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Cholesterol Enhances Phospholipid Binding and Aggregation of Annexins by Their Core Domain

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Annexins are Ca^{2+}-dependent phospholipid-binding proteins composed of two domains: A conserved core that is responsible for Ca^{2+}- and phospholipid-binding, and a variable N-terminal tail. A Ca^{2+}-independent annexin 2-membrane association has been shown to be modulated by the presence of cholesterol in the membranes. Herein, the roles of the core and the N-terminal tail on the cholesterol-enhancement of annexin 2 membrane binding and aggregation were studied. The results show that (i) the cholesterol-mediated increase in membrane binding and in the Ca^{2+} sensitivity for membrane aggregation were not modified by a N-terminal peptide (residues 15–26), and were conserved in mutants of the N-terminal end (S11 and S25 substitutions); (ii) cholesterol induced an increase in the Ca^{2+}-dependent membrane binding and aggregation of the N-terminally truncated protein (Δ 1–29); and (iii) annexins 5 and 6, two proteins with unrelated N-terminal tails and homologous core domains showed a cholesterol-mediated enhancement of the Ca^{2+}-dependent binding to membranes. These data indicate that the core domain is responsible for the cholesterol-mediated effects. A model for the cholesterol effect in membrane organisation, annexin binding and aggregation is discussed.

Key Words: **annexin; cholesterol; core domain; phospholipid microdomains.**

Annexins, Ca^{2+}-phospholipid-binding proteins that are expressed in different tissues, are able to associate to different intracellular membranes (1–10). However, the determinants of the specificity of intracellular localisation of annexins to date are unknown. *In vivo*, annexin 2 is associated with endosomes (11, 12), chromaffin granules (6) and the plasma membrane (13, 14), and has been implicated in several steps of membrane traffic, such as exocytosis (14–19), endocytosis (11, 12) and membrane fusion (12, 20, 21).

Annexin 2 is found as a monomer (p36), or as a heterotetramer (p90) when associated with p11, a member of the S100 family of proteins. Both annexin 2 forms bind to membranes and induce their aggregation in a Ca^{2+}-dependent manner, this association is mediated by the C-terminal core (residues 32–338) (22–24) and requires the presence of anionic phospholipids such as phosphatidylserine (PS) or phosphatidylinositol. The Ca^{2+} sensitivity of these reactions is regulated, in part, by the N-terminal domain (residues 1–31), which contains the binding site for the p11 light chain (residues 1–14) (25, 26). Two N-terminally deleted forms of monomeric annexin 2 (Δ28 and Δ44) have different Ca^{2+} requirements for chromaffin granule aggregation (24). The phosphorylation of the N-terminal residues Tyr23 and Ser25 decreases the aggregation capacity of the protein (27, 28), and mutant proteins with Ser-to-Glu replacements on positions 11 and 25 show a decrease in the extent of aggregation of chromaffin granules and an increase in the Ca^{2+} requirement for liposome aggregation (29).

Annexin 2, especially in its tetrameric form (p90), has been shown to bind to biological membranes in the absence of Ca^{2+} (1, 6, 30, 31). Moreover, this Ca^{2+}-independent bound annexin 2 is released from these membranes by treatment with cholesterol sequestering agents (31, 32). We previously showed that p36 and p90 are able to bind to phosphatidylcholine/phosphatidylserine (PC/PS) liposomes in a Ca^{2+}-free buffer, and that the percentage of bound protein, and the Ca^{2+} sensitivity for membrane aggregation are increased when cholesterol is present in the liposomes (31). Annexin 2 does not bind directly to cholesterol and these cholesterol-mediated effects are strictly dependent on the presence of PS in the liposomes (31). It has been suggested that cholesterol is involved in the formation of lipidic membrane domains (33–36). The

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specific presence of proteins in these microdomains has been documented for several proteins (37–40) including annexins (4, 7, 41, 42). Because the cholesterol-mediated enhancement of Ca^{2+}-independent membrane binding is strictly dependent on the presence of PS, it has been suggested that the cholesterol effect was due to an increase in the efficiency of PS accessibility for binding, possibly by formation of PS-rich microdomains (31). In this context, it can be suggested that the annexin domain involved in the PS-binding (i.e., the core), is the domain responsible for the cholesterol-mediated increase in membrane binding. In this case, the Ca^{2+}-dependent properties of annexin 2 and other annexins should be increased by the presence of cholesterol in the membranes. Here we have studied the role of the annexin 2 N-terminal and core domains in the cholesterol-mediated increase of membrane binding and aggregation.

MATERIALS AND METHODS

Materials. Bovine brain type III-B L-α-phosphatidylcholine (PC), L-α-phosphatidyl-L-serine (PS), and cholesterol were obtained from Sigma. Anti-annexin 2 antibody was from Transduction laboratories. The synthetic 15–26 peptide was a kind gift of Dr. M-F. Bader (Strasbourg, France). Purified annexin 5 was a kind gift of Dr. A. Hofmann (MD). Purified pig brain annexin 6 was a kind gift of Dr. L-A. Pradel (Paris, France). All other chemicals were of analytical grade purity.

Preparation of proteins. Subcloning of the human *annexin 2*, the N-terminal mutants (S11A, S11E, S25A, S25E, S11A-S25A, S11E-S25A, S11A-S25E, S11E-S25E), their expression in *Saccharomyces cerevisiae,* their purification and their characterisation have been described (29). Recombinant human p11, was expressed in *Escherichia coli* and purified as already described (29). The heterotetramer (p90) was obtained by mixing equimolar quantities of the heavy (p36) and the light (p11) chains. p33 was a degradation product of p36. p33 was produced by long term storage of p36 at 4°C. The core and the N-terminal peptide(s) were separated from samples devoid of p36. Three milligrams of protein were incubated for 30 min at 4°C in 1 ml of TES buffer (Tris 50 mM, pH 7.4, EDTA 1 mM, sorbitol 0.6 M, NaCl 125 mM, $CaCl_2$ 1 mM) containing 3 mg of NaOH-extracted chromaffin granules membranes prepared by the method of Steiner *et al.* (43). The p33 containing membranes were pelleted in an eppendorf tube (13,000 rpm for 20 min at 4°C). The pellet was washed in the same solution and recentrifuged, then p33 was solubilised by incubation in 1 ml of buffer Tris 25 mM, pH 7.5, $MgCl_2$ 2 mM, EDTA 0.1 mM, EGTA 10 mM, NaCl 150 mM, DTT 0.5 mM for 30 min at 4°C in agitation. After centrifugation, the supernatant was recovered, the buffer was changed to PBS DTT 1 mM and the protein was concentrated (≈2 mg ml^{-1}) by serial centrifugation cycles on Centricon 10 (Amicon). The protein was >98% pure as judged by SDS–PAGE. The sequence analysis indicated that residues 1–29 were deleted (29).

Liposomes. We previously showed that the most efficient lipid composition of liposomes to observe the cholesterol enhancement of binding and aggregation is PC/PS/cholesterol (50/25/25 by weight) (31). Cholesterol-free liposomes contained the same amount of PS, and PC replaced the cholesterol (PC/PS 75/25). Large unilamellar vesicles were obtained by extrusion. Briefly, lipids in chloroform were mixed, subjected to solvent evaporation and resuspended by vortexing vigorously in buffer A (40 mM Hepes pH 7, 30 mM KCl, 1 mM EGTA) to a total lipid concentration of 2.5 mg/ml. The multilamellar liposomes were then extruded by filtration through 0.4, 0.2, and 0.1 μm pore size polycarbonate filters (Sciema Technical Services Ltd., Richmond, BC, Canada), the last filtration was repeated three times.

Annexin binding to liposomes. Four micrograms of p33, annexin 5 or annexin 6 were added to 25 μg of liposomes in 50 μl of buffer A containing the indicated free Ca^{2+} concentrations, as calculated with the Calcv.22 program (44). After incubation for 20 min at room temperature, the mixtures were centrifuged in a Beckman Airfuge at 170,000*g* for 30 min. Pellets and supernatants were analysed for annexin 2 content by scanning Coomassie-stained SDS–PAGE gels. Results were corrected by substracting the faint insoluble annexin background when observed in liposome-free controls. For the experiments in the presence of the 15–26 peptide, and in order to obtain high peptide/protein ratios, p36 and p90 were used at a final concentration of 0.25 μM. Half of the peptide was incubated with the protein and the rest of the peptide with the liposome suspension for 10 min on ice. Mixing both solutions started the experiments. Analysis of pellets and supernatants was performed by western blot using the monoclonal anti-human annexin 2 and an HRP-coupled anti-mouse IgG (Sigma) as a secondary antibody. Western blots were revealed by chemiluminescence (Renaissance, Dupont-NEN). Band quantification of gels and films were performed by analysis of images with the NIH image 1.62 software. The Student's *t* test was performed with the SigmaPlot software.

Aggregation of liposomes. Liposome aggregation was performed in 500 μl of buffer A at different free Ca^{2+} concentrations as calculated with the Calcv.22 program (44). Liposomes were added at a lipid concentration of 20 μg/ml. To initiate the aggregation reaction, annexin 2 was added at a final concentration of 20 μg/ml. Aggregation was followed at 28°C, for seven min by monitoring the absorbance increase at 340 nm. For aggregations in the presence of the 15–26 peptide, prior to the experiment, half of the peptide was incubated with the protein for 10 min on ice, and the second half with the liposome solution for 1 min at room temperature. Aggregation curves were obtained by fitting the experimental data to the equation $y = a(kx)^n/1 + (kx)^n$ in which a is the maximal aggregation signal in y, k a constant, and n the Hill number (45).

RESULTS

Annexin 2-membrane binding and aggregation in the presence of the 15–26 peptide. To study the involvement of residues 15–24 of the N-terminal tail of annexin 2 in the cholesterol-mediated enhancement of annexin 2 binding and aggregation of membranes, we performed experiments in the presence of an active synthetic peptide (15–26) that has been shown to block annexin 2 translocation and catecholamine release in chromaffin cells (17). In the absence of Ca^{2+}, p90 and p36 bound to PC/PS liposomes at ≈20 and ≈10% (not shown), and to PC/PS/cholesterol liposomes at ≈50 and ≈40%, respectively (Fig. 1 and (31)). At a peptide/protein molar ratio of 640 (160 μM/0.25 μM), the 15–26 peptide showed no effect on the cholesterol-mediated increase in p90 and p36 liposome binding (Fig. 1A).

The extent of p90-mediated liposome aggregation in the absence of Ca^{2+} and the Ca^{2+} sensitivity for aggregation of p36 are increased by the presence of cholesterol in the liposomes (31). Therefore, the effect of the 15–26 peptide (100 molar excess) on liposome aggregation was investigated. The cholesterol-mediated increase in the extent of both; p90-mediated liposome aggregation in the absence of Ca^{2+}, and p36-mediated aggregation at 20 μM

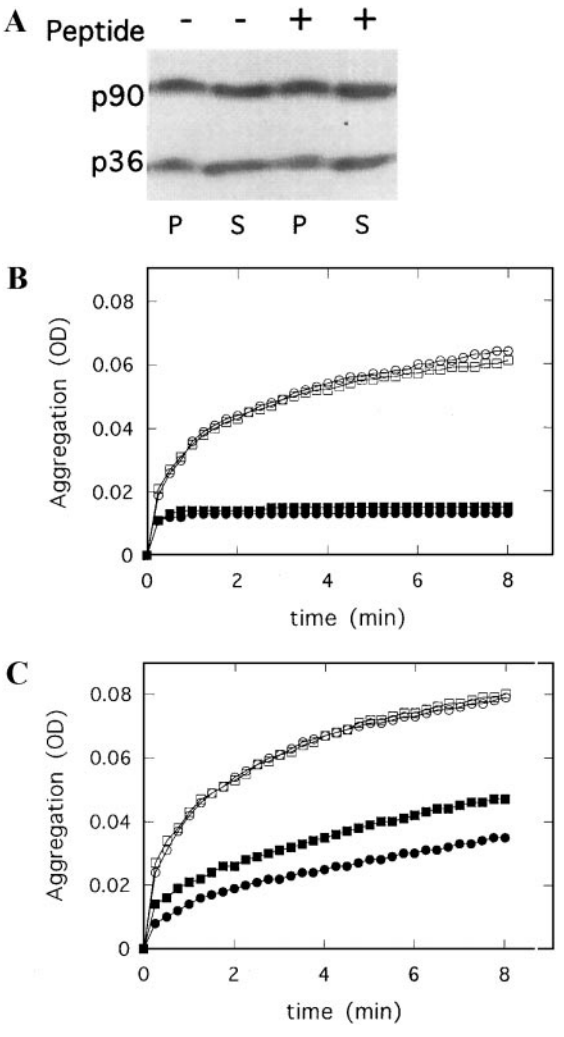

FIG. 1. p90 and p36 binding and aggregation of liposomes in the presence of the 15–26 peptide. (A) p90 and p36 (0.25 μM of p36) were incubated with PC/PS/cholesterol liposomes either in the absence or the presence of 640 molar excess of the peptide in a Ca^{2+} free buffer. After centrifugation, pellets (P) and supernatants (S) were analysed by Western blot. Figure shown is representative of three independent experiments. (B) Time course of p90-mediated aggregation in the absence of Ca^{2+} of PC/PS (filled symbols) and PC/PS/cholesterol liposomes (open symbols) in the absence (circles) or in the presence (squares) of the peptide (50 μM). (C) Time course of p36-mediated aggregation in the presence of 20 μM Ca^{2+} of PC/PS and PC/PS/cholesterol liposomes either in the absence or the presence of the peptide. Symbols and peptide concentration as in B. Protein concentration was 0.5 μM of p36. The peptide did not block the cholesterol effects on annexin 2-membrane binding and aggregation.

free Ca^{2+} were not significantly modified in the presence of the peptide (Figs. 1B and 1C).

Cholesterol-dependent increase in membrane aggregation of N-terminal mutants of annexin 2. In a second series of experiments, annexin 2 proteins harbouring point mutations on serines 11 and 25 were studied. Mutants containing Glu residues in positions 11 and 25 affect the conformation of the N-terminal tail as measured by chymotryptic sensitivity and show a smaller Ca^{2+} sensitivity for aggregation (29). The previously described effect of cholesterol on the Ca^{2+} dependency of liposome aggregation by p36 (31) was studied on the replacement mutants S11A, S11E, S25A, S25E, S11A-S25A, S11E-S25A, S11A-S25E, and S11E-S25E. Figure 2 shows the curves for wild type and four mutants. The wild-type protein and the Ala mutations (S11A, S25A, and S11A-S25A) showed a similar Ca^{2+} sensitivity for PC/PS liposome aggregation (half-maximal aggregation at $\approx$80 μM Ca^{2+}). Mutants with S25E and S11E replacements and the doubly substituted S11E-S25E mutant required more Ca^{2+} to aggregate the liposomes (half-maximal aggregation at $\approx$250 μM, $\approx$630 μM and $\approx$1.3 mM, respectively). This difference in the mutants' Ca^{2+} sensitivity (Fig. 2 and Table 1) is explained by a change in the conformation of the N-terminal tail that affect the protein's capacity to dimerise (29). The presence of cholesterol in the liposomes reduced significantly the Ca^{2+} concentration required for liposomes aggregation of the wild-type and all of the eight mutant proteins (some not shown), regardless of the number of changed residues (one or two), their position (11 or 25) or the type of mutation

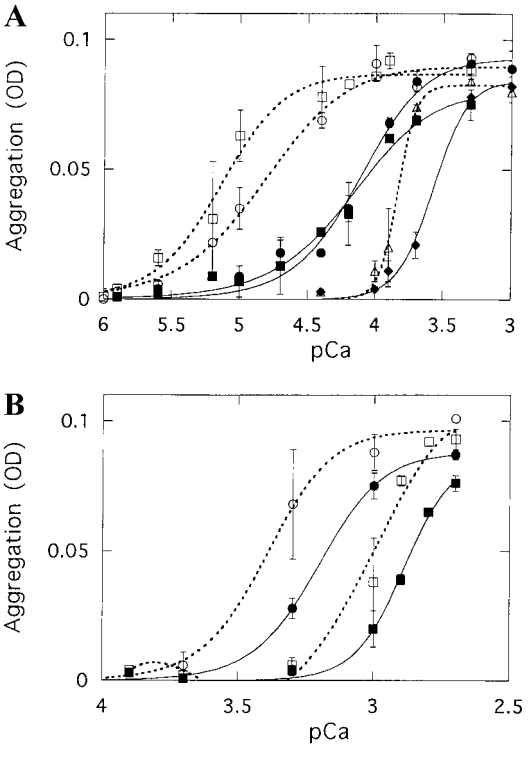

FIG. 2. Ca^{2+}-dependency of liposome aggregation of different annexin 2 mutants. PC/PS liposomes (filled symbols and continuous lines) and PC/PS/cholesterol liposomes (open symbols and discontinuous lines) were aggregated by p36 proteins. (A) Wild-type (●, ○), S25A (■, □), and S25E (◆, △). (B) S11E (●, ○) and S11E-S25E (■, □). Points are mean ± SEM of 2 to 7 experiments. In all cases, compared to PC/PS liposomes, the aggregation of PC/PS/cholesterol liposomes required lower Ca^{2+} concentration.

TABLE 1

Ca^{2+} Concentration for Half-Maximal Aggregation of PC/PS and PC/PS/Cholesterol Liposomes for p36 Wild-Type and Mutants and p33

Protein	Liposomes: PC/PS $\mu M\ Ca^{2+}$ (pCa)	Liposomes: PC/PS/cholesterol $\mu M\ Ca^{2+}$ (pCa)	$[Ca^{2+}]$ shift $\Delta\mu M$[a]
wt	80 (4.10)	13 (4.87)	67
S11A	65 (4.19)	8 (5.11)	57
S25A	70 (4.16)	8 (5.10)	62
S11A-S25A	88 (4.06)	45 (4.33)	43
S11E	620 (3.21)	350 (3.45)	270
S11E-S25A	650 (3.19)	500 (3.30)	150
S25E	280 (3.19)	210 (3.68)	70
S11A-S25E	250 (3.60)	150 (3.82)	100
S11E-S25E	1260 (2.90)	1050 (2.98)	210
p33	780 (3.11)	500 (3.30)	280

[a] Difference between the calcium concentrations required for half-maximal aggregation of PC/PS and PC/PS/cholesterol liposomes.

(S-to-A or S-to-E). Table 1 summarises the Ca^{2+} concentrations required for half-maximal aggregation of mutants for both, PC/PS and PC/PS/cholesterol liposomes. The Ca^{2+} concentration shift (between 50 to 250 μM) was largest for the glutamic acid containing mutants. Therefore, proteins harbouring point mutations that change the conformation of the N-terminal tail but conserve an intact core sustained the cholesterol-dependent enhancement of the Ca^{2+} sensitivity for liposomes aggregation.

Cholesterol-dependent enhancement of binding and aggregation of N-terminally deleted annexin 2. The precedent results suggested that the N-terminal tail was not directly involved in the cholesterol enhancement of membrane binding and aggregation either in the presence or the absence of Ca^{2+}. To test whether or not the core of the protein was the domain involved in the cholesterol effect, the properties of p33, a protein deleted of the first 1–29 residues were investigated. p33 membrane binding in the absence of Ca^{2+} was very small (1–2% of the protein, not shown and (30, 31)). However, because in the presence of Ca^{2+}, cholesterol enhances annexin 2 binding and membrane aggregation (31), the membrane binding of p33 at different Ca^{2+} concentrations was determined. When cholesterol was present in the liposomes p33 binding was increased from 63 ± 4 to 78 ± 4 at 200 $\mu M\ Ca^{2+}$ and from 73 ± 4 to 89 ± 3 at 500 $\mu M\ Ca^{2+}$ (Figs. 3A and 3B). However, at higher Ca^{2+} concentration (1 mM), this difference was abolished, a phenomenon probably due to the saturation of the PS-annexin 2 sites.

The Ca^{2+}-dependency of the liposome aggregation capacity of p33 was also studied. Similarly to p90 and the nondeleted p36, (wild-type and mutants), cholesterol increased the Ca^{2+} sensitivity for p33-mediated liposome aggregation (Fig. 3C). The effect on aggregation started around 400 $\mu M\ Ca^{2+}$ (pCa 3.4) a value that correlates with the Ca^{2+} concentration at which the cholesterol enhancement of binding was observed. The Ca^{2+} concentration for half-maximal aggregation was reduced from 780 μM for PC/PS to 500 μM for PC/PS/cholesterol liposomes (280 μM shift, Table 1). These experiments clearly demonstrate that the N-terminal tail is not necessary for the cholesterol-mediated enhancement of annexin 2 binding and membrane aggregation.

Cholesterol-dependent enhancement of binding of annexins 5 and 6 to liposomes. If the core of the protein mediated the cholesterol effect, it should be found for

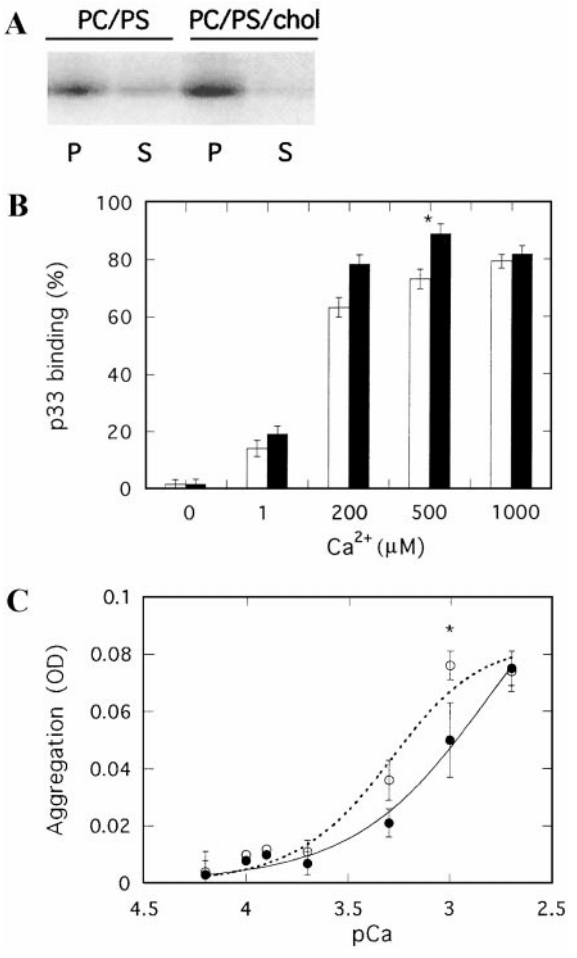

FIG. 3. Ca^{2+}-dependency of liposome binding and aggregation of the N-terminally deleted annexin 2 (p33). p33 was incubated with PC/PS or PC/PS/cholesterol liposomes as described in the methods section. (A) Representative Coomassie blue-stained gel of pellets (P) and supernatants (S) from PC/PS and PC/PS/cholesterol liposomes in the presence of 200 $\mu M\ Ca^{2+}$. (B) p33 binding to PC/PS liposomes (open bars) or PC/PS/cholesterol liposomes (filled bars) at different Ca^{2+} concentrations. Mean ± SEM from 5 to 7 experiments. $*P = 0.02$ ($n = 7$) by Student's t test. (C) p33-mediated aggregation of PC/PS (●) and PC/PS/cholesterol (○) liposomes. Points are mean ± SEM from 3 to 7 experiments. $*P = 0.04$ ($n = 5$) by Student's t test.

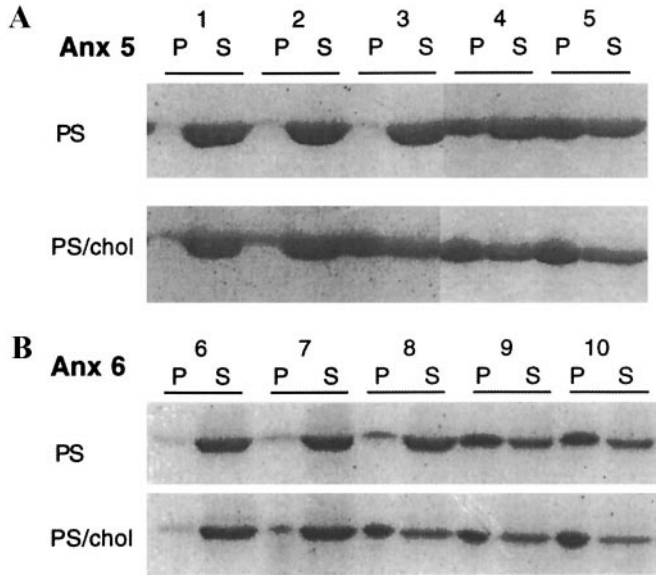

FIG. 4. Effect of cholesterol on the binding of annexins 5 and 6 to liposomes. Liposome binding of annexins 5 and 6 was performed as described in the methods section. (A) SDS–PAGE of pellets (P) and supernatants (S) of liposomes incubated with annexin 5 at different Ca^{2+} concentrations; 1 μM (lane 1), 40 μM (lane 2), 100 μM (lane 3), 200 μM (lane 4), 500 μM (lane 5). Figure is representative of three independent experiments. (B) Pellets (P) and supernatants (S) of liposomes incubated with annexin 6 at different Ca^{2+} concentrations; no Ca^{2+} (lane 6), 1 μM (lane 7), 2.5 μM (lane 8), 10 μM (lane 9), 40 μM (lane 10). Figure representative of four independent experiments.

other annexins. To test this hypothesis, the binding capacity of annexins 5 and 6 was tested at different Ca^{2+} concentrations. In the absence of Ca^{2+}, both annexins were unable to associate to liposomes. At low Ca^{2+} both proteins were not significantly bound and at high Ca^{2+} (>500 μM), they bound maximally (50–60% for annexin 5 and 80–90% for annexin 6). At intermediate Ca^{2+} concentrations, the amount of bound annexin was significantly higher for the PC/PS/cholesterol liposomes compared to the cholesterol-free liposomes. At 100 μM Ca^{2+}, the binding of annexin 5 was increased in the cholesterol containing liposomes from 16 ± 7% to 40 ± 9% and at 2.5 μM Ca^{2+} the binding increased from 20 ± 2% to 37 ± 8% for annexin 6 (Fig. 4). These data demonstrate that cholesterol in the liposome membrane is able to increase the Ca^{2+}-dependent binding of annexins with unrelated N-terminal tails.

DISCUSSION

A Ca^{2+}-independent binding of annexin 2 to chromaffin granules and early endosome membranes has been demonstrated (6, 30, 31). This binding is in part regulated by the presence of cholesterol (31, 32). However, annexin 2 does not bind to cholesterol and the cholesterol-mediated enhancement of annexin 2 binding to liposomes and their aggregation are strictly dependent on the presence of acidic phospholipids (PS) (31).

To study the roles of the N-terminal and core domains of annexin 2 on the cholesterol-mediated increase in membrane binding and aggregation, three sets of experiments were designed. PC/PS and PC/PS/cholesterol liposome binding and aggregation of p36 and p90 were performed in the presence of a peptide containing the 15–26 N-terminal residues. This peptide has been shown to block annexin 2 translocation from the cytosol to the cortical membranes and to reduce exocytosis of chromaffin cells (17). At a high peptide/protein molar ratios (100 to 640), the peptide showed no effect on the cholesterol-mediated increase in p36 and p90 membrane binding and aggregation. In the second series of experiments, the cholesterol enhancement of liposome aggregation for some mutants in the N-terminal domain was studied. We previously showed that replacements of serines 11 and 25 by glutamic acid residues modify the membrane aggregation properties of p36 and p90 (29). Although point mutations in two serine residues at positions 11 and 25 alter the Ca^{2+} dependence of the liposome aggregation, the cholesterol-induced shift of the Ca^{2+} sensitivity was observed in all cases.

These two sets of data, added to the facts that (i) acidic phospholipids are necessary for the cholesterol-mediated increase of membrane binding and aggregation in the absence of Ca^{2+} (31) and (ii) that the binding in the presence of Ca^{2+} and the Ca^{2+} sensitivity for aggregation, two properties known to reside in the core of the protein, are also increased by cholesterol, suggest that the core is the determinant domain in the cholesterol effect. It is clear from the data that addition of cholesterol to the liposomes cause a decrease in the amount of Ca^{2+} required for membrane binding and aggregation, and that the N-terminal region of annexin 2 appears to play a rather insignificant role in this process. The experiments showing that cholesterol enhanced the Ca^{2+}-dependent binding and aggregation of the 1–29 deleted protein (p33) are consistent with the essential role of the core in the cholesterol effect.

The possibility that the N-terminal tail interacts with cholesterol in the membranes seems unlikely and some points are in disagreement with this hypothesis: (i) Johnsson *et al.* (46) and Thiel *et al.* (47) showed that the regions 25–27 (of the tail) and 63–67 (of the core) form a discontinuous epitope for the H28 monoclonal antibody. Residues 63–67 are localised in the third helix of repeat I, that is localised in the concave face of the protein; (ii) Lambert *et al.* (48) have shown that in p90, p11 is in contact with the concave faces of two opposing annexin 2 molecules. Thus, with residues 1–14 interacting with p11 and residues 25–27 close to the third helix of repeat I, it seems very difficult that residues 15–24 could make a loop out from the concave

surface, near the third helix of domain I to contact the cholesterol in the membrane and return to join p11 in the concave interface of two annexin 2 molecules.

Jost *et al.* (30) have shown that the Ca^{2+}-independent endosome association of monomeric annexin 2 is not affected by introducing a deletion of residues 1–14. On the contrary, a deletion of the sequence spanning residues 1–24 abolishes this Ca^{2+}-independent association. They concluded that residues 15–24 of the N-terminal domain are necessary for the Ca^{2+}-independent binding to endosomes. The facts that the 1–24 deleted protein has lost the Ca^{2+}-independent binding to endosomes (30) and that a chimeric protein composed of the Anx2 N-terminal tail and the Anx1 core also showed a Ca^{2+}-independent binding to early endosomes (49) revealed an important role of the N-terminal domain of Anx2 in the Ca^{2+}-independent binding to endosomes. These facts could be explained by two hypotheses. First by the known fact that the N-terminal tail have a modulatory influence in the membrane binding capacity of the core and on the Ca^{2+}-sensitivity for membrane aggregation of annexin 2 (24, 31, 50). Second, by the existence of a proteinaceous receptor which could bind the N-terminal tail of annexin 2 (30). However, concerning chromaffin granules and liposomes, saturation curves indicate that the binding sites for protein-free liposomes are as numerous as for chromaffin granule membranes. Finally, a Ca^{2+}-independent binding on chromaffin granules (6, 31), endosomes (30) and adipocyte plasma membranes (1) has been demonstrated, and the existence of different cholesterol-dependent annexin 2 receptors for different organelles seems also unlikely. On the contrary, acidic phospholipids, the family of molecules present in all these membranes seem indicated for the role of a cholesterol-modulated 'receptor'. However, the cholesterol-PS effect does not preclude the possibility that annexin 2 could interact with other protein(s) in a Ca^{2+}-independent or -dependent manner as has been proposed for endosomes (30, 49) and demonstrated for actin (51, 52) and other proteins (10, 53).

There is strong evidence for the involvement of membrane microdomains in the Ca^{2+}-dependent binding of annexins to membranes: After stimulation of smooth muscle cells, annexins 2 and 6 reach a cholesterol and sphingomyelin rich fraction (4, 42); annexin 13b has been found associated to cholesterol rich domains in MDCK cells (7); in the presence of Ca^{2+}, annexins 2 and 7 were found in detergent insoluble fractions (5, 15, 41). All these data indicate that annexin 2 binding to specific membrane domains could be both Ca^{2+}-dependent and -independent.

The structural basis of the Ca^{2+}-dependent binding of annexin 5 to membranes implies that Ca^{2+} is coordinated with phospholipid head groups and amino acid residues (54). The Ca^{2+}-independent binding of annexins to membranes is not well understood at present;

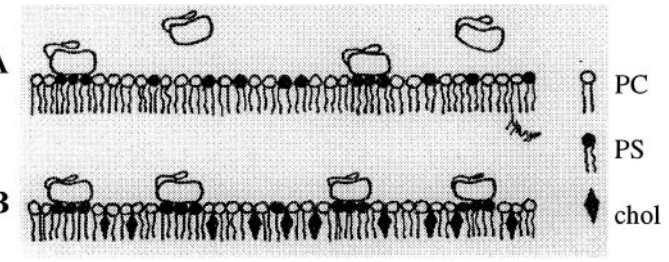

FIG. 5. Proposed model for cholesterol-mediated enhancement of annexin 2 binding to phospholipids. (A) In a cholesterol-free membrane, the PS distribution results in local PS concentrations allowing low annexin 2 binding to membranes. (B) In the presence of cholesterol, a reorganisation of the membrane lipids leads to an increase of PS local concentrations (microdomain formation), resulting in a more efficient annexin 2 binding. Notice that membrane binding is mediated by the core domain of the protein but modulated by the N-terminal tail.

however, contacts between phospholipid head groups and residues of the convex face of annexin 5 have been suggested by structural and mutagenesis studies (54, 55). Changes in residues T72, S144, S288 and S303 of annexin 5 alter the membrane binding capacity of the protein without modification of the structure and the Ca^{2+} binding capacity (55). For annexin 2, the corresponding residues S90, S162, and notably K246 and K321 could mediate electrostatic Ca^{2+}-independent contacts with the acidic phospholipids.

Taking these observations into account, we can speculate on the role of cholesterol in both Ca^{2+}-dependent and Ca^{2+}-independent binding of annexin 2 to membranes. Phospholipid microdomains have been observed in liposomes in the absence (56) or the presence of cholesterol (57). Cholesterol association with phospholipids is not only controlled by the nature of the fatty acids but also by that of the polar head groups. Using different combinations of phospholipid heads and tails, Huster *et al.* (58) showed that cholesterol associates preferentially to PC than to PS. Thus, by inclusion or exclusion of acidic phospholipids in cholesterol rich domains, cholesterol might induce or stabilise the formation of PS-rich domains, which in turn would favour the interaction of this phospholipid with annexin 2, at the level of the core (Fig. 5). According to this hypothesis, the core domain of annexin 2 would play an essential role by its affinity for acidic phospholipids. This model of PS-rich microdomain formation is strongly supported by the experiments in which the Ca^{2+} sensitivity for binding of annexins 5 and 6, other members of the family with homologous core but unrelated N-terminal tail, was also increased by the presence of cholesterol in the membrane (Fig. 4). The specificity of the intracellular localisation of annexins could result from the combination of their differential specificity of interaction with phospholipids, their difference in the Ca^{2+} sensitivity for binding, and by the role of

cholesterol in the organisation of phospholipids in microdomains.

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