Volume 285, number 1, 59–62 FEBS 09871 July 1991

ADONIS 001457939100577F

Characterization of a discontinuous epitope on annexin II by site-directed mutagenesis

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Received 24 April 1991

Recombinant annexin II mutants were generated to identify amino acids involved in the formation of the discontinuous epitope of the monoclonal antibody H28. Analysis of the various mutant proteins by immunoblotting and enzyme-linked immunosorbent assay revealed that residues Lys^{27}, Arg^{62}, Glu^{65}, and Arg^{67} are indispensable for H28 reactivity. Residues in equivalent positions are also in close proximity in the recently determined X-ray structure of annexin V, a different member of the same family of Ca^{2+}/lipid-binding proteins. Thus annexins II and V show a similar three-dimensional folding in this region of the molecule. Consequently, the Ca^{2+} binding sites and the residues phosphorylated by $pp60^{src}$ (Tyr^{23}) and protein kinase C (Ser^{25}) most likely reside on opposite sides of the annexin II molecule.

Ca^{2+}/phospholipid-binding protein; Calpactin; Lipocortin; Tyrosine phosphorylation

1. INTRODUCTION

Increasing interest has focused on a still growing family of Ca^{2+}- and phospholipid-binding proteins. Originally referred to as lipocortins, calpactins and endonexins, they are now generally termed annexins [1]. All members of this family consist of segments of 70–80 amino acids, which are repeated either 4 times (32–39 kDa annexins) or 8 times (68 kDa annexin) and comprise the so-called core (for review see [2–6]). The individual segments (also known as annexin repeats) share a high degree of sequence homology which is particularly pronounced over a stretch of 17 amino acids known as the 'endonexin' fold [7]. The annexin core is defined by its resistance to limited proteolysis. It displays a remarkably high α-helix content [8] and harbors the binding sites for Ca^{2+} and phospholipid [9–11] which partly reside within the endonexin fold [12,13]. In each annexin, the core is preceded by a protease-sensitive N-terminal tail which is variable in sequence and length.

Annexin II, one member of the family, was recognized as a major cellular substrate for the tyrosine protein kinase encoded by the *src* oncogene [14–16]. Phosphorylation occurs at Tyr^{23} in the N-terminal tail [17], which also harbors a protein kinase C site (Ser^{25}) [18,19]. The tail of annexin II also contains the binding site for the cellular protein ligand p11 [9,10,19–21]. Both p11 binding, which leads to the formation of a $p36_2p11_2$ heterotetramer, and phosphorylation by $pp60^{src}$ alter properties displayed by annexin II, e.g. annexin II phosphorylated at Tyr^{23} has an increased Ca^{2+}-requirement for phospholipid binding when compared with the non-phosphorylated form [22]. Thus the N-terminal tail seems to regulate physiological functions of annexin II, which are thought to include membrane fusion events during exocytosis and/or the membrane–cytoskeletal linkage (for review see [23,24]).

A structural basis for the regulatory role of the N-terminal tail on properties displayed by the core stems from the preliminary mapping of a discontinuous epitope. Johnsson et al. [25] showed that residues 25–28 of the tail, as well as residue 65, which is close to the first endonexin fold of the core, are involved in the formation of the epitope for the monoclonal antibody H28. Here we have generated site-specifically mutated annexin II molecules which allow the precise description of the H28 epitope. Our results show that in the region of the H28 epitope, the three-dimensional folding of annexin II must be highly similar to that of annexin V, which was recently determined by X-ray diffraction [8,12]. Thus the phosphorylation sites (Tyr^{23}, Ser^{25}) and the Ca^{2+}-binding sites most likely reside on different sides of the molecule, and an enhanced Ca^{2+}-requirement in phospholipid binding of the $pp60^{src}$ phosphorylated annexin II [22] must be explained by phosphorylation-induced conformational changes.

Abbreviations: cDNA, complementary DNA; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; $pp60^{v\text{-}src}$, tyrosine kinase encoded by the viral src gene; RT, room temperature; SDS, sodium dodecyl sulfate.

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2. MATERIALS AND METHODS

Cloning and purification of human recombinant annexin II have been described [13]. Briefly, the complete human cDNA was ligated into the expression vector pDS 10 [26] and transferred into *E. coli* JM 101 using standard procedures. The insoluble recombinant protein was recovered as inclusion bodies [27], dissolved in buffer containing 9 M urea and treated batchwise with Q-Sepharose (Pharmacia LKB) and CM-52 (Whatman). Following renaturation by dialysis against renaturation buffer (20 mM imidazole-HCl, pH 7.5, 100 mM NaCl, 2 mM NaN_3, 1 mM EGTA, 1 mM DTT) further purification was carried out as described for the isolation of annexin II from porcine intestine [16]. In vitro mutagenesis was performed following the method of Eckstein and co-workers [28] using an Amersham kit. Positive clones were characterized by dideoxy sequencing using a T7-sequencing kit (Pharmacia).

The α-chymotryptic core of human recombinant annexin II was obtained with an enzyme to protein ratio of 1:75 at room temperature for 20 min [10]. Digestion was stopped with 2 mM phenylmethylsulfonylfluoride.

Due to the cloning strategy, all recombinant proteins start with the sequence MRGSFK prior to the annexin II coding sequence. These additional 6 residues are introduced by the pDS 10 expression vector. The N-terminal sequences of the recombinant annexin II and the chymotryptic core were confirmed by direct protein sequencing on an Applied Biosystems gas-phase sequenator (model 470A). Immunoblotting [29] and enzyme-linked immunosorbent assays [30] were carried out using standard procedures. The murine monoclonal antibody H28 has been described [31]. Peroxidase labelled swine anti-mouse IgGs (Dako) were used as second antibodies.

3. RESULTS

The previous proposal that the murine monoclonal antibody H28 recognized a discontinuous epitope involving at least Glu^{65} and one or more residue(s) between Ser^{25} and Lys^{27} of porcine annexin II was essentially based on two sets of observations. Proteolytic derivatives lacking less than 25 residues at the N-terminus retained reactivity in Western blots, while the loss of an additional 2 residues yielded non-reactive annexin II cores. The limited cross-species reactivity of H28 seemed explained by the finding that the reactive

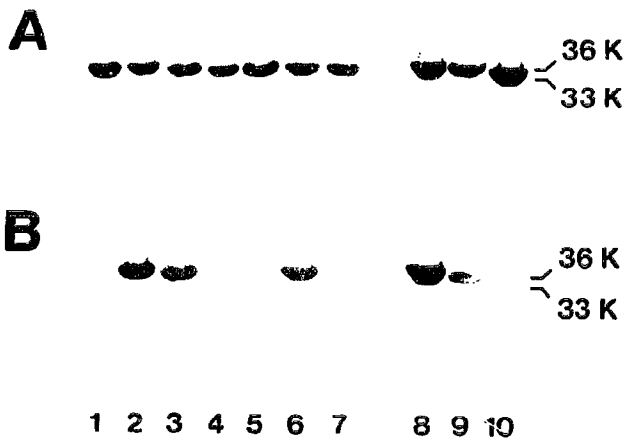

Fig. 2. Western blot analysis of different annexin II mutants. (A) Poinceau staining of the proteins separated in 12.5% SDS polyacrylamide gels and transferred to nitrocellulose, (B) immunostaining of the nitrocellulose filters shown in A with H28 and peroxidase coupled anti-mouse IgGs. Lanes: 1, human wild type A65; 2 S25D; 3, V26A; 4, K27A; 5, R62Q; 6, A65E; 7, R67M; 8 and 9 correspond to 6 and 7 on a second blot; 10, A65E chymotryptic core.

annexins II of pig, cow and chicken display a glutamic acid in position 65, while in the non-reactive annexins II of man and mouse, residues 65 is an alanine (in the former case) or a valine (in the latter) [25]. To assess these predictions and to identify the H28 epitope in more detail, we have generated recombinant human annexin II and several mutant derivatives (Fig. 1). Recombinant proteins were expressed in *E. coli*, purified to homogeneity and analyzed by immunoblotting and ELISA.

Fig. 2 shows that the recombinant human annexin II is not recognized by H28 in immunoblotting. When a single amino acid replacement, substituting glutamic acid for Ala^{65}, is introduced, the resulting protein (A65E) is fully reactive. This reaction is lost when the

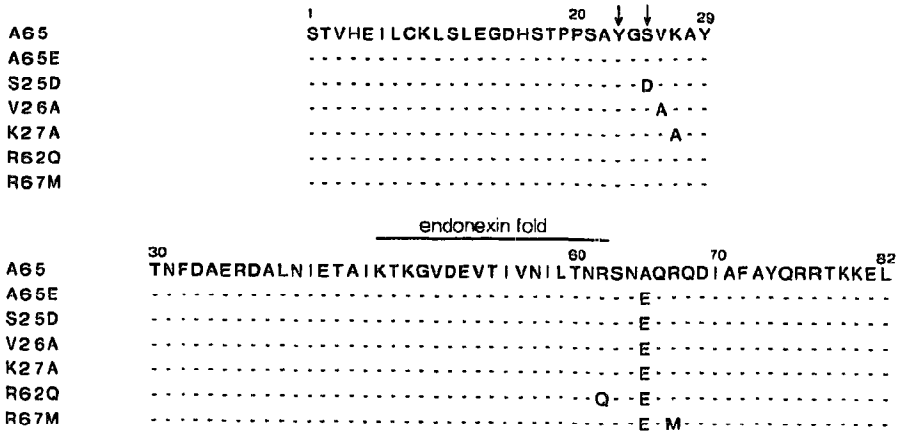

Fig. 1. Amino acid substitutions in different annexin II mutants. The N-terminal 82 residues of wild-type human annexin II are given in the upper row (A65). Substitutions present in the different mutants are indicated below.

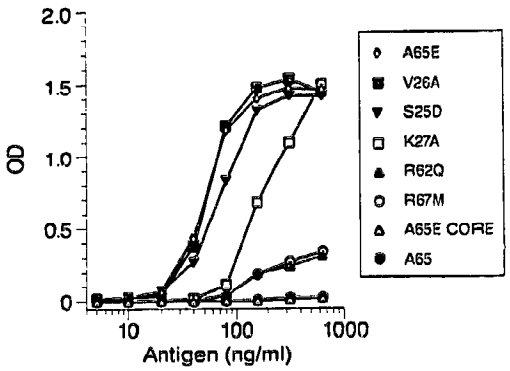

Fig. 3. ELISA of the different annexin II mutants and the H28 monoclonal antibody. Purified proteins were bound overnight at 4°C to Dynatech microtiter plates with concentrations ranging from 5 to 640 ng/ml. Saturation and antibody incubations were performed for 2 h in 10% horse serum. 2 mM PMSF was added at all steps. The dye reaction using 1.2-phenylenediamine was analyzed measuring the optical density (OD) with a Dynatech photometer.

N-terminal 28 residues are removed by chymotrypsin (compare slots 1,6,8 and 10). Based on this result, all further annexin mutants were derived from the A65E clone, i.e. position 65 was kept as glutamic acid. Fig. 2 shows that R62Q has no H28 reactivity, while R67M and K27A react only very weakly with the antibody. In contrast, S25D and V26A display a reactivity comparable to that of the A65E parent molecule.

All recombinant annexins were also directly assayed as native proteins in an ELISA (Fig. 3). The nearly identical titration curves of A65E and V26A were only moderately lowered in S25D. The two mutants R67M and R62Q showed a low reactivity, which became detectable only at high concentrations. Wild-type human annexin II (A65) and the core of A65E reacted only at background levels. The sole difference between immunoblotting and ELISA concerns mutant K27A, which showed moderate reactivity in the ELISA and hardly any reaction in blotting.

4. DISCUSSION

Use of recombinant annexin II molecules and single point mutations introduced by site-directed mutagenesis confirms and greatly extends the previous characterization of the discontinuous H28 epitope [25]. Most importantly, substitution of alanine 65 in the non-reactive human annexin by glutamic acid, the residue present in the reactive species from pig, cow and chicken, provides a fully reactive molecule. In addition to glutamic acid 65 we have now identified arginines 62 and 67 as further contact points. The second part of the epitope involving the segment around residues 25 to 27 is indicated by the finding that a proteolytic derivative starting at position 25 is fully active, while derivatives shortened by two or four additional residues have lost reactivity (see also Figs. 2 and 3). Point mutations in this region have either no effect (V26A), a very small reduction in affinity (S25D) or only a moderate effect (K27A). Thus in this region the antibody may detect the conformation of the protein and/or the protein backbone rather than a specific side chain.

The discontinuous H28 epitope is easily understood with the recently established X-ray structure of annexin V [8] which is a different member of the protein family showing 42% sequence identity to annexin II. This sequence homology, as well as the H28 epitope mapping, allow the prediction that annexin II has a very similar three-dimensional folding. In the annexin V structure, each of the four annexin repeats is built by 5 tightly packed helices (a to e) connected by short turns. The lower concave surface of the folded annexin V molecule carries the N-terminal tail [8], while presumptive Ca^{2+} binding sites locate to the upper convex surface [12]. Lys^{27}, Arg^{62}, Glu^{65} and Arg^{67}, the currently identified positions of the H28 epitope, correspond in the linear sequence alignment to residues 10,45,48 and 50 of annexin V [32]. In the crystal structure of annexin V, the α-carbon distances between positions 10 and 45 or 48 are 6.8 Å and 9.8 Å respectively (R. Huber, personal communication). Since this is well within the range of an antibody binding site (for review see [33]), the equivalent residues in annexin II (positions 27 and 62 or 65) are likely to be folded in a similar way. Thus Lys^{27} is situated 6 residues prior to helix 1a, Arg^{62} close to the C-terminal end of helix 1b and Glu^{65} and Arg^{67} in the N-terminal part of helix 1c. All residues involved in the formation of the epitope are located on the concave side of the molecule.

The similar structure of annexin II and V raises an interesting problem as to the enhanced Ca^{2+}-requirement in phospholipid binding of annexin II phosphorylated at Tyr^{23}. While this residue lies on the concave side, Ca^{2+} binding sites are thought to occur exclusively on the convex side [12]. Thus it seems that a conformational change induced by phosphorylation is responsible for the altered Ca^{2+}/lipid-binding properties. Whether this conformational change involves the pore, which transverses the annexin molecule and is thought to be a voltage dependent Ca^{2+} channel [8,12], is not known. It seems however important that all regulatory elements of annexin II, the phosphorylation sites for kinase C and $pp60^{src}$ at Ser^{23} and Tyr^{25}, as well as the p11 binding site at residues 1 to 14 are probably situated in close proximity to the entrance of the pore.

Acknowledgements: We thank Uwe Pleßmann for sequencing, H.-P. Geithe for synthesizing oligonucleotides and Dr. Nils Johnsson for discussion. This work was supported in part by the BMFT 'Schwerpunkt Bioprozeßtechnik' and a Kekulé-Stipend from the Verband der Chemischen Industrie to C.T.

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