BIOCHEMISTRY
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Biochemistry **2009,** *48,* 8171–8178 8171
DOI: 10.1021/bi9010623

Biochemical Characterization of Hyperactive β2-Chimaerin Mutants Revealed an Enhanced Exposure of C1 and Rac-GAP Domains†

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Received June 23, 2009; Revised Manuscript Received July 17, 2009

ABSTRACT: Recent studies established that the Rac-GAP β2-chimaerin plays important roles in development, neuritogenesis, and cancer progression. A unique feature of β2-chimaerin is that it can be activated by phorbol esters and the lipid second messenger diacylglycerol (DAG), which bind with high affinity to its C1 domain and promote β2-chimaerin translocation to membranes, leading to the inactivation of the small G-protein Rac. Crystallographic evidence and cellular studies suggest that β2-chimaerin remains in an inactive conformation in the cytosol with the C1 domain inaccessible to ligands. We developed a series of β2-chimaerin point mutants in which intramolecular contacts that occlude the C1 domain have been disrupted. These mutants showed enhanced translocation in response to phorbol 12-myristate 13-acetate (PMA) in cells. Binding assays using [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) revealed that internal contact mutants have a reduced acidic phospholipid requirement for phorbol ester binding. Moreover, disruption of intramolecular contacts enhances binding of β2-chimaerin to acidic phospholipid vesicles and confers enhanced Rac-GAP activity *in vitro*. These studies suggest that β2-chimaerin must undergo a conformational rearrangement in order to expose its lipid binding sites and become activated.

Rac, a small GTPase that belongs to the Rho family, mediates signals by tyrosine kinase and G-protein-coupled receptors and is known to play key roles in the regulation of actin dynamics, cell cycle progression, adhesion, and migration. This small G-protein cycles between active (GTP[1]-bound) and inactive (GDP-bound) conformations. Upon receptor stimulation, Rac guanine-nucleotide exchange factors (Rac-GEFs) promote GTP loading onto Rac, which adopts a conformation capable of activating downstream effectors such as Pak kinases. Rac inactivation is mediated through GTPase activating proteins (GAPs) that promote the hydrolysis of GTP from activated Rac (*1*, *2*). One of the Rac-GAP families that received significant attention in the last years is the chimaerin family, a group of four related GAPs encoded by two genes: CHN1 (α1- and α2-chimaerins) and CHN2 (β1- and β2-chimaerins) (*3*−*5*). Recent studies established that chimaerins play fundamental roles in development, neuritogenesis, and cancer progression (*6*−*11*). α2- and β2-chimaerins represent alternative spliced variants that possess an N-terminal SH2 domain. A Rac-GAP domain that is highly homologous in α- and β-chimaerins is present at the C-terminus. A unique feature of chimaerin Rac-GAPs is that they are regulated by the lipid second messenger diacylglycerol (DAG), a product of phospholipases generated in response to receptor activation (*12*). All four chimaerin isoforms have a single copy of the C1 domain, a 50 amino acid motif originally characterized as the binding site for DAG and phorbol esters in protein kinase C (PKC) isozymes. *In vitro* studies established that the C1 domain in chimaerins, like those in PKCs, has the ability to bind DAG and phorbol esters with high affinity in a phosphatidylserine- (PS-) dependent manner (*13*, *14*). Moreover, α- and β-chimaerins translocate to membranes in response to phorbol ester treatment or upon stimulation of receptors coupled to DAG generation such as the EGF receptor (*15*, *16*). Mutations of key C1 domain residues in chimaerins abolish phorbol ester/DAG binding and translocation, arguing for an essential role of this domain in intracellular targeting and activation of these Rac-GAPs (*12*).

X-ray crystallography analysis of full-length β2-chimaerin revealed that the C1 domain is buried in the structure with the DAG/phorbol ester binding site occluded by intramolecular interactions with both N- and C-terminal regions (*17*). Therefore, despite the similar nanomolar binding affinities for phorbol esters displayed by isolated C1 domains in *in vitro* radioligand assays (*14*), it is not surprising that a higher concentration of phorbol ester is required for promoting the translocation of β2-chimaerin to membranes relative to PKC isozymes with exposed C1 domains. Studies have shown that the EC_{50} for translocation of PKCα by phorbol 12-myristate 13-acetate (PMA) is ~10 nM, while the EC_{50} for β2-chimaerin is 2 orders of magnitude higher. Remarkably, disruption of key intramolecular interactions between the C1 domain and regions that wrap around the C1 domain renders β2-chimaerin more sensitive to phorbol ester-induced membrane translocation (*17*). For

†This work is supported by Grant R01-CA74197 from the NIH to M. G.K. and in part by the Intramural Program of the NIH, Center for Cancer Research, National Cancer Institute.

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[1]Abbreviations: biotin-X-DHPE, *N*-(6-(biotinoylamino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FRET, fluorescence resonance energy transfer; GAP, GTPase activating protein; GDP, guanosine diphosphate; GST, glutathione *S*-transferase; GTP, guanosine triphosphate; GEF, guanine nucleotide exchange factor; MDCC, *N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TCR, T-cell receptor.

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Published on Web 07/20/2009

example, an Ile to Ala mutation in position 130, a residue in the SH2 domain that makes contact with the C1 domain, increases the sensitivity to PMA-induced translocation by ~100-fold. Similarly, mutation in Leu 28, which is located in the N-terminus and also makes contact with the C1 domain, causes a remarkable sensitization for PMA-induced translocation (*17*). It is conceivable that these mutants adopt a conformation that is energetically favorable for ligand binding, thus arguing that chimaerin activation requires a major conformational change in order to expose the C1 and Rac-GAP domains as well as hydrophobic membrane binding surfaces. To date, there is no biochemical evidence that mutants hypersensitive to ligand-induced translocation in cells present enhanced access to ligand.

In this study, we characterized the phorbol ester binding properties of β2-chimaerin internal contact mutants using the radioligand [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu). We observed that mutants hypersensitive to phorbol ester-induced translocation possess a lower acidic phospholipid requirement for ligand binding. Moreover, these mutants present higher Rac-GAP activity *in vitro* than wild-type β2-chimaerin. Our studies suggest a model in which inactive β2-chimaerin exists in a conformation that is not favorable for translocation and predict that a significant structural rearrangement would be required in order to expose the C1 domain and translocate the protein to membranes where it inactivates Rac.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phospho-L-serine, and avidin high capacity agarose were obtained from Sigma. Biotin-X-DHPE was purchased from Molecular Probes (Eugene, OR). Thrombin, glutathione−Sepharose 4B, and benzamidine−Sepharose 6B were purchased from Amersham Biosciences (Pittsburgh, PA). 1,2-Dioleoyl-*sn*-glycerol was purchased from Avanti Polar Lipids (Alabaster, AL). [^{3}H]PDBu was purchased from Perkin-Elmer Life Sciences (Waltham, MA).

Expression and Purification of Recombinant Wild-Type β2-Chimaerin and Mutants. Generation of β2-chimaerin mutants is described elsewhere (*17*). Inserts encoding for β2-chimaerin mutants were isolated from their corresponding pEGB vectors and subcloned in-frame into the baculovirus vector pAcG2T (Pharmingen, San Diego, CA). Recombinant baculoviruses were generated with the BaculoGold transfection kit (Pharmingen). To express the recombinant proteins, Sf9 cells in spinner flasks (1 L) were infected at a multiplicity of infection of 1 pfu/cell with the corresponding recombinant baculoviruses. After 48 h, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 μg/mL aprotinin, 5 μg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1 μM pepstatin A. After sonication, cell homogenates were centrifuged at 20000*g* for 30 min, the supernatants collected, and GST-fusion proteins bound to glutathione−Sepharose 4B beads following the protocol recommended by the manufacturer. To cleave the GST partner, beads were incubated overnight with thrombin at 4 °C. Thrombin was removed with benzamidine−Sepharose 6B beads. Purified proteins were aliquoted and stored at −80 °C in 25% glycerol.

Binding of β2-Chimaerin to Lipid Vesicles. For the generation of lipid vesicles, phospholipids in chloroform were dried down under a stream of nitrogen, suspended in 50 mM Tris-HCl (pH 7.5) by brief vortexing, and sonicated using a model 100 Sonic dismembrator sonicator (10 s, 2 times, setting 5). When DAG was included in the assays, this lipid was dried down and resuspended together with the phospholipids. The total concentration of phospholipids in the vesicles was 100 μg/mL.

For lipid vesicle binding assays, biotin-X-DHPE (5 μg/mL) was included in the vesicles. Vesicles were incubated with 0.3 μg/mL recombinant β2-chimaerin (wild-type or mutants) for 30 min at 25 °C and then precipitated with avidin−agarose beads (25 μL of a 50% solution). After extensive washing with resuspension buffer, vesicles were subjected to SDS−PAGE. Proteins were transferred to nitrocellulose membranes and subjected to Western blot using an anti-β2-chimaerin antibody (*12*). Densitometric analysis was carried out using ImageJ software.

Expression and Purification of Wild-Type GST-Rac1 and GST-V12-Rac1. Expression of wild-type GST-Rac1 and GST-V12-Rac1 in *Escherichia coli* was performed using pGEX-2TK plasmids. GST-Rac1 wild type and GST-V12-Rac1 expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) added to the bacterial culture after it reached an A_{600} = 0.8. After 4 h at 37 °C, cells were pelleted at 6000*g*. GST-fusion proteins were purified as described above. Purity of proteins was >80%, as confirmed by Coomassie blue staining. Recombinant proteins were stored at −80 °C in 25% glycerol.

Radioligand Assays. [^{3}H]PDBu binding was measured using a poly(ethylene glycol) precipitation assay as described earlier (*18*), using 100 μg/mL phospholipids (phosphatidylserine, PS, and/or phosphatidylcholine, PC) for 10 min at 18 °C (unless otherwise indicated). A detailed description of the methodology for the binding assays can be found elsewhere (*19*).

In Vitro Rac-GAP Assays. GST-Rac1 wild type bound to glutathione−Sepharose 4B beads was incubated with 10 mM EDTA for 1 min at 4 °C and then loaded with GTP for 20 min (2 μg of GTPase:80 μM GTP). The reaction was stopped by the addition of 40 mM $MgCl_2$. The loaded GTPase was washed four times in 20 mM Tris (pH 7.5) and 1 mM $MgCl_2$ and eluted from the beads with 10 mM reduced gluthatione in 20 mM Tris (pH 8.0) and 1 mM $MgCl_2$. GTP hydrolysis was measured by determining the binding of released inorganic phosphate to the "Phosphate Sensor" (Invitrogen), a mutant-purified *E. coli* phosphate-binding protein labeled on Ala 197 with the fluorophore *N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) (*20*). Recombinant chimaerins were incubated with loaded GTPase at a concentration of 65 μg/mL. The phosphate-binding protein was then added at a concentration of 0.25 μM. The reaction was followed for 10 min at room temperuature using a Spectromax Gemini 96-well plate reader fluorometer (Molecular Devices). Wavelengths for excitation and emission were 426 and 464 nm, respectively.

RESULTS

We have previously established that β2-chimaerin requires ~100-fold higher concentration of PMA to translocate to membranes compared to PKCα despite the similar affinities of their isolated C1 domains for phorbol esters and DAG (*4*). This suggests a differential exposure of C1 domains in these proteins in their native conformations. According to structural predictions, Leu 28, Ile 130, and Ile 359 make hydrophobic contacts with the C1 domain and stabilize the protein in a conformation that limits the access of C1 ligands (*17*) (Figure 1). Disruption of these intramolecular contacts by site-directed mutagenesis greatly reduces the concentration of phorbol ester required for β2-chimaerin translocation (*17*). While wild-type β2-chimaerin requires low micromolar concentrations of PMA for translocation, as determined by subcellular fractionation assays, L28A-,

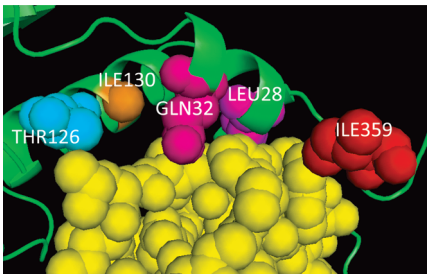

FIGURE 1: Structure of the DAG/phorbol ester binding pocket in β2-chimaerin. Residues implicated in interactions with the C1 domain are shown. The C1 domain is represented in yellow.

I130A-, and I359A-β2-chimaerin are hypersensitive for translocation by PMA in cells (*17*). Whether such mutations in β2-chimaerin facilitate ligand binding to the C1 domain remains unknown. We also used the mutant Q32A-β2-chimaerin, which has moderately enhanced sensitivity to phorbol ester-induced translocation, and T126A-β2-chimaerin, which behaves like wild-type β2-chimaerin in translocation assays. A detailed characterization of the membrane translocation properties of β2-chimaerin mutants in cells has been presented elsewhere (*15*, *17*) (see also Figure 3).

We first examined the phospholipid-dependent phorbol ester binding properties of internal contact mutants L28A-, I130A-, and I359A-β2-chimaerin. Proteins fused to GST were expressed in Sf9 insect cells and purified using glutathione−Sepharose 4B beads. The GST partner was cleaved with thrombin and the protease removed using benzamidine−Sepharose 6B beads. Typically, we obtained 0.5−1 mg of protein/L of Sf9 cells, and the purity of the preparation was in most cases greater than 80%. A representative Coomassie blue stained gel is depicted in Figure 2A.

To assess the phorbol ester binding properties of β2-chimaerin mutants, we used [^{3}H]PDBu as a radioligand. The assay was performed using 100 μg/mL PS vesicles at 37 °C for 5 min. We have previously established that [^{3}H]PDBu binding to β2-chimaerin was strongly dependent on acidic phospholipids (*13*). While little binding is observed in 100% PC vesicles, incorporation of PS into the vesicles greatly enhanced [^{3}H]PDBu binding in a concentration-dependent manner (*13*). Figure 2B shows typical Scatchard plots for the different mutants derived from the binding curves and their corresponding dissociation constants (K_d). In agreement with our previous study (*13*), wild-type β2-chimaerin bound [^{3}H]PDBu in 100% PS vesicles with high affinity ($K_d = 1.4 \pm 0.2$ nM, $n = 3$). β2-Chimaerin internal contact mutants showed similar high affinities for [^{3}H]PDBu in 100% PS vesicles. K_d's were in all cases ~1−2 nM (Figure 2B).

As these experiments were carried out under saturating acidic phospholipid conditions, next we decided to examine the binding properties of β2-chimerin mutants under a limiting PS concentration that resembles that of the inner leaflet of membranes. Binding in 10% PS vesicles was compared to maximum binding under saturating conditions (50% PS). Neutral PC was used to keep the total phospholipid concentration constant (100 μg/mL), as done in previous studies (*13*). [^{3}H]PDBu binding affinity of wild-type β2-chimaerin in 50% PS/50% PC vesicles was similar to that in 100% PS vesicles ($K_d = 1.2 \pm 0.1$ nM, $n = 3$). Figure 3 reveals that mutants L28A-, I130A-, and I359A-β2-chimaerin had significantly higher binding at 10% PS than wild-type or T126A-β2-chimaerin, a mutant with similar translocation properties as wild-type β2-chimaerin. Q32A-β2-chimaerin, which is partially hyperactive in cells and presents moderately enhanced translocation efficiency, shows only a marginal increase in [^{3}H]PDBu binding at 10% PS relative to wild-type β2-chimaerin. The figure clearly shows that mutants with enhanced [^{3}H]PDBu binding presented a lower PMA requirement for translocation. It is conceivable that internal contact β2-chimaerin mutants adopt a conformation that is energetically favorable for accessing to membrane lipids that drive translocation via the C1 domain.

β2-Chimaerin Internal Contact Mutants Have Enhanced Binding to Phospholipid Vesicles. The reduced phospholipid requirement for [^{3}H]PDBu binding of internal site contact mutants prompted us to examine their ability to bind directly to lipids. To address this issue, we developed a precipitation-based lipid binding assay using the neutral phospholipid biotin-X-DHPE. This allowed for the precipitation of vesicles with avidin−agarose beads and subsequent determination of β2-chimaerin in the precipitates by Western blot using an anti-β2-chimaerin antibody. Figure 4A shows a representative experiment in which binding of different concentrations of β2-chimaerin to either PC or PS phospholipid vesicles (100 μg/mL, 95% PC or PS, and 5% biotin-X-DHPE) was determined. Similar to PKC isozymes (*21*−*23*), β2-chimaerin preferentially bound to PS vesicles. Increasing the proportion of PS in the vesicles led to a marked enhancement in β2-chimaerin binding (Figure 4B). The percentage of PS in the vesicles required for achieving 50% binding ($EC_{50\%}$) was 16.8 ± 2.7% (n = 3). Maximum β2-chimaerin binding was observed at 40% PS. Addition of DAG, the natural C1 domain ligand, markedly enhanced β2-chimaerin binding to vesicles (Figure 4C), which is consistent with the established anchoring role of DAG for C1 domain-containing proteins, including β2-chimaerin (*12*, *15*).

Next, we examined the lipid binding properties of internal contact β2-chimaerin mutants. Recombinant wild-type β2-chimaerin as well as mutants L28A- and I359A-β2-chimaerin were incubated with vesicles containing increasing amounts of PS (0−25%, the remaining being 5% biotin-X-DHPE and PC). As shown in Figure 5A, while wild-type β2-chimaerin had a clear dependency on PS for binding to vesicles, L28A- and I359A-β2-chimaerin showed nearly maximum binding in the absence of PS. Indeed, hyperactive mutants L28A-, I130A-, and I359A-β2-chimaerin showed approximately 2.5-fold higher binding to PC vesicles than wild-type β2-chimaerin, while the effect was not observed with Q32A-β2-chimaerin (Figure 5B). This suggests, on one hand, that β2-chimaerin internal contact mutants adopt a conformation that favors their association with lipids. On the other hand, these results argue that neutral phospholipids are capable of fully supporting binding of β2-chimaerin when they adopt an "open" conformation.

Binding of β2-Chimaerin to Lipids Is Not Affected by Active Rac. β2-Chimaerin preferentially binds its target Rac when the GTPase is in its active, GTP-bound form. We have previously determined that β2-chimaerin strongly associates in cells with V12-Rac1, a constitutively active Rac1 mutant, but not with the inactive mutant N17-Rac1 (*15*). It is not known whether Rac1 contributes to the binding of β2-chimaerin to membrane phospholipids. To address this issue, we carried out phospholipid binding assays in either the presence or absence of purified recombinant active GST-V12-Rac1. GST-V12-Rac1 could be readily detected in PS vesicles (Figure 6A). Figure 6B shows that binding of β2-chimaerin to phospholipid vesicles was essentially identical in either the absence or presence of GST-V12-Rac1, suggesting that the activated form of the GTPase does not

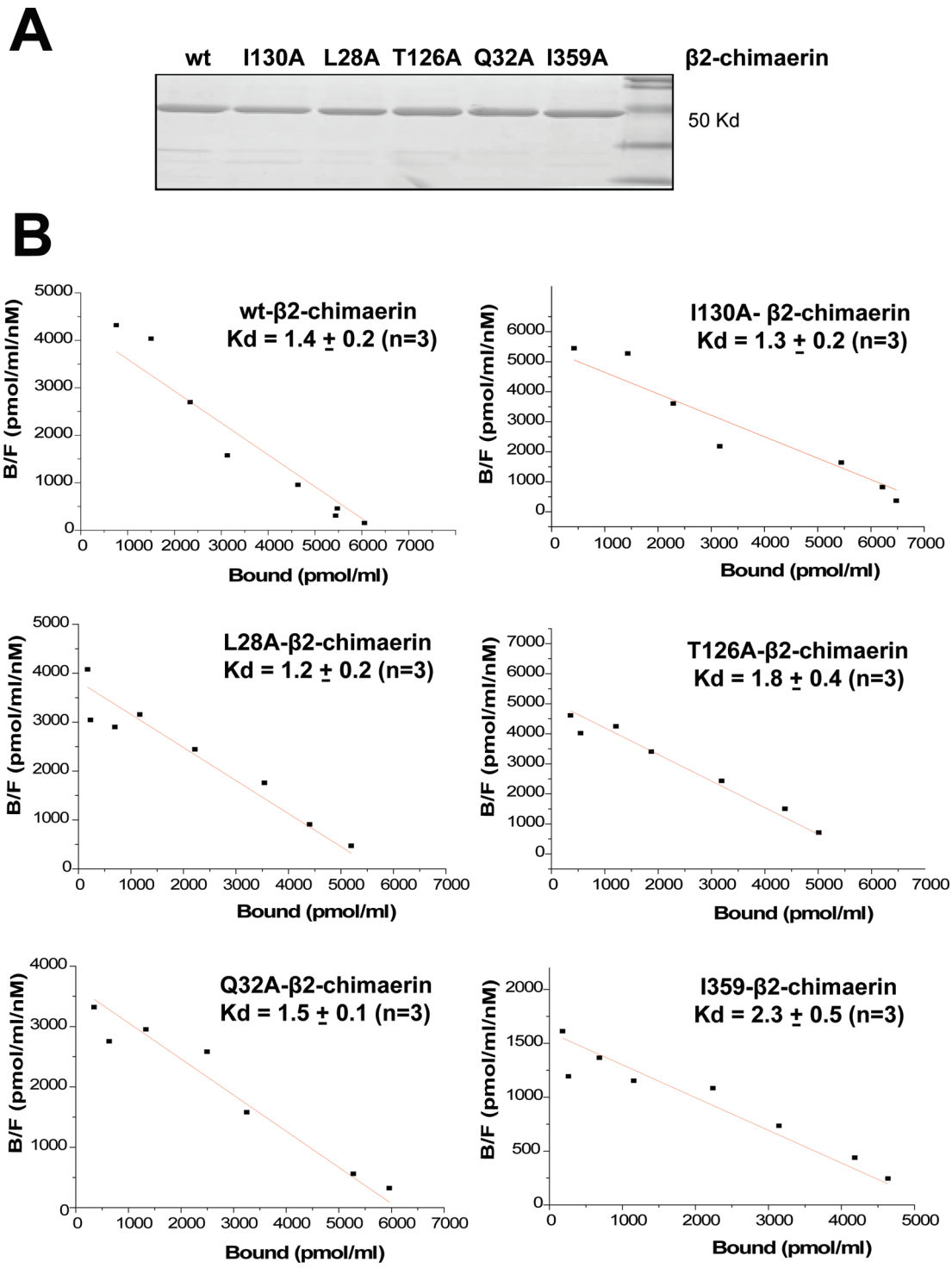

FIGURE 2: Binding of [^{3}H]PDBu to β2-chimaerin mutants. (A) Recombinant proteins were affinity purified with glutathione−Sepharose 4B and cleaved from GST with thrombin. Aliquots of samples were subjected to SDS−PAGE, and the gel was stained with Coomassie Blue. (B) Scatchard plots for [^{3}H]PDBu binding to purified recombinant β2-chimaerin (wild type and mutants) using 100% PS vesicles. The corresponding K_d's are indicated and expressed as mean ± SE ($n = 3$).

significantly contribute to anchoring β2-chimaerin to membrane lipids.

β2-Chimaerin Internal Contact Mutants Have Increased Rac-GAP Activity in Vitro. According to the β2-chimaerin 3-D structure obtained from X-ray crystallography, exposure of the C1 domain upon conformational change should create accessibility of the Rac-GAP domain to Rac. If this hypothesis were true, then internal contact mutants should be hyperactive and have enhanced Rac-GAP activity. In order to measure GTP hydrolysis from Rac1, we used an inorganic phosphate-binding protein coupled to the fluorophore MDCC. Phosphate binding to this phosphosensor causes maximum fluorescence emission at 464 nm. Rac1 was loaded with GTP, and GTP hydrolysis was monitored over time. Figure 7 shows that wild-type β2-chimaerin accelerated GTP hydrolysis from Rac1 with a $t_{1/2} = 3.6 \pm 0.8$ min ($n = 3$). Spontaneous GTP hydrolysis was negligible. Interestingly, β1-chimaerin, a spliced variant that lacks the autoinhibitory N-terminal domain, showed a very fast rate of

GTP hydrolysis, and nearly maximum GTP hydrolysis could be observed at the earliest time point of the assay. Remarkably, internal contact mutants showed a faster rate of GTP hydrolysis compared to wild-type β2-chimaerin. The $t_{1/2}$'s for L28A- and I130A-β2-chimaerin mutants were 0.16 ± 0.03 ($n = 3$) and 0.16 ± 0.05 min ($n = 3$), respectively. Q32A-β2-chimaerin, which is only partially hypersensitive for phorbol ester-induced translocation, hydrolyzed GTP from Rac1 slightly faster than wild-type β2-chimaerin, although with a rate that was slower than those of the L28A- and I130A-β2-chimaerin mutants ($t_{1/2} = 0.9 \pm 0.2$ min, $n = 3$). In all cases, maximum activity was observed at ~10 min. Taken together, these results suggest that mutations in key residues involved in intramolecular contacts not only facilitate the access of C1 domain ligands and lipid binding but also enhance the exposure of the Rac-GAP domain, leading ultimately to enhanced GTP hydrolysis from Rac1.

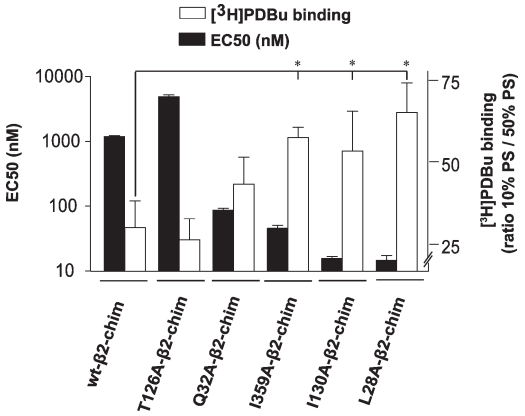

FIGURE 3: Enhanced [^{3}H]PDBu binding of β2-chimaerin internal contact mutants. Black bars: EC_{50} for PMA-induced translocation of β2-chimaerin (wild type and mutants) in Cos-1 cells (adapted from ref *17*). White bars: [^{3}H]PDBu binding ratio (10% PS/50% PS). Total lipid concentration was 100 μg/mL. Data are expressed as mean $\pm$ SE ($n = 7-9$ for EC_{50}; $n = 3$ for binding ratio). *, $p < 0.05$.

DISCUSSION

While PKC isozymes have been extensively characterized as receptors for the lipid second messenger DAG and phorbol esters, studies in the last years have established that other proteins with C1 domains can also bind DAG and phorbol esters with high affinity (*5*). The chimaerin Rac-GAPs represent a family of DAG/phorbol ester receptors that have recently gained signifi-

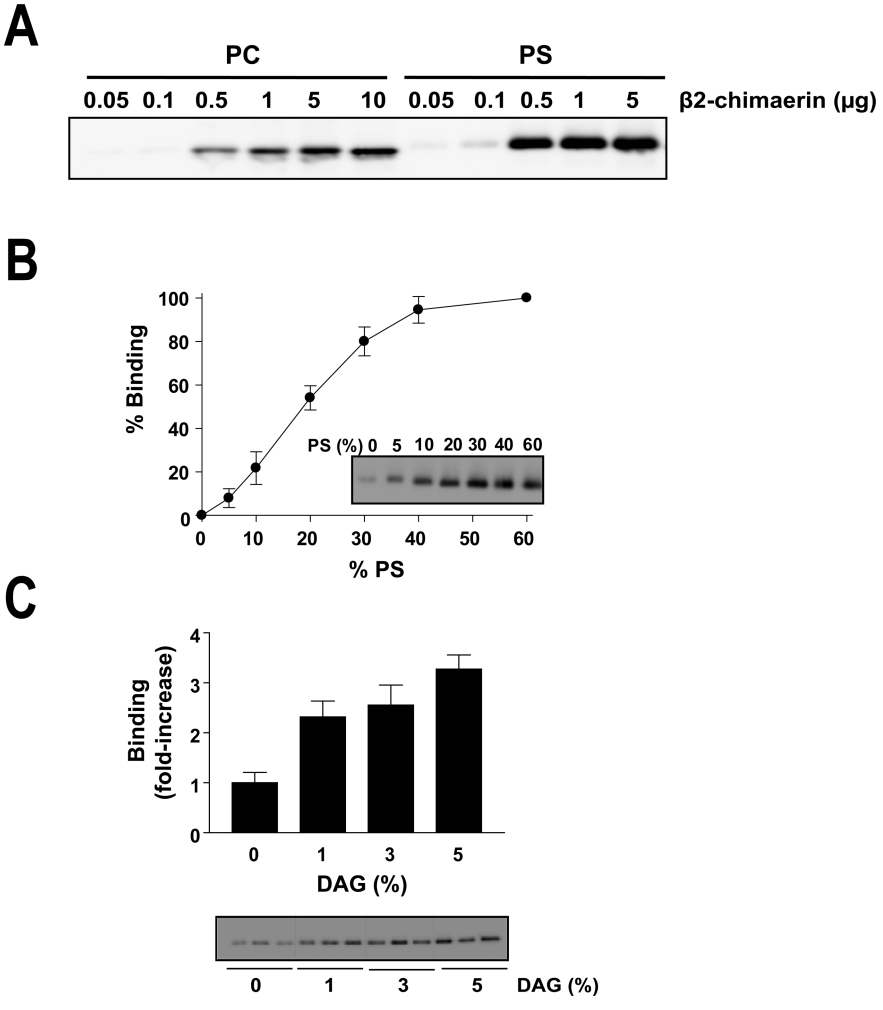

FIGURE 4: PS enhances binding of β2-chimaerin to vesicles. (A) Increasing amounts of purified β2-chimaerin were incubated with lipid vesicles containing 95% PC or 95% PS for 30 min at 25 °C. The remaining 5% was biotin-X-DHPE. Vesicles were precipitated with avidin–agarose beads and subjected to Western blot using an anti-β2-chimaerin antibody. (B) β2-chimaerin (0.3 μg) was incubated for 30 min at 25 °C with vesicles containing variable PS concentrations and 5% biotin-X-DHPE (the remaining phospholipid is neutral PC). Vesicles were precipitated with avidin–agarose beads and subjected to Western blot. Densitometric analysis of three independent experiments is shown. (C) β2-Chimaerin was precipitated from 7.5% PS vesicles containing increased DAG concentrations and subjected to Western blot. A representative experiment with triplicate samples is shown. Data are expressed as mean $\pm$ SD. Two additional experiments gave similar results.

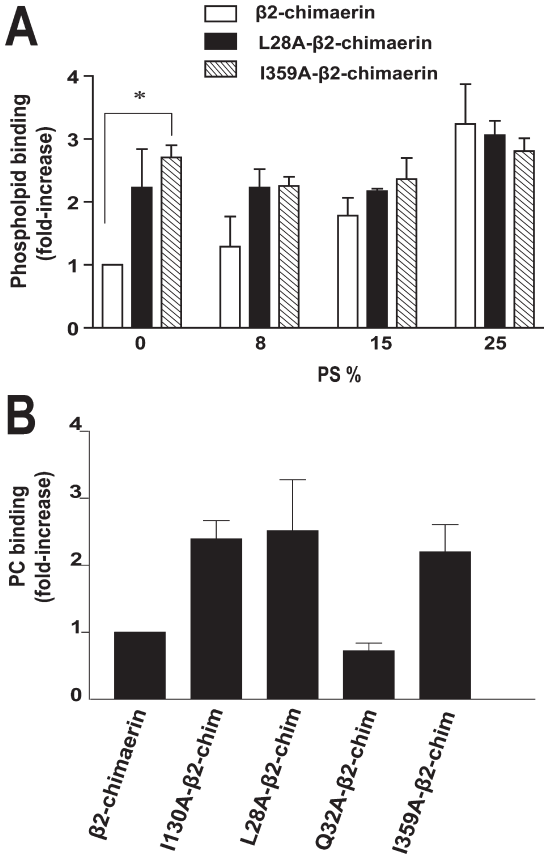

FIGURE 5: Enhanced binding to phospholipid vesicles of β2-chimaerin internal contact mutants. (A) Recombinant β2-chimaerin wild type and mutants were incubated with vesicles containing different proportions of PS and 5% biotin-X-DHPE (the remaining phospholipid is neutral PC). Vesicles were precipitated, and β2-chimaerins in the vesicles were determined by Western blot. (B) Vesicles containing 95% PC and 5% biotin-X-DHPE were incubated for 30 min at 25 °C in the presence of 0.5 μg of the indicated recombinant proteins. β2-Chimaerins in the precipitated vesicles were determined by Western blot. Densitometric analysis of three independent experiments is presented, expressed as mean ± SE. Results are normalized to β2-chimaerin wild type (0% PS). *, $p < 0.02$.

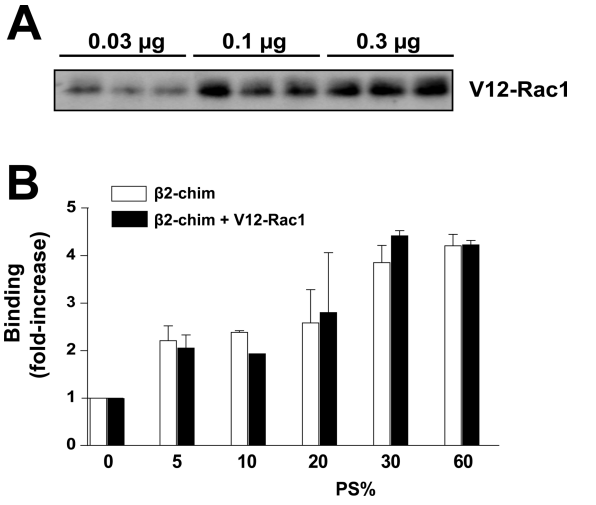

FIGURE 6: V12-Rac1 does not affect binding of β2-chimaerin to PS vesicles. (A) Increasing amounts of purified V12-Rac1 were incubated with vesicles containing 7.5% PS, 5% biotin-X-DHPE, and 87.5% PC for 30 min. After precipitation with avidin−agarose beads, V12-Rac1 levels in the precipitated vesicles were determined by Western blot using an anti-Rac antibody. (B) Purified V12-Rac1 was incubated for 30 min with lipid vesicles containing increasing amounts of PS and 5% biotin-X-DHPE (the remaining lipid is PC). β2-Chimaerin (0.5 μg) was added to lipid vesicles. β2-Chimaerin levels in precipitated vesicles were determined by Western blot. Densitometry analysis of a representative experiment is shown. Data are presented as mean ± SD of triplicate samples. An additional experiment gave similar results.

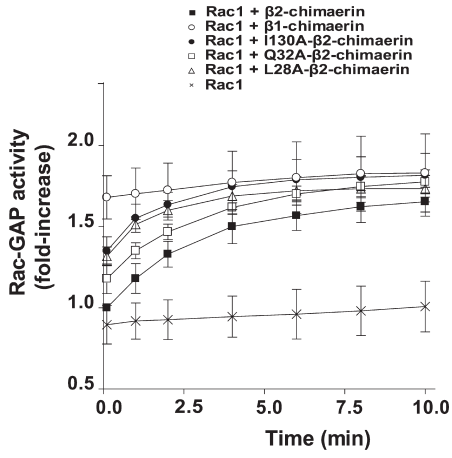

FIGURE 7: Enhanced Rac-GAP activity of β2-chimaerin internal contact mutants. GTP hydrolysis was determined by using a "phosphate sensor", as described in Experimental Procedures. Data are expressed as mean ± SE of three independent experiments.

cant attention due to their implications in development, neuritogenesis, and cancer progression (*6*−*11*). Similar to PKCs, chimaerins can redistribute to membranes in cellular models in response to phorbol ester stimulation, an effect mediated by direct ligand binding to the C1 domain (*4*, *12*, *15*). Moreover, stimulation of receptors that generate DAG via phospholipase C, such as the EGF receptor, promotes the translocation of chimaerins to membranes. This intracellular redistribution is essential for binding to activated Rac, as demonstrated in previous studies using a FRET approach (*15*). It is intriguing that despite the fact that the phorbol ester/DAG binding site in α2- and β2-chimaerins is not exposed, mutations in the C1 domain impair translocation (*12*, *16*), arguing that this domain is essential for protein relocalization. This led us to hypothesize that a conformational rearrangement would be needed in order to expose the ligand binding site. Since the Rac binding site in the GAP domain is not exposed either (*17*), such conformational change would also be required for the activation of Rac-GAP activity.

The present study supports the evidence that β2-chimaerin is regulated by an autoinhibitory mechanism. Lipophilic intramolecular interactions maintain β2-chimaerin in a conformation that is not favorable for C1 domain ligand binding. Structural analysis predicts that Leu 28 in the N-terminal region forms hydrophobic bonds with Trp 234 and Phe 232 in the C1 domain. Ile 130 is buried between the N-terminal and the C1 domains and stabilizes the tertiary structure through interaction with Trp 234. Ile 359 in the Rac-GAP domain region interacts with Trp 225 in the C1 domain to stabilize an autoinhibited conformation (Figure 1). Disruption of these key intramolecular contacts by site-directed mutagenesis sensitizes β2-chimaerin to translocation by phorbol esters and enhances the association to Rac1 as determined by FRET (*15*, *17*). Here we provide biochemical evidence that the C1 domain in β2-chimaerin is more accessible to lipid binding when intramolecular contacts are disrupted. When phorbol ester binding was determined using

[^{3}H]PDBu at saturating PS conditions (100% PS), maximum binding was obtained, in agreement with previous studies (*13*). Binding affinities are in the same range as those of PKC isozymes. However, when limited proportions of PS are used, it became evident that β2-chimaerin internal contact mutants show higher binding than wild-type β2-chimaerin, suggesting that the energetic requirement for ligand binding is lower for mutant chimaerins and that exposure of the C1 domain is facilitated when intramolecular contacts that occlude this domain are disrupted. Similarly, when binding of β2-chimaerin to PS vesicles was assessed, we found that less PS is required for the association of mutants compared to wild-type β2-chimaerin. Although the precise PS binding sites in chimaerins have not yet been determined, C1 domains are known to interact with membrane phospholipids, and acidic phospholipids are essential cofactors for phorbol ester and DAG binding to C1 domains (*12*, *13*). Altogether, our results argue that β2-chimaerin needs to undergo a conformational change to expose membrane binding sites and concomitantly become activated. A recent study with the related isoform α2-chimaerin revealed that mutations in hydrophobic residues that expose the C1 domain greatly facilitate its access to the plasma membrane and enhance Rac-GAP activity in cellular models (*16*), suggesting common regulatory mechanisms for α2- and β2-chimaerins, which both represent the larger spliced versions from chimaerin CHN1 and CHN2 genes, respectively.

While the precise mechanism of cellular activation of chimaerins remains to be elucidated, our studies suggest that association with membrane lipids contributes to triggering the conformational change that exposes the C1 domain for ligand binding. DAG greatly enhances β2-chimaerin binding to phospholipid vesicles. On the other hand, activated Rac has no impact on β2-chimaerin binding to lipid vesicles. This is consistent with studies in cellular models that revealed negligible direct contribution of V12-Rac1 to β2-chimaerin plasma membrane association (*15*). However, activated Rac can facilitate translocation of β2-chimaerin to the plasma membrane in an indirect manner through the activation of phospholipase C and DAG generation (*15*). It is remarkable that internal contact β2-chimaerin mutants bind efficiently to PC vesicles, while full binding for wild-type β2-chimaerin requires PS. This is not unexpected from structural predictions that suggest that multiple hydrophobic contacts would be required to sustain β2-chimaerin in an "open" active conformation. Previous studies from our laboratory showed that the *in vitro* Rac-GAP activity of β2-chimaerin was slightly increased in the presence of 20% PS vesicles but markedly augmented in 100% PS vesicles. Therefore, PS can act as a platform for β2-chimaerin activation by DAG, while neutral phospholipids may support binding of the active conformation to membranes. It cannot be ruled out that the differences observed relate to distinct structural properties of vesicles of different composition. The limited translocation of β2-chimaerin by a potent C1 domain ligand such as PMA also suggests that additional regulatory mechanisms may take place in a cellular context. We speculate that one such mechanism may involve protein−protein interactions. Recent studies have identified the adaptor Nck as a chimaerin-binding protein (*7*, *24*). We have recently found that both α2- and β2-chimaerins bind Nck preferentially when located in the cytoplasm and that upon translocation to membranes this interaction is lost (Colon-Gonzalez and M. G. Kazaniez, unpublished observations). When complexed with Nck, chimaerin relocalization may be impeded; thus the dissociation of the complex would be required to free chimaerin for membrane redistribution. Posttranslational modifications may also be implicated in the modulation of chimaerin relocalization. Such mechanisms have been extensively described for PKCs and RasGRP3, a DAG-regulated Ras-GEF (*25*, *26*). For example, Tyr phosphorylation in PKCθ leads to a conformational change that exposes the C1 domains for membrane interactions (*27*). Emerging evidence suggests that Tyr phosphorylation of α2- and β2-chimaerin modulates their Rac-GAP activity (*28*). Multiple inputs may be therefore necessary to achieve the structural rearrangement required for full activation of chimaerins, and these mechanisms may vary according to cell type and stimuli. For example, a recent study by Siliceo et al. reported that Tyr phosphorylation of β2-chimaerin in T cells in response to TCR receptor activation negatively regulates its Rac-GAP activity (*29*). DAG-independent activation of chimaerins has also been reported in T cells (*30*).

In summary, our data support a model for the lipid activation of β2-chimaerin that requires a conformational change in order to achieve full activation of this Rac-GAP. Point mutations in key residues responsible for autoinhibition lead to the exposure of the C1 domain for ligand binding and enhanced Rac-GAP activity. Interestingly, a recent study identified somatic mutations in Leu 20 in α2-chimaerin (equivalent to Leu 28 in β2-chimaerin), as well as in other residues involved in intramolecular contacts in patients with Duane's retraction syndrome, leading to Rac-GAP hyperactivation in motor neurons (*31*). Presumably this hyperactivation is driven by lipid signals via the C1 domain. Deciphering the biochemical basis for the activation of chimaerin Rac-GAPs may therefore have significant physiopathological relevance.

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