Purification, Identification and Phosphorylation of Annexin I from Rat Liver Mitochondria

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Annexin was purified from rat liver mitochondria to an apparent homogeneity with a molecular weight of 35kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified mitochondrial annexin (AXmito) was identified as annexin I by an immunoblot analysis using anti-annexin I antibody. The inhibitory effect of AXmito I on porcine pancreatic phospholipase A_2 activity was as potent as that of bovine lung annexin I. The presence of annexin I in mitochondria was confirmed by an electron-microscopic study. AXmito I was shown to be phosphorylated by intrinsic protein tyrosine kinases on its tyrosine residues. This annexin was also phosphorylated by protein kinase C.

Key words: annexin, mitochondria, protein tyrosine kinases, protein kinase C, phospholipase A_2

Annexins (also called lipocortins) are widely distributed in eukaryotic cells, and at least 13 structurally related proteins have been identified to date and studied in many cells and tissues (1-5). In addition other related proteins such as chromobindins (6), calcimedins (7), endonexins (8), calelectrins (9), calpactins (10), and proteins I-III (11) have also been described by many laboratories. Annexins have been implicated in cellular differentiation and cell growth (12-14), inflammation and glucocorticoid action (15-17, 18), interaction to actin and spectrin (19, 20), and secretion and exocytosis (21-23). We have also shown that annexin I is involved in the pathogenesis of human hepatocellular carcinomas (24). The anti-inflammatory action of annexins is attributed to their inhibititory activity on phospholipase A_2 (PLA_2), a Ca^{2+}-dependent enzyme producing arachidonic acid from membrane phospholipids, although there exists some controversy regarding this inhibition (25, 26). We previously reported that 3 types of annexins have the ability to inhibit to some extent PLA_2 activity *in vitro* (27, 28). Another characteristic of annexins is their susceptibility to phosphorylation by several protein tyrosine kinases (PTKs) (29, 30) or by Ca^{2+}-phospholipid-dependent protein kinase (C-kinase) (28).

We have studied the coupling mechanisms between mitochondrial functions and their configurational changes, and successfully demonstrated the existence of calmodulin, other Ca^{2+}-binding proteins, and actin in mitochondria (31-33). PLA_2 is present in mitochondria and plays an important role in mitochondria's configurational changes induced by phospholipid hydrolysis (32, 34-36). The presence of both actin and PLA_2 in mitochondria suggested the possibility that annexins may be present in mitochondria and regulate PLA_2 or actin-polymerization. The typical purification procedure (28) for annexins allowed us to purify mitochondrial annexin (AXmito).

In the present report, we describe the purification of AXmito and several of its properties, such as immunoreactivity against anti-annexin antibodies, anti-PLA_2 activity, and phosphorylation by PTKs and C-kinase.

Materials and Methods

Materials. Anti-annexin I and II antibodies were kindly provided by Dr. RB Pepinsky (Biogen Res. Co., MA, USA). Bovine lung annexins I and II were purified according to Khanna *et al.* (28). Anti-phosphotyrosine antibody was purchased from Amersham (Tokyo). Protein A-conjugated Sepharose 4B and Percoll were purchased from Pharmacia (Tokyo). Radioisotopes were obtained from Japan Radioisotope Association (Tokyo).

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Angiotensin II, and porcine pancreatic phospholipase A_2 were from Sigma Chemical Co. (Tokyo). Other chemicals were purchased from Wako Pure Chemical Ltd. (Osaka).

Purification of annexin from rat liver mitochondria. Crude mitochondria were purified from rat liver using the conventional differential centrifugation method, and washed 3 times with buffer A (0.25 M sucrose, 40 mM Tris-HCl [pH 7.4], 1 mM dithiothreitol [DTT], 3 mM benzamidine, 50 μM phenylmethylsulfonylfluoride [PMSF], 1 μg/ml leupeptin, and 1 mM EGTA [ethylene glycol bis (β-aminoethylether) N,N,N',N'-tetraacetic acid]). The mitochondria were then suspended in 33% (v/v) Percoll solution in buffer A, and centrifuged at 40,000 $\times$ g for 30 min without electric braking (37). The purified mitochondria were suspended in hypotonic buffer B (20 mM Tris-HCl [pH 7.4], 1 mM DTT, 3 mM benzamidine, 1 μg/ml leupeptin, and 1 mM $CaCl_2$), and sonicated at 19.5 KHz 1 mA for 30 sec with type T-A 4280 Ultrasonicator (Kaijo Denki, Tokyo). After removing the unbroken mitochondria by centrifugation at 8,500 $\times$ g for 10 min, the supernatant was centrifuged at 105,000 $\times$ g for 70 min to separate the soluble matrix and membrane fractions. The membrane fraction was then washed 3 times with buffer B. To extract annexin from membrane, buffer C (containing 1 mM EGTA instead of $CaCl_2$ in buffer B) was added to the membrane fraction and homogenized using a teflon homogenizer. After centrifugation at 105,000 $\times$ g for 70 min, the supernatant was collected as an annexin fraction. For further purification of annexins from this fraction, anion-exchange chromatography (DEAE-Sephacel, Pharmacia), high performance liquid chromatography (HPLC) gel permeation chromatography (TSKgel G3000SW, TOSOH, Tokyo), and HPLC cation-exchange chromatography (TSKgel CM-3SW) were performed according to methods previously described (28). At each step, the fraction was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to examine the annexin's purity.

Phospholipase A_2 assay. PLA_2 assay was performed according to Khanna *et al.* (27) using porcine pancreatic PLA_2 as the enzyme and L-α-palmitoyl-2-[^{14}C]oleyl phosphatidyl-choline as a substrate.

Assay of protein tyrosine kinase activity. The phosphorylation reaction was carried out according to the method previously described (38). The reaction medium contained 40 mM 3-[N-Morpholino] propanesulfonic acid [pH 7.4], 5 mM $MgCl_2$, 1 mM DTT, 7 mM p-nitrophenyl-phosphate, 1 μg/ml leupeptin, and 1 mM angiotensin II as a substrate. The reaction was initiated by the addition of [γ-^{32}P] ATP (5 μCi/ml) at a final concentration of 50 μM ATP, and was terminated by the addition of phosphoric acid after incubation for 30 min at 30 °C.

Phosphorylation of mitochondria. Pure mitochondria were suspended in buffer A, and the phosphorylation reaction was started by adding ^{32}P-labeled ortho-phosphate (^{32}Pi, 0.5 mCi/ml) at a concentration of 0.2 mM. Mitochondria were centrifuged and washed 3 times with buffer A to remove unincorporated free ^{32}Pi. Phosphopeptides were analyzed by SDS-PAGE (12.5% uniform or 7.5–20% gradient acrylamide gels). Gels were treated with 1N KOH for 2 h at 55 °C in order to hydrolyze phosphoserine or phosphothreonine residues, and then dried. Autoradiographic analysis was performed to demonstrate the presence of alkaline-resistant phosphorylation of peptides.

Phosphorylation of annexin by C-kinase. C-kinase was purified from bovine brain as described by Kikkawa *et al.* (39), and phosphorylation by C-kinase was performed as described previously (28).

Immunoprecipitation analysis. Phosphorylated mitochondria were solubilized with 0.5% Triton X-100 in buffer A and incubated with anti-annexin I antibody for 12 h at 4 °C to precipitate annexin I. The antigen-antibody complex was absorbed by Protein A-conjugated Sepharose 4B beads for 2 h at room temperature. The beads were collected by centrifugation and washed thoroughly with 40 mM Tris-HCl buffer [pH 7.4] containing 150 mM KCl, 0.5% Tween-20, and 1 mM DTT, and then boiled in SDS-PAGE sample buffer. After SDS-PAGE, autoradiographic analysis was performed to demonstrate the precipitated phosphopeptides.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed according to the method previously described (40). Phosphorylated peptide (not alkaline-treated) was cut from SDS-PAGE and electro-eluted with 2% agarose gel. Partial acid hydrolysis was performed and phosphoamino acids were separated on a cellulose plate by electrophoresis (1,000 V, 60 min) at pH 3.5 (pyridine/acetic acid/water [1:10:189, v/v]). Marker phosphoamino acids were phosphotyrosine (p-Tyr), phosphothreonine (p-Thr), and phosphoserine (p-Ser), identified by ninhydrin staining.

Autoradiography was carried out on Kodak X-Omatic AR film.

Immunoblot analysis. Immunoblot analysis using anti-annexin I and II antibodies was performed according to the method previously described (28). Bovine lung annexins I and II were used as positive standards for the reaction.

Immunoelectron microscopy. Immunoelectron microscopy was performed as previously described (33).

Others. SDS-PAGE was performed according to Laemmli (41). Protein concentration was measured by the method of Bradford (42) using bovine serum albumin as a standard.

Results

Purification and characterization of annexin from rat liver mitochondria. Percoll-purified mitochondria were separated into matrix and membrane fractions in the presence of 1 mM $CaCl_2$, and the membrane fraction was collected and washed extensively by centrifugation. A millimolar concentration of calcium ions allows annexins to associate to the membrane, and this protein can then be easily extracted by the addition of EGTA, a Ca^{2+}-chelator. Several peptide bands were observed in this extract on SDS-PAGE pattern as shown in Fig. 1A (lane b). An annexin-like peptide was purified to an apparent homogeneity with a molecular weight of 35kDa (lane c) using the typical method for annexins (28). The molecular weight of the AXmito was identical to bovine lung annexin I (lane d) and was smaller than bovine lung annexin II (lane e). From 100 g of rat liver, 10-15 μg of AXmito were purified.

An immunoblot analysis using anti-annexin I and II antibodies was performed (Fig. 1B). The purified peptide was immuno-reacted with anti-annexin I antibody but not

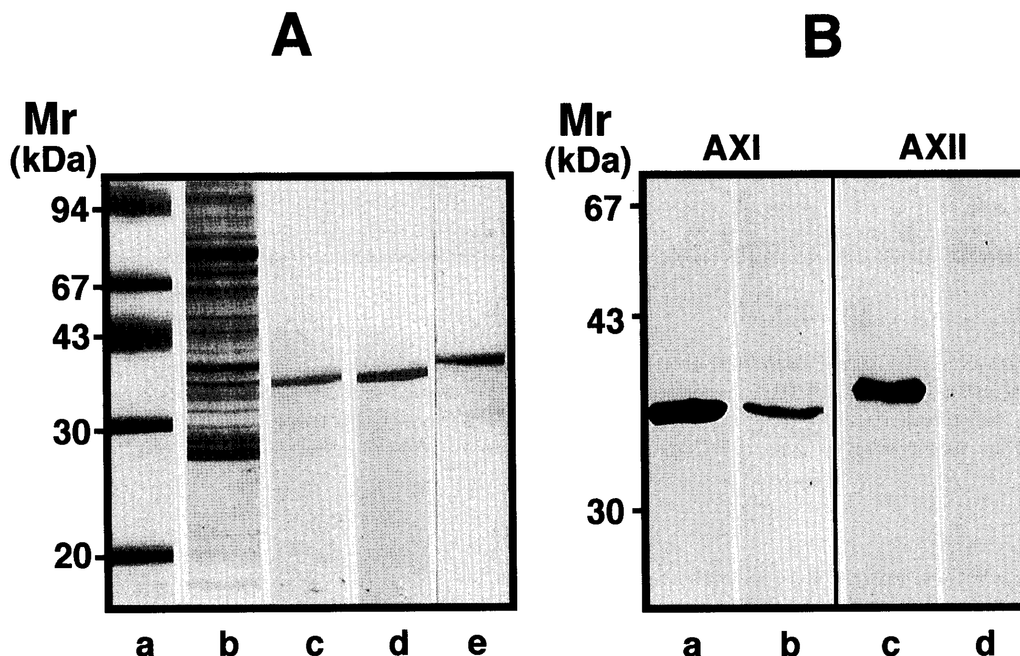

Fig. 1 Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of mitochondrial annexin (AXmito). **A**: SDS-PAGE analysis was performed as described in "Materials and Methods". Lane a, molecular weight markers in daltons (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100); lane b, ethylene glycol bis (β-aminoethylether) N, N, N', N'-tetraacetic acid-extract from mitochondrial membrane fraction; lane c, purified AXmito after high performance liquid chromatography-carboxy-methyl chromatography; lane d, purified bovine lung annexin I; lane e, purified bovine lung annexin II.

B: Western blot analysis was performed as described in "Materials and Methods". The first antibody used was either anti-annexin I antibody (AXI) or anti-annexin II antibody (AXII). Lane a, bovine lung annexin I; lanes b and d, AXmito; lane c, bovine lung annexin II.

with anti-annexin II antibody.

To confirm the presence of annexin I in rat liver mitochondria, an immunoelectron-microscopic study using anti-annexin I antibody was performed (Fig. 2). About 90 % of immunoreactive gold particles for annexin I were located in mitochondria (Fig. 2b). Most of the particles were located in the membranous structures and a few in the matrix. Only a few particles were observed when pre-immune serum and anti-annexin II antibody were used (Figs. 2a and 2c, respectively).

Anti-PLA$_2$ activity of mitochondrial annexin. We examined the inhibitory effect of AXmito on PLA$_2$ activity *in vitro* using 50 ng of porcine pancreatic PLA$_2$ as a target enzyme and 1 nmole of L-α-palmitoyl-2-[^{14}C] oleyl phosphatidylcholine as a substrate. As shown in Fig. 3, AXmito was as potent as bovine lung annexin I in inhibiting PLA$_2$ activity.

Presence of protein tyrosine kinases in

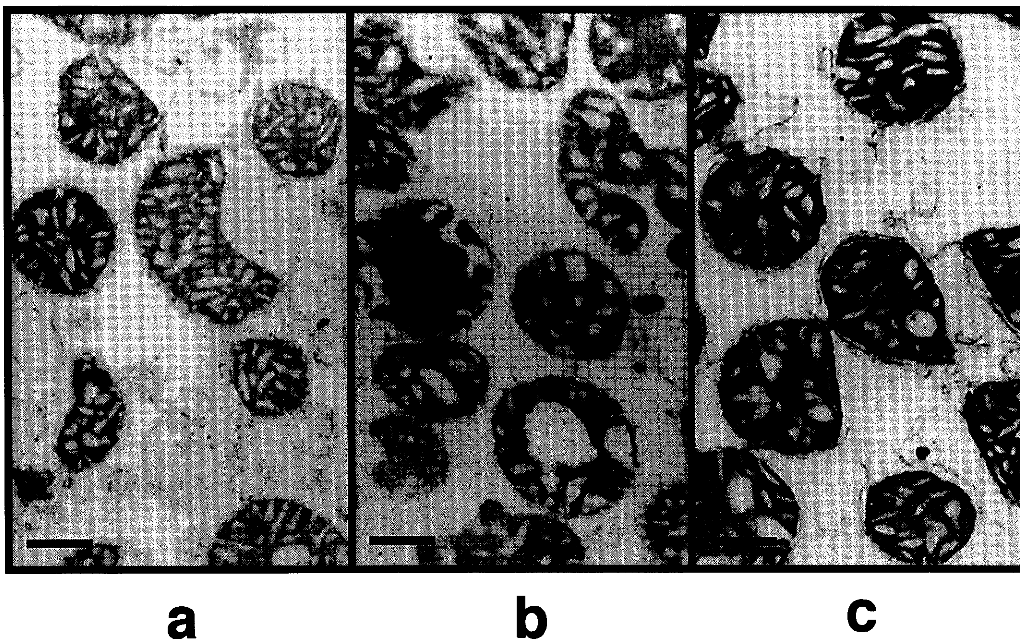

Fig. 2 Immunoelectron microscopic analysis of annexin I in rat liver mitochondria.
Percoll-purified mitochondria were subjected to immunoelectron microscopic analysis as described in "Materials and Methods". Pre-immune rabbit serum (**a**), anti-annexin I antibody (**b**) and anti-annexin II antibody (**c**) were used as the first antibody. Gold particle-conjugated anti-rabbit IgG was used as the second antibody. Bars in the figure indicate 0.5 μm.

Table 1 Protein tyrosine kinase (PTK) activity[a] in rat liver mitochondria

Fractions	Specific activity[b] (cpm/mg protein/min)	Protein (mg)	Total activity (cpm)	Yield (%)
Crude mitochondria	486	362	176,000	100
Percoll-purified mitochondria	392	258	72,500	57.4
Membrane fraction	426	96	40,900	23.2
Matrix fraction	86	105	9,030	5.1

a : PTK activity was measured by the method described in the "Materials and Methods".
b : Specific activity was estimated as the amount of incorporated ^{32}P-labeled ortho-phosphate (^{32}Pi, cpm) in angiotensin II for 1 min by PTKs contained in 1 mg protein of each fraction.

mitochondria. Another property of annexin is its susceptibility to phosphorylation by several PTKs such as $pp60^{v-src}$ (29) and epidermal growth factor receptor kinase (30). As shown in Table 1, PTK activity in mitochondria was shown using angiotensin II as a substrate. There was PTK activity in the crude mitochondrial fraction, and after washing 3 times with buffer A and subsequent Percoll purification, about 60% of the activity was retained in mitochondria. This activity was not significantly reduced by further washing with buffer A, and the membrane fraction was shown to contain 4 times higher PTK activity than the matrix fraction.

Phosphorylation of mitochondrial annexin by intrinsic PTKs. Intact mitochondria were incubated with ^{32}Pi in buffer A. Mitochondria were then solubilized and analyzed by SDS-PAGE. When succinate was added as a substrate for the Krebs cycle, tyrosine-phosphorylation of 2 peptides (about 300kDa and 35kDa) occurred (Fig. 4, right panel). Both peptides were phosphorylated at 15 min. The 300kDa peptide was quickly dephosphorylated by 85% at 30 min and by 100 % at 45 min. In contrast, 2.4 fold phosphorylation of 35kDa peptide occurred at 30 min and then dephosphorylation occurred gradually, and even after 60 min, about 25% of the maximum phosphorylation level (at 30 min) was retained. The fact that no other major tyrosil-phosphorylated bands were seen indicates that annexin I in mitochondria was the major substrate for mitochondrial PTKs. When succinate was not added, almost no phosphorylation was observed except for a trace phosphorylation of 35kDa peptide at 15 min (Fig. 4, left panel).

Immunoprecipitation experiment using anti-annexin I antibody revealed that the precipitated peptide was phosphorylated (Fig. 5A, lane b) and the phosphorylation was resistant to alkaline treatment (Fig. 5A, lane c). The 35kDa phosphopeptide was then extracted from a non-alkaline treated gel, and a phosphoamino acid analysis was performed. Autoradiography demonstrated that only tyrosine residue was phosphorylated (Fig. 5B).

Phosphorylation of mitochondrial annexin by C-kinase. Purified AXmito was phosphorylated by C-kinase as shown in Fig. 5C. AXmito was strongly phosphorylated by C-kinase in the presence of Ca^{2+} and phospholipid (lane b); however, no phosphorylation was observed in their absence (lane a).

Discussion

An annexin was purified from rat mitochondria to an apparent homogeneity and identified as annexin I based on the following evidence: (a) the apparent molecular weight of the purified protein was 35kDa which is identical to annexin I from bovine lung preparation (Fig. 1A); (b) the protein was immunologically recognized by anti-annexin I antibody but not by anti-annexin II antibody (Fig. 1B); (c) an inhibitory effect of the purified protein on PLA_2 activity *in vitro* was as potent as that of bovine lung annexin I (Fig. 3), and (d) the protein was tyrosine-phosphorylated by endogenous PTKs (Figs. 5A, 5B), and also phosphorylated by C-kinase (Fig. 5C).

One might argue that cytosolic annexin I was co-

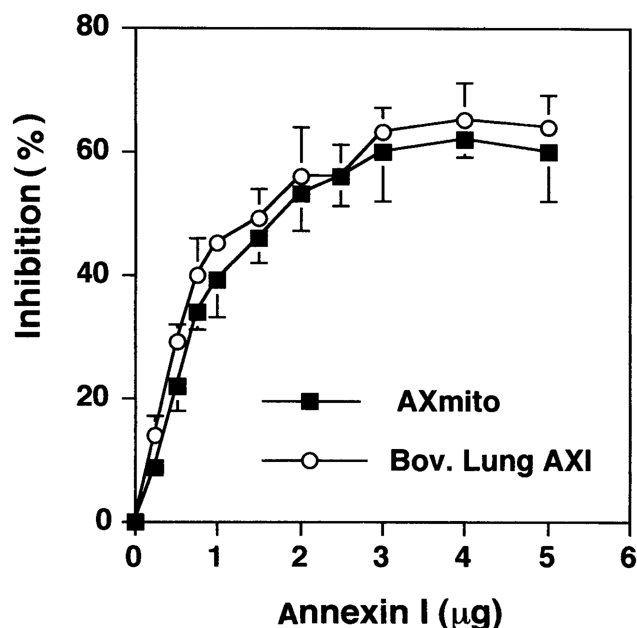

Fig. 3 Anti-phospholipase A_2 (PLA_2) activity of mitochondrial annexin (AXmito).
PLA_2 activity was assayed using the method described in "Materials and Methods". The reaction mixture contained 50 ng of porcine pancreatic PLA_2 and 1 nmole of L-α-palmitoyl-2-[^{14}C] oleoyl phosphatidylcholine (approximately 20,000 dpm). A specific amount of bovine lung annexin I (Bov. Lung AXI, ○) or AXmito (■) was added to the reaction mixture 10 min before the addition of the substrate, and the reaction was started by the addition of labeled substrate and incubated for 30 min at 6 °C. Percent inhibition indicates [(PLA_2 activity without annexin) − (PLA_2 activity with annexin)] ÷ [PLA_2 activity without annexin] × 100. All average values and standard deviations were calculated by 3 different experiments.

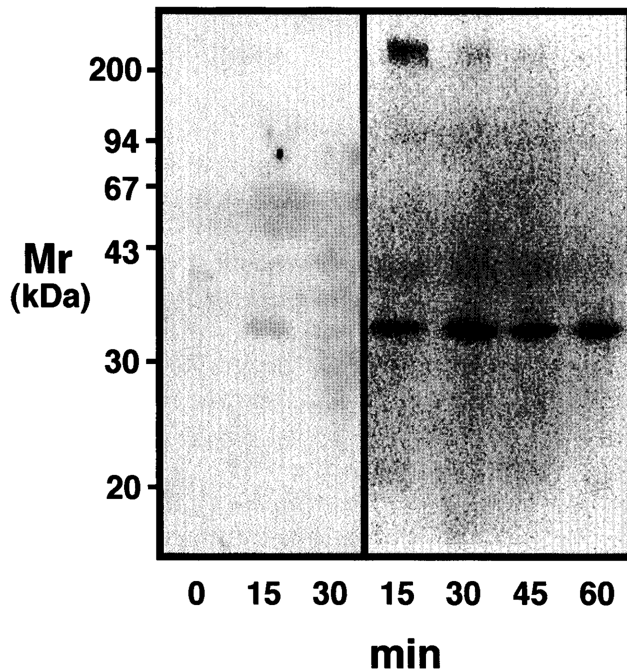

Fig. 4 Time course analysis of alkaline-resistant phosphopeptides in mitochondria.
Whole mitochondria were incubated with ^{32}Pi (0.5 mCi/ml) at 37 °C in the buffer A as described in "Materials and Methods", and the reaction was terminated each time by boiling in SDS-PAGE sample buffer. After SDS-PAGE analysis, the gel was stained and then treated with 1N KOH for 2 h at 55 °C, and was subjected to autoradiography. Molecular weight markers were the same as those used in Fig. 1A except for myosin (200,000) and α-lactalbumin (14,300). Left panel, without succinate; right panel, with succinate (2 mM).
^{32}Pi; SDS; PAGE, see legends to Table 1 and Fig. 1.

purified with mitochondria preparation. The mitochondria used for this study were washed at least 3 times in the presence of EGTA, and were further purified using the Percoll gradient method. The fact that both annexin I and II immunoreactivities were present in the 100,000 xg hepatocyte supernatant (data not shown), while only annexin I and not annexin II was detected in mitochondria (both EGTA extract and HPLC-CM-purified fractions: blotting data using anti-annexi II antibody not shown) indicates that the possibility of contamination of cytosolic annexins during the purification steps can be excluded. Furthermore, an immunoelectron microscopic analysis (Fig. 2) elucidated that annexin I was present in mitochondria and existed in trace amounts in contaminated structures such as microsomes.

Since 1 mM $CaCl_2$ was present at the beginning of the purification procedure for annexins to bind to membrane, Ca^{2+}-dependent proteases could have been active during the purification procedure, and it is possible that the protein was proteolyzed by these proteases. It is, therefore, very crucial to add leupeptin, a potent Ca^{2+}-dependent protease inhibitor, as well as other inhibitors such as benzamidine and PMSF to the preparation medium. Otherwise, proteolytic degradation would yield peptides with smaller molecular weights (30-34kDa, data not shown).

When the purified AXmito was applied on a HPLC gel-permeation chromatography column, it was eluted between ovalbumin (45kDa) and α-chymotrypsinogen A (24kDa), indicating that this protein existed as a monomeric form (data not shown). We did not detect any 11kDa peptide, which is a subunit of annexin-85 (28), by SDS-PAGE analysis of the purified protein.

It is unlikely that AXmito is coded in mitochondrial DNA because the entire sequence of mitochondrial DNA has been identified and no region provides for annexin. This fact suggests that annexin may be transported into mitochondria from cytosol. The possible mechanism of transport remains to be elucidated.

Annexins have been reported to have a potent inhibitory effect on PLA_2 activity (27, 28). AXmito was shown to be as potent as bovine lung annexin I on PLA_2 inhibition (Fig. 3), although the inhibitory action on pancreatic PLA_2 *in vitro* may not be a physiological function (26). Mitochondrial PLA_2 has been reported to be involved in mitochondrial configurational changes by altering ion or water permeability due to hydrolysis of membrane phospholipids (32, 34-36). AXmito I may have a role in the regulation of mitochondrial PLA_2.

Another possible role of annexin is to control actin-polymerization, although this has not been fully established (19, 20). We have demonstrated the existence of mitochondrial actin and its ability to polymerize (33). These data suggest that mitochondrial actin might be a cytoskeleton-like component involved in the configurational changes in mitochondria. AXmito may also regulate the configuration of mitochondria by affecting some intra-mitochondrial structure, possibly by its actin-binding property.

The evidence that PTK is present in mitochondrial membrane fraction supports the findings of Piedimonte *et al.* (43) who reported that PTK activity is localized in the outer mitochondrial membrane. According to our data, they might have detected both membrane-associated and

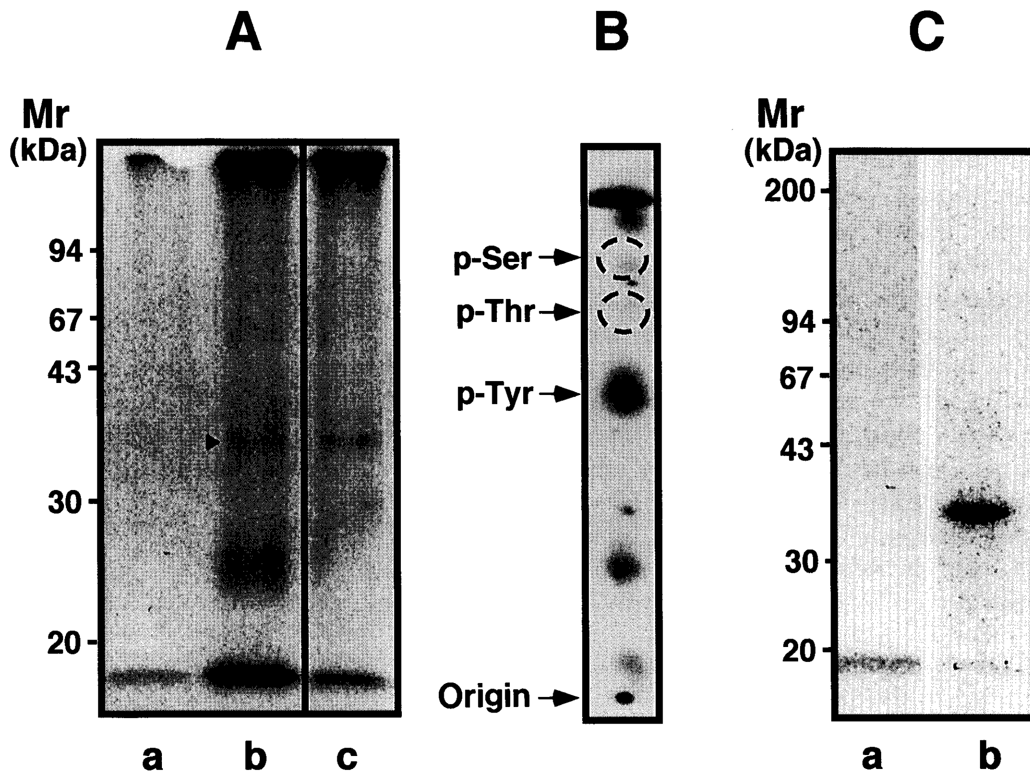

Fig. 5 Phosphorylation of mitochondrial annexin (AXmito) I by protein tyrosine kinase and C-kinase.
A: Immunoprecipitation analysis of phosphorylated mitochondria using anti-annexin I antibody. Phosphorylated mitochondria were solubilized with 0.5% Triton X-100 containing buffer and incubated using control pre-immune rabbit serum (lane a) and anti-annexin I antibody (lane b) for 12 h at 4 °C as described in "Materials and Methods". Antigen-antibody complexes were adsorbed to protein A-conjugated Sepharose 4B beads and then solubilized with sample buffer for SDS-PAGE analysis. Following SDS-PAGE analysis, the gel was stained and analyzed autoradiographically. The gel of lane b was then cut and treated with 1N KOH for 2 h at 55 °C, and was subjected to autoradiography (lane c). Arrowhead indicates AXmito. Molecular weight markers were the same as those in Fig. 1A.
B: Phosphoamino acid analysis of the phosphorylated AXmito. Phosphorylated AXmito was extracted from the gel (Fig. 5A, lane b) and analyzed by thin layer electrophoresis as described in "Materials and Methods". Markers are phosphotyrosine (p-Tyr), phosphothreonine (p-Thr) and phosphoserine (p-Ser).
C: Phosphorylation of AXmito I by C-kinase. AXmito I was phosphorylated by C-kinase as described in the "Materials and Methods", and was analyzed autoradiographically after SDS-PAGE. a, in the absence of Ca^{2+} and phospholipid; b, in the presence of Ca^{2+} and phospholipid. SDS; PAGE, see legend to Fig. 1.

membrane-integrated PTK activities in human fibroblasts. The former could be dissociated by washing, but the latter could not. After the removal of the dissociable PTK from the membrane fraction, enough PTK activity retained to phosphorylate intrinsic substrates such as AXmito and a 300kDa peptide. The 35kDa peptide which was immuno-precipitated with anti-annexin I antibody was phosphorylated by intrinsic kinases (Fig. 5A). Since the 300kDa peptide was not immuno-precipitated by anti-annexin I antibody, this peptide was not immunologically related to annexin I, and further identification of this protein was not performed. The heavily phosphorylated band with very high molecular weight observed in lanes b and c, as well as 26kDa phosphopeptide in lane b, were not identified either. The 35kDa peptide was extracted from the gel and subjected to phosphoamino acid analysis (Fig. 5B), and this assay revealed that AXmito was phosphorylated on tyrosine residue when mitochondrial respiration is stimulated by the addition of succinate which is a substrate for the Krebs cycle. The possible function of AXmito on the configurational change of mitochondria might be regulated by tyrosine phosphorylation in energetically stimulated mitochondria.

We have shown that annexin can be phosphorylated

by C-kinase with a better stoichiometry than PTK (about 1.0 mol Pi incorporated/mol protein by C-kinase as compared to 0.05-0.15 by PTK) (28). C-kinase could also phosphorylate AXmito (Fig. 5C). We analyzed the existence of C-kinase in mitochondria, but no activity of the enzyme was detected in Percoll-purified mitochondria (data not shown). C-kinase seems to be associated with the cytoplasmic side of the outer membrane in a Ca^{2+}-dependent manner. In this respect, the phosphorylation of AXmito by C-kinase may not play a physiologically important role in mitochondria.

On the other hand, tyrosine phosphorylation might be physiological, because mitochondria express intrinsic PTK activity. Although much data concerning annexin phosphorylation has accumulated, and there is some evidence for the alteration of calcium sensitivity by PTK phosphorylation (44), the definite role of its phosphorylation remains unclear.

Acknowledgements. We acknowledge the generous gift of anti-annexin I and II antibodies from Dr. RB Pepinsky (Biogen Research Co., MA, USA). We are grateful to Dr. Najma Janjua for her proof-reading of this manuscript. We would like to express our appreciation to the staff of the Institute for Radioisotopes and the Common Equipment Research Center in the Kagawa Medical University for their technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Received October 1, 1999; accepted December 2, 1999.