Phorbol Esters and Related Analogs Regulate the Subcellular Localization of β2-Chimaerin, a Non-protein Kinase C Phorbol Ester Receptor*

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The novel phorbol ester receptor β2-chimaerin is a Rac-GAP protein possessing a single copy of the C1 domain, a 50-amino acid motif initially identified in protein kinase C (PKC) isoforms that is involved in phorbol ester and diacylglycerol binding. We have previously shown that, like PKCs, β2-chimaerin binds phorbol esters with high affinity in a phospholipid-dependent manner (Caloca, M. J., Fernandez, M. N., Lewin, N. E., Ching, D., Modali, R., Blumberg, P. M., and Kazanietz, M. G. (1997) J. Biol. Chem. 272, 26488–26496). In this paper we report that like PKC isoforms, β2-chimaerin is translocated by phorbol esters from the cytosolic to particulate fraction. Phorbol esters also induce translocation of α1 (n)- and β1-chimaerins, suggesting common regulatory mechanisms for all chimaerin isoforms. The subcellular redistribution of β2-chimaerin by phorbol esters is entirely dependent on the C1 domain, as revealed by deletional analysis and site-directed mutagenesis. Interestingly, β2-chimaerin translocates to the Golgi apparatus after phorbol ester treatment, as revealed by co-staining with the Golgi marker BODIPY-TR-ceramide. Structure relationship analysis of translocation using a series of PKC ligands revealed substantial differences between translocation of β2-chimaerin and PKCα. Strikingly, the mezeiner analog thymeleatoxin is not able to translocate β2-chimaerin, although it very efficiently translocates PKCα. Phorbol esters also promote the association of β2-chimaerin with Rac in cells. These data suggest that chimaerins can be positionally regulated by phorbol esters and that each phorbol ester receptor class has distinct pharmacological properties and targeting mechanisms. The identification of selective ligands for each phorbol ester receptor class represents an important step in dissecting their specific cellular functions.

The phorbol ester tumor promoters are the most common tools for the activation of protein kinase C (PKC) in biological systems. These natural compounds exert a variety of effects in cells, which have been largely attributed to the calcium-dependent classical PKCs (cPKCs, β, βII, and γ) or calcium-independent novel PKCs (nPKCs, ε, η, and θ). Unlike cPKCs and nPKCs, the third class of PKC isoforms or atypical PKCs (aPKCs and PKCζ/σ) is phorbol ester-unresponsive. Phorbol esters also target PKCμ (PKD), a kinase related to PKC that has unique substrate specificity and regulation (1–4). The heterogeneity in the phorbol ester responses is probably related to the multiple phorbol ester receptors present in each cell type. An additional level of complexity in the phorbol ester responses is conferred by the unique pharmacological profile of each phorbol ester analog. In fact, ligands for PKCs not only include the typical dinetepene phorbol esters but also a large number of unrelated structural analogs such as nonphorbol ester diterpenes (e.g. mezereins, octahydro mezerein, and thymeleatoxin), macrocyclic lactones (e.g. bryostatins), and indole alkaloids (e.g. indolactams and teleocidins). The differential effects of phorbol ester analogs in cellular models suggest unique modes of interaction with different phorbol ester receptor classes and may explain the heterogeneous properties of the ligands (4–7).

One of the important novel concepts that has emerged in the past few years is that PKC isoforms are not the only receptors for the phorbol esters and related derivatives. In fact three novel families of phorbol ester receptors unrelated to PKCs have been isolated. These novel phorbol ester receptors, which lack a kinase domain in their structure, include the chimaerin isoforms, Caenorhabditis elegans Unc-13 and its mammalian homologs (Munc13 isoforms), and RasGRP. These proteins have in common a single copy of the C1 domain, a 50/51-amino acid motif that is duplicated in tandem in cPKC and nPKC, which is the binding site for the phorbol esters and diacylglycerol (DAG) in these PKCs (4, 6, 7). It has recently been reported that chimaerins, Unc-13, and RasGRP bind phorbol esters and related analogs with high affinity in vitro, thereby suggesting that a single copy of the C1 domain is sufficient to confer binding responsiveness. In all cases phorbol ester binding is phospholipid-dependent, and acidic phospholipids are the most efficient cofactors for reconstitution of binding (8–11). The identification of these “nonkinase” phorbol ester receptors suggests that phorbol esters and related analogs may regulate cellular pathways independently of the activation of PKC isoforms.

Chimaerins are a novel family of phorbol ester receptors with protein; MD, molecular dynamics; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; GFP, green fluorescent protein; GST, glutathione S-transferase.
Chimaerins as Phorbol Ester Receptors

Rac GTPase-activating protein (GAP) activity and therefore accelerate the hydrolysis of GTP to GDP leading to Rac inactivation. Chimaerins comprise at least four isozymes (α1- or n-, α2-, β1-, and β2-chimaerins) that are alternative spliced variants from the α- and β-chimaerin genes (14-16). The C1 domain in chimaerin isoforms has ~40% homology with those of PKC isozymes. Using the radioligand [3H]phorbol 12,13-dibutyrate, we have reported that β2-chimaerin is a high affinity receptor for phorbol esters in vitro (8). Competition assays revealed that different ligands have unique patterns of recognition for different phorbol ester receptors. In fact, although DAGs and indolactams have similar affinities for PKCs and β2-chimaerin, thymeleatoxin (a mezeerin analog) has ~60 times less affinity for β2-chimaerin (8). Therefore, it is likely that specific residues within individual C1 domains are critical for conferring binding specificity. Limited information is available on the ligand binding properties of these novel phorbol ester receptors. The regulation, localization, and function of the chimaerins are largely unexplored.

One of the hallmarks for the activation of cPKCs and nPKCs by phorbol esters is their change in subcellular localization or “translocation.” Translocation of PKC isoforms is a complex process that not only involves lipid-protein interactions mediated by the C1 domain and other motifs, but it is also dictated by protein-protein associations that may play a key role in determining function specificity for each PKC isoform (12, 13).

The aim of this study is to investigate whether chimaerins are subject to subcellular redistribution or translocation by phorbol ester analogs. The results presented in this paper show that chimaerins are subject to subcellular translocation by phorbol ester derivatives. Using a series of deletion mutants of β2-chimaerin, we determined that translocation is entirely dependent on the ligand binding to the C1 domain. Interestingly, we found that upon stimulation with different analogs, β2-chimaerin translocates to the Golgi apparatus. These data indicate that the chimaerin family of phorbol ester receptors has unique ligand recognition properties and suggests that phorbol esters have the potential to regulate additional targets in addition to PKC isoforms.

EXPERIMENTAL PROCEDURES

Materials—PMA, 4a-PMA, thymeleatoxin, (-)-octylindolactam V, 12-deoxyphorbol 13-phenyacetate, and GF 109203X were purchased from LC Laboratories (Woburn, MA). Drystain 1 was a kind gift from Dr. Peter M. Blumberg (NCI, National Institutes of Health). BODIPY-TR-ceramide was obtained from Molecular Probes, Inc. (Eugene, OR). Cell culture reagents and media were obtained from Life Technologies, Inc.

Cell Culture—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

Generation of β2-Chimaerin Expression Vectors—A 1.4-kilobase XhoI-MulI fragment comprising the full-length β2-chimaerin was ligated into the mammalian expression vector pCR3 to generate pCR3-β2-chimaerin, as we have previously described elsewhere (8). To generate a GFP construct for β2-chimaerin (pEGFP-β2-chimaerin), a 1.4-kilobase EcoRI-EcoRI fragment was isolated from pCR3-β2-chimaerin (8) and subcloned in-frame into the GFP plasmid pEGFP-C3 (CLONTECH, Ref. 17). Deletion mutants of β2-chimaerin were generated by PCR using pEGFP-β2-chimaerin as a template. The following oligonucleotides were used (EcoRI and SalI restriction sites are underlined): CATGAAATTCATGCCTCTCTCTCC and AGATGTGACAGCGG-AGCTCATTGGGAAC (amino acids 1-262), for pEGFP-β2-C1; GAGAATATCCACAACTTTAAGGTCC and AGATGTGACAGCGGACATGG-AGCTCATTGGGAAC (amino acids 213-262), for pEGFP-β2-C2; GAGAATATCCACAACTTTAAGGTCC and AGATGTGACAGCGGACATGG-AGCTCATTGGGAAC (amino acids 213-466), for pEGFP-β2-C1-GAP. The PCR products were ligated into pCRII using the TA cloning kit (Invitrogen). The corresponding EcoRI and SalI fragments were isolated and subcloned into the GFP plasmid pEGFP-C2 (CLONTECH). The construct pEGFP-β2-GAP was generated by PCR from pACG2T-β2-chimaerin (8) using the following oligonucleotides: CGCACGCGTGAATAAAAGCTTGTCGTTTTCATTTAAA and AGCTCCAGATGTTGCTAGCATGTCATTCGGAGA. The PCR product was ligated into pCRII, and a fragment comprising the GAP domain (amino acids 291-466) was isolated by reaction with EcoRI and ligated in frame into pEGFP-C2. β1-Chimaerin was isolated from human testis cDNA (CLONTECH) by PCR using the following oligonucleotides: GTGGGGTCGAGGAAATCCATGGTCTCTTC and AGATGTGACAGCGGACATGG-AGCTCATTGGGAAC (β1-chimaerin) and CGCACGCGTGAATAAAAGCTTGTCGTTTTCATTTAAA and AGCTCCAGATGTTGCTAGCATGTCATTCGGAGA (β2-chimaerin). The plasmid pEGFP-C2-β2-chimaerin and the plasmid pEGFP-C246A-β2-chimaerin was described elsewhere (17). In all cases, constructs were sequenced by the dyeoxy chain termination method.

Expression of β2-Chimaerin in COS-1 Cells and Subcellular Fractionation—Mammalian expression vectors for chimaerins or deletion mutants of COS-1 cells in 6-well dishes were transfected using EffectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol. 48 h after transfection, cells were treated with different concentrations of phorbol ester analogs. Experiments were performed in the presence of the PKC inhibitor GF 109203X (5 μM), added 30 min before and during the incubation with the phorbol ester analogs, as we have previously described (8).

Cells were harvested into lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 5 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pancreatic A) and lysed by sonication. Separation of cytosolic (soluble) and particulate fractions was performed by ultracentrifugation as described previously (8, 17). Equal amounts of protein (10 μg) for each fraction were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes that were immunostained with the following antibodies: anti-PKCα antibody (1:3,000; Upstate Biotechnology Inc., Lake Placid, NY), anti-β2-chimaerin antibody (1:1,000), and anti-GFP antibody (1:25,000). The intensity of the bands was determined by densitometry using a Scanner Control, version 1.00 (Molecular Dynamics, Inc., Sunnyvale, CA). Densitometric analysis was performed under conditions that yielded a linear response.

Visualization of GFP-β2-Chimaerin Translocation by Fluorescent Microscopy—COS-1 cells were transfected with the GFP expression vectors using FuGENE (Roche Molecular Biochemicals), according to the manufacturer’s protocol. 48 h after transfection, cells were treated with the phorbol ester analogs and fixed with 3.7% formaldehyde. Photomicrographs were taken with an Olympus fluorescent microscope—Confocal Assistant™, version 4.02. All the images shown are individual middle sections of projected Z series mounting.

Modeling Studies—A newly developed q jumping molecular dynamics simulation method, which has been used to successfully predict the PKC-ligand receptor complex (19), was used to predict the binding model of thymeleatoxin in complex with β2-chimaerin. The major advantage of this program is its ability to include both the ligand and receptor flexibility in the docking simulation. The q jumping method has been implemented in the CHARMm program, as described previously (19, 20). The q jumping protocol was carried out using the CHARMm script. The CHARMm force field (21) was used to describe the structure of the receptor, and all the necessary parameters for thymeleatoxin were generated by using the QUANTA program (Molecular Simulations Inc., San Diego, CA).

The three-dimensional structure of β2-chimaerin C1 domain was modeled based upon the x-ray structure of PKCs C1b, as described previously (17, 22). Starting from the phorbol 13-acetate structure in the x-ray crystal data (22), the initial conformation of thymeleatoxin was generated by QUANTA. All the appropriate hydrogen atoms were added to the ligand and minimized. In the docking simulation, the
ligand was allowed to be fully flexible, and all the side chains of the residues 8–12 and 20–27, which form the ligand binding site in β2-chimaerin, were allowed to be flexible; everything else in the receptor was fixed in our docking simulation. Then, the MD simulations described above were carried out in vacuum, using the following q jump:

\[ q = 1.02 - 1.03, \quad \epsilon = 800 \text{ and } P_f = 0.1. \]

The q jumping MD simulations were carried out at \( T = 300 \text{ K} \), using the constant temperature algorithm of Berendsen et al. (23). The SHAKE algorithm (24) was used to fix all the bonds containing hydrogen with a time step of 1 fs. For energy evaluations, a distance-dependent dielectric model was employed with the nonbonding interactions truncated at 8 Å. Finally, nuclear Overhauser effect constraints consisting of a central atom of the ligand, and each α-carbon atom in the β2-chimaerin residues 10 and 24 was introduced to prevent the ligand from escaping from the binding site completely. With the MD protocol, we performed several independent MD runs for 1–2 ns, and the binding mode was determined from the lowest energy conformation from each of the MD trajectories. Then, the resulting complex structures were further refined by a minimization consisting of 5000 steps of adopted Newton-Raphson method.

**Determination of Rac-GAP Activity**—To determine GTPase activity of β2-chimaerin, recombinant purified Rac was first incubated at 30 °C for 10 min with \( [γ-32P]GTP (60 \mu\text{Ci/mL}; \text{Amersham Pharmacia Biotech}) \) in loading buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM dithiothreitol, and 0.5 mM MgCl₂). The loading reaction was stopped by the addition of MgCl₂ (final concentration, 10 mM). Purified β2-chimaerin (expressed in SF9 cells) was then incubated with loaded Rac in reaction buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 10 mM MgCl₂, 1 mg/ml bovine serum albumin, 1 mM GTP) at 15 °C, and Rac GTPase activity was determined in filters by measuring the reduction of the remaining lipid is neutral phosphatidylcholine for 45 min at 30 °C in reaction buffer, prior to adding the loaded Rac. Experiments were performed in duplicate. In general, duplicate determinations differed by <10%. Expression and purification of recombinant β2-chimaerin from SF9 cells is described elsewhere (8).

**Association of β2-Chimaerin and Rac**—CO3-1 cells were co-transfected with pCR3e-β2-chimaerin and pEBG-Rac (a mammalian expression vector for GST-Rac) or pEBG (empty vector for expression of GST alone). pEBG vectors were a kind gift of Dr. Margaret M. Chou (University of Pennsylvania School of Medicine). 48 h after transfection cells were treated with GF 109203X (5 μM) for 30 min and then for 1 h with PMA (1 μM). After treatment, cells were harvested into lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 5 mM MgCl₂, 0.1 mM dithiothreitol, 5 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 μM leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin A), and the lysates were then incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 2 h at 4 °C. Beads were extensively washed with lysis buffer, resuspended in Laemmli’s sample buffer, and boiled. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and then probed with anti-β2-chimaerin antibody (1:1000). Aliquots of total lysates were also probed with an anti-β2-chimaerin antibody (1:1000) and an anti-GST antibody (Amersham Pharmacia Biotech; 1:1000).

**RESULTS**

**Translocation of β2-Chimaerin by Phorbol Ester Derivatives**—We have previously shown that β2-chimaerin, a RacGAP protein, is a high affinity phorbol ester receptor. Our previous work revealed that β2-chimaerin binds \(^{[3]H}\)phorbol 12,13-dibutyrate in a phospholipid-dependent fashion with an affinity that is in the same range as cPKCs and nPKCs (8). Cells were transfected with the mammalian expression vector pCR3e-β2-chimaerin, and high levels of expression of β2-chimaerins were observed 48 h later, as judged by Western blot analysis and by assessing phorbol ester binding levels (8). To assess the effects of phorbol esters on the subcellular distribution of β2-chimaerin, cells were treated with PMA and then subjected to subcellular fractionation. The levels of β2-chimaerin in soluble (cytosolic) and particulate fraction were then determined by Western blot. These experiments were carried out in the presence of the PKC inhibitor GF 109203X (5 μM) to rule out any involvement of PKCs in the effect of phorbol esters, as we have previously described (8, 17). Fig. 1 shows that PMA induced translocation of β2-chimaerin from the soluble to the particulate fraction in a dose-dependent manner. A GFP fusion construct for β2-chimaerin was also generated and responds to PMA in a similar fashion as the nonfused construct, suggesting that, as previously described for PKC isoforms (26–29), the GFP tag does not affect responsiveness to phorbol esters. As expected, nonfused GFP protein was unresponsive to PMA.

Phorbol ester derivatives and related analogs have unique binding properties for discrete PKC isoforms, and recent studies have shown that different derivatives have differential properties for translocating PKC isoforms (28, 29). Our previous in vitro studies on structure-activity analysis revealed striking differences in binding potency for β2-chimaerin and PKC isoforms. In fact, although phorbol esters and 12-deoxyphorbol esters have ~10-fold lower affinity for β2-chimaerin than for PKCs, indole alkaloids (indolactams) have similar binding potency, and mezerein derivatives such as the tumor promoter thymeleatoxin show ~60-fold less affinity for β2-chimaerin (8). To explore the structure-activity for translo-
of PKCα, we investigated the effect of four different analogs on the subcellular redistribution of this novel receptor and compared it with PKCα (Fig. 2). The macrocyclic lactone brystatin 1, an analog with an atypical spectrum of biological responses compared with the typical phorbol esters, was the most potent agent at inducing translocation of β2-chimaerin. The \( ED_{50} \) for translocation is ~30 nM. Bryostatin 1 is somewhat more potent in translocating PKCα (\( ED_{50} \) ~3 nM). The 12-deoxyphorbol ester derivative 12-deoxyphorbol 13-phe-nyacetate and the indole alkaloid (-)-octylindolactam V both induced translocation of β2-chimaerin. Despite the similar potency \( in vitro \) for binding recognition, (-)-octylindolactam V was very poor at inducing translocation of β2-chimaerin when compared with PKCα. Strikingly, the mezerein derivative thymeleatoxin was totally ineffective at inducing translocation of β2-chimaerin even at a concentration of 10 \( \mu \)M. On the other hand, thymeleatoxin fully translocated PKCα at a concentration of 10 nM, suggesting that this derivative is a selective agent for translocation of PKC.

**Studies with Deletion Mutants of β2-Chimaerin: Translocation Dependence on the C1 Domain**—The C1 domains in PKCs are the binding sites for phorbol esters and related derivatives and are essential for mediating the subcellular redistribution of PKCs. To ascertain whether the C1 domain in β2-chimaerin mediates its translocation after phorbol ester treatment, we constructed a series of deletion mutants for β2-chimaerin in pEGFP vector. The following constructs were generated (Fig. 3A): GFP-β2-N-C1 (from N terminus to C1-domain), GFP-β2-C1 (C1 domain alone), GFP-β2-C1-GAP (from C1 domain to C terminus), and GFP-β2-GAP (GAP domain alone). Cells were transfected with each of these constructs and subsequently treated with PMA. Western blot analysis of soluble and particulate fractions after subcellular fractionation revealed that PMA induced the translocation of GFP-β2-C1 and GFP-β2-C1-GAP from soluble to particulate fraction, as observed with the full-length β2-chimaerin (Fig. 3B). On the other hand, no changes in subcellular localization were observed after deletion of the C1 domain, as revealed by the absence of translocation of GFP-β2-GAP after PMA treatment. Unexpectedly, a deleted version of β2-chimaerin expressing only the N-terminal region and C1 domain (GFP-β2-N-C1) was found at the particulate fraction even in the absence of PMA treatment. The involvement of the C1 domain in translocation was confirmed by site-directed mutagenesis of the C1 domain. A point mutant of β2-chimaerin in which Cys in position 246 was replaced by Ala was generated and expressed as a GFP fusion protein (GFP-C246A-β2-chimaerin). Position 246 corresponds to the third Cys in the C1 domain, which is essential for the coordination of Zn\(^{2+}\), as determined in structural studies in C1 domains of PKCs (22). In a previous study we have determined that this β2-chimaerin mutant does not bind phorbol esters (17). In agreement with our results with the deletion mutants, GFP-C246A-β2-chimaerin does not translocate in response to PMA. All together, these results unambiguously indicate that the C1 domain mediates the translocation of β2-chimaerin.

**Translocation of Different Chimaerin Isoforms by PMA**—All chimaerin isoforms have in common a single C1 domain. The homology between the C1 domains of α- and β-chimaerins is ~94%. Like β2-chimaerin, α1-chimaerin (n-chimaerin) is a high affinity phorbol ester receptor \( in vitro \) and it binds \( ^{3}H \)phorbol 12,13-dibutyrate in a phospholipid-dependent manner with an affinity that is similar to that of PKC isoforms (18). To evaluate whether multiple chimaerin isozymes respond to PMA, we transiently expressed α1-, β1-, and β2-chimaerins in COS-1 cells and monitored subcellular distribution in each case after PMA treatment. Fractionation of untreated cells revealed that 25 and 45% of the α1- and β1-chimaerin immunoreactivity was present in the soluble fraction (cytosol), compared with 85% for β2-chimaerin. After PMA treatment, the immunoreactivity in the soluble fraction is sub-
stansitually reduced in all cases (Fig. 4). Unlike β2-chimaerin, an increase in the particulate fraction for α1- and β1-chimaerins was not evident, probably because most of the immunoreactivity is already present in the particulate fraction before stimulation. Remarkably, as observed with β2-chimaerin, thymeleatoxin did not induce any significant changes in the levels of soluble α1-chimaerin. Only a small fraction of β1-chimaerin was translocated by thymeleatoxin when compared with PMA. Therefore, thymeleatoxin is a much more potent agent in translocating PKCα than chimaerin isoforms, as we have demonstrated in Fig. 2.

**Molecular Modeling of Thymeleatoxin Binding to PKCα C1b and β2-Chimaerin C1 Domains**—The reduced binding affinity of β2-chimaerin for thymeleatoxin and the marked differences in translocation between PKCα and β2-chimaerin suggest that each receptor may interact differently with this ligand. We therefore decided to perform a molecular modeling analysis of the C1 domains of PKCα and β2-chimaerin in complex with thymeleatoxin. For the purposes of this study, we use the C1b domain of PKCα (Fig. 5). X-ray crystallographic studies of PKCα C1b domain and our previous molecular modeling and site-directed mutagenesis analysis have clearly demonstrated that the binding of ligands to PKC C1 domains is governed by two important interactions, namely hydrogen bonding and hydrophobic interactions (22, 30). Thymeleatoxin forms an identical hydrogen bond network with PKCα C1b and β2-chimaerin, suggesting that the selectivity between PKCα and β2-chimaerin for thymeleatoxin is not due to different hydrogen bonding interactions. In the predicted binding models, thymeleatoxin is in close contact with a number of hydrophobic residues, including residues at positions 11, 20, 22, and 24. Although the residues at positions 11 and 24 (Pro and Leu) are conserved between PKCα C1b and β2-chimaerin C1 domains, the residues at positions 20 and 22 are Leu in PKCα C1b and Phe in β2-chimaerin. The residue at position 22 is Tyr in PKCα C1b but is replaced by Trp in β2-chimaerin. Importantly, a Leu residue is conserved at position 20 among all the PKC C1b domains. Thus, these different hydrophobic residues at positions 20 and 22 in PKCα C1b and β2-chimaerin may explain differences in binding recognition for thymeleatoxin.

Furthermore, although the residue at position 9 is hydrophilic in nature in PKCα C1b domains, a positively charged Arg residue is present in position 9 of β2-chimaerin. Based upon the x-ray structure of PKCα C1b in complex with phorbol 13-acetate, it was proposed that a primary mechanism of PKC translocation and activation upon ligand binding is the transformation of a predominant hydrophilic surface to a hydrophobic surface, thus allowing the effective insertion into the membrane (22). The presence of a positively charged Arg residue at position 9 in β2-chimaerin makes the surface less hydrophobic upon ligand binding and may make β2-chimaerin intrinsically less capable of translocation than PKCα, as supported by our current results. Thus, the inability of thymeleatoxin to translocate β2-chimaerin may be due to the combination of the weaker binding affinity for thymeleatoxin binding to the C1 domain of β2-chimaerin and the intrinsic weaker ability of β2-chimaerin to translocate to the membrane.

**Localization Studies Using GFP-tagged β2-Chimaerin and Its Mutants**—PKC isozymes translocate to different intracellular compartments upon activation with phorbol esters and related derivatives. Evaluation of subcellular localization of GFP-β2-chimaerin by fluorescence microscopy revealed that this novel phorbol ester receptor was distributed in the cytoplasm. No nuclear staining was observed. Upon PMA stimulation, a significant fraction of GFP-β2-chimaerin distributed to a perinuclear region. We also observed faint plasma membrane staining upon PMA treatment (Figs. 6A and 7). No translocation was observed upon treatment of cells with the inactive PMA isomer 4α-PMA (Fig. 6A). Similar results were observed in immunofluorescence studies with β2-chimaerin nonfused to GFP using an anti-β2-chimaerin antibody (data not shown).

Recent studies by the Blumberg lab (28, 29) have shown a distinct pattern of redistribution of PKC isozymes depending on the phorbol ester analog used, suggesting that the PKC activator plays a key role in determining intracellular localization. We decided to evaluate whether a similar mechanism takes place for β2-chimaerin. Experiments using bryostatin 1, the 12-deoxyphorbol ester 12-deoxyphorbol 13-phenylacetate,
and (-)-octylindolactam V revealed similar perinuclear localization of β2-chimaerin in all cases (Fig. 6A). In agreement with our Western blot studies, thymeleatoxin was unable to translocate GFP-β2-chimaerin to the perinuclear region, thereby confirming the lack of an effect for this derivative.

In the next set of experiments we evaluated the localization of the β2-chimaerin mutants previously described. In agreement with the fractionation analysis (Fig. 3), the point mutant C246A-β2-chimaerin retained its cytoplasmatic localization after PMA treatment (Fig. 6B). Likewise, the β2-chimaerin GAP domain was unresponsive to PMA. The presence of a functional C1 domain confers responsiveness, as shown with GFP-β2-C1-GAP. Interestingly, the mutant expressing the N-terminal region of β2-chimaerin (GFP-β2-N-C1) was localized to the perinuclear compartment to which β2-chimaerin translocated after phorbol ester treatment (Fig. 3).

To determine the nature of the perinuclear compartment to which β2-chimaerin translocated after phorbol ester treatment, cells were treated with BODIPY TR ceramide, a red fluorescent probe that specifically labeled the Golgi network. This probe has been previously used to determine the Golgi localization of PKCδ after activation (27). We evaluated colocalization of GFP-β2-chimaerin before and after PMA treatment. As illustrated in Fig. 8, GFP-β2-chimaerin shows some degree of co-localization with the Golgi marker in the absence of PMA treatment. However, a marked increase in co-localization in the perinuclear region was observed after PMA treatment, as judged by superposition of the images. The mutant GFP-β2-N-C1, which was localized to the perinuclear region even in the absence of PMA, co-localized with BODIPY TR ceramide without and with PMA treatment (data not shown). Our results demonstrate that PMA induces the translocation of β2-chimaerin to the Golgi network.

Rac-GAP Activity of β2-Chimaerin—It has previously been demonstrated that α1-chimaerin has RacGAP activity, and, therefore, it accelerates the hydrolysis of GTP from Rac (31). As shown in Fig. 8A, β2-chimaerin also accelerates the hydrolysis of GTP from Rac in a concentration-dependent manner. No effect of β2-chimaerin on Cdc42 or RhoA was observed (data not shown). Interestingly, when we performed similar assays in the presence of phospholipid vesicles, we observed a marked increase in GAP activity of β2-chimaerin (1 ng/μl) as the concentration of phosphatidylserine in the vesicles increases (Fig. 8B). Phosphatidylserine did not induce any changes in GTP hydrolysis in the absence of β2-chimaerin (data not shown).

![FIG. 5. Docking of thymeleatoxin to the PKCα C1b and β2-chimaerin C1 domains.](image)

![FIG. 4. Multiple chimaerin isozymes are translocated by PMA. COS-1 cells were transfected with pEGFP, pEGFP-α1-chimaerin, pEGFP-β1-chimaerin, or pEGFP-β2-chimaerin. After 48 h cells were treated with PMA (P) or thymeleatoxin (T) (1 μM, 1 h) in the presence of GF109203X (5 μM). After subcellular fractionation by ultracentrifugation, soluble and particulate fractions were subjected to Western blot analysis using either an anti-GFP or anti-PKCα antibody. The molecular masses of GFP-α1-chimaerin, GFP-β1-chimaerin, and GFP-β2-chimaerin are 61, 59, and 78 kDa, respectively. Two additional experiments gave similar experiments. C, Control.](image)

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similar effect was observed with other acidic phospholipids, such as phosphatidic acid, but not with neutral phospholipids, such as phosphatidylethanolamine. Therefore, the Rac-GAP activity of chimaerins is regulated by its association to lipids.

PMA Promotes the Association of β2-Chimaerin to Rac—We hypothesize that regulation of β2-chimaerin by phorbol esters may promote its association to Rac. A similar model has been documented for PKC isozymes, where phorbol esters promote translocation of PKCs and their association to PKC regulatory proteins and substrates (4). To evaluate the association of Rac with β2-chimaerin, COS-1 cells were co-transfected with expression vectors for GST-Rac (or GST alone) and β2-chimaerin. GST-Rac (or GST) from cell extracts was then bound to glutathione-Sepharose 4B beads, and the presence of β2-chimaerin in the beads was evaluated by Western blot with an anti-β2-chimaerin antibody (Fig. 9). Association of Rac with β2-chimaerin was not detected in nonstimulated cells. In contrast, treatment of COS-1 cells with PMA for 1 h markedly increased the amount of β2-chimaerin associated to GST-Rac. No association to GST alone was found either in the absence or presence of PMA. Therefore, phorbol esters promote the association of β2-chimaerin with its effector Rac in cells.

DISCUSSION

In this study we have demonstrated that chimaerin isoforms, like cPKCs and nPKCs, are positionally regulated by phorbol esters. Translocation of chimaerins by phorbol esters is dependent on the chimaerin C1 domain and independent of PKC activation. We have previously demonstrated that these Rac-GAP proteins bind phorbol esters and DAG with low nanomolar affinity (8, 17). Like PKCs, phorbol ester binding to chimaerins...
is dependent on phospholipids as cofactors, which suggests that association to membranes may be crucial for regulating its activity and/or association to targets. In that regard, it was previously shown that the GAP activity of α1-chimaerin is activated by acidic phospholipids such as phosphatidylserine (31). In this paper we have observed a similar activation of β2-chimaerin by this acidic phospholipid. Therefore, as described for cPKCs and nPKCs, the association of β2-chimaerin to membrane phospholipids regulates its activity. In preliminary experiments we observed that phorbol esters do not promote RacGAP activity of β2-chimaerin in vitro and that they do not change the phosphatidylserine requirement for GAP activity. Although a small degree of activation of RacGAP activity by phorbol esters has been reported for α1-chimaerin (31), our results suggest that phorbol esters are responsible for targeting β2-chimaerin to membranes rather than triggering its allosteric activation. Our observation that β2-chimaerin associates with Rac upon phorbol ester treatment suggests that translocation of β2-chimaerin may be a key event in the regulation of Rac activity. We have also observed that β2-chimaerin does not affect GTP hydrolysis from Cdc42 or RhoA, and in addition it inhibits Rac-GTP levels upon stimulation of the EGF receptor in COS-1 cells, an indication that β2-chimaerin regulates Golgi stability during interphase (43). Others have also described the requirement of other organelles including mitochondria, lysosomes, and endo-

Fig. 8. Rac-GAP activity of β2-chimaerin. A, purified Rac was incubated with [γ-32P]GTP, and then purified β2-chimaerin (8) was added to loaded Rac. GTP hydrolysis was measured 4 min after the addition of β2-chimaerin. The reaction was carried out at 15 °C as described in Ref. 25. Results are expressed as the percentages of [γ-32P]GTP remaining relative to that in the absence of β2-chimaerin, B, GTP hydrolysis was assessed after 4 min (at 15 °C) in the presence of 100 μg/ml phospholipid vesicles having variable proportions of phosphatidylserine (the remaining phospholipid is neutral phosphatidylcholine) and 1 ng/μl β2-chimaerin. Two additional experiments gave similar results.

Fig. 9. Association of β2-chimaerin and Rac. COS-1 cells were transfected with different vectors as described in the figure. 48 h after transfection cells were treated for 1 h with PMA (3 μM) in the presence of the PKC inhibitor GF 109203X (5 μM). Cells were lysed and incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 2 h at 4 °C to bind GST or GST-Rac. Beads were washed with lysis buffer, re-suspended in Laemmli’s sample buffer and boiled. Samples were subject to Western blot and probed with either an anti-β2-chimaerin antibody or an anti-GST antibody. Similar results were observed in two additional experiments.

We have identified the C1 domain in chimaerins as the minimum domain required for phorbol ester binding and subcellular redistribution in cells. It is therefore clear that a single C1 domain is sufficient for binding of phorbol esters. These results support those from previous experiments using single C1 domains of PKCs, which show that this 50-amino acid domain is sufficient for ligand binding (30, 32, 33). Moreover, experiments using a single C1 domain of PKCγ fused to GFP have shown that this domain was capable of translocating to membranes (35). The notion that a single C1 domain is sufficient to confer translocation is supported by studies showing that functional inactivation of a single C1 domain in PKCs or PKCd renders mutant PKCs that are still able to translocate upon phorbol ester stimulation (36, 37). However, these mutants having a single functional C1 domain require higher phorbol ester concentration for translocation, as observed with β2-chimaerin. Therefore, two C1 domains may account for the full responsiveness for PKC translocation by phorbol esters. Our studies do not rule out the possibility that the reduced sensitivity of β2-chimaerin for translocation compared with PKCs is related to the absence of other domains present in PKC that are involved in protein-lipid and/or protein-protein interaction, such as the C2 domain.

It is interesting that β2-chimaerin associates with a perinuclear compartment upon phorbol ester activation. Co-staining with a specific Golgi marker reveals a Golgi localization for β2-chimaerin. It has been previously reported that PKCd and PKCe translocate to the Golgi network upon treatment with phorbol esters or DAGs (28, 38–41). Translocation of PKCe to the Golgi is mediated by its C1 domains (38) and involves the association with the coatomer protein β′-COP, a Golgi protein that acts as an intracellular receptor for PKCe (39). Interestingly, the novel phorbol ester receptor Munc13 also translocates from the cytosol to the Golgi apparatus after phorbol ester stimulation (42). In support of our localization studies, it was reported that α1-chimaerin regulates Golgi stability during interphase (43). Others have also described the requirement of a PKC-like molecule without phosphorylating activity for the production of post-Golgi vesicles (44). Association of PKCs to other organelles including mitochondria, lysosomes, and endo-
plasmic reticulum has also been reported (45–47). In analogy with the models proposed for PKC isozymes, translocation of chimaerins may involve protein-protein interaction mechanisms. In fact, we have recently isolated a chimaerin interacting protein (Tnp2p or p23) using the yeast two-hybrid system. This protein is expressed in the Golgi apparatus and endoplasmic reticulum. The association of β2-chimaerin and p23 in cells is markedly enhanced by phorbol ester treatment, suggesting that mechanisms of protein-protein interaction similar to those described for PKC isozymes can dictate the intracellular localization of chimaerins. It may be possible that, upon activation, a conformational change in β2-chimaerin occurs that exposes the protein-protein interacting sites, as also described for PKC isozymes. The domain in chimaerins interacting with p23 is in the N-terminal region, which may explain the constitutive association of the mutant β2-N-C1 to the perinuclear compartment. It is important to mention that a large pool of intracellular Rac, the target for chimaerins, is located in a perinuclear region and kept in an inactive state (48). An attractive hypothesis is that chimaerins may play a role in the maintenance of this small GTP-binding protein in an inactive, GDP-bound state at the perinuclear compartment. These important issues are currently being investigated in our laboratory.

Our experiments using different ligands show important differences in their potencies for translocating β2-chimaerin. We have previously reported that PKC ligands have a unique pattern for binding to this RacGAP protein (8). Remarkably, each receptor class (i.e., PKC isozymes, Unc-13, RasGRP, and chimaerins) interacts differently with phorbol esters and related analogs (6, 8, 9, 11, 49). Clearly, specific residues within the individual C1 domains confer unique properties to each receptor class. This is not surprising, because marked differences in ligand selectivity have been shown for individual C1 domains. Potent ligands for one C1 domain may be either weak ligands or may not bind at all to other C1 domains, such as the C1 domains of PKCζ, Vav, or Rac (6, 50, 51). These striking differences have been reported even for the C1a and C1b domain of a single PKC isozyme, such as PKCa, PKCe, or PKCμ, providing strong evidence that C1 domains are nonequivalent (6). Structural and biochemical studies with individual C1 domains have confirmed that these marked differences for ligand recognition exist, as described for unrelated ligands such as indolacetic acids, DAGs, and certain phorbol ester analogs (17, 34, 50, 52). In fact, a detailed structure-activity analysis of β2-chimaerin revealed unique binding properties for this novel phorbol ester receptor. The 60-fold difference in binding affinity for thymeleatoxin between PKCa and β2-chimaerin is the highest reported so far for two individual phorbol ester receptors. Although mutagenesis studies within the β2-chimaerin C1 domain have yet to be done, our modeling analysis with thymeleatoxin has revealed distinct binding recognition for PKCa and β2-chimaerin. It is also remarkable that thymeleatoxin is very potent in translocating PKCa but poorly translocates chimaerin isoforms. To our knowledge, this is the first example of a selective ligand for phorbol ester receptors, a finding that will be valuable for dissecting the biological functions of each receptor class.

In summary, the chimaerin isoforms represent a novel class of phorbol ester receptors that are subject to subcellular translocation by phorbol esters and related analogs through their C1 domain. The unique pharmacological properties and distinct localization of each receptor highlights the complexity of phorbol ester pharmacology and DAG signaling, as well as makes questionable the use of phorbol esters as specific ligands for PKC isozymes. The elucidation of the cellular functions of the novel chimaerin receptors will benefit from the rationale design of specific pharmacological agents.