Characterization of a Ca$^{2+}$-binding Site in Human Annexin II by Site-directed Mutagenesis*

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Annexin II, a major cytoplasmic substrate of the src tyrosine kinase, is a member of the annexin family of Ca$^{2+}$/phospholipid-binding proteins. It is composed of a short N-terminal tail (30 residues) and four so-called annexin repeats (each 70-80 residues in length) which share sequence homologies and are thought to form a new type(s) of Ca$^{2+}$-binding site(s). We have produced wild-type and site specifically mutated annexin II molecules to compare their structure and biochemistry. The recombinant wild-type annexin II displays biochemical and spectroscopical properties resembling those of the authentic protein purified from mammalian cells. In particular, it shows the Ca$^{2+}$-induced blue shift in fluorescence emission which is typical for this annexin. Replacement of the single tryptophan in annexin II (Trp-212) by a phenylalanine abolishes the fluorescence signal and allows the unambiguous assignment of the Ca$^{2+}$-sensitive spectroscopic properties to Trp-212. This residue is located in the third annexin repeat in a highly conserved stretch of 17 amino acids which are also found in the other annexin repeats characterized so far, a GT(D, N, or R) motif at positions 4-6 (relative counting of the 17 residues of the endonexin fold) and an arginine residue at position 17 are almost invariant. Limited proteolysis experiments revealed that binding sites for the common annexin ligands (Ca$^{2+}$, phospholipid) map to a protease-resistant core domain which comprises the sum of the annexin repeats. In the primary structure this core is preceded by a protease-sensitive N-terminal domain (known as the tail) which is variable in sequence and length. In annexin II, the N-terminal tail harbors the binding site for a unique protein ligand, p11, and also contains the phosphorylation sites for protein kinase C and the src tyrosine kinase (for recent reviews see Refs. 7-9). Since both phosphorylation events occur in vivo, it has been proposed that annexin II is involved in signal transduction during cellular growth and differentiation.

Although 10 different annexins have been identified and sequenced so far, structural analyses have not revealed details as to the architecture of the Ca$^{2+}$- and/or phospholipid-binding sites. While obvious sequence motifs known to form Ca$^{2+}$-binding sites, such as the helix-loop-helix structure (EF-hand) found in Ca$^{2+}$-binding proteins like calmodulin and parvalbumin, are absent from annexin core sequences, it has been speculated that the endonexin fold forms a loop-helix structure involved in Ca$^{2+}$-binding (6). The hypothetical assignment of a Ca$^{2+}$-binding site to the endonexin fold is supported by Tb$^{3+}$ fluorescence studies. Geisow et al. (6) suggested that a Tb$^{3+}$-binding site (presumably identical to a Ca$^{2+}$-binding site) in annexin IV (19) is in close proximity to a tryptophan residue within one endonexin fold. Similarly, Marriott et al. (11) used resonance energy transfer experiments to show that the sole tryptophan of annexin II (Trp-212), which is found in position 10 of the endonexin fold in repeat 3, is located within less than 8 Å of the Tb$^{3+}$ and, by implication, Ca$^{2+}$-binding site. Fluorescence spectroscopy also revealed that the emission maximum from annexin II undergoes a pronounced blue shift upon Ca$^{2+}$- (and Tb$^{3+}$) binding (11-13). Although 1-2 mM Ca$^{2+}$ are required to saturate this blue shift, the affinity for Ca$^{2+}$ is increased more than 100-fold in the presence of phospholipid (14). For the annexin II-p11 complex, membrane and phospholipid binding is observed at micromolar (15) or even submicromolar (16, 17) Ca$^{2+}$ concentrations, indicating that an annexin II-membrane interaction can be expected at intracellular (i.e. submicromolar) Ca$^{2+}$ levels.

Here we have employed the well-defined blue shift in the fluorescence emission maximum to characterize the molecular parameters of a unique Ca$^{2+}$-binding site in annexin II. Using site-directed mutagenesis, several amino acid substitutions were introduced in the endonexin fold of the third annexin repeat. Analysis of these mutants by fluorescence spectrosc-
copy and Ca\(^{2+}\)-dependent phospholipid binding reveals that Gly-206 and Thr-207 seem involved in the correct folding of the Ca\(^{2+}\)-binding site present in the third repeat.

**EXPERIMENTAL PROCEDURES**

**Annexin II cDNA Cloning and Expression in E. coli.—**A λgt 10 cDNA library prepared from HT29 (a human adenocarcinoma cell line) was screened by oligonucleotide-directed mutagenesis following the method of Eckstein and co-workers (21). Oligonucleotides carrying the desired mutations were synthesized on an 8750 Milligen Biosearch DNA synthesizer and purified on denaturing polyacrylamide gels. In *vitro* mutagenesis was performed with a mutagenesis kit (Amersham Buchler, Braunschweig, F. R. G.) according to the manufacturer’s protocol using the annexin II cDNA cloned into M13mp18 as single-stranded DNA template. DNA from recombinant plaques was analyzed by oligo sequencing (22) with T7-polymerase (Pharmacia, LKB, Uppsala, Sweden). Positive clones were amplified and the RF-DNA purified using Qiagen pack 500 (Qiagen Inc., City, Studio, CA). After confirmation of the desired mutation by sequence analysis the replicative form DNA was co-transfected with pBluescript II (after removal of mutations, which eliminated the internal HindIII site, HindIII was used to create the 3′ end of the annexin II cDNA insert). The annexin II cDNA insert was purified by agarose gel electrophoresis and cloned into the pDS 10 expression vector as described above. Other cloning steps were carried out following standard procedures (23).

**Purification of Recombinant Annexin II.—**E. coli JM 101 carrying expression constructs were grown for 14 h at 37 °C in 1.5 liters of LB medium containing 100 mg/ml ampicillin. Inclusion bodies were prepared following the method of Nagai and Thogersen (20) with some modifications. The lysis buffer contained 5 mM EGTA, 1 M sucrose, 50 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM TFA, pH 7.5, 1 mM dithiothreitol, 20 mM EGTA (Sigma Chemical GmbH, Munich, Federal Republic of Germany (F. R. G.)), and 2.5 μM E64 (1-L-trans-carboxyoxirane-2-carboxyl)-1-leu-agmatin; Peptide Institute, Osaka, Japan). Purified inclusion bodies containing the recombinant annexin II were resuspended in 80 mM NaCl, 20 mM imidazole-HCl, pH 7.5, 20 mM NaCl, 1 mM Tris, pH 7.5, and adjusted to a concentration of 1 mg/ml with a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, FRG) in water. Binding experiments were performed in 20 mM imidazole-HCl, pH 7.5, 100 mM NaCl at the Ca\(^{2+}\)-concentrations indicated. Each reaction contained 5 μg of protein and 28 μg of liposomes in a total volume of 150 μl. After incubation at room temperature for 15 min, samples were centrifuged at 200,000 × g for 12 min. Bound protein was extracted from the liposome pellet at room temperature for 20 min with 150 μl of buffer containing 10 mM EGTA instead of Ca\(^{2+}\). The fraction of bound and unbound proteins was determined quantitatively by SDS-polyacrylamide gel electrophoresis and subsequent densitometry of the Coomassie Blue-stained annexin II band.

**RESULTS**

Expression and Biochemical Characterization of Recombinant Human Annexin II.—A full-length CDNA clone for human annexin II was isolated from an HT 29 (an adenocarcinoma cell line) CDNA library made in λgt 10. For cloning
pursues, a unique BamHI site was introduced in the 5' nontranslated region (nucleotide position -11 with respect to the initiator methionine), whereas the internal HindIII site (position 343 of the coding region) was destroyed by site-directed mutagenesis without changing the amino acid sequence. Subsequently, the entire protein-coding region (contained in a BamHI-HindIII fragment) was cloned into the prokaryotic expression vector pDS 10. Transformation of E. coli with this construct leads to the efficient expression of recombinant human annexin II, which is driven by the coli T5 promoter of pDS 10 (Fig. 1A). When synthesized in bacteria, human annexin II is insoluble, but can be extracted from the inclusion bodies by 8 M urea. Following renaturation (achieved by the protocol outlined under "Experimental Procedures") final purification was obtained by ion-exchange chromatography on CM-52 and Mono-S (Fig. 1A). This approach routinely yields 10 mg of pure annexin II from 1 liter of bacterial culture. The recombinant protein is recognized by an annexin II rabbit antiserum (25) but not by the murine monoclonal antibody H28 (26). This antibody detects in immunoblots porcine, bovine, and chicken annexin II but not the murine or human protein. Based on a comparison of annexin II sequences from different species, it has been deduced that residue 65 represents an important contact site for the H28 monoclonal (glutamic acid in reactive annexin II molecules; valine or alanine in non-reactive proteins) (27). By introduction of glutamic acid in place of alanine at position 65, which was achieved by site-directed mutagenesis, we confirmed this prediction. The A65E variant of human annexin II is clearly recognized by the H28 monoclonal (Fig. 1B). For subsequent studies, the A65E variant was used as the wild-type annexin II control; all human annexin II mutations discussed below also contain glutamic acid at position 65.

The purified recombinant annexin II, i.e. the A65E molecule, shows the same biochemical and physicochemical properties as the protein isolated from mammalian cells. It binds to phospholipids in a Ca²⁺-dependent manner (cf. Table I), displays a characteristic CD spectrum which reveals the α-helical conformation (not shown) and shows Ca²⁺-induced conformational changes as documented by fluorescence and UV difference spectroscopy (see below). In addition, the protein core of recombinant annexin II exhibits the characteristic stability toward mild proteolytic attack. Chymotrypsin treatment, for example, converts the bacterially synthesized protein into a 33-kDa species (Fig. 2A). Direct protein sequence analysis revealed that this derivative starts at position 30 of the annexin II sequence (data not shown) and thus represents the typical chymotryptic core (28, 29). Similarly, limited trypsin cleavage produces two major fragments of 20 and 15 kDa that have already been described for porcine annexin II (Fig. 2B) (30). The N-terminal protein sequence of the two tryptic fragments starts at Ala-28 (20 kDa) and Lys-265 (15 kDa) (data not shown) again identical to the situation reported for annexin II purified from porcine intestinal epithelium (30).

Ca²⁺-induced Conformational Changes in Annexin II Can Be Monitored by Fluorescence and UV Absorption Properties of the Single Tryptophan (Trp-212)—Annexin II contains a single tryptophan at position 212. Previous studies have interpreted Ca²⁺-induced differences in UV absorption and fluorescence emission of annexin II solely in terms of this tryptophan (11, 13). In particular, this chromophore appeared responsible for the blue shift in fluorescence emission and a number of negative UV difference peaks which are observed upon Ca²⁺ binding (for discussion of these vibronic structures, see Marriott et al., 1990). All these spectroscopical analyses point to a close proximity of a Ca²⁺ site and the single tryptophan which seems to reside in a highly non-polar environment (11). In order to unambiguously identify Trp-212 as the residue responsible for the effects discussed, we employed site-directed mutagenesis to introduce a phenylalanine in place of the tryptophan at position 212 (Figs. 3 and 5). The bacterially synthesized mutant protein was purified following the protocol developed for wild-type, i.e. A65E, annexin II.

**Fig. 1. Synthesis and purification of recombinant annexin II.** Panel A, proteins present in total bacterial lysates (lanes 2 and 3) or different fractions of the purification protocol (lanes 4-9) were separated in SDS-polyacrylamide gels and stained with Coomassie Blue. Lane 1, porcine annexin II standard purified from intestinal epithelium and separated from its p11 ligand. Lane 2, total proteins from E. coli transformed with the expression plasmid pDS 10. Lane 3, total proteins from E. coli transformed with the pDS10-A65E construct. The identical E. coli clone, grown at increased aeration (lane 4, note the high level of annexin II synthesis under these conditions), was used as starting material for the inclusion body preparation. Soluble cellular proteins (lane 5) were separated from the inclusion bodies (lane 6). Annexin II present in the inclusion bodies was further purified on Q-Sepharose (lane 7) and CM-52 (lane 8) in the presence of 8 M urea. After renaturation, final purification was achieved by CM-52 and Mono-S chromatography (lane 9). Panel B, human annexin II (lanes 1 and 3) and the A65E variant (lanes 2-4) were synthesized in bacteria and purified as shown in panel A. Equivalent amounts were run in SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblotting using an annexin II rabbit antiserum (lanes 1 and 2) or the H28 monoclonal antibody (lanes 3 and 4). Note that the monoclonal only recognizes the A65E variant. Arrows mark the position of the annexin II (p36) polypeptide chain.
**TABLE I**

| Mutants | Ca" conc. at half-maximal binding |
|---------|----------------------------------|
| A65E    | 4 ᅹ M                             |
| G206A   | 15-20 ᅹ M                          |
| T207A   | 10 ᅹ M                            |
| D208A   | 4 ᅹ M                             |
| D208N   | 5-10 ᅹ M                           |
| S214A   | 4 ᅹ M                             |

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Wild-type (A65E) annexin II and different repeat 3 mutants were assayed for binding to phosphatidylserine (PS) liposomes. Reactions were performed in buffers containing increasing Ca\(^{2+}\) concentrations and the fraction of bound and unbound protein was determined by SDS-polyacrylamide gel electrophoresis (see "Experimental Procedures"). The Ca\(^{2+}\) concentrations required for binding to the PS-liposomes were determined in several independent experiments for each mutant protein. To eliminate a potential influence of the N-terminal tail of recombinant annexin II all assays were carried out with protein cores generated by limited n-chymotrypsin treatment (7 min at room temperature, enzyme/substrate ratio of 1:100).

Panel A, UV difference spectra of A65E in the absence (a) and presence (b) of Ca\(^{2+}\) which were added after recording of the base line (c). Note that the structural spectrum is only induced in the case of wild-type annexin II. Panel B, fluorescence emission spectra of A65E in the absence (a) and presence (b) of Ca\(^{2+}\) and of the W212F mutant (c). The excitation wavelength was set at 295 nm. Note the characteristic Ca\(^{2+}\)-induced blue shift in the fluorescence emission of A65E and the absence of any signal from W212F.
tryptophan residue in the same relative position of the endonexin folds in repeats 1, 2, and 4. All mutant proteins were constructed to contain a single tryptophan/molecule, i.e. the original tryptophan 212 was substituted by a phenylalanine (Fig. 5). The different mutant proteins were synthesized in bacteria and purified as described above. They displayed the same biochemical properties as wild-type annexin II. Only the protein core of I56W showed a somewhat reduced resistance toward chymotrypsin attack (data not shown). Fluorescence emission spectra of the tryptophan mutants are given in Fig. 5. With excitation at 295 nm, the emission maxima are found at 320 nm (I56W), 326 nm (L127W), and 333 nm (L287W), respectively. Thus, the tryptophan residues situated in the same relative position of the endonexin fold in repeats 1, 2, 3, and 4 (position 10 of the fold) reside in different environments. Interestingly, the three tryptophan mutants show no or only a very minor Ca²⁺-induced alteration in the fluorescence emission spectra. While the spectra of I56W and L287W remain unchanged, the intensity of the fluorescence emission of L127W is slightly reduced in the Ca²⁺-bound conformation (data not shown). Thus, only the naturally occurring tryptophan of annexin II (Trp-212) resides in an environment that is clearly different in the Ca²⁺-bound and the Ca²⁺-free molecule.

**Structural Characterization of the Ca²⁺-binding Site in the Third Annexin Repeat**—Since previous energy transfer studies had indicated a close proximity of a Ca²⁺-binding site and Trp-212 in annexin II (11), we chose the endonexin fold of the third repeat to study the effect of single amino acid substitutions on Ca²⁺ binding. The mutant proteins listed in Fig. 3 were purified from bacterial inclusion bodies and subjected to fluorescence spectroscopy with the excitation wavelength set at 295 nm. All spectra show maxima at around 321 nm in the absence of Ca²⁺ (Fig. 6). Thus, Trp-212 in the different repeat 3 mutants is located in a similar environment, indicating that all mutant proteins assume the correct conformation upon renaturation. This conclusion is also supported by the finding that the different mutants exhibit the same resistance toward proteolysis as wild-type annexin II, i.e. a typical protein core is produced by mild chymotryptic treatment. However, a remarkable difference between the wild-type molecule and some of the repeat 3 mutants is seen when the fluorescence emission spectra are recorded in the presence of varying Ca²⁺ concentrations. While wild-type annexin II (Fig. 6) as well as the S214A protein (data not shown) display the typical blue shift in the fluorescence emission at free Ca²⁺-concentrations of 1–2 mM, the other mutant molecules (G206A, T207A, D208A, and D208N) require considerably higher Ca²⁺ levels for the same effect (Fig. 6). The strongest difference is seen with the T207A mutant. Here, even the addition of 20 mM Ca²⁺ to the protein solution is not sufficient to produce a pronounced blue shift.

The combined data on the Ca²⁺ titration of the fluorescence emission shift are summarized in Fig. 7. Four different types of mutations can be distinguished. 1) The S214A mutation, which has eliminated the hydroxyl function of the conserved serine (or threonine) residue usually found in position 12 of the endonexin fold, does not cause significant perturbations in Ca²⁺ binding. Less than 2 mM Ca²⁺ are required to shift the fluorescence emission maximum of the S214A protein from 321 nm (Ca²⁺-free conformation) to 312 nm (Ca²⁺-bound conformation). The Ca²⁺ titration curve of S214A is almost identical to that of wild-type annexin II. 2) The D208A protein is still able to bind Ca²⁺, albeit with reduced affinity. It requires 8 mM Ca²⁺ to display the blue shifted emission maximum, i.e. to assume the Ca²⁺-bound conformation. 3) The D208N and, in particular, the G206A mutant proteins show a markedly reduced affinity for the divalent cation. In both cases, more than 20 mM are necessary to establish a significant blue shift. (4) The T207A mutation, finally, causes the most severe effect. Even 20 mM Ca²⁺ is not sufficient to induce a significant blue shift in the emission spectrum of the T207A protein.

To obtain a set of independent data which allow a direct comparison of Ca²⁺ affinities of the different repeat 3 mutants, we performed a series of Ca²⁺-dependent liposome pelleting assays. In these experiments, a Ca²⁺ titration of the liposome binding of different annexin II mutants was employed to evaluate their relative Ca²⁺ affinities in comparison to the wild-type molecule. We chose this approach instead of Ca²⁺-
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**Fig. 6.** Fluorescence emission spectra of different repeat 3 mutants. Emission spectra of three mutant proteins (G206A, T207A, D208N) are compared to wild-type (A65E) annexin II. The spectra were recorded for the different proteins in Ca\(^{2+}\)-free buffer (0.02 mM EGTA) or buffer containing increasing concentrations of Ca\(^{2+}\) (1, 2, 5, 10, 15, 20 mM Ca). Excitation wavelength was 295 nm. Excitation and emission band widths were set at 4 nm.

**Fig. 7.** Ca\(^{2+}\) titration of the blue shift in fluorescence emission of different repeat 3 mutants. Fluorescence emission spectra (with excitation at 295 nm) were recorded for wild-type annexin II (A65E) and the different repeat 3 mutants in buffers containing increasing levels of Ca\(^{2+}\). The position of the emission maximum is given as a function of the Ca\(^{2+}\) concentration.

Binding measurements by equilibrium dialysis since the latter method only revealed conflicting data for different annexins so far, e.g. annexins I and II were found to contain in the presence of phospholipids either four or only two Ca\(^{2+}\)-binding sites with dissociation constants of 75 and 4.5 \(\mu\)M, respectively (14, 33). Since the presence of phospholipid increases the affinity for the divalent cation by at least two orders of magnitude (14), Ca\(^{2+}\) levels in the micromolar range were employed in the liposome pelleting assay. Table I compares the Ca\(^{2+}\) concentrations that are required by the different mutants to promote half-maximal binding to phosphatidylserine liposomes. Experiments were performed with protein cores obtained by limited chymotrypsin treatment, i.e. the domain comprising the four annexin repeats (residues 30-338). Wild-type annexin II, as well as the D208A and the S214A mutants exhibit half-maximal binding at 4 \(\mu\)M Ca\(^{2+}\), a value also obtained with the chymotryptic core of porcine annexin II (not shown). The G206A, T207A, and D208N mutants, on the other hand, require 2-5-fold higher Ca\(^{2+}\) concentrations for half-maximal phospholipid binding. However, even the T207A protein, which most likely contains an inactive binding site in the endonexin fold of the third repeat (see above), is still able to interact with the phosphatidylserine liposomes in a Ca\(^{2+}\)-dependent manner.

**DISCUSSION**

Wild-type and site specifically mutated annexin II molecules were produced in *E. coli* to compare their structural and biochemical properties. The Ca\(^{2+}\)-dependent phospholipid binding, proteolytic cleavage pattern, as well as physicochemical properties of the recombinant wild-type annexin II resemble those of the authentic protein purified from mammalian cells. Thus, the approach presented, i.e. a mutational analysis of annexin II synthesized in bacteria, is valid to study the structure of this particular annexin.

The replacement of the single tryptophan in annexin II (Trp-212) by a phenylalanine led to the unambiguous assignment of the Ca\(^{2+}\)-sensitive spectroscopic properties to this tryptophan residue. Thus, our data confirm conclusions drawn in previous spectroscopical studies (11-13). With excitation at 295 nm, it is indeed the single tryptophan which absorbs energy and shows the characteristic fluorescence emission maxima at 321 nm in the absence and 311 nm in the presence of Ca\(^{2+}\). Using energy transfer experiments, Marriott et al. (11) located the Ca\(^{2+}\)-binding site, whose occupation induces the described shift in the fluorescence emission maximum, to within \(-8\AA\) of Trp-212. By studying both the fluorescence properties and the Ca\(^{2+}\) requirements for liposome binding of different annexin II point mutants, we now show that the residues Gly-206, Thr-207, and Asp-208 seem involved in the formation of this Ca\(^{2+}\)-binding site. However, with the experiments described here we are not able to distinguish whether (a) side-chain oxygen of Thr-207 and/or Asp-208 are coordinating the Ca\(^{2+}\) ion, (b) free carbonyl electrons of peptide bonds between amino acids 205 and 209 are involved in Ca\(^{2+}\) complexation, or (c) the GTD sequence (amino acids 206-208) is indispensable for the correct folding of the Ca\(^{2+}\) site. It seems likely, however, that at least the Asp-208 side chain...
is not directly involved in Ca\(^{2+}\) coordination since D208A shows only a mild defect in Ca\(^{2+}\) binding, whereas D208N (a mutant still containing a side chain with free electron pairs in position 208) is markedly impaired.

When our analysis was complete Huber et al. (31) reported the X-ray structure for human annexin V. Annexin V was shown to be an extraordinary compact molecule in which each annexin repeat is composed of five densely packed \(\alpha\)-helices. Within the repeats each endonexin fold follows an \(\alpha\)-helix (helix \(a\)) and describes a short loop (residues 1/2-5/6 of the fold) followed by another \(\alpha\)-helix (helix \(b\), residues 5/6-17). Interestingly, the side chain of the hydrophobic residue in position 10 of the endonexin fold is surrounded by amino acids of different hydrophobicity in each of the repeats 1, 2, 3, and 4. This aspect of the annexin V structure is in line with our data on the different tryptophan mutants of annexin II. Our fluorescence spectra reveal different emission maxima for I65W, L127W, L287W, and wild-type annexin II (Fig. 5), indicating that the tryptophan positioned as residue 10 of the endonexin fold clearly resides in different environments in the four annexin repeats.

Unfortunately, the conformation of the endonexin fold in repeat 3 of annexin II cannot be deduced from a simple extrapolation of the annexin V crystal structure. Despite a very good overall similarity, the primary and most likely also the secondary and tertiary structure of annexins II and V seem to diverge in the third repeat. In particular, the loop described by the first part of the endonexin fold and the two flanking helices is clearly different. While helix \(a\), i.e. the helix preceding the endonexin fold, ends in an ELK sequence in annexin V, the corresponding region shows a cluster of basic residues (KRK) in annexin II. In addition, the beginning of helix \(b\), which is characterized by a row of acidic residues (DEE) in annexin V, reads DVP in annexin II. Although these differences leave ambiguities in interpreting the annexin II structure in the third repeat, our data suggest that Gly-206 and Thr-207 are part of a loop likely to be involved in Ca\(^{2+}\) binding. This view is also supported by the finding that the peptide bond between residues Arg-204 and Lys-205, i.e. the 2 residues directly preceding Gly-206, is the only bond in the annexin II core which is susceptible to limited trypsin treatment (30). Similar loops which are described by the other three endonexin folds reside in relatively close proximity on one side of the molecule (31). This configuration could explain the mutual influence of Ca\(^{2+}\) and phospholipid binding and might also be the basis for some cooperativity between Ca\(^{2+}\) sites. Future experiments have to reveal whether in the three-dimensional conformation of annexin II phospholipid and additional Ca\(^{2+}\) sites are indeed found in the vicinity of Gly-206 and Thr-207.

Acknowledgments—We thank Dr. T. Jovin for access to the spectrofluorometer, U. Plessmann for help with protein sequence analysis, and H.-P. Geithe for oligonucleotide synthesis. Drs. D. Louvard and M. Arpin (Pasteur Institute, Paris) kindly provided the HT29 cDNA library. Thanks also to Dr. N. Jonsson and members of the annexin laboratory for fruitful discussions.

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