Role of the Amino-Terminal Domain in Regulating Interactions of Annexin I with Membranes: Effects of Amino-Terminal Truncation and Mutagenesis of the Phosphorylation Sites†

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ABSTRACT: Phosphorylation of the N-terminal tail by protein kinase C strongly inhibits the ability of bovine or human annexin I to aggregate chromaffin granules by increasing the calcium requirement 4-fold (Wang, W., & Creutz, C. E. (1992) *Biochemistry 31*, 9934–9936). In the present study three forms of human annexin I truncated in the amino terminus at residue Trp-12, Lys-26, or Lys-29 exhibit dramatic differences in their sensitivities to calcium in a chromaffin granule aggregation assay, while the $[Ca^{2+}]_{(1/2)max}$ values for binding of the truncated proteins to granule membranes are similar. Cleavage at Trp-12 causes a 3-fold decrease in calcium sensitivity in the membrane aggregation assay, while cleavage at Lys-26 causes a 4-fold enhancement of calcium sensitivity. In contrast, cleavage at Lys-29 results in virtually no change in calcium sensitivity. Mutagenic substitution with negatively charged amino acids of Ser-27, a site for phosphorylation by protein kinase C, or Tyr-21, a site for phosphorylation by the epidermal growth factor receptor kinase, mimics the inhibition of granule-aggregating activity seen with phosphorylation by protein kinase C. When bovine chromaffin cells are stimulated to secrete by nicotine, annexin I is phosphorylated in the amino terminus. Thr-24 and Ser-28, which are sites for phosphorylation by protein kinase C *in vitro*, are two of the sites phosphorylated *in vivo* in stimulated chromaffin cells. These data demonstrate that the ability of annexin I to promote membrane aggregation is highly sensitive to changes in the structure of the N-terminal domain of the protein. Phosphorylation and proteolysis may therefore be potent mechanisms for regulation of annexin I function *in vivo*.

Annexin I is a member of the annexin family of calcium-dependent membrane binding proteins (Creutz, 1992). These proteins possess a similar core domain with four or eight conserved 70 amino acid repeats and an amino-terminal domain which varies in length and sequence among different members. Annexin I is a substrate for protein kinase C (PKC)[1] (Michener et al., 1986; Summers & Creutz, 1985) and for the epidermal growth factor receptor kinase (Pepinsky & Sinclair, 1986; Sawyer & Cohen, 1985) both *in vitro* and *in vivo*. The sites phosphorylated by these kinases *in vitro* have been localized to the N-terminal domain (Schlaepfer & Haigler, 1988; Varticovski et al., 1988). Annexins have been implicated in calcium-regulated exocytosis, on the basis of their ability to aggregate secretory granules in a calcium-dependent manner (Creutz et al., 1987; Drust & Creutz, 1988). Annexin I has also been shown to be the predominant cytosolic mediator of Ca^{2+}-dependent fusion of liposomes with isolated neutrophil plasma membranes (Meers et al., 1992). In bovine chromaffin cells, annexin I becomes rapidly phosphorylated when the cells are stimulated to secrete (Michener et al., 1986). It has been hypothesized that phosphorylation might modulate annexin functions related to membrane trafficking during exocytosis. It has also been demonstrated that annexin I is a major substrate for the EGF receptor kinase in multivesicular bodies, suggesting that annexin I may play a role in mediating inward vesiculation (Futter et al., 1993).

The X-ray crystal structure of annexin V has shown that the four tandem repeats of the annexin core domain are folded into four bundles of α-helices (Huber et al., 1990a,b). These four bundles are arranged in a planar array, with the calcium binding sites located on a slightly convex surface, which also binds membranes. The N-terminal tail, however, is located at the concave surface of the molecule, opposite the membrane attachment site. We previously reported that phosphorylation of annexin I at the amino terminus by protein kinase C strongly inhibits its ability to aggregate chromaffin granules while mildly promoting its binding to the granule membrane (Wang & Creutz, 1992). This suggests that membrane binding and membrane aggregation are sequential events involving different parts of the annexin I molecule. Consistent with this model is the observation that a monoclonal antibody to the amino terminus of annexin I inhibits vesicular aggregation but not binding (Meers et al., 1992). It was also reported (Andree et al., 1993) that a chimeric protein with the N-terminus of annexin I and the core of annexin V has a similar Ca^{2+} requirement for binding to phospholipid vesicles. However, the chimeric protein promotes aggregation of phospholipid vesicles, while annexin V does not, suggesting a regulatory role of the N-terminus of annexin I in membrane aggregation.

In this report, we present further evidence that the membrane-aggregating activity of annexin I is highly sensitive

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PKC, protein kinase C; PNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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to changes in the structure of the N-terminal domain of the protein through phosphorylation, proteolysis, or mutagenesis. In addition, we demonstrate that phosphorylation of annexin I in the chromaffin cell in response to secretagogue stimulation occurs exclusively in the N-terminus, on Thr-24, Ser-28, and possibly other sites.

EXPERIMENTAL PROCEDURES

Materials. Cathepsin D and calpain (calcium-activated neutral protease) were purchased from Sigma. Human plasmin was provided generously by Dr. S. L. Gonias (University of Virginia). PNPGB was from Sigma and was kindly provided by Dr. S. L. Gonias. ^{32}P-Orthophosphate was from ICN Biomedical, Inc. HPTLC-Fertigplatten cellulose plates were obtained from EM Separations.

Purification of Recombinant Human Annexin I. Human annexin I was expressed in *Saccharomyces cerevisiae* under control of the *GAL10* promotor as described previously (Creutz et al., 1992), using plasmid YEpDB60-lipocortin and strain C^{2}Y6. The recombinant protein was isolated from yeast extract by calcium-dependent lipid binding using endogenous yeast membranes as the lipid source. A 4-L yeast culture was grown to a density of 7×10^7 to 1×10^8 cells/mL in synthetic galactose minimal medium supplemented with histidine (2% galactose, 1.6 g/L yeast nitrogen base without amino acids (DIFCO), 11.1 g/L succinic acid, 0.5% (w/v) ammonium sulfate, 20 mg/L histidine, and ~8 g/L NaOH, pH 7.3). Cells were harvested by centrifugation at 4000*g* for 5 min, washed once, resuspended in 30 mL of buffer H (20 mM Hepes, pH 7.4, at 4 °C, 150 mM KCl, 2 mM $MgCl_2$, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, 0.5 mM DTT, 1 mM benzamidine, and 4 mM iodoacetic acid), and lysed by milling with glass beads in a Beadbeater (Biospec Products, Bartlesville, OK) packed in ice. The homogenate was centrifuged at 14400*g* for 10 min. Calcium was added to the supernatant to 4 mM final concentration, which was followed by centrifugation at 200000*g* for 45 min. The pellet was washed three times with buffer H supplemented with 2 mM calcium by resuspension in a Dounce homogenizer and recentrifugation at 200000*g* for 30 min. The final pellet was extracted with buffer H plus 5 mM EGTA and centrifuged at 300000*g* for 30 min. The extraction was repeated two more times. The first extract, which usually contained most of the annexin I, was dialyzed extensively against 25 mM MES, pH 6.0 at 4 °C, 0.5 mM DTT, and 1 mM EGTA before being applied to an FPLC Mono S column (1 mL) equilibrated with 25 mM MES (pH 6.0 at 4 °C). A linear gradient of 0–500 mM KCl in 25 mM MES was applied. Annexin I eluted around 350 mM KCl as a single peak. Mutant annexin I proteins were purified with the same procedures and eluted at a similar KCl concentration from the Mono S column. Using these purification methods, 2 mg of annexin I was routinely purified from a 4-L yeast culture. The preparation was estimated to be more than 95% pure by SDS–PAGE.

Limited Proteolysis. Recombinant human annexin I (500 μg) was incubated with one of the following proteases as previously described (Ando et al., 1989; Schlaepfer & Haigler, 1988): (1) 62.5 μg of cathepsin D in 50 mM Tris–acetate, pH 4.5, in a total volume of 1.5 mL at room temperature for 30 min; (2) 135 μg of calpain in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM DTT, and 0.1 mM EDTA in a total volume of 1.5 mL at room temperature for 30 min, initiated by adding calcium to 4 mM final concentration; (3) 500 μg of plasmin in 200 mM Hepes, pH 7.4, in a total volume of 4 mL at 37 °C for 1 h. The reactions were terminated by dialysis against 25 mM MES, pH 6.0 at 4 °C, and the mixtures were applied to an FPLC Mono S column (1 mL) equilibrated in the same buffer. A linear gradient of 0–500 mM KCl was applied. The truncated forms of annexin I eluted at around 350 mM KCl, similar to intact annexin I.

Chromaffin Granule Binding and Aggregation Assays. Annexin-induced aggregation of chromaffin granule membranes was monitored by measuring turbidity at 540 nm as previously described (Drust & Creutz, 1988; Wang & Creutz, 1992). In the aggregation assay, binding of the annexins to the surface of the granules does not cause a significant change in the turbidity, nor does calcium only, with no annexins. The granule suspension had an initial absorbance at 540 nm of 0.15, corresponding to 35–45 μg of granule protein/mL (25 μg of granule lipid/mL) (Creutz, 1981). Protein titrations were done to determine the amount of annexin required for maximal levels of aggregation (OD ≃ 0.26). Annexin I at 4 μg/mL gave maximal aggregation at a high calcium level (2.5 mM), and this concentration was used for all calcium titrations of granule aggregation. The Ca^{2+}-EGTA buffer employed in all calcium titrations had a final concentration of 2.5 mM EGTA and the desired free Ca^{2+} concentration, checked with a Ca^{2+}-selective electrode (Radiometer, Copenhagen, Denmark) standardized with solutions of known Ca^{2+} concentrations (Orion, Boston, MA).

After aggregation was assayed for 10 min, the granules were immediately centrifuged in a microfuge for 5 min. The granule pellets were subjected to 10% SDS–PAGE and stained with Coomassie Blue. The annexins associated with granule membranes at various levels of calcium were quantitated by two-dimensional densitometry, using the MCID image analysis program (Imaging Tech, St. Catherines, Canada). A standard curve, using 0.5–10 μg of bovine serum albumin, gives a correlation coefficient of 0.9756. The amount of annexins measured was in the linear range of the standard curve.

Mutagenesis of Phosphorylation Sites. Codons for Tyr-21 and Ser-27 were altered to encode Glu or Asp, respectively, by polymerase chain reaction (PCR) after the annexin I cDNA was subcloned into a more convenient plasmid, Bluescript II SK + (Stratagene). The annexin cDNA coding sequence was excised from the expression plasmid YEpDB60-lipocortin (Creutz et al., 1992) with *Sal*I and *Bam*HI, and the fragment was purified by electrophoresis in agarose and electroelution. The fragment was ligated into Bluescript cut with the same enzymes. This construct, Bluescript–lipocortin, was used as the template for amplification of a mutagenized partial cDNA. Upstream primers were designed to introduce the mutations as follows.

Y21E (56-mer):

```
5  S  E  F  L  K  Q  A  W  F  I  E  N  E  E  Q  E
14 CAGAATTCCTCAAGCAGGCCTGGTTTATTGAAAATGAAGAGCAGGAA
     Eco RI

21  E  V  Q
61 GAGGTACAA
      Rsa I
```

This primer spans nucleotides 14–69 of the annexin I cDNA, corresponding to amino acids 5–23. The codon for Tyr-21 (TAT) is replaced by a codon for Glu (GAG). A silent change has been introduced in the codon for Val-22 (GTT to GTA) in order to introduce an *Rsa*I site, as marked above, to facilitate identification of the mutant construct. The *Eco*RI site indicated is unique in the coding sequence.

S27D (71-mer):

```
5  S  E  F  L  K  Q  A  W  F  I  E  N  E  E  Q  E
14 CAGAATTCCTCAAGCAGGCCTGGTTTATTGAAAATGAAGAGCAGGAA
     Eco RI

21 Y  V  Q  T  V  K  D  S
61 TATGTACAAACTGTGAAGGACTCC
     Rsa I
```

This primer spans nucleotides 14–84 of the annexin I cDNA, corresponding to amino acids 5–28. The codon for Ser-27 (TCA) is replaced by a codon for Asp (GAC). As above, a silent change was introduced in the codon for Val-22 in order to create an *Rsa*I site.

The downstream primer for both mutagenesis procedures was

```
5'-CCAGATCTCTCTTCAGTTCCTCTC-3'
     Bgl II
```

This is the complement of nucleotides 521–544 of the annexin I cDNA and incorporates the unique *Bgl*II site of the cDNA.

By use of these primer pairs, 539-bp fragments were amplified from the annexin I cDNA in the Bluescript plasmid. The reaction products were washed by chloroform–phenol extraction and ethanol precipitation and cut at the unique *Eco*RI and *Bgl*II sites. After cleavage, the samples were washed again and then incubated with T4 ligase. The vast excess of mutant product favored the insertion of the mutant cDNA back into the Bluescript plasmid. After ligation and transformation of *Escherichia coli* XL1-Blue, minipreps of plasmid DNA were examined for the presence of the *Rsa*I site introduced by the primers. Candidate mutagenized clones were selected and then subjected to sequencing of the 539-bp amplified segment by the Sanger method. The mutations were confirmed, and no other changes were detected in the amino acid sequence encoded by the constructs. The mutagenized cDNAs were then excised from Bluescript by digestion with *Sal*I and *Bam*HI and then ligated into the expression plasmid YEpDB60 cut at these sites.

Stimulation of Bovine Chromaffin Cells and ^{32}P Labeling. Bovine chromaffin cells were cultured as previously described (Ely et al., 1990) and were kindly provided by Dr. Sarah J. Parsons (University of Virginia). The cells were plated on 100-mm petri dishes at a density of 2×10^7 cells/dish. Three day old cultures were washed three times with KRB–Hepes buffer (120 mM NaCl, 4.75 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 24 mM $NaHCO_3$, and 10 mM Hepes, pH 7.5 at 22 °C). The cells were then labeled for 2 h at 37 °C with 5 mL of KRB-Hepes buffer containing 2.5 mCi of carrier-free ^{32}P-orthophosphate. The cells were stimulated by adding nicotine directly to the labeling media for either 5 or 20 min at a final concentration of 20 μM. ^{32}P-Labeled annexin I contained in the cytosolic fraction was purified by its calcium-dependent binding to lipid vesicles as previously described (Michener et al., 1986). It was further purified by 10% SDS–PAGE and either excised directly from the gel for plasmin treatment or transferred to a nitrocellulose membrane before excision for trypsin digestion.

Other Procedures. In vitro phosphorylation of annexin I was carried out essentially as previously described (Wang & Creutz, 1992). For tryptic phosphopeptide mapping, ^{32}P-labeled proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes, visualized by autoradiography, excised, and subjected to trypsin digestion. Labeled peptides were separated on thin-layer cellulose plates according to Luo et al. (1991). For amino acid analysis, either labeled peptides from trypsin digests or individual phosphopeptides recovered from thin-layer cellulose plates were treated according to Boyle et al. (1991). Automated Edman degradation was performed by Dr. J. D. Shannon, University of Virginia, on an Applied Biosystems Model 470A gas-phase sequencer according to the manufacturer's instructions except that the phosphopeptide was coupled to a Sequelon aryl amine membrane and extracted with 90% methanol and 1 mM phosphate, pH 7.

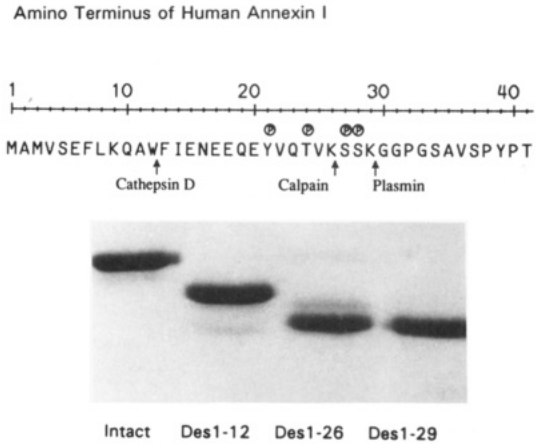

FIGURE 1: Amino terminal truncation of annexin I by proteases. The top panel shows the sequence of the amino terminus of human annexin I. Ⓟ denotes the *in vitro* phosphorylation sites as previously reported (Haigler et al., 1987; Schlaepfer & Haigler, 1988); ↑ marks the positions of protease cleavage. The bottom panel shows recombinant human annexin I treated with various proteases as described in Experimental Procedures. Five micrograms of each of the N-terminus-truncated forms des 1–12, 1–26, and 1–29 and the intact form was run on 10% SDS–PAGE and stained with Coomassie Blue.

RESULTS

Different N-Terminus-Truncated Forms of Annexin I Exhibit Different Calcium Sensitivities in Aggregating Chromaffin Granules. We previously reported that phosphorylation on the N-terminus of bovine annexin I inhibited its ability to aggregate chromaffin granules at low levels of Ca^{2+}, even though the phosphorylation slightly enhanced the binding of annexin I to granule membranes (Wang & Creutz, 1992). To further test whether the N-terminus is more directly involved in membrane aggregation than in membrane binding, recombinant human annexin I was cleaved specifically at Trp-12, Lys-26, and Lys-29 with cathepsin D, calpain, or plasmin, respectively (Ando et al., 1989; Huang et al., 1987). The truncations generated single polypeptides lacking part of the amino terminus, as illustrated in Figure 1. The protein truncated at position Lys-26 (des 1–26) exhibited greatly increased calcium sensitivity in aggregating chromaffin granules (Figure 2A). The Ca^{2+} concentration required to induce half-maximal aggregation ($[Ca^{2+}]_{(1/2)max}$) decreased from 175.46 ± 42.42 μM (n = 7) for the intact form to 40.55 ± 3.96 μM (n = 3) for des 1–26. The protein truncated at Trp-12 (des 1–12), however, required a higher level of calcium in the aggregation assay. The $[Ca^{2+}]_{(1/2)max}$ was 487.59 ± 202.41 μM (n = 3), similar to that of the phosphorylated form, 458.13 ± 80.67 μM (n = 4). Interestingly, when the first 29 amino acid residues were cleaved, the truncated form, des 1–29, required a level of calcium, $[Ca^{2+}]_{(1/2)max}$ = 133.69 ± 56.48 μM (n = 2), similar to that required by the intact unphosphorylated form. Although the half-maxima for the calcium titration curves varied somewhat with different chromaffin granule preparations, leading to the large standard errors given here, the relative positions of the titration curves

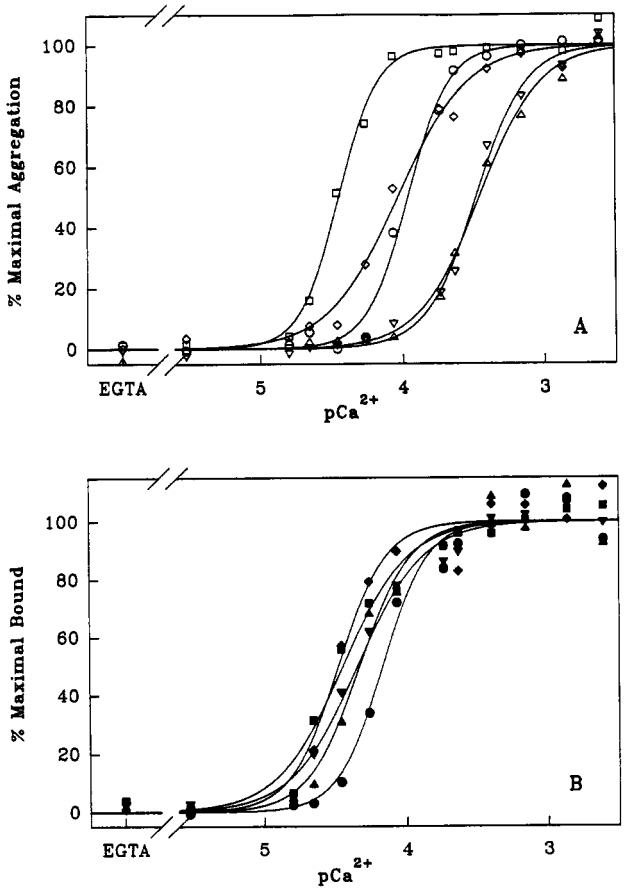

FIGURE 2: Ca^{2+} titration curves for chromaffin granule aggregation (A) and binding (B) by annexin I: (○, ●) intact annexin I; (△, ▲) phosphorylated annexin I; (▽, ▼) des 1–12; (□, ■) des 1–26; (◇, ◆) des 1–29. The data shown are from a representative experiment in which different forms of annexin I were assayed at the same time to minimize variations among different chromaffin granule preparations. The continuous lines represent the best fit of the expression $y = 100(kx)^n/[1 + (kx)^n]$ to the normalized data (k, constant; n, Hill coefficient).

did not vary if different forms were tested at the same time using the same granule preparation (Figure 2A).

The differences in calcium requirement for different N-terminus truncated forms of annexin I to aggregate chromaffin granules did not correlate with the abilities of these forms to bind granule membranes. Following each aggregation reaction, the chromaffin granules were centrifuged immediately in a microfuge and the protein bound to granule membranes was separated by SDS–PAGE and quantified by densitometry. As shown in Figure 2B, the intact unphosphorylated form required the highest level of calcium to bind granule membranes, $[Ca^{2+}]_{(1/2)max} = 77.72 \pm 8.08$ μM (n = 3). Both the phosphorylated form and des 1–12 bound granule membranes at similar levels of calcium as the intact form, $[Ca^{2+}]_{(1/2)max} = 62.14 \pm 21.50$ (n = 2) and 53.95 ± 12.09 μM (n = 2), respectively, although both of these forms required much higher levels of calcium than the intact unphosphorylated form to aggregate granules. The des 1–26 and des 1–29 forms required lower levels of calcium to bind granule membranes than the intact form, $[Ca^{2+}]_{(1/2)max} = 30.87 \pm 7.05$ (n = 2) and 33.76 ± 0.59 μM (n = 2), respectively, compared to 77.72 ± 8.08 μM (n = 3) for the intact form.

Mutational Analysis of Human Annexin I Phosphorylation Sites. It has been suggested that membrane binding and membrane aggregation are sequential events involving different parts of the annexin I molecule (Wang & Creutz, 1992).

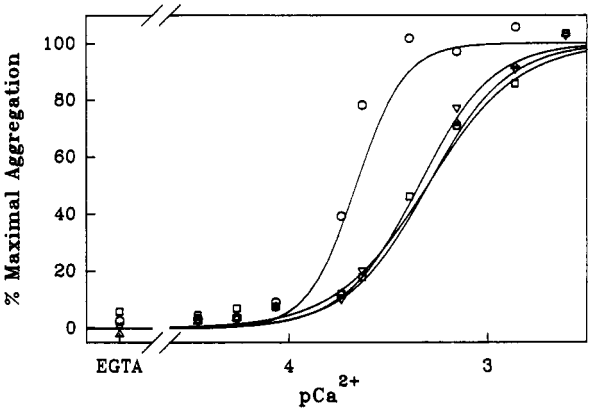

FIGURE 3: Ca^{2+} titration curves of aggregation of chromaffin granules by S27D and Y21E mutant proteins: (○) native recombinant human annexin I; (△) phosphorylated annexin I; (▽) S27D mutant protein; (□) Y21E mutant protein.

Since both phosphorylation and proteolysis of the amino terminus greatly affect the granule aggregating activity of annexin I without significantly changing the membrane binding activity, it appears that the amino-terminal domain plays a regulatory role in the membrane aggregating activity of the protein. Ser-27 has been mapped previously by two groups as a primary site of phosphorylation of human annexin I by protein kinase C (Schlaepfer & Haigler, 1988; Varticovski et al., 1988). Tyr-21 is the site of phosphorylation by EGF receptor kinase both *in vitro* and *in vivo* (Fava & Cohen, 1984; Sawyer & Cohen, 1985). To model the effects of phosphorylation on individual amino acids, either Ser-27 or Tyr-21 was mutated to negatively charged Asp or Glu, respectively, creating S27D or Y21E mutant proteins to mimic the "constitutively" phosphorylated state. The mutant proteins were purified to homogeneity by using the *GAL10* promoter in yeast as the expression system. When tested in the chromaffin granule aggregation assay, both mutant proteins showed an increased requirement for calcium to aggregate granules, virtually indistinguishable from the calcium requirement of the native form phosphorylated by protein kinase C (Figure 3). However, the calcium dependence of chromaffin granule binding was not significantly altered by the mutations (data not shown).

Phosphorylated annexin I was shown to act as an inhibitor of granule aggregation by the unphosphorylated protein (Wang & Creutz, 1992). Although the S27D mutant protein showed an increased requirement for calcium to aggregate granules, similar to the native phosphorylated form, it did not inhibit the native unphosphorylated form to aggregating granules (Figure 4) at pCa^{2+} 3.6. The Y21E mutant protein also failed to inhibit the native protein from aggregating granules at pCa^{2+} 3.6.

Comparison of in Vivo and in Vitro Phosphorylation of Annexin I. Since annexin I is phosphorylated in bovine chromaffin cells in a stimulus-dependent manner (Michener et al., 1986), we wanted to examine whether the same site(s) were phosphorylated both *in vivo* and *in vitro* by protein kinase C. The recently published sequence of bovine annexin I (Ernst, 1993) reveals that the sequences of bovine and human annexin I are 90% identical. In the first 41 amino acids, only three substitutions occur: an isoleucine (bovine) in place of Val-22 (human), a lysine in place of Gln-23, and a glycine in place of Ser-27.

Cultured bovine chromaffin cells were stimulated with 20 μM nicotine for 5–20 min as previously described (Michener et al., 1986). ^{32}P-Labeled annexin I from stimulated chro-

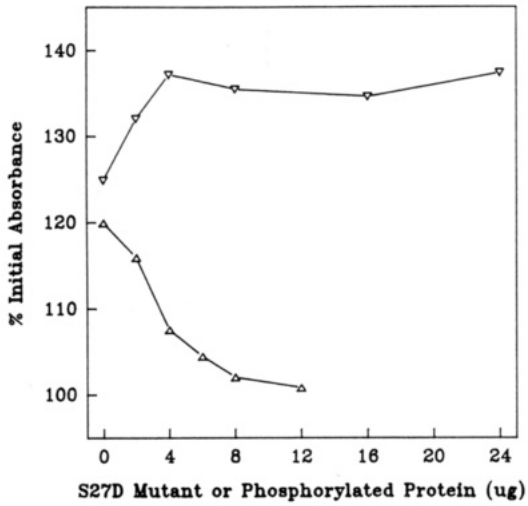

FIGURE 4: Interaction between S27D mutant protein and native annexin I. Granule aggregation was assayed at pCa^{2+} 3.632. Four micrograms of unphosphorylated native annexin I was mixed with increasing amounts of either S27D mutant protein (▽) or native protein phosphorylated *in vitro* by protein kinase C (△). The turbidity (A_{540}) of the granule suspension after 8 min is plotted as a function of the amount of S27D mutant protein or phosphorylated protein in the mixture. The absorbance at 540 nm is expressed as percent of initial value (100%).

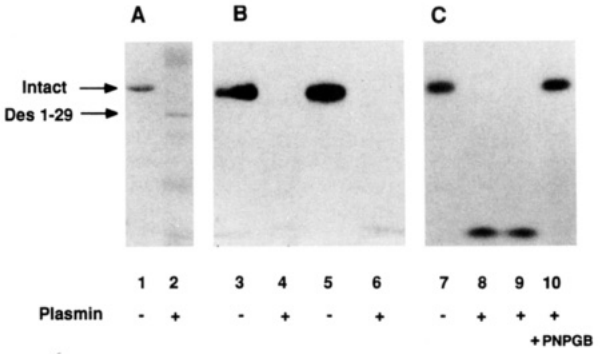

FIGURE 5: Plasmin digestion of phosphorylated bovine annexin I. (A) Coomassie Blue staining; (B) autoradiograph. Lanes 1–4, *in vitro* phosphorylated annexin I; Lanes 5 and 6, *in vivo* phosphorylated annexin I. Lanes 1-6 contained equal amounts of phosphorylated material (400 cpm) before plasmin digestion, with 2 μg of native annexin I added as a carrier; 1 μM plasmin was added in lanes 2, 4, and 6. (C) Autoradiograph of the wet gel, identifying the radioactive digestion product. Exposure time was 90 min at room temperature. Equal counts (17 000 cpm) were loaded in lanes 7–10, with 5 μg of native annexin I added in each lane as a carrier. Lanes 8, 9, and 10 contained 0.75, 1.5, and 1.5 μM plasmin, respectively. Lane 10 contained 0.1 mM PNPGB in addition. Total reaction volume was 50 μL.

maffin cells was purified by using its calcium-dependent binding to phosphatidylserine vesicles. Annexin I phosphorylated either *in vivo* or *in vitro* was subjected to plasmin digestion, which has been shown to cleave at a single site, Lys-29 (Huang et al., 1987), and generate the truncated form, des 1–29 (Figure 5A). Figure 5B shows that there was complete loss of phosphate when annexin I was cleaved by plasmin. Since the protein was transferred to an Immobilon membrane before autoradiography, the radioactivity was not fully recovered. In a control experiment (Figure 5C) in which the wet gel was immediately analyzed by autoradiography, most of the radioactivity was recovered in the amino-terminal peptide. In the presence of *p*-nitrophenyl *p'*-guanidinobenzoate (PNPGB), a specific inhibitor of plasmin, annexin I remained intact and phosphorylated, excluding the possibility that the plasmin was contaminated with phosphatase activities. These results indicate that all the phosphorylation sites reside in the

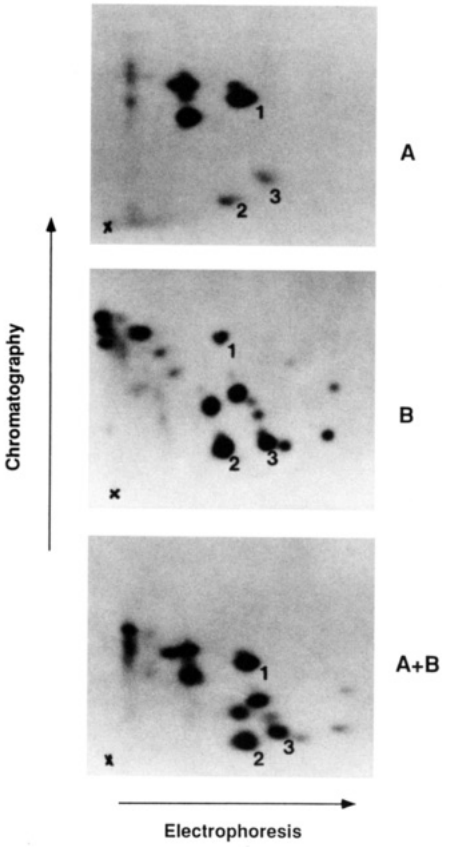

FIGURE 6: Tryptic phosphopeptide maps of bovine annexin I: (A) *in vivo* and (B) *in vitro* phosphorylated annexin I. Equal amounts of *in vivo* and *in vitro* phosphorylated annexin I (1000 cpm) were mixed in the third map. × denotes the origin. The spots labeled 1, 2, and 3 are common to maps of both the *in vivo* and *in vitro* phosphorylated annexin I.

first 29 amino acids of the N-terminus of bovine annexin I, whether the phosphorylation occurs *in vivo* or *in vitro*. Phosphoamino acid analysis of bovine annexin I labeled *in vitro* revealed that it contained both phosphoserine (71%) and phosphothreonine (29%). However, annexin I isolated from stimulated bovine chromaffin cells contained mostly phosphoserine with only a small amount of phosphothreonine (data not shown).

The tryptic phosphopeptide map of the *in vivo* phosphorylated annexin I was compared to that of the *in vitro* phosphorylated protein (Figure 6). There were more spots derived from *in vitro* labeled protein than from the *in vivo* labeled protein. Because of the limited amount of ^{32}P-labeled material that could be obtained from primary chromaffin cell cultures, we focused our effort on analyzing phosphopeptides that were labeled *in vitro* which appeared to correspond to phosphopeptides labeled *in vivo*. On the basis of comigration in two dimensions on thin-layer cellulose (TLC) plates, we determined that three spots (labeled 1, and 2, and 3 in Figure 6) are common to both *in vivo* and *in vitro* phosphorylated annexin I. Phosphoamino acid analysis of *in vitro* labeled protein (Figure 7) revealed that spot 1 contained predominantly phosphothreonine; spot 3, predominantly phosphoserine; and spot 2, both phosphoserine and phosphothreonine. The phosphothreonine detected in spot 1 must be Thr-24 because (i) all the phosphorylation sites are localized to the N-terminal 29 amino acids (Figure 5) and (ii) Thr-24 is the only threonine present in these 29 amino acids (see the amino-terminal sequence in Figure 8).

We determined that spot 3 contained phosphorylated Ser-28 on the basis of the following observations: (i) Spot 3

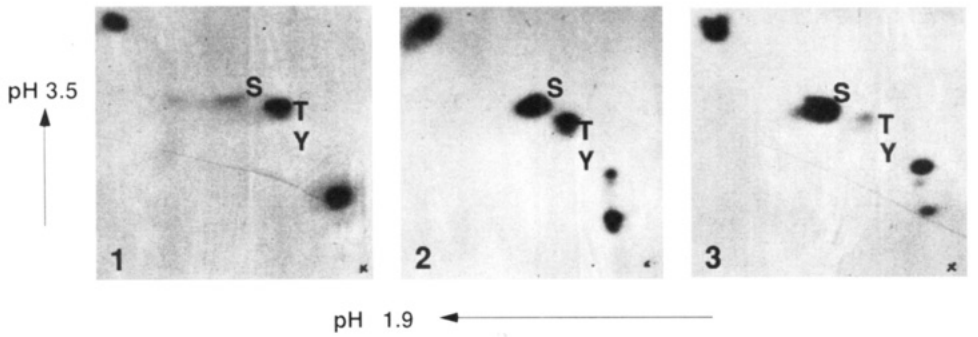

FIGURE 7: Phosphoamino acid analysis. The three spots labeled 1, 2, and 3 in Figure 6B were scraped off the TLC plate according to Boyle et al. (1991). Two-dimensional phosphoamino acid analysis was performed on each spot as described in Experimental Procedures. × marks the origin.

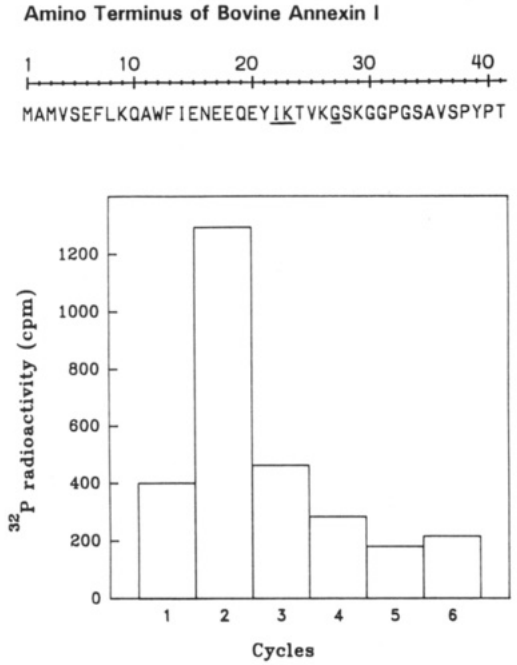

FIGURE 8: Edman degradation of phosphopeptide 3. Spot 3 derived from annexin I phosphorylated *in vitro* was recovered from the TLC plate with a total initial activity of 8670 cpm; ~50% of the activity was successfully coupled to a Sequelon aryl amine membrane. Radioactivity detected in the first six cycles of degradation is plotted here; 900 cpm remained on the membrane. The top panel shows the sequence of the amino terminus of bovine annexin I (Ernst, 1993). The three residues that differ from those of the human sequence are underlined.

contained predominantly P-Ser. The potential candidates in the N-terminal 29 amino acids are Ser-5 and Ser-28 according to the sequence of bovine annexin I (Ernst, 1993). (ii) Sequential Edman degradation of spot 3 released $^{32}P_i$ at the second cycle (Figure 8). Because there is a trypsin digestion site at Lys-26, this establishes Ser-28 as a site phosphorylated in stimulated chromaffin cells. Sequential Edman degradation of spot 2 did not yield any $^{32}P_i$ peaks in the first ten cycles.

DISCUSSION

Annexin I has been purified from various cells and tissues such as A-431 cells (Fava & Cohen, 1984), porcine lung (De et al., 1986), human placenta (Haigler et al., 1987), and rat peritoneal exudates (Pepinsky et al., 1986). In these preparations, all (De et al., 1986) or a significant fraction (Haigler et al., 1987; Pepinsky et al., 1986) of the protein is cleaved at the amino terminus. Although intracellular proteases which produce these annexin derivatives have not yet been identified, various proteases have been used *in vitro* to generate such N-terminus-truncated forms (Ando et al., 1989; Huang et al., 1987). In this paper, we demonstrate that these proteolytic modifications of annexin I lead to significant changes in the Ca^{2+} sensitivity of the protein in aggregating membranes but not in the membrane-binding capabilities of the protein. These differences in the calcium sensitivities of the truncated forms in aggregating membranes do not have a direct correlation with changes in the net electric charge of the amino-terminal upon various proteolytic cleavages. Mechanisms involving structural changes of the amino terminal domain might be necessary to explain these differences.

The three-dimensional structures of annexin V (Huber et al., 1990a,b) and annexin I lacking the first 32 residues (Weng et al., 1993) show that all the Ca^{2+} and membrane binding sites are located on one side of the molecule, while the N-terminus is located on the opposite side of the molecule. The membrane–membrane aggregation event may occur through protein–protein interaction between annexins bound to different membranes. The N-terminal domain may play an important role in this interaction and be subject to regulation by phosphorylation and proteolysis. If phosphorylation blocks an interaction between the tail and the core domain, the tail might be released from the bulk of the protein, allowing it to act as an inhibitor of membrane aggregation. This type of structural change has been suggested by the observation that when the N-terminal domain of annexin I is phosphorylated on tyrosine, it is 10-fold more sensitive to trypsin cleavage at Lys-26 than is the unphosphorylated N-terminal domain (Haigler et al., 1987). We observed that when the N-terminal 26 residues are removed, the truncated form, des 1–26, aggregates membranes at the lowest level of calcium (Figure 2A), consistent with the possibility that residues 1–26 act in an inhibitory fashion. Residues 27–29 appear to actively participate in promoting membrane aggregation, since further removal of these three residues lowers the calcium sensitivity of des 1–29 to the same level as that of the intact form.

It is intriguing that des 1–12 exhibits the same increased requirement for calcium as the phosphorylated form to aggregate membranes (Figure 2A). Residues 1–12 might be critical for holding the entire amino-terminal domain (residues 1–41) folded securely against the core of the molecule. Deletion of these 12 residues might alter the structure and release the rest of the amino-terminal tail, just as phosphorylation might render the tail mobile. Further proof of this will rely heavily on determination of the three-dimensional structure of the N-terminal domain of annexin I.

Ando et al. (1989) previously reported that annexin I truncated at either Trp-12 or Lys-26 requires less calcium than the intact form to bind a phospholipid affinity column (PS:PC:PE:cholesterol = 4:4:1:1, w/w). Although we ob-

served here that N-terminus-truncated forms of annexin I generally bind chromaffin granule membranes at lower levels of calcium than the native form, the difference is small. This might be due to a different sensitivity of the assay used here compared to that used by Ando et al. (1989), or it may be due to a lower content of phosphatidylserine in the chromaffin granule membranes.

Annexin I can be phosphorylated on Tyr-21 by the EGF receptor kinase (Pepinsky & Sinclair, 1986; Sawyer & Cohen, 1985). It was previously reported that phosphorylation of Tyr-21 reduces by 5-fold the amount of Ca^{2+} required for half-maximal binding of the protein to phosphatidylserine vesicles (Schlaepfer & Haigler, 1987). Because of the low stoichiometry of tyrosine phosphorylation, it was not previously feasible to assess the effect of tyrosine phosphorylation on the membrane-aggregating activity of the protein. We used site-specific mutagenesis to change Tyr-21 into a negatively charged residue, glutamic acid. Interestingly, this mutant protein was found to be extremely sensitive to proteolysis during regular purification procedures, and a significant portion of the protein was cleaved to a 32 kDa fragment, possibly at Lys-26 (data not shown). No *in vivo* tyrosine phosphorylation of annexin I was observed in bovine chromaffin cells. It is possible that tyrosine phosphorylation is obscured *in vivo* because annexin I phosphorylated on tyrosine is highly susceptible to proteolysis. The Y21E mutant, as well as the S27D mutant, shows an increased requirement for Ca^{2+} to aggregate granules, indistinguishable from the effects of phosphorylation by protein kinase C. The des 1–26 truncated form, however, requires a much lower level of calcium to aggregate granules. There might be successive modifications of the N-terminus of annexin I *in vivo*: Phosphorylation would down regulate its membrane-aggregating activity; subsequent proteolysis of the phosphorylated form would reactivate the protein.

In contrast to phosphorylated annexin I, neither the S27D nor the Y21E mutant protein inhibited the native unphosphorylated annexin I from aggregating granule membranes at pCa^{2+} 3.6 (Figure 4). It is interesting to note that 4 μg/mL S27D mutant protein, when combined with 4 μg/mL native annexin I, caused membrane aggregation to a greater extent than 4 μg/mL native annexin I alone (Figure 4). At very high protein concentrations (>16 μg/mL), the S27D mutant protein alone can aggregate membranes to the same level that lower concentrations of the native protein would (data not shown). This degree of aggregation is not observed with high concentrations of the phosphorylated native annexin I (Wang & Creutz, 1992). One explanation might be that the mutant proteins interact with the native form to aggregate membranes, while they do not interact with each other with high affinity at low levels of calcium to promote membrane aggregation. This apparent low affinity of interactions between mutant annexin I molecules appears to be overcome by increasing the total amount of the mutant protein. In contrast, the phosphorylated protein appears to be unable to interact either with itself or with the unphosphorylated protein and thus acts as an inhibitor of the interactions between membrane-bound annexins necessary for membrane aggregation. These differences between the phosphorylated form and the mutant protein might be due to (i) multiple sites of phosphorylation on one molecule or (ii) negatively charged amino acids not completely mimicking the effect of phosphorylation. The latter explanation seems more likely, because the 1:1 stoichiometry of phosphorylation of annexin I (Schlaepfer & Haigler, 1988; Wang & Creutz, 1992) argues against the first possibility. Neither Ser-27 nor Tyr-21 was mutated to amino acids other than negatively charged ones. It is possible that mutagenic substitution of these two residues might have caused other structural changes, besides addition of a negative charge, that are responsible for the effect we observe here.

Annexin I serves as a good substrate for protein kinase C *in vitro* (Summers & Creutz, 1985). Schlaepfer and Haigler (1988) have shown that human annexin I is phosphorylated *in vitro* to equal extents on Thr-24, Ser-27, and Ser-28 by protein kinase C. However, these phosphorylation sites are not entirely conserved among different species (Varticovski et al., 1988). In the bovine annexin I sequence, Ser-27 is replaced by a glycine (Ernst, 1993). On the basis of comigration of *in vivo* and *in vitro* labeled phosphopeptides on two-dimensional TLC plates, we infer that Thr-24 and Ser-28 are two of the sites phosphorylated in the secretagogue-stimulated chromaffin cells. The additional tryptic phosphopeptides derived from *in vivo* labeled protein (Figure 6A) might contain Ser-5, although this residue is not in a preferred PKC recognition site. It is possible that other kinases, besides protein kinase C, may also be involved in phosphorylating annexin I in the cell. Alternatively, these peptides may be the result of incomplete trypsin digestion. Since all the phosphorylation sites are located in the first 29 amino acids of the N-terminus (Figure 5) and mutation at either Tyr-21 or Ser-27 of human annexin I has an inhibitory effect on the membrane-aggregating activity of the protein, we speculate that various kinases, in response to different extracellular signals, might phosphorylate different residues on the N-terminus to provide similar effects on annexin I activities.

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