Phospholipase D (PLD) and small GTPases are vital to cell signaling. We report that the Rac2 and the PLD2 isoforms exist in the cell as a lipase-GTPase complex that enables the two proteins to elicit their respective functionalities. A strong association between the two molecules was demonstrated by co-immunoprecipitation and was confirmed in living cells by FRET with Rac2 and YFP-PLD2 fluorescent chimeras. We have identified the amino acids in PLD2 that define a specific binding site to Rac2. This site is composed of two CRIB (Cdc42-and Rac-interactive binding) motifs that we have named “CRIB-1” and “CRIB-2” in and around the PH domain in PLD2. Deletion mutants PLD2-ΔCRIB-1/2 negate co-immunoprecipitation with Rac2 and diminish the FRET signal in living cells. The PLD2-Rac2 association was further confirmed in vitro using affinity-purified recombinant proteins. Binding was saturable with an apparent $K_d$ of 3 nM and was diminished with PLD2-ΔCRIB mutants. Furthermore, PLD2 bound more efficiently to Rac2-GTP than to Rac2-GDP or to a GDP-constitutive Rac2 N17 mutant. Increasing concentrations of recombinant Rac2 in vitro and in vivo during cell adhesion inhibit PLD2. Conversely, Rac2 activity is increased in the presence of PLD2-WT but not in PLD2-ΔCRIB. We propose that in activated cells PLD2 affects Rac2 in an initial positive feedback, but as Rac2-GTP accumulates in the cell, this constitutes a “termination signal” leading to PLD2 inactivation.

The ARF was the first G-protein identified to activate PLD1 (3, 4). Other small GTPases such as RhoA, Rac1, and Cdc42 in the presence of GTPyS directly associate with and stimulate PLD1 activity (5, 6), as mutation of the Rho binding site on PLD1 prevents PLD1/ARF interaction (7, 8). The Rho family of GTPases is also involved in indirect regulation of PLD1 enzymatic activity through stimulation of PI(4,5)P$_2$ kinase, Rho kinase, or by intracellular translocation of the PLD isoforms (9–12).

In contrast to PLD1, PLD2 is constitutively active, although it can be further activated by phosphatidylinositol 4,5-bisphosphate (PIP$_2$). PLD2 localizes to cell membranes where its PH domain binds to PIP$_2$ (13, 14). PLD2 can also be activated in intact cells by growth factors and agonists (8, 15, 16). On the other hand, Rac1 does not regulate the activity of the other PLD isoform, PLD2 (13, 17). The role of Rac2, which is more directly implicated in chemotaxis than Rac1, in the modulation of PLD2 activation, if any, is unknown.

Rac is a member of the Rho family of small GTPases that regulate cell growth, transformation, and motility. Rac cycles between a GDP-bound inactive form and a GTP-bound active form. Activation occurs due to GTP exchange factors such as Sos, and inactivation is mediated by the intrinsic GTPase enzymatic activity of Rac2 greatly aided by GTPase activating proteins. Further regulation of the cycle is mediated by guanosine nucleotide dissociation inhibitors that antagonize both GTP exchange factors and GTPase activating proteins. Further regulation of the cycle is mediated by guanosine nucleotide dissociation inhibitors that antagonize both GTP exchange factors and GTPase activating proteins (18–21). Rac2 plays an important role in cell migration with activation of membrane ruffling, whereas the other members of the Rho family, like RhoA and Cdc42, mediate stress fibers and filopodia formation, respectively (22, 23).

Once activated, Rho proteins act on substrates or effector molecules and initiate signaling cascades further downstream. The conserved CRIB (for Cdc42-and Rac-interactive binding) motif is the hallmark of an important subset of effectors downstream of Cdc42 and Rac, such as the serine/threonine kinase PAK-1 (p21-activated kinase 1) and the tyrosine kinase ACK-1 (activated Cdc42-associated kinase 1). The CRIB motif forms part of a larger binding domain for Cdc42 and Rac, the “PBD” (p21 binding domain), also called “GBD” (GTPase binding domain), which is essential for the interaction of the PAK-1 and ACK-1 effectors with GTP-bound (but not GDP-bound) Cdc42 or Rac (24, 25). The short region was first identified by (26). Subsequently, a host of candidate Cdc42/Rac effector proteins...
were identified (27) on the basis of sequence homology to this region, and CRIB motif was coined. PAK, ACK, and a third Cdc42 effector, WASP (Wiscott-Aldrich Syndrome protein), are the most well studied CRIB motif-containing proteins and provide an important basis for the understanding of Cdc42/Rac-mediated signaling (28).

The PLD activity is regulated by small GTPases, whereas PLD also acts upstream to regulate the activities of some small GTases. PLD1 and PLD2 are dependent on PIP2 for activity (29, 30), whereas the synthesis of PIP2 by the three phosphatidylinositol 4-phosphate 5-kinase isoforms is controlled by RhoA as well as Rac1 and Cdc42, implicating the regulation of PIP2 synthesis is central to signaling of these three Rho GTases (31, 32). Therefore, Rac, PIP2, and PLD are probably involved in the same signaling pathways and positively regulate a variety of cellular functions (i.e. Rac activates the synthesis of PIP2 and PIP2 controls PLD activity). Furthermore, Rho family proteins including Rac1 are also well characterized activators of PLD (2, 7, 15, 33). Interestingly, Rac1 small GTPase, which is well known for its activity in promoting actin polymerization and the formation of lamellipodia, was also reported to be regulated by PLD through PA generated by the PLD-hydrolyzing membrane phosphatidylcholine (34) or through direct binding of PLD1 to Rac1 and/or Rho with its C terminus (Rho family proteins appear to bind to PLD1 between amino acids 984 and 1000 in human and rat PLD1) (10, 35). These findings indicate a potential signaling feedback loop between Rac GTase and PLDs.

In neutrophils, PLD activation has been implicated in agonist-induced cell migration, exocytosis, phagocytosis of opsonized particles, and activation of NADPH oxidase (36, 37). In Madin-Darby canine kidney epithelial cells, expression of ARNO (a guanine nucleotide exchange factor for ARF) GTases leads to increased activation of endogenous Rac1 and that Rac activation is required for ARNO-induced cell motility. On the other hand, ARNO-induced activation of ARF6 also results in increased activation of PLD, and inhibition of PLD activity also inhibits cell motility (38).

Based on the literature evidence shown above, we suspected that an interacting mechanism exists between PLD2 and Rac2. For this to occur, we hypothesized that the two proteins are in close spatial proximity in the cells where they can regulate each other and show evidence for this in the present study. In regard to the site of binding, and as far as we know, there were not previously any CRIB domains described in PLD2 that could account for it being an “effector” molecule that Rac uses for signaling. In fact, automatic searches looking for a CRIB domain PLD2 failed to turn up one; however, manual, more detailed comparative searches did uncover two CRIB-like domains in PLD2. We present evidence in this paper for the existence of two new CRIB domains in PLD2 that the protein uses to bind to Rac2 and modulate its biological activities.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers were synthesized by Integrated DNA Technology (IDT) (Coralville, IA). DNA sequencing was provided by Retrogen, Inc. (San Diego, CA). Pfu DNA polymerase was purchased through Stratagene (Santa Clara, CA). QIAquick gel extraction kit was purchase from Qiagen (Valencia, CA). The In-Fusion Ready BacPAK Vector Set and the In-Fusion Dry-Down PCR Cloning kit were from Clontech (Mountain View, CA). Tetramethyl benzidine (TMB) reagent (chromagen solution) was from Invitrogen. Ninety-six-well ELISA plates were from BD Biosciences. Precise protein gels were purchased from Fisher.

**Generation of PLD2-ΔCRIB Mutants**—The construct pcDNA3.1-myc-PLD2aWT was used as a template to create ΔCRIB deletion mutants (ΔCRIB-1 and ΔCRIB-2) after the QuikChangeXL site-directed mutagenesis protocol (Stratagene, La Jolla, CA) using the following sets of primers:ΔCRIB-1 forward (5′-CTCCGAGACAGTGCCCAAAGGA-GGACGGAGGC-3′) and ΔCRIB-1 reverse (5′-GCTCCTTTGGCCACCTGCTCAGG-3′); ΔCRIB-2 forward (5′-GTTGGGGGAAAAGAGGAGCTACGCCCCC-3′) and ΔCRIB-2 reverse (5′-GGGGGGTAGCTGTCCCTTGG-GCACCACC-3′). All oligonucleotides and their reverse complements were PAGE/HPLC-purified (Integrated DNA Technology). Molecular identity of the two pcDNA3.1-myc-PLD2 mutants was confirmed by direct sequence analysis (Agencourt Bioscience Group, Beverly, MA).

**Fluorescence Resonance Emission Transfer (FRET) Analysis**—For the donor of the FRET pair, we subcloned PLD2 into monomeric (m) C-Citrine (a derivative of YFP) (a gift from Joel Swanson, University of Michigan School of Medicine), and the new fluorescent chimera plasmid was named “YFP-PLD2.” For the donor of the FRET pair, we subcloned Rac2 into C-mCerulean (a derivative of CFP), and the new fluorescent chimera plasmid was named “CFP-Rac2.” Both plasmids were verified by direct sequencing, and their activities were verified by PLD activity and GTP binding (PBD pulldown) and found to be within the efficiencies of the parental plasmids (both N- and C-terminal end clones were generated, but the C-terminal clones consistently yielded activity levels closer to wild type). COS-7 cells were co-transfected with PLD2-mCitrine and Rac2-mCFP using FuGENE 6 reagent for 48 h (the cells were serum-starved for the last 16 h). When ready for microscopy, cells were left unstimulated. Fluorescence microscopy was performed with appropriate excitation and emission filter sets (Chroma Corp.) for CFP, YFP, and FRET (CFP excitation and YFP emission). Images were collected on a 14-bit Cascade HQII digital camera and were processed as described in Hoppe et al. (39) to determine the FRET stoichiometry of the interaction between PLD2 and Rac2. Fluorescence imaging showed basic wide field FRET microscopy: IA (PLD2-mCitrine), ID (Rac2-mCFP), and IF (FRET) images. Calculated FRET images are EA, E D, and RM. The mean average FRET efficiency over the entire cell (Eavg) was determined using Metamorph and Matlab software and reached a maximum of 0.15. For comparison, the linked CFP-Citrine positive control used for calibration of the microscope yields an Eavg of 0.37. Control experiments co-expressing PLD2-mCitrine and free CFP showed no FRET with or without EGF stimulation.

**Baculovirus Cloning and Vector Construction**—An upstream Sall restriction enzyme digestion (RED) site and a downstream HindIII RED site were introduced into the homologous fragments for each polymerase chain reaction (PCR) product due to
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

**TABLE 1**

| DNA | Sequence (5’-3’) | Function | Length |
|-----|-----------------|----------|--------|
| PLD2 | TAAGGCCTCTCAGGAG | PCR forward primer | 59 nt |
| PLD2 | CAGAATTCGCAAGCTT | PCR reverse primer | 31 nt |
| Rac2 | TAAGGCCTCTCAGGAG | PCR forward primer | 55 nt |
| Rac2 | CAGAATTCGCAAGCTT | PCR reverse primer | 29 nt |

The sequences of the PCR primers. The PCR products for both PLD2 and Rac2 were amplified with Pfu DNA polymerase using the primers shown in Table 1. The PCR protocol was as follows for 35 cycles: predenaturing at 95 °C for 45 s, denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, elongation at 72 °C for 6 min, and then one final cycle of elongation at 72 °C for 10 min. The PCR products were purified with QIAquick gel extraction kit. In-Fusion Ready BacPAK Vector Set contained prelinearized vectors for adding either N- or C-terminal 6xHN tags to our proteins of interest. The recombinant reaction between the linearized vector and the PCR product was performed with In-Fusion Dry-Down PCR Cloning kit. The recombinant baculovirus plasmids were sent to Retrogen, Inc. for sequencing and verification. The recombinant baculovirus vector maps for PLD2 and Rac2 are depicted in supplemental Fig. 1, A and B, respectively.

**Sf21 Cell Culture**—Sf21 insect cells were maintained at 2 × 10⁶ cells/ml in Complete Grace’s Insect Cell media containing 3.3 g/liter yeastolate, 3.3 g/liter lactalbumin hydrolysate, and 10% fetal calf serum at 30 °C in the absence of CO₂ using spinner flasks. For in vivo or in vitro assays, insect cells were maintained in monolayer cultures using 35-mm wells at 2 × 10⁶ cells/well.

**Generation of Purified, Recombinant Proteins from a Baculovirus/Insect Cell Expression System**—A large scale overexpression of both PLD2 and Rac2 was set up from baculovirus, starting from pBacC1 clones infected in Sf21 insect cells. We selected virulent Bac-C1-HA-Rac2, Bac-C1-myc-PLD2-WT, and Bac-C1-myc-PLD2-ΔCRIB-1 recombinant viruses to infect Sf21 cells. Lysates from Sf21 cells (2 × 10⁶ cells/ml) in a spinner of Complete Grace’s Insect Cell Culture Media were used to bind 6xHN-tagged proteins in TALON resin (Clontech) according to the manufacturer’s instructions. Washing buffer was 50 mM sodium phosphate, pH 7.0–7.5, 5 mM imidazole, 300 mM NaCl, and elution buffer was 50 mM sodium phosphate, pH 7.0–7.5, 500 mM imidazole, 300 mM NaCl. Optical density at 280 nm was read from eluates of columns. Fractions were then dialyzed (5 mM HEPES, pH 7.8, 50 mM NaCl, 1 mM DTT, 5% glycerol) for 2 h. Aliquots were used for PAGE gels and for immunoblots that showed the prevalence of a protein of ~22 kDa for Rac2 and, in separate experiments, of a protein of ~110 kDa for PLD2.

**Binding in an ELISA Plate Setting**—Purified 6xHN-myc-PLD2 (WT and deletion mutants) or Rac2 were incubated with PVC grade, non-tissue culture-treated 96-well plates. After blocking with 2% BSA and 0.2% Tween 20, the coated plates were then incubated with buffer only or with increasing amounts of the protein partner to be tested. After incubation at 37 °C, extensive washes of the 96-well plate followed. Anti-HA (or anti-Myc) monoclonal mouse antibodies were then added to the ELISA plate and allowed to interact with the purified, recombinant protein bound to the plastic. After a treatment with a secondary, anti-mouse IgG HRP conjugate and tetramethyl benzidine (chromagen solution) treatments for 15 min at room temperature, the PLD2/Rac2 binding was quantified at 620 nm in a microplate spectrophotometer.

**PLD Activity Assay**—Purified baculoviral PLD2 was processed for PLD2 activity in PC8 liposomes and n-[³H]butanol beginning with the addition of the following reagents (final concentrations): 3.5 mM PC8 phospholipid, 45 mM HEPES (pH 7.8), and 1.0 μCi of n-[³H]butanol in a liposome form as indicated in Liscovitch et al. (40). Samples were incubated for 20 min at 30 °C with continuous shaking. The addition of 0.3 ml of ice-cold chloroform/methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by thin layer chromatography. The amount of [³H]phosphatidylbutanol ([³H]PBut) that comigrated with PBut standards was measured by scintillation spectrometry.

**Rac2 PBD Pulldown Assays**—2 × 10⁶ Sf21 insect cells were infected with no Rac2 baculovirus or with Rac2 baculovirus at a multiplicity of infection of 0.5:1 (number of virus particles to number of insect cells). PLD2-WT baculovirus was also simultaneously co-infected using increasing multiplicities of infections from 5:1 to 50:1. Infections occurred for 48 h, after which time cells were activated using 100 ng/ml EGF for 7 min at 37 °C. Cell lysates were prepared in special lysis buffer containing digitonin. Next, 5 μl of PAK-1-PBD-agarose was added to each lysate sample and incubated on a tube rotisserie at 4 °C for 30 min in the presence of the following magnesium lysis buffer (final concentrations): 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Samples were sedimented and washed 3 × in magnesium lysis buffer and resuspended in 1 × SDS sample loading buffer. Samples were then denatured at 95 °C for 7 min. The supernatant of each sample was loaded onto 4–20% gradient Precise Protein gels and electrophoresed at 100 V for ~1 h. Gels were transferred onto Whatman PVDF membranes for 1–2 h at 70 V. PVDF membranes were blocked for a few hours in 2% BSA, 0.2% Tween 20 in Tris-buffered saline at room temperature. PVDF membranes were then probed overnight at 4 °C with either α-HA antibodies to detect recombinant, GTP-bound Rac2 or α-Myc antibodies to detect recombinant PLD2 that interacted with Rac2 in the pulldown assay. HRP-conjugated secondary antibodies were incubated with PVDFs, and then
products were visualized using enhanced chemiluminescence reagents (ECL) and autoradiography.

Statistical Analysis—Data are presented as the mean ± S.E. The difference between means was assessed by the single factor analysis of variance test. Probability of \( p < 0.05 \) was considered to indicate a statistically significant difference.

RESULTS

Rac2 and PLD2 Overexpression Increase Cell Adhesion—PLD and Rac small GTPases are integral to cell signaling (37, 41), and PLD activity and Rac-GTP loading are both activated by the same stimuli and to approximately the same extent. Fig. 1A indicates that overexpression of cells with Rac2 and PLD2 leads to an increased cell adhesion functionality signifying that these two molecules appear to cooperate in the cell in some fashion. This happens, however, as long as the PLD2:Rac2 ratio is \( \approx 1 \) (PLD2 DNA is in excess compared with Rac2 DNA in the transfection reactions). Conversely, using overexpression of cells with a PLD2:Rac2 ratio of \( < 1 \), we found the opposite effect (Fig. 1B), that is, a decrease in cell adhesion. To understand the mechanism for these physiological events, we undertook a series of experiments with the hypothesis in mind that both proteins must be in close spatial proximity.

Rac2 and PLD2 Form a Dimeric Complex—As PLD and Rac are both activated with a profound effect on the kinetics of cell adhesion, it is reasonable to assume that both proteins must be in close spatial proximity. Fig. 2 demonstrates that Rac2 and PLD2 are associated in the cell, as they can both be immunoprecipitated with antibodies specific for the other protein. Co-immunoprecipitation was performed using two complementary approaches. First, COS-7 cells were transfected with tagged expression plasmids (Myc-Rac2 and HA-PLD2) (Fig. 2A) alone or in combination. In Fig. 2B, the top two panels are positive controls showing appropriate expression of transfected proteins. I.P. with \( \alpha\)-Myc antibody and subsequent W.B. with \( \alpha\)-HA antibody pulled down the HA-tagged PLD2 protein associated with Myc-tagged Rac2 (third panel). I.P. with the \( \alpha\)-HA antibody and subsequent W.B. with the \( \alpha\)-Myc antibody pulled down the Myc-tagged PLD2 protein associated with HA-tagged Rac2 (fourth panel). The fifth panel is a control to show equal loading in all lanes as ascertained with anti-actin antibodies.

FIGURE 1. Overexpression of both Rac2 and PLD2 (with an excess of the latter) cooperatively increases cell adhesion. RAW264.7 mouse macrophages were transfected with either Myc-Rac2 (filled triangles) or HA-PLD2 (open spheres) alone or in combination (filled spheres). Forty-eight hours post-transfection cells were allowed to adhere to collagen-coated coverslips in the presence of 3 nM EGF for varying amounts of time. A, the ratio of PLD2:Rac2 DNA used for transfection is at molar equivalence and results in increased cell adhesion. B, the ratio of PLD2:Rac2 DNA used for transfection is \( < 1 \) (Rac2 is in excess compared with PLD2) and results in decreased cell invasion.

FIGURE 2. Demonstration of a molecular association between Rac2 and PLD2. A, Myc- and HA-tagged constructs are shown. COS-7 cells were transfected with either Myc-Rac2 or HA-PLD2 alone or in combination. After 2 days, cells were incubated in the absence of EGF, and lysates were immunoprecipitated (I.P.) with the indicated antibodies. Resulting Western blots (W.B.) were probed with the indicated antibodies (B). C, fluorescent (YFP and CFP)-tagged constructs were transfected in COS-7 and analyzed as previously described by immunoprecipitation and WB (D). The bottom row of each panel is a control that shows equal loading in all lanes as ascertained with anti-actin antibodies.
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

named “PLD2-YFP.” This chimera showed strong protein expression and PLD2 enzymatic activity (not shown) when transfected in COS-7 cells. For the donor of the FRET pair, we chose Rac2-CFP. COS-7 cells were co-transfected with PLD2-YFP and Rac2-CFP for 48 h followed by fluorescence microscopy. Although ED varied slightly in different cells, the existence of FRET (Fig. 3) indicates that Rac2 and PLD2 indeed form a heterocomplex in the cell. Control experiments co-expressing PLD2-YFP and free CFP showed no FRET with or without EGF stimulation (data not shown). In summary, Rac2 and PLD2 indeed form a heterocomplex in the cell. Control experiments co-expressing PLD2-YFP and free CFP showed no FRET with or without EGF stimulation (data not shown). In summary, Rac2 and PLD2 indeed form a heterocomplex in the cell.

The Specific Site of PLD2 Binding to Rac2 Involves Two Previously Uncharacterized CRIB Domains on the Lipase—The PLD2–Rac2 interaction begged the question as to the location of the Rac2 binding site on PLD2. To ascertain this, we generated several PLD2 mutants where we hypothesized that binding occurred. The rationale for choosing target sites was based on previous knowledge of sequences found in Rac2 binding partners. A CRIB motif has been shown to specifically bind the GTP-bound form of Cdc42 or Rac with a preference for Cdc42. The prototypes are the non-receptor-tyrosine kinase, ACK, and the serine/threonine kinase, PAK (Fig. 4A). The length of the consensus CRIB motif is ~16 amino acids, containing a region of variable length between the two halves of the binding motif. The CRIB motif contains eight core amino acids with the sequence ISX<sub>1</sub>PXXXXF<sub>2</sub>HXXHVG. However, proteins with one or two differences within the core sequence can still show binding to Cdc42/Rac. By standard data base searches, no obvious CRIB motif was immediately present in PLD2. Nevertheless, a manual search of the PLD2 amino acid sequence yielded two putative CRIB-like candidate amino acid segments that contained the key amino acids of the consensus in the appropriate order. One such putative Rac2 binding site is located inside the PH domain of PLD2 and the other is located mostly just outside of and downstream from the PH domain (Fig. 4B). We have named the first domain CRIB-1 (253ISFVQLDPGLFEVQGV<sup>270</sup>) and the second domain...
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

CRIB-2 (ITELAQGRDFLQLHRHD$^{306}$). Fig. 4C shows a schematic representation of the motifs studied here whereby the deletion mutants PLD2ΔCRIB-1 and PLD2ΔCRIB-2 were generated.

With the new CRIB deletion mutants, we repeated I.P./W.B. experiments similar to those of Fig. 1 to ascertain if the PLD2-Rac2 interaction was affected by deletion of the putative CRIB domains. The results are presented in Fig. 5, A and B (for CRIB-1) and C and D (for CRIB-2). In Fig. 5A, the top and bottom panels are positive controls to show appropriate expression of proteins. In Fig. 5B, I.P. with α-Myc antibody and subsequent W.B. with HA antibody pulled down a Rac2-associated PLD2 (top panel, left lane) when cells were co-transfected with PLD2-WT and Rac2-WT. However, a much-diminished PLD2 signal was pulled down or detected (70% decrease) in lysates of cells that were co-transfected with PLD2ΔCRIB-1 and Rac2-WT (top and middle panels, right lane). These data suggest that the PLD2-CRIB association occurs at the level of the CRIB-1 domain, shown to be present on PLD2 for the first time in this study. Additionally, we present the relative levels of protein-protein binding using either Myc- or HA-tagged immunoprecipitation (Fig. 5E). Thus, CRIB-1 and CRIB-2 are two binding sites that were ascertained in the in vitro binding experiments of Fig. 3. Additionally, the effect of removal of the PLD2 CRIB domain on Rac2 was also characterized through the use of FRET. Average FRET efficiency of Fig. 5F indicates that FRET was diminished ~50% with PLD2ΔCRIB-1 and PLD2ΔCRIB-2, respectively, when compared with PLD2-WT.

Our next goal was to manufacture large-scale quantities of purified PLD2 and Rac2 to be utilized for in vitro studies. We chose to explore the feasibility of protein production in the eukaryotic baculovirus-insect cell system for protein overexpression because our conventional cell model (transfection of bacterial plasmids into COS-7 cells) suffered low protein yields. This pitfall could limit our ability to use PLD2 or Rac2 proteins in binding assays that required viable amounts of protein for analysis to maintain native structure and function. Therefore, we chose the baculoviral expression system for the ease of purification with few purification steps.

Cloning and Production of a Recombinant Baculovirus-expressing Human PLD2 or Rac2—The production schematic of the recombinant baculovirus vector maps for PLD2 and Rac2 are depicted in supplemental Fig. 1, A and B, respectively, and additional methodology is presented in the supplemental Experimental Procedures. The results presented in supplemental Fig. 1C show an increase in Rac2-WT expression. The results presented in supplemental Fig. 1D indicate that PLD2-WT expression was augmented. Proteins were eluted from the TALON batch matrix (supplemental Fig. 2, A and C). Elution #2 contained categorically the greatest concentration of purified protein. A portion of each elution was
used for SDS-PAGE and stained with Coomassie Blue (Fig. 6, A and B) revealing relatively pure Rac2 and PLD2. A final yield of protein obtained from the 200-ml cultures was in excess of 90% pure for Rac2 and >80% pure for PLD2 as determined by SDS-PAGE and densitometric scanning of the accompanying gels (Fig. 6, A and B). Western blot analyses revealed a HA-tagged Rac2 protein of approximate molecular mass of 26 kDa that reacted with an anti-HA
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

monoclonal antibody and a Myc-tagged PLD2 protein of approximate molecular mass of 110 kDa that reacted with an Myc-tagged monoclonal antibody (Fig. 6, A and B). A constant amount of purified, baculoviral Rac2 in the absence or presence of increasing GTPγS was utilized to determine Rac2 GTPase activity using PAK-1 PBD-agarose that specifically binds to GTP-bound Rac2 via the p21 binding domain of PAC-1 (supplemental Fig. 2F). Increasing amounts of purified, baculoviral PLD2 results in increased PLD activity (supplemental Fig. 2D).

The Interaction of Rac2-PLD2 Binding—To ascertain the further interaction of PLD2 and Rac2 binding, we used the recombinant proteins from the baculovirus/insect cell expression system. First, we determined the levels of saturation binding of PLD2-WT and PLD2-ΔCRIB1 to the ELISA plate alone (Fig. 6C). As shown, both wild-type and mutant PLD2 protein have essentially the same binding capacity for the ELISA plate, and saturation binding was achieved at 9.1 pmol each. Next, Rac2 was used to determine binding to either PLD2-WT or PLD2-ΔCRIB1, which were seeded in separate ELISA plates at 9 pmol (Fig. 6D). After blocking, the PLD2-coated plate was then incubated with buffer only or with serial dilutions of increasing amounts of Rac2 protein ranging from 0.019 to 42.7 pmol. If Rac2 directly bound to Rac2 to the CRIB mutant.

Because the input protein was 9.1 pmol, the stoichiometry of binding on the binding stoichiometry with PLD2 from the pre-bound to Rac2 than the co-immunoprecipitations (Fig. 5 KD

Effect of Rac2 Activation on PLD2 Activity

Effect of Rac2 Activation on PLD2/Rac2 Dimerization and PLD2 Activity—Next, we determined the effect of Rac2 activation on the binding stoichiometry with PLD2 from the previous experiment using two different approaches. First, when preincubated with either GTPγS or GDP, Rac2 would be in either its active or inactive form, respectively, and binding of 9.1 pmol of PLD2 to GTP-bound Rac2 (open circles or untreated, control Rac2 (filled circles) resulted in differing binding capacities but with similar efficiencies (Fig. 7A). PLD2 bound less effectively to GDP-bound Rac2 (open triangles) when compared with either GTP-bound or control Rac2. The presence of GTP-bound Rac2 increased binding by 40% when compared with that of the GDP-bound Rac2 negative control. These data indicate that the protein-protein binding interaction between PLD2 and Rac2 is dependent on Rac2 activation. The apparent KD values for control Rac2-WT, GTP-bound Rac2-WT, and GDP-bound Rac2 binding to PLD2-WT are 10, 8, and 10 nm, respectively, are virtually the same and of similar efficiency. Second, we utilized a constitutively GDP-bound Rac2 mutant (Rac2-T17N or “Rac2N17”) in comparison with the wild-type and GTP-bound Rac2 (Fig. 7B). As shown, PLD2 binds better to Rac2-WT than to the Rac2-N17 mutant protein, which yielded a 25% decrease in Rac2 binding. Apparent KD values are similar at 10 and 13 nm for Rac2-WT and Rac2-N17 binding to PLD2-WT, respectively. These two sets of data show that PLD2 binds to Rac2 and with preference to GTP-bound Rac2.

As shown in Fig. 7, binding between PLD2-WT and Rac2 is dependent on Rac2 activation. As such, we then determined the effect of Rac2 activation on PLD2 activity (Fig. 8A). Increasing concentrations of GTP-bound Rac2 (filled circles) inhibit PLD2-WT activity in vitro by ~25% at maximal values. This negative effect is partially reversed when GDP-bound Rac2 is used (open circles). The difference in lipase activity between GTP-bound Rac2 and GDP-bound Rac2 is ~10% at maximal values of Rac2. Additionally, when constitutively GDP-bound Rac2-N17 mutant was included in the lipase reaction, PLD2 activity was virtually unaffected (filled triangles). The presence of GDP in the reaction by using either GDP-bound Rac2-WT or Rac2-N17 reversed this inhibitory effect on PLD activity. This data suggests that when PLD2 and Rac2 bind to one another, lipase activity is inhibited by the presence of active Rac2 in the reaction, which parallels the binding data of Fig. 7A and implicates reduced binding of Rac2 to PLD2 results in less of a biological effect of Rac2 on PLD2 activity.

As shown in Fig. 6D, removal of the CRIB domain from PLD2 interfered with the ability of the phospholipase to bind Rac2. Next, we determined the effect this binding interference had in vivo on PLD2 activity of baculoviral-infected SF21 insect cells stimulated with 50 ng/ml PMA (Fig. 8B). Increasing multiplicity of infection ratios of Rac2:PLD2 inhibit wild-type PLD2 activity in vivo by ~50% at maximal values. This negative effect is almost completely reversed when PLD2-ΔCRIB1 is used and

FIGURE 5. CRIB deletion mutants of PLD2 affect binding and FRET to Rac2. COS-7 cells were transfected with Myc-Rac2, HA-PLD2-WT, or HA-PLD2-ΔCRIB1 alone or in combination. After 2 days, cells were incubated in the presence of 10 nM EGF for 10 min, and lysates were immunoprecipitated (I.P.) with the indicated antibodies. Resulting Western blots (W.B.) were probed with the indicated antibodies. A, top panel is a positive control for PLD2. The bottom panel is a control positive for Rac2. B, the top panel is the amount of PLD2 that was bound to immunoprecipitated Rac2. The middle panel is the amount of Rac2 that was bound to the immunoprecipitated PLD2. The bottom panel is a control that shows equal loading in all lanes as ascertained with anti-actin antibodies. C and D, I.P./W.B. of COS-7 cells transfected with Myc-Rac2, HA-PLD2-WT, or HA-PLD2-ΔCRIB2 alone or in combination is shown. E, graphic representation of relative levels of protein-protein binding using either Myc- or HA-tagged immunoprecipitation is shown. F, shown is a demonstration of diminished FRET. Average FRET efficiency of YFP-PLD2ΔCRIBs is compared with WT. COS-7 cells were transfected with CFP-Rac2 (the donor in the FRET pair) and with YFP-PLD2 (the acceptor of the FRET pair). The mean average FRET efficiency over the entire cell (Eavg) was determined using Metamorph and Metlab software. The error bars are S.E. (n = 15–26). The (*) denotes a significant increase (p < 0.05) in FRET with respect to the CFP-Rac2 and YFP-PLD2. The (#) denotes a significant decrease (p < 0.05) in FRET with respect to the WT control.
FIGURE 6. Purification of recombinant Rac2 and PLD2 proteins from recombinant baculovirus-infected Sf21 insect cells and binding interaction. Recombinant proteins were purified as outlined under “Experimental Procedures.” A, Rac2-WT purification is shown. B, PLD2-WT purification is shown. A and B, Coomassie Blue-stained SDS-polyacrylamide gels of (left to right) blank (non-infected) control sample, crude cell lysate, and elution #2 and densitometric scans of SDS-polyacrylamide gels of elution #2 are shown. Each major peak corresponds to the molecular weight signature of the protein of interest. A and B, Western blot analyses of Rac2 or PLD2 purification were performed as mentioned under “Experimental Procedures.” The monoclonal antibodies used to detect either Rac2 or PLD2 were directed against the N-terminal HA (Rac2) or Myc (PLD2) tag of each recombinant protein, respectively. Lanes are (left to right): blank (non-infected) control sample, crude cell lysate and elution #2. Numbers to the left indicate molecular weight mass in kDa. Protein notation is indicated on the right.

C, varying amounts of Myc-PLD2-WT or -H9004 CRIB-1 (0.14 –18 pmol) were incubated with the 96-well ELISA plate and detected using an anti-Myc antibody followed by a HRP secondary antibody as described under “Experimental Procedures.” Protein binding was measured by spectrometry at $A_{620}$ nm. Results represent the mean ± S.E. for four independent experiments.

D, increasing amounts of myc-Rac2 (0.17–380 pmol) were laid on top of the PLD2-coated wells (with the amount of PLD2 remaining constant at 9.1 pmol) and washed extensively after incubation. Plates were probed with anti-HA primary antibody to detect bound Rac2 followed by a HRP secondary antibody. Protein binding was measured by spectrometry at $A_{620}$ nm. Results represent mean ± S.E. for four independent experiments.
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

with either Myc-Rac2 or HA-PLD2ΔCRIB mutants alone or in combination. Forty-eight hours post-transfection, cells were allowed to adhere to collagen-coated coverslips in the presence of 3 nM EGF for ∼21 min. As shown in (Fig. 8C), we found that when the ratio of PLD2ΔCRIB:Rac2 used for transfection is <1 (Rac2 is in excess compared with PLD2), this resulted in an improved cell invasion.

Effect of PLD2 on Rac2 GTP Loading Activity—All the data so far have been performed looking at the effect of Rac2 on PLD2 activity (and with Rac2 in excess). Finally, we infected PLD2-ΔCRIB-1 into insect cells and studied the effect of mutant PLD2-ΔCRIB on Rac2 activity using in vitro experiments with lysates from these infected Sf21 cells. The left three lanes in this figure are positive controls using PLD2-WT and showing a reproducible and increasing effect of PLD2 on basal GTP-loading of Rac2 (Fig. 9A). Conversely, when PLD2-ΔCRIB-1 was used, no such positive effect was found at any of the multiplicity of infection concentrations tested. The last two lanes are positive and negative controls showing GTP/GDP exchange activity. These data reveal that an intact CRIB domain on PLD2 is necessary to increase the GTP loading of its target, Rac2.

In summary, the binding of Rac2 to PLD2 has a functional consequence in that PLD activity is decreased by increasing concentrations of recombinant Rac2 in vitro. Conversely, Rac2 activity (p21 binding-domain pulldown) is increased in the presence of PLD2-WT but not with the PLD2-ΔCRIB mutant. This would explain the biphasic effect of Rac2 and PLD2 as shown in cell adhesion of Fig. 1 and will be further elaborated on in the discussion.

DISCUSSION

We have demonstrated using FRET, cell transfection, and in vitro experiments with recombinant proteins the existence of a PLD2/Rac2 interaction both in vivo and in vitro and the site of Rac2 binding to PLD2, which consists of two previously uncharacterized CRIB domains in PLD2 and around its PH domain that we have named CRIB-1 and CRIB-2. The production of milligram quantities of soluble, active recombinant PLD2 and Rac2 using a baculovirus-insect cell expression system have facilitated the finding that these proteins form a protein complex. When Rac2 overwhelms PLD2, the net effect is less adhesion. Conversely, when PLD2 overwhelms Rac2, the net effect is a positive feedback. We propose that in activated cells, PLD2 affects Rac2 in an initial positive feedback, but as Rac2-GTP accumulates in the cell, this can be a termination signal leading to PLD2 inactivation (Fig. 9B) via one of two different scenarios. The first scenario might allow for 2 Rac2 molecules to interact with PLD2 via each of its two CRIB domains. Alternatively, the second scenario might allow for 1 Rac2 molecule to interact with PLD2 via simultaneous interaction with both CRIB domains.

could be due to the mutant PLD2 not binding to Rac2 equally as efficiently as the wild-type PLD2, and thus, the inhibitory effect of Rac2 on PLD2 is less pronounced. The difference in lipase activity between PLD2-WT and PLD2-ΔCRIB-1 is ∼40% at maximal values of Rac2. This negative effect of Rac2 on PLD2-WT activity was almost totally abrogated if PLD2-ΔCRIB-1 was used, indicating that the physical interaction between PLD2 and Rac2 is dependent in vivo on binding to the CRIB domains. To correlate this effect to a physiological process, we performed an adhesion experiment similar to Fig. 1 using RAW264.7 mouse macrophages that were transfected...
which PA can activate Rac2 is by helping it to get the autoinhibitory domain unlocked. This was demonstrated in the effector of Rac1, p67Phox, in the NADPH oxidase complex, which interacted directly with Rac1 in a GTP-dependent manner (43). In general, Rac effectors exist in a folded inactive conformation in which an autoinhibitory domain overlaps with the CRIB domain. This interaction is disrupted upon binding of the activated GTPase, bringing about an open conformation that allows the effector to establish further interactions or display catalytic activity (44–46).

As far as the negative effect of Rac2 on PLD2 that we have also observed, the mechanism could be related to a steric difficulty of PLD binding to the membrane, as Rac2 is present in the reaction once it is activated in the GTP-bound mode. The PH domain of PLD is responsible for the binding of PIP2 to the membrane and docking of the protein, and then the relevant cell functionality is triggered. It is entirely possible that when Rac2 binds to each PLD2 molecule at the CRIB-1 and/or the CRIB-2 sites, the removal of PLD from its natural membrane environment ensues. Subsequent availability of cofactors (chiefly PIP2) results in the cessation of relevant cell function.

In this study we have identified and defined two new CRIB domains in PLD2 that bind to Rac2 using co-immunoprecipitation and FRET experiments. CRIB1 is located within the PH domain of PLD2, whereas CRIB2 is just downstream of the PH domain and overlaps five amino acids of the PH domain. The CRIB deletion mutants, PLD2−/CRIB1 and PLD2−/CRIB2, abrogate the Rac2-PLD2 binding. There is no precedent for the existence of CRIB domains on PLD2 or on any PLD for that matter; therefore, this is the first such report uncovering CRIB domains on a phospholipase that mediate enzymatic regulation. The minimal conserved region consists of 16 amino acids of the Cdc42 binding site of a murine p65PAK isoform, which was then verified and used to search for other CRIB domain proteins (27). Automatic searches looking for a CRIB domain on PLD2 failed to turn up one; however, manual, more detailed
Evidence for Two CRIB Domains in PLD2 That Bind Rac2

Comparative searches did uncover two CRIB-like domains in PLD2 when searching for the CRIB domain (PubMed conserved domain cl00113) of (ISFVQLPDPGFQYEVKSTVEARHGVIDTSH) and CRIB2 (ITELAQQPGRDFQHLHRHD), the “.XGXID” part is relatively more variable among the proteins containing CRIB domains. There are many more potential effector molecules with a more or less conserved motif that can be found in data base searches (27, 47).

In conclusion, this is the first report of a direct GTPase-phospholipase binding with the identification of the precise amino acid sites involved, namely, the existence of two new CRIB domains in PLD2. We also provide evidence for the $K_D$ of the various binding interactions, and the discovery of a novel mechanism of interregulation between these two proteins and their biological activities that should be able to explain adhesion and chemotaxis of leukocytes during innate immune defense.

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Evidence for Two CRIB Domains in PLD2 That Bind Rac2

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