The regulation and function of chimaerins, a family of "non-protein kinase C" (PKC) phorbol ester/diacylglycerol receptors with Rac-GAP activity, is largely unknown. In a search for chimaerin-interacting proteins, we isolated Tmp21-I (p23), a protein localized at the perinuclear Golgi area. Remarkably, phorbol esters translocate β2-chimaerin to the perinuclear region and promote its association with Tmp21-I in a PKC-independent manner. A deletional analysis revealed that the C1 domain in chimaerins is required for the interaction with Tmp21-I, thereby implying a novel function for this domain in protein-protein associations in addition to its role in lipid and phorbol ester binding. Our results support the emerging concept that multiple pathways transduce signaling by phorbol esters and revealed that, like PKC isozymes, chimaerins are subject to a posttranscriptional regulation. In this setting, Tmp21-I serves as an anchoring protein that determines the intracellular localization of these novel phorbol ester receptors.

Protein kinase C (PKC) isozymes, a family of related serine/threonine kinases, were the first receptors isolated for the phorbol ester tumor promoters and the second messenger diacylglycerol (DAG) (1, 2). It is now well established that phorbol esters and DAG also bind to multiple proteins lacking kinase activity, including the Rac GTPase-activating proteins (GAPs) α- and β-chimaerins, the Ras exchange factor RasGRP, and the Unc-13/Munc13 family of scaffolding proteins. These novel "non-kinase" phorbol ester receptors possess a single copy of the C1 domain, the cysteine-rich motif responsible for binding of phorbol esters and DAG. Two copies of the motif (C1a and C1b) are present in the regulatory domain of phorbol ester-responsive PKCs (classical PKCs, cPKCs, and novel PKCs, nPKCs). The 50 or 51 amino acid C1 domains have the motif HX_{13-14}CX_4CX_4HX_4CX_2C, where X is any other amino acid (3–5). C1 domains are required for the association of PKCs and novel non-kinase phorbol ester receptors with membranes (6–8).

The chimaerin family of phorbol ester receptors includes four related isoforms (α1- or n-, α2-, β1-, and β2-chimaerin), which are spliced variants from the α- and β-chimaerin genes. The C1 domain in chimaerin isoforms has ~40% identity with those in PKC isozymes. The identity between the C1 domains of α- and β-chimaerin is 94% (3, 9–11). The C-terminal region of chimaerins possesses high homology to BCR, the breakpoint cluster region protein involved in Philadelphia chromosome translocation in chronic myelogenous leukemia. This domain was reported to have GAP activity for Rac (12), a small GTP-binding protein that plays a key role in actin cytoskeleton organization, adhesion, migration, gene expression, and mitogenesis (13). The main structural difference among chimaerin isoforms is the presence of a putative N-terminal SH2 domain in the spliced forms α2- and β2-chimaerin. Although the distribution of α1- and β1-chimaerin is restricted mainly to brain and testis, respectively, α2- and β2-chimaerin isoforms are widely expressed (3, 9–11).

We have established that chimaerin isoforms bind phorbol esters with affinities that are in the same range as those of cPKCs and nPKCs (low nanomolar). Using the radioligand 3H-labeled phorbol 12, 13-dibutyrate, we have determined that binding is phospholipid-dependent and that phosphatidylserine is the most efficient phospholipid for the reconstitution of binding (14). Interestingly, phorbol esters and related ligands promote the subcellular redistribution or translocation of β2-chimaerin, a mechanism that has been extensively described for the phorbol ester-responsive cPKCs and nPKCs. Translocation of β2-chimaerin by phorbol esters and DAG is entirely dependent on the binding of the ligand to the C1 domain and is abrogated by the mutation of essential cysteines within the C1 domain (8, 15). Translocation of β2-chimaerin requires higher concentrations of phorbol esters than PKCs, suggesting a differential sensitivity for redistribution. A distinctive feature of β2-chimaerin is that phorbol esters promote its translocation from the cytosol to a perinuclear compartment in addition to the plasma membrane. Indeed, recent co-localization experiments revealed that β2-chimaerin translocates to the Golgi network, suggesting a specialized function for this novel family of phorbol ester/DAG receptors (8).

The regulation and function of chimaerin isoforms are basically unknown. It has been reported that chimaerins accelerate the hydrolysis of GTP to GDP from Rac in in vitro assays (8, 12), although evidence for their Rac-GAP activity in cellular models is limited. To begin elucidating the function and regulation of chimaerins, we sought to isolate proteins that interact...
with this novel family of phorbol ester/DAG receptors using a yeast two-hybrid approach. In this screen we have identified positional mechanisms similar to those reported for PKC-β isoforms may regulate the function of these Rac-GAP proteins.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA) and GF 109203X were purchased from LC Laboratories (Woburn, MA). Cell culture reagents were obtained from In VitroGen. Reagents for the expression and purification of recombinant glutathione S-transferase (GST) fusion proteins and Gammabind G-Sepharose were purchased from Amersham Biosciences, Inc. Yeast culture reagents and media were obtained from CLONTECH (Palo Alto, CA). ONPG (O-nitrophenyl-β-d-galactopyranoside) was obtained from Sigma. Reduvia t-(35)S-methionine was purchased from PPL Biochemicals, Inc. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Plasmid DNA was sequenced on an ABI 377 sequencer.

Plasmid Construction— Constructs for the yeast two-hybrid screening were made in pLexA vector (CLONTECH), p1-α-Chimaerin (aa 1–57, aa 1–80, aa 1–120, aa 1–147, aa 140–334, aa 1–334), β1-chimaerin (aa 1–120, aa 113–295, aa 1–295), and β2-chimaerin (aa 1–291, aa 1–446) were amplified by PCR from cDNA clones in pCR3 vector (Stratagene) and subcloned in frame as EcoRI-BamHI or EcoRI-XhoI fragments into pLexA. pCR3e (15), pcDNA3.1/V5-His (Invitrogen), pEGFP-C2/C3 (for expression of green fluorescent protein (GFP) fusion proteins, CLONTECH), and pEGF were used as mammalian expression vectors. pEGF (generous gift from Dr. Margaret M. Chou, University of Pennsylvania School of Medicine) is a mammalian expression vector for the expression of GST fusion proteins. pCR3e-α1-chimaerin (aa 1–147, aa 140–334, aa 1–334), pCR3e-β1-chimaerin (aa 1–120, aa 113–295, aa 1–295), and pCR3e-β2-chimaerin (aa 1–291, aa 1–466) constructs were obtained by ligating the corresponding inserts in-frame into either Xhol-MluI or EcoRI-XhoI sites in pCR3e. pEGFP-α1-chimaerin (aa 1–147, aa 140–334, aa 1–334), pEGFP-β1-chimaerin (aa 1–120, aa 113–295, aa 1–295), and pEGFP-β2-chimaerin (aa 1–291, aa 1–466) constructs were generated by inserting the corresponding fragments into EcoRI-BamHI HI sites in pCR3-C2 vectors (8). His6-α-chimaerin (aa 219) was generated by reverse transcriptase-PCR from human smooth muscle mRNA using the following primers (sense 5′-CCGCGCTGGAACATGTTCTGGTTGTTGCGCC, antisense: 5′-CCGGAGATCTCCTAACATTAATTCGGGACC, BamHI and EcoRI sites underlined), pcDNA3.1-V5/Temp21-I (full-length; a 219, aa 1–291, aa 1–334) were obtained by ligating the corresponding inserts in-frame into either Xhol-MluI or EcoRI-XhoI sites in pCR3e. pEGFP-α1-chimaerin (aa 1–147, aa 140–334, aa 1–334), pEGFP-β1-chimaerin (aa 1–120, aa 113–295, aa 1–295), and pEGFP-β2-chimaerin (aa 1–291, aa 1–466) constructs were generated by inserting the corresponding fragments into EcoRI-BamHI HI sites in pCRII vectors (8). His6-β1-chimaerin (aa 219) was performed under conditions that yielded a linear response. The intensity of the β2-chimaerin immunoreactivity in the precipitates was normalized by the intensity of the corresponding bands in total lysates.

Co-immunoprecipitation Studies—COS-1 cells at ~50% confluency were co-transfected with pEGF-Bmp21-I (aa 1–219 or aa 1–219) and empty vector (pEGF) and pCR3e-β2-chimaerin. Sixty hours later, cells were washed twice with cold PBS and then lysed at 4 °C for 30 min in 300 μl of lysis buffer containing 50 μM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (5 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml peptatin A). Twenty μl of glutathione-Sepharose 4B beads were added to the lysate and incubated overnight at 4 °C. The beads were extensively washed in lysis buffer and boiled. The samples were then resolved in a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane for Western blot analysis. In some experiments, cells were treated with different concentrations of PMA for 1 h in the presence or absence of the PKC inhibitor GF 109203X (added 30 min before and during PMA incubation). Ethanol (vehicle) was used as a control for PMA. The intensity of the bands was determined by densitometry using a ScanJet Scanner, version 1.00 (Molecular Dynamics, Inc., Sunnyvale, CA). Western blot analysis was performed under conditions that yielded a linear response. The intensity of the β2-chimaerin immunoreactivity in the precipitates was normalized by the intensity of the corresponding bands in total lysates.

Cell Culture and Transfections—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 atmosphere at 37 °C. Cells in 6-well plates at ~50% confluence were transfected with different mammalian expression vectors (1–2 μg) using FuGENE (Roche Molecular Biochemicals) according to the manufacturer’s protocol.

Expression of GST Fusion Proteins in E. coli—GST fusion proteins were expressed in bacteria after induction for 4 h with 1 mM isopropanol-1-galactopyranoside. One unit of β-galactosidase activity was defined as the amount that hydrolyzes 1 μmol of ONPG to O-nitrophenol and β-galactose per minute and per cell.

Yeast Two-hybrid Screen—The full-length human β1-chimaerin cDNA was used as a bait, pLexA-β2-chimaerin, which contains a His Marker, was co-transformed with a human fetal brain cDNA library (Trp marker, gift from Dr. E. Golemis, Fox Chase Cancer Center) and p50OP-LaUz reporter vector (Ura marker) into the yeast strain EGY48. Transformants were plated on yeast drop-out medium lacking tryptophan, uracil, histidine, and leucine, thereby selecting for the plasmids encoding proteins capable of two-hybrid interaction as evidenced by transactivation of leucine prototrophy and the LacZ reporter gene (16). β-Galactosidase Liquid Assays—Yeast was cultured in galactose/raffinose/-His/-Ura/-Trp liquid SD selection medium until the cells were in mid-log phase (A600 = 0.5–0.8). Cells were pelleted at 14,000 × g for 30 s and resuspended in 300 μl of a buffer (pH 7.0) containing 60 mM Na2HPO4, 40 mM NaH2PO4·H2O, 10 mM KCl, 1 mM MgSO4, and 0.27% (100 mM) 2-mercaptoethanol. One hundred μl of each sample were then frozen and thawed three times in liquid nitrogen and a 37 °C water bath, respectively, and an additional 700 μl of resuspension buffer were added. ONPG was then added (final concentration: 670 μM/glucose) and the reaction was initiated by the addition of Na2CO3 (final concentration: 300 μM/glucose). β-Galactosidase activity was determined as described by Miller (17). One unit of β-galactosidase activity is defined as the amount that hydrolyzes 1 μmol of ONPG to O-nitrophenol and β-galactose per minute and per cell.
Association of Chimaerins with Tmp21-I

rin mAb (1:1,000, CaloCA et al. (8), anti-V5 mAb (1:5,000, Invitrogen, Carlsbad, CA), anti-Rac mAb (1:2,000, Upstate Biotechnology, Lake Placid, NY), anti-GST polyclonal Ab (1:5,000, kind gift from Dr. Margaret M. Chou, University of Pennsylvania School of Medicine), or anti-GFP mAb (1:25,000, Berkeley Antibody Company). Membranes were then washed three times with 0.1% Tween 20PBS and incubated with anti-mouse (1:3,000, Bio-Rad), anti-rat (1:3,000, Jackson Immunoresearch Laboratory), or anti-rabbit antibodies (1:3,000, Bio-Rad) conjugated to horseradish peroxidase. Bands were visualized by the ECL Western blotting detection system (Amer sham Biosciences, Inc.).

Immunocytochemistry and Confocal Microscopy—Plasmids encoding for chimaerin isoforms or their truncated mutants in pGEX-CorC2 vector (0.2 μg) and Tmp21-I in the V5 epitope-tagged pcDNA3.1 vector (1.8 μg) were co-transfected into COS-1 cells using FuGENE. After 60 h, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After washing once with PBS containing 0.5% SDS and 5% β-mercaptoethanol (37 °C, 30 min) and twice with PBS alone, cells were incubated with an anti-V5 mAb (1:1,500, Invit rogen). As a secondary antibody, a donkey anti-mouse antibody conjugated with Cy3 was used (1:1,000). Slides were mounted using Vectashield and viewed with a Bio-Rad MRC-1024ES laser scanning confocal microscope. The confocal images were processed using Confo cal Assistant™ version 4.02. All the images shown are individual middle sections of projected Z-series mounting.

For co-localization of the endogenous proteins, cells were seeded overnight in 6-well plates with coverslips, and after fixation, they were stained with an anti-beta-chimaerin antibody (8) and an anti-Tmp21-I antibody (kind gift of Dr. Irene Schultz, Homburg-Saar, Germany) at 1:200 and 1:2,000 dilution, respectively. As second antibodies, we used goat anti-rat Cy3-conjugated (1:1, 500) for beta-chimaerin and goat anti-rabbit fluorescein isothiocyanate-conjugated (1:1, 500) for Tmp21-I (Jackson Immunoresearch Laboratory). Confocal analysis was performed as described above.

Determination of Rac-GTP Levels—We used a pull-down assay to isolate Rac-GTP by binding to the p21 binding domain of PAK1 (18). Cells were lysed in a buffer containing 8 μg of GST-p21 binding domain protein, 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl2, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM 3-mercaptopropanol, and protease inhibitors (5 μg/ml 4-1-2-aminoethyl benzenesulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin A). Lysates were centrifuged at 14,000 × g, 4 °C, 10 min) and then incubated with glutathione-Sepharose 4B beads (4 °C, 1 h). After extensive washing, the beads were boiled in loading buffer. The samples were resolved in a non-reducing 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane for Western blot analysis using an anti-rac antibody.

RESULTS

Isolation of Tmp21-I as a Chimaerin-interacting Protein—To identify chimaerin-interacting proteins, we screened a human fetal brain cDNA library using a LexA yeast two-hybrid system. We used as a bait full-length human beta-chimaerin. The tester strain of the screen, EGY48, contained two reporters, LEU2 and LacZ. Among the 1 × 107 cDNA clones screened, 81 positive clones were isolated by the two selection criteria (their ability to turn blue in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and the ability to grow in the leucine-deficient medium). One of the most abundant cDNAs, isolated four times, corresponded to a partial sequence (aa 108–219) of Tmp21-I (p23), a type I transmembrane protein of the p24 family involved in intracellular vesicular trafficking (GenBank™ accession no. X97442).

To confirm the specificity of this interaction and determine the domain of Tmp21-I required for their association, different LexA-fused constructs were generated (Fig. 1A). These plasmids were co-transfected with pB42AD/Tmp21-I (aa 108–219) into the EGY48 (p80P-LacZ) yeast strain. Although pLexA bait proteins are expressed after induction (galactose/raffinose plates) or without induction (glucose plates) (Fig. 1C), Tmp21-I (aa 108–219) in vector pB42AD (HA-tagged) is only expressed in galactose/raffinose plates (Fig. 1D). As shown in Fig. 1B, Tmp21-I strongly interacts with full-length alpha-chimaerin and with its N-terminal region. The interaction of Tmp21-I with a fragment of beta-chimaerin comprising its N-terminal region (β-GAP domain deleted) was also detected, although it seems to be somehow weaker. The interactions are only detected under conditions in which both bait and prey protein were expressed in yeast. Unexpectedly, the full-length beta-chimaerin did not interact with Tmp21-I in yeast. It is likely that in the library screening, Tmp21-I was isolated by interaction with a degradation fragment of beta-chimaerin comprising its N-terminal region and that the interaction site is masked when the full-length protein is expressed in yeast. Indeed, a degradation fragment of beta-chimaerin similar in size to the N-terminal region was observed in Western blots of yeast lysates (Fig. 1C, lane 4 versus lane 5). The interaction of full-length beta-chimaerin and Tmp21-I was nevertheless detected in vitro binding assays and in mammalian cells (see below). In yeast, we were unable to test the interaction with pLexA-β1-chimaerin (full-length) or pLexA-β1-chimaerin (N-terminal region) because yeast transformed with either of those plasmids grew extremely slowly. The reason for this reduced growth is not known. A number of LexA constructs for unrelated proteins (lamin, 5-lipoxigenase, p53, and pRFH2M-1) did not show any interaction with Tmp21-I. The positive interactions were confirmed using assays for β-galactosidase activity in yeast growing in liquid cultures (Fig. 1E).

Direct in Vitro Interaction between the N-terminal Region of Chimaerins and Tmp21-I—In the next set of experiments, we evaluated whether chimaerins associate directly with Tmp21-I using in vitro binding assays. Tmp21-I (aa 108–219) was expressed as a GST fusion protein in E. coli and immobilized on glutathione-Sepharose 4B beads. The beads were incubated with 35S-labeled, in vitro-translated α-, β-, or β-chimaerins, and the presence of bound protein was determined by autoradiography. As shown in Fig. 2, all three chimaerin isoforms interact with in vitro-translated GST-Tmp21-I but do not interact with GST alone.

Similar experiments were performed with truncated forms of chimaerins comprising either the N-terminal or the C-terminal regions. In all cases, chimaerin N-terminal regions (aa 1–147 for α1-chimaerin, aa 1–120 for β1-chimaerin, and aa 1–291 for β2-chimaerin) associate with GST-Tmp21-I but not with GST alone. The C-terminal region of chimaerins (α-GAP or β-GAP domains) did not interact with GST-Tmp21-I or GST (Fig. 2). These results confirm that the interaction occurs at the N-terminal region of α- and β-chimaerins. Furthermore, these data also suggest that the interaction is direct.

Mapping of the Tmp21-I Interacting Site in Chimaerins, the C1 domain—The N-terminal region of α1-, β1-, and β2-chimaerins has a stretch of 90 amino acids that is highly homologous among all isoforms. This region includes the C1 domain, which is almost identical (94% identity) in α- and β-chimaerins (Fig. 3A). To further define the minimum region in chimaerins involved in the interaction with Tmp21-I, we performed a deletion analysis of the N-terminal region of α1-chimaerin. These deletion mutants, shown in Fig. 3B, were subcloned in pLexA, and the LexA fusion proteins were analyzed for their ability to interact with Tmp21-I in yeast. All the mutants were readily detected with an anti-LexA antibody when expressed in yeast (Fig. 3D). Mutants expressing the C1 domain (aa 1–147 and aa 1–130) strongly associate with Tmp21-I. The interaction only occurs when Tmp21-I is expressed in yeast in the presence of galactose/raffinose. Interestingly, the deletion of amino acids 81–130 (C1 domain) totally abolished the interaction with Tmp21-I (Fig. 3, B and C).

Association of β2-Chimaerin with Tmp21-I in COS-1 Cells—To confirm our yeast two-hybrid and in vitro interaction data, we next assessed the interaction in mammalian cells. As a first approach, we co-transfected pEBG/Tmp21-I (aa 108–
or pEBG alone (empty vector) together with pCR3/H9280/H9252/2-chimaerin. pEBG vectors encode for GST fusion proteins that can be purified from cell extracts using glutathione-Sepharose 4B beads. The presence of GST and GST-Tmp21-I was detected in lysates and beads using an anti-GST antibody. After probing the resulting precipitates with an anti-/H9252/2-chimaerin antibody, /H9252/2-chimaerin was detected only in GST-Tmp21-I beads but not in GST beads (Fig. 4A). The interaction of /H9252/2-chimaerin with full-length Tmp21-I in mammalian cells was also detected using a similar approach (see Fig. 5C).

As a second strategy, COS-1 cells were co-transfected with pCR3/H9280/H9252/2-chimaerin and pcDNA3.1/V5-Tmp21-I (aa 108–219), a V5 epitope-tagged plasmid. As a control, we used either pcDNA3.1/V5 (empty vector) or pcDNA3.1/V5-LacZ. Sixty h after transfection, immunoprecipitation with an anti-V5 antibody was performed. As shown in Fig. 4B, /H9252/2-chimaerin was detected in immunoprecipitates from cells expressing V5-Tmp21-I but not in those from control cells. These results confirm the interaction of Tmp21-I with /H9252/2-chimaerin in mammalian cells.

PMA Promotes the Association of /H9252/2-Chimaerin with Tmp21-I in a PKC-independent Manner—Like PKC isozymes, /H9252/2-chimaerin is subject to translocation by phorbol esters and DAGs. We have previously established that after PMA treatment, /H9252/2-chimaerin redistributes from the cytosol to a perinuclear compartment, where it co-localizes with a Golgi network marker. Translocation was not observed when an essential cysteine (cysteine 246) in the /H9252/2-chimaerin C1 domain was mutated to alanine (8, 15), suggesting that the C1 domain in /H9252/2-chimaerin is essential for phorbol ester binding and phorbol ester-induced translocation. An attractive hypothesis is that

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**Fig. 1.** Yeast two-hybrid analysis reveals an interaction between Tmp21-I and chimaerin isoforms. EGY48 yeast were co-transformed with pLexA-fused chimaerins, a LacZ reporter plasmid (p8OP-LacZ), and HA-tagged Tmp21-I (aa 108–219) in pB42AD. A, schematic representation of the different chimaerin constructs. FL, full-length; N, N-terminal; C, C-terminal. B, assay of β-galactosidase activity on non-induction (left) or induction (right) plates. Gal/Raf, galactose/raffinose. C, expression of bait proteins in yeast cell lysates using an anti-pLexA antibody. D, expression of HA-Tmp21-I (aa 108–219) in yeast using an anti HA-tag antibody. E, assay of β-galactosidase activity in liquid cultures using ONPG as a substrate. Experiments were performed in triplicate, and results were expressed as mean ± S.E.

**Fig. 2.** Interaction of Tmp21-I with in vitro translated α1-, β1-, and β2-chimaerins. pCR3e plasmids for full-length or deleted chimae-rin isoforms were transcribed and translated in vitro using a TNT T7 coupled reticulocyte lysate system. One μg of GST or GST-Tmp21-I (aa 108–219) was immobilized on glutathione-Sepharose 4B beads and incubated with the different 35S-labeled products as described under “Experimental Procedures.” The associated proteins were detected by autoradiography. FL, full-length; N, N-terminal; C, C-terminal. Similar results were observed in 2 additional experiments.
association of β2-chimaerin with the perinuclear compartment involves its binding to Tmp21-I. To explore this issue, we evaluated whether PMA promotes the association of β2-chimaerin with Tmp21-I in COS-1 cells. The interaction was determined both by the GST-precipitation method and by co-immunoprecipitation using the anti-V5 antibody, as described above. Fig. 5A shows that the amount of β2-chimaerin associated with GST-Tmp21-I (aa 108–219) in the glutathione-Sepharose 4B beads increases in a dose-dependent manner when cells were treated with PMA (0.03–3 μM, 1 h). Densitometric analysis reveals the following changes in β2-chimaerin immunoreactivity in the beads (fold increase, normalized by total levels in lysates): 0.03 μM PMA, 1.3 ± 0.3; 0.3 μM PMA, 1.5 ± 0.2; 3 μM PMA, 3.3 ± 1.1 (n = 3). Co-immunoprecipitation assays using the anti-V5 antibody also revealed that PMA dose-dependently increases the amount of β2-chimaerin associated with Tmp21-I (aa 108–219) (Fig. 5B). No changes in association were observed when cells were treated with 4α-PMA, an inactive isomer of PMA (data not shown). To rule out the involvement of PKC isozymes in the phorbol ester effect, we used the PKC inhibitor GF 109203X (5 μM). Under this experimental condition, PKC-mediated responses, such as ERK MAPK activation by PMA in COS-1 cells (data not shown) or PMA-induced apoptosis (19), were completely abrogated. We have previously shown that this PKC inhibitor does not affect the perinuclear translocation of β2-chimaerin, which suggested that phorbol ester-induced translocation of chimaerins was independent of PKC (8, 14). Fig. 5B shows that similar results were observed...
both in the absence or presence of the PKC inhibitor, suggesting that the PMA-induced association of β2-chimaerin with Tmp21-I is not mediated by PKC activation but rather by a direct effect of PMA on β2-chimaerin. We performed association experiments using full-length Tmp21-I fused to GST. PMA dose-dependently increases the association of β2-chimaerin to full-length Tmp-21-I bound to glutathione-Sepharose 4B beads (Fig. 5C). Densitometric analysis of these last experiments reveals the following changes in β2-chimaerin immunoreactivity in the beads (fold increase, normalized by total levels in lysates): 0.03 μM PMA, 1.3 ± 0.2; 0.3 μM PMA, 3.1 ± 1.3; 3 μM PMA, 5.7 ± 2.6 (n = 3).

Co-localization of Chimaerin Isoforms and Tmp21-I—To further confirm the association of chimaerins with Tmp21-I, we performed localization studies by confocal microscopy. COS-1 cells were transfected with pcDNA3.1/V5-Tmp21-I (full-length) and plasmids encoding for chimaerins fused to GFPs. As shown previously by others (22), Tmp21-I was localized to a perinuclear Golgi-like structure in COS-1 cells. Fig. 6 reveals a cytoplasmic localization with a significant perinuclear staining for β1-, β2- and β2-chimaerin. Interestingly, Tmp21-I co-localized with GFP-β1-, β2- and β2-chimaerin, as judged by the yellow color observed in the overlapped images. A remarkable finding is that N-terminal fragments of chimaerins (aa 1–147 for β1-chimaerin, aa 1–120 for β1-chimaerin, and aa 1–129 for β2-chimaerin) show a poor cytoplasmic staining and that those proteins were mainly localized to the perinuclear region. These results agree with those from our previous studies showing that the N-terminal region of β2-chimaerin has a perinuclear localization even in the absence of phorbol ester treatment (8). The N-terminal regions of α1-, β1-, and β2-chimaerins fully co-localized with Tmp21-I. In agreement with our experiments...
in yeast and with our in vitro association studies, neither the C-terminal α-GAP domain nor the β-GAP domain co-localize with Tmp-21-I.

In the next set of experiments, we evaluated by confocal microscopy whether PMA could promote the association of β2-chimaerin with Tmp-21-I. In agreement with our co-precipitation and co-immunoprecipitation results, PMA markedly enhanced the co-localization of Tmp-21-I and GFP-β2-chimaerin (Fig. 7A, left panels). The ability of PMA to redistribute β2-chimaerin was confirmed by subcellular fractionation analysis, as we have reported previously (8, 15, 16). These experiments reveal a shift of β2-chimaerin immunoactivity from the soluble (cytosolic) to the particulate fraction after PMA treatment. As expected, Tmp-21-I localized only to the particulate fraction (Fig. 7B). A mutated form of β2-chimaerin (C246A) that is unresponsive to phorbol esters (Fig. 7B, see also Ref. 8) did not co-localize with Tmp-21-I in either untreated or PMA-treated cells (Fig. 7A, right panels). In conclusion, translocation of β2-chimaerin to the perinuclear region results in its association with Tmp-21-I, and it requires an intact C1 domain.

We have also evaluated the co-localization of endogenous β2-chimaerin and Tmp-21-I in COS-1 cells (Fig. 7C). Cells were treated with either 3 μM PMA or vehicle (ethanol) for 1 h and then immunostained with anti-β2-chimaerin (8) and anti-Tmp-21-I antibodies (kind gift of Dr. Irene Schultz, Germany). Although some co-localization was observed in the perinuclear region in the absence of PMA treatment, co-localization was markedly enhanced by the phorbol ester.

Association of β2-Chimaerin with Tmp-21 Regulates Intracellular Rac-GTP Levels—α- and β-chimaerins accelerate GTP hydrolysis from Rac and thereby down-regulate Rac function. We transfected a mammalian expression vector for β2-chimaerin into COS-1 cells and observed a significant reduction in Rac-GTP levels (Fig. 8, A and C). Densitometric analysis shows a 57% reduction in Rac-GTP levels upon β2-chimaerin transfection (Fig. 8B). Because transfection efficiency is ~50% under our experimental conditions, β2-chimaerin may suppress the normal levels of Rac activation. Similar reductions in Rac-GTP levels were observed after transfection of C246A-β2-chimaerin, which has an intact GAP domain. However, expression of mutant (N-terminal, 1–291)-β2-chimaerin (which has the GAP domain deleted) was not able to reduce Rac-GTP levels (Fig. 8, A and B).

Using in vitro GAP assays, it has been demonstrated that the Rac-GAP activity of chimaerins can be enhanced by phospholipids and/or phorbol esters (8, 20), which suggests that upon translocation, chimaerins may be allosterically activated. Interestingly, treatment of COS-1 cells with PMA in the presence of the PKC inhibitor GF 109203X reduces the cellular levels of Rac-GTP (Fig. 8, lanes 1 and 2), suggesting that non-PKC phorbol ester/DAG receptors may be responsible for this effect. To evaluate the functional relevance of the interaction, we assessed the effects of overexpressing Tmp-21-I on Rac-GTP levels. Interestingly, in cells expressing V5-Tmp-21-I, PMA failed to produce a reduction in Rac-GTP levels (Fig. 8, lanes 5 and 6). This suggests that increasing the levels of Tmp-21-I may reduce the availability of chimaerins to access active Rac, probably by sequestering chimaerins at the perinuclear region. The effect of Tmp-21-I can be overcome by overexpression of β2-chimaerin (Fig. 8, lanes 7 and 8). Thus, the availability of β2-chimaerin may be limited by its binding to Tmp-21-I, therefore suggesting that the Tmp-21-I/β2-chimaerin association may be critical to determine the activation status of Rac.

**DISCUSSION**

The isolation of receptors for the phorbol esters and DAGs unrelated to the PKC family suggests a complex regulation of proteins possessing DAG-responsive C1 domains. Although very little information is available on the regulation of chimaerins, it has been shown that these Rac-GAPS are high affinity receptors for phorbol esters and DAG (4, 5, 14, 21). In this study, we have identified Tmp-21-I, a protein localized at the cis-Golgi network, as an interacting protein for chimaerins. The isolation of a Golgi/endoplasmic reticulum protein as a chimaerin-interacting protein was indeed expected based on our previous results showing a perinuclear localization of chimaerins and co-localization with a Golgi network marker (8). Tmp-21-I is a member of the p24 family of transmembrane proteins involved in sorting/trafficking in the early secretory pathway. Although the precise function of p24 proteins remains unclear, it is thought that they function as receptors for cargo exit from the endoplasmic reticulum as well as in trans-

**FIG. 6.** Co-localization of chimaerin isoforms and Tmp21-I by confocal microscopy. COS-1 cells were co-transfected with pEGFP plasmids encoding for different chimaerin isoforms or truncated mutants together with pcDNA3.1/V5-Tmp21-I (full-length). Sixty h later, cells were fixed with 4% paraformaldehyde and processed for immunofluorescence using an anti-V5 antibody. V5-Tmp21-I is visualized as red after staining with Cy3-conjugated donkey anti-mouse secondary antibody. Slides were analyzed by confocal microscopy. Upper images, green fluorescence from GFP fusion proteins; middle images, red fluorescence from Tmp21-I; lower images, overlapped images. FL, full-length; N, N-terminal; C, C-terminal. Similar results were observed in at least 3 independent experiments.
A

GFP-β2-chimaerin (wt) - PMA + PMA
GFP-β2-chimaerin (C246A) - PMA + PMA

GFP-fusion protein
Tmp21-I
Overlapped

B

<table>
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<tr>
<th></th>
<th>Tmp21-I</th>
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<tbody>
<tr>
<td>Total</td>
<td>+</td>
<td>-</td>
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<td>Soluble</td>
<td>+</td>
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<td>Particulate</td>
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C

β2-chimaerin
Tmp21(p23)
Overlapped

- PMA
+ PMA

Fig. 7. Co-localization of β2-chimaerin and Tmp21-I after PMA treatment. A, COS-1 cells were co-transfected with pEGFP-β2-chimaerin or pEGFP-C246A-β2-chimaerin and pcDNA3.1/V5-Tmp21-I (full-length). Sixty h later, cells were treated for 1 h with 3 μM PMA (+ PMA) or vehicle (- PMA) in the presence of 5 μM GF 109203X. Upper images, green fluorescence from GFP-β2-chimaerin; middle images, red fluorescence from Tmp21-I; lower images, overlapped images. B, COS-1 cells were co-transfected with pEGFP-β2-chimaerin or pEGFP-C246A-β2-chimaerin and pcDNA3.1/V5-Tmp21-I (full-length). Sixty h later, cells were treated for 1 h with 3 μM PMA (+ PMA) or vehicle (- PMA) in the presence of 5 μM GF 109203X. After subcellular fractionation, soluble and particulate fractions were subjected to Western blot using anti-GFP (left) or anti-V5 antibodies (right) for the detection of chimaerins and Tmp21-I, respectively. Similar results were observed in at least 3 independent experiments. C, co-localization of endogenous β2-chimaerin and Tmp21-I. COS-1 cells were treated for 1 h with either PMA (+ PMA) or vehicle (- PMA) in the presence of 5 μM GF 109203X and then immunostained with anti-β2-chimaerin and anti-Tmp21-I antibodies, as described under “Experimental Procedures.” The figure shows representative immunofluorescence images. Similar results were observed in 2 additional experiments. wt, wild type.

Interestingly, a PKC-like molecule without phosphorylating activity is required for the production of post-Golgi vesicles (26). In addition, it has been shown that α1-chimaerin regulates Golgi stability during interphase (27). Although our studies do not look into the potential role of chimaerins in vesicular trafficking or Golgi/endoplasmic reticulum function, they reveal a novel role for Tmp21-I as an intracellular receptor for chimaerins in addition to serving as a receptor for cargo exit. Because Tmp21-I may select diverse proteins for inclusion in coatomer-coated vesicles, an attractive possibility is that Tmp21-I participates in the intracellular trafficking and sorting for chimaerins and/or other relevant molecules in Rac signaling. The cross-talk between signaling cascades and the transport machinery is widely recognized (28, 29). Indeed, actin cytoskeletal organization, cell shape, and migration, all Rac-mediated responses, are linked to intracellular transport (30, 31).

As described for PKC isozymes, it is likely that positional regulation of chimaerins dictates their access to substrates and/or regulatory proteins. In the case of cPKCs and nPKCs, phorbol esters and DAGs translocate these PKC isozymes to discrete intracellular compartments, and the association with specific interacting proteins may be the key for determining isozyme localization and substrate specificity. Indeed, a large number of PKC-binding proteins (substrates and/or regulatory proteins) have been isolated that may associate with PKCs either in an active or an inactive state (4, 32, 33). For example, upon activation with phorbol esters or DAG, PKC isozymes can interact with receptors for activated PKCs (RACKs) (32). Interestingly, PKCε interacts with the Golgi protein β-COP, a RACK that localizes this nPKC to the Golgi network (34). The novel phorbol ester receptor Munc13 also translocates from the cytosol to the Golgi network as a consequence of phorbol ester treatment (35). Our results strongly suggest that similar targeting mechanisms may regulate the subcellular redistribution of chimaerins.

Our deleterional analysis revealed that chimaerins require an intact C1 domain for their interaction with Tmp21-I. Thus, C1 domains may have dual roles, both as modules for ligand/lipid recognition and for protein-protein interaction. As described for PKC C1 domains, the C1 domain in chimaerins is required for membrane association. Moreover, phorbol ester binding to the C1 domain of α- and β-chimaerins is phospholipid-dependent (14, 21). It has been reported that PKC C1 domains can interact with proteins such as actin (for PKCα) or PAR4 (for nPKCs) (36–38). Moreover, the C1 domains of PKCε are required for its translocation to Golgi upon phorbol ester stimulation (39). Whether discrete C1 domains of PKC isozymes or chimaerins have distinctive specificities and whether Tmp21-I serves a role as an anchoring protein for PKCs is not known at the present time.

A remarkable finding in this study is that PMA promotes the association of β2-chimaerin with Tmp21-I, as revealed in our co-precipitation and localization studies. It may be possible that, upon activation, a conformational change in β2-chimaerin occurs that exposes the protein-protein interacting sites, a well described mechanism for PKC isozymes. Previous results using deletion mutants of β2-chimaerin have shown that the N-terminal region comprising the C1 domain is necessary for its perinuclear localization. In fact, we found that mutants of chimaerins comprising only the N-terminal region are constitutively localized to the perinucleus. Another important implication from these studies is that a single C1 domain is capable of conferring full responsiveness to phorbol esters. We have identified the C1 domain in chimaerins as the minimum domain required for phorbol ester binding and subcellular redistribution in cells. Previous experiments using single C1 do-
mains of PKCs showed that this 50-amino acid domain is sufficient for ligand binding (40, 41). Moreover, functional inactivation of a single C1 domain in PKC isozymes renders proteins that are still phorbol ester/DAG-responsive (42). Using a single C1 domain of PKCγ fused to GFP, Oancea and Meyer (43) have shown that this domain alone was capable of translocating to membranes upon ligand binding.

An important question is how chimaerins regulate Rac function in cells. Studies from our laboratory revealed that β2-chimaerin accelerates GTP hydrolysis from Rac but not from Cdc42 or RhoA in vitro (8). Overexpression of β2-chimaerin in Rat-1 fibroblasts has profound effects on cell growth, similar to those observed with a dominant negative form of Rac (N17Rac) (44). Chimaerins also inhibit the increase in Rac-GTP levels observed upon stimulation of EGF receptor. Overexpression of β2-chimaerin leads to a reduction in Rac-GTP levels in serum-stimulated COS-1 cells (Fig. 8). The effect is entirely dependent on the β2-chimaerin GAP domain. Interestingly, Tmp21-I overexpression counteracts the chimaerin effect, probably by retaining β2-chimaerin at its perinuclear site. This observation further supports a functional role of Tmp21-I as a chimaerin-anchoring protein. Rac is generally thought to cycle between a cytosolic inactive form and an activated plasma membrane form. Because a pool of β2-chimaerin localizes to the plasma membrane upon activation (8), it is possible that this pool of β2-chimaerin deactivates Rac at that location.

Interestingly, recent studies have shown that a large pool of Rac is located in the perinuclear region and that this pool of Rac is in its inactive, GDP-bound form (45, 46). It is tempting to speculate that chimaerins play a role in the maintenance of this perinuclear pool of Rac in an inactive state before this Rac-GTPase moves to the plasma membrane. An interesting finding is that Rac co-localizes in the perinucleus with ARF6, a member of the ADP-ribosylation factor (ARF) family of GTPases, suggesting a link between membrane traffic and Rac function. Moreover, ARF6 activation is required for Rac to induce its membrane effects (45). Another interesting observation is that other Rho GTPases, such as Cdc42 or Rac/Cdc42 effectors (i.e. IQGAP and Fgd1), also localize to the Golgi apparatus. Indeed, Cdc42 binds to coatomer subunits, and this association is necessary for the transforming activity of Cdc42 (47, 48). Taken together, these observations highlight the potential involvement of transport mechanisms in signaling by small GTPases.

In summary, our findings suggest that Tmp21-I serves as an intracellular receptor for chimaerin isofoms. Our results strongly support a role for C1 domains as targeting modules through lipid-protein and protein-protein interactions.

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Chimaerins, Novel Non-protein Kinase C Phorbol Ester Receptors, Associate with Tmp21-I (p23): EVIDENCE FOR A NOVEL ANCHORING MECHANISM INVOLVING THE CHIMAERIN C1 DOMAIN
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