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Mutational analysis of the putative PLA_2-inhibiting sequence of annexin 1

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Annexin 1 has been proposed to inhibit phospholipase A_2 by direct interaction through a specific amino acid sequence spanning residues 246–254. The possible role of this region was investigated by protein engineering. Three point mutations and a deletion have been performed. The four mutant proteins have been expressed in *E. coli*, purified and tested for calcium and lipid binding, and for phospholipase inhibition. All mutant proteins conserved the properties of the wild-type recombinant protein. This result clearly demonstrates that this part of the molecule is not involved in the inhibition of phospholipase A_2.

Annexin 1; Site-directed mutagenesis; Phospholipase A_2 inhibition

1. INTRODUCTION

Annexins are a family of proteins known to bind acidic phospholipids in a calcium-dependent manner [1]. Annexin 1 (lipocortin1) has been reported to inhibit phospholipase A_2 in vitro [2]. As it is suspected to be inducible in some cell lines by glucocorticoids, (although this observation is largely controversial [3,4]), annexin 1 has been proposed to be the mediator of the anti-inflammatory effect of these drugs. To test this hypothesis some authors initiated very precise studies on the kinetics of PLA_2 inhibition by annexins. The insoluble nature of the phospholipid substrate complicates this kind of studies so that different experimental models were developed. Davidson et al. [5] demonstrated that the inhibition could be overcome by increasing the phospholipid substrate concentration, suggesting that annexins inhibit PLA_2 by substrate depletion rather than by direct interaction with the enzyme. On the other hand, Miele et al. [6] designed 3 short synthetic peptides exhibiting potent PLA_2-inhibiting properties in vitro. These nonameric, α-helical peptides were deduced from sequence comparison between annexin 1 and uteroglobulin, another PLA_2 inhibitor. The authors suggested that the peptides inhibited PLA_2 by direct interaction with the enzyme.This result is not easy to reproduce [7], and it strongly depends on the assay conditions [8]. These last observations raise the question of real significance of this sequence inside the whole molecule of annexin 1. In order to answer this question we performed mutations on the specific zone corresponding to the peptides. The mutant proteins have been produced by genetic engineering, purified and checked for PLA_2 inhibition.

Abbreviations: PLA_2, phospholipase A_2; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *cDNA cloning*

A cDNA λgt10 library was constructed using the U937 cell line. This library was screened using two oligonucleotides deduced from the published cDNA sequence of human annexin 1 [9]: GGAATTCTGATACCATTGCC (5′ region) and CTCCACAAAGAGCCACCAGG (3′ region). Two overlapping clones were obtained and used to reconstruct the complete cDNA with the common central site*Bgl*II.

2.2. *Mutant construction*

Mutants were constructed using the PCR-mediated method as described [10], by means of 2 oligonucleotides containing a *Nco*I site and corresponding to the 5′ and 3′ extremities, and a third oligonucleotide containing the required mutation. The PCR fragment obtained was digested and cloned into the *Nco*I site of pKK233-2 (Pharmacia). The mutation and the whole sequence of each clone were verified by the Sanger dideoxy method using different oligonucleotides spred over the cDNA sequence.

2.3. *Purification of recombinant annexins*

Wild type and mutant annexins were expressed in *E. coli* and purified from the soluble fraction of the bacterial sonicate by the liposomes-mediated method of Kaplan et al. [11]. The liposomes were composed of an equimolar mixture of phosphatidyl serine, phosphatidyl ethanolamine and cholesterol, and were obtained as described [12]. The relative quantities of the proteins purified in this manner were estimated by scanning SDS-PAGE-migrations of each protein using a Shimatzu apparatus.

2.4. *Purification of the calcium and lipid binding fraction of U937 cells*

Differentiated U937 cells (results not yet published) were pelleted, then resuspended in 5 mM Tris, pH 7.5, 0.5 mM EGTA, 1 mM PMSF

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at 0°C. This mixture was gently vortexed to allow only partial disruption of the cells, then centrifuged at 30 000 × g. The supernatant was then treated exactly like the soluble bacterial fraction in section 2.3.

2.5 *Dose–response measurements of anti-PLA$_2$ activity*

The EGTA eluates from liposomes were tested for PLA_2 inhibition by the method of Rothut et al. [13], using [^{3}H]-labeled *E. coli* membranes as a substrate. The conditions were those described [5] with minor modifications: 10 µl of substrate and 150 ng of PLA_2 were used. There were controls to test the influence of the EGTA elution buffer and of PLA_2 or inhibitor alone. The inhibition was quantified by the following ratio:

$$I = [R(S+P) - R(S+P+I)]/[R(S+P) - R(S)]$$

Where $R(S)$ = ^{3}H cpm released in an assay with substrate alone; $R(S+P)$ = ^{3}H cpm released in an assay with substrate plus phospholipase; and $R(S+P+I)$= ^{3}H cpm released in an assay with substrate plus phospholipase plus inhibitor.

3. RESULTS AND DISCUSSION

Miele et al. [6] have defined a 'core' tetrapeptide Lys–Val–Leu–Asp common to all their active peptides. This sequence is a fragment of a classical amphipatic α-helix with 2 charged residues facing the solvent and 2 hydrophobic residues buried inside the protein. An extrapolation, by sequence comparison, of the recently published cristallographic structure of annexin 5 [14] for annexin 1 confirms the peripheral position of this helix. In the hypothesis of an inhibition mediated by protein–protein interaction the 2 charged residues should be extremely important since they are exposed to the solvent and they are able to engage ionic interactions. We thus performed the double point mutation Ly^{250}→Thr, Asp^{253}→ Asn, turning the 2 charged residues into neutral but polar ones. This mutation allows the removal of the charges without affecting the local helical structure. Two other point mutations affect the NH_2 extremity of the sequence described by Miele et al [6]. One is a single mutation abolishing a negative charge, Asp^{247}→ Asn; the other is a double mutation that abolishes the same residue but conserves the local charge, Asp^{247}→ Gly and Asn^{249}→ Asp. The fourth mutation is a deletion of five amino acids, Asn^{249}–Lys–Val–Leu–Asp^{253}. Four of these constitute the 'core tetrapeptide' Lys–Val–Leu–Asp common to uteroglobin, annexin 1, and all the active peptides synthesized. If this sequence is really involved in PLA_2 inhibition, its deletion is expected to induce noticeable changes to the activity of annexin 1.

The 4 mutant proteins, and the wild-type recombinant protein as a control, were expressed and purified on liposomes. As shown in Fig. 1, the 5 proteins were recovered in the same yield, indicating that these mutations affect neither the expression, the stability, nor the binding of the protein to lipids and calcium under the conditions we used.

The ability of the different liposome-purified proteins to inhibit PLA_2 was studied by dose–response experiments using ^{3}H-labeled *E. coli* membranes. To ensure that we really measured the activity of recombinant annexins and not that of some bacterial contaminant we made 2 kinds of controls. As a negative control we performed the liposome-mediated calcium-dependent extraction on the same bacterial strain containing the wild-type expression plasmid pKK233-2 devoid of the insertion coding for annexin 1: no PLA_2-inhibiting activity was found in the extract. As a positive control the

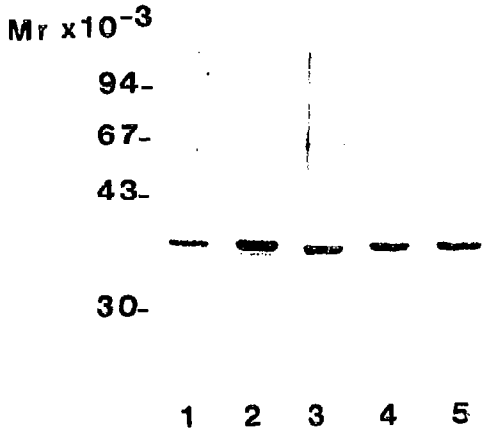

Fig. 1. SDS-PAGE migration of the purified mutant proteins. The mutant proteins were purified from *E. coli* using their calcium-dependent liposome binding properties as described in Materials and Methods. The proteins were analysed on a 12.5% gel stained with Coomassie brilliant blue. Each sample corresponds to the quantity obtained from 1 ml of an IPTG-induced *E. coli* culture. Lane 1, wild-type recombinant annexin 1; lane 2, mutation (K^{250}→T, D^{253}→N; lane 3, deletion (N^{249}KVLD^{253}); lane 4, mutation (D^{247}→N); lane 5, mutation (D^{247}→G, N^{249}→D).

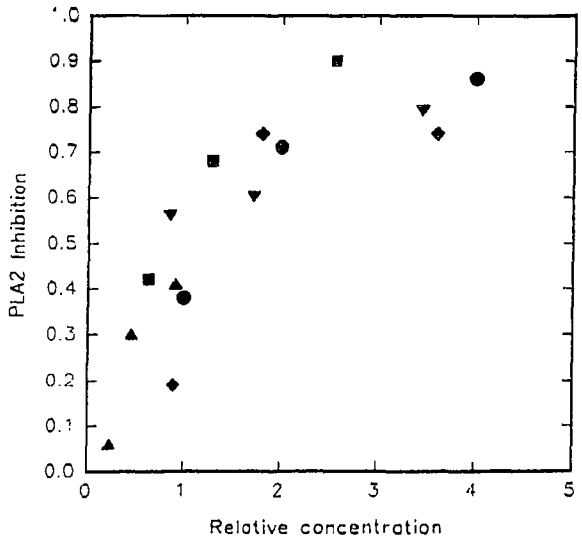

Fig. 2. Dose-response of phospholipase inhibition by the mutant proteins. The inhibition values are calculated from one representative experiment as described in Materials and Methods. At least 3 experiments were performed on different days for each protein. (♦) Wild-type recombinant annexin 1; (●) mutation (K^{250}→T, D^{253}→N; (▼) deletion (N^{249})KVLD^{253}); (▼) mutation (D^{247}→ N); (■) mutation (D^{247}→ G, N^{249}→D).

same kind of extraction was performed on differentiated U937 cells. We thus obtained a calcium-dependent lipid-binding fraction containing the annexin 1 expressed in these cells. SDS-PAGE of this fraction showed 3 major bands between 32 and 35 kDa. As expected, this fraction exhibited PLA_2-inhibiting activity. For a same quantity of protein, estimated by scanning analysis of the gel, the bacterial extract was found to be three time more active than the cellular extract. This is a logical result considering that annexin 1, as shown by electrophoresis, should represent only 30% of the extract since it is contaminated by other proteins which are not all phospholipase inhibitors.

Unexpectedly, the profiles we observed are identical for the 4 mutant proteins and the bacterial control (Fig. 2). This result unambiguously indicates that the different mutations we made on the inhibitor peptide-related sequence have no effect on the in vitro inhibition of PLA_2 by annexin 1. If this region was (even partly) implicated we might have noticed some modifications. The fact that the deleted mutant proteins conserve the intact properties of the wild-type protein specifically argues against an inhibition by contact through this zone as was suggested by Miele et al. [6]. On the other hand, our results could be explained by the substrate-depletion hypothesis, since no other interacting region has yet been proposed. Another interesting point is how the peptide itself inhibits PLA_2 in some in vitro and in vivo assays. Newman et al. [8] recently demonstrated that the ability of the synthesized peptides to inhibit PLA_2 was linked to their capacity to penetrate the lipid monolayer. One must keep in mind that all the residues of a peptide are able to engage interactions, although the same sequence included inside a protein has only a few residues exposed. In the synthesized peptides, the hydrophobic residues (which are buried in annexin 1), are probably involved in the penetration of the lipid monolayer and perhaps in some perturbations of the interaction PLA_2/substrate. To check this possibility further experiments can be carried out such as testing the PLA_2-inhibiting ability of other amphipathic peptides.

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