N-Terminal Domain of Annexin 2 Regulates Ca^{2+}-Dependent Membrane Aggregation by the Core Domain: A Site Directed Mutagenesis Study†

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ABSTRACT: Annexin 2 binds and aggregates biological membranes in a Ca^{2+}-dependent manner. This protein exists as a monomer (p36) or as a heterotetramer (p90) in which two p36 chains are associated with a dimer of p11, a member of the S100 protein family. Protein kinase C phosphorylates the protein at the level of the N-terminal tail on serines 11 and 25, thereby modifying its oligomeric structure and its properties of membrane aggregation. To analyze these effects, the properties of a series of mutants in which serines 11 and 25 were replaced by alanine and/or glutamic acid were investigated. The affinity for p11 light chain was decreased in the S11E mutants. Glutamic acid residues in positions 11 or 25 did not change membrane binding, either in the tetrameric or in the monomeric form. On the other hand, these mutations affected the aggregation properties of the two forms. For the tetramer, the aggregation efficiency was decreased but not the Ca^{2+} sensitivity, whereas the latter was affected in the case of the monomer. The effects were stronger in the S11E mutants, and they were cumulative in the double mutant. They suggest a different conformation of the N-terminal domain in the mutants (and in the phosphorylated protein), a hypothesis which is supported by proteolysis experiments. This conformational change would affect aggregation by the monomer through a dimerization step.

Annexins are a family of proteins which have in common the property to bind membranes containing acidic phospholipids in a Ca^{2+}-dependent manner. These proteins possess a conserved core domain with four or eight conserved 70 amino acid repeats in which the Ca^{2+} sites responsible for membrane binding have been identified (*1*). On the other hand, there is a large diversity in the length and the sequence of the N-terminal extremity of the various annexins (*1*). This diversity is largely responsible for their different biochemical properties and biological functions. The structure of several annexins has been solved by X-ray crystallography (*2*−*5*). However, these data have been obtained on proteins with short or truncated N-terminal extremity. The structure of annexin 2 has been obtained on such a truncated protein, and the first visible residue is threonine 30 (*3*). The N-terminal tail of annexin 2 has been involved in three types of biochemical reactions. First, the p36[1] monomer associates with a dimer of p11, a member of the S100 family to form a heterotetramer $(p36-p11)_2$ or p90 (*6*). The p11 binding site resides in the first 12 residues of annexin 2 (*7*, *8*). The heterotetramer aggregates membrane vesicles in the low micromolar Ca^{2+} concentration range. Second, the protein is phosphorylated by $pp60^{src}$ tyrosine kinase on tyrosine 23 (*9*), by protein kinase C (PKC) on serine 25 (*10*) and on another site recently proposed to be serine 11 (*11*), and by calmodulin and cAMP serine/threonine kinases (*12*). Third, proteolysis of the N-terminal extremity generates two fragments, lacking the first 28 or 44 residues. Both are still able to aggregate chromaffin granules at high Ca^{2+} concentrations, but with differences between the two proteins (*13*), thus indicating a role for the N-terminal domain in membrane aggregation induced by monomeric annexin 2.

Among these reactions, phosphorylation by protein kinase C is especially interesting since annexin 2 is phosphorylated by this enzyme in chromaffin cells stimulated by nicotine (*14*), though the physiological role of annexin 2 is still unknown. In vitro, phosphorylation by PKC of the tetrameric form of annexin 2 has marked effects on its aggregation properties (*15*, *16*). However, there are some discrepancies among these observations. For instance, annexin 2 tetramer was reported to aggregate liposomes at 0.18 μM Ca^{2+}, and the phosphorylated annexin 2 at high calcium concentration (0.65 mM) (*16*), whereas in a different report (*15*), chromaffin granules were not aggregated by Ca^{2+} concentrations up to 1 mM. In the latter observation, the phosphorylation did not affect the binding capacity of the protein to chromaffin granules and had limited effect on the equilibrium binding constant (*15*). There are also some discrepancies on the effect of phosphorylation on the dissociation of the tetramer (*12*, *15*, *16*). Since PKC-mediated phosphorylation of annexin 2 is complex, these discrepancies might be explained by the presence of the unphosphorylated and/or different hyperphosphorylated forms (*10*, *12*, *15*, *16*). It should be noted that the effect of phosphorylation on the properties of the p36 monomer has not been described.

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[1] Abbreviations: p36, annexin 2 monomer; p90, annexin 2 tetramer; PKC, protein kinase C.; DBH, Dopamine β-hydroxylase.

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To investigate the effect of phosphorylation on the regulation of the biochemical properties of the phosphorylated monomeric and tetrameric annexin 2 with a homogeneous preparation, site-directed mutagenesis was applied to serines 11 and 25 in the N-terminal tail. Mutants with alanine and glutamic acid substituted for serine were constructed and analyzed for their biochemical properties, tetramer formation, binding, and aggregation of membranes by the monomer and the tetramer.

MATERIALS AND METHODS

Subcloning of Human Annexin 2. Yepsec vector containing the human annexin 2 gene was kindly provided by Dr. E. Solito and Dr. F. Russo-Marie from Institut Cochin de Génétique Moléculaire, Paris. The annexin 2 coding sequence devoid of the ATG start codon was subcloned in pBlueScript (*Sma*I, *Xba*I sites), yielding pBSAxII plasmid. The ATG start codon was inserted by PCR using the oligonucleotides ATTCCTGCAGCCCGGG**ATG**TCGACTGTTCAC and TATCCTGTCTCTGTTCATTGCTGCGGTT. The PCR fragment was digested by *Pst*I and *Nde*I. The ATG fragment was purified and subcloned in pBSAxII plasmid cleaved by *Nde*I and partially digested with PstI, thus yielding the complete annexin 2 ORF (pBSAxATG). Annexin 2 coding region was cloned in the yeast expression vector pYeDP60, kindly provided by Dr. D. Pompon (CNRS, Gif sur Yvette). This vector contains a *Bam*HI site just after the promoter. To avoid long PCR amplifications during the introduction of flanking *Bam*HI sites of the annexin 2 coding region, a mini-annexin 2 gene was first constructed. pBSAxATG was digested by *Bcl*I, eliminating an internal annexin fragment of 818 bp. The plasmid was religated to obtain mini-annexin 2 with the N-terminal and C-terminal portions of the protein (pMNAx). To introduce the *Bam*HI sites beside the start and stop codons by PCR, we used the oligonucleotides AACGCGGATCC**ATG**TCGACTGTTCAC and GAGGAGGATCC**TCA**GTCATCTCCACC. The amplified mini-annexin fragment was digested with *Bam*HI and cloned in pBlueScript to obtain pMNAxBam. The entire annexin 2 gene was obtained by reinsertion of the 818 bp *Bcl*I fragment in the right orientation in the *Bam*HI-flanked mini-annexin (pBSAxBam). The complete *Bam*HI-flanked annexin 2 gene was cloned in the BamHI site of pYeDP60 (pYeAxII). The orientation of fragments was checked by restriction patterns and all the PCR cloned products and fragments junctions were verified by sequencing (Sequenase, Amersham).

Mutagenesis. To mutate serine 25 to Ala or Glu residues, we used a one-step PCR mutation strategy. The primers containing the S25 substitutions were CCCCAAGTGCCATATGGG**GCT**GTCAA for S25A and CCCCAAGTGCCATATGGG**GAG**GTCAAAGCCTA for S25E. The 3′ primer used for both S25 mutations was CCCTTCATGGAAGCTTTTAGCTCAGA (Hind-primer). The amplified PCR fragments were cleaved by *Nde*I and *Hin*dIII and inserted into the pBSAxATG plasmid devoid of the corresponding fragment of the annexin 2 gene. The Ser11 mutations were performed by the overlap extension method as described by Ho et al. (*17*). The overlap primers for S11A substitution were CTGTGCAAGCTC**GCC**TTGGAGGGTGATCA and GATCACCCTCCAA**GGC**GAGCTTGCACAG, and the primers for S11E replacement were CTGTGCAAGCTC**GAA**TTGGAGGGTGATCA and GATCACCCTCCAA**TTC**GAGCTTGCACAG. The distal primers for both mutations were the *Hin*d-primer and the upstream oligonucleotide CAAGGTACCCGGGGAATGTCTACTGT. After PCR, the amplified products were digested with *Sma*I and *Hin*dIII and inserted into the pBSAxATG plasmid devoid of the corresponding sequence. To verify the mutations, the whole amplified regions were sequenced. To obtain the yeast expression vectors for the mutants S11A and S11E, the strategy described for the subcloning of the wild-type gene was used. For the S25A and S25E mutants, the corresponding 818 bp *Bcl*I fragments were inserted in the wt pMNAxBam plasmid. For the double mutants S11A−S25A, S11A−S25E, S11E−S25A and S11E−S25E, the 818 bp *Bcl*I fragments with the S25 replacements were inserted into the pMNAxBam plasmids containing S11 mutations. All the resulting pBSAxBam plasmids were used to construct the corresponding pYeAxII expression vectors as explained for the wild-type.

Expression and Purification of Recombinant Annexin 2. Yeast culture and gene expression induction were performed as described by Pompon et al. (*18*). After galactose induction, cells were centrifuged for 6 min at 6000 rpm in a GSA Sorvall rotor. They were washed once in water and once in TEK buffer (50 mM Tris, pH 7.4, 1 mM EDTA, and 100 mM KCl). Finally, the pellet from 1 L of culture was resuspended in 10 mL of TES buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.6 M Sorbitol) containing protease inhibitors (0.5 mM PMSF, 20 μg/mL soybean trypsin inhibitor, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 0.2 mM Pefabloc, 5 μg/mL aprotinin, and 0.5 mM benzamidine). The cell suspension (10−15 mL) was poured into 40 mL centrifuge tubes. Glass beads (0.45 mm diameter B. Braun Scientific) were added to skim the surface of the cell suspension. The suspension was shaken vigorously by hand to break the cells (up and down movements at approximately two per second and for a total time of 5 min in the cold room). Then 5 mL of TES buffer was added to each tube and mixed thoroughly. The liquid phase was transferred to another tube, and the washing of the beads was repeated three times. Lysed cells were centrifuged for 10 min at 8000 rpm in a SS34 Sorvall rotor. The supernatant containing the cytosol and light membranes was recovered. NaCl and $CaCl_2$ were added to, respectively, 125 and 1 mM final concentration and the mixture incubated for 15 min on ice. This step induced association of annexin 2 to membranes. The membranes were recovered by centrifugation for 30 min at 40 000 rpm in a Beckman 70 Ti rotor. The pellet was washed in 25 mM Tris, pH 7.5, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 1 mM $CaCl_2$, and 150 mM NaCl buffer containing protease inhibitors. To detach the annexin from membranes, the pellet was resuspended in the same buffer without calcium, but with 10 mM EGTA. After a 1 h incubation with stirring in the cold room, membranes were centrifuged and the supernatant dialyzed against 10 mM imidazole, pH 7.4, 1 mM EGTA, 0.1 mM EDTA, and 0.5 mM DTT buffer. The solution was passed through a DEAE-cellulose column (DE52) equilibrated in the same buffer, and the flow through containing annexin 2 was collected. Annexin 2 was more than 98% pure as judged by SDS−PAGE. The protein was dialyzed against PBS containing 1 mM DTT, concentrated with Centricon 30 (Amicon), and stored at −20 °C in aliquots.

Expression and Purification of p11. The p11 expression vector derived from pET-23a was kindly provided by Dr. Volker Gerke (Münster, Germany). B834 (DE3) *Escherichia coli* cells were cultured in LB medium containing 100 μg/mL of ampicilin at an optical density of 0.6, measured at 600 nm, and then expression of p11 was induced by addition of 1 mM IPTG for 4 h at 37 °C. Cells were pelleted (6000 rpm for 6 min) and resuspended in lysis buffer (100 mM Tris pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT) with the protease inhibitors described in the previous section. They were lyzed by sonication (six 1 min pulses). After centrifugation for 15 min at 10 000 rpm in a SS34 Sorvall rotor, the supernatant was saved and precipitated by $(NH_4)_2SO_4$ at 50% saturation. The mixture was centrifuged at 15 000 rpm for 20 min, and the supernatant was applied onto a Butyl-Sepharose column equilibrated with the same buffer. p11 was eluted by a linear gradient of $(NH_4)_2SO_4$ from 50% to 0% and recovered in the last fractions. After dialysis against 10 mM imidazole, pH 7.4, 1 mM EGTA, 0.1 mM EDTA, and 0.5 mM DTT buffer, the protein was applied onto a DEAE cellulose column equilibrated in the same buffer. The flow through containing p11 was dialyzed against PBS containing 1 mM DTT and concentrated with Centricon 10 (Amicon), and stored at −20 °C in aliquots. At the end of the purification, the p11 protein was more than 98% pure as judged by SDS−PAGE.

Annexin 2-p11 Affinity Measurement by Surface Plasmon Resonance (SPR). The affinity of the different p36 proteins for p11 was measured by SPR (BiacoreX system, Upsala, Sweden). p11 was immobilized on a carboxylated dextran matrix (CM5) according to the manufacturer's instructions. The resonance signal for the immobilized material ranged from 920 to 1150 RU, corresponding to 0.9−1.2 ng/mm^2. Interaction with p36 was measured by injection of six different concentrations of the protein in the 25−800 nM range in 10 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% P-20 surfactant (V/V). Data were analyzed with the BIA 2.2 evaluation software, assuming a single site interaction between p11 and p36. Association and dissociation rate constants were used to obtain the equilibrium dissociation constant K'_D.

Chromaffin Granule Aggregation and Binding. Bovine chromaffin granules were prepared using a Percoll density gradient as described (*19*). To monitor chromaffin granule aggregation, annexin 2 monomer or tetramer was added to a cuvette containing chromaffin granules at a OD of 0.3 (measured at 540 nm) in 40 mM Hepes buffer, pH 7, containing 30 mM KCl, 0.3 M sucrose, 1 mM EGTA, and $CaCl_2$ adjusted to give the desired free Ca^{2+} concentration calculated with the calcv.22 program (*20*). Aggregation was measured as the absorbance increase at 540 nm after 7 min at 28 °C. When the tetramer was used, p90 was reconstituted before addition to the assay by mixing equimolar amounts of p36 and p11 (1−5 μg/μL). Annexin 2 binding to chromaffin granules was measured on the samples used for the aggregation assay. The mixture (0.5 mL) was centrifuged at 12 000 rpm for 3 min and the pellets were resuspended in the electrophoresis sample buffer (*21*). Quantification was achieved by scanning Coomassie stained gels and normalizing the data to the main protein band (75 kDa, identified as DBH) of the chromaffin granule preparation.

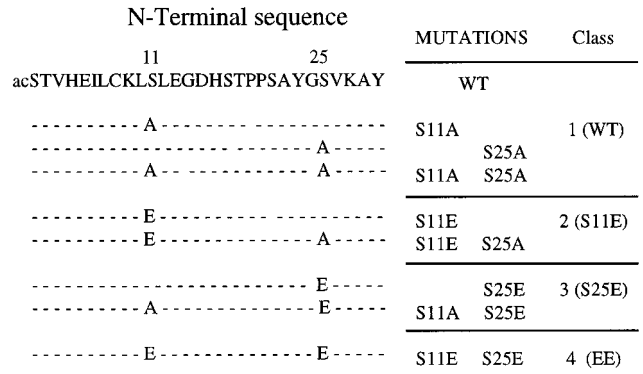

FIGURE 1: Annexin 2 mutations and their classification in four classes.

Aggregation curves have been obtained by fitting the experimental data to the equation $y = a(kx)^n/1 + (kx)^n$ in which a is the maximal aggregation signal y, k a constant, and n a constant representing the Hill number (*22*). The numerical modeling was performed with Kaleidagraph software (Synergy).

Liposome Preparation, Aggregation, and Binding. Liposomes containing phosphatidylcholine and phosphatidylserine (75/25) were obtained by extrusion as previously described (*23*). Liposome aggregation by annexin 2 was monitored for 7 min in a calcium buffered solution as described in ref *23*. Annexin 2 binding was measured on the samples used for the aggregation assay. The mixture was centrifuged at 40 000 rpm in a Beckman 70.1 Ti rotor for 30 min at 4 °C. The pellets were solubilized in the electrophoresis sample buffer and the gels were quantified after Coomassie blue staining using a standard of annexin 2.

Chymotryptic Digestion. α-Chymotrypsin (1 μg) was added to samples containing monomeric annexin 2 (3 μg) in 50 μL of 40 mM Hepes, pH 7, 30 mM KCl, and 1 mM EGTA at various calcium concentrations, and the mixtures were incubated at 25 °C for various periods of time. The reaction was stopped by addition of 2 μL of 250 mM PMSF solution in ethanol; after chilling, SDS−PAGE loading buffer was added and the samples were analyzed by SDS−PAGE (*21*).

Miscellaneous Procedures. Immunodetection of proteins by western blot was performed after electrotransfert of SDS−PAGE gels on Immobilon membranes. Two different rabbit polyclonal anti-bovine annexin 2 and one monoclonal anti-human annexin 2 (Transduction laboratories) were used. Blots were revealed by chemiluminescence (Renaissance from Amersham).

RESULTS

Characterization of Recombinant Annexin 2 and of Various Mutants. Since protein kinase C was reported to phosphorylate serine 25 (*10*, *12*) and 11 (*11*) of annexin 2 heavy chain (p36), these residues were mutated to glutamic acid to introduce a negative charge mimicking the phosphate group. They were also mutated to alanine to see whether serine had a specific function. Nine constructs corresponding to the wild-type, to the single mutants, and to the double ones were prepared. The proteins were grouped in four classes (Figure 1): (1) wild-type protein (wt) and mutants with the same charge (S11A, S25A, and S11A−S25A); (2)

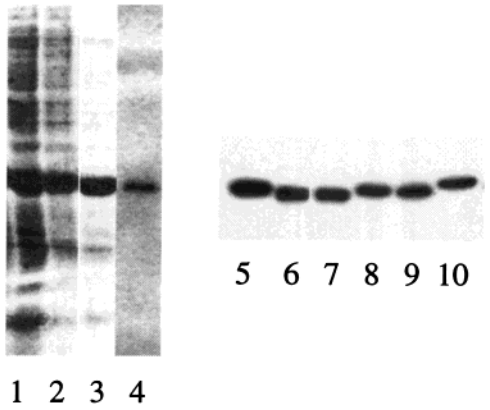

FIGURE 2: Purification and characterization of recombinant proteins. (Left) Purification of wild-type annexin 2. Lane 1, light membrane pellet; lane 2, EGTA-washed light membrane pellet, and lane 3, supernatant from the EGTA-washed membranes; lane 4, annexin 2 preparation. Right, immunoreactivity of some recombinant p36 mutants with the monoclonal anti-human annexin 2 antibody: lane 5, S11E; lane 6, S25A; lane 7, S11A−S25A; lane 8, S11A−S25E; lane 9, S11E−S25A and lane 10, S11E−S25E.

mutants with the S11E substitution (S11E, S11E−S25A); (3) mutants with the S25E replacement (S25E, S11A−S25E); and (4) the double acidic mutant (S11E−S25E).

The recombinant proteins were expressed in the yeast *Saccharomyces cerevisiae.* Since yeast does not express annexins, purification was simple and rapid, yielding 5−10 mg of highly purified protein per liter of culture. The purity, assessed by scanning Coomassie stained gels, was higher than 98% (Figure 2, lanes 1−4). The wt and the mutants were detected by immunoblot with two different rabbit anti-bovine annexin 2 polyclonal antibodies and with a mouse monoclonal anti-human annexin 2 antibody (Figure 2, lanes 5−10). No degradation product was detected in the freshly prepared proteins. The absence of proteolyzed material was also shown by sequence analysis. Edman degradation indicated that the N-terminal extremity was blocked and only traces of a sequence corresponding to p36 proteolyzed at position 29−30 could be detected. This result indicated acetylation of the N-terminal serine, as reported for annexin 1 expressed in yeast (*24*). Acetylation has been reported to be required for p36/p11 association (*7*, *8*), though this point has been disputed (*25*). The heavy-chain p36 expressed in yeast associated with recombinant p11 expressed in *E. coli* (reconstituted tetramer) had properties identical to that of the bovine lung tetramer in the chromaffin granule aggregation assay (not shown).

Phosphorylation decreased the mobility of p36 analyzed by SDS−PAGE. Two components have been observed which have been ascribed to the mono- and the diphosphorylated protein (*10*, *12*, *15*, *16*). Consistent with this view, the mutants of class 2 (Figure 2, lanes 5 and 9) and class 3 (lane 8) migrated more slowly than the wild-type and class 1 proteins (lanes 6 and 7) and the double mutant (lane 10) was slower than those with a single acidic mutation.

Aggregation of Chromaffin Granules by Tetrameric Annexin 2 Mutants. Aggregation of chromaffin granules by annexin 2 is affected by phosphorylation of the tetramer by PKC (*15*, *16*). We tested the calcium dependency of chromaffin granules aggregation by the tetrameric annexin 2 (p90) mutants. At 0.4 μM p90 final concentration, a classical aggregation curve was observed (Figure 3), which

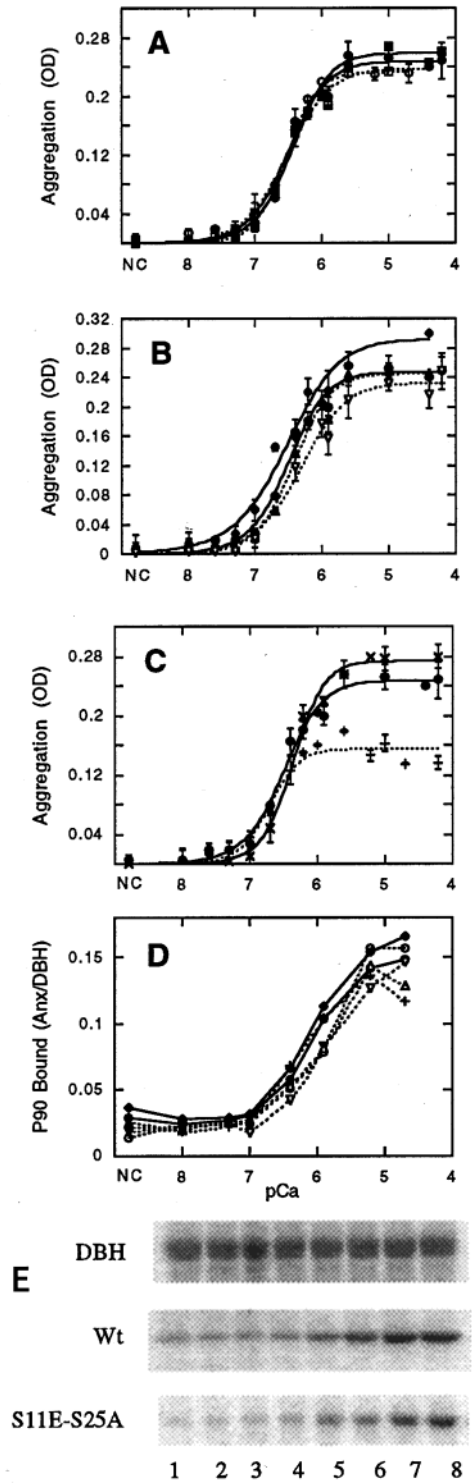

FIGURE 3: Calcium dependency of p90-mediated chromaffin granule aggregation and binding. In each panel, a class of mutants is compared to wt and corresponding Ala mutants (class 1). (A) Class 3 (S25E) mutants: (●) wt; (■) S25A; (□) S25E; (○) S11A−S25E. (B) Class 2 (S11E) mutants: (●) wt; (◆) S11A; (▽) S11E; (△) S11E−S25A. (C) Class 4 (S11E−S25E) mutant: (●) wt; (×) S11A−S25A; (+) S11E−S25E. Points are means ± SEM of two to five experiments. NC, no calcium added. (D) Calcium dependency of p90 binding to chromaffin granules. Symbols as described for aggregation. For the quantification of the annexin 2 bound to chromaffin granules, DBH was used as an internal control for chromaffin granule content. Data are presented as the annexin 2/DBH ratio. (E) SDS−PAGE of DBH, wt and S11E−S25A mutant samples at: no calcium added (lane 1), pCa 8 (lane 2), pCa 7.3 (lane 3), pCa 7 (lane 4), pCa 6.4 (lane 5), pCa 6 (lane 6), pCa 5.2 (lane 7), and pCa 4.7 (lane 8).

Table 1: Calcium Dependency (pCa for half-maximal aggregation) and Cooperativity (Hill number, *n*) of Chromaffin Granules (ch-gr) and Liposomes (lipos) Binding and Aggregation by Tetrameric (p90) and Monomeric (p36) Annexin 2 Mutants

	mutant classes							
	class 1 and wt		class 2 (S11E)		class 3 (S25E)		class 4 (S11E−S25E)	
	pCa	n^a	pCa	n^a	pCa	n^a	pCa	n^a
p90 ch-gr aggregation	6.49	1.29 ± 0.26	6.47	1.31 ± 0.22	6.05	1.55 ± 0.25	6.50	1.61 ± 0.31
p90 ch-gr binding	6.09	0.73 ± 0.32	5.93	0.68 ± 0.22	5.90	0.73 ± 0.23	6.23	1.00 ± 0.24
p36 ch-gr aggregation	4.03	0.80 ± 0.19	4.19	0.62 ± 0.19	4.22	0.79 ± 0.23	4.15	0.38 ± 0.06
p36 ch-gr binding	5.58	0.72 ± 0.17	5.52	0.59 ± 0.11	5.50	0.49 ± 0.32	5.70	0.58 ± 0.17
p36 lipos aggregation	4.01	1.87 ± 0.81	3.19	3.13 ± 1.46	3.59	2.51 ± 0.42	2.93	2.84 ± 1.00
p36 lipos binding	5.73	0.59 ± 0.13	5.60	0.49 ± 0.10	5.50	0.70 ± 0.16	5.60	0.40 ± 0.06

[a] Mean ± SD.

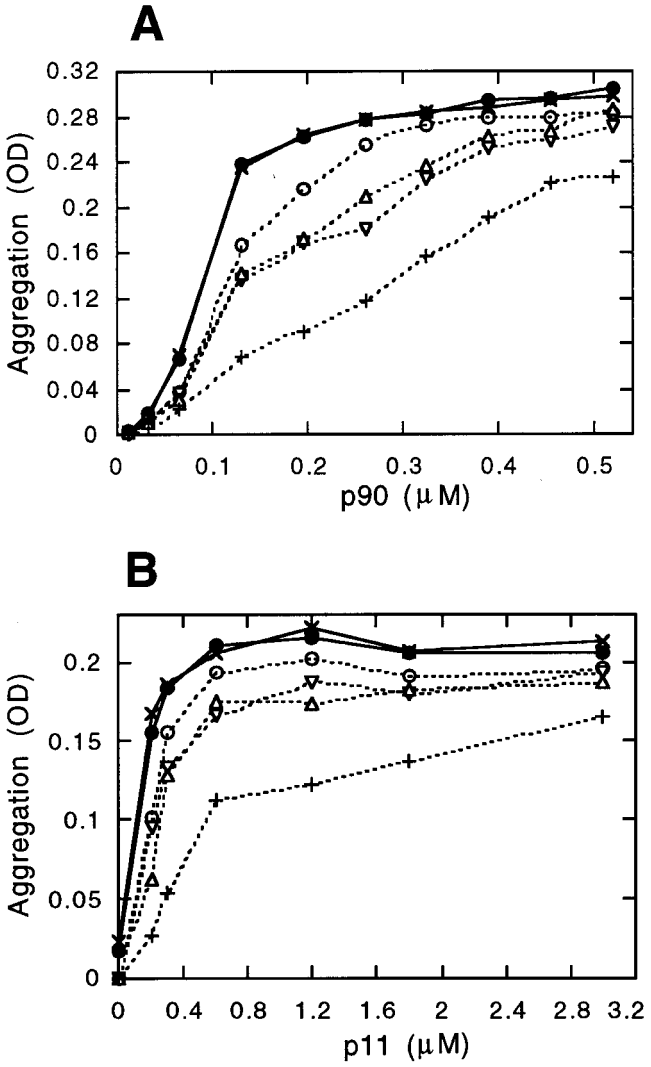

FIGURE 4: Chromaffin granule aggregation as a function of p90 and p11 concentrations. (A) aggregation at variable p90 concentrations. (B) chromaffin granule aggregation at constant p36 (600 nM), and variable p11 concentrations. Assays were performed in the presence of 10 μM Ca^{2+}: (●) wt; (×) S11A−S25A; (○) S11A−S25E; (▽) S11E; (△) S11E−S25A; (+) S11E−S25E.

was characterized by half-maximal aggregation at pCa 6.5 (0.3 μM). All mutants showed a similar Ca^{2+} dependency (Figure 3 and Table 1) and similar cooperativity (Hill number $\approx$ 1.4). However, differences were observed in the plateau value of the double mutant S11E−S25E (Figure 3C), for which the plateau value is only 60% of that of the wild-type and of a group 1 mutant (S11A−S25A). In addition, examination of the plateau value indicated a slight decrease for class 2 and class 3 mutants (clearly visible at lower protein concentrations, see Figure 4).

Table 2: Equilibrium Dissociation Constants (K'_D Values) of p36−p11 Interaction

mutant protein	K'_D (nM)[a]	class of mutants	class K'_D (nM)[a]
Wt	175 ± 10 (3)		
S11A	170 ± 28 (2)	1 (Wt)	176 ± 8 (9)
S25A	180 ± 14 (2)		
S11A−S25A	170 ± 42 (2)		
S11E	391 ± 25 (3)	2 (S11E)	363 ± 24 (6)
S11E−S25A	334 ± 42 (3)		
S25E	195 ± 10 (3)	3 (S25E)	187 ± 9 (6)
S11A−S25E	179 ± 19 (3)		
S11E−S25E	348 ± 34 (3)	4 (S11E−S25E)	348 ± 34 (3)

[a] Mean ± SEM (*n*).

A possible interpretation of the plateau effect observed with the acidic mutants was that association of the heavy and light chains was affected, thus resulting in a decreased level of tetrameric p90, the molecular species responsible for aggregation at low calcium level. Preliminary p11 overlay tests and coprecipitation of p11 with p36 in the presence of membranes and Ca^{2+} showed that mutated p36 bound to p11. However, to obtain a more quantitative description of the association of p36 mutants with p11, affinity measurements were performed by the Biacore plasmon surface resonance technique. Equilibrium dissociation constants (K'_D) were in the same range (170−195 nM) for class 1 and class 3 (S25E) proteins whereas all proteins bearing the S11E mutation (classes 2 and 4) showed a significantly lower affinity for p11 ($K'_D \approx 350$ nM) (Table 2).

These results were not consistent with the proposed interpretation of the aggregation data (Figure 3), since mutants of class 2 (S11E) and the double mutant S11E−S25E had similar K'_D but different plateau values in the aggregation assay. The small effect of the class 2 (S11E) and class 3 (S25E) mutants compared to that of the double mutant indicated that p11 binding was not the only factor involved in the phenomenon. The implication of p90 dissociation was further tested in two experiments. In the first one (Figure 4A), chromaffin granule aggregation was measured as a function of p90 concentration in the presence of 10 μM free Ca^{2+}, a concentration sufficient for maximal aggregation with class 1 proteins (wt and S11A/S25A), aggregation leveled off at a p90 concentration of 200 nM. With the S25E mutant, which had the same affinity for p11 than the wild-type, the plateau was reached at 300 nM. With class 2 proteins (S11E, S11E−S25A) and with the double

mutant, higher p90 concentrations were required (around 450 nM). Moreover, the double E mutant did not reach the same plateau value. In the second experiment (Figure 4B), a constant concentration of p36 was used (0.6 μM) to which increasing p11 concentrations (0−3 μM) were added. In all cases, a titration point could be observed corresponding to a stoichiometry of 1 for the p36/p11 complex (likely to reflect tetramer formation), but the plateau values of the glutamic acid containing mutants, specially that of the double E mutant were lower than that of the wild-type, even in the presence of a large excess of the light chain. These results show that acidic substitutions at both serine 11 and 25 induced a conformation change which affected the aggregation capacity of the tetramer.

Another interpretation of the aggregation experiments (Figures 3 and 4) might be an effect on the binding of tetrameric annexin 2 to chromaffin granule membranes. Therefore, we analyzed binding on the samples used for aggregation experiments (Figure 3, panels D and E). Compared to aggregation, binding required the same minimal Ca^{2+} concentration of 0.1 μM, but the curve was less cooperative (Hill number $\approx$ 0.8) and half-maximal binding was shifted toward higher Ca^{2+} concentrations by about 0.5 pCa unit (pCa 6, Table 1). It should be noted that all the mutations tested were without any effect on the binding curve.

Aggregation of Chromaffin Granules by Monomeric p36 Mutants. Aggregation by monomeric annexin 2 was investigated under the same experimental conditions, using Ca^{2+} in the 0.1 μM-1 mM concentration range and the same p36 concentration (Figure 5). The monomeric protein was less efficient than the tetramer, requiring higher Ca^{2+} concentrations. In addition, the curves were less cooperative, and defining a plateau value was difficult. Consequently, pCa at half-maximal aggregation and Hill number (derived from the theoretical equation) are only indicative. However, comparison of the various proteins is quite significant. The minimal concentration required for aggregation is not changed by the various mutations. The cooperativity of the curve is unchanged in class 1 and class 3 (Hill number $\approx$ 0.8), but it is decreased in class 2 (S11E), $n \approx 0.6$ and for the double mutant S11E−S25E, $n \approx 0.4$. This effect can also be appreciated by comparing the absorbance measured at 1 mM Ca^{2+}. For class 1 proteins (wt and S-to-A), the absorbance was 0.115. Class 3 mutants (S25E and S11A−S25E) were slightly affected (0.08 OD, corresponding to a 25% inhibition), whereas class 2 (S11E, S11E−S25A) and class 4 (S11E−S25E) were strongly inhibited (60 and 70% inhibition, respectively).

As for p90, binding was also analyzed under the conditions used for the aggregation assay (Figure 5, panels D and E). For the monomer as for the tetramer, the mutations tested had no obvious effect on the binding. When the binding of the heavy chain, p36 (Figure 5D), was compared to that of the tetramer (Figure 3D), a shift was noted. pCa at mid-binding decreased by about 0.5 pCa unit (Table 1). On the other hand, for the monomer, when the binding curve (Figure 5D) and the aggregation curves (Figure 5, panels A−C) were compared, a clear difference was observed since binding leveled off at pCa 5 whereas aggregation still increased at pCa 3. The cooperativity of binding (Hill number $\approx$ 0.6) was similar to that of the tetrameric complex.

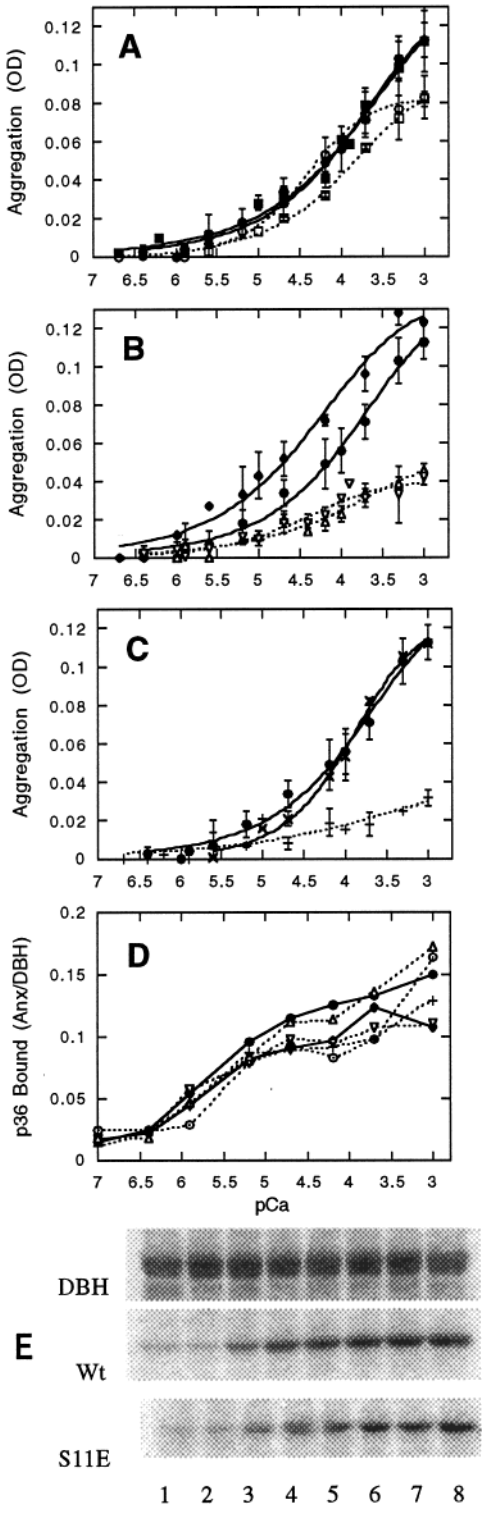

FIGURE 5: Calcium dependency of p36-mediated chromaffin granule aggregation and binding. The wt and the null Ala mutant corresponding to each class are presented in each panel. (A) Class 3 (S25E) mutants: (●) wt; (■) S25A; (□) S25E; (○) S11A−S25E. (B) Class 2 (S11E) mutants: (●) wt; (◆) S11A; (▽) S11E; (△) S11E−S25A. (C) Class 4 (S11E−S25E) mutant: (●) wt; (×) S11A−S25A; (+) S11E−S25E. Points are means ± SEM of two to five experiments. (D) Calcium dependency of p36 binding to chromaffin granules. Symbols as described for aggregation. For the quantification of the annexin 2 bound to chromaffin granules, see Figure 3 legend. (E) SDS−PAGE of DBH, wt, and S11E mutant samples at no calcium added (lane 1), pCa 8 (lane 2), pCa 7.3 (lane 3), pCa 7 (lane 4), pCa 6.4 (lane 5), pCa 6 (lane 6), pCa 5.2 (lane 7), and pCa 4.7 (lane 8).

Aggregation of Liposomes by Monomeric p36 Mutants. Because aggregation of chromaffin granules by p36 did not reach a clear plateau, these experiments were repeated with liposomes containing phosphatidylcholine/phoshatidylserine (75/25). With this procedure, even though plateau values were not accurately defined, maximal aggregation values were similar for all mutants (0.08−0.1 OD). As a result, curves were more cooperative. At variance with the results observed on the aggregation of chromaffin granules, mutants differed from one another in the pCa at half-maximal aggregation (Figure 6). The pCa of class 1 proteins was 4.05 (90 μM) whereas those of the mutants of classes 2, 3, and 4 were, respectively, 3.2 (650 μM), 3.6 (260 μM), and 2.9 (1.17 mM) (Table 1). Under the same conditions, the p33 core where residues 1−29 were deleted was characterized by an aggregation curve similar to that of the S11E mutants (Figure 6C).

Binding of p36 to liposomes was also investigated for two proteins of class 1 and 2 and one of class 3 and 4 (Figure 6, panels D and E). As for binding to chromaffin granules, all the proteins tested had a similar profile with a half-maximal pCa value of 5.6 (Table 1) and reached a plateau value at pCa 4.4 (40 μM). It is interesting to note that, at that Ca^{2+} concentration, class 1 proteins start to aggregate liposomes and other classes have no effect at all. As for p36 and p90 binding to chromaffin granules, the Hill number of liposome binding was in the range of 0.5, indicating that binding properties were unaltered by the mutations.

Sensitivity to Chymotrypsin Cleavage of the Heavy Chain p36. Since the aggregation experiments suggested a conformational change induced by the acidic mutation, chymotrypsin sensitivity of the protein was investigated in order to find an independent evidence supporting this hypothesis. Chymotrypsin cleaves the p36 N-terminal extremity between Tyr 29 and Thre 30, generating a 33 kDa fragment. When the S11E, S11E−S25A, and S11A−S25E mutants were compared to the wild-type, they were significantly more rapidly degraded (Figure 7), a result which indicated that the cleavage site was more exposed in the mutant. The experiment was performed in the presence or in the absence of calcium at concentrations which would have induced liposome binding and aggregation. Under the conditions used, Ca^{2+} had no effect on chymotrypsin sensitivity.

DISCUSSION

In the present work, recombinant annexin 2 (p36) expressed in yeast was used since acetylation of the N-terminal serine is important for p36-p11 interaction (*7*, *8*). The system used allowed high level of expression and rapid purification of a protein with a blocked N-terminal extremity. The biochemical properties of recombinant p36 protein (immunoreactivity, electrophoretic mobility) and those of the reconstituted p90 (chromaffin granule aggregation) were indistinguishable from those of the bovine lung protein. Annexin 2 mutants in which glutamic acid was substituted for serine at positions 11 and 25 were also prepared. Surprisingly, the acidic mutations induced a detectable change in the electrophoretic mobility of the protein, which was more intense in the S11E−S25E double mutant. This effect is similar to that reported for the protein phosphorylated by PKC (*10*, *12*, *15*, *16*).

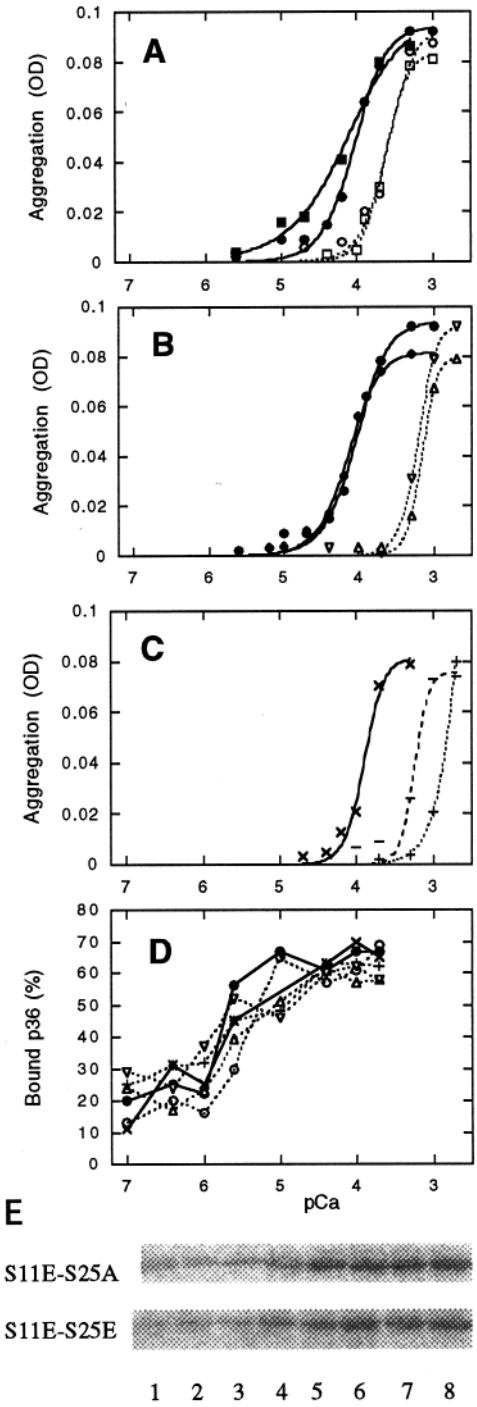

FIGURE 6: Calcium dependency of p36-mediated liposomes aggregation and binding. The wt and the null Ala mutant corresponding to each group are presented in each panel. (A) Class 3 (S25E) mutants: (●) wt; (■) S25A; (□) S25E; (○) S11A−S25E. (B) Class 2 (S11E) mutants: (●) wt; (◆) S11A; (▽) S11E; (△) S11E−S25A. (C) Class 4 (S11E−S25E) mutant: (×) S11A−S25A; (+) S11E−S25E; and (−) p33. (D) calcium dependency of p36 binding to liposomes. Percent of protein bound to liposomes. Symbols as for aggregation. (E) SDS−PAGE of liposome pellets of S11E−S25A and S11E−S25E mutant samples at pCa 7 (lane 1), pCa 6.4 (lane 2), pCa 6 (lane 3), pCa 5.6 (lane 4), pCa 5 (lane 5), pCa 4.4 (lane 6), pCa 4 (lane 7), and pCa 3.7 (lane 8).

The present work sheds some light on the properties of both the phosphorylated and unphosphorylated protein. First, association of the heavy chain p36 with the light chain p11 is not affected by the S25E mutation and only moderately by the S11E one. This view is consistent with measurements

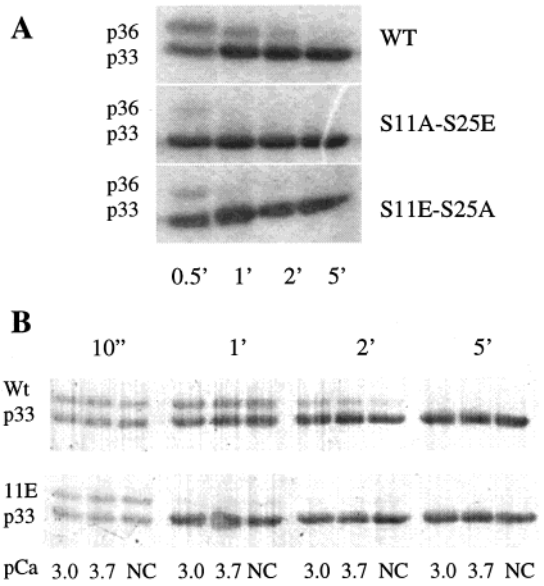

FIGURE 7: Sensitivity to α-chymotrypsin of p36. (A) wt, S11A−S25E and S11E−S25A mutants were subjected to mild chymotrypsin digestion in the absence of calcium. (B) Wild-type and S11E mutant were incubated in the absence (NC) or in the presence of calcium: pCa 3 (1 mM), and pCa 3.7 (0.2 mM). Digestions were performed for the indicated periods of time at 25 °C.

of the interaction between p11 and peptides derived from p36 N-terminal extremity (*8*), which showed a limited effect for a peptide with the S11D mutation. It is also consistent with the crystal structure of a complex of p11 with a peptide corresponding to the first 13 residues of the annexin 2 N-terminal extremity (*26*), which confirmed the existence of a strong hydrophobic interaction involving the first 10 residues of p36 and could not precise the position of serine 11, thus indicating that this residue is not essential for the interaction with p11. In addition, phosphorylation by protein kinase C of the tetramer induced limited dissociation of p11 (*15*, *16*), and reassociation was only partially affected by phosphorylation of the heavy chain (*12*). These rather confused results can be explained by either a limited effect on the equilibrium dissociation constant or the heterogeneity of the preparation which might contain the unphosphorylated and several phosphorylated forms of the protein.

A second and a more important observation is the effect of acidic mutants on the aggregation properties of the monomer and of the tetramer. The results are perhaps easier to discuss in the case of the aggregation of liposomes by monomeric annexin 2 (Figure 6). Clearly, substitution of an acidic residue for serine 25 or serine 11 raises the calcium ion concentration required for half-maximal aggregation $[Ca^{2+}]_{1/2}$ from 90 μM to, respectively, 260 and 650 μM. For the double mutant, this concentration attained 1.17 mM. The effect is restricted to Ca^{2+} sensitivity, with no significant change in plateau value or cooperativity. It is noteworthy that p36 binding is not affected by the mutations and is characterized by $[Ca^{2+}]_{1/2}$ of 10 μM, significatively, lower than the concentrations required to observe aggregation. Therefore, aggregation implies a reaction, different from binding to liposomes, which requires a higher concentration of Ca^{2+} and in which the N-terminal extremity is involved.

The same conclusion can be reached from the results of experiments on chromaffin granule aggregation by monomeric annexin 2 (Figure 5), though the shape of the aggregation curves is somehow different. In this case, the change in the calcium sensitivity of the aggregation reaction appeared as a change of the cooperativity of the curve. The difference observed between the two experimental models originates probably in the relative concentration and mobility of acidic phospholipids and in the complexity of the aggregation reaction between two membranes. Interestingly, the behavior of monomeric annexin 2 mutants is very similar to that of phosphorylated annexin 1, the closest paralogue of annexin 2. Annexin 1 is phosphorylated by PKC (*27*). This phosphorylation has a limited effect on its membrane binding properties (*28*, *29*), but decreases markedly the calcium sensitivity of aggregation of chromaffin granules (*22*, *29*) and liposomes (*28*). Moreover, site-directed mutagenesis studies on serine 27, the main serine phosphorylated by PKC, showed that membrane binding of the S27D (*22*) and S27E (*30*) mutants is identical to that of the wild-type and to the PKC-phosphorylated annexin 1. On the contrary, the calcium sensitivity for membrane aggregation of the S27D mutant is the same as that of the phosphorylated protein (*22*).

Analysis of the images of liposome aggregation by monomeric annexin 1 or annexin 2 obtained by cryo-electron microscopy (*31*) showed the presence in most instances of two protein layers at the interface between contacting liposomes and suggested the existence of p36 dimers as the aggregation unit. Since p36 cleaved at residue 44 aggregates efficiently chromaffin granules with a $[Ca^{2+}]_{1/2}$ of 24 μM (*13*), dimerization should more probably be viewed as a property of the core domain. How is the dimerization Ca^{2+}-dependent? Though structural evidence is missing, this reaction is likely to involve contacts between the concave faces of p36 and no Ca^{2+} binding sites are present on these faces. Contacts might involve the α-helices present on the concave face and having their axe parallel to the plan of p36. A first hypothesis is that high concentrations of Ca^{2+} on the convex side induces a conformation change on the concave one, exposing the structure involved in the dimerization. This hypothesis is supported by experimental data obtained by measurements of time-resolved and anisotropy of fluorescence in the presence of Ca^{2+} [J.A.-S., Vincent, Sopkova, and Gallay, *Biochemistry* (in press)], and by the previously reported annexin 2-annexin 2 interaction (*32*). Another hypothesis, which is not exclusive, is a Ca^{2+}-dependent negative modulation of the core domain dimerization by the N-terminal domain. Such a negative effect would account for the decreased aggregation efficiency of p36 lacking the 28 first residues compared to that lacking the first 44 ones (*13*). It would also explain the absence of aggregation by annexins with short N-terminus such as annexin 5, where the extremity of the chain is in contact with the core domain (*5*). On the other hand, this interaction would be prevented in annexins with a long N-terminus which would fold autonomously, thus liberating the dimerization site. To account for the decreased aggregation efficiency of the mutants, we propose that negative charges at positions 11 or 25 induce a different conformation, allowing a negative effect of the N-terminus on the core. Experiments on chymotrypsin sensitivity support the existence of such a conformation change induced by negative charges (Figure 7). This model is also supported by experiments performed on annexin 1 (*22*, *33*).

We also observed that the acidic mutations had an effect on chromaffin granule aggregation by tetrameric annexin 2. This effect was more clearly seen at low protein concentration, but it did not originate from a dissociation of the tetramer (Figure 4). With the tetramer, dimerization occurs through the p11 light chain and it is not a Ca^{2+} requiring step. This is shown by the fact that aggregation and binding started to be detected at the same calcium concentration. It can thus be proposed that, in the S11E and S25E mutants, the proposed conformation change results in an organization of the tetramer which is less efficient for membrane aggregation. The effects observed with the mutants are less marked than those reported for the tetramer phosphorylated by protein kinase C, where the calcium sensitivity of the aggregation reaction was dramatically affected. Two hypotheses might account for this difference. A first possibility is that phosphorylation is not fully mimicked by glutamic acid replacement of serine. This hypothesis is difficult to rule out, however, comparison of the results obtained on p36 monomer and on annexin 1 supports the relevance of the approach. A second hypothesis might be to question previous results on the effects of phosphorylation of annexin 2 because of the heterogeneity of the phosphorylated annexin 2 preparations, which might involve unphosphorylated as well as hyperphosphorylated molecules in an active or inactive state. To reach clear conclusions, it will be necessary to phosphorylate mutants in order to have define species bearing only one phosphoryl group.

At the present time, the use of mutant proteins gives indications on the effect of phosphorylation by PKC. The main site of phosphorylation is serine 25 (*10*), but in vitro, hyperphosphorylation has been noted, presumably on serine 11 (*11*). With the mutations used, modification of serine 25 has no effect on the association of p36 with p11, consistent with the localization of the p11 binding site on p36 heavy chain (*7*, *8*, *25*). This mutation seems to have also quite discrete effects on the aggregation reaction, compared to the S11E one which was the only one to alter the association of p36 with p11. However, these effects are unquestionable since the double mutant S11E−S25E has a phenotype markedly different from the S11E one. Therefore, we propose that introduction of a negative charge in position 25 by mutagenesis or phosphorylation alters the conformation of the N-terminal domain and favors its interaction with the core domain. This interaction blocks membrane aggregation by preventing protein−protein contacts at the concave faces of monomeric annexin 2 and possibly by changing the orientation of the core domains for the heteroteramer. The mechanism by which the N-terminus of annexin 2 interacts with the core domain remains to be clarified and should await further structural data.

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REFERENCES

1. Raynal, P., and Pollard, H. B. (1994) *Biochim. Biophys. Acta 1197*, 63−93.
2. Weng, X., Luecke, H., Song, S., Kang, D. S., Kim, S.-H., and Huber, R. (1993) *Protein Sci. 2*, 448−458.
3. Burger, A., Berendes, R., Liemann, S., Benz, J., Hofmann, A., Göttig, P., Huber, R., Gerke, V., Thiel, C., Römisch, J., and Weber, K. (1996) *J. Mol. Biol. 257*, 839−847.
4. Huber, R., Römisch, J., and Paques, E.-P. (1990) *EMBO J. 9*, 3867−3874.
5. Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Römisch, J., and Paques, E. (1992) *J. Mol. Biol. 223*, 683−704.
6. Glenney, J. R., Boudreau, M., Galyean, R., Hunter, T., and Tack, B. (1986) *J. Biol. Chem. 261*, 10485−10488.
7. Johnsson, N., Marriott, G., and Weber, K. (1988) *EMBO J. 7*, 2435−2442.
8. Becker, T., Weber, K., and Johnsson, N. (1990) *EMBO J. 9*, 4207−4213.
9. Glenney, J. R., and Tack, B. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7884−7888.
10. Gould, K. L., Woodgett, J. R., Isake, C. M., and Hunter, T. (1986) *Mol. Cell. Biol. 6*, 2738−2744.
11. Jost, M., and Gerke, V. (1996) *Biochim. Biophys. Acta 1313*, 283−289.
12. Johnsson, N., Van, P. N., Söling, H.-D., and Weber, K. (1986) *EMBO J. 5*, 3455−3460.
13. Drust, D. S., and Creutz, C. E. (1988) *Nature 331*, 88−91.
14. Delouche, B., Pradel, L.-A., and Henry, J.-P. (1997) *J. Neurochem. 68*, 1720−1727.
15. Regnouf, F., Sagot, I., Delouche, B., Devilliers, G., Cartaud, J., Henry, J.-P., and Pradel, L.-A. (1995) *J. Biol. Chem. 270*, 27143−27150.
16. Johnstone, S. A., Hubaishy, I., and Waisman, D. M. (1992) *J. Biol. Chem 267*, 25976−25981.
17. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
18. Pompon, D., Louerat, B., Bronine, A., and Urban, P. (1996) *Methods Enzymol. 272*, 51−64.
19. Carty, S. E., Johnson, R. G., and Scarpa, A. (1980) *Analytical Biochem. 106*, 438−445.
20. Föhr, K. J., Warchol, W., and Gratzl, M. (1993) *Methods Enzymol. 221*, 149−157.
21. Laemmli, U. K. (1970) *Nature 227*, 680−685.
22. Wang, W., and Creutz, C. E. (1994) *Biochemistry 33*, 275−282.
23. Ayala-Sanmartin, J., Henry, J. P., and Pradel, L. A. (2000) *Biochim. Biophys. Acta* (in press).
24. Giga-Hama, Y., Tohda, H., Okada, H., Owada, M. K., Okayama, H., and Kumagai, H. (1994) *Biotechnology 12*, 400−404.
25. Kang, H.-M., Kassam, G., Jarvis, S. E., Fitzpatrick, S. L., and Waisman, D. M. (1997) *Biochemistry 36*, 2041−2050.
26. Réty, S., Sopkova, J., Renouard, M., Osterloh, D., Gerke, V., Tabaries, S., Russo- Marie, F., and Lewit-Bentley, A. (1999) *Nat. Struct. Biol. 6*, 89−95.
27. Summers, T. A., and Creutz, C. E. (1985) *J. Biol. Chem. 260*, 2437−2443.
28. Johnstone, S. A., Hubaishy, I., and Waisman, D. M. (1993) *Biochem. J. 294*, 801−807.
29. Wang, W., and Creutz, C. E. (1992) *Biochemistry 31*, 9934−9939.
30. Porte, F., de Santa Barbara, P., Phalipou, S., Liautard, J.-P., and Sri Widada, J. (1996) *Biochim. Biophys. Acta 1293*, 177−184.
31. Lambert, O., Gerke, V., Bader, M.-F., Porte, F., and Brisson, A. (1997) *J. Mol. Biol. 272*, 42−55.
32. Liu, L. (1999) *Cell. Signal. 11*, 317−324.
33. Bitto, E., and Cho, W. (1999) *Biochemistry 38*, 14094−14100.

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