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# ATP and GTP Hydrolytic Function of N-terminally Deleted Annexin I

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**Annexin I is a 37 kDa member of the annexin family of calcium-dependent phospholipid binding proteins. Annexin I plays regulatory roles in various cellular processes including cell proliferation and differentiation. Recently we found that annexin I is a heat shock protein (HSP) and displays a chaperone-like function. In this paper we investigated the function of annexin I as an ATPase using 1 to 32 amino acids deleted annexin I (Δ-annexin I). Δ-Annexin I hydrolyzed ATP as determined by thin layer chromatography. The ability of ATP hydrolysis was inhibited by ADP, GTP and GDP, but not by the AMP, GMP and cAMP. In view of the ATP hydrolyzing function of HSP, the results support the function of annexin I as a HSP.**

**Keywords:** Annexin I, ATPase, ATP binding protein, Heat shock protein.

## Introduction

Annexins (also called lipocortins) are a family of intracellular proteins that bind phospholipids and membranes in a $Ca^{2+}$-dependent manner (reviewed in Raynal and Pollard, 1994). Various members of the annexin family have been implicated in various cellular processes including membrane fusion, cell growth and differentiation, inhibition of phospholipase $A_2$, and anti-inflammation (Schlaepfer and Haigler, 1990; Kim *et al.*, 1994; Kang *et al.*, 1996; Raynal *et al.*, 1997). However, despite many functional possibilities, well-characterized biological functions have not been determined for any of the annexins.

The structure of annexins consists of C-terminal core domain and a N-terminal tail (Meers, 1990). The core domain consists of four or eight repeats of about a 70 amino acids sequence that shares a high homology. This domain is known to be a common primary structure for $Ca^{2+}$ ion and phospholipids binding sites. The N-terminal domains of the annexins have great diversity in both length and sequence. Since this domain contains the sites for selective proteolytic cleavage, phosphorylation and interaction with other proteins, it is considered to be the regulatory region of the protein and seems to be important for specific cellular functions of each annexin (reviewed in Raynal and Pollard, 1994).

Recently, we found that some properties of annexin I are consistent with the characteristics of stress or heat shock proteins (HSP). Annexin I displays a chaperone-like function (Kim *et al.*, 1997). In cultured cells, annexin I is induced by environmental stresses, such as heat and oxidative stress (Rhee *et al.*, 2000). Since many stress proteins show ATP hydrolyzing activity we questioned if annexin I is an ATPase. This hypothesis is supported by our recent observation by NMR spectroscopy that an ATP bining site exists in the first domain of annexin I (Han *et al.*, 1998). Studies by fluorescence spectroscopy indicated an interaction of the porcine liver annexin VI with ATP (Bandorowicz-Pikula *et al.*, 1997; Bandorowicz-Pikula, 1998; Danieluk *et al.*, 1999). Here we investigated the function of annexin I as an ATPase.

## Materials and Methods

**Materials** [α-$^{32}$P]ATP (400 Ci/mmol) and [α-$^{32}$P]GTP (400 Ci/mmol) were purchased from Amersham International (UK). $Na_2$ATP, $Na_2$ADP, $Na_2$AMP, $Na_2$GTP, $Na_2$GDP, $Na_2$GMP, cAMP, and IPTG were purchased from Sigma (St. Louis, MO, USA). Polyethyleneimine (PEI)-cellulose for thin layer chromatography (TLC) was purchased from Merck Co. (USA). All other chemicals were reagent grade or higher.

**Preparation of Δ-Annexin I** Recombinant Δ-annexin I, in which 1 to 32 amino acids were deleted was produced in *E. coli* and purified as described previously (Weng *et al.*, 1993). The concentration of Δ-annexin I was determined according to the methods of Bradford using bovine serum albumin (BSA) as a standard (Bradford, 1976).

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**Photoaffinity labeling of the Δ-annexin I with [α-$^{32}$P]ATP** Δ-Annexin I was labeled by UV light irradiation using [α-$^{32}$P]ATP according to the methods as described previously, but with slight modifications (Suzich *et al.*, 1993; Tamura *et al.*, 1993). Δ-annexin I (1 μg), purified from *E. coli* was incubated at 25°C in a 20 μl volume of a reaction mixture containing 10 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 20% glycerol, and 25 μCi [α-$^{32}$P]ATP. A sample was irradiated using a UV illuminator for 2 min at 254 nm (25 J/cm$^2$). Bovine serum albumin (BSA) (1 μg) was incubated and exposed to UV light under identical conditions. The mixtures were subjected to 7.5% SDS-PAGE. Protein bands were visualized by Coomassie blue staining and autoradiography.

**ATPase (or GTPase) activity assay** ATP hydrolysis was determined by the appearance of the hydrolyzed products of [α-$^{32}$P]ATP according to the methods as described previously, the with slight modifications (Suzich *et al.*, 1993; Tamura *et al.*, 1993). The reaction mixture contained 1 μg of Δ-annexin I and 2.5 μCi of [α-$^{32}$P]ATP, 20 mM MOPS, pH 6.5, 1 mM $MgCl_2$ in a 10 μl reaction mixture. The reaction was performed at 25°C for 40 min and was stopped by adding 1 μl of 500 mM EDTA. Then 0.5 μl of sample from the reaction mixture was spotted onto a PEI-cellulose thin layer chromatography (TLC) plate (Merck Co.) and was then developed by ascending chromatography using 0.375 M potassium phosphate, pH 3.5. The TLC plate was visualized by autoradiography. The GTPase activity of Δ-annexin I was also determined by the same procedure using [α-$^{32}$P]GTP as a substrate.

**Inhibition of ATPase activity by various nonradioactive nucleotides** The reaction was performed under identical conditions except 100 μM of nonradioactive nucleotide was supplemented.

## Results

**Photoaffinity labeling of the Δ-annexin I with [α-$^{32}$P]ATP** Δ-annexin I was incubated with [α-$^{32}$P]ATP and then exposed to UV light. As shown in Fig. 1, Δ-annexin I was radiolabeled (lane 2) suggesting that Δ-annexin I binds ATP. On the other hand, BSA was not labeled under the same experimental conditions (lane 1). This result shows that binding of ATP to Δ-annexin I is specific.

**ATP hydrolyzing activity of Δ-annexin I** [α-$^{32}$P]ATP was incubated with Δ-annexin I in the hydrolysis buffer and the products were analyzed by TLC. The results are shown in Fig. 2A. While BSA showed no ATP hydrolysis activity (lane 2), Δ-annexin I hydrolyzed ATP in a concentration dependent manner. Under the present reaction conditions, ADP was produced in presence of more than 0.5 μg of Δ-annexin I (lanes 5-8). In presence of a larger amount of Δ-annexin I, AMP was also produced (lanes 7, 8).

The time dependence of this reaction was studied using a fixed amount of Δ-annexin I (1 μg). As shown in Fig. 2B, ADP appeared at 10 min and increased with time. However, AMP was not produced until 80 min and the reaction reached the maximum at 40 min. This result indicates that the protein concentration, but not the reaction time, is an important factor for the complete hydrolysis of ATP into AMP under the present conditions.

**Inhibition of ATPase activity of Δ-annexin I by nonradioactive nucleotides** In order to verify that the appearance of ADP and AMP resulted from a specific reaction by Δ-annexin I, an inhibition study was performed with various cold (nonradioactive) nucleotides. As shown in Fig. 3, in the presence of an excess amount of cold ATP the hydrolysis of [α-$^{32}$P]ATP was completely inhibited, indicating a competitive inhibition (lane 4). In presence of an excess amount of cold ADP, the hydrolysis of [α-$^{32}$P]ATP was also inhibited. Since ADP is not a substrate under the present experimental conditions (Fig. 2B), the inhibition is likely to be the product inhibition. Interestingly, the ATPase activity of Δ-annexin I was also inhibited in the presence of an excess amount of GTP and GDP, but not inhibited by AMP, GMP, and c-AMP. These results led us to speculate that Δ-annexin I might also have GTP hydrolysis activity.

**GTPase activity of Δ-annexin I** The GTP hydrolyzing ability of Δ-annexin I was determined under identical reaction conditions as in the ATPase experiment using [α-$^{32}$P]GTP as a substrate. As shown in Fig. 4A, a TLC pattern, which was similar to the ATPase experiment, was obtained. The hydrolysis of GTP by Δ-annexin I was observed in the reactions with more than 0.5 μg of Δ-annexin I. In the reactions containing more than 5 μg of Δ-annexin I, both GDP

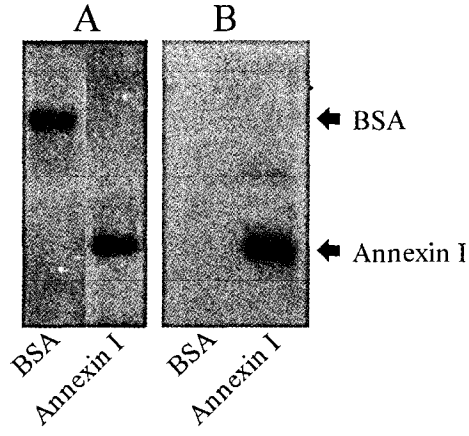


**Fig. 1.** Photolabeling of the Δ-annexin I with [α-$^{32}$P]ATP. Δ-Annexin I (1 μg) was incubated at 25°C in 20 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 20% glycerol, and 25 μCi [α-$^{32}$P]ATP. A sample was irradiated using a UV illuminator for 2 min at 254 nm (25 J/cm$^2$). BSA (1 μg) was identically treated by UV light. The mixtures were the subjected to 7.5% SDS-PAGE. Protein bands were visualized by Coomassie blue staining (A) and autoradiography (B).

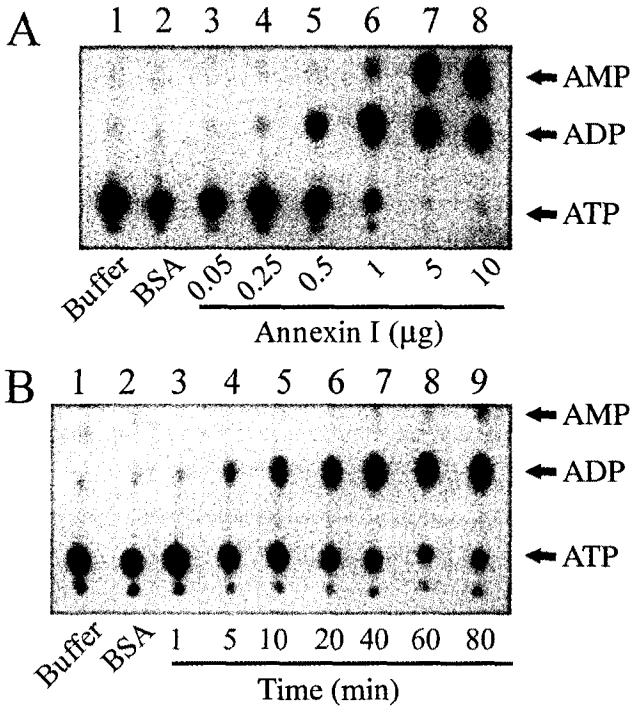


**Fig. 2.** ATPase activity of Δ-annexin I. (A) Concentration dependence of the ATPase activity. Various amounts of recombinant Δ-annexin I and 2.5 μCi [α-$^{32}$P]ATP was incubated in 20 mM MOPS pH 6.5, 1 mM $MgCl_2$ at 25°C for 40 min. BSA (10 μg) and buffer only, which were used as controls, were identically treated. Products were analyzed by TLC and visualized by autoradiography. Lane 1, buffer only (no Δ-annexin I control); lane 2, 10 μg of BSA. Lanes 3 to 8 shows samples with different amounts (in μg) of Δ-annexin I. (B) Time dependence the ATPase activity. Δ-Annexin I (1 μg) and 2.5 μCi [α-$^{32}$P]ATP was incubated in 20 mM MOPS pH 6.5, 1 mM $MgCl_2$ at 25°C. Aliquots (0.5 μl) were removed from the reaction mixtures at each time point and directly spotted onto a PEI-cellulose plate. BSA (1 μg) and buffer (no Δ-annexin I control) were incubated for 80 min under the same reaction conditions. Products were analyzed by TLC and visualized by autoradiography. Lane 1, no Δ-annexin I control; lane 2, 1 μg of BSA. Lanes 3 to 9 show the reactions with Δ-annexin I for various times (in min).

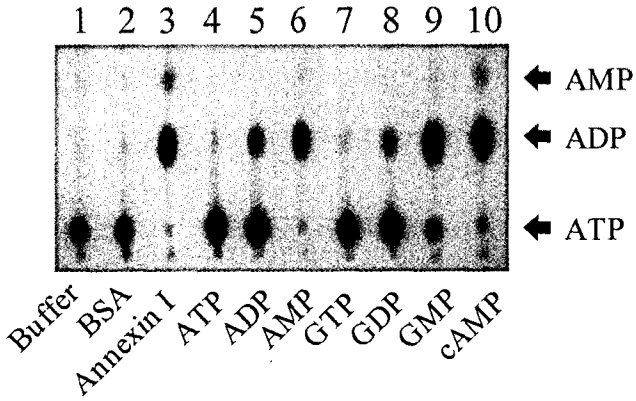


**Fig. 3.** Inhibition of the ATPase activity of Δ-annexin I by nonradioactive nucleotides. Δ-annexin I (1 μg) and 2.5 μCi [α-$^{32}$P]ATP were incubated in 20 mM MOPS, pH 6.5, 1 mM $MgCl_2$ at 25°C containing 100 μM of indicated nonradioactive nucleotides. After a 40 min reaction, EDTA (50 mM) was added to the reaction mixture in order to terminate the reactions. Lane 1, buffer only; lane 2, BSA; lane 3 Δ-annexin I. Lanes 4 to 10 show the reactions of Δ-annexin I in the presence of 100 μM cold nucleotides.

and GMP were produced and no GTP remained.

The time dependence of the GTPase activity was examined using a fixed amount of Δ-annexin I (1 μg). As shown in Fig. 4B, GDP appeared at 10 min and increased with time. As in the case for the ATP hydrolysis (Fig. 2B) GMP was not produced until 80 min and the reaction reached to a plateau after 60 min.

## Discussion

In this paper, we demonstrated that (1) Δ-annexin I binds ATP as revealed by photoaffinity labeling, and (2) Δ-annexin I catalyzes the hydrolysis of ATP as well as GTP. The hydrolyzing activity was shown by the appearance of ADP and AMP (or GDP and GMP) in the presence of Δ-annexin I. The hydrolysis of ATP was inhibited by excess amounts of ADP, GTP and GDP, but not by AMP, GMP, and cAMP.

The results are consistent with our recent observation by NMR spectroscopy that the Δ-annexin I binds ATP (Han *et al.*, 1998). Several studies have also shown the binding of nucleotides to annexins. Annexin VI from porcine liver binds ATP, as revealed by fluorescence spectroscopic studies and photoaffinity labeling (Bandorowicz-Pikula and Awasthi, 1997; Bandorowicz-Pikula *et al.*, 1997; Bandorowicz-Pikula, 1998; Danieluk *et al.*, 1999). Annexin I purified from bovine lung interacts differently with cAMP and ATP (Cohen *et al.*, 1995). Interstingly, Δ-annexin I does not have a classical Walker A consensus sequence (GXXXXGKT; X is hydrophobic residues) for the binding of ATP or GTP, which is a characteristic feature of the nucleotide binding proteins including regulatory factors, heat shock proteins and RNA hericases (Mian *et al.*, 1993). Our study by NMR spectroscopy revealed that the ATP binding site is located in the first domain of Δ-annexin I, and $His^{52}$ is involved in the binding (Han *et al.*, 1998).

Since specific functions of each annexin reside in the N-terminal tail, the results that Δ-annexin I is capable of ATP/GTP hydrolysis suggest that the ATPase/GTPase activity may not be a specific function of annexin I, but may rather be a general function of annexins. ATPase/GTPase activity of partially purified annexins from maize (Zea Mays), tomato, and cotton fiber supports this notion (McClung *et al.*, 1994; Calvert *et al.*,1996; Shin and Brown, 1999). The ATPase activity of a native annexin I that is purified from human placenta was similar to that of Δ-annexin I (data not shown).

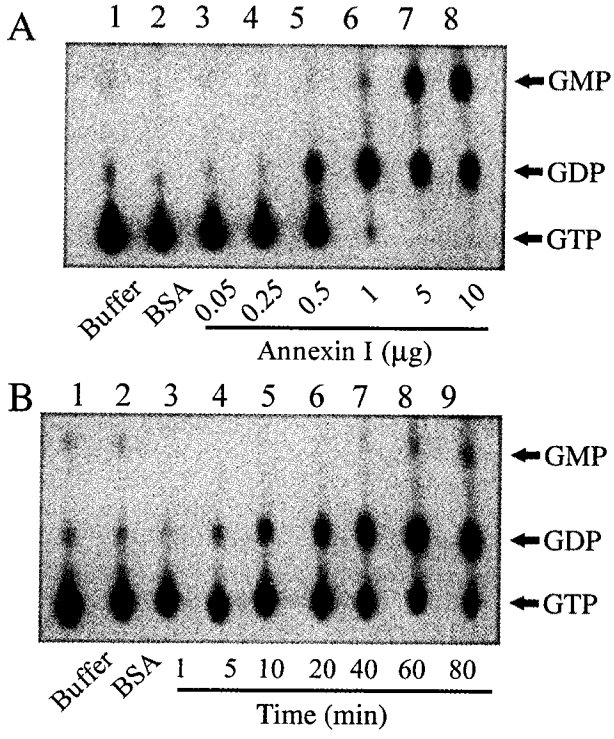


**Fig. 4.** GTPase activity of Δ-annexin I. (A) Concentration dependence of the GTPase activity. Experiments were performed as in Fig. 2A using 2.5 μCi [α-$^{32}$P]GTP as a substrate. Lanes 1 and 2 indicate the controls: Lane 1, no Δ-annexin I control; lane 2, 10 μg of BSA. Lanes 3 to 8 shows samples with different amounts (in μg) of Δ-annexin I. All other details were the same as in Fig. 2A. (B) Time dependence of GTPase activity. Experiments were performed as in Fig. 2B using 2.5 μCi [α-$^{32}$P]GTP as a substrate. Lane 1, no Δ-annexin I control; lane 2, 1 μg of BSA. Lanes 3 to 9 show the reactions with Δ-annexin I for different times (in min).

The inhibition of ATPase activity by GTP and GDP suggests that the binding sites for ATP and GTP are in close proximity, if not identical. On the other hand ATPase activity of Δ-annexin I was not inhibited by cAMP, despite the fact that the cAMP is known to interact with annexin I (Cohen *et al.*, 1995). It is likely that the cAMP binding site is different from the ATP binding site. A cAMP binding motif (Arg-Ala-Ala) that corresponds to the classical cAMP binding sequences exists in annexin I that spans the first and second repeats, especially Arg$^{97}$ (Cohen *et al.*, 1995).

The biological meaning of the nucleotide binding to annexins, and the nucleotide hydrolyzing activity of annexins, are unclear. Modulation of annexin VI-driven aggregation of liposome by ATP (Bandorowicz-Pikula and Pikula, 1998), and regulation of annexin I-driven chromaffin granule membrane aggregation and liposome fusion by ATP and cAMP (Cohen *et al.*, 1995), have been proposed. Since annexins have pleiotropic cellular functions other than interaction with membranes such as proliferation and differentiation, it is important to know whether or not the nucleotide binding is related to these functions. We have shown that annexin I is a HSP and exhibited a chaperone-like function (Kim *et al.*, 1997; Rhee *et al.*, 2000). The ATPase activity of annexin I provides another similarity to HSP.

It has been shown that annexin I relocates to the nucleus and peri-nucleus region during exposure to various cellular stress by heat, $H_2O_2$, or arsenite (Rhee *et al.*, 2000). Relocation of annexin I to the nucleus by mitogenic signal in cultured cells or in the neuronal cells of the animal under immobilization stress have also been observed (Park *et al.*, 1998; Rhee *et al.*, 1999). Although functional significance of the nuclear or peri-nuclear redistribution annexin I during physiological stress is not clear at present, the process may require energy source. Therefore it is tempting to speculate that the ATP hydrolyzing activity of annexin I is related to the relocation.

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