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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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 isoforms, chimaerins are subject to a positional 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)1 isoforms, 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 HX12CX2CX13/14CX4CX4HX4CX7C, 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 isoforms. The identity between the C1 domains of α- and β-chimaerins 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 phosphatidylinerine 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 the first chimaerin-interacting protein, Tmp21-I (p23), a transmembrane protein with type I topology localized in the cis-Golgi network and involved in intracellular vesicular trafficking. Remarkably, the association of chimaerins with Tmp21-I is promoted by phorbol esters in a PKC-independent manner. This interaction requires an intact chimaerin C1 domain. The isolation of an anchoring protein for the chimaerin family of non-kinase phorbol ester receptors raises the possibility that positional mechanisms similar to those reported for PKC isozymes 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 Invitrogen. Reagents for the expression and purification of recombinant glutathione S-transferase (GST) fusion proteins and Gambamid 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. Redivia t-(35)S-methionine was purchased from New England Nuclear Biochemicals.

**Plasmid Construction**—Constructs for the yeast two-hybrid screening were made in pLexA vector (CLONTECH). α1-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) constructs were obtained from Sigma. Redivia t-(35)S-methionine was purchased from New England Nuclear Biochemicals.

**Co-precipitation Using Glutathione-Sepharose 4B Beads**—Cells were harvested at ~50% confluence and were co-transfected with pEBG-Tmp21-I (aa 1–219 or aa 1–219) or empty vector (pEBG) and pCR3-β2-chimaerin. Initially, 1, 10, 100, or 1000 units/ml of PMA (proliferator-activated receptor-α agonist) were added to the cell cultures 30 min before and during PMA incubation. After incubation overnight at 4 °C, bound GST fusion proteins were eluted from the GST-Sepharoses by mixing the samples with 2× SDS-PAGE sample buffer and electrophoresis 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**—Primary NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/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 E. coli cells after induction for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside and then bound to glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.) according to the manufacturer’s instructions.

**In Vitro Translation and in Vitro Binding Assay**—pCR3β vectors containing different coding regions of α1, β1, and β2-chimaerin were transcribed and translated in vitro using a TNT T7-coupled reticulocyte lysate system kit (Promega) according to the manufacturer’s instructions. Association of the 35S-labeled products with GST or GST-Tmp21-I (aa 108–219) bound to glutathione-Sepharose 4B beads was performed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Tween 20. After incubation overnight at 4 °C, bound GST fusion proteins were eluted from the GST-Sepharoses by mixing the samples with 2× SDS-PAGE sample buffer and electrophoresis 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.

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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 (Amersham Biosciences, Inc.).

Immunocytochemistry and Confocal Microscopy—Plasmids encoding for chimaerin isoforms or their truncated mutants in pEGFP-C1 vector (10.2 µg) and Tmp21-I in the V5 epitope-tagged pDNA3.1 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 (1:500, Invitrogen). 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 Confocal 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 cover slips, 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 a 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 PKAI (18). Cells were lysed in a buffer containing 8% of GST-p21 binding domain protein, 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl2, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM β-glycerophosphate, and protease inhibitors (5 µg/ml 4-1’-amocephyl benzeneasulfonil 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 loaded in loading buffer. The samples were resolved in a 12% 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 β2-chimaerin. The tester strain of the screen, EGY48, contained two reporters, LEU2 and LacZ. Among the 1 × 108 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 domains in chimaerins involved in the association, different LexA fusions constructs were generated (Fig. 1A). These plasmids were co-transfected with pEGFP/Tmp21-I (aa 108–219) into the EGY48 (p8OP-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 α1-chimaerin and with its N-terminal region. The interaction of Tmp21-I with a fragment of β2-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 β2-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 β2-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 β2-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 β2-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-lipoxygenase, p53, and prFHM-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 α1-, β1-, or β2-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 associate 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 α-, 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 cloned 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–
219) 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
Fig. 3. Mapping of the C1 domain as the interaction site in chimaerins. A, sequence homology between the N-terminal regions of α- and β-chimaerins. B, interaction in yeast of Tmp21-I (aa 108–219) with different deletion mutants of the N-terminal region of α1-chimaerin fused to LexA. The panel shows a schematic representation of the LexA fusion constructs. The interaction was assayed either in glucose plates (−Gal/Raf) or in galactose/raffinose plates (+Gal/Raf). Positive interactions were detected as blue in +galactose/raffinose plates. C, 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. 4. Association of Tmp21-I with β2-chimaerin in COS-1 cells. A, COS-1 cells were co-transfected with pEBG/Tmp21-I (aa 108–219) or pEBG alone (empty vector) together with pCR3-β2-chimaerin. Sixty h later, cells were lysed, and GST or GST-Tmp21-I were recovered with glutathione-Sepharose 4B beads. Samples of lysates and beads were analyzed by Western blot using anti-GST and anti-β2-chimaerin antibodies (Ab). Two additional experiments gave similar results. B, COS-1 cells were co-transfected with pcDNA3.1/V5-Tmp21-I (aa 108–219) and pCR3-β2-chimaerin. pcDNA3.1/V5 or pcDNA3.1/V5-LacZ were used as controls. Sixty h later, immunoprecipitation with an anti-V5 antibody was performed as described under “Experimental Procedures.” Representative Western blots for cell lysates and immunoprecipitates (IP) are shown in the figure. Two additional experiments gave similar results.

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 Tmp21-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 α1-, β1-, and β2-chimaerin. Interestingly, Tmp21-I co-localized with GFP-α1-, β1-, and β2-chimaerins, 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 α1-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 Tmp21-I.

In the next set of experiments, we evaluated by confocal microscopy whether PMA could promote the association of β2-chimaerin with Tmp21-I. In agreement with our co-precipitation and co-immunoprecipitation results, PMA markedly enhanced the co-localization of Tmp21-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 immunoreactivity from the soluble (cytosolic) to the particulate fraction after PMA treatment. As expected, Tmp21-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 Tmp21-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 Tmp21-I, and it requires an intact C1 domain.

We have also evaluated the co-localization of endogenous β2-chimaerin and Tmp21-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-Tmp21-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 Tmp21-1 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. 8D). 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 Tmp21-I on Rac-GTP levels. Interestingly, in cells expressing V5-Tmp21-I, PMA failed to produce a reduction in Rac-GTP levels (Fig. 8, lanes 5 and 6). This suggests that increasing the levels of Tmp21-I may reduce the availability of chimaerins to access active Rac, probably by sequestering chimaerins at the perinuclear region. The effect of Tmp21-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 Tmp21-I, therefore suggesting that the Tmp21-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 Tmp21-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). Tmp21-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-
Association of Chimaerins with Tmp21-I

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). For example, upon activation with phorbol esters or DAG, PKC isozymes can interact with receptors for activated PKCs (RACKs) (32). Interestingly, PKCe interacts with the Golgi protein β2-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 deletional 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 lipid recognition and for protein-protein interaction. As described previously for PKC C1 domains, the C1 domain in chimaerins is required for phorbol ester binding and subcellular redistribution. 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 PKCε) or PAR4 (for aPKCs) (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 isoforms 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 isoforms. Previous results using deletion mutants of β2-chimaerin have shown that the N-terminal region comprising the C1 domain is necessary for its interaction with Tmp21-I (14). Moreover, the C1 domains of PKCε are required for its translocation to Golgi upon phorbol ester stimulation (39). Whether discrete C1 domains of PKC isoforms 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.

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/ER 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 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).

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Association of Chimaerins with Tmp21-I

Fig. 8. Effect of Tmp-21-I and β2-chimaerin on Rac-GTP levels. A, COS-1 cells growing in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum transfected with 1 μg of pEGFP (vector), pEGFP-β2-chimaerin, pEGFP-N1, or pEGFP-C246A-β2-chimaerin. Rac-GTP levels were determined 60 h later as described under “Experimental Procedures.” The levels of total Rac and GFP fusion proteins were monitored by Western blot using anti-Rac and anti-GFP antibodies, respectively. Two additional experiments gave similar results. B, densitometric analysis of Rac-GTP levels expressed as percentages of the values observed in control (vector-transfected) cells. Data are expressed as mean ± S.E. of 3 independent experiments. C, COS-1 cells growing in Dulbecco’s modified Eagle’s supplemented with 10% fetal bovine serum were co-transfected with 0.2 μg of pcDNA3-β2-chimaerin and 0.8 μg of pDNA3.1/V5-Tmp21 (or the corresponding empty vectors). Sixty h later, cells were treated for 1 h with 3 μM PMA or vehicle in the presence of PKC inhibitor GF 109203X (5 μM). Rac-GTP levels were determined as described under “Experimental Procedures.” N, N-terminal; C, C-terminal. Two additional experiments gave similar results.

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 isoforms renders proteins that are still phorbol ester/DAG-responsive (42). Using a single C1 domain of PKCy 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,2 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.3 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 isoforms. Our results strongly support a role for C1 domains as targeting modules through lipid-protein and protein-protein interactions.

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