p11, a Unique Member of the S100 Family of Calcium-binding Proteins, Interacts with and Inhibits the Activity of the 85-kDa Cytosolic Phospholipase A₂*

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Using a two hybrid system screen of a human cDNA library, we have found that p11, a unique member of the S100 family of calcium-binding proteins, interacts with the carboxyl region of the 85-kDa cytosolic phospholipase A₂ (cPLA₂). p11 synthesized in a cell-free system interacts with cPLA₂ in vitro. The p11-cPLA₂ complex is detectable from a human bronchial epithelial cell line (BEAS 2B). Furthermore, p11 inhibits cPLA₂ activity in vitro. Selective inhibition of p11 expression in the BEAS 2B cells by antisense RNA results in an increased PLₐ₂ activity as well as an increased release of prelabeled arachidonic acid. This study demonstrates a novel mechanism for the regulation of cPLA₂ by an S100 protein.

Phospholipase A₂₉ (PLA₂₉, EC 3.1.1.4) are a group of enzymes that catalyze the hydrolysis of the sn-2-ester bond of phospholipids, resulting in the production of free fatty acid and lysophospholipids. These lipid products may serve as intracellular second messengers or can be further metabolized to potent inflammatory mediators. The release of arachidonic acid (AA) from membranes by PLA₂ and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids play a key role in the process leading to inflammation. Several mammalian PLA₂₉, including the 14-kDa secreted form PLA₂ and the 85-kDa cytosolic PLA₂ (cPLA₂), have been proposed to mediate AA release and eicosanoid generation in various cell types (1–3).

The mammalian low molecular mass 14-kDa PLA₂₉ mainly include two types, type I and type II. Although the type I PLA₂ is mainly found in pancreatic secretion, it has also been found in other mammalian tissues, and the physiological role of this extra-pancreatic type I PLA₂ remains unclear. The type II PLA₂ has been suggested to be involved in the pathogenesis of inflammatory reactions (1–2). It is found associated with several cells and tissues or is present extracellularly when released in response to proinflammatory mediators such as interleukin-1, interleukin-6, or tumor necrosis factor. The soluble type II 14-kDa PLA₂ found in inflammatory fluids, tissue exudates, or serum has been suggested to propagate inflammation through membrane digestion and liberation of proinflammatory lipid mediators. The cell-associated type II PLA₂ has been assumed to release AA for lipid mediator generation (1–3). However, because the 14-kDa PLA₂ lacks selectivity for the sn-2 fatty acids of phospholipids and requires much higher Ca²⁺ (µM) than normal cellular Ca²⁺ level (nM–µM) for activation, it was not certain whether this type of PLA₂ contributes to the release of AA and production of eicosanoids. Studies using antisense inhibition and selective inhibitors of different forms of PLA₂ have demonstrated that the type II PLA₂ participates in AA release and prostaglandin generation in the P388D₁ macrophages (4–6). These findings have established an important role of the 14-kDa PLA₂ for AA metabolism.

The high molecular mass 85-kDa cPLA₂ exhibits a preference for arachidonic acid in the sn-2 position of the substrate phospholipids (1–3, 7–10). The enzyme activity is regulated by phosphorylation (11–14), G-proteins (15), and physiologically relevant concentrations of Ca²⁺ (7–9, 16). These characteristics make the 85-kDa cPLA₂ a strong candidate for participation in the liberation of AA from membrane phospholipids for subsequent metabolism to eicosanoids. The cPLA₂ is expressed in a wide variety of cells (1–3). Although the cPLA₂ gene lacks a TATA box in its promoter (17–19), its expression is induced by various cytokines in several cell types (20–26). The cPLA₂ has two functionally distinct regions, the NH₂-terminal region (amino acids 1–178) containing the homologous Ca²⁺-dependent lipid binding motif (amino acid 36–81) and the COOH-terminal region (amino acids 179–749) containing the catalytic domain (8, 27). The NH₂-terminal region Ca²⁺-dependent lipid binding motif mediates the Ca²⁺-dependent translocation of cPLA₂ from cytosol to membrane substrate. The COOH-terminal region mediates the Ca²⁺-independent enzyme catalysis, which likely involves Ser-228 at the active site (28). Several phosphorylation sites (Ser-437, Ser-454, Ser-505, and Ser-727) have also been identified in the COOH-terminal region (29). Ser-505, located at the consensus site for mitogen-activated protein kinase, is phosphorylated by mitogen-activated protein kinase (12–13). Other protein kinases such as protein kinase C and cyclic AMP-dependent kinase (protein kinase A) also cause phosphorylation of cPLA₂ in vitro (12–13, 30). Thus, both the calcium-dependent translocation mediated by the NH₂-terminal Ca²⁺-dependent lipid binding domain and phosphorylation of the COOH-terminal region play an important regulatory role in the activation of cPLA₂ and regulation of AA release.

In an effort to further investigate the regulation of the 85-kDa cPLA₂, this study was designed to identify proteins interacting with the NH₂-terminal or COOH-terminal region of cPLA₂ protein by screening a human cDNA library using the yeast two hybrid system. An 11-kDa protein (p11), the unique member of S100 family of calcium-binding proteins, was found to interact with the COOH-terminal region of cPLA₂. Further studies demonstrated that p11 associated with cPLA₂ and in-
hibited the cPLA2 activity in vitro. The p11-cPLA2 complex was precipitated from a human bronchial epithelial cell line (BEAS 2B cell) expressing both p11 and cPLA2. Reduction of p11 expression in the BEAS 2B cells by antisense RNA reversed the inhibition of p11 on cPLA2, which resulted in an increased cPLA2 activity as well as an increased AA release. This study revealed a previously unrecognized mechanism of cPLA2 regulation by the 101 protein family.

EXPERIMENTAL PROCEDURES

Construction of Plasmids for Two Hybrid System Screen—Plasmid pGBT9 (CLONTECH, Palo Alto, CA), containing the sequences for the GAL4 DNA-binding domain (amino acids 1–147) as well as sequences for tryptophan (TRP1), was used to express the fusion proteins. Two cPLA2 cDNA fragments (540 bp corresponding to the NH2 terminus and 1707 bp corresponding to the COOH terminus) were amplified by polymerase chain reaction using human cPLA2 cDNA plasmid as the template (8). The obtained cPLA2 cDNA fragments were cloned into pGBT9 vector in the correct orientation and with the correct reading frame to express the GAL4-cPLA2 fusion proteins. To obtain the cPLA2 cDNA fragments, two primer pairs were synthesized according to the cPLA2 cDNA sequence (8). The primer pair for the 540-bp cPLA2 cDNA fragment was composed of the following sequences: 5′-primer-GTCGACTCTTTAGATTTATGG-3′ (corresponding to the +540 to +511 of the coding region; containing the underlined SaI site and TG dinucleotides for in frame cloning); 3′-primer-GTCGACTCTTTAGATTTATGG-5′ (corresponding to the +540 to +511 of the coding region; containing the underlined SaI site and TG dinucleotides for in frame cloning); 3′-primer-GTCGACTCTTTAGATTTATGG-3′ (corresponding to the +2476 to +2518 of the coding region and containing the underlined SaI site). The cDNA was amplified via polymerase chain reaction utilizing Thermus aquaticus DNA polymerase. The amplified fragments were initially cloned into the pCR9TOPO vector using the TA cloning kit (Invitrogen). The inserts were then released from the pCR9TOPO vector by restriction enzyme digestion with SaI and cloned into the pGBT9 vector. The identity and orientation of the constructs were confirmed by DNA sequencing. The plasmid containing the 1707-bp cPLA2 cDNA fragment was designated as plasmid pGBT9-cPLA2. The plasmid containing the 1707-bp cPLA2 cDNA fragment corresponding to the COOH-terminal cPLA2 protein was designated as plasmid pGBT9-cPLA2C. These plasmids were used to transform host E. coli cells. The transformants containing the pGBT9-cPLA2N or pGBT9-cPLA2C were selected on tryptophan prototrophy and examined for beta-galactosidase activity using a filter assay. The beta-galactosidase activity was undetectable in colonies containing only pGBT9-cPLA2N or pGBT9-cPLA2C. The His7 cells containing pGBT9-cPLA2N or pGBT9-cPLA2C were then lysed for Western blotting analysis using the rabbit anti-cPLA2 antibody (Genetics Institute, Boston, MA). The identity of the expressed fusion proteins was confirmed by the observation that both the NH2-terminal cPLA2 fusion protein (approximately 40 kDa) and COOH-terminal cPLA2 fusion protein (approximately 90 kDa) reacted with the polyclonal anti-cPLA2 antibody.

Two Hybrid System Screening of Human cDNA Library—A human leukocyte MATCHMAKER cDNA library constructed in pGAD10 plasmid (CLONTECH) was screened to identify proteins interacting with cPLA2. The Saccharomyces cerevisiae HF7c reporter strain was used for transformation with the library and plasmid pGBT9-cPLA2N or plasmid pGBT9-cPLA2C by the lithium/acetate method (31). The HF7c transformants were plated on MM9 agar medium containing 50 μg/ml ampicillin, 150 μg/ml ampicillin, 40 μg/ml ampicillin, and 1 mg thiamine HCl. Individual colonies were isolated, and the plasmid DNA was extracted using a standard plasmid mini-prep procedure (33).

Sequence Analysis of pGAD10-cDNA Clones—Initial sequence of the cDNA inserts was obtained by dyeoxy sequencing using a 17-mer oligonucleotide primer (5′-TACACCTAAGGATG-3′), which corresponds to the GAL4 activation domain sequence and reads toward the junction of the GAL4 activation domain and the cloned candidate interacting protein. Specific oligonucleotide primers corresponding to the insert sequence were subsequently generated to obtain the full-length sequence of the inserts.

Expression of p11 Protein in Bacteria—To construct plasmid for p11 protein expression in bacteria, human p11 cDNA was amplified by reverse transcription polymerase chain reaction using human lung poly(A) RNA. The primer pair, synthesized according to the human p11 cDNA sequence (34), was composed of the following sequences: 5′-primer-GGTGTTGCATTCTGCACGTGATGTGCCTGTG-3′ (corresponding to 115–138 of the cDNA sequence; adjacent to the translation start site) and TG dinucleotides for in frame cloning); 3′-primer-GGTGTTGCATTCTGCACGTGATGTGCCTGTG-5′ (corresponding to 27 of the coding region and containing the underlined SaI site). The cDNA was amplified via polymerase chain reaction utilizing Thermus aquaticus DNA polymerase. The amplified fragments were initially cloned into the pCR9TOPO vector using the TA cloning kit (Invitrogen). The inserts were then released from the pCR9TOPO vector by restriction enzyme digestion with SaI and cloned into the pGBT9 vector. The identity and orientation of the constructs were confirmed by DNA sequencing. The pET30a-p11 vector expressed p11 protein as a fusion protein containing a stretch of 6 consecutive histidine residues (His-tag) at the NH2 terminus. The His-tag, removable by digestion with protease (e.g. enterokinase), was used for efficient purification of the recombinant p11 proteins under gentle, native conditions for maintaining activity of soluble 10-kDa fusion protein (31). The plasmid was transformed into E. coli strain BL21 (DE3), a low mutant strain containing the T7 polymerase under the control lacUV5 promoter. Addition of isopropyl-β-D-thiogalactopyranoside induces the expression of p11. The expressed p11 was purified using histidine-binding resin (Novagen) according to the manufacturer’s instruction. The His-tag-p11 was digested with enterokinase, and the released p11 without His-tag was collected and further purified by gel filtration using Bio-Gel P-30 (Fig. 1). The purified p11...
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was used for subsequent studies of the regulatory effects of p11 on cPLA2 enzyme activity.

In Vitro PLA2 Activity Assay Using Purified cPLA2 and p11—As the in vitro assayed cPLA2 activity is dependent on the composition of the lipids (36), we used the natural membrane vesicles prepared from the BEAS 2B cells (37). To maintain the natural distribution of AA in the membrane vesicles, the BEAS 2B cells grown in 175-cm² flasks were labeled with [3H]AA for 18 h to allow equilibration among phospholipids. Under these conditions, phosphatidylethanolamine accounts for the majority of esterified [3H]AA in phospholipids, the remainder being esterified in phosphatidylcholine, phosphatidylserine, and phosphatidylinositol (Sigma). The phospholipids were visualized by development with chloroform/methanol/acetone/water, 50:30:8:4, using standards of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Alltech, Dearfield, IL) developed with chloroform/methanol.

LipofectAMINE Reagent with 20 μg DNA/μl (200 μg, protein) of the obtained cytosolic protein was subjected to SDS-PAGE and Western blotting analysis using a 1:1000 dilution of mouse anti-p11 (Transduction Laboratories) and a 1:1000 dilution of mouse anti-p11 monoclonal antibody (protein A-agarose). Following washing of the Protein G/Protein A-agarose pellet with PBSTDS, 100 μl of loading buffer was added to the pellet, and the sample was boiled for 5 min before electrophoresis of 20 μl on 6% polyacrylamide gels (Novex) using Tris glycine/SDS buffer. The separated proteins were electrochemiluminescently transferred onto a nitrocellulose membrane, which was blocked with 5% nonfat milk overnight, and then probed with a 1:1000 dilution of mouse anti-p11 monoclonal antibody. The blots were then probed with a 1:1000 dilution of horse-radish peroxidase-labeled goat anti-mouse IgG and detected using the enhanced chemiluminescence Western Blotting Detection System.

Stable Transfection of Antisense p11 Plasmid in BEAS 2B Cells—To construct the antisense p11 expression vector, full-length human p11 cDNA was released from one of the three isolated plasmids encoding the fusion protein G4A activation domain/p11 protein and then cloned into the expression plasmid. The antisense plasmid was transfected into the selected cells. For the antisense vector, the luciferase gene and the SV40 origin of replication to allow efficient replication in cell lines that express SV40 large T antigen (e.g. BEAS 2B cells). The BEAS-2B cells grown in 175-cm² flasks were exposed to 250 μl of LipofectAMINE Reagent with 20 μg of p11AS-pCI-neo plasmid. Control cells were transfected with pCI-neo expression plasmid alone. Cells were exposed to the mixture of LipofectAMINE and plasmid for 2 h. Following removal of the transfection reagent, fresh medium was added and the incubation was continued for 24 h. The cells were then plated in medium containing 800 μg/ml geneticin (G418 sulfate). Colonies of resistant cells appeared after approximately 12 days, and the cells were subcultured at 16 days. Subsequent cultures of the selected cells were routinely grown in the presence of selective pressure.

To determine the protein levels of p11, cytoplasmic extracts were prepared as described above using BEAS 2B cells. 100 μl (200 μg, protein) of the cytosol was immunoprecipitated with 2.5 μg of mouse anti-p11 monoclonal antibody (transduction science) and Protein G/Protein A-agarose. Following washing of the Protein G/Protein A-agarose pellet with PBSTDS, 100 μl of loading buffer was added to the pellet, and the sample was boiled for 5 min before electrophoresis of 20 μl on 6% polyacrylamide gels using Tris glycine/SDS buffer. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane that was blocked with 5% nonfat milk overnight, and then probed with a 1:1000 dilution of rabbit anti-cPLA2 antibody (Genetics Institute) and then probed with a 1:1000 dilution of horse-radish peroxidase-labeled goat anti-rabbit IgG and detected using the Pierce SuperSignal detection system.

Binding of Purified p11 and cPLA2 in Vitro—The 58kDa-labeled p11 (carrying the His-tag at the NH2-terminal) was synthesized from the pET30a-p11 vector by the coupled in vitro transcription and translation using the Single Tube Protein Expression System 2 (Novagen). For each synthesis reaction, 0.5 μg of pET30a-p11 plasmid template was transcribed in 10 μl at 37 °C for 15 min, followed by the addition of 40 μl of translation mixture with [35S]methionine and continued incubation for 60 min. Products from individual synthesis reactions were pooled and stored in aliquots at −20 °C for future use.

To allow formation of the p11-cPLA2 complex, 5 μl of the synthesized [35S]p11 (3.4 × 10⁶ dpm) was incubated with 100 ng of purified human cPLA2 in 1 ml of HBSS (with calcium and magnesium) at 4 °C for 30 min. The cPLA2 was provided by Genetics Institute, Boston, MA, and is purified from bovine brain. Binding of p11 to cPLA2 complex (Genetics Institute) and 25 μl of Protein G Plus/Protein A-agarose (Pierce) were then added, and the mixture was incubated at 4 °C on a rotating device for 4 h, followed by centrifugation in a microcentrifuge at 2500 rpm for 5 min at 4 °C. The supernatant was aspirated, and the pellet was washed four times with 1.0 ml of PBSTDS (1 × phosphate-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) by centrifugation. Following four washings with PBSTDS, 20 μl of protein loading buffer was added to the pellet, and the sample was boiled for 10 min before electrophoresis on 18% polyacrylamide gels (Novex, San Diego, CA) using Tris glycine/SDS buffer. The gel was then dried under vacuum and exposed to the x-ray film.
tion Laboratories), rabbit anti-cPLA<sub>2</sub>, or mouse anti-annexin II (Transduction Laboratories) antibodies. The blots were then probed with a 1:1000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG (for detection of p11 and annexin II) or Protein A (for detection of cPLA<sub>2</sub>) and finally detected using the enhanced chemiluminescence Western Blotting Detection System.

**Immunoprecipitation of Native p11 Protein and Annexin II Heavy Chain from Transfected BEAS 2B Cells Using Anti-human cPLA<sub>2</sub> Antibody—**Crude cytosolic extracts were prepared, and immunoprecipitation with anti-cPLA2 antibody was performed as described above using BEAS 2B cells stably transfected with either the antisense p11 plasmid or the control pCI neo plasmid (vector). Following washing of the Protein G/Protein A-agarose pellet with PBSTDS, 100 µl of loading buffer was added to the pellet, and the sample was boiled for 5 min before electrophoresis on 18% polyacrylamide gels using Tris glycine/SDS buffer. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane that was blocked with 5% nonfat milk overnight and then probed with a 1:1000 dilution of either mouse anti-p11 monoclonal antibody or mouse anti-annexin II monoclonal antibody.

The blots were then probed with a 1:1000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG and detected using the Pierce Super Signal<sup>®</sup> detection system.

**PLA<sub>2</sub> Activity and Arachidonic Acid Release from Cells Transfected with Antisense p11 Plasmid—**Cellular PLA<sub>2</sub> activity was assayed as described previously (23–24). Equal numbers of the control cells transfected with pCI-neo vector alone or cells transfected with the plasmid pCI-neo-p11AS were grown on 175-cm<sup>2</sup> culture flasks coated with type I rat tail collagen. Each flask contained approximately 3 × 10<sup>6</sup> cells when culture reached confluence. The number of cells in each culture dish was monitored by measuring cellular DNA level using bis-benzimidazole (Hoechst 33258) (39).

For [3H]AA release, equal numbers of the control cells transfected with pCI-neo vector alone and the cells transfected with the antisense p11 plasmid pCI-neo-p11AS were grown in 175-cm<sup>2</sup> culture flasks and, when the cells were nearly confluent, labeled for 18 h with 1 µCi/ml [5,6,8,9,11,12,14,15-3H]arachidonic acid (214 Ci/mmole) in LHC-8 media. For studies of basal release the cells were then washed with culture media and fresh media replaced and collected after 4 hours of culture. The samples were extracted by Sep-Pak C<sub>18</sub> cartridges and chromatographed by reverse phase high pressure liquid chromatography (HPLC) as described previously (23–24). The arachidonic acid fraction of HPLC elution was collected and measured for radioactivity. For studies of [3H]AA release after calcium ionophore stimulation, equal numbers of the control cells transfected with pCI-neo vector alone and the cells transfected with the antisense p11 plasmid pCI-neo-p11AS were cultured on 60-mm culture dishes coated with type I rat tail collagen. Each dish contained approximately 4 × 10<sup>5</sup> cells when culture reached confluence. Experiments were performed when the cells were nearly confluent. The cells were labeled for 18 h with 1 µCi/ml [5,6,8,9,11,12,14,15-3H]arachidonic acid (214 Ci/mmole) in LHC-8 media. Following repeated washing with HBSS, 5 ml of HBSS with calcium ionophore A23187 (10 µg/ml) was added to each dish, and the cells were incubated at 37 °C for 15 min. The supernatants collected from four dishes were pooled as one sample for further analysis. The samples were extracted by Sep-Pak C<sub>18</sub> cartridges and chromatographed by reverse phase high pressure liquid chromatography (HPLC) as described above. The arachidonic acid fraction of HPLC elution was collected and measured for radioactivity.

**RESULTS**

**Two Hybrid Screen Indicated that p11 Binds to the C-terminal Region of cPLA<sub>2</sub>—**In an effort to identify proteins that interact with either the NH<sub>2</sub>-terminal regulatory Ca<sup>2+</sup>-binding domain or the COOH-terminal catalytic domain of the 85-kDa cPLA<sub>2</sub> protein, different regions of the cPLA<sub>2</sub> cDNA were cloned into pGBT9 vector to express the fusion proteins containing the common GAL4 DNA-binding domain and the NH<sub>2</sub>-terminal or COOH-terminal fragment of the cPLA<sub>2</sub> catalytic domain. Either the plasmid pGBT9-clpA2N (containing the 540-bp cPLA<sub>2</sub> cDNA fragment corresponding to the NH<sub>2</sub>-terminal cPLA<sub>2</sub> protein) or pGBT9-clpA2C (containing the 1707-bp cPLA<sub>2</sub> cDNA fragment corresponding to the COOH-terminal cPLA<sub>2</sub> protein) was used to transform the host HF7c cells. The HF7c strain containing pGBT9-clpA2N or pGBT9-clpA2C was used for subsequent transformation with DNA prepared from a human leukocyte MATCHMAKER cDNA library constructed in the pGAD10 plasmid. By this approach, four clones were identified to interact with the COOH-terminal region of cPLA<sub>2</sub> protein. However, no clone was found to interact with the NH<sub>2</sub>-terminal region of the cPLA<sub>2</sub>.

To verify the true positives, the isolated four plasmids were retransformed back into the original HF7c yeast host strain in the presence or absence of plasmid pGBT9 containing GAL4 DNA-binding domain alone, plasmid pGBT9-clpA2N, or plasmid pGBT9-clpA2C. All four positive clones exhibited positive results in the β-galactosidase activity assay only when cotransformed with the plasmid pGBT9-clpA2C. These plasmids were also cotransformed into another host S. cerevisiae strain SFY526. Similar results in the β-galactosidase activity assay were obtained in SFY526. In SFY526, the lacZ reporter gene is under the control of a promoter different from that used to control lacZ in HF7c. While these two promoters share only GAL4-responsive elements in common, the rest of the two promoter sequences differ significantly. Any positive two hybrid interaction observed in both HF7c and SFY526 reporter strains likely requires binding of the GAL4 DNA-binding domain specifically to the GAL4 responsive elements (40). Therefore, we concluded that the isolated four clones represent the true positive.

DNA sequences were obtained for inserts of the four clones identified in the genetic screen. Three of the clones were identical and contained the full-length cDNA sequence of human p11 (34), a member of the S100 protein family.

**p11 Inhibits cPLA<sub>2</sub> Enzyme Activity—**The findings from the two hybrid screen indicated that p11 binds to cPLA<sub>2</sub> protein. To study the possible regulatory effect of p11 on cPLA<sub>2</sub> enzyme activity, experiments were designed to examine the direct effect of p11 on cPLA<sub>2</sub> enzyme activity in vitro. The natural membrane substrate was incubated with 100 ng of purified human cPLA<sub>2</sub> with various concentrations of recombinant human p11. Cytochrome c was used as a control protein for the in vitro PLA<sub>2</sub> activity assays as described previously (41). As shown in Fig. 2, while cytochrome c exhibited no effect, p11 caused a dose-dependent inhibition of the cPLA<sub>2</sub> activity. These results demonstrate that p11 interacts with the cPLA<sub>2</sub>.
enzyme and inhibits the cPLA₂ activity. To test whether the inhibitory effect of p11 is related to substrate concentrations, the inhibition of cPLA₂ by p11 was evaluated under various amounts of substrate (2.5–20 μM phospholipid). As shown in Fig. 3A, p11 exhibited similar inhibitory effects under different substrate concentrations. These findings suggest that the inhibition of cPLA₂ by p11 is unlikely through an interaction between p11 and the phospholipid substrate.

As p11 is often associated with the 36-kDa annexin II to form annexin II heterotetramer in cells, we also examined whether the inhibitory effect of p11 on cPLA₂ activity may be altered by the presence of annexin II. The effect of annexin II alone or combined annexin II and p11 on cPLA₂ activity was studied under the same in vitro conditions. As shown in Fig. 3B, annexin II alone exhibited some inhibitory effect on cPLA₂ activity. This effect was not unexpected because annexin II might sequester substrate from its access to various PLA₂s under the in vitro conditions. While annexin II alone exhibited certain inhibitory effect on cPLA₂ activity, this effect was less than that of p11. The combination of annexin II and p11 appeared to exhibit additive inhibition. These results indicate that annexin II does not significantly alter the inhibitory effect of p11 on cPLA₂ activity.

Identification of p11-cPLA₂ Complex from a Human Bronchial Epithelial Cell Line (BEAS 2B Cell)—The above results demonstrated that p11 binds to cPLA₂ in vivo (based on two hybrid screen) and in vitro (based on the in vitro binding of synthesized p11 and purified cPLA₂). To further investigate the interaction between p11 and cPLA₂ in human cells, additional experiments were designed to detect the existence of p11-cPLA₂ complex in cells expressing both p11 and cPLA₂. Our previous studies revealed the existence of cPLA₂ in the BEAS 2B human bronchial epithelial cell line (23–24). Using immuno-blotting analysis, the expression of p11 was also found in the BEAS 2B cells (see below, Fig. 6). Therefore, we reasoned that the p11-cPLA₂ complex might be detected from the BEAS 2B cell line.
FIG. 5. Immunoprecipitation of p11-cPLA₂ complex from BEAS 2B cells. Different amounts of the isolated crude cytosolic protein (lanes 1, 2, and 4, 300 μg of crude cytosolic protein; lanes 5, 6, and 8, 75 μg of crude cytosolic protein; lanes 3 and 6, no crude cytosolic protein) were preincubated with 10 μl of rabbit anti-human cPLA₂ (lanes 3, 4, 7, and 8) or 10 μl of rabbit IgG (lanes 2 and 6) in 1 ml of HBSS at 4 °C for 30 min. Lanes 1 and 4 contained only the crude cytosolic protein without rabbit anti-human cPLA₂ or rabbit IgG. 25 μl of Protein G Plus Protein A-agarose beads was then added to each sample, and the sample was incubated at 4 °C on a rotating device for 4 h. The beads were collected and washed four times with 1.0 ml of PBS/0.5% Tween 20 by repeated centrifugation. Protein loading buffer was then added to the beads, and the samples were boiled for 10 min and subjected to SDS-PAGE. The precipitated p11 protein was then detected by Western blotting analysis using mouse anti-human p11 monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG as described under “Experimental Procedures.” The position of p11 protein is indicated.

Antisense Inhibition of p11 Increases PLA₂ Activity and Arachidonic Acid Release from the BEAS 2B Cells—We have shown that p11 binds to cPLA₂ and inhibits the cPLA₂ activity assayed in vitro. To study further the effect of p11 on PLA₂ activity and AA release in human cells, we used the antisense RNA approach to selectively inhibit the expression of p11 and then to examine the PLA₂ activity and AA release in the BEAS 2B cells. The expression of p11 protein was successfully reduced by the antisense inhibition (Fig. 6). The antisense inhibition of p11 expression did not alter cellular production of annexin II. In addition, the interaction of p11 and of annexin II heavy chain with cPLA₂ from vector control cells and from cells transfected with the p11 antisense vector was studied. Immunoprecipitation of cytosol preparations from vector control cells and from cells transfected with the antisense vector was performed using the polyclonal anti-cPLA₂ antibody. Western blotting was then performed, and the blots were probed with antibody to p11 or to annexin II heavy chain. While the antisense cells contained reduced p11 but comparable amounts of annexin II heavy chain, immunoprecipitation of cytosol with anti-cPLA₂ antibody demonstrated less p11 and less annexin II bound to cPLA₂ in the antisense cells than in vector control cells (Fig. 7). We then determined the PLA₂ activity and AA release from these permanently transfected cells. The in vitro assay of PLA₂ activity in cells transfected with pCI-neo-p11AS vector (91.7 ± 5.8 pmol/mg protein) was significantly higher than in cells transfected with pCI-neo vector alone (46.8 ± 3.4 pmol/mg protein) (p < 0.01, n = 3). The release of prelabeled AA from cells transfected with pCI-neo-p11AS was studied under two conditions. First, unstimulated or basal release of AA from both cell lines was studied over a period of 4 h after labeling with [3H]AA for 18 h. Without stimulation, cells permanently transfected with vector alone released 68 ± 8% of the arachidonate released from cells transfected with the p11-AS vector (n = 6, p < 0.01). Second, because cPLA₂ may be activated by increases in intracellular calcium concentrations, AA release after treatment with the calcium ionophore A23187 was studied. After treatment with A23187, the release of prelabeled AA from cells transfected with pCI-neo-p11AS was significantly higher than from cells transfected with pCI-neo vector alone (Fig. 8). These results indicate that p11 inhibits PLA₂ activity and thus decreases AA release in normal BEAS 2B cells. Decreased expression of p11 in the antisense cells reduces the inhibitory effect of cPLA₂ by p11, which results in an increase in PLA₂ activity and AA release.

FIG. 6. Protein expression of p11, annexin II, and cPLA₂ in BEAS 2B cells permanently transfected with antisense p11 expression vector. The crude cytosolic protein was isolated and subjected to SDS-PAGE and Western blotting analysis as described under “Experimental Procedures.” The protein levels of p11 (11 kDa), annexin II (36 kDa), and cPLA₂ (110 kDa) are shown from the left as follows: wild type BEAS 2B cells (WT), individual flasks of cells transfected with pCI-neo vector alone (lanes 1–3), or pCI-neo-p11AS containing the antisense p11 cDNA sequence (lanes 4–6).

FIG. 7. Western blot of p11 and annexin II. Crude cytosolic protein from cell lysates of BEAS 2B cells permanently transfected with antisense p11 expression vector or with vector alone was immunoprecipitated with rabbit polyclonal anti-cPLA₂ antibody and Protein G/Protein A-agarose. Following electrophoresis of the washed immunoprecipitates on 18% polyacrylamide gels using Tris glycine/SDS buffer, the separated proteins were electrophoretically transferred onto a nitrocellulose membrane that was blocked and then probed with a 1:1000 dilution of either mouse anti-p11 monoclonal antibody or mouse anti-annexin II monoclonal antibody. The blots were then probed with a 1:10000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG and detected using the Pierce Super Signal™ detection system.

DISCUSSION

This study reveals a novel mechanism for the regulation of 85-kDa cPLA₂. We demonstrate that p11, a unique member of the S100 family of calcium-binding proteins, interacts with the
carboxyl region of cPLA\(_2\) and inhibits the cPLA\(_2\) activity. The interaction between p11 and cPLA\(_2\) was recognized by screening a human cDNA library using a yeast two hybrid system. Further experiments demonstrated that p11 associated with cPLA\(_2\) in vitro. Using natural membrane vesicles as substrate, p11 was found to inhibit the activity of purified cPLA\(_2\) in vitro. The inhibition of cPLA\(_2\) activity by p11 was independent of substrate concentrations. These results demonstrated that p11 directly interacts with cPLA\(_2\) and inhibits the cPLA\(_2\) enzyme activity. Additional experiments were performed to study the role of p11 in intact human cells. The p11-cPLA\(_2\) complex was identified from the BEAS 2B human bronchial epithelial cell line expressing both cPLA\(_2\) and p11. Selective inhibition of p11 expression in the BEAS 2B cells by antisense RNA caused a reduction of cellular p11 and resulted in an increased PLA\(_2\) activity as well as an increased release of prelabeled AA. Taken together, the above in vitro and in vivo observations demonstrate a novel mechanism for the regulation of cPLA\(_2\) by the S100 protein family.

p11, a Unique Member of the S100 Family of Calcium-binding Proteins, Interacts with the COOH-terminal Region of cPLA\(_2\) — The S100 proteins are a group of low molecular mass (approximately 10–12 kDa) acidic Ca\(^{2+}\)-binding proteins, so named after the solubility of the first isolated protein in 100% saturated ammonium sulfate (42–44). The most striking conserved feature of these proteins is the presence of EF-hands (a term representing the two α-helices, E and F, and the intervening Ca\(^{2+}\)-binding loop), which function as the Ca\(^{2+}\)-binding sites. While other Ca\(^{2+}\)-binding proteins such as the parvalbumin protein family contain three EF-hands and the calmodulin protein family and troponin C protein family contain four EF-hands per monomer molecule, the S100 proteins have two EF-hands (NH\(_2\)-terminal and COOH-terminal EF-hands). The evident amino acid sequence homology, conservation of calcium-binding sites, and similarities of gene organization suggest that this group of proteins evolved from a common ancestral EF-hand gene (42–44). The biological functions of the S100 proteins are less clear. No specific enzymatic property has been ascribed to any of the proteins to date. The binding to calcium induces a conformational change in the S100 proteins, and this may then affect the secondary effector proteins. This mode of protein–protein interaction and modulation of the activity of the secondary effector protein is similar to that seen with calmodulin, another family of calcium-binding proteins containing the EF-hands (42–44). As the distribution of particular S100 proteins is dependent on specific cell types, the S100 proteins may be involved in transducing the signal of an increase in intracellular calcium in a cell type-specific fashion (42–44).

p11 was first isolated as a potential substrate for the epidermal growth factor-receptor tyrosine kinase (45). Although p11 shares considerable sequence homology with the S100 family, it does not bind calcium because of amino acid deletions and substitutions in the two EF-hand motifs (46). This is an exception to the general Ca\(^{2+}\)-binding properties displayed by other S100 proteins. Instead, p11 interacts with another protein, the 36-kDa annexin II (47–48). The p11 and annexin II proteins are linked to each other in a tight heterotetrameric complex that is present in many eukaryotic tissues and cells. p11 itself forms a tight dimer that bridges two annexin II molecules thus mediating the formation of a heterotetrameric annexin II-p11 complex. While annexin II exists as a monomer or binds to p11 forming the heterotetrameric complex, p11 has often been found as part of a complex with annexin II in many p11-positive tissues where annexin II is present in equimolar amounts or in excess (47–48). However, this phenomenon may vary with different cell types. The expression of p11 and annexin II is not always coordinated, and the ratio of p11 to annexin II varies with different cell types (49). In the F9 teratocarcinoma cells, there exists a significant amount of p11 but almost no annexin II both at the RNA and protein levels, with the unbound p11 distributed in the cytoplasm (50). Differentiation of these cells results in a large increase in annexin II and the formation of the annexin II-p11 heterotetramer (50). The formed heterotetrameric complex becomes associated with plasma membrane and submembranous cytoskeleton through the Ca\(^{2+}\)-dependent interaction of annexin II with the negatively charged phospholipids and components of the cytoskeleton. The interaction of annexin II with actin and membrane phospholipids is dependent upon Ca\(^{2+}\) and p11. p11 binding alters the Ca\(^{2+}\) and/or phospholipid-binding properties of annexin II, with the tetrameric complex having a more than 100-fold increased affinity toward Ca\(^{2+}\)/phospholipid compared with monomeric annexin II (51).

It remains unknown whether p11 is also able to associate with any other protein. Our results demonstrate that the 85-kDa cPLA\(_2\), a unique calcium-binding enzyme, is a novel protein that interacts with p11. Results from the two hybrid screen demonstrated that p11 associated with cPLA\(_2\) in vivo. While the exact amino acid residues involved in the interaction is unknown, only the COOH-terminal region of the cPLA\(_2\) is found to interact with the p11. The association of p11 and cPLA\(_2\) was also found under in vitro conditions. By using purified human cPLA\(_2\) protein and the in vitro translated human p11 protein, we found that p11 and cPLA\(_2\) interact with each other in vitro. Furthermore, the formation of p11-cPLA\(_2\) complex was also identified from cells expressing both p11 and cPLA\(_2\) by immunoprecipitation. Taken together, these results establish a direct interaction between p11 and the COOH region of cPLA\(_2\). In addition to annexin II, cPLA\(_2\) represents the only other protein that binds to p11.

p11 Inhibits cPLA\(_2\) Activity and Decreases the Release of Prelabelled AA From BEAS 2B Cells—To examine how the binding of p11 to cPLA\(_2\) may alter the catalytic activity of cPLA\(_2\), we studied the effects of recombinant p11 on the activity of purified human cPLA\(_2\) under in vitro assay conditions. Two aspects of the assays deserve comment. The first aspect is the use of

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**FIG. 8.** \[^{3}H\]AA release from BEAS 2B cells permanently transfected with antisense p11. The cells grown on 60-mm dishes were labeled for 18 h with 1 μCi/ml \[^{3}H\]AA in 5 ml of LHC-8 media. After repeated washing, the cells were incubated with 10\(^{-8}\) M ionophore A23187 in 5 ml of HBSS (containing 1.3 mM Ca\(^{2+}\)) for 15 min, and the supernatants were collected for future analysis. The supernatants collected from four dishes were pooled to generate each individual sample. The samples were extracted by Sep-Pak C\(_{18}\) cartridges and chromatographed by HPLC as described under “Experimental Procedures.” The peak shown in the figure corresponds to the retention time of AA (fraction 59). The chromatogram shown is a representative of three separate experiments.
membrane vesicles containing a mixture of phospholipids as cPLA2 substrate to mimic the in vivo interaction between cPLA2, p11, and membrane. This method was developed by Clark and colleagues (8) who used the U937 cell membrane vesicles as cPLA2 substrate. In this study, the membrane vesicles labeled with [3H]AA were prepared from the BEAS 2B cells. Another aspect of the assay is the relatively low Ca\(^{2+}\) concentration used in the assay. As the cPLA2 is activated by micromolar concentrations of Ca\(^{2+}\) and the physiological cellular Ca\(^{2+}\) level is normally at micromolar level as well (0.1 μM in normal resting cells and 0.3–1.0 μM in activated cells), we used the physiologically relevant Ca\(^{2+}\) concentration (0.5 μM free Ca\(^{2+}\) in the Ca\(^{2+}\)-EGTA buffer) for all the assays. Under these assay conditions, we found that p11 inhibited cPLA2 activity in a dose-dependent fashion (Fig. 2), and this inhibitory effect is not dependent on substrate concentration (Fig. 3A). These results demonstrate that p11 binds to cPLA2 and inhibits the cPLA2 enzyme activity in vitro. Although the mechanism for the inhibition of cPLA2 by p11 is not yet known, it is unlikely that this effect is due to the interaction between p11 and phospholipid substrate because 1) p11 does not bind to phospholipids (46–47) and 2) the p11-mediated cPLA2 inhibition is not dependent on substrate concentration. Instead, we demonstrate a direct interaction between p11 and cPLA2, and it is likely that the binding of p11 to cPLA2 results in the inhibition of cPLA2 activity. However, it remains to be determined how the binding of p11 to cPLA2 inhibits the cPLA2 activity. The possibilities may include blockage of the catalytic process of cPLA2, conformational change of cPLA2, or alteration of calcium-dependent association of cPLA2 with membrane substrate.

To study further the effect of p11 on PLA2 activity and AA release in human cells, we used the antisense RNA approach to selectively inhibit the expression of p11 and then to examine the PLA2 activity and AA release in the BEAS 2B cells. The release of prelabeled AA from cells with reduced p11 expression was significantly higher than from the control cells (Fig. 7). The crude cytosolic fraction isolated from the p11-reduced cells also showed an increased PLA2 activity. These results demonstrate that p11 inhibits PLA2 activity and decreases AA release in the BEAS 2B cells. Reduced p11 expression reverses the inhibitory effect of p11 on cPLA2 and thus increases the PLA2 activity and increases the release of [3H]AA from the [3H]AA-prelabeled cells. These results suggest that p11 decreases the release of AA for eicosanoid production in intact cells by inhibiting cellular cytosolic PLA2 activity.

p11-induced cPLA2 Inhibition Does Not Depend on Annexin II—It is well known that p11 binds to annexin II, a member of the annexin protein family, which includes at least 13 relatively abundant and structurally related proteins (47–48, 52–57). The annexins are widely distributed in eukaryotic cells. The roles of the annexins are not well understood, but they have been suggested to function in a broad range of physiological processes including inhibition of PLA2 (54). The biochemical hallmark of members of the annexin protein family is their Ca\(^{2+}\)-dependent binding to negatively charged phospholipids, most likely reflecting their association with cellular membranes in vivo. This common property is mediated by the conserved Ca\(^{2+}\)/phospholipid-binding sites that comprise four to eight repeats of 70–80 amino acid segments with a highly conserved 17-amino acid consensus sequence in each repeat. Typically, the annexins are monomeric proteins with the exception of annexin II which can exist as monomer or heterotetramer. The annexin II heterotetramer is composed of two subunits of 36-kDa annexin II and two subunits of p11 (47–48). The annexin II consists of two functional domains. The aminoterminal domain contains the first 30 amino acids and includes the binding site for p11 and both the serine and tyrosine phosphorylation sites. The first NH\(_2\)-terminal 14 amino acids of annexin II molecule bind to p11 forming an amphipathic α-helix upon complex formation. The serine and tyrosine residues can be phosphorylated by protein kinase C and the tyrosine kinase encoded by the src oncogene, respectively (45). The remaining carboxyl domain of annexin II contains four repeats of the conserved amino acid segments that bind to Ca\(^{2+}\)-phospholipids, and F-actin. Although p11 does not directly bind to calcium and phospholipids, annexin II has all Ca\(^{2+}\)- and lipid-binding properties typical of members of the annexin family.

For the treatment of inflammatory diseases, considerable research efforts have been made to identify selective inhibitors of PLA2. The annexins, formerly called lipocortins or calcapacins, were found to inhibit cellular PLA2 activity in various cell types (54, 58). In vitro studies using the 14-kDa PLA2 demonstrated that the PLA2 activity was inhibited by Annexin II, and this led to the hypothesis that the inhibition of 14-kDa PLA2 by annexins was the mechanism of the anti-inflammatory action for glucocorticoids (54, 58–61). However, subsequent studies failed to demonstrate any direct interaction between the 14-kDa PLA2 and annexins. Instead, it was found that the in vivo inhibition of 14-kDa PLA2 by annexins could be overcome by increasing the substrate concentrations (41, 62–64). The extent of inhibition was more closely related to the inhibitor:substrate ratio rather than the inhibitor:enzyme ratio, and the kinetics of inhibition were not compatible with a simple competitive or noncompetitive pattern. Based on these observations, the inhibitory action of annexins toward 14-kDa PLA2 was ascribed to substrate sequestration rather than to direct interaction with the 14-kDa PLA2.

We have demonstrated that p11 interacts with the COOH-terminal region of cPLA2 and inhibits the cPLA2 enzyme activity. As the inhibition of cPLA2 by p11 is independent of substrate concentration, this effect is likely due to a direct interaction between p11 and cPLA2. Because p11 is often associated with the 36-kDa annexin II to form annexin II heterotetramer in cells, we also examined whether the inhibitory effect of p11 on cPLA2 activity may be altered by the presence of annexin II. The effect of annexin II alone or combined annexin II and p11 on cPLA2 activity was studied under the in vitro conditions (Fig. 3B). We found that annexin II alone exhibited some inhibitory effect on cPLA2 activity. This effect was as expected because annexin II might sequester substrate from its access to various PLA2s under the in vitro assay conditions. While annexin II alone exhibited certain inhibitory effect on cPLA2 activity, this effect was less remarkable than the inhibition by p11. The combination of annexin II and p11 appeared to exhibit additive inhibition. These results indicated that annexin II does not significantly alter the inhibitory effect of p11 on cPLA2 activity. p11 itself is sufficient to bind and inhibit cPLA2.

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