Phosphatidylserine (PS) on apoptotic cells is a target for diagnosis and therapy using annexin A5 (anxA5). Pretargeting is a strategy developed to improve signal to background ratio for molecular imaging and to minimize undesired side effects of pharmacological and radiotherapy. Pretargeting relies on accessibility of the target finder on the surface of the target cell. anxA5 binds PS and crystallizes in a two-dimensional network covering the PS-expressing cell surface. Two-dimensional crystallization is the driving force for anxA5 internalization by PS-expressing cells. Here, we report structure/function analysis of anxA5 internalization. Guided by structural bioinformatics including protein–protein docking, we revealed that the amino acids Arg63, Lys70, Lys101, Glu138, Asp139, and Asn160 engage in intermolecular salt bridges within the anxA5 trimer, which is the basic building block of the two-dimensional network. Disruption of the salt bridges by site-directed mutagenesis does not affect PS binding but inhibits trimer formation and cell entry of surface-bound anxA5. The anxA5 variants with impaired internalization are superior molecular imaging agents in pretargeting strategies as compared with wild-type anxA5.

Cell surface-expressed phosphatidylserine (PS) is an attractive biomarker for diagnosis of diseases and evaluation of efficacy of treatment (1). PS is absent in the exofacial leaflet of the plasma membrane of healthy living cells and is confined to membrane leaflets facing the cytosol due to the aminophospholipid translocase (2, 3). During apoptosis, PS is translocated to the exofacial leaflet of the plasma membrane where it triggers phagocytes to engulf the dying cell (4). Phagocytosis of dying cells is a rapid and efficient process in healthy tissue (5). Pathological tissue, on the other hand, is characterized by impaired clearance of apoptotic cells and, consequently, a sustained presence of PS-expressing cells (6). In addition to apoptotic cells, cell surface expression of PS has also been observed for living endothelial cells of tumor vasculature and stressed tumor cells (7).

The 35-kDa human protein annexin A5 (anxA5) binds PS in a Ca\(^{2+}\)-dependent manner and has established itself as a molecular imaging probe to measure PS expressing cells in vitro and in vivo in animal models and in patients (8). Molecular imaging of PS in patients employs radioisotope labeled anxA5 that is injected intravenously and monitored by single photon emission computed tomography (9). A disadvantage of the current anxA5 imaging protocol is the low signal to background ratio reducing sensitivity and specificity. In addition, intravenous administration of radiolabeled anxA5 imposes a risk for nephrotoxicity because biodistribution and dosimetry studies show a high uptake and long residence of radiolabeled anxA5 in the kidneys (10, 11). These drawbacks can be circumvented by pretargeting procedure that injects anxA5 and the radioactive moiety separately into the subject (12). The small radioactive compound reacts with anxA5 bound to PS-expressing cells, whereas unreacted compounds are rapidly cleared from the circulation. Favorable biodistribution and low background signals are thus achieved.

Success of pretargeting strongly depends on accessibility of anxA5 for the small radioactive compound. Recently, we have reported that anxA5 is internalized by PS-expressing cells through a novel portal of cell entry (13). anxA5 binds cell surface-expressed PS and induces membrane invagination through a nanomechanical mechanism involving the two-dimensional crystallization of anxA5 trimers (13). We embarked on a study to unravel the relationship between anxA5 structure and two-dimensional crystallization to generate anxA5 variants that bind cell surface expressed PS without opening the portal of cell entry. This paper reports on elucidation of amino acids involved in anxA5 trimerization and generation and analyses of anxA5 variants that bind PS without being internalized. These anxA5 variants are superior in pretargeting strategy to measure apoptosis as compared with wild-type anxA5.

**EXPERIMENTAL PROCEDURES**

**Structural Bioinformatics and Protein-Protein Docking**—We employed protein–protein docking as implemented in the ICM package (MolSoft). The high resolution (1.9 Å) structure of human anxA5 (14) was the starting structure. The ICM-protein–protein rigid body docking procedure, called ICM-
Disruption of Annexin A5 Trimer Inhibits Internalization

docking and interface side-chain optimization (see Ref. 15 for details), was used as follows. First, one monomeric unit was docked onto a second identical monomeric unit. About 400 dimers were thus generated and clustered. Only those poses that corroborated published experimental data and gave a 3-fold axis of symmetry (C3) when assembled into a trimeric complex were accepted after visual inspection. Next, after generation of the trimer (two identical dimers were superposed on one of their monomeric units), energy minimization was applied to remove clashes from the trimeric complex.

This procedure resulted in the selection of one hypothetical trimeric organization of annexin monomeric units.

Cloning of anxA5 and Its Variants—Wild-type human anxA5 cDNA was cloned into pQE30Xa (Qiagen). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). On the basis of bioinformatics, the following variants were generated: 2D1–6 with amino acid replacements R63A, K70A, K101A, E138A, D139G, and N160A; 2D2–6 with K70A, K101A, E138A, D139G, and N160A; and 2D1–5 with R63A, K70A, K101A, E138A, and D139G. In addition, all two-dimensional variants contained a GI66C substitution for allowing site-specific conjugation with maleimide derivatized fluorochromes. Gly166 is within the linker connecting helices IIE and IIIA and protruding at the concave side of the molecule (16). The GI66C substitution was also introduced in wild-type anxA5 (J-anxA5). cDNAs were fully sequenced using the ABI prism sequencing kit.

Expression and Labeling of anxA5 and Its Variants—Escherichia coli M15 (Qiagen) were transformed with pQE30Xa containing cDNA of anxA5 and its variants. Bacteria were grown to an optical density of 6 (measured at 600 nm, and expression was induced by adding 1 mM isopropyl 1-thio-β-d-galactopyranoside. After 3 h, bacteria were harvested and suspended in phosphate buffer containing 500 mM NaCl, 20 mM imidazole, and 1% Triton X-100. Bacteria were lysed by sonification at 9 μm amplitude for 6 × 10 s. Lysis was continued at room temperature for 2.5 hours. Cell debris was removed by centrifugation. His-tagged proteins were isolated from the supernatant by chromatography using Ni2+ columns (GE Healthcare/Amersham Biosciences) and a two-step imidazole gradient (125 and 500 mM imidazole). His tags were removed by factor Xa cleavage in 5 mM 2-mercaptoethanol. The latter was added to prevent dimerization through intermolecular disulfide bridge formation. Dimerization hampered cleavage by factor Xa severely. J-anxA5 and two-dimensional anxA5 were further purified by a second run over a Ni2+ column. Purity was assessed by SDS-PAGE with silver staining and Western blotting with polyclonal anti-anxA5 (Hyphen Biomed) as well as MALDI TOF/TOF analysis (Applied Biosystems).

J-anxA5 and two-dimensional variants were fluorescently labeled with maleimide-fluorescein (F) and maleimide-Alexa Fluor 568 (A) (Invitrogen). This yielded conjugates of 1:1 stoichiometry as verified by 400 MALDI TOF/TOF analyzer (Fig. 3A). The maleimide-derivatized fluorophores reacted specifically with Gly166 because pretreatment of anxA5 variants with 5 mM iodoacetamide blocked labeling with maleimide-derivatized compounds (data not shown).

Electron Microscopic Analysis of Two-dimensional Crystallization—Procedure and experimental conditions for growing annexin A5 two-dimensional crystals on a phospholipid monolayer are described in detail elsewhere (17). Briefly, 17 μl of 60 μg/ml J-anxA5 and two-dimensional variants in 10 mM HEPES, 150 mM NaCl, 2 mM CaCl2, and 3 mM NaN3, pH 7.4, were deposited in a Teflon well (d, 4 mm; depth, 1 mm). 0.6 ml of lipid solution containing 150 mM dioleoyl-PS/450 mM dioleoyl-PC in chloroform/n-hexane (1:1 w/w) was deposited on top of the solution to form the functionalized lipid monolayer. The two-dimensional protein-lipid domains were formed on the lipid/buffer interface after overnight incubation at 21 °C in a humid chamber. The two-dimensional protein-lipid domains were transferred to carbon-coated electron microscopy grids and negatively stained with 1% uranyl acetate solution as described previously (17, 18). Grids were observed in a JEOL 2010 transmission electron microscope operated at 200 kV, equipped with a LaB6 and 2 × 2000 CCD UltraScan digital camera (Gatan, Inc., 15 micron/pixel resolution). All data were collected as 2048 × 2048 digital images at a total magnification of 38,700× and low electron dose conditions (<1500 electron·nm−2·s−1). Digital micrograph images were converted in MRC (Medical Research Council, UK) format using the proc2d program from the EMAN software package (National Center for Macromolecular Imaging) (19) and further analyzed with the CRISP two-dimensional crystallography software (Calidris) (20).

Binding to PS Containing Synthetic Phospholipid Membranes and Apoptotic Cells—Binding of unlabeled and fluorescently labeled anxA5 variants was investigated by ellipsometry as described previously (21). Binding of fluorescently labeled anxA5 and its variants to apoptotic Jurkat cells was measured by flow cytometry (22).

FRET—FRET of phospholipid bound anxA5 and its variants was determined by measurement of fluorescence excitation spectra as described elsewhere (23). Briefly, large unilamellar vesicles (LUVs, φ = 100 nm) of 20:80 PS/PC (molar/molar) were prepared in the absence of calcium to avoid clustering of PS. anxA5-variant F/annxA5-variant iodoacetamide/annxA5-variant A were mixed in a ratio of 2:1:0 and 2:0:1 and supplemented with 3 mM EDTA or 1 and 3 mM CaCl2. LUVs and anxA5 mixtures were added, and binding was allowed to take place for 10 min. Annexin A5:phospholipid ratios were chosen such that 1 mol anxA5 covers 1360 mol phospholipid. Excitation scans ranging from 450 to 590 nm in 1-nm steps at an emission wavelength of 600 nm were measured at a speed of 5 nm/s and an integration time of 0.15 s (SLM Aminco). Spectra of donor without acceptor were subtracted from spectra of donor with acceptor to correct for the contribution of fluorescein to the FRET signal. Corrected spectra were normalized against the 570-nm fluorescence signal. FRET was determined as the increase in fluorescence intensity at 490 nm in the presence of calcium (Fig. 3B).

FRET of anxA5 and its variants bound to apoptotic Jurkat cells was determined using flow cytometry. Jurkat cells at a cell density of 106 cells·ml−1 in DMEM were stimulated to go into apoptosis by incubation with 200 ng/ml Fas ligand (Clone 7C11, Beckman Coulter) for 3 h. Cells were pelleted
Disruption of Annexin A5 Trimer Inhibits Internalization

and resuspended in buffer at a cell density of 5 × 10⁶ cells/ml⁻¹, anxA5-variant F/anxA5-variant iodoacetamide/ anxA5-variant A were mixed in ratios of 0:2:1 and 2:0:1 and supplemented with 3 mM EDTA and 1 or 3 mM CaCl₂. 10⁶ cells/ml⁻¹ were incubated with 33 nM anxA5 variant for 10 min. FRET was determined as the increase of mean fluorescence intensity in channel FL3.

Internalization—Binding and internalization of anxA5 variants by apoptotic Jurkat cells was visualized with two photon laser scanning microscopy as described elsewhere (22). Briefly, Jurkat cells were incubated with 200 ng/ml⁻¹ anti-Fas and 6 μg/ml⁻¹ fluorescein-labeled anxA5. After 3 h, the cells were washed with 25 mM HEPES/NaOH, 140 mM NaCl, 10 mM EDTA, pH 7.4, to remove noninternalized anxA5. The cells were then incubated with 1 μg/ml⁻¹ Alexa Fluor 568-labeled WT anxA5 in 25 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4, for 5 min. Subsequently, the cells were fixed and visualized with two photon laser scanning microscopy. Quantification of anxA5 internalization by Jurkat cells executing apoptosis was performed as follows. Cell were incubated with 200 ng/ml⁻¹ anti-Fas and 6 μg/ml⁻¹ fluorescein-labeled anxA5 variants. After 3 h, cells were pelleted, and membrane-bound anxA5 was washed from the cellular surface with 10 mM EDTA. Cells were resuspended in distilled water and lysed by snap-freezing three times in liquid nitrogen. Fluorescence intensity was measured with a fluorimeter (SLM Aminco) and quantified using reference curves prepared with each anxA5 variant. Total protein concentrations were determined using micro-BCA according to the manufacturer’s protocol (Pierce). Internalization was determined as the increase of the ratio of anxA5/total protein (ng/t)

RESULTS

Identification of Amino Acids Engaging in Trimer Formation—The protein-protein docking strategy returned >400 possible conformations for the anxA5 homotypic dimer. This set was reduced in number by applying the criteria that dimers should have the conserved basic cluster Arg²⁵, Lys²⁹, Arg⁶³, and Arg¹⁵¹ at the interface between the monomers (24). Possibilities were further decreased by restricting that only those dimers were eligible that could build a trimer with C3 symmetry. Applying these criteria yielded one top-ranking trimer complex (Fig. 1). The trimer complex shows a convex shape at the membrane binding side supporting the nanomechanical mechanism of anxA5 internalization by PS-expressing cells (13).

The top-ranking trimer complex was analyzed on proximity of amino acids of interacting domains. Arg⁶³, part of the basic cluster and present in the linker connecting helices IC and ID, contacts Asp¹⁷² (present in the linker connecting helices IIE and IIIA) at 3.1 Å (NH₂-OD1). Lys⁷⁹, present in helix ID contacts Glu¹³⁸ (present in helix IID) at 2.8 Å (NZ-OE2). Lys¹⁰¹ (present in linker connecting helices IIA and IIB), Glu¹³⁸, and Asp¹³⁹ (both present in helix IID) position near the 3-fold axis of symmetry; these residues have multiple contacts with neighboring residues and together form a cluster of oppositely charged residues. Asn¹⁶⁰ (present in linker connecting helices IIE and IIIA) opposes the amino acid stretch comprising the linker connecting helices IC and ID and helix
Disruption of Annexin A5 Trimer Inhibits Internalization

![Graph A](image)

**FIGURE 2.** Ca\(^{2+}\)-dependent binding of anxA5 to a bilayer of PS/PC. **A,** binding isotherm measured with ellipsometry. Time points of anxA5 (final concentration, 1 \(\mu\)g/ml) and EDTA (final concentration, 5 mM) addition are indicated by arrows (21). **B,** table summarizing the key characteristics of binding of anxA5 variants (final concentration, 10 \(\mu\)g/ml) to a bilayer of PS/PC as determined with ellipsometry. **C,** dot plot of a flow cytometric measurement of binding of anxA5-F (final concentration, 1 \(\mu\)g/ml) to apoptotic Jurkat cells (10^6 cells/ml). R1 events comprised the Jurkat cells with viable forward and side scatter that bind anxA5-F. These are the early apoptotic cells. E, dose response of anxA5-F binding to apoptotic Jurkat cells (10^6 cells/ml). Mean fluorescence intensity (MFI) was determined with statistics of the R1 events. **D,** Ca\(^{2+}\)-dependence of anxA5-F binding (final concentration, 6 \(\mu\)g/ml) to apoptotic Jurkat cells (10^6 cells/ml). Mean fluorescence intensity was determined with statistics of R1 gated events. J-anx, J-anxA5.

ID. Thus, Asn\(^{160}\) is in close vicinity to residues Arg\(^{63}\), Asp\(^{64}\), Asp\(^{67}\), and Asp\(^{68}\) with the shortest distance of 2.3 Å between HD2 (Asn\(^{160}\)) and backbone C=O (Asp\(^{68}\)). Based on the above considerations, the following amino acids were selected for indicated substitutions because of high probability of disrupting stabilizing interactions between monomers in the trimer: R63A, K70A, K101A, E138A, D139G, and N160A.

**PS Binding**—Single and double substitutions of the putative two-dimensional amino acids did not change two-dimensional crystallization significantly (data not shown). Therefore, we decided to analyze in depth only 2D1–5, 2D2–6, and 2D1–6. Purified J-anxA5 and two-dimensional variants were >95% pure and showed no significant differences in molecular weight and overall α-helix content (data not shown). To assess biological activities, we measured calcium-dependent PS binding by ellipsometry using phospholipid bilayers consisting of 5/95 and 20/80 mol % PS/PC. J-anxA5 and all three two-dimensional variants exhibit similar binding isotherms for binding to 20/80 PS/PC at 3 mM Ca\(^{2+}\) (Fig. 2A). Maximal binding is comparable, and binding is fully reversible upon addition of EDTA. Ca\(^{2+}\) titration was performed to determine differences in affinity between the anxA5 variants for binding to PS/PC membranes. At low PS densities, 2D2–6 showed a Ca\(^{2+}\) dependence similar to wild-type and J-anx5 (Fig. 2B). 2D1–5 and 2D1–6 required slightly more Ca\(^{2+}\) for half-maximal binding. Maximal binding was achieved at 1 mM (20/80 PS/PC surface) and above 5 mM Ca\(^{2+}\) (5/95 PS/PC surface) for wild-type, J-anxA5, and the three variants (Fig. 2B).

To assess binding to PS embedded in biological membranes, we determined binding of fluorescein-labeled anxA5 variants to anti-Fas-stimulated Jurkat cells using flow cytometry and the anxA5 affinity assay protocol (22). Early apoptotic cells were analyzed, and binding of fluorescein-labeled anxA5 variants is expressed as mean fluorescence intensity. There were no significant differences in dose responses and Ca\(^{2+}\) sensitivities of apoptotic cell binding of the anxA5 variants (Fig. 2, C–F).

**Two-dimensional Crystallization**—The property of trimerization on a phospholipid surface was assessed by a FRET protocol that was previously used for measuring two-dimensional crystallization of annexins A1, A2, A5, and B12 (23). For this purpose, J-anxA5 and the two-dimensional variants were labeled with maleimide-fluorescein as donor and maleimide-Alexa Fluor 568 as acceptor. Labeling efficiencies were >95% and yielded 1:1 stoichiometric complexes as confirmed by MALDI TOF/TOF analyses (Fig. 3A). Addition of Ca\(^{2+}\) to a mixture of 45 \(\mu\)M LUVs and 22 nM J-anxA5-F and 11 nM J-anx5-A induced an additional peak in the excitation spectrum (measured at 600 nm) with a maximum around \(\lambda = 495\) nm (Fig. 3B). The ratio phospholipid/J-anxA5 in the mixture was chosen such that maximally 18% of the phospholipid surface could be covered with anxA5 indicating that the additional peak arises from clustering of phospholipid bound anxA5 as reported previously (23). Subtracting the spectrum in the absence of Ca\(^{2+}\) from the one in the presence of Ca\(^{2+}\) results in a FRET signal at 495 nm in arbitrary units (Fig. 3B). FRET analyses of the anxA5 variants show that 2D1–5 and 2D1–6 generate significantly less FRET on 20/80 PS/PC surfaces in the presence of 1 and 3 mM Ca\(^{2+}\) as compared with J-anxA5 (Fig. 3C). 2D2–6 generated a FRET signal compara-
ble with J-anxA5 indicating that Arg63 is the dominant amino acid of the six in respect of two-dimensional organization.

The next step was to determine the organization of anxA5 variants on the surface of apoptotic cells. Apoptotic Jurkat cells were incubated either with J-anxA5-A alone or with a mixture of J-anxA5-F and J-anxA5-A and analyzed by flow cytometry. The presence of J-anxA5-F increases fluorescence in the FL3 channel having a 600-nm long-pass filter indicating FRET of cell-bound anxA5. 2D1–5 and 2D1–6 but not 2D2–6 generate significantly less FL3 fluorescence as compared with J-anxA5 (Fig. 3D). The reduction in fluorescence signal cannot be explained by reduced amounts of apoptotic cell bound 2D1–6 and 2D1–5 (Fig. 2, E and F). Together, these results strongly suggest that 2D1–6 and 2D1–5 have lost their ability to form organized structures on a membrane surface.

This was confirmed for 2D1–6 with transmission electron microscopy. J-anxA5 and 2D1–6 formed well defined flat protein domains when bound to PS in the presence of 2 mM Ca$^{2+}$. No domains were observed in the presence of EDTA. Only J-anxA5 domains were ordered well enough to allow further crystallographic analysis. The order could be visually seen and assessed by Fourier transform of the digital images (Fig. 3E). The Fourier transform of these two-dimensional areas showed characteristic hexagonal pattern, previously reported for two-dimensional crystals of anxA5 obtained at similar conditions (25, 26). We have carried out only at low resolution and from negatively stained specimen to show that J-anxA5 has a typical trimeric organization (Fig. 3F), which was not observed for the protein domains formed with 2D1–6. The protein domains of 2D1–6 were not ordered, and no diffraction peaks were observed in Fourier transform of the recorded digital images. These findings corroborate the results of the FRET analyses using synthetic phospholipid membranes. We are the first to show that anxA5 organizes on a cellular membrane in a manner that depends on the same structure/function relationship as the trimer organization on a synthetic phospholipid surface.

Internalization—anxA5 binds PS-expressing cells and is subsequently internalized by these cells in a PS- and anxA5-specific mechanism (13). It is hypothesized that anxA5 crystallizes on the PS-expressing surface and that the energy released from crystallization molds the membrane along the convex shape of the molecule causing invagination of the membrane. The trimer, which was used to resolve structure/function relationship of trimerization, displays clearly an...
overall convex shape of the phospholipid binding side supporting the hypothesis (Fig. 1). Microscopic analysis of Jurkat cells that were incubated with anti-Fas and fluorescein-labeled anxA5 variants revealed that only J-anxA5 and 2D2–6 were internalized by PS expressing cells (Fig. 4). PS-expressing Jurkat cells bound 2D1–5, 2D1–6, anxA1, and lactadherin without significant internalization. The latter two proteins can bind to PS without the formation of a two-dimensional crystal and served as negative controls. The microscopic findings were underscored with quantitative measurements. A clear difference was measured between J-anxA5 and 2D2–6 on the one hand and 2D1–5, 2D1–6, anxA1, and lactadherin on the other hand (Fig. 4).

**Pretargeting**—The pretargeting potential of the anxA5 variants in vitro was determined as described under “Experimental Procedures.” Total fluorescence of R1 events (Fig. 2C) was calculated as the area under the anxA5-FL1 histogram (Fig. 2D). The total fluorescence of 2D1–5 and 2D1–6 increased relative to J-anxA5 with increasing time of internalization, whereas 2D2–6 showed no significant difference with J-anxA5 (Fig. 4C). The total fluorescence reflects pretargeting capacity of a variant. Hence, we have demonstrated that 2D1–5 and 2D1–6 are superior to J-anxA5 in pretargeting protocols, and we conclude that superiority results from attenuated internalization due to impaired two-dimensional crystallization of 2D1–5 and 2D1–6.

**DISCUSSION**

In this study, we have investigated the structure/function relationship of anxA5 internalization by cells executing apoptosis. The purpose was to unravel structural information for the generation of anxA5 variants that are not internalized and that are suitable for pretargeting strategies. Our hypothesis for internalization of anxA5 purports a nanomechanical mechanism involving trimerization of anxA5 on the cell surface (13, 27). anxA5 is a monomer in solution but when bound to a PS-expressing membrane, it forms a typical trimer by protein-protein interactions (25). anxA5 trimers assemble in large two-dimensional crystals with various symmetries on the PS-expressing membrane (23, 28). The convex shape of the phospholipid-interacting surface of the anxA5 molecule (16, 29) is maintained in membrane bound trimers (26). If such a trimer would form on a PS-expressing cellular membrane, the two-dimensional crystal would be able to mold the membrane along its bended shape and induce invagination. Such a mechanism is realistic as anxA5 binding to PS is accompanied by release of free energy (23, 30). Furthermore, our internalization experiments with anxA1 and lactadherin demonstrate that PS binding can occur without internalization. Hence, an additional step/signal is mandatory to trigger internalization. We hypothesized that this additional step involves trimerization and two-dimensional crystallization. anxA5 binds to the PS-expressing cell surface and forms a two-dimensional crystal of trimers that cause invagination of the PS-expressing membrane patch. Subsequently, an as-yet-unresolved mechanism is activated that triggers endocytic vesicle fission (27). Indications in support of the two-dimensional crystallization hypothesis came from the
Disruption of Annexin A5 Trimer Inhibits Internalization

anxA5 variant M23, which has D142Q and E228A substitutions that disrupt Ca\(^{2+}\)-binding of domains 2 and 3 (13). In this study, we searched for amino acids mediating the homotypic protein interactions in the trimer.

Previous reports have suggested that the basic cluster Arg\(^{25}\), Lys\(^{79}\), Arg\(^{83}\), and Arg\(^{151}\) is a structural motif that mediates anxA5 trimerization (24). This basic cluster is present in annexins (anxA4, A5, A6, and B12) that trimerize and are absent in nontrimerizing annexins (anxA1 and anxA2). However, replacement of the basic cluster in anxB12 had no effect on trimerization (23). Therefore, we searched for other amino acids that mediate trimerization. Our approach involved in silico structural bioinformatics and protein-protein docking to identify candidate amino acids. Protein-protein docking with the structural data from Sopkova et al. (14) and a set of selection criteria returned a top-ranking anxA5 trimer with an overall convex shape of the PS-interacting side. The top-ranking trimer revealed six amino acids as the prime candidates responsible for trimer stabilization. Among them is Arg\(^{63}\), which forms part of the basic cluster. We generated anxA5 variants in which one or two candidate amino acids were replaced by others that do not establish trimer-stabilizing salt bridges. None of the single and double replacements caused significant changes in the FRET signal. On the basis of these findings, we decided to make three variants: 2D1–6, which has all six candidate amino acids replaced; 2D2–6, which has five replacements and Arg\(^{63}\) retained; 2D1–5, which has five replacements and Asn\(^{160}\) retained. Arg\(^{63}\) and Asn\(^{160}\) were picked to inspect their role in two-dimensional crystallization because Arg\(^{63}\) is part of the basic cluster and Asn\(^{160}\) is a possible partner of Arg\(^{63}\) in stabilizing the trimer. The amino acid replacements of 2D1–6 are accompanied neither with changes in overall α-helix content nor with loss of affinity of PS binding or with loss of affinity of PS binding to a 20 mol % PS/80 mol % PC bilayer and to apoptotic cells at Ca\(^{2+}\) levels of 1 mM and higher. 2D1–6, however, generated less FRET signals on both a synthetic phospholipid bilayer and an apoptotic cell surface as compared with J-anxA5. Because this could not be explained by reduced binding of 2D1–6, we inferred a decreased propensity of 2D1–6 to form trimers and to organize in a two-dimensional crystal. 2D1–6 indeed lacks the ability to form trimers as was demonstrated with transmission electron microscopy analysis of membrane bound 2D1–6 indicating that the selected six amino acids do play a role in trimer formation. The FRET signal of 2D1–6 was not fully obliterated. Residual FRET activity probably results from trimerization-independent clustering of 2D1–6 due to Ca\(^{2+}\)-induced clustering of PS binding sites. The phenomenon of Ca\(^{2+}\)-induced PS clustering was an argument to doubt trimerization of anxA5 as a result of intermolecular interactions (30). Clustering of anxA5 on the surface as measured by FRET was hypothesized to arise from clustering of binding sites and crowding on the surface (30). This hypothesis, however, is not in agreement with our findings that 2D1–6 generates less FRET signal as compared with J-anxA5, whereas surface density of both is comparable on a synthetic as well as a cellular surface. In addition, the hypothesis does not explain the findings that mixtures of anxB12 and anxA5 generate FRET, whereas mixtures of anxA2 and anxA5 do not under the same conditions (23). Hence, we conclude that anxA5 trimerizes on a synthetic as well as a cellular membrane and that trimerization results from protein-protein interactions in which the identified six amino acids play a crucial role. Among them, Arg\(^{63}\) appears to be a governing amino acid with the annotation that Arg\(^{63}\) is alone or in combination with one amino acid of the other five appears to be insufficient to mediate trimerization.

Identification of the six amino acids involved in trimerization allowed to address the relationship between trimer formation and internalization. We have demonstrated that the ability of anxA5 to form trimers is causally linked to its internalization by cells executing apoptosis. The anxA5 variants with the lowest FRET generating potential, 2D1–6 and 2D1–5, are the least internalized anxA5 variants. Consequently, the targeting procedure revealed that 2D1–6 and 2D1–5 have the highest pretargeting potential, confirming that disrupting trimerization without affecting PS binding causes a decrease in internalization with a concomitant higher density of anxA5 at the cell surface.

In summary, this paper presents successful application of in silico bioinformatics and protein-protein docking to resolve the structure/function relationship of anxA5 internalization. We are the first to show that anxA5 forms trimers at the surface of an apoptotic cell and that trimerization is responsible for internalization supporting our nanomechanical explanation for the first step of membrane invagination. Importantly, the study produced an anxA5 variant that bears the potential to improve the anxA5 imaging concept for measuring apoptosis noninvasively in animals and patients using a pretargeting strategy.

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Disruption of Annexin A5 Trimer Inhibits Internalization