Epitope mapping of annexin I: antibodies that compete with phospholipids and calcium recognize amino acids 42–99

Joel D. ERNST*

Department of Medicine and Division of Infectious Diseases, University of California, San Francisco, and The Rosalind Russell Arthritis Research Laboratories, San Francisco General Hospital, San Francisco, CA, U.S.A.

To understand further the structural basis of phospholipid binding by annexin I, three monoclonal antibodies that compete with Ca^{2+} and phospholipids for binding of annexin I were used to screen an expression library containing fragments of bovine annexin I cDNA. In all, 15 clones were isolated, and all contain overlapping fragments of the cDNA. The smallest unit common to all of the clones encodes amino acids 42–99 of annexin I, representing a portion of the first repeat domain. This demonstrates that recognition of a single domain of annexin I is sufficient to completely block phospholipid binding, and implies that the first repeat may contribute to phospholipid binding by annexin I.

INTRODUCTION

Annexins are abundant cytosolic proteins that bind Ca^{2+} and anionic phospholipids. In the presence of phospholipid, the affinity of annexins for Ca^{2+} increases two to three orders of magnitude [1], which suggests that Ca^{2+} and phospholipid binding are functionally closely associated. Furthermore, determination of the structure of annexin V by X-ray crystallography suggests that protein-bound Ca^{2+} ions and phospholipid head groups interact directly [2,3]. Although the Ca^{2+}-binding sites have been identified in annexin V, our understanding of the mechanism of phospholipid binding by annexins is incomplete. For example, it is unclear whether phospholipids also interact with annexins at sites distinct from the Ca^{2+}-binding sites, and it is not known whether all Ca^{2+}- and phospholipid-binding sites are functionally independent and equivalent. Individual annexins contain four or eight structurally related tandem repeat domains of 70–75 amino acids each, and the Ca^{2+}- and phospholipid-binding sites are located within the repeat-containing 'core' of the proteins [1,2]. While the repeat domains are structurally similar, there is emerging evidence that individual repeats exhibit functional diversity [4], and some do not directly bind Ca^{2+} and/or phospholipids [2].

Glenney and Zokas [5] prepared and characterized 14 monoclonal antibodies that recognize bovine annexin I. Of these, most recognized residues in the N-terminal tail, while three recognized a 10 kDa tryptic fragment derived from the core domain. In the latter group, two of the antibodies (designated II65 and II77) blocked binding of Ca^{2+} and phosphatidylserine (PtdSer) by annexin I, and a third (II85) failed to recognize annexin I in the presence of Ca^{2+} and PtdSer vesicles. These properties suggest that these antibodies recognize annexin I near one or more Ca^{2+}- and phospholipid-binding domains. We have mapped the recognition sites of these monoclonal antibodies, and found that all three recognize a minimum unit of 58 amino acids in the first repeat domain.

EXPERIMENTAL

Bovine annexin I cDNA cloning

Approx. 1000000 plaques of a Madin–Darby bovine kidney (MDBK) cDNA library in Lambda ZAP II (Stratagene) were screened with a labelled 1309 bp *Eco*RI fragment of human annexin I cDNA [4,6] at 68 °C in 6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) with 0.05% BLOTTO [7]. Filters were washed at 68 °C in 1 × SSC with 0.1% SDS prior to autoradiography. All positive clones were plaque-purified, and plasmids were excised according to procedures recommended by the manufacturer. Nested deletions were constructed using exonuclease III and mung-bean nuclease, and sequences were determined on both strands by the dideoxy technique using Sequenase (U.S. Biochemicals).

Isolation and identification of annexin I fragments recognized by monoclonal antibodies

A library containing random fragments of the bovine annexin I cDNA was constructed in Lambda ZAP II using procedures identical with those previously described for a human annexin I fragment library [4]. The fragment library (approx. 1×10^5 recombinants) was screened by pooling three monoclonal antibodies that recognize bovine annexin I at or near the phospholipid-binding site [5]. The antibodies, purified as previously described [5], were used at a concentration of 0.1 μg/ml. Bound antibodies were detected by using alkaline-phosphatase-conjugated goat anti-mouse IgG (Stratagene). After plaque purification, individual antibodies were used to examine samples of each clone to determine which clones produced fusion proteins recognized by the individual antibodies. Sequences of the fragment-containing clones were determined using double-stranded plasmid templates and T3 and T7 promoter primers (Stratagene).

RESULTS

Bovine annexin I cDNA cloning

Since the monoclonal antibodies that compete with phospholipids for annexin I binding do not recognize human annexin I, it was necessary to clone a cDNA encoding bovine annexin I in order to identify the binding sites of these antibodies. The initial screen of an MDBK library with a human annexin I cDNA probe at moderate stringency yielded 60 duplicate positive plaques. Of

Abbreviations used: PtdSer, phosphatidylserine; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate; MDBK, Madin–Darby bovine kidney.

* Correspondence address: University of California, Box 0868, San Francisco, CA 94143-0868, U.S.A.

```
Bovine  MAMVSEFLKQAWFIENEEQEYikTVKgSKGGPGSAVSPYPTFNPSSDVeALHKAItVKGVDEATIIeILTKRNNAQRQQI  80
        ||||||||||||||||||||||  ||| ||||||||||||||||||||| |||||| |||||||||| ||||||||||||
Human   MAMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFNPSSDVAALHKAIMVKGVDEATIIDILTKRNNAQRQQI

        KAAYLQEkGKPLDEvLKKALlGHLEEVVLALLKTPAQFDAeELRAAMKGLGTDEDTLnEILASRTNrEIReINRVhREEL 160
        ||||||| |||||| ||||| |||||||||||||||||| |||||||||||||| |||||||| ||| |||| ||||
        KAAYLQETGKPLDETLKKALTGHLEEVVLALLKTPAQFDADELRAAMKGLGTDEDTLIEILASRTNKEIRDINRVYREEL

        KRDLAKDIaSDTSGDyekALLaLAKGDRSEelaVNdDLADSDARALYEAGERRKGTDVNVFtTILTTRSYPhLRRVFQKY 240
        |||||||| |||||| ||| |||||||| || |||||||||||||||||||||||| ||||||||| ||||||||
        KRDLAKDITSDTSGDFRNALLSLAKGDRSEDFGVNEDLADSDARALYEAGERRKGTDVNVFNTILTTRSYPQLRRVFQKY

        sKYSKHDMNKVLDLELKGDIEKCLTvIVKCATSqPmFFAEKLHQAMKGiGTRHKtLIRIMVSRSEIDMNDIKAcYQKlYG 320
         |||||||||||||||||||||||| ||||||| | |||||||||||| ||||| |||||||||||||||| ||| ||
        TKYSKHDMNKVLDLELKGDIEKCLTAIVKCATSKPAFFAEKLHQAMKGVGTRHKALIRIMVSRSEIDMNDIKAFYQKMYG

        ISLCQAILDETKGDYEKILVALCGrd  346
        |||||||||||||||||||||||
        ISLCQAILDETKGDYEKILVALCGGN
```

Figure 1 Deduced amino acid sequence of bovine annexin I compared with that of human annexin I

Identical amino acids are indicated by vertical bars between the aligned sequences. The amino acid sequences are identical at 90% of positions. (The nucleotide sequence of the bovine annexin I cDNA from which the amino acid sequence shown was deduced has been deposited in EMBL, DDBJ and GenBank Nucleotide Sequence Databases under the accession number X56649.)

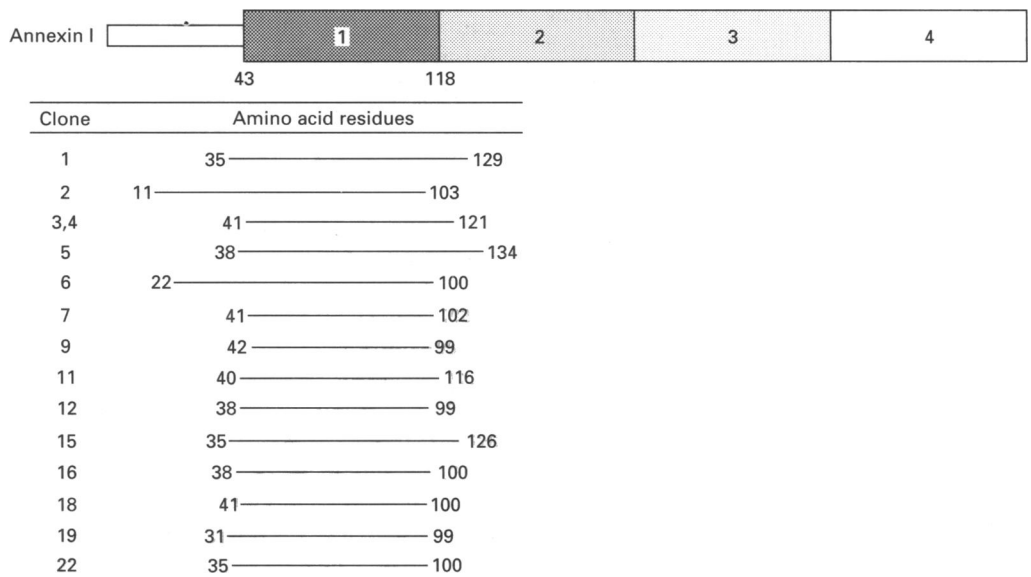

Figure 2 Fragments of bovine annexin I recognized by monoclonal antibodies

The fragments represented by individual clones are shown aligned with annexin I. The smallest insert (clone 9) encodes amino acids 42–99, and all of the clones isolated encode fragments that include these residues.

these, 18 remained positive after a higher-stringency wash (0.2 × SSC/0.1% SDS, 68 °C), and initial sequencing revealed that 13 of the 18 were closely related to human annexin I. One, termed Q1, was sequenced in its entirety on both strands. The deduced amino acid sequence of the bovine annexin I protein is 90% identical with the sequence of the human protein, and is shown in Figure 1.

Isolation and identification of annexin I fragments recognized by monoclonal antibodies

To identify the domain of annexin I that is recognized by the selected monoclonal antibodies, we constructed an expression library containing random fragments of the bovine annexin I cDNA and screened it with three monoclonal antibodies that bind annexin I at or near a phospholipid-binding site. In all, 20 clones were consistently recognized by the antibodies and were plaque-purified and analysed further. Digestion with *Eco*RI revealed that five had more than one insert each, and these were discarded. The remaining 15 had inserts ranging in size from 180 to 280 bp. Sequencing of each insert revealed that these 15 clones represented 14 distinct overlapping fragments (Figure 2). Alignment of the sequence of these inserts with the cDNA revealed that all of the clones contained residues in the first repeat domain

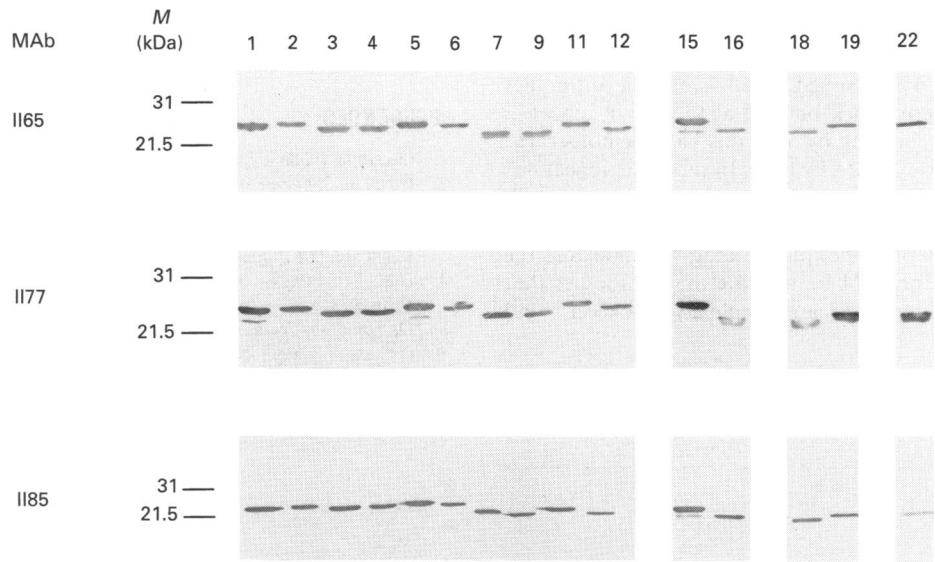

Figure 3 Analysis of individual clones with individual monoclonal antibodies

E. coli containing Bluescript plasmids corresponding to the clones isolated by screening the fragment library with the pooled monoclonal antibodies were grown to early exponential phase, and expression of fusion proteins (containing 36 amino acids of β-galactosidase fused to the fragments of bovine annexin I) was induced with 1.0 mM isopropyl thio-β-D-galactoside. After 3 h, bacteria were lysed by boiling in Laemmli sample buffer, and proteins were resolved by SDS/PAGE and transferred to nitrocellulose. Membranes were prepared in triplicate and were individually probed with the designated monoclonal antibodies, which were used at 0.1 μg/ml. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG. All of the clones that were isolated using the pool of monoclonal antibodies encoded fragments that were recognized by the individual antibodies. Abbreviation: *M*, molecular mass.

of bovine annexin I (Figure 2). The shortest insert recognized by the pool of antibodies encodes amino acids 42–99, and these residues are common to all of the clones isolated by screening the fragment library with these antibodies.

To determine which clones were recognized by individual antibodies, lysates of *E. coli* expressing each of the cloned fragments as fusion proteins were examined by immunoblotting (Figure 3). This revealed that all of the clones were recognized by all three of the antibodies. In order to determine whether the antibodies recognized domains of annexin I in addition to that isolated from the fragment library, we constructed plasmids that directed expression of repeats 2, 3 and 4 of bovine annexin I individually and in combination, and examined them by immunoblotting. None of the monoclonal antibodies recognized these fragments, which confirms that these antibodies only recognize a portion of repeat 1.

DISCUSSION

We have identified the domain of bovine annexin I recognized by three well-characterized monoclonal antibodies that apparently compete with Ca^{2+} and phospholipids for binding annexin I. Two of these antibodies block binding of Ca^{2+} and PtdSer by annexin I, while the other antibody fails to recognize annexin I when it has bound PtdSer vesicles. We found that all three of these antibodies recognize a fragment containing amino acids 42–99, which reside in the first repeat of annexin I. Indeed, by alignment with annexin V, these residues are predicted to correspond exactly to the first four of the five α-helices in repeat 1 [3]. While the simplest interpretation of these results is that this domain of annexin I binds Ca^{2+} and phospholipid, experimental evidence (J. D. Ernst and G. Chew, unpublished work), as well as predictions based on structural analysis [2], strongly suggest that repeat 1 of annexin I does not possess a Ca^{2+}-binding site. Determination of the structure of annexin V by X-ray crystallography has identified three Ca^{2+}-binding sites, all of them on the convex surface of the protein, in repeats 1, 2 and 4. In contrast, annexin I was predicted to possess Ca^{2+}-binding sites in repeats 2 and 4, but not in repeat 1 [2], since repeat 1 differs significantly in sequence from the consensus Ca^{2+}-binding sites found in annexin V. Consequently, we conclude that these antibodies recognize a linear sequence that is not directly involved in Ca^{2+} and phospholipid binding. We suggest that these antibodies bind residues that are exposed on the same (convex) surface of annexin I as the Ca^{2+}- and phospholipid-binding sites and so impede binding of these ligands. The antibodies that block Ca^{2+} and PtdSer binding by annexin I (II65 and II77) presumably exhibit a higher affinity for the protein than do Ca^{2+} and PtdSer, whereas the antibody that does not recognize annexin I in the presence of Ca^{2+} and PtdSer (II85) is likely to have a lower affinity for the protein than do these ligands.

We have previously characterized a monoclonal antibody (EH7a) that specifically blocks annexin I-mediated phospholipid vesicle aggregation. This monoclonal antibody also recognizes residues in repeat 1 of human annexin I (amino acids 41–118) [4]. Since this antibody does not block annexin I binding of phospholipid vesicles, it is likely that it binds residues in repeat 1 that are not on the (convex) surface that possesses the Ca^{2+}- and phospholipid-binding sites. Since EH7a does not bind bovine annexin I and the antibodies described in the present paper do not bind human annexin I, it is not currently possible to determine directly whether these antibodies with disparate actions compete with one another for binding or whether they bind to distinct surfaces of repeat 1.

The structural model of annexins derived from X-ray crystallography indicates that the proteins fold so that repeats 1 and 4 are closely packed and repeats 2 and 3 are closely packed. Therefore antibodies that recognize residues in repeat 1 are most likely to overlie the Ca^{2+} binding site in repeat 4. Our observation that monoclonal antibodies that completely block Ca^{2+} and phospholipid binding recognize repeat 1 is compatible with at least two models. In one, the binding sites in repeats 2 and 4 are

both sufficiently close to repeat 1 that antibody binding impedes access of Ca^{2+} and phospholipid to both sites. Alternatively, it is possible that the site in repeat 2 cannot bind Ca^{2+} or phospholipid, unless the site in repeat 4 is occupied. In this event, the antibodies may only need to directly block binding at the site in repeat 4. Indeed, studies of Ca^{2+} binding by annexins have demonstrated that binding exhibits co-operativity [1,8]. In this case, repeat 4 is likely to be the site with higher affinity for Ca^{2+} and phospholipid. While further experimental work is necessary to fully understand the molecular basis of phospholipid binding by annexins, the results of the studies reported here indicate that antibodies that recognize a portion of repeat 1 can completely block Ca^{2+} and phospholipid binding.

I thank John Glenney for providing the purified monoclonal antibodies and Tina Lee Mok for excellent technical assistance. This work was funded by grants from the Arthritis Foundation, from the American Heart Association (California Affiliate), and the National Institutes of Health (AI23697).

Received 10 July 1992; accepted 5 August 1992

REFERENCES

1. Glenney, J. (1986) J. Biol. Chem. **261**, 7247–7252
2. Huber, R., Schneider, M., Mayr, I., Römisch, J. and Paques, E.-P. (1990) FEBS Lett. **275**, 15–21
3. Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Römisch, J. and Paques, E. (1992) J. Mol. Biol. **223**, 683–704
4. Ernst, J. D., Hoye, E., Blackwood, R. A. and Mok, T. L. (1991) J. Biol. Chem. **266**, 6670–6673
5. Glenney, J. and Zokas, L. (1988) Biochemistry **27**, 2069–2076
6. Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. **132**, 6–13
7. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
8. Schlaepfer, D. D. and Haigler, H. T. (1987) J. Biol. Chem. **262**, 6931–6937