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GTPase Activity and Biochemical Characterization of a Recombinant Cotton Fiber Annexin[1]

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A cDNA encoding annexin was isolated from a cotton (*Gossypium hirsutum*) fiber cDNA library. The cDNA was expressed in *Escherichia coli*, and the resultant recombinant protein was purified. We then investigated some biochemical properties of the recombinant annexin based on the current understanding of plant annexins. An "add-back experiment" was performed to study the effect of the recombinant annexin on β-glucan synthase activity, but no effect was found. However, it was found that the recombinant annexin could display ATPase/GTPase activities. The recombinant annexin showed much higher GTPase than ATPase activity. Mg^{2+} was essential for these activities, whereas a high concentration of Ca^{2+} was inhibitory. A photolabeling assay showed that this annexin could bind GTP more specifically than ATP. The GTP-binding site on the annexin was mapped into the carboxyl-terminal fourth repeat of annexin from the photolabeling experiment using domain-deletion mutants of this annexin. Northern-blot analysis showed that the annexin gene was highly expressed in the elongation stages of cotton fiber differentiation, suggesting a role of this annexin in cell elongation.

Annexins constitute a family of at least 13 structurally related proteins in mammals that interact with phospholipid membranes in a Ca^{2+}-dependent manner. These proteins contain four or eight characteristic structural repeats consisting of 70 to 75 amino acids each, including the 17-amino acid "endonexin motif" or "consensus sequence" (see Raynal and Pollard, 1994). Annexins have diverse biological functions related to their Ca^{2+}- and phospholipid-binding properties. In plants the first annexin-like proteins were identified from tomato (Boustead et al., 1989). Since then, relatively abundant annexins have been identified and isolated from a number of plants (Smallwood et al., 1990; Blackbourn et al., 1992; Clark et al., 1992; Andrawis et al., 1993). Although the general functions of plant annexins are still in question (for review, see Clark and Roux, 1995; Delmer and Potikha, 1997), recent isolation of partial or complete annexin cDNA clones from higher plants, including monocots (Battey et al., 1996) and dicots (Pirck et al., 1994; Gidrol et al., 1996; Proust et al., 1996; Potikha and Delmer, 1997), provides some insight into the structural characteristics of plant annexins. The endonexin repeats found in animal annexins are also observed in plant annexins; however, it seems that plant annexins contain fewer predicted Ca^{2+}-binding sites than animal annexins (see Clark and Roux, 1995).

Progress has been made recently in understanding the biochemical properties of plant annexins. Most importantly, some plant annexins have been found to be associated with enzyme activities. Maize annexins were first found to be associated with ATPase activity (McClung et al., 1994). It was later found that tomato annexins could display a similar ATPase activity that was inhibited by phospholipid binding (Calvert et al., 1996). These observations indicate that annexins may be involved in the energy-dependent cellular processes in plant cells. Recently, an annexin from Arabidopsis was found to possess a peroxidase-like activity (Gidrol et al., 1996). Because peroxidase is known to be involved in plant-defense mechanisms during the oxidative burst (see Lamb and Dixon, 1997), it would be interesting to determine whether the peroxidase-like annexins really function in plant-defense mechanisms associated with the oxidative burst. Taken together, these discoveries suggest that plant annexins are multifunctional and play a role in a variety of cellular processes, as is the case for mammalian annexins (Raynal and Pollard, 1994).

In cotton (*Gossypium hirsutum*) fibers, annexins were first identified by Andrawis et al. (1993) during β-glucan synthase purification, and these authors suggested that these proteins might be responsible for inhibition of β-glucan synthase activity. We also isolated annexins associated with β-glucan synthase activity from cotton fiber membranes (Shin et al., 1995). In this report we demonstrate that cotton fibers contain the so-called "annexin doublet" with apparent molecular masses of 35 and 35.5 kD that is similar to other plants (see Clark and Roux, 1995), whereas the cotton annexins reported by Andrawis et al. (1993) were identified as a single band at 34 kD on SDS-PAGE. We also report the cloning of a cDNA encoding the 35.5-kD annexin from a cotton fiber cDNA library, which allowed us to express the cotton annexin cDNA in *Escherichia coli*. Here we show some biochemical properties of the resultant recombinant annexin purified from *E. coli* in relation to the current understanding of plant annexins. Our present study focused on the GTPase activity displayed by the recombinant annexin.

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Abbreviation: PME, plasma membrane-enriched.

MATERIALS AND METHODS

Materials

Cotton (*Gossypium hirsutum* cv Texas marker 1) fibers at different stages of development after anthesis were removed from the locules and immersed in liquid nitrogen as described previously (Okuda et al., 1993). Unless indicated otherwise, all chemicals for the preparation and assay of β-glucan synthases were purchased from the sources described previously (Okuda et al., 1993; Kudlicka et al., 1995). A PCR kit with *Taq* polymerase was purchased from GIBCO-BRL. A TA cloning kit was from Invitrogen (San Diego, CA). The expression vector pET21a(+), *Escherichia coli* BL21 (DE3), and His-bind resin/buffer kit for His-tag chromatography were from Novagen (Madison, WI).

β-Glucan Synthase Preparations

The PME fraction from cotton fibers was prepared as described previously (Okuda et al., 1993; Kudlicka et al., 1995). Cotton fibers ground to a fine powder in liquid nitrogen were extracted with cold buffer consisting of 50 mM Mops, pH 7.5, 5 mM EDTA, 0.25 M Suc, and a combination of protease inhibitors (0.5 mM PMSF, 10 μM leupeptin, 0.1 mM *N*-α-*p*-tosyl-L-Lys chloromethyl ketone, and 0.1 mM L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone). The extract was filtered through a 210-μm Spectra mesh screen (Spectrum Medical Industries, Houston, TX) to remove cell walls and then was centrifuged at 8,000*g* for 10 min over a 60% Suc cushion. The PME fraction was collected at the buffer-Suc interface, resuspended in a 1:5 dilution of extraction buffer, and recentrifuged at 100,000*g* for 30 min. The membrane pellet was resuspended in a small volume of resuspension buffer consisting of 50 mM Mops, pH 7.5, and 0.25 M Suc.

The fractions of digitonin-solubilized enzymes (SE1 and SE2) were obtained from the PME fraction as described previously (Kudlicka et al., 1995). The PME fraction was mixed with an equal volume of the first solubilization buffer containing 50 mM Mops, pH 7.5, 0.25 M Suc, and 0.1% digitonin. The mixture was centrifuged over a 30% (w/v) glycerol cushion at 100,000*g* for 1 h at 4°C. The supernatant was collected and denoted as SE1. The pellet was resuspended with the second solubilization buffer containing 50 mM Mops, pH 7.5, 0.25 M Suc, and 1% digitonin. The suspension was centrifuged at 100,000*g* for 1 h at 4°C over 30% (w/v) glycerol, and the supernatant was denoted as SE2. SE1 and SE2 were concentrated using Centriprep-10 (Amicon, Beverly, MA) before use.

The membrane fraction precipitated in the second solubilization step was resuspended in 50 mM Mops, pH 7.5, and 0.25 M Suc and denoted as the pellet (P) fraction.

Protein assay for the different enzyme fractions was performed using a modification of the Lowry procedure (Markwell et al., 1978).

β-Glucan Synthase Assay

The assay mixture was composed of 8 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM cellobiose, 100 μM cyclic 3′-5′-GMP, 0.5 mM UDP-[U-^{14}C]Glc (specific activity, 12.5 mCi/mmol), 10 mM bis-Tris-propane-Hepes buffer (pH 7.6), and approximately 40 μg of protein in a final volume of 200 μL. The reaction was conducted for 30 min at 25°C and terminated by placing the reaction mixture in a boiling-water bath for 1 min. The radioactive product was collected by filtration on a GF/C glass filter (Whatman) and washed three times with distilled water and once with methanol. The radioactivity on the filter was dissolved in Ready Organic cocktail and counted with a liquid scintillation system (model LS 6800, Beckman).

Isolation of Annexins by Product Entrapment from Cotton Fibers

Annexins were isolated from the SE1 fraction by a modification of the product-entrapment procedure according to the method of Kudlicka et al. (1995). The SE1 fraction was incubated in the β-glucan synthase assay mixture as described above, except that 1 mM UDP-Glc was used as the substrate. After incubation at 25°C for 2 h, the reaction product was pelleted by centrifugation at 6000*g* for 10 min. The pellet was resuspended in a small volume of buffer (10 mM Mops, pH 7.5, and 0.25 M Suc) by vigorous vortexing and centrifuged again at 6000*g* for 10 min. Annexins of 35 and 35.5 kD were highly enriched in the supernatant. The supernatant was subjected to SDS-PAGE and the proteins were visualized with Coomassie blue R-250 staining.

Protein Sequencing

The Coomassie blue-stained 35- and 35.5-kD bands were excised from SDS-polyacrylamide gels and pooled. The gel slices were incubated in neutralization buffer and subjected to enzyme digestion with V-8 protease (P2922, Sigma) as described by Cleveland (1983). The resulting peptides were separated by electrophoresis on a 16% SDS-polyacrylamide gel and blotted onto PVDF membranes according to the method of Matsudaira (1987). Automated N-terminal sequencing of the PVDF-blotted peptides was performed on a sequencer (model 477A, Applied Biosystems) at the Protein Sequencing Laboratory at the University of Texas, Austin.

Cloning of an Annexin cDNA

The complete cDNA was obtained in two steps using the peptide-sequence information obtained by protein sequencing. In the first step, a partial cDNA fragment encoding the C terminus of the 35.5-kD annexin was synthesized by PCR using a cotton fiber cDNA library constructed in the Uni-ZAP XR vector (Stratagene). This library (provided by Xiaojiang Cui, University of Texas at Austin) was constructed using mRNAs obtained from cotton fibers 14 d after anthesis. For PCR a 24-mer degenerate oligonucleotide designed from the partial amino acid sequence KAYSDDDV of the 35.5-kD annexin was used as a 5′ upstream primer, and a poly(dT)$_{12–18}$ oligonucleotide was used as a 3′ downstream primer. The PCR product was subcloned into the pCRII vector using a TA-cloning sys-

tem, and the nucleotide sequence was determined by DNA sequencing. A second PCR was then performed with a new set of primers on the same cDNA library to obtain a full-length cDNA. An SK sequence flanking the 5′ ends of cDNA inserts in the Uni-ZAP cDNA library vector was synthesized as a 5′ upstream primer, and a 21-mer antisense oligonucleotide from the 3′ untranslated region of annexin cDNA was synthesized as a 3′ downstream primer. The amplified product was ligated into the pCRII vector using a TA-cloning system, and it was used to transform *E. coli* XL1 Blue. The identity of the annexin clone (pCRII-Ann) was verified by DNA sequencing.

Amplification of Annexin cDNAs for Expression Cloning

The plasmid (pCRII-Ann) containing the full-length annexin cDNA was used as a template for PCR to amplify cDNAs encoding a full-length and seven domain-deletion mutants of cotton annexin. Eight primers (1F, 2F, 3F, 4F, 1R, 2R, 3R, and 4R) were synthesized for PCR, where the numbers indicate the identity of annexin domains and the letters F and R indicate the site and the orientation of primers on each annexin domain, respectively (see Fig. 5). For example, 1F is a forward 5′ primer corresponding to the N-terminal site of annexin domain 1, and 1R is a reverse 3′ primer corresponding to the C-terminal site of annexin domain 1. All forward primers incorporated an *Nde*I restriction site at the 5′ ends of the DNAs, and all reverse primers incorporated an *Xho*I restriction site at the 3′ ends of the DNAs. A total of eight amplified products were obtained from PCR performed with eight combinations of forward and reverse primers. These products were designated 1F4R, 1F3R, 1F2R, 1F1R, 2F4R, 2F3R, 3F4R, and 4F4R by the combination of primers used in the PCR.

Expression and Purification of Recombinant Annexin Proteins

The eight amplified products (1F4R, 1F3R, 1F2R, 1F1R, 2F4R, 2F3R, 3F4R, and 4F4R) were subcloned into the *Nde*I and *Xho*I sites of pET21a(+), which were used to transform *E. coli* XL1 Blue. Ampicillin-resistant colonies were used to isolate plasmids, the identities of which were verified by DNA sequencing. The sequence-verified plasmids were used to transform *E. coli* BL21 (DE3), and the positive transformants were cultured overnight at 37°C in 4 mL of Luria-Bertani medium including 100 μg/mL ampicillin. These precultures were used to inoculate 200 mL of Luria-Bertani-ampicillin medium, and cells were grown for approximately 2 h at 37°C until the A_{600} of cultures reached approximately 0.6. Protein expression was then induced by the addition of isopropyl 1-thiol-β-D-galactopyranoside to a final concentration of 1 mM and incubation was continued for 3 h. The cells were collected by centrifugation at 5000*g* for 5 min.

Because all of the recombinant constructs were designed to express proteins with a fusion of six His residues at their C termini, the purification of expressed proteins from the collected cells was performed by the protocol provided with His-bind resin and a buffer kit for His-tag chromatography from Novagen. The His-tagged recombinant proteins eluted from the His-tag column were dialyzed for 24 h at 4°C against 25 mM Mops buffer, pH 7.0, using Slide-A-Lyzer dialysis kits (Pierce). Protein concentrations were determined by the Lowry assay with BSA as a standard and confirmed by SDS-PAGE with Coomassie blue staining. All eight recombinant annexin proteins were purified and designated Ann1F4R(1–316), Ann1F3R(1–241), Ann1F2R(1–163), Ann1F1R(1–82), Ann2F4R(76–316), Ann2F3R(76–241), Ann3F4R(157–316), and Ann4F4R(235–316), where the numbering system indicates the amino acid residues of cotton annexin that are included in the recombinant protein (see Fig. 5).

β-Glucan Synthase Assay with Recombinant Annexin

Various amounts of the affinity-purified recombinant annexin were added to the β-glucan synthase assays performed with different enzyme fractions. The assay for β-glucan synthase was performed as described above.

Antibody Production and Western-Blot Analysis

The full length of a recombinant annexin (Ann1F4R) purified as described above was sent to HTI Bio-Products, Inc. (Ramona, CA) to raise polyclonal antisera in rabbits.

For western-blot analysis, 30 μg of total protein per lane was separated on a 10% SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by semidry electroblotting. After blocking overnight in PBS buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5, and 0.02% Tween 20) with 5% nonfat, dried milk (Carnation, Glendale, CA) at 4°C, the membrane was incubated with antiserum in PBS buffer at 1:250 dilution for 2 h. After the blot was washed in PBS buffer four times, it was incubated with a horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution for 1 h at room temperature. The protein complexes were detected by chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

GTPase and ATPase Assays

GTPase and ATPase activities were determined by the method of Ames (1996) with some modifications. The 200-μL reaction mixtures containing 10 μg of purified recombinant annexin, 5 mM Mops buffer, pH 6.5, 50 mM KCl, 3 mM $MgCl_2$, and 3 mM GTP (or ATP) were incubated at 37°C for 30 to 60 min. The reactions were terminated by the addition of 0.6 mL of Ames reagent and allowed to stand for 30 min at room temperature for the color development. A_{820} was measured against the blank buffer control containing no annexin protein.

Photoaffinity Labeling with [α-^{32}P]GTP

Purified annexin proteins were incubated with 5 μCi of [α-^{32}P]GTP in 20 mM Hepes, pH 7.6, 2 mM $MgCl_2$, and 0.1 mg/mL BSA, in a final volume of 100 μL, for 5 min at room temperature, then placed in an ice bath, and irradiated with

UV light (254 nm) for 20 min. For competition assays varying concentrations of ATP and GTP were added into this reaction mixture, which was incubated and irradiated under the same conditions. After irradiation, proteins were concentrated with methanol/chloroform extraction and mixed with SDS-sample buffer for polyacrylamide gel analysis. Autoradiographs of gels were exposed for 2 to 5 d.

Northern Blotting

Total RNAs from different cotton tissues were isolated and quantitated by general protocol. For northern-blot analysis, total RNAs (20 μg) from each tissue were electrophoresed in alkaline agarose gels and transferred to nylon membranes (Hybond-N, Amersham) according to the manufacturer's protocol. The probe was synthesized by the Prime-a-Gene labeling kit (Promega) with [α-^{32}P]dCTP using full-length annexin cDNA (1149 bp) as the template.

RESULTS

Copurification of Annexins with β-Glucan Synthase

During the process of β-glucan synthase purification from cotton fibers 18 to 20 d after anthesis, we noticed that two polypeptides with apparent molecular masses of 35 and 35.5 kD were highly enriched by product-entrapment reaction from the low SE1 fraction. It is interesting that increasing the Ca^{2+} concentration in the entrapment reactions increased the yield of these two proteins along with that of β-glucan synthase activity (Fig. 1), indicating a Ca^{2+}-dependent interaction of β-glucan synthase with these proteins. The identity of these two bands was investigated by sequencing the 18- and 16-kD peptides generated from V-8 protease digestion of the 35- and 35.5-kD peptides, respectively. The partial amino acid sequences obtained from the two peptides showed significant homology with annexins (Fig. 1) from Arabidopsis (Gidrol et al., 1996), maize (Battey et al., 1996), and cotton fibers (Delmer and Potikha, 1997). Our experiments demonstrate for the first time, to our knowledge, the presence of the so-called "annexin doublet" in the cotton fibers similar to those observed in other plants (see Clark and Roux, 1995). The cotton annexins reported by Andrawis et al. (1993) were observed as a single band at 34 kD on SDS-PAGE.

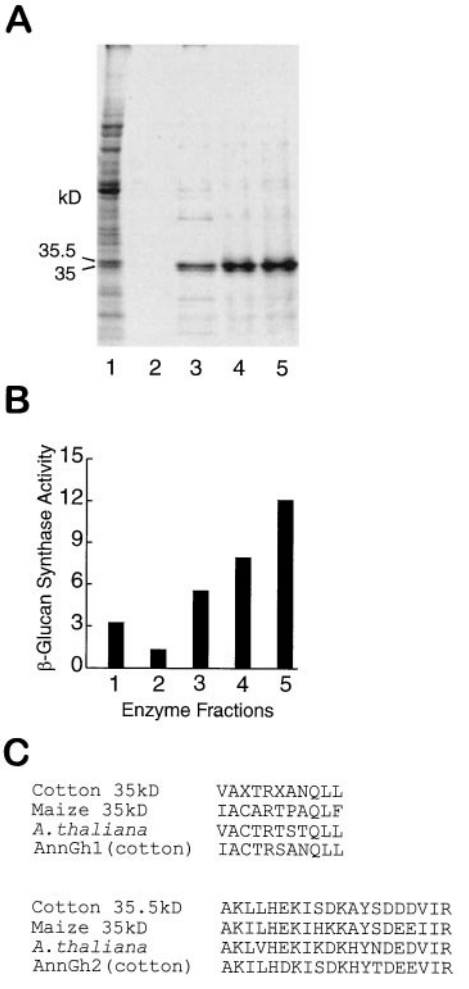

Figure 1. Copurification of annexins with β-glucan synthase from cotton fibers. A, Protein profiles of different enzyme fractions on an SDS-PAGE gel stained with Coomassie blue. Lane 1, SE1, 15 μg/lane. Lanes 2, 3, 4, and 5, Proteins released from product entrapment of the SE1 fraction that was performed with different Ca^{2+} concentrations of 0, 1, 2, and 4 mM, respectively. B, β-Glucan synthase assay for the fractions in A. β-Glucan synthase activity is represented as units, where 1 unit = 1 nmol Glc incorporated min^{-1}. C, Partial amino acid sequences obtained from the 35- and 35.5-kD bands are aligned with homologous regions in annexins from maize (35 kD; Battey et al., 1996), Arabidopsis (*A. thaliana*; Gidrol et al., 1996), and cotton fibers (AnnGh1 and AnnGh2; Delmer and Potikha, 1997).

Cloning of an Annexin cDNA Encoding the Cotton 35.5-kD Polypeptide

Because the 35.5-kD polypeptide was always more abundant than the 35-kD polypeptide in our purification, an attempt was made to isolate a cDNA clone encoding the 35.5-kD polypeptide. From two rounds of PCR, we obtained a full-length cDNA clone (1149 bp) for the 35.5-kD annexin (accession no. U89609). The complete open reading frame encodes a protein of 316 amino acids, and its deduced molecular mass and pI are 36 kD and 6.62, respectively. The deduced amino acid sequence showed good homology to annexins, and the sequence could be divided into four repeat domains based on the alignment with other plant annexins (Fig. 2). All plant annexins have the type-II Ca^{2+}-binding site in the first repeat (Clark and Roux, 1995). The 35.5-kD cotton annexin also has the predicted type-II Ca^{2+}-binding site in the first repeat, which is defined as a Gly-X-Gly-Thr loop with an acidic Asp or Glu residue 42 amino acids downstream of the first Gly (Chen et al., 1993). There is another type-II-like conserved sequence in repeat 4 of the cotton annexin, in which the first Gly is substituted with an Arg residue. A search of the PROSITE database (www.expasy.ch, Swiss Institute of Bioinformatics, Geneva, Switzerland) for motifs revealed that there are many potential posttranslational modification sites in the deduced cotton annexin sequence (Fig. 2).

A

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Cot1     MATLTVPTTV PSVSEDCEQL RKAFSGWGTN EGLIIDILGH RNAEQRNLIR KTYAETYGED PLKALDKELS NDFERLVLLW     80
Cot2     ALDPAERDAL LANEAtkrWT SSNQVLMEIA CTRSANQLLH ARQAYHARYK KSLEEDVAHH TTGDFRKLLL PLVSsyrYEG    160
Cot3     EEVNMNLAKT EAKLLHEKIs dkAYSDDDVI RVLATRSKAQ InatlNHYKN EYGNDINKDL KADPKDEFLA LLRStvkCLV    240
Cot4     YPEKYFEKVL RLAINRRGTD EGALTRVVCT RAEVDLKIIA DEYQRRNSVP LTRAIVKDTH GDYEKLLLVL AGHVEN       316
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B

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                                    KGhGTDEx-xLIPILApR
Cot1     1 MATLTVPTTVPSVSEDCEQLRKAFSGWGTNEG-LIIDILGHRNAEQRNLIRKTYAETYGEDPLKALD-KELSNDFERLVLLW--------  80
Ara1     1 MATLKVSDSVPAPSDDAEQLRTAFEGWGTNED-LIISILAHRSAEQRKVIRQAYHETYGKDLLKTLD-KELSNDFERAILLW--------  80
Zea1     1 MATLTVPSSVPAVAEDCEQLHKAFEGWGTNEK-LIISILAHRNAAQARAIRRGYAEAYGKELLRALG-DEIHGKFERAVILW--------  80

Cot2    81 --------ALDP-AERDALLANEATKRWTSSNQVLMEIACTRSANQLLHARQAYHARYKKSLEEDVA-HHTTGDFRKLLLPLVSSYRYEG 160
Ara2    81 --------TLEP-GERDALLANEATKRWTSSNQVLMEVACTRTSTQLLHARQAYHARYKKSLEEDVA-HHTTGDFRKLLVSLVTSYRYEG 160
Zea2    81 --------TLDP-AERDAVLANEAKKSHPGGRALVEIACARTPAQLFAVKQAYHDRFKRSLEEDVA-AHVTGDFRKLLVPLVSAYRYDG 160

Cot3   161 -----EEVNMNLAKTEAKLLHEKISDKAYSDD-DVIRVLATRSKAQINATLNHYKNEYGNDINKDLK-ADPKDEFLALLRSTVKCLV--- 240
Ara3   161 -----DEVNMTLAKQEAKLVHEKIKDKHYNDE-DVIRILSTRSKAQINATFNRYQDDHGEEILKSLEEGDDDDKLPCTLRSTIQCL---- 240
Zea3   161 -----PEVNTSLAHSEAKILHEKIHKKAYSDE-EIIRILTTRSKAQLLATFNSYKDQFTHAINKDLK-ADPKDEFLSTLRAIIRCFT--- 240

Cot4   241 ----------YPE-KYFEKVLRLAINRRGTDEG-ALTRVVCTRAEVDLKIIADEYQRRNSVPLTRAIV-KDTHGDYEKLLLVLAGHVEN-- 316
Ara4   241 --------TRPE-LYFVDVLRSAINKTGTDEG-ALTRIVTTRAEIDLKVIGEEYQRRNSIPLSKAIT-KDTRGDYEKMLVALLGEDDA-- 317
Zea4   241 ----------CPD-RYFEKVIRLALGGMGTDED-DLTRVVTTRAEVDLKLIKEAYQKRNSVPLERAVA-KDTTRDYEDIMLALLGAE---- 314
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Figure 2. A, The deduced amino acid sequence of a 35.5-kD cotton annexin cDNA. Potential posttranslational modification sites on the deduced sequence are emphasized as follows: Uppercase, underlined letters, *N*-myristoylation sites; lowercase, underlined letters, *N*-glycosylation site; boldface, uppercase letters, cAMP-dependent kinase phosphorylation sites; and boldface, lowercase letters, protein kinase C phosphorylation sites. B, Alignment of annexin repeat domains from cotton (Cot), Arabidopsis (Ara) (Gidrol et al., 1996), and maize (*Zea*) (Battey et al., 1996). Annexin repeat domains 1 to 4 from Arabidopsis, cotton, and maize are designated *Ara1–4*, *Cot1–4*, and *Zea1–4*, respectively. Underlined are amino acid residues conserved in the type-II Ca^{2+}-binding site. The 17-amino acid annexin "endonexin fold" or "consensus sequence" is shown above the alignment, in which h = hydrophobic residue, P = polar residue, and x = variable residue. The 12 domains were aligned by the ClustalW1.7 program, which is available as an Internet service provided by the Human Genome Center at Baylor College of Medicine (Houston, TX).

It was found that there are a total of 16 potential phosphorylation sites in the deduced annexin sequence, including 4 protein kinase C phosphorylation sites, 8 casein kinase II phosphorylation sites, 1 Tyr kinase phosphorylation site, and 3 cAMP-dependent protein kinase phosphorylation sites. The potential protein kinase C sites are located in the repeat domains 2 and 3 of the cotton annexin, whereas no potential protein kinase C site is found at the N- and C-terminal domains of the cotton annexin (Fig. 2). In maize the PROSITE search with p33 and p35 annexins revealed a potential protein kinase C phosphorylation site at the most N-terminal Thr residue in p33 annexin (Battey et al., 1996). Other potential modification sites include one *N*-glycosylation site and three *N*-myristoylation sites.

Recently, two cDNA sequences encoding cotton fiber annexins, designated AnnGh1 and AnnGh2, were reported (Potikha and Delmer, 1997). Our annexin amino acid sequence deduced from cDNA showed 95% identity with AnnGh1 protein and 65% identity with AnnGh2 protein, indicating that AnnGh1 from cotton cv Acala SJ-2 corresponds to our annexin.

Recombinant Annexin and β-Glucan Synthase Activity

A purified recombinant annexin was prepared after expression of the cotton annexin cDNA in *E. coli* (Fig. 3), and it was used in an "add-back experiment" to observe the effect of annexin on β-glucan synthase activity (Table I). As summarized in Table I, no effect on β-glucan synthase activity was noted after addition of the recombinant annexin. In this experiment different enzyme fractions were used to avoid the possibility of endogenous annexins interfering with the assay.

Western-Blot Analysis of β-Glucan Synthase Fractions

To investigate whether the endogenous annexins were depleted from the β-glucan synthase fractions used in the add-back experiment, we performed western-blot analysis with a polyclonal antibody raised against the 35.5-kD recombinant annexin. As shown in Figure 4, the 35.5-kD annexin was present in the PME and the SE1 fractions, but was almost completely depleted in the SE2 fraction and was completely absent in the solubilization-resistant P fraction.

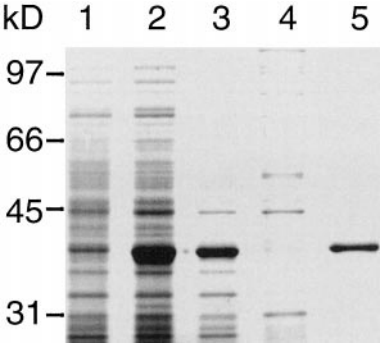

Figure 3. Purification of recombinant cotton annexin. The cotton annexin cDNA was expressed in *E. coli* BL21 (DE3) as His-tagged protein and purified by affinity chromatography as described previously. Proteins were separated by 10% SDS-PAGE and stained with Coomassie blue. Lane 1, Crude extract from uninduced cell; lane 2, crude extract from isopropyl 1-thiol-β-D-galactopyranoside-induced cell; lane 3, soluble fraction obtained from lane 2 fraction; lane 4, washed-out fraction from His-tag chromatography; and lane 5, eluted fraction from His-tag chromatography. Note the highly purified annexin band in lane 5 with an apparent molecular mass of 36 kD.

Table I. *Influence of recombinant cotton annexin on β-glucan synthase activity*

In each experiment, three separate reactions were carried out with individual enzyme fractions by using 1, 5, and 10 μg of purified recombinant annexin. The same experiment was repeated three times to obtain SD values. 1 unit = 1 nmol Glc incorporated min^{-1}. SE1, The first solubilized fraction with 0.05% digitonin; SE2, the second solubilized fraction with 1% digitonin; P, the pellet fraction that remained after solubilization.

Enzyme Fraction	100% of β-Glucan Synthase Activity before Adding Annexin	β-Glucan Synthase Activity after Adding Annexin
	units/mg	*%*
PME	20.1	100 ± 6.2
SE1	3.1	100 ± 5.5
SE2	12.5	100 ± 4.2
P	15.2	100 ± 5.1

Table II. *Recombinant annexin and ATPase/GTPase activity*

ATPase and GTPase assays were performed at pH 6.5, with 10 μg of purified recombinant annexin proteins and 3 mM $MgCl_2$ in the presence of 3 mM ATP or GTP. At least three replicates were carried out for each assay to obtain SD values.

Samples	ATPase Activity	GTPase Activity
	nmol Pi mg^{-1} h^{-1}	
Purified whole annexin		
Ann1F4R	42.5 ± 3.3	400.1 ± 5.2
Heat-treated whole annexin		
Ann1F4R	5.1 ± 2.1	8.3 ± 2.5
N-terminal deletion mutants		
Ann2F4R	2.1 ± 1.1	1.5 ± 2.2
Ann3F4R	3.2 ± 1.3	2.1 ± 0.7
C-terminal deletion mutants		
Ann1F3R	1.3 ± 1.5	2 ± 1.5
Ann1F2R	2.5 ± 1.6	1.7 ± 0.5

We found that a 42-kD polypeptide in the SE1 fraction appeared to interact with the antibody against the 35.5-kD annexin (Fig. 4). Although we lack biochemical evidence for the identity of this 42-kD band, it is possible that this 42-kD protein may be a new annexin in cotton fibers. So far, a vacuole-associated annexin from tobacco cells (Seals et al., 1994) has been identified as the only 42-kD annexin from plants.

Recombinant Annexin Displays GTPase Activity

Recently, annexins prepared from maize (McClung et al., 1994) and tomato (Calvert et al., 1996) tissues were found to exhibit ATP/GTPase activity. These discoveries prompted us to determine whether our recombinant annexin possessed such activity. As summarized in Table II, the full-length (Ann1F4R) recombinant cotton annexin displayed an enzyme activity for the hydrolysis of ATP and GTP. Significantly, it was found that GTPase activity was much greater than ATPase activity, indicating that GTP is the preferred substrate over ATP for cotton annexin activity. The specific activity of GTPase by cotton annexin was about 400 nmol Pi mg^{-1} h^{-1}, which is almost 10 times greater than that of ATPase (42 nmol Pi mg^{-1} h^{-1}). For the controls, four domain-deletion mutants of annexin were assayed for their ATPase/GTPase activity. We found no significant activity from either N-terminal (Ann2F4R and Ann3F4R) or C-terminal deletion mutants (Ann1F3R and Ann1F2R) (see also Fig. 5). This result clearly disproves the possibility that the ATPase/GTPase activity displayed by a whole recombinant annexin may be from *E. coli* contamination during purification. In addition, boiling abolished the ATPase/GTPase activity from the recombinant annexin, indicating that the annexin activity is conformation dependent.

To determine whether Ca^{2+} and/or Mg^{2+} can affect the annexin GTPase activity, varying concentrations of both cations were added during the enzyme assays. It was

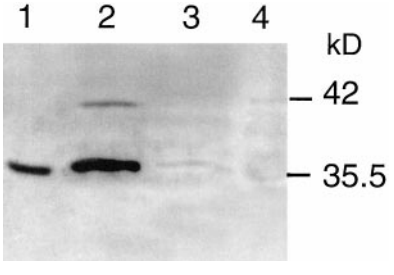

Figure 4. Immunoblot analysis of different β-glucan synthase fractions prepared from cotton fibers. The proteins of different enzyme fractions (PME, SE1, SE2, and P) were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The proteins were probed with polyclonal annexin antiserum diluted in PBS buffer (1:250 dilution). Lanes 1 to 4, PME, SE1, SE2, and P, respectively.

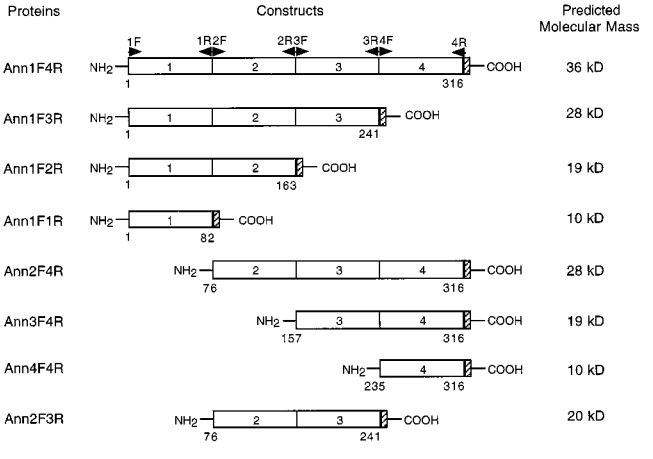

Figure 5. Strategy for the construction of domain-deletion mutants of cotton annexin. The four annexin repeat domains are designated inside of the boxes. The numbers below the boxes indicate the first and last numbers of amino acid residues of the cotton annexin expressed by the constructs. The hatched boxes represent the 6xHis tags at the C-terminal ends of recombinant proteins. The eight PCR primers synthesized for mutant construction are designated 1F, 2F, 3F, 4F, 1R, 2R, 3R, and 4R above the arrows that show the sites and orientations of these primers.

shown that Mg^{2+} was essential for the annexin GTPase activity and that a high concentration of Ca^{2+} was inhibitory to the activity (Fig. 6). Addition of less than 1.5 mM Mg^{2+} increased the GTPase activity of annexin, but more than 1.5 mM Mg^{2+} did not affect the activity. Addition of less than 3 mM Ca^{2+} decreased the GTPase activity in an inverse manner, but more than 3 mM Ca^{2+} did not further inhibit the GTPase activity. The maximum inhibition level of GTPase activity by Ca^{2+} was about 60% of the full activity.

A photoaffinity-labeling experiment was performed with [α-^{32}P]GTP to determine the substrate preference of cotton annexin GTPase/ATPase activity (Fig. 7). As shown in Figure 7, the photolabeling of annexin with [α-^{32}P]GTP was more sensitive to the presence of unlabeled GTP than to unlabeled ATP in the reaction mixture, indicating a greater binding specificity of annexin to GTP than to ATP. Quantitatively, at least 100 μM unlabeled ATP was required to completely inhibit the labeling, whereas 50 μM unlabeled GTP could achieve complete inhibition.

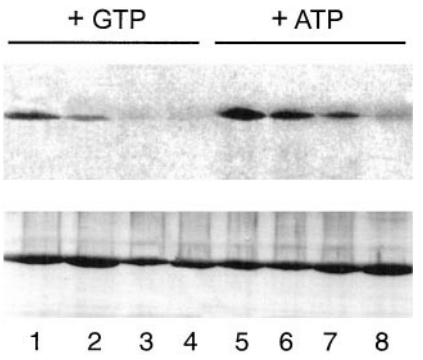

Figure 7. Competition analysis of photoaffinity labeling. The whole recombinant cotton annexin was incubated with [α-^{32}P]GTP for 10 min under UV irradiation in the presence of unlabeled GTP or ATP at variable concentrations. The protein was extracted and subjected to 10% SDS-PAGE. Top, Autoradiograph of a photolabeled SDS gel. Lanes 1 to 4, Competition of photolabeling by adding unlabeled GTP to a final concentration of 10, 20, 50, and 100 μM, respectively; lanes 5 to 8, competition of photolabeling by adding unlabeled ATP to a final concentration of 10, 20, 50, and 100 μM, respectively. Bottom, Coomassie blue staining of the same gel.

Mapping of the GTP-Binding Site on Annexin

Because the predicted amino acid sequence of the 35.5-kD cotton annexin lacked a common motif for nucleotide binding such as a Walker-type nucleotide-binding site (Walker et al., 1982), we decided to determine the GTP-binding site on cotton annexin. Domain-deletion mutants of annexin were constructed, expressed, and purified from *E. coli* as described in "Materials and Methods," and the mutant proteins were designated Ann1F1R, Ann1F2R, Ann1F3R, Ann1F4R, Ann2F3R, Ann2F4R, Ann3F4R, and Ann4F4R (Fig. 5). All mutant proteins migrated at their predicted molecular mass on SDS-PAGE (Fig. 8). Photoaffinity labeling with [α-^{32}P]GTP was performed to determine the GTP-binding ability of each of the deletion mutants. BSA was included in all of the reactions as the internal control. As shown in Figure 8, three annexin mutants, Ann2F4R, Ann3F4R, and Ann4F4R, all including the C-terminal fourth domain of annexin, were labeled with [α-^{32}P]GTP, whereas any mutant missing the C-terminal domain was not labeled. Therefore, the result of this experiment clearly suggests that the GTP-binding site on annexin is located in the C-terminal region of the protein.

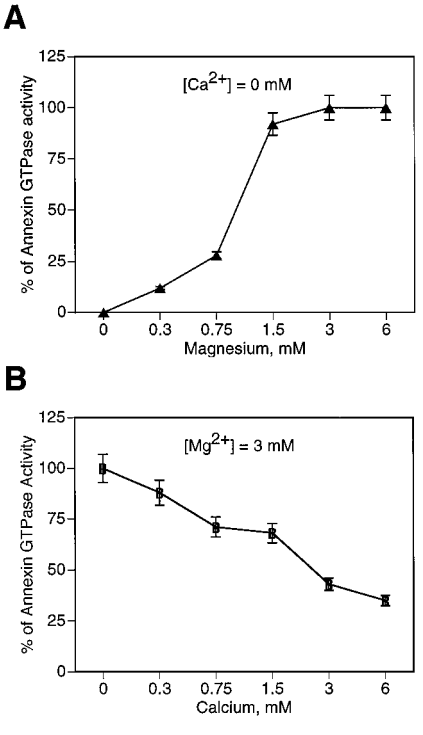

Figure 6. Effects of Ca^{2+} and Mg^{2+} ions on the annexin GTPase activity. The assay was performed according to the method of Ames as described previously. One-hundred percent GTPase activity represents 400 nmol Pi mg^{-1} h^{-1}. A, Effect of Mg^{2+} concentrations in the absence of Ca^{2+}. B, Effect of Ca^{2+} concentrations in the presence of Mg^{2+} at 3 mM.

Cotton Annexin Gene Expression

To understand the physiological significance of our data, we performed a northern-blot analysis using a probe generated from the full-length cotton annexin cDNA. High levels of annexin transcript at 1.2 kb were detected in roots, flowers, and fibers, whereas almost none was detected in leaves (Fig. 9). In cotton fibers the expression of the annexin gene was high during the early primary stage of development, and it gradually decreased as fibers entered the secondary stage. The expression was very low during the secondary stage beyond 24 d after anthesis. These results suggest that the expression of the annexin gene is regulated temporally and spatially in cotton tissues.

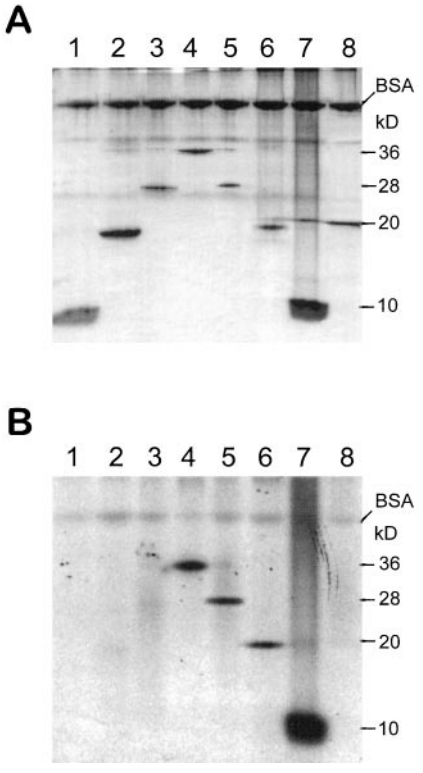

Figure 8. Mapping of the GTP-binding site on annexin by photolabeling experiment. Recombinant annexin proteins were incubated with [α-^{32}P]GTP for 10 min under UV irradiation, and the proteins were extracted and subjected to 10% SDS-PAGE. A, SDS-polyacrylamide gel stained with Coomassie blue. Lanes 1 to 8, Purified recombinant annexin proteins of Ann1F1R, Ann1F2R, Ann1F3R, Ann1F4R, Ann2F4R, Ann3F4R, Ann4F4R, and Ann2F3R, respectively. BSA was added to the reactions as the internal control. B, Autoradiograph of the same gel. Note that any annexin mutant missing the C-terminal domain 4 was not labeled.

DISCUSSION

Annexins and β-Glucan Synthase Activity

In cotton fibers annexins were first identified by Andrawis et al. (1993) as the proteins that may be responsible for inhibition of β-glucan synthase activity. Andrawis et al. (1993) also suggested that the interaction between annexins and β-glucan synthase is Ca^{2+} dependent. Our data in Figure 1 show an essential role of Ca^{2+} in the successful entrapment of β-glucan synthase with annexins, in accordance with the results of Andrawis et al. (1993). The data in Figure 1 appear to show a positive correlation between the amount of entrapped annexins and the activity of β-glucan synthase.

In our experiment for the β-glucan synthase assay with a recombinant annexin (Table I), we provide clear evidence that the 35.5-kD recombinant annexin has no effect on the β-glucan synthase activity. In addition, the endogenous 35.5-kD annexins are not thought to have interfered with our assay, because they were virtually not present in the SE2 and P fractions as shown by western-blot analysis (Fig. 4). However, it may be premature to conclude that annexins are not responsible for inhibition of β-glucan synthase activity because (a) we lack the data with all of the different annexins (Potikha and Delmer, 1997) and (b) posttranslational modifications may be required for the functions of endogenous annexins.

Recombinant Annexin and GTPase Activity

The PROSITE analysis shows that the deduced amino acid sequence of the 35.5-kD cotton annexin cDNA contains a variety of potential phosphorylation sites (Fig. 2), suggesting that phosphorylation may determine the specific function of this annexin in cotton fibers. However, it remains to be determined whether these potential sites are actually phosphorylated and whether phosphorylation affects the biochemical properties of this annexin in vitro.

We have demonstrated that a cotton annexin can display enzyme activities for the hydrolysis of ATP and GTP. This result is important because this is the first evidence, to our knowledge, of ATPase/GTPase activities obtained from a recombinant plant annexin. The tomato annexins (p34 and p35) that were recently demonstrated by Calvert et al. (1996) to have similar activities were directly purified from tomato tissues. Therefore, using a highly purified recombinant protein, we provided evidence that plant annexins can possess inherent ATPase/GTPase activities. Most important, we noted that several biochemical characteristics of cotton annexin activities are different from those of tomato annexins. First, most significantly, the substrate preference for GTP over ATP by cotton annexin was surprisingly high. Cotton annexin showed GTPase activity almost 10 times greater than ATPase activity (Table II). The substrate preference for GTP over ATP by cotton annexin was further demonstrated by competition photolabeling assay (Fig. 7). In tomato, annexins displayed ATPase activity almost equal to GTPase activity. Second, whereas the tomato annexins did not require Mg^{2+} for the activity, it was essential for the cotton activity (Fig. 6). Third, although it was not shown clearly in the tomato annexins whether only Ca^{2+} can inhibit the tomato annexin activity, our results demonstrate that cotton annexin activity can be inhibited only by Ca^{2+} (Fig. 6). It was also shown that a correct conformation of the annexin protein is necessary for the activity, because boiling the protein could destroy the activities.

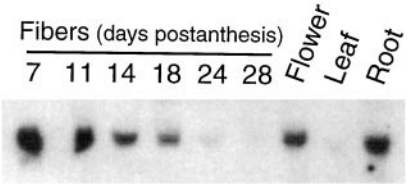

Figure 9. Northern-blot analysis of the annexin gene expression in cotton. Radioactive probe was generated from the full length of cotton annexin cDNA. Note that the expression of annexin gradually decreases as cotton fibers develop into the secondary stage. Annexin is highly expressed in flower and root tissues, but not in leaves (Leaf). Total RNA was quantitated by spectrophotometry, and equal loading (20 μg/lane) was confirmed by ethidium bromide staining.

Although a color assay modified from the Ames method (McClung et al., 1994) was used as the major GTPase assay, the GTPase activity of the recombinant cotton annexin was confirmed by using a radioactive assay (de Boer et al., 1991) that resulted in the formation of ^{32}Pi from [γ-^{32}P]GTP and that was determined by scintillation counting (data not shown here).

We demonstrated that the GTP binding to cotton annexin occurs in a sequence-specific manner. From photoaffinity-labeling experiments using domain-deletion mutants of cotton annexin, it was evident that the GTP-binding site is present in the C-terminal domain 4 of cotton annexin (Fig. 8). To our knowledge, this is the first evidence showing the importance of the C-terminal domain in the functions of plant annexins.

Potential Functions of Annexin in Cotton Fibers

We demonstrated by northern-blot analysis that the expression of the cotton annexin gene is regulated temporally and spatially (Fig. 9). Notably, cotton annexin was highly expressed during the early primary stages of cotton fiber development. During these early stages in cotton fiber differentiation, cotton fibers grow and elongate rapidly by forming the central vacuole, plasma membrane, and primary cell wall (Ramsey and Berlin, 1976). According to Ryser (1979), dictyosome-associated coated vesicles are especially numerous during the fiber-elongation stages, whereas they are not conspicuous in the secondary stages of cotton fibers. Taken together, it is tempting to suggest that annexins may be associated with dictyosome-derived coated vesicles participating in the elongation of cotton fibers.

Recently, Seals and Randall (1997) showed that a vacuole-associated annexin, VCaB42, was related to the expansion of tobacco cells. Other plant annexins that may have a similar role in cell expansion are maize annexins that are strongly transcribed in the cell-elongation region of root tips (Battey et al., 1996). Although the expansion of tobacco cells was suggested to occur by the vacuolation process in which annexins may play a certain role (Seals and Randall, 1997), the exact role of annexins in these cell-expansion processes is still unknown.

More recently, Carroll et al. (1998) provided evidence that annexins modulate exocytosis in maize root-cap cells. This is the only direct evidence that suggests that plant annexins may regulate exocytosis, because so far only circumstantial evidence has been obtained from several studies with plant annexins (see Clark and Roux, 1995). Significantly, exocytosis from maize root-cap cells was also shown to be modulated by Ca^{2+} and GTP (Carroll et al., 1998). In this context, our data that cotton annexins possess GTPase activity inhibited by Ca^{2+} could provide some insights into the potential role of annexins in the exocytotic process.

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