Cell Surface-expressed Phosphatidylserine and Annexin A5 Open a Novel Portal of Cell Entry*

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Expression of phosphatidylserine (PtdSer) at the cell surface is part of the membrane dynamics of apoptosis. Expressed phosphatidylserine functions as an “eat me” flag toward phagocytes. Here, we report that the expressed phosphatidylserine forms part of a hitherto undescribed pinocytic pathway. Annexin A5, a phosphatidylserine-binding protein, binds to and polymerizes through protein-protein interactions on membrane patches expressing phosphatidylserine. The two-dimensional protein network of annexin A5 at the surface prevents apoptotic body formation without interfering with the progression of apoptosis as demonstrated by activation of caspase-3, PtdSer exposure, and DNA fragmentation. The annexin A5 protein network bends the membrane patch nanomechanically into the cell and elicits budding, endocytic vesicle formation, and cytoskeleton-dependent trafficking of the endocytic vesicle. Annexin A1, which binds to PtdSer without forming a two-dimensional protein network, does not induce the formation of endocytic vesicles. This novel pinocytic pathway differs from macropinocytosis, which is preceded by membrane ruffling and actin polymerization. We clearly showed that actin polymerization is not involved in budding and endocytic vesicle formation but is required for intraacellular trafficking. The phosphatidylserine-annexin A5-mediated pinocytic pathway is not restricted to cells in apoptosis. We demonstrated that living tumor cells can take up substances through this novel portal of cell entry. This opens new avenues for targeted drug delivery and cell entry.

Programmed cell death eliminates cells from tissues in a silent and non-provocative manner. It is essential for development and tissue homeostasis of the multicellular organism. Apoptosis is the most abundant form of programmed cell death (1). Its well ordered biochemistry produces the hydrolysis of the constituent macromolecular structures of the cell (2) and the appearance and elicits budding, endocytic vesicle formation, and cytoskeleton-dependent trafficking of the endocytic vesicle. Annexin A1, which binds to PtdSer without forming a two-dimensional protein network, does not induce the formation of endocytic vesicles. This novel pinocytic pathway differs from macropinocytosis, which is preceded by membrane ruffling and actin polymerization. We clearly showed that actin polymerization is not involved in budding and endocytic vesicle formation but is required for intraacellular trafficking. The phosphatidylserine-annexin A5-mediated pinocytic pathway is not restricted to cells in apoptosis. We demonstrated that living tumor cells can take up substances through this novel portal of cell entry. This opens new avenues for targeted drug delivery and cell entry.

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1 The abbreviations used are: PtdSer, phosphatidylserine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; CSLM, confocal scanning laser microscopy; FRET, fluorescence resonance energy transfer; MFI, mean fluorescence intensity; TRITC, tetramethylrhodamine isothiocyanate; Z, benzoylcarboxylic acid; fmk, fluoromethyl ketone.
The M1234 cDNA was recloned to remove the 5'-11032-11033-base pairs responsible for the three extra N-terminal amino acids after expression (21). The annexin A5 mutant M23 was generated by subcloning the mutations D144N and E228A into the annexin A5 cDNA. Annexin A5, M23, and M1234 cDNAs were cloned into the bacterial expression vector pET-5a (Novagen). Proteins were expressed in Escherichia coli and purified to homogeneity as assessed by silver-stained SDS-PAGE and Western blotting. Annexin A5, M23, and M1234 were labeled with fluorescein-isothiocyanate and Alexa568-succinimidylester according to the manufacturer’s protocol (Molecular Probes). The labeled proteins with 1:1 stoichiometry were purified from the mixtures by MonoQ chromatography with A¨ cta Explorer (Amer- sham Biosciences). Annexin A1 was kindly provided by Dr. E. Solito (London, UK). Annexin A1 was labeled with Alexa568-succinimidylester according to the manufacturer’s protocol. The PtdSer-binding capacity of annexin A1-Alexa568 was verified by ellipsometry.

Analysis of Apoptotic Body Formation—Jurkat cells were washed and resuspended at 10^6 cells ml^-1 in medium 199 (Invitrogen) supplied with 0.5 mM CaCl_2. Apoptosis was induced with anti-Fas (200 ng ml^-1) in the absence or presence of annexin A5 and M1234. The course of apoptosis was determined by flow cytometry using the annexin A5-FITC staining protocol (Nexins). Annexin A5 inhibits the formation of apoptotic bodies. a, CSLM of early apoptotic cells (R1), late apoptotic cells (R2), and apoptotic bodies (R3) stained with annexin A5-Oregon Green and propidium iodide. b, apoptotic body formation of Jurkat cells after treatment with 200 ng ml^-1 anti-Fas antibody for 3 h in the absence and presence of 10 µg ml^-1 annexin A5 and M1234. c, time course of caspase-3 activation of anti-Fas-treated Jurkat cells in the absence (open circles) and presence (filled circles) of 10 µg ml^-1 annexin A5. d, time course of PtdSer exposure of anti-Fas-treated Jurkat cells in the absence (open circles) and presence (filled circles) of a mixture of 7.5 µg ml^-1 annexin A5 and 2.5 µg ml^-1 annexin A5-FITC to allow determination of PtdSer exposure by flow cytometry. e, DNA laddering after 3 h of anti-Fas treatment in the absence and presence of 10 µg ml^-1 annexin A5. f, time course of ROCK-1 cleavage of Jurkat cells treated with anti-Fas antibody in the presence and absence of 10 µg ml^-1 annexin A5.

Caspase-3 Activity, PtdSer Exposure, ROCK-1 Cleavage, and DNA Laddering—Caspase-3 activity was determined in 2.5 × 10^6 Jurkat cells by resuspending the cells in 100 µl of ice-cold buffer (25 mM Hepes/NaOH, 140 mM NaCl, 1 mM EDTA, pH 7.4) after stimulation with anti-Fas. Cells were disrupted by ultrasonication, and cellular debris was removed by centrifugation (140,000 × g/min, 4 °C). 5 µl of chromogenic substrate (10 mM DEVD-p-nitroanilide (BioSource)) was added to 70 µl of the cellular supernatant. The mixture was incubated at 37 °C for 30 min and optical density (OD) was measured at 405 nm.

PtdSer exposure of anti-Fas-stimulated Jurkat cells was measured by flow cytometry and subsequent off-line calculation of the percentage of events with reduced forward and sideward scatter. Off-line analysis of the flow cytometer files was performed with WinMDI 2.8 (free share software designed by Joseph Trotter).

FIG. 1. Annexin A5 inhibits the formation of apoptotic bodies. a, CSLM of early apoptotic cells (R1), late apoptotic cells (R2), and apoptotic bodies (R3) stained with annexin A5-Oregon Green and propidium iodide. b, apoptotic body formation of Jurkat cells after treatment with 200 ng ml^-1 anti-Fas antibody for 3 h in the absence and presence of 10 µg ml^-1 annexin A5 and M1234. c, time course of caspase-3 activation of anti-Fas-treated Jurkat cells in the absence (open circles) and presence (filled circles) of 10 µg ml^-1 annexin A5. d, time course of PtdSer exposure of anti-Fas-treated Jurkat cells in the absence (open circles) and presence (filled circles) of a mixture of 7.5 µg ml^-1 annexin A5 and 2.5 µg ml^-1 annexin A5-FITC to allow determination of PtdSer exposure by flow cytometry. e, DNA laddering after 3 h of anti-Fas treatment in the absence and presence of 10 µg ml^-1 annexin A5. f, time course of ROCK-1 cleavage of Jurkat cells treated with anti-Fas antibody in the presence and absence of 10 µg ml^-1 annexin A5.

FIG. 2. Annexin A5 is internalized via a PtdSer-dependent pathway during apoptosis. a, CSLM of anti-Fas-treated Jurkat cells in the presence of 20 µg ml^-1 annexin A5-Alexa568. b, immunoelectron microscopy of anti-Fas-treated Jurkat cells in the presence of 20 µg ml^-1 annexin A5. Immunolabeling was performed with anti-annexin A5 antibody and protein A conjugated with 10 nm gold.

Phosphatidylserine-dependent Internalization of Annexin A5
by flow cytometry using annexin A5-FITC. If apoptosis was executed in the presence of annexin A5, a mixture of 7.5 μg ml⁻¹ annexin A5 and 2.5 μg ml⁻¹ annexin A5-FITC was added to the cells prior to the addition of anti-Fas.

ROCK-1 activation was determined by lysing Jurkat cells in Tris buffer containing 50 mM Tris, 50 mM EDTA, 0.1% Triton X-100, and 0.5% protease inhibitor mixture (Sigma). Lysates of 10⁶ cells were loaded in each well and submitted to SDS-PAGE and Western blotting using anti-ROCK-1 antibody (Santa Cruz).

DNA fragmentation was analyzed by resuspending 5 x 10⁶ cells in 600 μl of ice-cold lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). The mixture was incubated for 10 min on ice. The lysates were cleared by centrifugation (140,000 x g/min, 4 °C). DNA was extracted from the supernatant with an equal volume of phenol:chloroform:isoamyl alcohol (16:16:1). The water phase was adjusted to contain 20 mM Tris-HCl, pH 8.0, with the addition of 2% 1,4-diazobicyclo-(2,2,2)-octane (Merck). Slides were examined with a confocal scanning laser microscope (Bio-Rad) equipped with a krypton/argon mixed gas laser (Ion Laser Technology). Images were recorded and analyzed with NIH Image, Imaged, and Adobe PhotoShop. Variations on this standard protocol were used to study the internalization of annexin A5-Alexa568 (100 nm), M234-Alexa568 (100 nm), annexin A1-Alexa568 (100 nm), M23-Alexa568 (100 nm), transferrin-Oregon Green 488 (100 nm), and Oregon Green-labeled Tris (20 μM) with the succinimidyl ester group inactivated.

**Plasmid and Transfection**—The plasmid caveolin-1-green fluorescent protein was a kind gift of Dr. D. Mundy (University of Texas, Southwestern Medical Center, Dallas, TX). The plasmid was transfected in HeLa cells with the transfection agent FuGENE 6 (Roche Applied Science).

**Immuno-electron Microscopy**—2 x 10⁶ Jurkat cells were stimulated with 200 ng ml⁻¹ anti-Fas in the presence of 20 μg ml⁻¹ annexin A5. The cells were pelleted by centrifugation (15,000 x g/min) and resuspended in Ca²⁺-buffer containing 2% paraformaldehyde, 0.2% glutaraldehyde. After 10 min the cells were transferred to Ca²⁺-buffer containing 1% paraformaldehyde. The fixed cells were washed with PBS, resuspended in 20 μl of PBS containing 10% gelatin, and solidified by cooling down. The gelatin drops were stored overnight at 4 °C in PBS containing 2.3 mM sucrose and then vitrified in liquid nitrogen for subsequent cryosectioning (± 90 nm) on a Leica EM PCS Cryo-Microtome. The ultrathin sections were stained with anti-annexin A5 antibody (Hyphen Biomed) and protein A-conjugated with 10 nm gold (a kind gift of Dr. G. Postuma, University Medical Center Utrecht, Department of Cell Biology), washed with PBS containing 0.1% w/v bovine serum albumin, postfixed in 1% glutaraldehyde, rinsed in ice-cold 1.8% methylcellulose, 0.4% uranylacetate, and stained in ice-cold 1.8% methylcellulose, 0.4% uranylacetate for 5–10 min. The grids were air-dried and examined in a Philips CM 10 microscope at 80 keV.

**Transmission Cryo-electron Microscopy**—Extruded phospholipid vesicles of dioleoylphosphatidylcholine, dioleoylphosphatidylserine, and cholesterol (molar ratio 63:3.3:33.3) were prepared by passing a mixture of 10 mM hand-shaken liposomes of the above composition at least three times through a 100-nm filter (Anotop 10, Whatman). The extruded vesicles were submitted to a mild hypotonic shock to obtain 100-nm diameter vesicles with a perfect spherical shape.

Annexin A5 or M23 was added to the vesicles at a weight ratio of 1:4 in the absence of Ca²⁺ ions. At this ratio maximally 50% of the phospholipid surface can be covered with annexin A5 upon binding (15). Annexin A5 binding to the vesicles was achieved by adding Ca²⁺ ions to the mixture such that the final Ca²⁺ ion concentration was 2.5 mM, and the solution became hypertonic relative to the lumen of the vesicles. The vesicles were prepared for cryoelectron microscopy analysis on a Philips CM12 microscope (Philips) using the Vitrobot™ (36).

**Fluorescence Resonance Energy Transfer (FRET)**—FRET was determined with FITC and Alexa568-labeled proteins as donors and acceptors, respectively. Mixtures of annexin A5-FITC:annexin A5-Alexa568

![FIG. 3. Effect of cytoskeletal agents on apoptotic body formation and on the internalization of annexin A5. a, CSLM of anti-Fas-treated Jurkat cells in the presence of annexin A5-Alexa568 and stained with phalloidin-TRITC according to the manufacturer's protocol. b, CSLM of anti-Fas-treated Jurkat cells in the presence of annexin A5-Alexa568 and stained with phalloidin-TRITC. c, CSLM of anti-Fas-treated Jurkat cells in the presence of annexin A5-Alexa568 and stained with phalloidin-TRITC. d–f, CSLM of Jurkat cells treated with 0.5 μM latrunculin B, 100 μM Y-27632, and 1 μM colchicine 1 h prior to anti-Fas stimulation in the presence of annexin A5-Alexa568 and counterstained with 250 ng ml⁻¹ annexin A5-Oregon Green.](image)
A5-Alexa568 or M23-FITC:M23-Alexa568 were prepared in ratios 100:100:0, 100:80:20, 100:60:40, 100:40:60, 100:20:80, and 100:0:100. Mixtures of annexin A5:annexin A5-Alexa568 or M23:M23-Alexa568 were prepared in ratios 200:0, 180:20, 160:40, 140:60, 120:80, and 100:100. These mixtures were incubated with M-PVA-12 beads (Chemagen), which were coated with a phospholipid layer consisting of 35% dioleoylphosphatidylcholine, 20% dioleoylphosphatidylethanolamine, 10% dioleoylphosphatidylserine, and 35% cholesterol in Ca²⁺/H₂O buffer (22). The fluorescence bound to the beads was measured on a Coulter Epics XL-MLC™ flow cytometer. The mean fluorescence intensity (MFI, arbitrary units) was calculated off-line for the Alexa568 signal in channel 3 (MFI-FL3). FRET was calculated as the MFI-FL3 of the mixtures containing FITC divided by MFI-FL3 of the mixtures without FITC.

**RESULTS**

**Annexin A5 Is Internalized via a PtdSer-dependent Pathway during Apoptosis**—Apoptosis is characterized by membrane dynamics, including the exposure of PtdSer, membrane blebbing, and apoptotic body formation. Surface expression of Ptd-
FIG. 5. Annexin A5 is not internalized by HeLa cells via a known endocytic pathway. a, CSLM of living HeLa cells that have internalized annexin A5-Alexa568. b, CSLM of HeLa cells that have internalized annexin A5-Alexa568 and the macropinocytosis probe Oregon Green. The two different vesicle populations show that annexin A5-Alexa568 is not internalized via macropinocytosis. c, CSLM of HeLa cells that have internalized annexin A5-Alexa568 and transferrin-Oregon Green 488. Vesicles positive for annexin A5-Alexa568 do not colocalize with vesicles positive for transferrin-Oregon Green 488, showing that different pathways are activated for each protein. d, HeLa cells were transfected with caveolin 1-enhanced green fluorescent protein and 24 h later incubated with annexin A5-Alexa568. Annexin A5-Alexa568 does not co-localize with caveolin 1-enhanced green fluorescent protein demonstrating that annexin A5-Alexa568 does not enter the cell via caveolin 1-mediated endocytosis.

Ser can be measured with fluorescently labeled annexin A5 and starts rapidly after the onset of apoptosis (4). Short incubations of anti-Fas-stimulated Jurkat cells with annexin A5-Oregon Green result in plasma membrane staining of early apoptotic cells, massively blebbing apoptotic cells, and apoptotic bodies (Fig. 1a). We observed that co-incubation of Jurkat cells with anti-Fas and annexin A5 changed the apoptotic membrane dynamics such that apoptotic body formation was inhibited. This inhibition depends on the annexin A5 dose and its ability to bind to PtdSer, because its non-binding mutant M1234 was without effect (Fig. 1b). Co-incubation of Jurkat cells with anti-Fas and annexin A5 did not affect other parts of the apoptotic program such as caspase-3 activation (Fig. 1c), PtdSer expression (Fig. 1d), DNA fragmentation (Fig. 1e), and ROCK-1 activation (Fig. 1f). The latter is involved in membrane blebbing, which precedes apoptotic body formation (25).

Inspection of the co-incubated Jurkat cells with CSLM and transmission electron microscopy revealed apoptotic cells that had internalized annexin A5 in translucent vesicles of various sizes (Fig. 2, a and b). We demonstrated that these vesicular structures are endocytic vesicles and not part of an open connexicullar system by (i) washing the cells with EDTA and (ii) incubating them shortly with annexin A5-Oregon Green prior to CSLM analysis. This procedure neither removed annexin A5-Alexa568 from the vesicular structure nor resulted in the localization of annexin A5-Alexa568 and annexin A5-Oregon Green. The latter bound to the plasma membrane only. We concluded that annexin A5 is taken up in endocytic vesicles via a PtdSer-dependent mechanism. On the basis of our results so far, we reasoned that annexin A5 first binds to PtdSer expressing membrane patches prone to become blebs and second induces their invagination and budding.

A Novel Pinocytic Pathway Internalizes Annexin A5 into Cells Executing Apoptosis—The size distribution of the annexin A5 endocytic vesicles suggests that the uptake is a macropinocytic process. This process is preceded by membrane ruffling and dependent on the polymerization of actin (26). We never observed membrane ruffling when Jurkat cells were co-incubated with anti-Fas and annexin A5. The staining of F-actin with phalloidin-TRITC revealed the presence of an actin coat around the annexin A5-containing vesicles (Fig. 3, a–c), suggesting the involvement of the actin cytoskeleton. Latrunculin B (Sigma), an inhibitor of actin polymerization, did not prevent the uptake of annexin A5 in endocytic vesicles (Fig. 3d). These vesicles remained localized underneath the plasma membrane indicating that actin polymerization is involved in intracellular trafficking but is not required for invagination and budding.

The actomyosin contractile apparatus is involved in bleb formation during apoptosis via ROCK-1 activation (25). Y-27632 (Tocris), an inhibitor of ROCK-1, did not inhibit the internalization of annexin A5-Alexa568 (Fig. 3e). Instead, ROCK-1 inhibition led to the formation of large annexin A5-containing vesicles suggesting that the actomyosin counteracts annexin A5-induced internalization thereby limiting vesicle size. Co-incubation of Jurkat cells having colchicines (Sigma) disrupted microtubules with anti-Fas, and annexin A5-Alexa568 resulted in annexin A5-containing vesicles that remained attached to the plasma membrane (Fig. 3f), indicating that intracellular trafficking depends on microtubules. Taken together our data showed that annexin A5 is internalized through a process that differs mechanistically from macropinocytosis.

Two-dimensional Crystalization of Annexin A5 Is the Driving Force for Internalization—On basis of our results hitherto we reasoned that annexin A5 reverses membrane fission from blebbing into invagination. Because the annexin A5-PtdSer complex has no transmembrane orientation, direct intracellular signaling by this complex could be ruled out to be the cause for this reversion. Therefore, we started to look for an explanation on a nanomechanical basis. The tertiary structure of annexin A5 shows that its phospholipid binding side has a convex shape (27). When bound to the membrane annexin A5 forms trimers of which each monomer retains the convex shape.

FIG. 6. Annexin A5 and not annexin A1 opens the novel portal of cell entry. A–C, CSLM analyses of Jurkat cells that were co-incubated with anti-Fas and annexin A5-Alexa568 (A), annexin A1-Alexa568 (B), or the combination of annexin A5-Oregon Green and annexin A1-Alexa568 (C). D–F, CSLM analyses of living HeLa cells that were incubated with annexin A5-Alexa568 (D), annexin A1-Alexa568 (E), or the combination of annexin A5-Oregon Green and annexin A1-Alexa568 (F).
at its phospholipid binding side (19, 28). We hypothesized that the trimers bend the membrane and provide the driving force for the reversion of membrane movement. To test this hypothesis, we generated the annexin A5 mutant M23 (21), which by prediction from the available structural data (19, 27) binds to PtdSer but lacks the ability to form trimers. Using a novel FRET assay, we showed that annexin A5 but not M23 develops FRET when bound to a phospholipid surface (Fig. 4a). Atomic force microscopic analysis of phospholipid-bound annexin A5 and M23 revealed the ordered array formation of the former and the disordered organization of the latter (Fig. 4b).

The convex shape of phospholipid-bound annexin A5 trimers was demonstrated to induce invagination of part of the membrane using large unilamellar vesicles. Structures with typically invaginated cups were obtained (see “Experimental Procedures”) (Fig. 4c). The thickness of both the cup membrane with a negative curvature (Fig. 4d, ne) and the outer membrane with a positive curvature (pe) were 4.96 ± 0.75 nm. Incubating these structures with annexin A5 at subsaturating conditions resulted in thicknesses of 9.57 ± 1.46 and 5.26 ± 0.76 nm for negative and positive curvature, respectively. This showed that the annexin A5 network bound to the membrane is bent with the convex shape of its phospholipid binding side. Incubation of the vesicles with M23 yielded thicknesses of 5.91 ± 0.96 and 5.66 ± 0.75 nm for negative and positive curvature, respectively. All together, it is evident that annexin A5 and not M23 organizes at the phospholipid surface in a bent network. Co-incubation of Jurkat cells with anti-Fas and M23 resulted in the generation of large surface blebs with M23 at the outside and F-actin at the inside (Fig. 4e). No intracellular vesicles containing M23 were observed. Summarizing these results, we demonstrated that annexin A5 opens a PtdSer-dependent novel portal of cell entry by bending the membrane inward into the cell through two-dimensional crystallization.

Living Cells Bear a PtdSer-dependent Portal of Entry—The execution of apoptosis results in the surface expression of PtdSer, which functions as a determinant for the entry of annexin A5. To determine whether apoptosis is an essential element for internalization, we incubated HeLa cells that were not subjected to apoptotic stimuli with annexin A5-Alexa568. After 3 h of incubation more than 50% of the HeLa cells had internalized annexin A5 in vesicles of varying sizes, similar to those observed in apoptotic Jurkat cells (Fig. 5a). The internalization was not coupled to the execution of the apoptotic program because (i) Z-VAD-fmk (IDN 1529, Idun Pharmaceuticals) did not inhibit annexin A5 internalization, and (ii) the cells with internalized annexin A5 were viable (data not shown). Apart from this discrepancy viable HeLa cells share the modus operandi with Jurkat cells executing apoptosis concerning the entry of annexin A5 into the cell. Annexin A5 internalization is dependent on the PtdSer binding and two-dimensional crystallization properties of annexin A5, because HeLa cells did not internalize fluorescently labeled M1234 or M23. Furthermore, Latrunculin B did not inhibit the internalization of annexin A5. Both Latrunculin B and colchicine prevented the intracellular trafficking of the annexin A5 containing vesicles. Further examination of the pathway of uptake demonstrates that it differs from known portals of cell entry (26). Annexin A5 uptake occurs neither through fluid phase internalization (Fig. 5b) nor through clathrin- and caveolin-mediated entry (29) (Fig. 5, c and d). Taken together our data showed that both cells executing apoptosis and living tumor cells have the same PtdSer-dependent portals of cell entry.

The Specificity of the Novel Portal of Cell Entry—To investigate further how mandatory the two-dimensional crystallization is for endocytosis, we used annexin A1. This annexin binds to PtdSer but does not form a two-dimensional protein network on the membrane surface (30). Co-incubation of Jurkat cells with anti-Fas and annexin A1-Alexa568 results only in plasma membrane staining of the apoptotic Jurkat cells but not in the formation of endocytic vesicles typically seen with annexin A5 (Fig. 6). Similar results were obtained with living HeLa cells. Interestingly, annexin A5 shuttles annexin A1 into the cell, demonstrating that this novel pathway leads to the internalization of proteins bound to or in the vicinity of surface-expressed PtdSer. The latter has been demonstrated for tissue factor, which is internalized in PtdSer-expressing cells by annexin A5.2

DISCUSSION

Plasma membrane dynamics during apoptosis include the expression of PtdSer at the cell surface and membrane blebbing. Both phenomena occur during apoptosis downstream of caspase-3 activation (4) but are regulated independently. PtdSer expression occurs under conditions wherein membrane blebbing is inhibited (25). With fluorescently labeled annexin A5 it was shown that membrane blebs have surface-expressed PtdSer, indicating that the PtdSer-expressing membrane patches are flexible and bendable. Recently, it was reported that annexin A5 inhibits apoptotic body formation by applying a physical constraint on the plasma membrane and inhibiting the apoptotic program (20). We confirmed that annexin A5 inhibits apoptotic body formation, but it does so in our system without interfering with the key steps of the apoptotic program such as caspase-3 activation, PtdSer expression, ROCK-1 cleavage, and DNA fragmentation. Because the apoptotic body formation is downstream of membrane blebbing (25), the inhibitory mechanism may operate on membrane blebs. Our investigations into this phenomenon revealed the existence of an endocytic pathway that is activated by annexin A5 and surface-
expressed PtdSer. We demonstrated that annexin A5 reverses the movement of the PtdSer-expressing membrane patch from blebbing into invagination. This results in vesicle formation and the intracellular trafficking of the endocytic vesicle (Fig. 7). From the experiments with M23 and annexin A1 we concluded that the process of invagination is driven nanomechanically by the formation of an annexin A5 two-dimensional crystal that bends the membrane, which is likely due to the bent shape of the trimeric blocks (19). The mechanism of membrane bending differs from that operating in clathrin-coated pits. Here, the insertion of proteins into the lipid layer pushes lipid head groups of one leaflet apart thereby inducing curvature of the bilayer (31). If annexin A5 would operate comparably it is expected that annexin A5 facilitates blebbing instead of inducing invagination. Therefore, it is concluded that annexin A5 binds extrinsically and molds the membrane patch according to the convex shape of its phospholipid binding side. The energy required for this action is likely released by the two-dimensional crystallization process.

The size distribution of the annexin A5 containing endocytic vesicles suggests macropinocytosis rather than clathrin- or caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (26). Despite a morphological resemblance the two endocytic pathways are mechanistically distinct. An-

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Annexin-related Pathologies

Annexin-A5-induced endocytosis is neither actin-driven nor preceded by membrane ruffling. Hence, we concluded that the PtdSer-expressing membrane patch forms a novel portal of cell entry, which is attractive mechanism for targeted delivery and cell entry of drugs designed to kill (tumor) cells or to rescue ischemic/reperfu-

Annexin A5-induced endocytosis is neither actin-driven nor preceded by membrane ruffling. Hence, we concluded that the PtdSer-expressing membrane patch forms a novel portal of cell entry, which is likely attributed to the convex shape of its phospholipid binding side. The energy required for this action is likely released by the two-dimensional crystallization process. The size distribution of the annexin A5 containing endocytic vesicles suggests macropinocytosis rather than clathrin- or caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (26). Despite a morphological resemblance the two endocytic pathways are mechanistically distinct. Annexin A5-induced endocytosis is neither actin-driven nor preceded by membrane ruffling. Hence, we concluded that the PtdSer-expressing membrane patch forms a novel portal of cell entry, which is an attractive mechanism for targeted delivery and cell entry of drugs designed to kill (tumor) cells or to rescue ischemic/reperfusion cardiomyocytes.

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Annexin-A5-induced endocytosis is neither actin-driven nor preceded by membrane ruffling. Hence, we concluded that the PtdSer-expressing membrane patch forms a novel portal of cell entry, which is an attractive mechanism for targeted delivery and cell entry of drugs designed to kill (tumor) cells or to rescue ischemic/reperfusion cardiomyocytes.

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