Purification and characterization of nuclear alkaline phospholipase A_2 in rat ascites hepatoma cells

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Abstract The alkaline phospholipase A_2 (PLA_2) was purified from nuclei of rat ascites hepatoma cells (AH7974) by column chromatography with a Sephacryl S-300 column and an immunoadsorbent using anti-group II PLA_2 monoclonal antibody. From these two columns, the alkaline PLA_2 was eluted in parallel with a 17-kDa protein which is reactive to another anti-group II PLA_2 polyclonal antibody. Approximately 80% of nuclear PLA_2 was inhibited by this antibody. The alkaline PLA_2 was found in association with the chromatin fraction among subnuclear fractions. By an immunocytochemical staining, the nuclei of AH7974 were stained more strongly than other parts of cells with anti-group II PLA_2 antiserum.

Key words: Phospholipase A_2; Group II; Nucleus; Rat hepatoma; Purification

1. Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the sn-2-ester bond of phospholipids and produces the precursors of potent lipid mediators, such as leukotrienes, prostaglandins and platelet activating factor. In mammalian cells, PLA_2 has been found in various subcellular fractions, plasma membrane [1,2], Golgi membrane [3], microsome [4], mitochondria [5], lysosome [6], and cytosol [7,8]. To date, little information concerning nuclei is available, even inclusive of our previous report [9]. PLA_2 species of mammalian origin have been classified into three groups, group I (pancreatic), group II (non-pancreatic) and cytosolic, according to their primary structures. The former two are often termed 'secretory PLA_2'. In the last few years, the secretory PLA_2 species, especially group II PLA_2, have been found in the particulate fractions of many cells and tissues in spite of the presence of a typical signal sequence as a secretory enzyme in its molecule [5,10–12]. Further, the subcellular localization of group II PLA_2 seems to be various in tissues and cells, i.e. mitochondria in liver [5] and secretory granules of latent platelets [13] and of mast cells [14], while group II PLA_2 in rat mesangial cells has been shown to localize to the Golgi area by immunocytochemical study [15]. Thus, the membrane-binding mechanism and the subcellular localization of secretory PLA_2 remain to be investigated.

We have reported the growth-associated changes of fatty acid composition of nuclear phospholipids: the decrease in arachidonic and docosahexanoic acids and the increase in oleic acid at S phase of liver regeneration [16]. We also reported the presence of neutral and alkaline PLA_2 in the nuclei of rat ascites hepatoma cells (AH7974) [9]. These results raise the possibility that an acive acylation-deacylation cycle in nuclei alters the fatty acid moieties of nuclear phospholipids, and that it leads to the production of various bioactive substances from the released fatty acids and lysophospholipids, responding to the diverse growth signals. In the present study, we found that the alkaline PLA_2 in nuclei of rat ascites hepatoma AH7974 cells, was closely related to group II PLA_2 and associated with chromatin-rich fraction.

2. Materials and methods

2.1. Materials

1-Stearoyl-2-[1-^{14}C]arachidonyl-sn-glycero-3-phosphocholine, 1-acyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine ([^{14}C]-arachidonyl PE), 1,2-dioleoyl-sn-glycero-phospho-[2-^{14}C]ethanolamine, 1,2-dioleoyl-sn-glycero-phospho-[3-^{14}C]serine, 1-palmitoyl-2-[1-^{14}C]linoleoyl phosphatidylethanolamine, and 1-stearoyl-[1-^{14}C]arachidonyl-sn-glycero-3-phosphoinositol were purchased from Amersham Int. plc, UK (specific radioactivity of all lipids, 1.85–2.2 GBq/mmol). Anti-group II PLA_2 serum was raised in rabbits immunized with purified group II PLA_2 from rat platelets. The polyclonal (R377) and monoclonal (MD7.1) antibodies specific for rat group II PLA_2 were prepared as described previously [17,18]. The polyclonal antibody specific for rat pancreatic group I PLA_2 was generously gifted by Dr. M. Okamoto of Osaka University, Japan. The antibody MD7.1-conjugated Sepharose CL-413 was prepared as described previously [17]. Rat group II PLA_2 was purified from rat platelets according to the method of Mizushima et al. [19].

2.2. Preparation of nuclei

Nuclei were prepared from rat ascites hepatoma cells (AH7974) as described previously [9]. The purity of nuclei was estimated to be more than 90% based on the activities of 5′-nucleotidase as a plasma membrane marker, glucose-6-phosphatase as a microsomal marker and cytochrome oxidase as a mitochondrial marker, and also on electron microscopic observation [9].

2.3. Preparation of subnuclear fractions

Nuclei were fractionated according to the method of Payrastre et al. [20] with some modifications. In brief, the highly purified nuclei (1.5–2 ml of packed volume, approximately 100–150 mg protein) were incubated for 7 min in 10 ml of ice-cold solution containing 1% Triton X-

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Abbreviations: PLA_2, phospholipase A_2; [^{14}C]arachidonyl PE, 1-acyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol

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100, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at $650\times g$ for 5 min. The supernatant was used as nuclear membrane fraction. The pellet (membrane-depleted nuclei) was resuspended and incubated for overnight at 4°C in 10 ml of a solution containing 50 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 0.1 mM PMSF, 400 units of pancreatic DNase I (Takara-Shuzo Co., Japan) and 500 μg of pancreatic RNase (Boehringer-Mannheim Biochemicals, Germany). Then, 2 M $(NH_4)_2SO_4$ was added dropwise to the incubation mixture to a final concentration of 0.25 M. Nuclear matrices were pelleted at $10000\times g$ for 10 min and the resulting supernatant was used as chromatin fraction.

2.4. Purification of PLA_2

All procedures were carried out at 0–4°C. Ten ml of chromatin fraction prepared from 2 ml of packed nuclei as described above was put into a cellophane dialysis tube and concentrated to 2 ml with solid sucrose. Two ml of the solubilized PLA_2 solution thus obtained was applied on a column of Sephacryl S-300 (1.2×85 cm) equilibrated with a solution containing 0.25 M $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.2) and 0.1 mM PMSF. The column was then eluted with 200 ml of the same solution used for equilibration. The active fractions were pooled and applied onto an immunoaffinity CNBr-Sepharose column (1.5×2 cm) conjugated with a monoclonal antibody against rat group II PLA_2 (MD7.1) which was equilibrated with a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1 M KCl. The column was washed with 100 ml of the same solution used for equilibration and the enzyme was eluted with 20 ml of 20 mM glycine/HCl (pH 2.5). The eluate was immediately neutralized with 1 M Tris base.

2.5. Determination of PLA_2 activity

The standard assay for PLA_2 activity with [^{14}C]arachidonyl PE, except where otherwise noted, was carried out at 37°C for 30 min in a reaction mixture (100 μl) containing an appropriate amount of enzyme source, 100 mM glycine/NaOH (pH 9.5), 10 mM $CaCl_2$, 0.06% taurodeoxycholic acid, 100 μM [^{14}C]arachidonyl PE (specific radioactivity was adjusted to 3000 cpm/nmol by the addition of unlabeled PE). The assays with other phospholipids as substrates were carried out under the same conditions as described above. The released ^{14}C-labeled fatty acid was determined by a modification of Dole's procedure [21]. On the other hand, the released radioactive lysophospholipid was separated by thin-layer chromatography and its radioactivity determined as described previously [9].

For the neutralization of PLA_2 activity by the antibodies, the pooled active fractions from the Sephacryl S-300 column were preincubated with various amounts of polyclonal antibodies against rat group II PLA_2 and rat group I PLA_2 in a solution containing 100 mM glycine/NaOH (pH7.0) and ovalbumin (1 mg/ml) for 30 min on ice. The remaining activity was determined under the same conditions as described above.

2.6. SDS-PAGE and immunoblotting analyses

SDS-PAGE was performed under non-reducing condition on 20% polyacrylamide gel according to the method of Laemmli [22]. Individual protein bands were visualized using the 2D-SILVER STAIN II Kit (Daiichi Pure Chemicals Co., Ltd., Japan). Molecular mass markers (Bio-Rad) were lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31.0 kDa) and ovalbumin (45.0 kDa). For immunoblotting analysis, the electrophoresed proteins were transferred to a nylon membrane (Immobilon, Millipore Co., Bedford, MA) and probed with the specific polyclonal antibodies against PLA_2. The immune complex was then visualized with [^{125}I]protein A and analyzed on a Fujix BAS 2000 image analyzer (Fujix Co. Ltd., Tokyo).

2.7. Immunocytochemical analysis

Exponentially growing AH7974 cells were fixed with 4% paraformaldehyde and smeared on the slide glass coated with gelatin. The cells were immersed in 0.3% Triton X-100 for 30 min, treated with 10% normal goat serum for 1 h and then with anti-group II PLA_2 serum or non-immune rabbit serum (each dilution, 1:1000 with PBS) overnight at room temperature. Subsequent treatments were performed in the following orders: (1) biotinized anti-rabbit IgG (dilution, 1:200 with PBS) for 1 h, (2) streptavidin-biotin-peroxidase kit (Histofine, Nichirei, Japan) (dilution, 1:100 with PBS) for 1 h, (3) a solution containing 0.03% diaminobenzidine and 0.005% H_2O_2 for 5 min. The immunostained cells thus obtained were dehydrated and mounted.

3. Results

3.1. Subnuclear distribution of alkaline PLA_2

As shown in Fig. 1A, almost all of the alkaline PLA_2 activity in intact nuclei was recovered in the chromatin-rich fraction, i.e. the 0.25 M $(NH_4)_2SO_4$ extract from the 1% Triton X-100-treated, DNase- and RNase-digested nuclei. Further, the immunoreactive 17-kDa protein was detected by a polyclonal antibody against rat group II PLA_2 in the lysates of intact nuclei, membrane-depleted nuclei, and chromatin-rich fractions (Fig. 1B). The total activity in membrane-depleted nuclei was always higher than that in intact nuclei, suggesting the existence of an inhibitor for PLA_2 in nuclear extract that is removed by 1% Triton X-100 treatment. In connection with this, we found annexin I and II in 1% Triton X-100-soluble fraction of nuclei by immunoblotting analysis (data not shown). The alkaline PLA_2 found in nuclei is not due to contamination of endoplasmic reticulum and/or cytosol because the denuded nuclei by the treatment with 1% Triton X-100 completely retain both the alkaline PLA_2 activity and immunoreactivity with a polyclonal antibody against group II PLA_2 (Fig. 1A,B).

3.2. Purification of alkaline PLA_2 from nuclei

The efficient solubilization of the nuclear alkaline PLA_2 of rat ascites hepatoma AH7974 cells was achieved by the extraction in 0.25 M $(NH_4)_2SO_4$ of the 1% Triton X-100-treated nuclei after digestion with DNase and RNase as shown in Fig. 1A. Although the nuclear phospholipase C of AH7974 cell was well solubilized with 2 M KCl from intact nuclei [23], 70% of the nuclear PLA_2 remained insoluble under the same conditions (data not shown). The solubilized PLA_2 preparation (0.25 M $(NH_4)_2SO_4$ extract) thus obtained was concentrated, and was subjected to Sephacryl S-300 column gel filtration. The activity was eluted as a broad peak, corresponding to a molecular mass of 40 kDa and lower (Fig. 2A). On SDS-PAGE/immunoblotting with a polyclonal antibody against rat group II PLA_2, an immunoreactive 17-kDa protein appeared in the fractions that contained PLA_2 activity (data not shown). The broad peak was clearly separated into two peaks by rechromatography (Fig. 2B), and only the latter peak corresponded to alkaline PLA_2 that immunoreacted with a polyclonal antibody against group II PLA_2 (Fig. 2C). The former peak was identified as neutral PLA_2 by its pH optimum [9] and molecular mass which had already been estimated to be 33 kDa (unpublished data). These results suggest that the broad peak in Fig. 2A may be a mixture of neutral and alkaline PLA_2. The alkaline PLA_2 activity in the pooled active fraction (collected from fractions 54–68 in Fig. 2A) was inhibited to 20% of original activity by a polyclonal antibody against group II PLA_2, but not by a polyclonal antibody against group I PLA_2 under the conditions described in Section 2 (data not shown). The alkaline PLA_2 activity in intact nuclei was also dose-dependently inhibited by a polyclonal antibody against group II PLA_2 to 20% at maximum. These results indicate that most of the nuclear alkaline PLA_2 may belong to group II PLA_2. The pooled active fraction was

subsequently subjected to immunoaffinity column chromatography using an anti-rat group II PLA_2 monoclonal antibody-conjugated Sepharose. The SDS-PAGE of active fractions, eluted from the column with 20 mM glycine/HCl (pH 2.5), revealed protein bands at the position of molecular mass 17 kDa when visualized by silver staining (Fig. 3A). The protein bands were in parallel with the elution pattern of PLA_2 activity, which was highest in fractions corresponding to lanes 6–8 of Fig. 3A, assayed as described in Section 2 (data not shown). Further, as assessed by SDS-PAGE/immunoblotting, the 17-kDa protein which co-migrated with group II PLA_2 purified from rat platelets was recognized by anti-rat group II PLA_2 antibody (Fig. 3B). From these results, it is concluded that the alkaline PLA_2 in AH7974 cell nuclei is closely related to group II PLA_2. The enzyme was finally purified 735-fold. The apparent K_m and V_{max} were determined with various concentrations of [^{14}C]arachidonyl PE and were estimated to be 0.2 mM and 6600 nmol/mg/30 min, respectively.

3.3. Characterization of nuclear alkaline PLA_2

The Sephacryl S-300 fraction was used for enzymological analyses because the further purified enzyme obtained by immunoaffinity column was very labile and its activity was decreasing day by day. The optimum pH was estimated to be 9–10 and the free Ca^{2+} concentration for maximal activity was 1 mM (data not shown). The substrate preference was in the order PE, PS, PC, and PI (100:42:23:5). As to the 2-acyl moiety of PE, linoleic acid and oleic acid were hydrolyzed twice as efficiently as arachidonic acid, indicating that the specificity of the 2-acyl moiety is not as strict as that of cytosolic PLA_2 [24]. This specificity of the 2-acyl moiety of PE was also true in the enzyme purified from immunoaffinity column. These characteristics of nuclear alkaline PLA_2 were quite similar to those of rat group II PLA_2 [13,24].

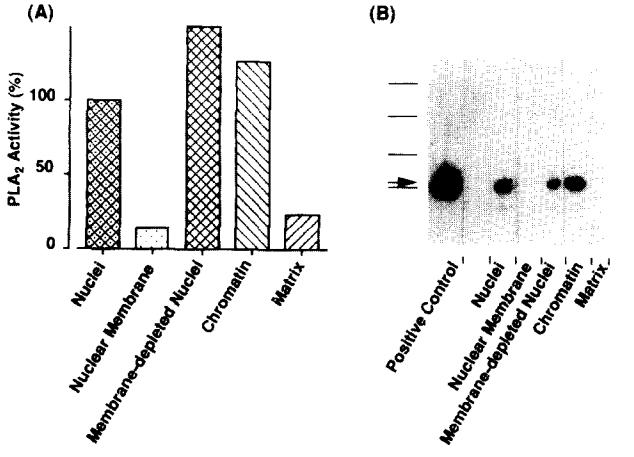

Fig. 1. Distribution of alkaline PLA_2 activity in different subnuclear fractions. A: Alkaline PLA_2 activity was determined under the conditions as described in Section 2, except that 5 μl aliquots of each 10 ml subnuclear fractions derived from 2 ml packed intact nuclei were used as an enzyme source. Relative activity was expressed as percentage of the total activity of PLA_2 in intact nuclei. Nuclei, intact nuclei; Nuclear Membrane, 1% Triton X-100 soluble fraction from intact nuclei; Membrane-depleted Nuclei, 1% Triton X-100 insoluble fraction from intact nuclei; Chromatin, 0.25 M $(NH_4)_2SO_4$ soluble fraction from DNase I and RNase digested membrane-depleted nuclei; Matrix, 0.25 M $(NH_4)_2SO_4$ insoluble fraction from DNase I and RNase digested membrane-depleted nuclei. B: Each 1 μl from the same nuclear fractions as described in A and rat platelet group II PLA_2 (5.6 ng) as a positive control were subjected to SDS-PAGE/immunoblot analysis using a polyclonal antibody against rat group II PLA_2 (R377) as described in Section 2. The lines show molecular mass standards, from top to bottom: ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The arrow shows the immunostained bands (17 kDa). The result is representative of those obtained from three experiments.

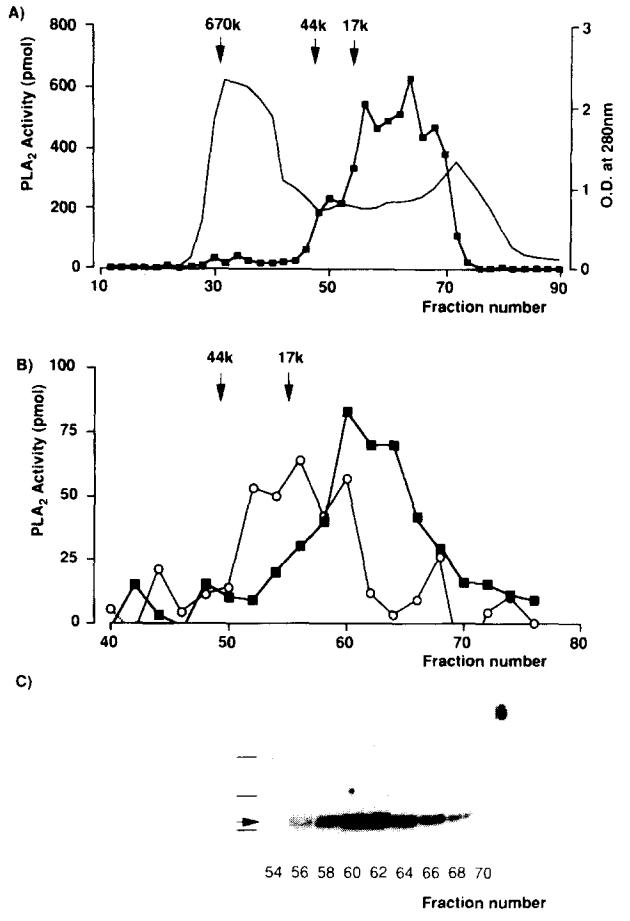

Fig. 2. Sephacryl S-300 column chromatography. A: 10 ml of 0.25 M $(NH_4)_2SO_4$ soluble fraction (56 mg protein) from the digested membrane-depleted nuclei was concentrated to 2 ml and applied to the Sephacryl S-300 column (1.2×85 cm) equilibrated with a solution containing 0.25 M $(NH_4)_2SO_4$, 20 mM Tris/HCl (pH 7.2) and 0.1 mM PMSF. The column was eluted with 200 ml of the same solution as described above and 10 μl aliquots of each fraction (2 ml) were subjected to the assay of PLA_2 activity as described in Section 2. Solid line without symbols, optical density at 280 nm; solid line with symbols, PLA_2 activity. The result is representative of those obtained from six experiments. B: The pooled active fraction (fractions 50–70 in Fig. 1A) was concentrated to 2 ml and subjected to rechromatography using the same column as in A. 10 μl aliquots of each fraction (2 ml) were assayed for PLA_2 activity at pH 7.0 (○) or pH 9.5 (■) as described in Section 2. C: 2 μl aliquots of each fraction (2 ml) were subjected to SDS-PAGE/immunoblot analysis using a polyclonal antibody against rat group II PLA_2 (R337) as described in Section 2. The lines show molecular mass standards, from top to bottom: carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The arrow shows the immunostained bands (17 kDa).

3.4. Immunocytochemical detection of nuclear alkaline PLA_2

AH7974 cells are island-forming type hepatoma cells and they grow as aggregates of several cells to form islands (Fig. 4C). When immunostained with anti-group II PLA_2 serum,

nuclei were clearly stained, but either plasma membrane or cytoplasm was stained only weakly (Fig. 4A). In contrast, they were not stained with non-immune serum (Fig. 4B).

4. Discussion

We previously described that rat ascites hepatoma AH7974 cells contain a modest level of PLA_2 [9], while normal rat liver hepatocytes have been reported to contain very low amounts of PLA_2 undetectable by immunocytochemical analysis [10,25]. Group II PLA_2 was shown to be expressed in human hepatoma [26]. It was also revealed that two kinds of PLA_2, alkaline and neutral PLA_2, exist in nuclei of rat ascites hepatoma AH7974 cells [9]. In the present study, it was found that the nuclear alkaline PLA_2 reacted well with antibody raised against rat platelet group II PLA_2 [17]. Taking advantage of this cross-reactivity, the alkaline PLA_2 was purified from nuclear extract using an immunoaffinity column with a monoclonal antibody against rat group II PLA_2 [17]. The purified enzyme was very similar to rat group II PLA_2 with respect to substrate specificity, Ca^{2+} requirement and apparent molecular mass estimated from its mobility on SDS-PAGE [13,24]. Although the fatty acid-releasing activity under alkaline condition was detected in all subcellular fractions of AH7974 cells, the total activity was highest in nuclei (52%, data not shown). Since the alkaline PLA_2 activity in the intact nuclei was recovered more than 100% in the denuded nuclei by 1% Triton X-100 treatment (Fig. 1A), it was strongly suggested that the activity was of nuclear origin. In support of this, immunostaining of AH7974 cells with anti-group II PLA_2 antibody preferentially stained nuclei (Fig. 4A). The alkaline PLA_2 activities in both intact nuclei and partially purified preparation were inhibited dose-dependently by an anti-group II PLA_2 antibody to 20% of the original activity. These results indicate that the larger part of alkaline PLA_2 activity in AH7974 nuclei is closely related with group II PLA_2.

Aarsman et al. [5] reported that PLA_2 from rat liver mitochondria has 100% sequence homology to the N-terminal 24 amino acids of rat group II enzyme. Our results combined with previous reports by other groups [24] may be relevant to an important question: whether very similar but distinct types of group II PLA_2 isozyme are expressed in the organ-specific manner and localize in different organelles in different cells, or a single type of group II PLA_2 exists in all tissues but

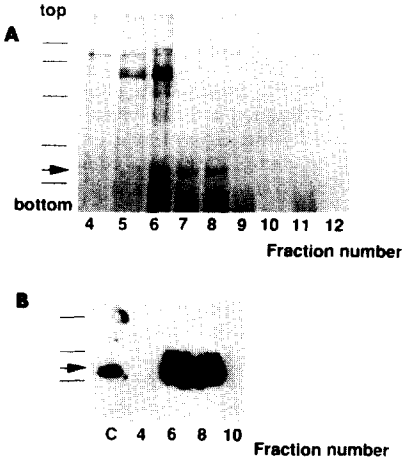

Fig. 3. Immunoaffinity column chromatography. A: The active fractions obtained from Sephacryl S-300 column chromatography (Fig. 1A) were pooled and applied to monoclonal antibody against rat group II PLA_2 (MD 7.1)-coated Sepharose column (1.5×2 cm) equilibrated with a solution containing 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 1 M KCl. The column was eluted with 20 ml of 20 mM glycine/HCl (pH 2.5) and 50 μl aliquots of each fraction (1 ml) were subjected to SDS-PAGE and stained with a silver reagent as described in Section 2. The arrow shows the protein band of which the intensity parallels PLA_2 activity. The lines indicate molecular mass standards, from top to bottom: bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). B: 50 μl aliquots of each fraction (1 ml) and rat group II PLA_2 purified from rat platelets (2 ng, lane C) were subjected to SDS-PAGE/immunoblot analysis using a polyclonal antibody against rat group II PLA_2 (R377) as described in Section 2. The lines indicate molecular mass standards, from top to bottom: carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The arrow shows the immunostained band (17 kDa). The result is representative of those obtained from three independent experiments.

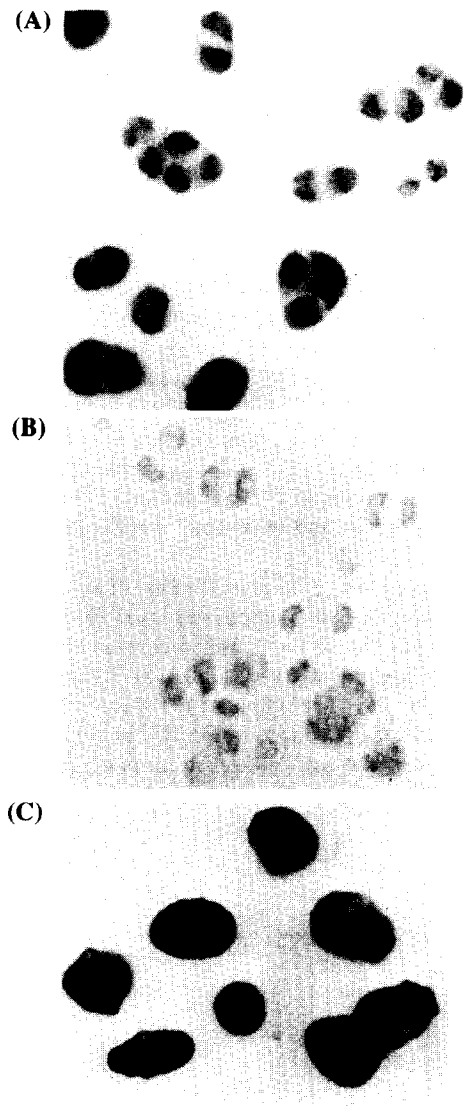

Fig. 4. Immunocytochemical localization of group II PLA_2 in AH7974 cells. AH7974 cells were immunostained with anti-group II PLA_2 rabbit serum (A) and a non-immune rabbit serum (B) as described in Section 2. AH7974 cells were also stained with hematoxylin-eosin (C). 1400×.

localizes differently in organelles depending on each cell type. The fact that the nuclear alkaline PLA_2 described here is immunochemically indistinguishable from group II PLA_2 of rat platelets that have no nuclei may support the latter possibility, but the conclusion must wait for further study. The mechanism of transportation of the group II-like PLA_2 into nuclei from either outside or inside cells also remains unknown.

It should be noted that the nuclear group II-like PLA_2 could be solubilized only from nuclease-digested nuclei with 0.25 M $(NH_4)_2SO_4$. The existence of group II PLA_2 in nuclei-containing fractions has been reported in human placenta [12] and rat spleen and liver cells [10,27] and its solubilization was reported to be rather difficult. These facts suggest that nuclear group II-like PLA_2 transported from either outside or inside cells tightly binds to negatively charged nucleic acids, just as in the case of the secreted group II PLA_2 which binds to negatively charged heparan sulfate proteoglycan in the surface of the target cells [28]. Recently, Bennett et al. [29] have reported that phosphorothionate oligonucleotides containing G-quartet directly inhibited human group II PLA_2 activity in a sequence-specific manner. Whether the nuclear group II-like PLA_2 binds to a specific DNA sequence or not is under investigation in our laboratory.

The exact mechanism how this enzyme protein carrying signal peptide distributes in nuclei is yet to be clarified, although several mechanisms including post-translational modification of enzyme protein have been hypothesized [25,30].

We have previously reported that the rapid turnover of the acyl chain at the 2 position of nuclear phospholipids can be induced by proliferating stimuli: the transient increase of oleic acid at the expense of arachidonic acid and docosahexaenoic acid [16]. Neufeld et al. [31] reported that [^{3}H]arachidonate was rapidly incorporated into the nuclear membrane of murine fibrosarcoma cells. Capriotti et al. [32] also reported that, in mouse fibrosarcoma cells, the most recently incorporated pool of arachidonate in the nuclear membrane was preferentially released upon stimulation by bradykinin. Further, the prostaglandin- and leukotriene-synthesizing enzymes have been reported to exist in the nuclei [33–39]. Interestingly, cyclooxygenase-2, which is induced by agonist stimulation, has been found largely in nuclei, while a constitutive enzyme, cyclooxygenase-1, exists only in endoplasmic reticulum and not in nuclei [39]. Since phospholipids exist in chromatin [40] as well as in the nuclear membrane, it is strongly suggested that nuclear group II-like PLA_2 associated with chromatin may play an important role in the regulation of nuclear function through the production of various bioactive substances by hydrolyzing the phospholipids of chromatin and/or the nuclear membrane.

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