Identification of the Calmodulin-binding Domain of Recombinant Calcium-independent Phospholipase A2β

IMPLICATIONS FOR STRUCTURE AND FUNCTION

Calcium-independent phospholipase A2 (iPLA2) is the major phospholipase A2 activity in many cell types, and at least one isoform of this enzyme class is physically and functionally coupled to calmodulin (CaM) in a reversible calcium-dependent fashion. To identify the domain in recombinant iPLA2β (riPLA2β) underlying this interaction, multiple techniques were employed. First, we identified calcium-activated CaM induced alterations in the kinetics of proteolytic fragment generation during limited trypsinolysis (i.e. CaM footprinting). Tryptic digests of riPLA2β (83 kDa) in the presence of EGTA alone, Ca<sup>2+</sup> alone, or EGTA and CaM together resulted in the production of a major 68-kDa protein whose kinetic rate of formation was specifically attenuated in incubations containing CaM and Ca<sup>2+</sup> together. Western blotting utilizing antibodies directed against either the N- or C-terminal regions of riPLA2β indicated the specific protection of riPLA2β by calcium-activated CaM at a cleavage site ~15 kDa from the C terminus. Moreover, calcium-activated calmodulin increased the kinetic rate of trypsin cleavage near the active site of riPLA2β. Second, functional characterization of products from these partial trypptic digests demonstrated that ~90% of the 68-kDa riPLA2β trypptic product (i.e. lacking the 15-kDa C-terminus) did not bind to a CaM affinity matrix in the presence of Ca<sup>2+</sup>, although >95% of the noncleaved riPLA2β as well as a 40-kDa C-terminal peptide bound tightly under these conditions. Third, when purified riPLA2β was subjected to exhaustive trypsinolysis followed by ternary complex CaM affinity chromatography, a unique trypptic peptide (684<sup>AWSEM-VG1QYFR705</sup>) within the 15-kDa C-terminal fragment was identified by RP-HPLC, which bound to CaM-agarose in the presence but not the absence of calcium ion. Fourth, fluorescence energy transfer experiments demonstrated that this peptide (684–705) bound to dansylcalmodulin in a calcium-dependent fashion. Collectively, these results identify multiple contact points in the 15-kDa C terminus as being the major but not necessarily the only binding site responsible for the calcium-dependent regulation of iPLA2β by CaM.

The phospholipase A<sub>2</sub>-catalyzed release of arachidonic acid from its phospholipid storage depots is a critical component of intra- and intercellular signal transduction. In most noncircularizing cells (e.g. cardiac myocytes, pancreatic islet β-cells, hippocampal neurons, and vascular smooth muscle cells), calcium-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>)<sup>1</sup> is the major but not the only phospholipase A<sub>2</sub> activity present (1–6). Multiple lines of experimental evidence implicate iPLA2 as an important mediator of arachidonic acid release in several cell types including: 1) the inhibition of the large majority of AVP-induced arachidonic acid release in A-10 smooth muscle cells by 1–2 μM BEL (7); 2) the attenuation of the release of arachidonic acid in lipopolysaccharide-stimulated macrophages by either BEL or antisense DNA targeted to iPLA2 (8, 9); 3) the robust release of ligand-stimulated arachidonic acid in cells whose rise in cytosolic calcium is ablated by EGTA and 1,2-bis(o-amino phosphon)ethane-N,N',N''-N''-tetracetic acid (tetracetoxyethyl)ester (i.e. the release of arachidonic acid occurs in the absence of Fura 2-detectable changes in intracellular Ca<sup>2+</sup> levels; Refs. 10 and 11); 4) the release of arachidonic acid by agents that deplete intracellular Ca<sup>2+</sup> stores in the absence of receptor occupancy (e.g. thapsigargin, cyclosporin A, 2, 5-di(t-butyl)-1,4-hydroquinone, and A23187) when alterations in [Ca<sup>2+</sup>]<sup>i</sup> are prevented (10, 11); and 5) the ionophore-induced release of arachidonic acid from HEK cells expressing riPLA2β (but not in wild type HEK cells) with its subsequent directed conversion to prostaglandin E<sub>2</sub> by cyclooxygenase I but not cyclooxygenase II (12).

To gain insight into the biochemical mechanisms responsible for the activation of iPLA2 in stimulated cells, an early observation was re-explored that demonstrated that the addition of calcium ion to myocardial cytosol inhibited iPLA2 activity (1). Subsequently, this inhibition was shown to be due to a protein factor that was identified as calmodulin after purification to homogeneity (13). These results were confirmed by reconstitution of calcium-dependent iPLA2 inhibition utilizing authentic calmodulin (13). Through detailed analysis of the interaction of myocardial iPLA2 with CaM, we demonstrated the formation of a catalytically inactive ternary complex of CaM-Ca<sup>2+</sup>-iPLA2 that could be reversibly dissociated by chelation of calcium ion with EGTA to regain full catalytic activity. Subsequent work demonstrated that agents that deplete intracellular calcium

<sup>1</sup> The abbreviations used are: iPLA<sub>2</sub>, calcium-independent phospholipase A<sub>2</sub>; iPLA2β, calcium-independent phospholipase A2β; riPLA2β, recombinant calcium-independent phospholipase A2β; BEL, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; CaM, calmodulin; DTT, dithiothreitol; RP-HPLC, reverse-phase high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyle.
Calmodulin-binding Domain of iPLA$_2$$\beta$

ion pools ($e.g.$, thapsigargin and cyclopiazonic acid), even in the absence of receptor occupancy or alterations in cytosolic calcium ion concentration, resulted in arachidonic acid release in intact cells (10). Furthermore, agents that inhibited the interaction of calmodulin with its target proteins through prevention of calcium-induced conformational changes in calmodulin ($e.g.$, W-7) resulted in the release of $[^3]$H]arachidonic acid in resting A-10 smooth muscle cells (10). These observations indicate that the majority of iPLA$_2$ activity in cells is tonically inhibited and that dissociation of calmodulin from iPLA$_2$ is the major mechanism that transforms a calcium-independent catalytic activity into an enzyme that is responsive to alterations in intracellular calcium ion homeostasis. Collectively, these results gave rise to the hypothesis of "calcium pool depletion-mediated iPLA$_2$ activation" (10). To further define the physiologic relevance of this hypothesis, it became important, from both a mechanistic perspective as well as a therapeutic strategy, to determine the site(s) of interaction of iPLA$_2$$\beta$ with calmodulin.

Since the first x-ray crystal structure (14) and NMR solution structure (15) of CaM-peptide complexes became available, it was apparent that calcium-induced conformationally activated calmodulin interacted with its regulatory targets at multiple contact points. No universal consensus sequences mediating the interaction of calmodulin with its extremely diverse protein targets have been identified, although the importance of positionally conserved hydrophobic and basic amino acid residues has been demonstrated in some cases (16–20). Accordingly, unambiguous identification of the loci of interaction of CaM with iPLA$_2$$\beta$ cannot be recognized a priori from comparisons of the iPLA$_2$$\beta$ primary sequence with other known calmodulin-regulated target proteins. To identify the site of interaction of calcium-activated calmodulin with iPLA$_2$$\beta$, analysis of direct protein-protein interactions (protein footprinting), functional interactions (ternary complex affinity chromatography), and biophysical interactions (fluorescence resonance energy transfer) were pursued. Herein, we demonstrate that incubation of iPLA$_2$$\beta$ with trypsin in the presence of calmodulin and calcium ion protects against tryptic cleavage at a C-terminal site (at or near residue 630) and increases hydrolytic cleavage near the active site of riPLA$_2$$\beta$. Moreover, complete tryptic digestion of riPLA$_2$$\beta$ resulted in a peptide within this 15-kDa C-terminal region (AWSEMVQIQYFR corresponding to residues 694–705) that binds to CaM in a calcium-dependent manner. Collectively, these results identify the 15-kDa C-terminal region of riPLA$_2$$\beta$ as necessary and sufficient for the calcium-induced binding of calmodulin to riPLA$_2$$\beta$ and conformational alteration of riPLA$_2$$\beta$ near the active site.

**EXPERIMENTAL PROCEDURES**

**Materials**—The peptide corresponding to residues 23–42 (CRVKIESVADYTSHERVKEG) near the N terminus of iPLA$_2$$\beta$ (GenBank accession number II5470) was synthesized by Alpha Diagnostic International (San Antonio, TX). The peptide corresponding to residues 731–744 (CTEYHHEKIQYFR) near the C terminus of iPLA$_2$$\beta$ was synthesized by the Protein Chemistry Laboratory at Washington University. Both peptides contain N-terminal cysteine residues for covalent linkage to maleimide-activated keyhole limpet hemocyanin as a carrier protein for generating antibodies as well as to activated thiol-Sepharose 4B for subsequent affinity purification of the antibodies. The synthetic 12-amino acid peptide (AWSEMVQIQYFR) corresponding to amino acid residues 694–705 of the iPLA$_2$$\beta$ sequence was synthesized by Research Genetics. Sequencing grade modified trypsin used for trypsinization of iPLA$_2$$\beta$ was purchased from Fromega. Kaledoscope pretested SDS-PAGE protein standards were obtained from Bio-Rad. The $\mu$RC2/C18 SC 2.1/10 column, CNBr-activated Sepharose resin, and ECL reagents were purchased from Amersham Pharmacia Biotech. $\alpha$-l-1-Palmityl-2-[1-3$^{14}$C]arachidonyl-phosphatidylcholine, used for measurements of riPLA$_2$$\beta$ activity, was purchased from PerkinElmer Life Sciences. High purity bovine brain calmodulin was purchased from Calbiochem. Calmodulin-agarose and most other reagents were obtained from Sigma.

**Generation and Affinity Purification of Anti-iPLA$_2$$\beta$ Antibodies—**Recombinant calcium-independent phospholipase A$_2$ was purified to homogeneity from an S9 cell expression system by sequential affinity chromatographic steps with CNBr-activated Sepharose 4B and heparin affinity resins as described previously (21, 22). Affinity purified polyclonal antibodies directed against riPLA$_2$$\beta$ were prepared by initial injection of 50 $\mu$g of purified riPLA$_2$$\beta$ in 0.5 ml of Freund’s complete adjuvant and boosted every 2 weeks with 50 $\mu$g of riPLA$_2$$\beta$ in Freund’s incomplete adjuvant until seroconversion was detected by immunoblotting. Purified antibodies were eluted by sequential application of solutions containing either 4.9 M MgCl$_2$ or 150 mM glycine, pH 2.5. After elution, the antibody was dialyzed against ammonium bicarbonate buffer prior to lyophilization for storage at $-$20 °C.

Antibodies directed against the peptides corresponding to residues 23–42 (N-terminal) and residues 731–744 (C-terminal) regions of iPLA$_2$$\beta$ were generated by immunizing rabbits with conjugates of the peptides with keyhole limpet hemocyanin. Brieﬂy, maleimide-activated keyhole limpet hemocyanin was reacted with the peptides (2 mg) according to the manufacturer’s instructions and dialyzed against 83 mM sodium phosphate, pH 7.2, containing 0.9 M NaCl. Rabbits were immunized/boosted with the conjugates using Freund’s complete/incomplete adjuvants as described above. For affinity purification of the antibodies, each peptide (2 mg) was covalently linked to activated thiol-Sepharose 4B (1 ml) in the presence of an equal volume of 0.1 × sodium citrate buffer, pH 4.5. Following an overnight incubation with mixing at room temperature, the extent of the reaction was monitored spectrophotometrically by the displacement of the 2-pyridyl groups as $N$-tosyl-$L$-phenylalanine chloromethyl ketone-modified trypsin (1:30 w/w trypsin:iPLA$_2$$\beta$) according to the manufacturer’s instructions and dialyzed against 83 mM sodium phosphate, pH 7.2, prior to application to the appropriate affinity resin equilibrated with the same buffer. The resins were extensively washed with 10 column volumes of 10 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl prior to elution of bound antibodies with 0.1 M glycine, pH 2.5, into collection tubes containing 1 × Tris-HCl, pH 9.0 (1:10 fraction volume), for investigation. Antibodies were dialyzed against phosphate-buffered saline and stored at 4 °C prior to use.

**Partial Trypsinolysis of Purified riPLA$_2$$\beta$—**Purified recombinant iPLA$_2$$\beta$ (9 $\mu$g) in 25 mM imidazole, pH 8.0, containing 100 mM NaCl and 2 mM DTT was partially digested with $N$-tosyl-$L$-phenylalanine chloromethyl ketone-modified trypsin (1:30 w/w trypsin:iPLA$_2$$\beta$) in the presence (1 mM CaCl$_2$) or absence (1 mM EGTA) of calcium ion and/or CaM (9 $\mu$g, 5-fold molar excess of CaM:iPLA$_2$$\beta$) at room temperature. Each reaction was terminated at the indicated time intervals (i.e., 1, 3, and 5 min) by SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue, and 100 mM DTT). The proteolyzed iPLA$_2$$\beta$ products were subsequently separated by SDS-PAGE on 10% polyacrylamide gels according to the method of Laemmli (23), transferred to polyvinylidene difluoride membranes by electroelution in 100 mM CAPS buffer, pH 11, and analyzed by ECL Western blotting with polyclonal antibodies directed against the iPLA$_2$$\beta$ holoprotein, the N-terminal region of iPLA$_2$$\beta$, or the C-terminal region of iPLA$_2$$\beta$ in conjunction with an anti-rabbit IgG horse-radish peroxidase conjugate.

**CaM-Agarose Affinity Chromatography of Partially Trypsinized iPLA$_2$$\beta$—**Purified recombinant iPLA$_2$$\beta$ (50 $\mu$g) was partially digested with trypsin (1:30 w/w trypsin:iPLA$_2$$\beta$) for 4–20 min at room temperature in 25 mM imidazole buffer, pH 8.0, containing 0.1 M EGTA, 1 mM DTT, and 50–150 mM NaCl. The reaction was terminated by addition of 1 mM 4-[2-aminoethyl]benzenesulfonfylfluoride. Calcium chloride was added to a final concentration of 5 mM, and the sample was applied to a 25-ml CaM-agarose column equilibrated with 25 mM imidazole, pH 8.0, containing 5 mM CaCl$_2$, 150 mM DTT, and 50 mM NaCl. The column was washed with equilibration buffer containing 10-fold less (0.5 mM) CaCl$_2$, followed by elution of bound protein buffer (without CaCl$_2$) containing 10 mM EGTA. Column fractions were analyzed by Western analysis as described above using polyclonal antibodies directed against the iPLA$_2$$\beta$ holoprotein.

**Tryptic Digestion, Isolation, and Characterization of the CaM-binding Peptide from riPLA$_2$$\beta$—**A 25-$\mu$g sample of highly purified riPLA$_2$$\beta$ was exhaustively digested with 1 $\mu$g of $N$-tosyl-$L$-phenylalanine chloro-
romethyl ketone-modified trypsin in 1.5 ml of 25 mM imidazole buffer, pH 8.0, containing 1 mM EGTA for 18 h at 37 °C. The resultant proteolytic fragments were adjusted to a final calcium ion concentration of 5 mM and loaded onto a 0.5-ml column of CaM-agarose. The column was washed with 5 column volumes of a buffer containing 25 mM imidazole, pH 8.0, and 0.5 mM CaCl2 prior to the elution of bound peptides with a buffer containing 25 mM imidazole, pH 8.0, and 4 mM EGTA. Aliquots of the tryptic peptides (50 μl) in the CaM-agarose load and void fractions were directly analyzed by RP-HPLC using a μRPC C2/C18 SC 2.1/10 Smart System column with UV absorbance detection at 215 nm. The peptides from the EGTA eluent of the CaM-agarose column (2.0 ml) were concentrated by lyophilization, resuspended in 100 μl of deionized H2O containing 0.07% trifluoroacetic acid (buffer A), and loaded onto a μRPC C2/C18 SC 2.1/10 RP-HPLC column. Peptides were resolved utilizing a discontinuous linear gradient from 0 to 38% buffer B (80% acetonitrile and 0.06% trifluoroacetic acid) for 60 min, 38 to 75% buffer B for 30 min, and 75 to 100% buffer B for 15 min. The affinity purified peptide from the RP-HPLC purification of the calmodulin-agarose eluent (denoted in Fig. 4B) was collected, lyophilized, and sequenced by automated Edman degradation utilizing an Applied Biosystems Procise Sequencer. RP-HPLC of the synthetic 12-amino acid peptide corresponding to the obtained sequence (vida infra) was conducted employing the chromatographic conditions described above.

**Fluorescence Spectrometry of Dansyl-Calmodulin—**Experiments employing dansyl-calmodulin were conducted utilizing an Aminco SLM 4800C fluorescence spectrometer (SLM Instruments, Inc.) using established methods (24–27). Briefly, the indicated amounts of synthetic peptide were added to a 3-ml cuvette containing 2 ml of dansyl-Calmodulin (1 μg/ml) in the presence of 20 mM HEPES, pH 7.2, 130 mM KCl, and 500 μM CaCl2 or 1 mM EGTA. Emission spectra were recorded from 400 to 550 nm utilizing an excitation wavelength of 340 nm.

**Deletional Mutagenesis of iPLA2β—**Mutants of iPLA2β were prepared by polymerase chain reaction-directed mutagenesis techniques as previously described (28). Three truncated mutants of iPLA2β containing amino acid residues 1–381, 1–600, and 1–690 were prepared. Briefly, antisense primers containing a stop codon after amino acids 381, 600, and 690 followed by an SphI restriction site were prepared for use in polymerase chain reaction with a sense primer containing an EcoRI restriction site prior to the ATG start site of the iPLA2β coding sequence. EcoRI/SphI-digested polymerase chain reaction products were cloned into similarly digested pFAST vectors for expression of recombinant protein in the baculovirus expression system as previously described (22). All constructs were sequenced on both strands to ensure fidelity of the sequence within the constructs prior to use in the baculovirus expression system.

**RESULTS**

Previous work has demonstrated that riPLA2β reversibly binds to calmodulin through a ternary complex comprised of calcium, calmodulin, and unidentified domains of riPLA2β (22). Accordingly, we sought to identify the specific regions of riPLA2β involved in calmodulin binding and modulation of iPLA2β enzymic activity. Historically, one approach through which protein-protein contact points have been identified is through binding of the regulatory protein to the target protein and protection of the target protein from proteolysis at the binding site (i.e. protein footprinting). Accordingly, we analyzed the kinetics of the production of individual proteolytic fragments formed during timed incubations of trypsin with riPLA2β, calcium, and calmodulin. Incubation of trypsin with riPLA2β produced six major proteolytic fragments (A-F) within 9 min under the conditions employed (Fig. 1). Similar kinetic analysis of tryptic digests of riPLA2β in the presence of EGTA alone, Ca2+ alone, or CaM and EGTA together produced similar amounts and ratios of proteolysis products. Remarkably, tryptic digests of riPLA2β in the presence of CaM and calcium together resulted in the nearly complete disappearance of the major proteolytic product at 68 kDa and the increased intensity of the band at 40 kDa (Fig. 1, band E). These results demonstrate that calcium-activated CaM specifically protected riPLA2β from trypsinolysis at a single cleavage site within ~15 kDa of either its N or C terminus. Moreover, they suggest that a site near the center of riPLA2β (i.e. near the active site) undergoes a conformational alteration in the presence of calcium and calmodulin together but not with either alone. To determine the locus of the protected site, immunoaffinity purified polyclonal antibodies directed against either the N- or C-terminal regions of iPLA2β were utilized to discriminate between the proteolytic products of riPLA2β (and undigested riPLA2β) were resolved by electrophoresis on 10% polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. Immune reactive bands were detected by ECL utilizing immunoaffinity-purified polyclonal antibodies directed against the riPLA2β holoprotein and an anti-rabbit IgG-horseradish peroxidase conjugate as described under “Experimental Procedures.” The first lane is riPLA2β alone incubated in buffer containing EGTA for 9 min at 22 °C in the absence of calcium (–T). Similar profiles were obtained in three independent preparations. Molecular masses of marker proteins are shown on the right in kilodaltons.

**Fig. 1. Time course analysis of the effect of Ca2+-CaM on limited trypsinolysis of riPLA2β.** Purified recombinant iPLA2β (~9 μg) was incubated at 22 °C with trypsin in 25 mM imidazole buffer (pH 8.0, containing 100 mM NaCl and 1 mM DTT) containing either Ca2+ (1 mM CaCl2), EGTA (1 mM), EGTA (1 mM) and CaM (~9 μg), or Ca2+ (1 mM CaCl2) and CaM (~9 μg). Aliquots from each reaction were taken at the indicated times, and proteolysis was terminated by boiling each aliquot in SDS-PAGE loading buffer for 3 min. Proteolytic products of riPLA2β (and undigested riPLA2β) were resolved by electrophoresis on 10% polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. Immune reactive bands were detected by ECL utilizing immunoaffinity-purified polyclonal antibodies directed against the riPLA2β holoprotein and an anti-rabbit IgG-horseradish peroxidase conjugate as described under “Experimental Procedures.” The first lane is riPLA2β alone incubated in buffer containing EGTA for 9 min at 22 °C in the absence of calcium (–T). Similar profiles were obtained in three independent preparations. Molecular masses of marker proteins are shown on the right in kilodaltons.

Previous work has demonstrated that riPLA2β reversibly binds to calmodulin through a ternary complex comprised of calcium, calmodulin, and unidentified domains of riPLA2β (22). Accordingly, we sought to identify the specific regions of riPLA2β involved in calmodulin binding and modulation of iPLA2β enzymic activity. Historically, one approach through which protein-protein contact points have been identified is through binding of the regulatory protein to the target protein and protection of the target protein from proteolysis at the binding site (i.e. protein footprinting). Accordingly, we analyzed the kinetics of the production of individual proteolytic fragments formed during timed incubations of trypsin with riPLA2β, calcium, and calmodulin. Incubation of trypsin with riPLA2β produced six major proteolytic fragments (A-F) within 9 min under the conditions employed (Fig. 1). Similar kinetic analysis of tryptic digests of riPLA2β in the presence of EGTA alone, Ca2+ alone, or CaM and EGTA together produced similar amounts and ratios of proteolysis products. Remarkably, tryptic digests of riPLA2β in the presence of CaM and calcium together resulted in the nearly complete disappearance of the major proteolytic product at 68 kDa and the increased intensity of the band at 40 kDa (Fig. 1, band E). These results demonstrate that calcium-activated CaM specifically protected riPLA2β from trypsinolysis at a single cleavage site within ~15 kDa of either its N or C terminus. Moreover, they suggest that a site near the center of riPLA2β (i.e. near the active site)
proteolytic fragments from the 4-min digestion reaction demonstrated that the overwhelming majority (−90%) of the 68-kDa polypeptide did not bind to the affinity matrix, whereas >95% of the holoenzyme (83 kDa) avidly bound to the CaM column (Fig. 3A). Interestingly, the 40-kDa fragment (band E in Figs. 1 and 2B) corresponding to the C-terminal half of riPLA₂β containing the active site appeared to bind as tightly to the Ca²⁺-CaM resin as did the 83-kDa holoprotein (Fig. 3A). The small residual amount of binding of the 68-kDa fragment could be due to the presence of riPLA₂β heteromultimers, which still possess at least one intact C-terminal portion of riPLA₂β (either as an intact 83-kDa component or as a noncovalently associated C-terminal fragment). To further address this issue, riPLA₂β was trypsinized for 20 min to eliminate (as much as possible) the 83-kDa holoprotein and then subjected to CaM-agarose chromatography as described above. Under these conditions, virtually all of the 68-kDa fragment failed to bind to the column (Fig. 3B, upper panel). In addition, N-terminal fragments D and F were also not retained by CaM-agarose in the presence of calcium ion (Fig. 3B, upper panel), although untrypsinized riPLA₂β bound tightly to calmodulin-agarose in control experiments (Fig. 3B, lower panel). Notably, the 68-kDa fragment retained the ability to bind to ATP-agarose, demonstrating that its folding and structural integrity had not been significantly compromised during limited trypsinolysis (data not shown). We specifically point out that the present results do not exclude the possibility that some portions of the 68-kDa N-terminal polypeptide do contact calmodulin. However, the results do demonstrate that the C-terminal portion is both necessary (i.e. >90% of the 68-kDa peptide was not bound by calmodulin) and sufficient (i.e. the 40-kDa C-terminal peptide bound in the presence of calcium ion and was released by EGTA) for binding of riPLA₂β to calmodulin in a calcium-dependent manner. Collectively, these results demonstrate that the major domain responsible for riPLA₂β binding to calcium-activated calmodulin is located between residues 620 and 752.

To identify specific portions of the 15-kDa C-terminal fragment (or potentially other regions) that interacted with CaM in a calcium-dependent manner, riPLA₂β was exhaustively trypsinized. SDS-PAGE of these samples demonstrated the completeness of trypsinolysis (data not shown). We specifically point out that the present results do not demonstrate that the overwhelming majority (−90%) of the 68-kDa polypeptide did not bind to the affinity matrix, whereas >95% of the holoenzyme (83 kDa) avidly bound to the CaM column (Fig. 3A). Interestingly, the 40-kDa fragment (band E in Figs. 1 and 2B) corresponding to the C-terminal half of riPLA₂β containing the active site appeared to bind as tightly to the Ca²⁺-CaM resin as did the 83-kDa holoprotein (Fig. 3A). The small residual amount of binding of the 68-kDa fragment could be due to the presence of riPLA₂β heteromultimers, which still possess at least one intact C-terminal portion of riPLA₂β (either as an intact 83-kDa component or as a noncovalently associated C-terminal fragment). To further address this issue, riPLA₂β was trypsinized for 20 min to eliminate (as much as possible) the 83-kDa holoprotein and then subjected to CaM-agarose chromatography as described above. Under these conditions, virtually all of the 68-kDa fragment failed to bind to the column (Fig. 3B, upper panel). In addition, N-terminal fragments D and F were also not retained by CaM-agarose in the presence of calcium ion (Fig. 3B, upper panel), although untrypsinized riPLA₂β bound tightly to calmodulin-agarose in control experiments (Fig. 3B, lower panel). Notably, the 68-kDa fragment retained the ability to bind to ATP-agarose, demonstrating that its folding and structural integrity had not been significantly compromised during limited trypsinolysis (data not shown). We specifically point out that the present results do not exclude the possibility that some portions of the 68-kDa N-terminal polypeptide do contact calmodulin. However, the results do demonstrate that the C-terminal portion is both necessary (i.e. >90% of the 68-kDa peptide was not bound by calmodulin) and sufficient (i.e. the 40-kDa C-terminal peptide bound in the presence of calcium ion and was released by EGTA) for binding of riPLA₂β to calmodulin in a calcium-dependent manner. Collectively, these results demonstrate that the major domain responsible for riPLA₂β binding to calcium-activated calmodulin is located between residues 620 and 752.

To identify specific portions of the 15-kDa C-terminal fragment (or potentially other regions) that interacted with CaM in a calcium-dependent manner, riPLA₂β was exhaustively trypsinized. SDS-PAGE of these samples demonstrated the completeness of trypsinolysis (Fig. 4A). RP-HPLC of the exhaustively digested riPLA₂β revealed the presence of ~44 peptide peaks (Fig. 4B, Load). When riPLA₂β peptides generated by exhaustive trypsinolysis were applied to CaM-agarose in the
presence of calcium ion, a single peptide (retention time = 62 min) was absent in the void volume (Fig. 4B). Moreover, when peptides that bound to the CaM matrix were subsequently eluted with buffer containing EGTA, the same unique peptide (retention time = 62 min) was selectively concentrated. Of the ~44 peaks in the load and void fractions that did not interact with CaM (i.e. they eluted in the void volume), the peak at 62 min was the only peak enriched in the EGTA elute (Fig. 4B).

The amino acid sequence of the unique calmodulin binding peptide was determined by automated Edman degradation and identified as the 12-amino acid fragment AWSEMVGHQYFR, which is within the previously identified 15-kDa C-terminal fragment from calmodulin footprinting experiments (Figs. 1 and 2). This peptide corresponds to tryptic cleavage between residues Arg<sup>693</sup> and Arg<sup>705</sup>.

To determine whether this peptide interacted with calmodulin in a calcium-dependent fashion, fluorescence resonance energy transfer experiments were performed. First, a peptide with this 12-amino acid sequence was synthesized and demonstrated to possess an elution profile by RP-HPLC similar to that of the peptide resulting from exhaustive trypsinolysis of riPLA<sub>2</sub>β (Fig. 5). Next, the 12-amino acid synthetic peptide was incubated with dansyl-CaM and shown to interact in a calcium-dependent manner as demonstrated by fluorescence energy transfer (Fig. 6). The increase in fluorescence and blue shift of the emission maxima of dansyl-CaM, which occurs only in the presence of calcium ion and the peptide, is indicative of their close association, as has been observed with other calcium-dependent peptide-mediated shifts in the fluorescence intensity and emission wavelength. The fluorescence intensity is measured in arbitrary units.

we considered the possibility that strict conservation of the 15-kDa C-terminal region was necessary for appropriate folding and retention of protein conformation to resist proteolysis of the incompletely folded protein. One way to gain insight into the importance of specific regions of primary sequence in protein structure and function is to identify highly conserved areas of amino acid homology across species lines. Absolute complexity alignment analysis (29) demonstrated that the CaM-binding domain was the longest conserved region in the protein over four known species lines (Fig. 7). Thus, this region may be necessary for the proper folding and tertiary structure of iPLA<sub>2</sub>β, and its absence may make the protein more susceptible to Sf9 cell degradative proteases.

**DISCUSSION**

Intracellular phospholipases A<sub>2</sub> are comprised of two distinct families of proteins that catalyze the serine-mediated nucleophilic attack of ester linkages in cellular phospholipids resulting in the release of arachidonic acid from its endogenous phospholipid storage depots. One family, the cytosolic phospholipase A<sub>2</sub> family, of enzymes possesses a GXSGGS sequence,
whereas the other family, the iPLA₂ family, possesses a GXSTG sequence at their active site. The iPLAβ family originated as an evolutionary distant archetype (i.e., iPLAα) (30) that subsequently developed specialized domains to fulfill specific intracellular functions. For example, iPLAβ contains eight N-terminal ankyrin repeat sequences (31) and is modulated by calmodulin (13), whereas iPLAγ contains a C-terminal peroxisomal localization sequence (32). The present study demonstrates that the calmodulin-binding domain of iPLAβ is comprised of multiple contact points in the 15-kDa C-terminal region. Calmodulin footprinting of riPLAβ with trypsin dramatically diminished the rate of generation of the 68-kDa riPLAβ proteolysis product (which lacks the C-terminal 15-kDa polypeptide) in the presence of calcium and CaM together but not with either alone. This strongly suggests that calcium-activated CaM directly binds to this region of iPLAβ, protecting it from proteolysis. Moreover, the large majority (~90%) of the 68-kDa trypsinolysis product (lacking the 15-kDa C-terminus) failed to bind CaM in the presence of CaCl₂ (Fig. 3). Importantly, the calmodulin binding peptide identified after exhaustive trypsinolysis is also present within this 15-kDa fragment and directly interacts with CaM as demonstrated by fluorescent energy transfer experiments. Collectively, these results identify the calmodulin-binding domain in iPLAβ as the 15-kDa C-terminal portion through both direct physical protein-protein interactions and the calcium-dependent functional association of this region with calmodulin.

The molecular mechanisms underlying the regulation of intracellular phospholipases A₂ have been an area of intense investigation. Alterations in cellular calcium ion flux are an integral part of signal transduction processes in most cell types. In the case of cytosolic phospholipase A₂ α, an internal C2 domain binds calcium and facilitates its translocation to specific membrane compartments after cellular activation (33–35). In prior studies, we have demonstrated that recombinant iPLAβ reversibly binds to CaM in the presence of calcium ion and calcium-activated calmodulin modulates iPLAβ enzymic activity (13). Calcium-dependent binding of CaM to iPLAβ is an intrinsic property of this polypeptide, which was previously exploited to obtain highly purified riPLAβ via CaM-agarose affinity chromatography (22). Thus, iPLAβ differs from cytosolic phospholipase A₂ α by requiring an exogenous protein, calmodulin, to integrate alterations in cellular calcium ion homeostasis during cellular activation with release of fatty acids and the generation of lysolipids. In the present study, multiple contact points in the 15-kDa C-terminal portion of iPLAβ have been identified (Fig. 8) as important determinants of the calcium-dependent interaction of iPLAβ with CaM. The demonstration of specific Ca²⁺-dependent CaM footprinting during limited trypsinolysis clearly identifies one site of calcium-dependent binding of calmodulin at or near residue 630 in iPLAβ. Similar CaM footprinting experiments with myosin light chain kinase (38), calcineurin A (37), and with endothelial (38) and neuronal nitric oxide synthases (38–40) have each provided insight into the domain structure and CaM binding sites of these proteins.

Remarkably, addition of calcium-activated CaM to riPLAβ induced a conformational change near the active site as demonstrated by an increase in the kinetic rate of the appearance of band E in the presence of calcium and calmodulin together but not with either alone (Figs. 1 and 2B). Production of band E results from hydrolysis at a site ~200 amino acids from the CaM-binding domain. The most likely reason underlying this increased rate of production of band E is that this site interacts through space with the CaM-binding region. Accordingly, we propose a model in which the active site of iPLAβ interacts with the CaM-binding domain (in the absence of CaM) leading to a catalytically competent enzyme, whereas the reversible disruption of this interaction by binding of CaM to the 15-kDa C-terminal region abrogates this interaction with resultant loss of enzyme activity. In this model, association of calcium-activated CaM to the C terminus of riPLAβ putatively prevents important catalytic interactions between the CaM-binding domain and the active site. Results from site-directed mutagenesis near this region have suggested its importance for catalytic activity of iPLAβ.² Intriguingly, absolute complexity analysis of the four known iPLAβ sequences (human, rat, mouse, and hamster) reveals that the amino acids residues between the 1-9-14 (vide infra) and IQ motifs represent one of the most highly conserved regions in the entire iPLAβ protein (Fig. 7). Collectively, these results provide the first specific evidence of a calcium-activated CaM-induced conformational alteration of the active site of iPLAβ.

Proteins that interact with CaM have evolved to preserve critical residues necessary for the recruitment and binding of calcium-activated calmodulin. Based on the analysis of calmodulin binding sites (16, 18, 19), various CaM-binding sequences have been identified that, when modeled as an α-helix, form a predicted amphipathic structure with hydrophobic and basic amino acid residues positioned on opposite sides of the helix. Several calcium-dependent CaM-binding proteins (e.g., Ras-GRF (41), CDC25m (42), and IRS-1 (43)) contain an IQ CaM-binding motif (IQXXRGXXXR) (19, 44). Notably, the 12-residue CaM-binding peptide (AWSEMVGIQYFR) identified in

---

² D. J. Mancuso and R. W. Gross, unpublished observations.
this study as an important determinant in CaM-iPLA2β interactions possesses a shortened version of the first half of the consensus IQ motif (I701Q702X8X705) (Fig. 8). The apparent close proximity of the tryptophan in the 12-mer peptide (Tryp695 in iPLA2β) to dansyl-calmodulin as demonstrated by fluorescence resonance energy transfer further substantiates the presence of a contact point between CaM and iPLA2β mediated by this peptide. Interestingly, iPLA2β contains arginine residues at positions 691 and 693 that contribute to the overall positive charge of this region and likely participate in electrostatic interactions with CaM that contribute to the stability and high affinity binding of the complex.

Analysis of the iPLA2β sequence at the CaM protected sites shows the presence of a cluster of positively charged residues (623R624K625Q626G627N628K629), one or more of which is likely a site of trypsinolysis (Fig. 8). Extensive digestion of iPLA2β would be expected to destroy this site, thus explaining why it is not present in the RP-HPLC profile of the exhaustively digested protein loaded onto the CaM affinity column and absent in the EGTA Elute fraction (Fig. 4). Further analysis of this region indicates a 1-8-14 pseudo-consensus sequence (I192-X193-XX94XX95XX96XX97XX98XX99XX100X101X102X103X104X105X106X107X108X109X110X111X112X113X114 = 1-9-14) in which the numbers refer to the relative position of hydrophobic residues within the sequence (19). Notably, Ile622 and Ile635 are separated by 12 amino acids, a distance that is believed to be critical for anchoring these residues to the two Ca2+ binding lobes of CaM (15, 16). Some variability has been demonstrated in the location of the central hydrophobic residue of the 1-8-14 sequence, suggesting that the flexible central helix of CaM is able to accommodate minor alterations in the position of this residue (45, 46). The high net positive charge (+5) of this putative 1-9-14 motif in iPLA2β and the positioning of these positively charged residues (Arg623, Lys624, Lys628, Lys631, and Lys632) adjacent to two of the hydrophobic residues (Ile622 and Val630) may function to stabilize the iPLA2β-Ca2+-CaM complex through electrostatic charge pairing with the two glutamate clusters of calcium-activated CaM (14, 46).

In conclusion, these results illustrate: 1) the direct physical interaction of calmodulin with riPLA2β within the 15-kDa C-terminal region; 2) calcium-activated calmodulin-induced conformational alterations near the iPLA2β active site; and 3) the conservation of hydrophobic and positively charged motifs identified in other proteins present in the calmodulin binding motif of iPLA2β. It is hoped that identification of the domain of riPLA2β that interacts with CaM and the demonstration of calmodulin-induced conformational alterations of the active site will facilitate the development of therapeutic strategies that will modulate the activity of iPLA2β in disease processes.

REFERENCES

1. Wolf, R. A., and Gross, R. W. (1985) J. Biol. Chem. 260, 7295–7303
2. McHowat, J., and Creer, M. H. (1998) J. Am. Physiol. 274, C447–C454
3. Gross, R. W., Ramanadham, S., Kruszka, K. K., Han, X., and Turk, J. (1993) Biochemistry 32, 327–336
4. Ramanadham, S., Wolf, M. J., Jett, P. A., Gross, R. W., and Turk, J. (1994) Biochemistry 33, 7442–7452
5. Wolf, M. J., Izumi, Y., Zuroniski, C. F., and Gross, R. W. (1995) FEBS Lett. 377, 358–362
6. Miske, R., and Gross, R. W. (1992) Biochim. Biophys. Acta 1165, 167–176
7. Lehman, J. J., Brown, K. A., Ramanadham, S., Turk, J., and Gross, R. W. (1993) J. Biol. Chem. 268, 20713–20716
8. Gross, R. W., Rudolph, A. E., Wang, J., Sommers, C. D., and Wolf, M. J. (1995) J. Biol. Chem. 270, 14555–14565
9. Akiba, S., Mizunaga, S., Kume, K., Hayama, M., Sato, T. (1999) J. Biol. Chem. 274, 19906–19912
10. Wolf, M. J., Wang, J., Turk, J., and Gross, R. W. (1997) J. Biol. Chem. 272, 1522–1526
11. Nowatzke, W., Ramanadham, S., Ma, Z., Hsu, F.-F., Bohrer, A., and Turk, J. (1998) Endocrinology 139, 4073–4085
12. Murakami, M., Kambe, T., Shimbara, S., Kudo, I. (1999) J. Biol. Chem. 274, 3103–3115
13. Wolf, M. J., and Gross, R. W. (1996) J. Biol. Chem. 271, 20989–20992
14. Meador, W. E., Means, A. R., Quiocio, F. A. (1992) Science 257, 1251–1255
15. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., Bax, A. (1992) Science 256, 632–638
16. O’Neil, K. T., and DeGrado, W. F. (1999) Trends Biochem. Sci. 15, 58–64
17. Jones, P., Vorherr, T., and Carafoli, E. (1995) Trends Biochem. Sci. 20, 38–42
18. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 85–116
19. Rholds, A. R., and Friedberg, F. (1997) FASEB J. 11, 331–340
20. Zhang, M., and Yuan, T. (1998) Biochem. Cell Biol. 76, 313–323
21. Hazen, S. L., Stuppy, R. J., and Gross, R. W. (1990) J. Biol. Chem. 265, 10625–10630
22. Wolf, M. J., and Gross, R. W. (1996) J. Biol. Chem. 271, 38079–38085
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Kincaid, R. L., Vaughan, M., Osborne, J. C., Jr., Tkachouk, V. A. (1982) J. Biol. Chem. 257, 10638–10643
25. Johnson, J. D., and Wittenauer, L. A. (1983) Biochem. J. 211, 473–479
26. Vorherr, T., Knopfel, L., Hofmann, F., Moller, S., Pfeuffer, T., and Carafoli, E. (1993) Biochemistry 32, 6081–6088
27. Anagali, J., Hofmann, F., Quadralli, M., Vorherr, T., Carafoli, E. (1995) Eur. J. Biochem. 233, 701–708
28. Delilov, B. C. (1993) in Methods in Molecular Biology: PCR Protocols: Current Methods and Applications (White, B. A., ed) Vol. 15, pp. 217–222, Humana, Totowa, NJ
29. InforMax, Inc. (2000) Vector NTI Suite, version 6.0, AlignX, Bethesda, MD
30. Andrews, D. L., Beames, B., Summers, M. D., and Park, W. D. (1988) Biochem. J. 252, 199–206
31. Tang, J., Kriz, R. W., Wolfman, N., Shaffer, M., Seehra, J., and Jones, S. S. (1997) J. Biol. Chem. 272, 5867–5875
32. Mancuso, D. J., Jenkins, C. M., and Gross, R. W. (2000) J. Biol. Chem. 275, 9937–9945
33. Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) J. Biol. Chem. 269, 18239–18249
34. Glover, S., de Carvalho, M. S., Baybutt, T., Jonas, M., Chi, E., Leslie, C. C., and Gelb, M. H. (1995) J. Biol. Chem. 270, 15359–15367
35. Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L.-L. (1995) J. Biol. Chem. 270, 30749–30754
36. Delilov, B. C. (1993) in Methods in Molecular Biology: PCR Protocols: Current Methods and Applications (White, B. A., ed) Vol. 15, pp. 217–222, Humana, Totowa, NJ
37. Andrews, D. L., Beames, B., Summers, M. D., and Park, W. D. (1988) Biochem. J. 252, 199–206
38. Andrews, D. L., Beames, B., Summers, M. D., and Park, W. D. (1988) Biochem. J. 252, 199–206
39. Andrews, D. L., Beames, B., Summers, M. D., and Park, W. D. (1988) Biochem. J. 252, 199–206
40. Andrews, D. L., Beames, B., Summers, M. D., and Park, W. D. (1988) Biochem. J. 252, 199–206