Calcium-dependent interaction of annexin I with annexin II and mapping of the interaction sites

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Abstract Annexins are multifunctional intracellular proteins with Ca^{2+}- and phospholipid-binding properties. Their structures consist of four conserved repeat domains that form the core and a diverse N-terminal tail, from which their functional differences may arise. We searched for cellular proteins that interact with the N-terminal tail plus domain I of annexin I (ANX1) by using the yeast two-hybrid method. Screening of a HeLa cell cDNA library yielded annexin II (ANX2) cDNA. The interaction between ANX1 and ANX2 also occurred in vitro in a Ca^{2+}-dependent manner. Mapping of the interaction sites revealed that interaction between domain I of ANX1 and domain IV of ANX2 was stronger than the other combinations.

Key words: Annexin I; Annexin II; Ca^{2+} dependence; Specific binding; Two-hybrid assay

1. Introduction

Annexins (ANXs, also called lipocortins) are a family of intracellular proteins that bind reversibly to phospholipids in a Ca^{2+}-dependent manner. The structures of ANXs, which are distinct from the EF-hand family of Ca^{2+}-binding proteins, consist of the core domains and the N-terminal tails. The core domains consist of four repeat domains that share approximately 50% amino acid similarity between different ANXs, and have binding sites for Ca^{2+} ions and phospholipids [1,2]. Each ANX family member contains a specific N-terminal tail that is not structurally related to the other ANXs. Various members of the ANX family proteins have been implicated in a number of different intracellular processes including vesicular trafficking [3], membrane fusion during exocytosis [4], signal transduction [1], and ion channel formation [5,6]. The different ANX genes show cell-specific expression [1], thereby implying that each ANX performs a different biological function. Since the N-terminal tails are not related to each other, these may impart functional specificity. In fact, the N-terminal tails of ANX1, ANX2 and ANX11 are involved in the interaction with S100C [7], p11 [8] and calcyclin [9], respectively, which are members of the S100 subgroup of the EF-hand family of Ca^{2+}-binding proteins. Both the interaction of ANX1 with S100C and the interaction of ANX11 with calcyclin are Ca^{2+}-dependent. In contrast, the ANX2 and p11 interaction is Ca^{2+}-independent. ANX1 is structurally more similar to ANX2 than the other ANXs, and p11 is structurally more similar to S100C than the other S100 proteins. Another similarity between ANX1 and ANX2 is that they are both phosphorylated by various kinases such as tyrosine kinase, epidermal growth factor receptor protein kinase [10,11], $pp60^{src}$ [12] and protein kinase C [13,14]. The N-terminal region of ANX1 is the site of selective proteolytic cleavage [15], and seems to be important for cellular functions.

In this study we searched for the cellular proteins interacting with the N-terminal tail plus domain I of ANX1 using the yeast two-hybrid system. The bait plasmid contained the fusion gene encoding the LexA DNA-binding domain and amino acids 1–113 of ANX1.

2. Materials and methods

2.1. Construction of bait plasmid for two-hybrid system

pEG202 (developed by Brent and coworkers [16]), which was used as a vector to express the LexA-$ANX1_{1-113}$ fusion protein, contains the *his3* selectable marker, yeast 2μ origin, *Escherichia coli* pBR origin, and LexA DNA-binding domain. The ANX1 cDNA fragment containing amino acids 1–113 was PCR amplified and cloned into *Bam*HI-*Sal*I sites of pEG202. The resulting plasmid, named pEGLexA-$ANX1_{1-113}$, was used as the bait. Yeast strain EGY48 [*MATa, his3, trp1, ura3-52, leu::pLeu-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] was transformed with the plasmid, and expression of the fusion protein was identified by Western blot analysis.

2.2. Two-hybrid screening of human HeLa cell cDNA library

A human HeLa cell cDNA library in the transcription activator B42 fusion vector pJG4-5 [16] was screened. Plasmid pJG4-5 contains the TRP1 selectable marker, yeast 2μ origin, and *E. coli* pUC origin. Expression of the fusion protein in this plasmid is under the control of GAL1, a galactose-inducible promoter. Yeast strain EGY48/pEGLexA-$ANX1_{1-113}$ was transformed with HeLa cell cDNA library by the lithium acetate method. Transformants were selected for tryptophan prototrophy on synthetic agar medium (Ura^-, His^-, Trp^-) containing 2% glucose. All of the transformants were pooled and respread on synthetic medium (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the introduced DNA. Cells growing on the selection media were retested on the synthetic medium (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose (inducing condition) and 2% glucose (non-inducing condition) to confirm the dependence of their growth on the presence of galactose. Cells growing only on the galactose media were subjected to further characterization. The selected cells were also streaked on synthetic medium (Ura^-, His^-, Trp^-) containing 2% galactose or 2% glucose with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to test for β-galactosidase activity. The cells expressing both reporter genes only in the presence of galactose were finally chosen to isolate the plasmids. The isolated plasmids were transformed into *E. coli* K12 strain KC8 (*pyrF::Tn5, hsdR, leuB600, trpC9830, lac D74, strA, gslK, hisB436*), and the transformants containing the recombinant cDNAs were selected by their growth on M9 minimal medium (Thi^+, His^+, Ura^+, Leu^+, Trp^-) containing ampicillin. The plasmids were then isolated from Trp^+ *E. coli* transformants and used to confirm the selection results and to sequence the inserted cDNA by using the B42 primer (5′-CC AGC CTC TTG CTG

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Abbreviations: ANX1, annexin I; ANX2, annexin II; X-gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside; MBP, maltose-binding protein

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AGT GGA GAT G). The LexA-IRS (isoleucyl tRNA synthetase) and B42-EPRS (glutamyl and prolyl tRNA synthetase) hybrid proteins were used as a positive control of interaction [17]. Other hybrid proteins (B42-β-tubulin, B42-ribosomal phosphoprotein, LexA-ALG2 (apoptosis-linked gene [18])) were used as negative controls.

2.3. In vitro protein binding of ANX1 and ANX2

Full length ANX2 cDNA was cloned into the maltose-binding protein (MBP) fusion protein expression vector pMAL-P2X 9 (New England BioLabs, USA) to produce pMAL-ANX2. The plasmid was transformed into *E. coli* XL-1 Blue, and expression of the fusion protein was induced by 0.3 mM IPTG. The fusion protein was purified using amylose resin (New England BioLabs, USA), according to the manufacturer's directions. ANX1 was in vitro translated from plasmid pCDNA3-ANX1, which was constructed by inserting full length ANX1 cDNA into pCDNA3 (Invitrogen, USA), using the TnT rabbit reticulocyte lysate in vitro transcription-translation system (Promega, USA). Reactions were performed in the presence of 20 μCi [^{35}S]methionine (Amersham, UK) to label the protein. Multiple aliquots of 50 μl reactions were performed and pooled after incubation at 30°C for 90 min. 20 μl of the reaction mixture was mixed with MBP-ANX2 (or MBP for control), and the binding buffer (20 mM Tris-HCl pH 8.0, 30 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 0.4 mM PMSF) was added up to 300 μl. The reaction mixtures were incubated on a rotating incubator at 4°C for 12 h. 20 μl of amylose resin beads was added to each reaction mixture, and the reaction mixtures were incubated for an additional 2 h at 4°C. Beads were washed four times in 1 ml of the binding buffer and resuspended in 20 μl of 10 mM maltose. The eluate was separated by 15% SDS-PAGE, and the labeled protein was visualized by autoradiography.

2.4. Quantitation of interaction

The strength of the interaction between different domains of ANX1 and ANX2 was determined by measuring the expression level of the lacZ reporter gene. The β-galactosidase activity was determined according to the method previously reported [17]. Yeast cells containing different sets of plasmids were cultured in yeast synthetic media containing 2% glucose until they reached mid-log phase. Cell growth was monitored by the absorbance at 600 nm. The culture broth (0.2 ml) was taken and mixed with Z buffer (0.7 ml) containing 2-mercaptoethanol. Chloroform (50 μl) and 0.1% SDS (50 μl) were added to the mixture, and the mixture was vortexed for 30 s. The reaction was started by adding the substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG) (0.16 ml), incubated at 30°C until the yellow color appeared, and then stopped by adding 0.4 ml of 1 M Na_2CO_3. Cell debris was removed by centrifugation, and the absorbance of the supernatant was measured at 420 nm. Enzyme activity was also measured by a filter method. Briefly, the cells grown on the yeast plate were patched onto a filter paper (Whatman 3MM) and this was submerged into liquid nitrogen for 10 s to freeze the cells. Then the cells were thawed at room temperature for 20 min to permeabilize the cell wall and the filter was incubated in the X-gal-containing Z buffer until a blue color appeared. The expression level of the reporter gene LEU2 was determined by the growth rate of the yeast cells in synthetic media lacking leucine.

3. Results

3.1. Screening for ANX1 interacting proteins

$ANX1_{1-113}$ interacting proteins were screened from a human HeLa cell cDNA library in plasmid pJG4-5, a transcription activator B42 fusion vector, using pEGLexA-$ANX1_{1-113}$ as the bait (Fig. 1A) as described in Section 2. Approximately 1×10^6 independent transformants were pooled and respread on selection media (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose. Of the many colonies that grew on the selection plate, 100 colonies were chosen and screened for expression of the two reporter genes (LEU2 and lacZ). The colonies were streaked on synthetic medium (Ura^-, His^-, Trp^-) containing 2% galactose or 2% glucose with X-gal to test for β-galactosidase activity. Expression of LEU2 was checked by the growth rate on synthetic medium lacking leucine. Fifteen colonies expressing both reporter genes only in the presence of galactose were analyzed further using several positive and negative control plasmids to verify the specificity of the interaction between the bait and the selected clones. All 15 clones showed specificity. The nucleotide sequences were determined and used for GenBank search.

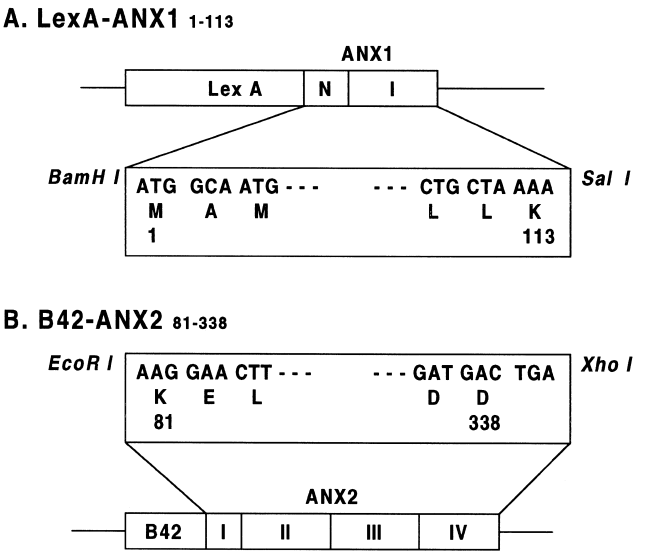

Fig. 1. Schematic representation of the LexA-$ANX1/_{1-113}$ fusion protein, which was used as the bait (A), and the B42-$ANX2_{81-338}$ fusion protein obtained from the screening (B). A: The DNA fragment encoding amino acids 1–113 of ANX1 ($ANX1_{1-113}$) was cloned into the *Bam*HI/*Sal*I sites of plasmid pEG202 [16] to produce the bait plasmid pEGLexA-$ANX1_{1-113}$. B: The clone was selected from screening of a HeLa cell cDNA library in the B42 fusion vector pJG4-5 [16]. Nucleotide sequencing of the selected clone revealed an ANX2 cDNA fragment encoding amino acids 81–338 (named pJG-$ANX2_{81-338}$).

3.2. Specific interaction of ANX1 with ANX2

Of the 15 clones, 14 turned out to be different portions of ANX2 cDNA, and one was a novel gene. One of the 14 ANX2 cDNA clones encoded amino acids 81–338

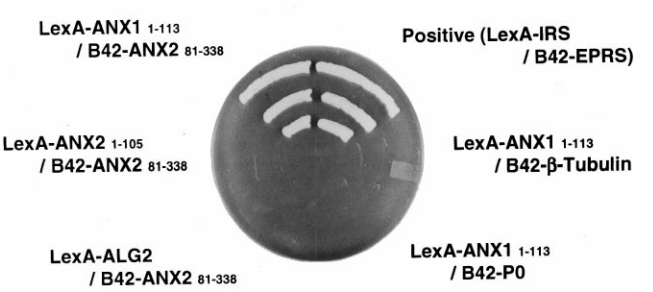

Fig. 2. Specific interaction between $ANX1_{1-113}$ and $ANX2_{81-338}$ in yeast. Yeast strain EGY48 transformed with plasmids expressing LexA-$ANX1_{1-113}$ and B42-$ANX2_{81-338}$ was grown on synthetic medium (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to verify the interaction between the two proteins. Different combinations of plasmids expressing LexA or B42 fusion proteins were used as positive and negative controls. Top right, positive interacting control (LexA-IRS and B42-EPRS) [17]; top left, LexA-$ANX1_{1-113}$ and B42-$ANX2_{81-338}$; middle right, LexA-$ANX1_{1-113}$ and B42-β-tubulin; middle left, LexA-$ANX2_{1-105}$ and B42-$ANX2_{81-338}$; bottom right, LexA-$ANX1_{1-113}$ and B42-P0 (ribosomal phosphoprotein); bottom left, LexA-ALG2 and B42-$ANX2_{81-338}$.

($ANX2_{81-338}$), which covers a portion of the I and all of domains II–IV (Fig. 1B). The results indicate that $ANX1_{1-113}$ interacts with $ANX2_{81-338}$. On the other hand, $ANX1_{1-113}$ did not interact with β-tubulin or ribosomal phosphoprotein. Also $ANX2_{81-338}$ did not interact with ALG-2 or $ANX2_{1-105}$ (middle left) (Fig. 2). Therefore the interaction between $ANX1_{1-113}$ and $ANX2_{81-338}$ is specific for each other.

3.3. In vitro protein-protein interaction

The interaction between ANX1 and ANX2 was further investigated by using in vitro translated ^{35}S-ANX1 and bacterially expressed MBP-ANX2 fusion protein as described in Section 2. As shown in Fig. 3, ^{35}S-ANX1 interacted with MBP-ANX2 in the presence but not the absence of Ca^{2+} ion (lane 4 vs. lane 2). When MBP was used in place of MBP-ANX2, the interaction was not observed (lanes 1 and 3). The results demonstrate that ANX1 specifically interacts with ANX2 in a Ca^{2+}-dependent manner.

3.4. Refining the interacting site between ANX1 and ANX2

ANX2 core domain contains four homologous repeat sequences (domains I, II, III, and IV) like other ANX family proteins (Fig. 4). To obtain a refined map for the interaction between ANX1 and ANX2, DNA fragments encoding different domains were cloned into the yeast two-hybrid vectors and their interaction activities were compared by using the expression of the reporter gene, lacZ. Fig. 4A shows the results when interaction between $ANX1_{1-113}$ and different domains of ANX2 was determined. The interaction was stronger in the domain IV-containing peptides (domains II–IV, domains III, IV and domain IV) compared to the other domain-containing peptides. Fig. 4B shows the interaction between $ANX2_{81-338}$ and different ANX1 fragments. The interaction of $ANX2_{81-338}$ with domain I was stronger than that with the N-terminal tail or domains II–IV. Fig. 4 demonstrates that interaction between domain I of ANX1 and domain IV of ANX2 was stronger compared to other combinations.

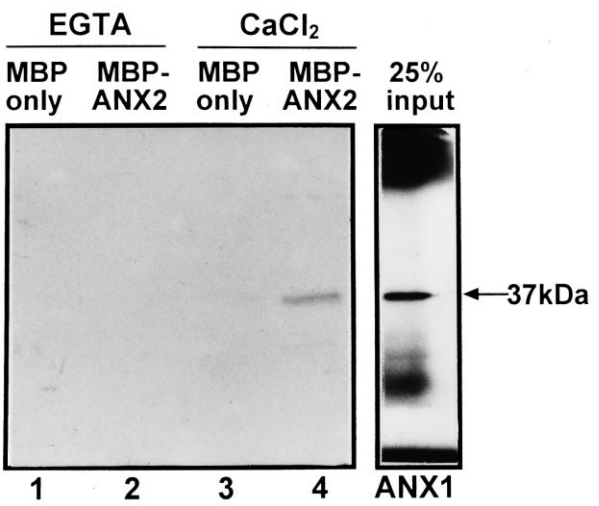

Fig. 3. Ca^{2+}-dependent interaction between ANX1 and ANX2. ^{35}S-ANX1, prepared by in vitro transcription and translation, was incubated with bacterially expressed MBP (lanes 1 and 3) and MBP-ANX2 (lanes 2 and 4) in the presence of EGTA (lanes 1 and 2) or Ca^{2+} (lanes 3 and 4). Amylose resin beads were added to each reaction mixture and this was incubated to allow binding of the beads to MBP. The bound proteins were eluted with maltose, analyzed by SDS-PAGE and autoradiography.

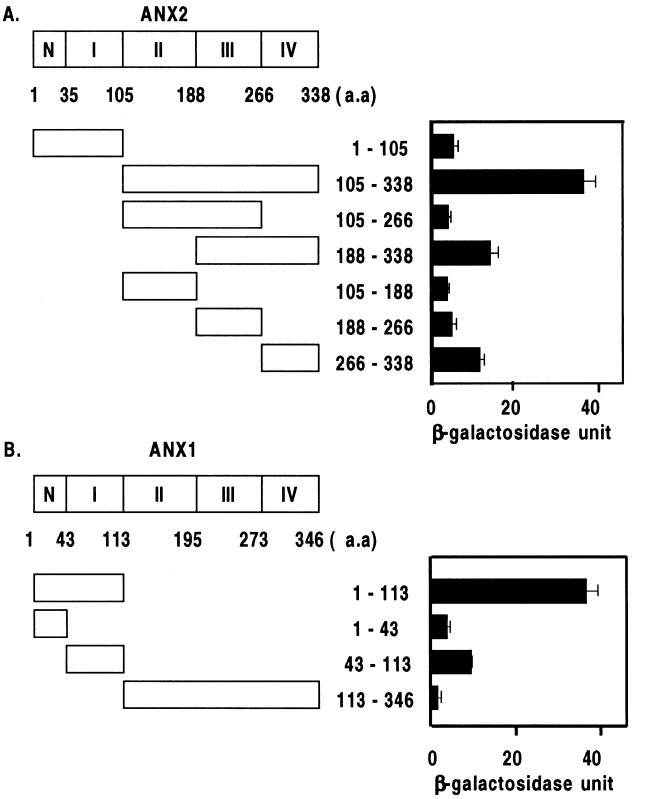

Fig. 4. Mapping of the interaction sites of ANX1 and ANX2. A: Interaction of $ANX1_{1-113}$ with different domains of ANX2. Various mutants expressing different domains of ANX2 (left) were introduced into the yeast cells expressing LexA-$ANX1_{1-113}$, and their interactions were compared by induction of β-galactosidase (right). B: Mutants expressing different regions of ANX1 (left) were introduced into the yeast cells expressing $ANX2_{81-338}$, and their interactions were compared by the induction of β-galactosidase (right). N, N-terminal tail. Roman numerals represent the repeat domains of the core domain. Arabic numbers represent the amino acid numbers. The enzymatic activities are shown by the means ± S.D. ($n = 4$).

4. Discussion

Although several physiological roles have been proposed for ANX1, the mechanism by which it displays its functions is still unknown. In this study, we attempted to identify proteins interacting with ANX1. ANX1 interacted with ANX2 in a Ca^{2+}-dependent manner, and the interaction occurred specifically between domain I of ANX1 and domain IV of ANX2. It is rather surprising that even though the four repeat domains of ANX1 and ANX2 are homologous to each other the interaction between the two domains is specific. It is reasonable to assume that the micro-heterogeneity between domain structures influences the binding. It is worth mentioning that the N-terminal tail of ANX1 binds to $ANX2_{81-338}$ to some extent and enhances the binding of domain I (Fig. 4B).

The N-terminal tail of ANX1 is known to be important for binding to another protein, S100C, and to be involved in the physiological function [7]. The first 12 amino acids of ANX1 are reported to be the S100C-binding site. Both ANX2 and

S100C interacts with ANX1 in a Ca^{2+}-dependent manner, but their interaction sites on ANX1 are different. ANX2 binds to domain I as well as to the N-terminal tail and S100C binds to the N-terminal tail of ANX1.

Many proteins undergo a conformational change upon Ca^{2+} binding, which often results in the exposure of a hydrophobic region and interaction with other proteins [7,19]. Binding of Ca^{2+} to domain IV of ANX2 may induce exposure of the hydrophobic amino acids, which are likely to interact with the α-helical structure of domain I of ANX1 [7]. It is known that ANX family proteins form polymers by themselves or with other proteins [20,21]. Whether the polymerization of other ANX family proteins also involves the same type of interaction is not known yet. It is likely to occur, at least in some cases, because amino acid sequences of the repeat domains are homologous and have the ability to bind Ca^{2+}.

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References

[1] Moss, S.E. (Ed.) (1992) The ANXs, Portland Press, London.
[2] Flower, R.J. and Rothwell, N.J. (1994) Trends Pharmacol. Sci. 15, 71–76.
[3] Gruenberg, J. and Emans, N. (1993) Trends Cell Biol. 3, 224–227.
[4] Creutz, C.E. (1992) Science 258, 924–931.
[5] Demange, P. (1994) Trends Biochem. Sci. 19, 272–276.
[6] Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C. and Burns, A.L. (1990) J. Biol. Chem. 265, 21207–21215.
[7] Mailliard, W.S., Haigler, H.T. and Schlaepfer, D.D. (1996) J. Biol. Chem. 271, 719–725.
[8] Johnsson, N., Marriott, G. and Weber, K. (1988) EMBO J. 7, 2435–2442.
[9] Tokumitsu, H., Mizutani, A. and Hidaka, H. (1993) Arch. Biochem. Biophys. 303, 302–306.
[10] Haigler, H.T., Schlaepfer, D.D. and Burgess, W.H. (1987) J. Biol. Chem. 262, 6921–6930.
[11] Schlaepfer, D.D. and Haigler, H.T. (1987) J. Biol. Chem. 262, 6931–6937.
[12] Glenney, J.R. and Tack, B.F. (1985) Proc. Natl. Acad. Sci. USA 82, 7884–7888.
[13] Schlaepfer, D.D. and Haigler, H.T. (1988) Biochemistry 27, 4253–4258.
[14] Gould, K.L., Woodgett, J.R., Isacke, C.M. and Hunter, T.H. (1986) Mol. Cell. Biol. 6, 2738–2744.
[15] Chuar, S.Y. and Pallen, C.J. (1989) J. Biol. Chem. 264, 21160–21166.
[16] Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993) Cell 75, 791–803.
[17] Rho, S.B., Lee, K.H., Kim, J.W., Shiba, K., Jo, Y.J. and Kim, S. (1996) Proc. Natl. Acad. Sci. USA 93, 10128–10133.
[18] Vito, P., Lacana, E. and D'Adamio, L. (1996) Science 271, 521–525.
[19] Donato, R. (1991) Cell Calcium 12, 713–726.
[20] Luecke, H., Chang, B.T., Mailliard, W.S., Schaepfer, D.D. and Haigler, H.T. (1995) Nature 378, 512–515.
[21] Langen, R., Isas, J.M., Luecke, H., Haigler, H.T. and Hubbell, W.L. (1998) J. Biol. Chem. 273, 22453–22457.