved regions involved in calcium binding and lipid interaction are marked (*green box*). Unique amino acids of AnxA8 within the conserved regions are marked (*red box*). *Bottom*, the location of the conserved regions is indicated (*green*) in an overview of the crystal structure of rat annexin A5 (39). Calcium ions bound to the convex surface of the protein are indicated (*gray balls*).

tected in the unbound fractions. Only a slightly increased binding was detected for the double mutant A8 (K112D, R272D). In contrast, about 80% of the quadruple mutant A8 (N40D, K112D, M194W, R272D) bound to the liposomes. When increasing the $CaCl_2$ concentration to 2.5 mM, a concentration used for flow cytometry experiments, increased amounts of mutants were able to bind, with about 30% of the wild-type AnxA8, 60% of the single mutants A8(K112D) and A8(R272D) or 80% of A8(K112D, R272D) found in the bound fractions. At this increased $CaCl_2$ concentration, the quadruple mutant A8(N40D, K112D, M194W, R272D) bound quantitatively to PS-containing liposomes. Hence, reintroducing homologous sequences of other annexins into AnxA8 protein increased the calcium-dependent binding to PS of mutant A8(N40D, K112D, M194W, R272D) to a level comparable to other annexins like AnxA5.

To analyze the interaction of AnxA5, AnxA8, and mutated forms of AnxA8 with dying cells, the proteins were labeled with the fluorescent Dye490 and used in apoptosis experiments. Single cell suspensions were stained with the labeled proteins and PI and binding to cell surface exposed PS of dying NIH3T3 cells was assessed by analytical flow cytometry in the presence of 2.5 mM or 1 mM $CaCl_2$ (Fig. 6). In the presence of 2.5 mM $CaCl_2$ non-induced cells showed only a weak binding to AnxA5-Dye490, whereas after staurosporine induction 11 and 7% of the cells were found to be apoptotic (AnxA5-Dye490$^{(+)}$/PI$^{(-)}$) or secondary necrotic (AnxA5-Dye490$^{(+)}$/PI$^{(+)}$), respectively (Fig. 6*A*, 6h NIH3T3, Q3, Q2). No binding to apoptotic cells was observed for AnxA8-Dye490 (Fig. 6*A*, 6h NIH3T3, *Q3*) or AnxA8 mutants A8(K112D)-Dye490 or A8(R272D)-Dye490 (Figs. 5*B* and 6*A*, 6h NIH, *Q3*). In contrast, labeled mutant proteins A8(K112D, R272D) and A8(N40D, K112D, M194W, R272D) show a similar staining pattern as AnxA5, with 10% or 9% of cells being either A8(K112D, R272D)-Dye490$^{(+)}$/PI$^{(-)}$ or A8(N40D, K112D, M194W, R272D)-Dye490$^{(+)}$/PI$^{(-)}$ (Figs. 5*B* and 6*B*, 6 h NIH3T3, *Q3*). Similar staining patterns were obtained at 1 mM $CaCl_2$ (Fig. 6*C*), although the proportion of apoptotic cells was slightly decreased for mutants A8(K112D, R272D) and A8(N40D, K112D, M194W, R272D). No binding to dying cells was seen with any labeled protein in the presence of 5 mM EDTA (supplemental Fig. S2). In conclusion, the absence of aspartic acids in positions 112 and 272 in AnxA8 contributes to the lack of phosphatidylserine recognition on the cell surface of apoptotic cells. This binding depends on the presence of sufficient amounts of calcium in the extracellular environment.

DISCUSSION

Multiple proteins have been implicated as binding partners for cell surface-presented PS and among those annexin A5 is widely accepted as a specific ligand and used as a marker for cell death (11–13). Surprisingly, prior to this study relatively little was known about the role of other members of the annexin family in cell death recognition. Annexins lack a classical signal peptide for cellular export and in the past extracellular functions of annexins were controversially discussed. Only more recently it became accepted that annexins can be secreted, are detectable in serum and act extracellularly on coagulation and leukocyte extravasation processes (26, 27).

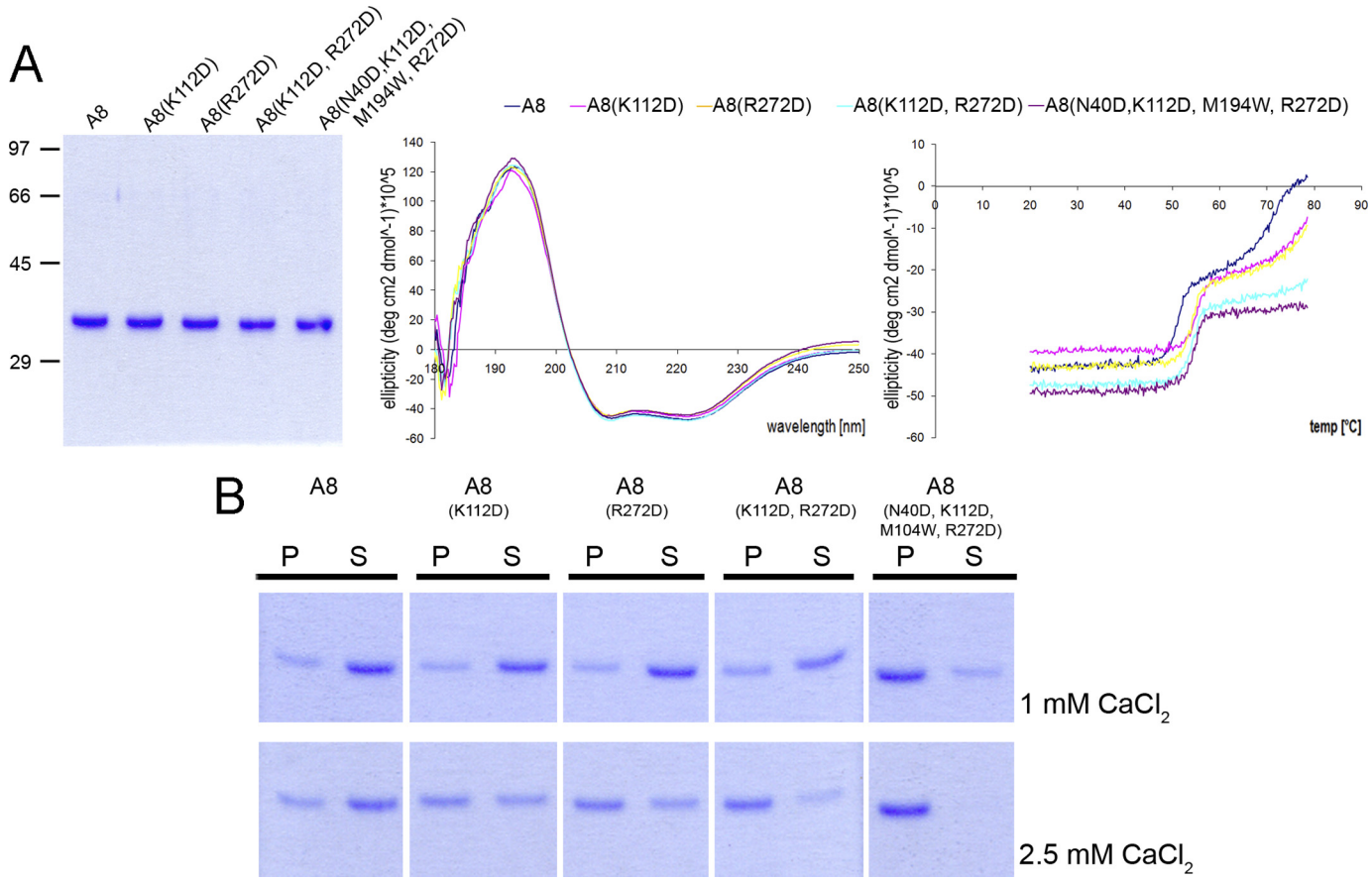

FIGURE 5. **Interaction of AnxA8 mutants with PS-enriched liposomes.** *A*, purified mutated and wild type AnxA8 (A8) proteins were separated in a Coomassie-stained SDS-PAGE gel. Circular dichroism spectra show a high content of α-helical structures and melting curve analysis indicates the thermal stability of the generated mutants. *B*, interactions of mutated AnxA8 proteins with PS were studied in a binding assay using liposomes containing (PC/PE/PS (7:2:1, w/w)) or lacking PS (PC/PE (8:2, w/w)). Bound proteins (pellet fraction, *P*) and unbound annexins (supernatant, *S*) were detected in Coomassie-stained SDS-PAGE gel.

Therefore, the individual annexin repertoire of dying cells and phagocytes may contribute to the process of clearance of dying cells in normal and pathological situations.

Comparison of the PS-binding annexins AnxA3, AnxA4, AnxA5, and AnxA13 with AnxA8, which revealed a significantly lower affinity to PS, identified the amino acid residues essential for apoptotic cell death recognition. The presence of two aspartic acid residues in position GXGT**D**-$[X]_{37}$-(D/E) in repeat domains two and four is required for efficient binding of annexins to the PS-signature of apoptotic cells at physiological calcium concentrations. Previous *in vitro* studies showed that single and multiple changes within the conserved acidic residues (D/E) of the type 2 calcium-binding motif GXGT**D**-$[X]_{37}$-(D/E) of AnxA4 to alanine critically influence membrane binding and aggregation (28). It was further shown that single mutations of any of the four calcium-binding motifs within the AnxA5 protein substantially decreased the affinity for calcium and PS-containing membranes (29). The conserved negatively charged aspartic acid residues in the GXGT**D**-$[X]_{37}$-(D/E) motif in domains two and four in other annexins are in AnxA8 replaced by positively charged lysine or arginine residues and disturb the integrity of the calcium binding sites. This reduces the affinity of AnxA8 to PS below a threshold level needed for interacting with the cell surface of apoptotic cells at physiological calcium concentrations.

This is further reflected by the decreased affinity of AnxA8 to PS as detected in surface plasmon resonance-based binding assays. In liposome binding tests, the introduction of four mutations in A8 (N40D, K112D, M194W, R272D) is needed for maximum binding to PS, whereas replacement of lysine 112 or arginine 272 residues with aspartic acid (K112D, R272D) shows only minor effects. As in flow cytometry experiments, the mutants A8(K112D, R272D) and A8(N40D, K112D, M194W, R272D) bind to a similar extent to apoptotic cells. The phospholipid composition of liposomes used in biochemical assays may not fully reflect the situation on cell membranes.

Interestingly, the phylogenetically older AnxA13 and the younger AnxA5 both contain an intact GXGT**D**-$[X]_{37}$-(D/E) motif and bind efficiently to dying cells (22). This may point to a unique loss of function mutation in the calcium binding motifs of AnxA8 during evolution. Earlier studies on the evolution of annexin calcium-binding domains in annexins already raised the question about the functional relevance and physiological roles of modifications within these calcium binding domains (30). The differences in the recognition of the PS signal by individual annexins now links the structural conservation of the GXGT**D**-$[X]_{37}$-(D/E) motif to annexin-specific roles in the recognition of apoptotic cells.

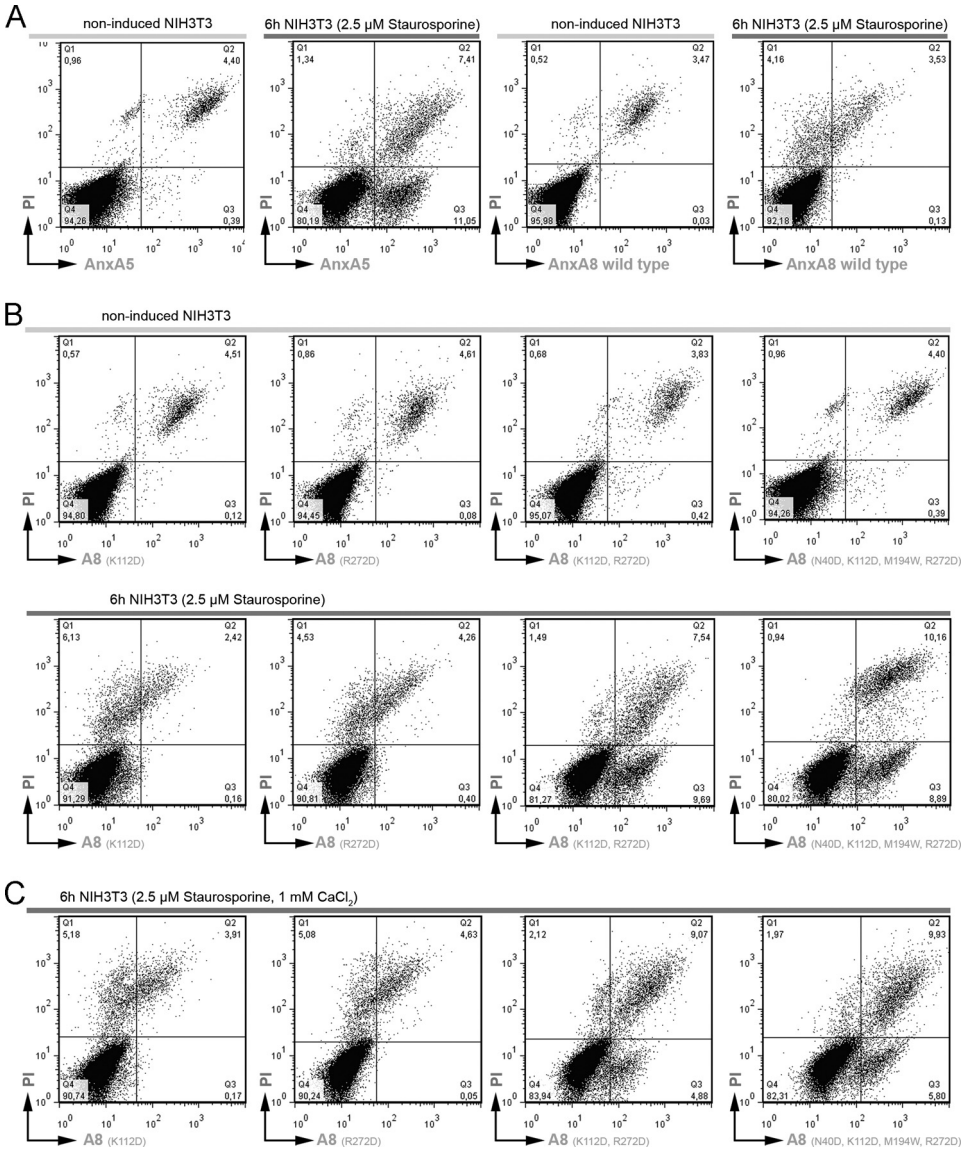

FIGURE 6. **Interaction of AnxA8 mutants with dying cells.** Binding of AnxA8 mutants to vital (non-induced) and staurosporine-treated (6 h) apoptotic NIH3T3 cells was compared with AnxA8 and AnxA5 by flow cytometry. Vital and apoptotic NIH3T3 cells were stained with the conjugated (Dye490) (*A*) AnxA5, AnxA8, or (*B*) AnxA8 mutants and PI in the presence of 2.5 mM $CaCl_2$ or (*C*) 1 mM $CaCl_2$. The dot plots are representative of two independent experiments.

Considering the conservation of the PS signal and the calcium binding motif of annexins throughout evolution, it is intriguing to speculate that the need of the innate immune defense to diversify the interpretation of the "eat me" signal PS at the cell surface of dying cells in higher organisms is paralleled by the diversification of calcium binding annexins. This view is supported by the fact that AnxA1 and AnxA5 both bind to the cell surface of apoptotic cells, but induce opposing effects in macrophages upon engulfment. The mediator of glucocorticoid action AnxA1 enhances the efficient clearance of apoptotic cells (19, 31) and prevents the production of proinflammatory cytokines by macrophages (20). In contrast, AnxA5 interferes with the immunosuppressive effects of apoptotic cells by inhibiting phagocytosis and eliciting an inflammatory response in macrophages (17). Impairment of phagocytosis by AnxA5 preferentially targets irradiated lymphoma cells to dendritic cells which enhances immune reactions and even improves protection against the tumor (32). The diversification of annexins during evolution may be linked to the need of the immune system to interpret the PS signal in the local microenvironment to facilitate an appropriate response toward dying cells and prevent inflammation and autoimmune responses.

Based on this *in vitro* evidence a pronounced immunological phenotype in annexin deficient mice could be anticipated. In contrast, the phenotypes of AnxA1- and AnxA5-deficient mice are rather unexceptional (17, 33). AnxA1$^{-/-}$ mice show an increased sensitivity to acute and chronic inflammatory stimuli, whereas in AnxA5-deficient mice no dramatic effects on the normal *in vivo* immune response toward apoptotic cells were observed (15).[4] However, when challenging the im-

[4] B. Brachvogel, unpublished data.

mune system by allogeneic cells, a retarded regression of tumors is observed in AnxA5 deficient mice (17). Deficiencies in receptor/bridging molecules involved in the recognition and ingestion of dying cells often show very specific but mild *in vivo* effects. Macrophage receptors of the scavenger family directly interact with phosphatidylserine on the cell surface of dying cells (6, 34) and isolated thymic macrophages of scavenger receptor AI (SR-AI)-deficient mice display a 50% reduction in the uptake of apoptotic thymocytes. Nevertheless, the frequency of dying cells in the thymus of SR-AI$^{(-/-)}$ is not impaired by the deficiency (35). It is therefore not surprising that clearance of dying cells in AnxA5-deficient mice is not strongly affected, considering the functional redundancy between AnxA5 and other annexins.

Irrespective of their redundancy, annexins may also play a significant role during pathological processes and are thought to be involved in the onset and progression of chronic autoimmune disorders, like systemic lupus erythematosus (SLE). For instance, AnxA1 autoantibodies of the IgM and IgG classes have been detected in patients with SLE (36) and may not only be a symptom, but also a disease promoting factor. Such autoantibodies may interfere with AnxA1 bound to apoptotic cell surfaces and decrease the anti-inflammatory removal of dying cells by macrophages. The absence of AnxA1 together with the presence of the PS signal on remaining dying cells may promote uptake by dendritic cells and the presentation of self-antigens which will then break self-tolerance. Similar scenarios are discussed for AnxA5 (16). Considering that annexins fulfill both redundant and unique functions in binding and clearance of dying cells, the situation may be far more complex. The annexin repertoire found at the surface of apoptotic cells as well as engulfing phagocytes contributes to the interpretation of apoptotic patterns by macrophages to facilitate an appropriate response of the innate immune system. As a consequence, autoantibodies against any annexin with the capacity to bind to dying cells may modify the immune response toward dying cells and define a risk factor for inflammatory and autoimmune diseases.

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