

Characterization and ultrastructural localization of annexin VI from mitochondria

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Abstract Annexin VI, a member of a family of related intracellular proteins that associate reversibly with membrane phospholipids in a Ca^{2+} -dependent manner, has been purified from bovine liver mitochondria and characterized. Moreover, biochemical and immunocytochemical lines of evidence are presented which strongly suggest that annexin VI is closely associated with the cristae in the inner membrane of mitochondria. These findings are consistent with a calcium channel activity of annexin VI in mitochondria.

Key words: Annexin VI; Mitochondria

1. Introduction

The annexin family consists of a growing number of closely related water-soluble proteins that associate reversibly with membrane phospholipids in a Ca^{2+} -dependent manner (for reviews, see [1,2]). They are characterized by homologous domains of 70 amino acids in length which contain a highly conserved 17-amino acid consensus sequence termed endonexin fold. Annexin VI, a 67 kDa member of this family, consists of eight domains flanked by a unique amino terminal region. The true physiological function of annexin VI is not yet fully understood. Nevertheless, it has been postulated that it may underlie endocytosis of surface membrane through the formation and pinching off of clathrin-coated vesicles [3]. This hypothesis is in agreement with the localization of annexin VI along the cytoplasmic face of the plasma membrane in ameloblasts and odontoblasts [4] and in enterocytes and hepatocytes [5]. Moreover, annexin VI has also been immunodetected in the mitochondria of a variety of cells: male germ cells [6], ameloblasts and odontoblasts [4], enterocytes and hepatocytes [5]. It was therefore felt that the knowledge of the precise location of annexin VI in mitochondria might clarify its function in these organelles. In this study, we have purified annexin VI from bovine liver mitochondria and demonstrated that it is identical to the other known bovine annexins VI. Moreover, we have examined the ultrastructural localization of annexin VI in mitochondria from rat enterocytes and shown that it is closely associated with the cristae of the inner membrane. This finding is in agreement with the reported calcium channel activity of purified annexin VI [7].

2. Materials and methods

2.1. Purification of annexin VI and production of antibodies to annexin VI

Annexin VI was purified from rat or bovine liver according to Mathew et al. [8]. Antibodies to rat liver annexin VI were developed in sheep and affinity-purified as described by Smith and Dedman [9]. Using the same procedures, affinity-purified antibodies were also elicited in rabbit against bovine liver annexin VI.

2.2. Immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using the antibody to annexin VI from bovine liver were performed according to directions provided by BioRad which were compiled from the methods of Laemmli [10] and Towbin et al. [11].

2.3. Isolation of mitochondria and microsomes from bovine liver

Bovine liver was homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 0.25 M sucrose, referred to as the sucrose buffer, and the homogenate was fractionated by differential centrifugation as described by Johnson and Lardy [12]. After centrifugation at $750 \times g$ for 10 min, the post-nuclear supernatant was subjected to $15,000 \times g$ for 15 min to pellet the mitochondria. The pellet was given three washes using sucrose buffer and re-centrifugation. The mitochondrial fraction was resuspended in sucrose buffer at a concentration of 40 mg protein/ml and referred to as crude mitochondria. Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at $100,000 \times g$ for 60 min. The microsomal fraction was resuspended in sucrose buffer at a concentration of 40 mg protein/ml. The crude mitochondria were purified by Percoll density gradient centrifugation according to Broekemeier et al. [13]. One ml of the crude mitochondrial preparation was layered onto 23 ml of 30% Percoll in sucrose buffer and subjected to centrifugation in a Beckman 60 Ti rotor at $50,000 \times g$ for 30 min, at 4°C . The mitochondria collected at the bottom of the tube were given three washes using sucrose buffer and re-centrifugation at $15,000 \times g$ for 10 min at 4°C . The purity of the mitochondrial fraction was assessed by measurement of the activities of citrate synthase, a mitochondrial enzyme, and 5'-nucleotidase, a plasma membrane enzyme. Citrate synthase activity was determined according to Srere [14] in Tris-HCl buffer, pH 7.8, at 30°C and expressed in international units (IU). It was found to be about 24 and 35 mU/mg protein in the crude and purified mitochondrial fractions, respectively. The latter value was close to that reported by Shepherd and Garland [15] for purified mitochondrial fraction from rat liver (about 50 mU/mg protein). 5'-Nucleotidase activity was performed by using the reagents from Sigma Diagnostics (St. Louis, MO) (kit ref. 265), according to directions provided by the manufacturer. No activity was found in both the crude and purified mitochondrial fractions. Moreover, for control experiments, mitochondria were further purified by digitonin treatment, according to Colbeau et al. [16]. Mitochondria (40 μg protein/ml) were incubated in sucrose buffer containing 50 μg digitonin/mg protein for 10 min, at 4°C and collected by 10-fold dilution with sucrose buffer and centrifugation at $15,000 \times g$ for 10 min, at 4°C . All samples were stored at -20°C .

2.4. Purification of annexin VI from mitochondria of bovine liver

Mitochondria of bovine liver (100 g) purified on Percoll gradient were homogenized 1:2.5 in 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 7 mM 2-mercapto-ethanol, 0.25 M NaCl, 1 mM phenylmethylsulfonyl fluoride in a Waring Blender. The homogenate was sonicated by means

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of a Branson sonifier at full-power for 2 min, and centrifuged at $100,000 \times g$ for 60 min. The pellet was re-extracted under the same conditions. The supernatants were pooled and dialysed against 10 mM Tris-HCl, 1 mM EGTA. The dialysate was centrifuged at $20,000 \times g$ for 30 min and mixed with 300 ml of phospholipid saturated phenyl-Sepharose (Pharmacia) [17]. CaCl_2 was added to a final concentration of 1 mM. The resin was stirred overnight and poured in a column (5×30 cm). Following washing with Tris-HCl, pH 7.4, 1 mM CaCl_2 , 0.5 M NaCl, annexin VI was eluted with Tris-HCl, pH 7.4, 10 mM EDTA, 0.5 M NaCl. Annexin VI was further purified by gel filtration on a Sephacryl S200 (Pharmacia) column (1×120 cm) equilibrated in 50 mM HEPES, pH 7.4, 2 mM EGTA, 0.2 M NaCl. At each step, the presence and purity of annexin VI were monitored by SDS-PAGE and immunoblot analysis.

2.5. Thermolysin peptides of mitochondrial annexin VI

Limited proteolysis of annexin VI from bovine liver mitochondria by thermolysin (Serva) was carried out according to Martin et al. [18]. It was performed with an enzyme/substrate ratio of 1:40 (w/w), in the presence of 2 mM CaCl_2 , for 3 h, at 37°C . The thermolysin peptides were separated on a DE 52 (Whatman) column using a discontinuous NaCl gradient from 0 to 0.4 M. A plateau was made when the first peptide was eluted in order to prevent an overlapping of the two protein peaks.

2.6. Amino acid and sequence determination

Amino acid analysis of annexin VI from bovine liver mitochondria was performed after hydrolysis of samples in 6 N HCl plus 1% phenol in evacuated tubes, in a Pico Tag Work Station (Waters), at 110°C , for 20 or 70 h. The samples were analyzed on a Beckman 6300 amino acid analyser, as described by Mansuelle et al. [19]. The thermolysin peptides (300 pmol) were desalted using ProSpin Samples Preparation Cartridges (Applied Biosystem) and sequenced on an Applied Biosystem 476A. The program FSTBLT was used.

2.7. Incubation of mitochondria and microsomes with proteolytic enzymes

Samples of mitochondria purified by Percoll gradient or Percoll gradient and digitonin treatment, and samples of microsomes were suspended in 1 ml of 10 mM HEPES, pH 7.4, 1 mM EGTA, 0.3 M sucrose and incubated in the presence of 1 mg trypsin per 10 mg protein for 0, 20, or 80 min, at 4°C . Alternatively, samples of the same materials were suspended in 10 mM HEPES, pH 7.4, 2 mM Ca^{2+} , 0.3 M sucrose and incubated in the presence of 1 mg thermolysin for the same periods of

time and at the same temperature. Thereafter, 1 ml of hot (60°C) electrophoresis sample buffer was added to each sample and the mixture submitted to SDS-PAGE and immunoblot analysis. Alternatively, after a 10-fold dilution with the fixation buffer, mitochondria incubated in the presence of proteolytic enzymes for 80 min were pelleted by centrifugation at $15,000 \times g$ for 10 min and processed for immunoelectron microscopy, as described below. For control, 50 μg purified annexin VI from bovine liver were proteolysed under the same experimental conditions.

2.8. Immunoelectron microscopy

Samples of bovine liver mitochondria and rat duodenum were fixed in 0.5% glutaraldehyde, 5 mM CaCl_2 , 0.1 M cacodylate buffer, pH 7.2, for 1 h, at 4°C , embedded in Lowicryl K4M, and processed for immunogold labeling, as described by Weinman et al. [20], using the primary antibodies at an IgG concentration of 25–50 $\mu\text{g}/\text{ml}$ and the appropriate gold (5 or 10 nm)-labeled secondary antibodies at a dilution of 1:50. The specificity of immunolabeling was assessed by comparison with labeling patterns obtained from antibodies preabsorbed with a 10 M excess of the complementary antigen. Gold grids were viewed in a Philips EM 300 electron microscope operated at 80 kV.

2.9. Data analysis

All experiments presented in this paper were performed at least three times using independent preparations from different animals. Similar patterns were observed, and the trends and ratios were the same in each case.

3. Results

3.1. Demonstration of the presence of annexin VI in different fractions of bovine liver

Homogenates of whole bovine liver, bovine liver mitochondria purified by Percoll density gradient centrifugation or bovine liver microsomes were submitted to SDS-7.5% PAGE and reacted with the antibody to bovine annexin VI. In homogenates of whole liver (Fig. 1, L) or microsomes (Fig. 1, m), the antibody bound specifically to a closely spaced polypeptide doublet, the upper band of which was lighter than the lower. In mitochondria (Fig. 1, M), only one band could be observed at the level of the upper band of the doublet.

Table 1

Comparison of the amino acid composition (mol %) of the 67 kDa protein isolated from bovine liver mitochondria with those of annexins VI isolated from Bovine liver [22,23], bovine brain [24], or bovine aorta [18], and with the amino acid composition calculated from the sequence of the human annexin VI [25].

Amino acid	67 kDa protein from bovine liver mitochondria	Bovine liver annexin VI		Bovine brain annexin VI	Bovine aorta annexin VI	Human annexin VI
Asx	11.0	10.3	10.4	10.5	11.2	10.8
Thr ^a	5.5	7.3	5.0	5.3	5.7	5.6
Ser ^a	7.6	11.9	6.2	8.3	5.8	5.9
Glx	11.6	11.7	11.4	13.2	13.0	12.0
Pro	2.2	3.8	4.0	2.6	2.4	2.2
Gly	7.2	7.1	7.0	9.0	8.5	6.7
Ala	9.3	11.9	9.2	8.3	9.0	8.6
Val	3.5	3.6	5.4	2.6	4.0	3.9
Cys	0.9	1.0	0.3	1.1	0.7	0.9
Met	3.0	5.9	2.8	2.6	2.3	3.9
Ile	6.1	6.3	5.3	4.9	6.2	7.0
Leu	9.6	8.2	10.7	9.8	9.5	9.8
Tyr	3.5	2.7	3.5	2.6	3.4	3.4
Phe	3.7	3.2	3.6	3.0	3.1	3.7
Lys	7.7	5.2	7.1	9.0	7.9	7.7
His	1.7	1.5	2.1	1.5	1.4	1.9
Arg	5.8	6.5	6.3	5.6	5.2	6.2
Trp	nd	nd	nd	nd	0.4	0.3

^a After extrapolation at zero time hydrolysis. nd: not determined.

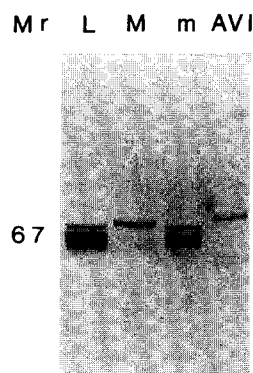


Fig. 1. Western blot analysis of different cellular fractions of bovine liver. Homogenates of whole liver (L), purified mitochondria (M) or microsomes (m) are co-electrophoresed on a SDS-7.5% polyacrylamide gel with annexin VI purified from bovine liver (AVI). M_r = molecular weight.

3.2. Purification and characterization of annexin VI from bovine liver mitochondria

When bovine liver mitochondria were treated by the procedure described in section 2, a pure protein of M_r 67 kDa was obtained, as determined by SDS-13% PAGE and immunoblot analysis (not shown). Its amino acid composition is presented in Table 1, in comparison with those of some known bovine or human annexins VI. Its N-terminus was blocked. Its thermolysin digestion yielded two major fragments of 33 and 34 kDa, which were eluted from a DE 52 column at 0.2 and 0.3 M NaCl, respectively (Fig. 2). Their partial sequences are shown in Table 2, in comparison with those of other known bovine or human annexins VI.

3.3. Proteolytic digestion of annexin VI in mitochondria

Purified mitochondria were incubated in the presence of trypsin or thermolysin for 0, 20 and 80 min and submitted to SDS-13% PAGE and Western blot analysis for annexin VI and its proteolytic fragments. As can be seen in Fig. 3, annexin VI was sensitive to trypsin or thermolysin proteolysis neither in intact nor in sonicated mitochondria. In contrast, trypsin and thermolysin caused the almost complete digestion of annexin VI in sonicated mitochondria treated with Triton X-100, as they did with microsomes or solution of purified annexin VI.

Table 2

Comparison of the sequences of 33 and 34 thermolysin fragments from the 67 kDa protein isolated from the bovine liver mitochondria with those of known annexin VI.

33 kDa fragment	AQGAKYRGSIRDFFDFNPSQDAETL
Bovine aorta annexin VI [18]	AQGAKYRGSIRDFFDFNPSQDAETL
Human annexin VI [25]	⁴ AQGAKYRGSIHDFPGFDPNQDAEAL ²⁸
34 kDa fragments	VARVELKGTVRPAGDFNPDADAKA LKGTVRPAGDFNPDADAKA VRPAGDFNPDADAKA
Bovine aorta annexin VI [18]	VARVELKGTVRPAGDFNPDADAKA
Bovine liver annexin VI [26]	³⁴⁷ VARVELKGTVRPAGDFNPDADAKA ³⁷⁰
Human annexin VI [25]	³⁴⁷ VARVELKGTVRPANDFNPDADAKA ³⁷⁰

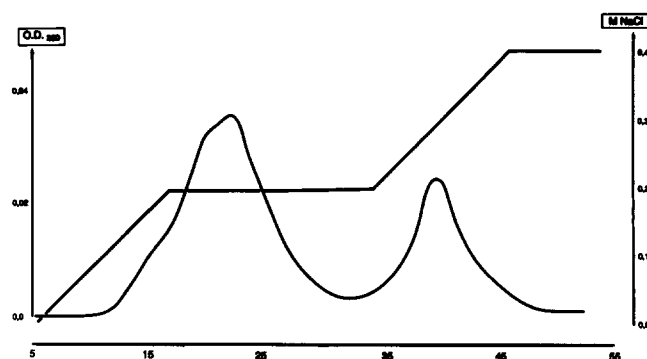


Fig. 2. Purification of peptides from a thermolysin digest of the 67 kDa protein isolated from bovine liver mitochondria on a DE 52 column. Following an initial wash with equilibration buffer, peptides were eluted with a discontinuous NaCl gradient from 0 to 0.4 M.

3.4. Ultrastructural localization of annexin VI in mitochondria

Purified mitochondria isolated from bovine liver were processed for immunogold electron microscopy. With the affinity-purified antibodies to the rat liver annexin VI and appropriate gold (5 nm)-labeled secondary antibody, numerous gold particles were localized on the electron dense cristae in the mitochondria purified by Percoll gradient or Percoll gradient and digitonin treatment (Fig. 4a and b). The same labeling was also found in mitochondria incubated in the presence of trypsin for 80 min (Fig. 4c). Similar results were obtained with the antibodies to the bovine liver annexin VI (Fig. 4e and f), although, due to the 10 nm diameter of the gold particles of the secondary antibody, the staining was much less intense than that obtained with the gold (5 nm)-labeled secondary antibody. The specificity of immunolabeling was assessed by comparison with labeling obtained with antibodies preabsorbed with an excess of complementary antigen. Under these conditions, very few background signals could be observed (Fig. 4d and g). Contamination by plasma membrane fragments or other organelles was negligible (data not shown). In rat duodenal enterocytes, the cristae of the mitochondria (Fig. 4h and i) showed intense labeling with the antibody to annexin VI.

4. Discussion

Annexin VI has been detected in mitochondria of a variety of tissues by immunogold electron microscopy [4,5,6]. As two forms of annexins VI have been described by Moss and Crumpton [21], the first goal of this study has been to characterize mitochondrial annexin VI. Mitochondrial annexin VI from bovine liver has the same electrophoretic migration as the form exhibiting the hydrophobic hexapeptide VAAEIL [21]. Its amino acid composition is very similar to those of previously described bovine [18,22,23,24] or human annexins VI [25] and its N-terminus is blocked. Proteolysis of mitochondrial annexin VI gives rise to peptides identical to those of bovine annexins VI from other sources, as determined by sequence determination [18,25,26]. Thus, it may be assumed that the bovine liver mitochondrial annexin VI is not different from the hydrophobic form of the annexins VI found in other bovine tissues.

An approach to understanding the physiological role of mitochondrial annexin VI has been to determine its relation with submitochondrial structures. The effect of protease digestion

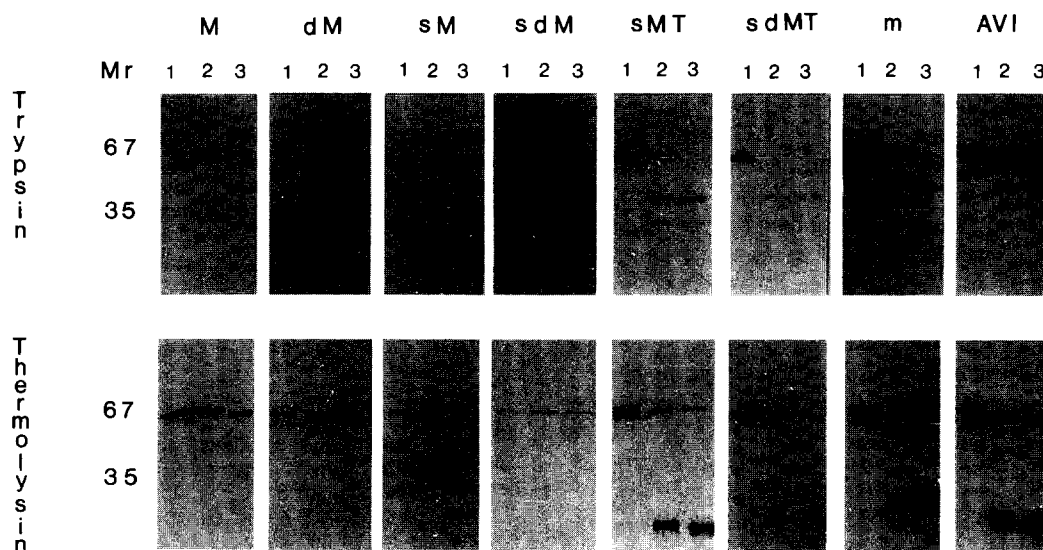


Fig. 3. Immunoblot analysis of the proteolytic digestion of annexin VI in bovine liver mitochondria. M and dM = intact mitochondria purified by Percoll density gradient or Percoll density gradient and digitonin treatment, respectively; sM and sdM = sonicated mitochondria; sMT and sdMT = sonicated mitochondria treated with Triton X-100; m = microsomes, or AVI = purified bovine liver annexin VI, were incubated with trypsin or thermolysin for 0, 20 or 80 min (lanes 1, 2 and 3, respectively) and subjected to SDS-13% PAGE and immunoblot analysis. Only mitochondria sonicated and treated with Triton X-100 (sMT and sdMT), microsomes and purified annexin VI were sensitive to proteolysis. M_r = molecular weight.

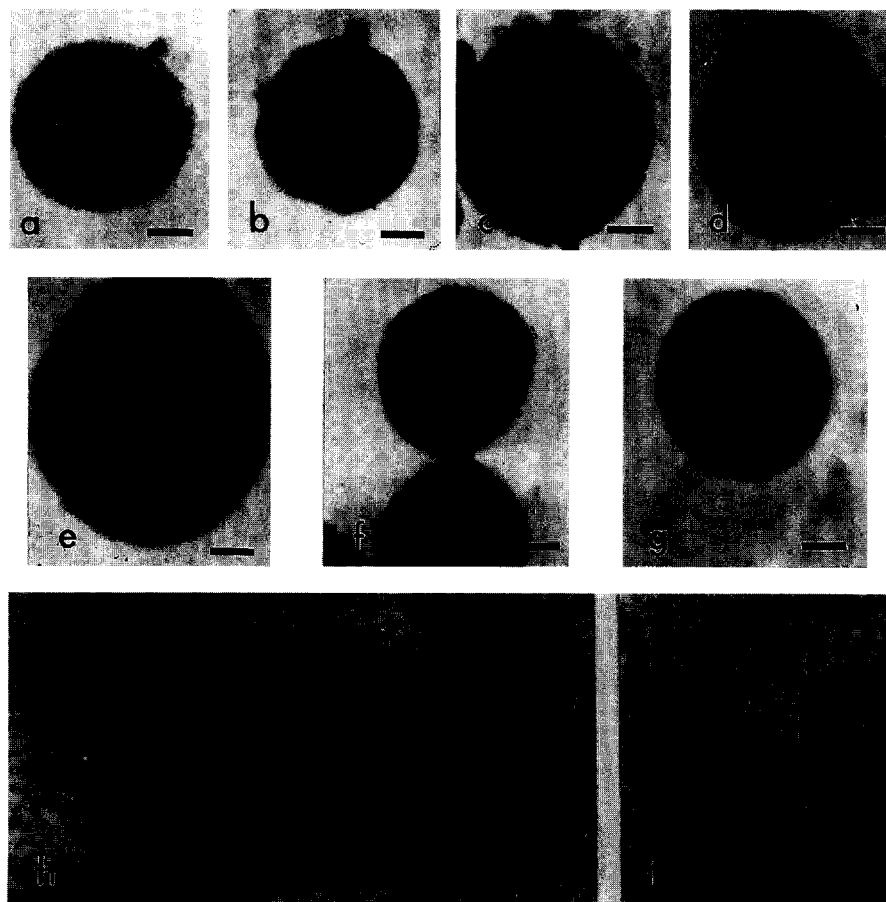


Fig. 4. Immunogold electron micrographs of bovine liver (a–g) and rat duodenal (h–i) mitochondria using antibodies against rat liver annexin VI and the appropriate gold (5 nm)-labeled secondary antibody (a–d and h–i) or antibodies against bovine liver annexin VI and the appropriate gold (10 nm)-labeled secondary antibody (e–g). (a) and (e) Mitochondria purified by Percoll density gradient. (b) Mitochondria purified by Percoll density gradient and digitonin treatment. (c) and (f) Mitochondria incubated in the presence of trypsin for 80 min. (d) and (g) Control experiments with the preabsorbed antibodies to annexin VI. Gold particles are found over the cristae in the mitochondria. Original magnification: $\times 20,000$. Bar = $0.2 \mu\text{m}$.

on binding of annexin VI in mitochondria has been determined. Annexin VI cannot be cleaved in intact purified mitochondria, before or after treatment by digitonin. This result demonstrates that the occurrence of annexin VI on the outer surface of mitochondria is unlikely. Moreover, annexin VI can be proteolyzed only in sonicated mitochondria by using Triton X-100 in addition to trypsin or thermolysin. These experiments show that proteases have access to annexin VI only in broken mitochondria where the inner membrane has been disrupted with detergent. In addition, immunogold labeling by specific antibodies to annexin VI reveals that annexin VI is located on the mitochondrial cristae. Thus, these lines of data demonstrate that annexin VI is firmly bound to mitochondrial inner membrane. The finding is in agreement with the known property of annexins to associate reversibly with membrane phospholipids in a Ca^{2+} -dependent manner. Moreover, the close association of annexin VI with a highly differentiated submitochondrial structure suggests a role of this protein in a specialized mitochondrial function. Mitochondria are considered to have a very high capacity for accumulating Ca^{2+} despite a poor affinity for the cation (10^{-6} to 10^{-5} M) (for a review, see [27]). As annexin VI exhibits calcium channel activity [6], it may well be a good candidate to play a role in Ca^{2+} gating in mitochondria between the intermembrane space and the matrix through the inner membrane. This hypothesis is reinforced by our finding that, in mammals, annexin VI is found inside mitochondria of all tissues examined [4,5,6], where it is the only calcium-binding protein detected so far. The possible role of annexin VI in mitochondrial Ca^{2+} gating can be compared with recently reported functions of this protein in other organelles. Diaz-Munoz *et al.* [28] have demonstrated that, in excitation-contraction coupling, annexin VI is able to modify the gating of the Ca^{2+} release channel from the luminal surface of the sarcoplasmic reticulum. Cao *et al.* [29] have shown the presence of annexin VI in cartilage matrix vesicles where it could be involved in Ca^{2+} gating through the membrane of these calcifying structures. In the three organelles, where the Ca^{2+} concentration is high, annexin VI binds Ca^{2+} and becomes highly hydrophobic. It could be thereafter recruited into the organelle membranes. The mechanism by which Ca^{2+} -activated annexin VI causes calcium channel activity could involve penetration of membranes by a hydrophobic annexin VI multimer able to span the phospholipid bilayer. Thus, construction of calcium channels from annexin VI would appear to conform to a theme first recognized in ion channels of excitable cells: a functional channel is built from repeats of a structural motif, i.e. the tetrad repeats of the so-called endonexin fold.

In conclusion, we ascertain the tight association of annexin VI with the inner mitochondrial membrane. This localization suggests a physiological role of annexin VI in the Ca^{2+} gating occurring in mitochondria. This finding reinforces the concept that annexin VI might well play a role in the modulation of the Ca^{2+} fluxes taking place in organelles where the Ca^{2+} concentration is high, such as in sarcoplasmic reticulum, cartilage matrix vesicles or mitochondria.

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