Class-Specific Binding of Two Aminoacyl-tRNA Synthetases to Annexin, a Ca²⁺- and Phospholipid-Binding Protein

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ABSTRACT. **Annexins are a family of Ca²⁺/phospholipid-binding proteins that have diverse functions. To understand the function of annexin in *Physarum polycephalum*, we searched for its binding proteins. Here we demonstrate the presence of two novel annexin-binding proteins. The homology search of partial amino acid sequences of these two proteins identified them as aminoacyl-tRNA synthetases (ARSs). Furthermore, antibody against aminoacyl-tRNA synthetases cross-reacted with one of two proteins. Our results imply the interaction between intracellular membrane dynamics and protein translation system, and may give a clue to understand the mechanism of some myositis diseases, which have been known to produce autoantibodies against ARSs.**

Key words: annexin/aminoacyl-tRNA synthetase/*Physarum polycephalum*/myositis

Introduction

Annexins are a family of Ca²⁺/phospholipid-binding proteins, which have diverse functions (Rescher and Gerke, 2004; Gerke and Moss, 2002). Many of the annexin family members are involved in membrane dynamics in cells, such as membrane transport or membrane fusion (Hayes and Moss, 2004). In our previous study, we demonstrated the presence of annexin in a true slime mold, *Physarum polycephalum* (Ogihara and Sesaki, 1992). *Physarum* annexin was identified biochemically, and later its whole nucleotide sequence was determined. *Physarum* annxin is a 32 kDa protein, and has a high homology with annexin VII, XI and XIII in higher eukaryotes. Judging from the differences in molecular mass of these proteins and the evolutionary distance, *Physarum* annexin seems to be highly specific to *Physarum*. *Physarum* annexin is a Ca²⁺-dependent lipid-binding protein, and a list of biochemical properties available for this protein was determined experimentally *in vitro*

(Caohuy and Pollard, 2002). Of most significance, the dissociation constants of *Physarum* annexin for Ca²⁺, determined by fluorescence spectrophotometry using Ca²⁺ titration, were found to be 0.32 μM, 1 μM and 3.2 mM. By considering the mM range dissociation constant, it is natural to assume that *Physarum* annexin may play some role in the extracellular space, because the intracellular Ca²⁺ concentration is usually maintained in sub-μM range. The μM range dissociation constants for *Physarum* annexin obviously indicate that annexin works in the intracellular space as well. Thus, *Physarum* annexin seems to have multiple functions and may work in multiple cellular spaces.

To understand the cellular function of *Physarum* annexin, we attempted to identify annexin-binding proteins in the plasmodia of *Physarum polycephalum*. Annexin family proteins have various kinds of binding proteins, which may reflect the diversity in function and localization of annexins (Moss and Morgan, 2004). Here we demonstrate the presence of two novel annexin-binding proteins; they are two different types of ARSs, proteins that work in protein translation. Our results suggest that there may be some interaction between intracellular membrane dynamics and protein translation system.

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Abbreviation: ARS, aminoacyl-tRNA synthetase.

Materials and Methods

Culture of the Physarum plasmodia

The plasmodia of *Physarum polycephalum* were cultured by the method of Camp (Camp, 1936). Briefly, the plasmodia were cultured on wet paper towels paved on glass Petri dishes in a plastic box. Oatmeal was fed daily.

Immunoprecipitation

Affi-gel protein A beads (Bio-Rad, NY, USA) were washed three times with Triton lysis buffer (50 mM HEPES-KOH, 20 mM EDTA-2Na, 1 mM DTT, 1 mM PMSF, pH 7.0) supplemented with 1% BSA. Fifty μl of Affi-gel protein A gel slurry was used for one immunoprecipitation sample. After washes, 2 volumes of 1% BSA/Triton lysis buffer and 15 μg of affinity-purified anti-annexin antibodies (autoimmune antisera from myositis patients) were added to the gel slurry. For immunoprecipitation using anti-ARS antibodies, 1 μl of anti-ARS serum was used per one immunoprecipitation sample. The mixtures of the gel slurry and the antibodies were incubated on a rotary shaker (TOMY, Tokyo, Japan) for 30 min at 4°C to make the antibody bind to the beads.

Physarum plasmodia were scraped into a small tube from a culture bucket and weighed. For 1 g of the plasmodia, 10 ml of ice-cold Triton lysis buffer was used. The plasmodia were homogenized by Physcotron (New Generation Instrument Technique, Chiba, Japan) and the lysate was centrifuged at 33,000 r.p.m. for 30 min at 4°C. One ml of the supernatant was added to 50 μl of the antibody-bound Affi-gel beads, and incubated on the rotary shaker for 30 min at 4°C. The beads were washed three times with Triton lysis buffer and collected by centrifugation. Seven μl of 2 × sample buffer for SDS-PAGE was added to the beads, boiled for 5 min, and run on SDS-PAGE (5–15% gradient gel).

Western blotting

Affinity-purified anti-annexin antibody (0.3 μg/ml) in 5% skim milk/PBS-T was overlaid onto the PVDF membrane and the membrane was incubated for 1 h at 37°C. The membrane was washed with PBS-T twice for 15 min each and twice for 5 min each. HRP-conjugated goat anti-rabbit IgG in 1:8000 dilution in 5% skim milk/PBS-T was overlaid and the membrane was incubated for 1 h at RT. The membrane was washed with PBS-T twice for 15 min each and twice for 5 min each. Then the chemiluminescent signal of HRP was detected using ECL-Plus (Amersham Biosciences, NJ, USA). The images were taken using LAS-1000 system (Fujifilm, Tokyo, Japan) and enhanced using Adobe Photoshop 5.0 (Adobe, NY, USA).

Peptide sequencing

About 50 pmol of the proteins were purified by SDS-PAGE after immunoprecipiation, cut out from SDS-PAGE gels, and digested to peptide fragments by in gel digestion. The peptide fragments yielded were purified by HPLC. Then the purified peptide fragments were sequenced with traditional Edman method. These partial sequences were analyzed using NCBI BLAST (http://www.ncbi.nih.gov/BLAST/), and homologous proteins were detected.

Far-Western blotting

For far-Western blotting, the same method was used as Western blotting except that 10 μg/ml biotinylated r-annexin was used as the first probe, and HRP-conjugated streptavidin in 1:8000 dilution was used as the second probe. Biotinylation of r-annexin was done using ECL™ Protein Biotinylation Module (Amersham Biosciences).

Results

Specific co-precipitation of two proteins with annexin

We screened annexin-binding proteins using a co-immunoprecipitation method with the affinity-purified anti-annexin antibody. Precipitation of annexin was confirmed by immunoblotting using anti-annexin antibody (Fig. 1, lane 3, 4, 6, and 7). In the co-precipitate, two proteins with molecular
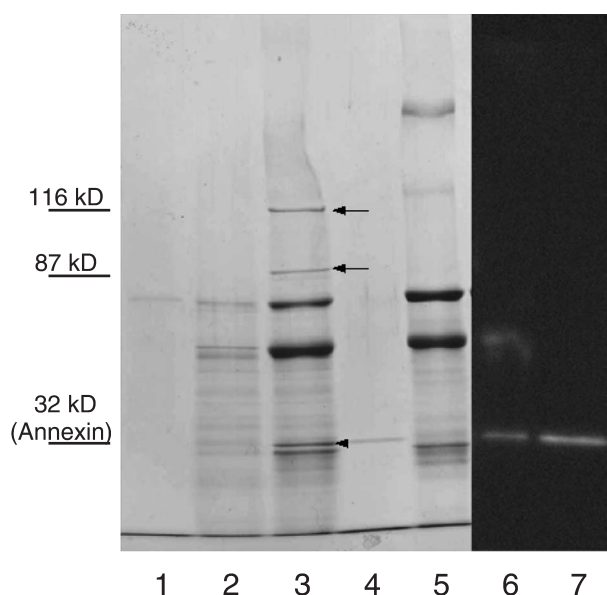

Fig. 1. Co-precipitation of two proteins with annexin in immunoprecipitation by affinity-purified anti-annexin antibody. Lanes 1–5: CBB stain. Lanes 6–7: Immunoblotting with anti-annexin antibody. Lane 1: No immunobeads for immunoprecipitation. Lane 2: Immunobeads only, without the primary antibody. Lane 3: Immunoprecipitate with 15 μg of anti-annexin antibody. Lane 4: Purified annexin. Lane 5: Antibodies only, without lysate. Lane 6: Immunoprecipitate prepared in the same way as in lane 3 was stained with anti-annexin antibody. Lane 7: Purified *Physarum* annexin (lane 4) was detected by anti-annexin antibody. In comparison with lane 1, 2 and 5, annexin (arrowhead) and the two proteins (116 kDa and 87 kDa, arrows) specifically appeared only in lane 3.

mass of 116 kDa and 87 kDa precipitated with annexin (Fig. 1, lane 3); these two proteins did not appear in the control experiments (Fig. 1, lane 1–3 and 5). The two proteins were designated p116 and p87 according to their molecular mass. Next, we determined the stoichiometry of these three proteins in the precipitate by changing the amounts of antibody used for immunoprecipitation. The amounts of p116, p87 and annexin in the precipitate increased depending on the amount of anti-annexin antibody used (Fig. 2, a.), and were almost in a fixed molar ratio of 1:1:2 (Fig. 2, b.), irrespective of the antibody concentration. The stoichiometry of these three proteins strongly indicated that p116 and p87 are annexin-binding proteins. Furthermore, to demonstrate the annexin-binding properties of p116 and p87 more directly, far-Western blotting was performed. To enhance the detectability of the probe annexin, biotinylated recombinant annexin was used. p87 showed very distinct and very specific signal of annexin-binding, indicating direct annexin-binding of p87, while p116 did not show annexin-binding by this method (Fig. 3).
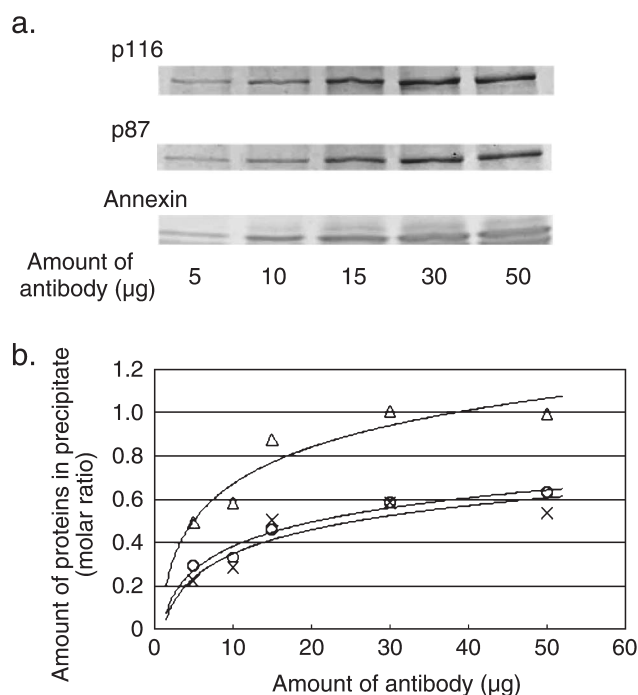
a.

p116

p87

Annexin

Amount of antibody (μg)   5   10   15   30   50

b.

Fig. 2. Stoichiometry of p116, p87 and annexin. (a) Immunoprecipitation with various amounts of anti-annexin antibody (5, 10, 15, 30 and 50 μg). The amount of p116, p87 and annexin in the precipitate increased in proportion to the amount of antibody used. (b) The bands in (a) were quantitated and plotted against the amount of antibody used. The amount of p116 (circle), p87 (cross) and annexin (triangle) in the precipitate were almost proportional to the amount of the antibody. The molar ratio of p116:p87:annexin was approximately 1:1:2, regardless of the amount of antibody.

p116 and p87 are different kinds of ARS

To identify p116 and p87, their partial amino acid sequences were determined and the homologous proteins with these partial sequences were searched using BLAST. (The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the following accession numbers; P84178 for p116, and P84179 for p87.) As shown in Fig. 4, the amino acid sequences of the four peptide fragments from p116 showed homologies with isoleucyl-tRNA synthetase from four different species. Similarly, two peptide fragments from p87 showed homologies with glutaminyl-, glutamyl- and glutamyl-proryl-tRNA synthetases. To further demonstrate that p116 and p87 are ARSs, immunoblotting was done using anti-isoleucyl-tRNA synthetase antibody and the antibody specifically bound to p87 (Fig. 5, lane 2). As this antibody is known to crossreact not only with isoleucyl-tRNA synthetase but shows specific interactions with leucyl- and glutaminyl-tRNA synthetase as well (Targoff *et al.* 1993), these data support the idea that p87 is glutaminyl-tRNA synthetase and that the p116 is isoleucyl-tRNA synthetase. Absence of the detection of p116 band by the antibody will be given explanations in Discussion.

Immunoprecipitation using the same anti-isoleucyl-tRNA synthetase antibody co-precipitated annexin (Fig. 6). As lanes 2–5 show, annexin was co-precipitated only with this antibody. These data imply that anti-isoleucyl-tRNA synthetase antibody bound to p87, and p87 bound to annexin in the precipate. This is another line of evidence for the annexin-binding property of p87.
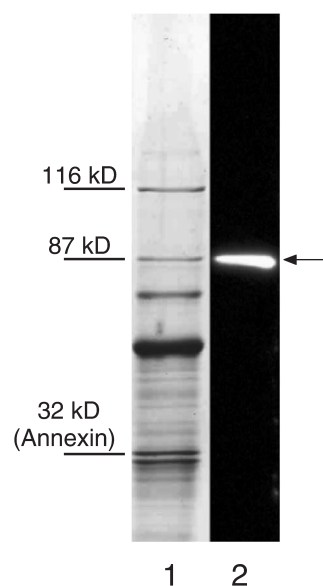
Fig. 3. Annexin-binding of p87. Far-Western blotting with biotinylated annexin showed direct binding of p87 to annexin. Lane 1: CBB stain of immunoprecipitate with 15 μg of anti-annexin antibody. Lane 2: Far-Western blotting. Annexin bound specifically to p87 (arrow).

a.

Homo sapiens	172	V Y R G V K V M P F S T A C N T P L S N F E	193	
Mus musculus	172	V Y R G V K V M P F S T A C G T P L S N F E	193	
Drosophila melanogaster	180	V Y Q G V K V M P Y S T A C T T S L S N F E	201	
Anopheles gambiae	204	V Y Q G V K V M P Y S T A C T T A L S N F E	225	
p116		- - - - - - V M P Y S T V L T T S - - - - -		
		VY GVKVMP STAC T LSNFE		

283	Y L K G K K Y R P L F D Y F L K C K - E N G	303
283	S L K G K K Y K P L F D Y F I K C K - E N G	303
291	T L K D L H Y K P L F P Y F A K R G A E V K	312
316	K L V G V R Y E P L F D Y F R K Y - - E S V	335
	- - - - - K Y T P L F N Y F L K - - - - - -	
	L Y PLF YF K E	

485	A E L E E L S G A K I S D L H R E S V D H L	506
485	A E L E E L S G T K I S D L H R E S I D H L	506
484	K Q L A E L S G V Q V E D L H R E S I D H I	515
517	D E L E R Y S G V R V T D L H R E S I D H I	538
	- - - - - L S G V R V T D L H - - - - - - -	
	L LSG DLHRES DH	

997	L R K K C N L V P T D E I T V Y Y K A K S E	1018
997	L R K K C N L V P T D E I T V Y Y N A K S E	1018
1009	L K K K A Q L I P T D P V L I F H E L A A D	1030
1024	L K K K A K L I P T D P V L I Y Y T V S G G	1045
	- - - - A G V N P G D P V E V F Y A V - - -	
	L KK L PTD Y	

b.

Homo sapiens	46	G H A K A A L L N Q H Y Q V N F K G K L I M	67	
Mus musculus	213	G H A K A A L L N Q H Y Q V N F K G K L I M	234	
Drosophila melanogaster	219	G H A K A A L L N Q Y Y A L A F Q G T L I M	240	
Oryza sativa	222	G H A K A A L L N K Y F A E R Y Q G R L I V	243	
p87		- - - - A A M L N S F Y A T S Y K P - - - -		
		GHAKAALLN Y G LI		

118	Q E G K A Y V D D T P A E Q M K A E R E Q R	139
265	Q E G K A Y V D D T P A E Q M K A E R E Q R	306
291	K E S K A Y V D D T P P E Q M K L E R E Q R	312
294	K Q G K A Y V D D T P K E Q M R S E R M D G	315
	- - - - A F I D D D P L E Q M R A E R - - -	
	KAYVDDTP EQM ER	

Fig. 4. Multiple alignment of partial amino acid sequences of p116 and p87 with their homologous protein sequences. (a.) For four amino acid sequences of (putative) isoleucyl-tRNA synthetase and p116, multiple alignment was done. The four peptide fragments of p116 shows homologies with the following four sequences. *Homo sapiens*: isoleucyl-tRNA synthetase (GenBank accession number NP_002152) *Mus musculus*: isoleucyl-tRNA synthetase (GenBank accession number NP_742012) *Drosophila melanogaster* isoleucyl-tRNA synthetase CG11471-PA (GenBank accession number NP_730716) *Anopheles gambiae*: ENSANGP00000017588 (GenBank accession number XP_320939) (b.) For four amino acid sequences of (putative) glutaminyl- or glutamyl-tRNA synthetase and p87, multiple alignment was done. The two peptide fragments of p87 show homologies with the following four sequences. *Homo sapiens*: glutaminyl-tRNA synthetase (GenBank accession number CAA 30354) *Mus musculus* glutamyl-prolyl-tRNA synthetase (GenBank accession number XP_129647) *Drosophila melanogaster*: glutamyl-prolyl-tRNA synthetase CG5394-PA (GenBank accession number NP_524471) *Oryza sativa*: putative glutamyl-tRNA synthetase (GenBank accession number NP_921080). In both the figures, the lower columns with residues in gray shadings indicate identical amino acids for at least four of the compared sequence. A pair of numbers sandwiching the amino acid sequences for individual species represent the position of amino acids from the N-terminal.

Discussion

In this study, we demonstrated the presence of the two annexin-binding proteins, p116 and p87. p87 shows direct binding (Fig. 3) and p116 indirect binding most plausibly via p87. Both proteins were identified as class I ARSs (Burbaum and Schimmel, 1991). Before the present study, it was not known that ARS binds to annexin. Thus, the class I ARSs, or at least one of them, is a novel annexin-binding protein. It is surprising that annexin and ARS interact with each other, because they seem to work in utterly different cellular compartments. The finding that annexin binds to ARSs, one directly and the other indirectly, may imply that there may be some interaction between membrane dynamics and protein translation systems.

ARS was identified as novel annexin-binding protein

Two proteins, p116 and p87, co-precipitated with annexin in a stoichiometric manner (Fig. 1 and 2). The amounts of these three proteins were approximately 1:1:2 in molar ratio. This stoichiometry strongly indicates that the annexin-binding of p116 and p87 is not non-specific but specific. In addition, biotinylated recombinant annexin bound to p87 directly (Fig. 3). By these experiments, it was strongly demonstrated that p87 is an annexin-binding protein, and forms a protein complex with annexin and p116. It is most likely that annexin exists as a dimer while the other two proteins are monomers. It is well known that class I ARSs, such as isoleucyl-, leucyl-, glutaminyl- and glutamyl-tRNA synthetase, bind to each other to form a complex (Cerini *et al.*, 1997). In this multisynthetase complex, isoleucyl- and glutaminyl-tRNA synthetases are present in 1:1 ratio, consistent with the 1:1 stoichiometry between p87 and p116.

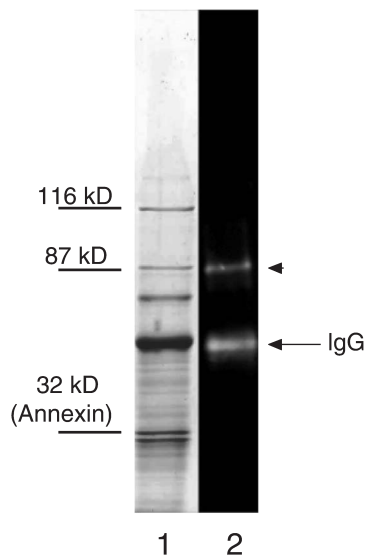ARS is well known as a member of the protein translation

Fig. 5. Anti-isoleucyl-tRNA synthetase binding to p87. Anti-isoleucyl-tRNA synthetase antibody cross-reacted with p87 in immunoprecipitate with anti-annexin antibody. Lane 1: CBB stain of immunoprecipitate with 15 μg of anti-annexin antibody. Lane 2: Western blotting. Anti-isoleucyl-tRNA synthetase antibody binds specifically to p87 (arrowhead). Arrow indicates the secondary antibody bound to anti-annexin antibody.

Fig. 6. Class-specific co-precipitation of annexin with anti-isoleucyl-tRNA synthetase antibody. Immunoprecipitation with anti-annexin antibody, as control (lane 1), 1 μl of anti-isoleucyl-tRNA synthetase antibody (lane 2), 1 μl of anti-glycyl-tRNA synthetase antibody (lane 3), 1 μl of anti-histidyl-tRNA synthetase antibody (lane 4), 1 μl of anti-tyrosyl-tRNA synthetase antibody (lane 5). Isoleucyl-tRNA synthetase is a class I ARS, while the others are class II ARSs. Annexin was detected by Western blotting of the immunoprecipitates with anti-annexin antibodies. In four anti-ARS antibodies, only anti-class I ARS antibody but not anti-class II ARS antibody precipitated annexin (arrowhead). The dense band above the annexin band in lane 1 is the anti-annexin antibody light-chain reacted with the secondary antibody used in Western blotting.

system, and also known as an autoantigen in some kind of myositis. Patients with dermatomyositis or polymyositis produce autoantibodies against ARSs and consequently this is called 'ARS syndrome'. In the present immunoprecipitation and the immunoblotting experiments, anti-serums from myositis patients were used (Fig. 5 and 6). The anti-isoleucyl-tRNA synthetase antibody cross-reacted with p87, indicating p87 is ARS, as the homology search results predicted. Though p116 did not show cross-reactivity with the antibody against isoleucyl-tRNA synthetase, this does not necessarily mean that p116 is not ARS. Some of the ARSs are known to lose their antigenecity by processing for SDS-PAGE (Dang *et al.*, 1988). Immunoprecipitation using anti-isoleucyl-tRNA synthetase antibody precipitated annexin, giving another evidence of the annexin-binding property of p87.

Possible cooperation of annexin and ARS

The interaction of annexin and ARS provides two possibilities: firstly, annexin may play some role in the protein translation machinery; secondly, ARS may be involved membrane fusion or other membrane dynamics where annexin is involved. Although there is no direct evidence regarding the physiological function of the annexin-ARS complex, there are two findings that may imply the significance of the interaction of annexin with ARS. About 10% of the myositis patients produce the autoantibody against annexin as well as ARS (Mimori, 1996). Furthermore, the
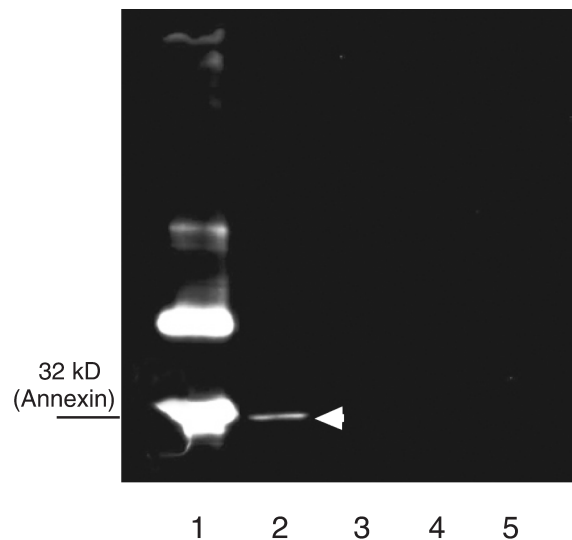
expression of annexin and some ARSs are similarly elevated by γ-interferon at the same time (Shaw *et al.*, 1999). These findings may suggest that annexin and ARS share some physiological role in processes relevant to myositis and immune responses.

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