Characterization of the Rac-GAP (Rac-GTPase-activating protein) activity of β 2-chimaerin, a 'non-protein kinase C' phorbol ester receptor

Maria Jose CALOCA^{1,2}, HongBin WANG¹ and Marcelo G. KAZANIETZ³

Center for Experimental Therapeutics and Department of Pharmacology, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104-6160, U.S.A.

The regulation and function of β 2-chimaerin, a novel receptor for the phorbol ester tumour promoters and the second messenger DAG (diacylglycerol), is largely unknown. As with PKC (protein kinase C) isoenzymes, phorbol esters bind to β 2-chimaerin with high affinity and promote its subcellular distribution. β 2-Chimaerin has GAP (GTPase-activating protein) activity for the small GTP-binding protein Rac1, but for not Cdc42 or RhoA. We show that acidic phospholipids enhanced its catalytic activity markedly *in vitro*, but the phorbol ester PMA had no effect. β 2-Chimaerin and other chimaerin isoforms decreased cellular levels of Rac-GTP markedly in COS-1 cells and impaired GTP loading on to Rac upon EGF (epidermal growth factor) receptor stimulation. Deletional and mutagenesis analysis determined that the β 2-chimaerin GAP domain is essential for this effect. Interestingly, PMA has a dual effect on Rac-GTP levels in COS-1 cells. PMA increased Rac-GTP levels in the absence of a PKC

inhibitor, whereas under conditions in which PKC activity is inhibited, PMA markedly decreased Rac-GTP levels and potentiated the effect of β 2-chimaerin. Chimaerin isoforms co-localize at the plasma membrane with active Rac, and these results were substantiated by co-immunoprecipitation assays. In summary, the novel phorbol ester receptor β 2-chimaerin regulates the activity of the Rac GTPase through its GAP domain, leading to Rac inactivation. These results strongly emphasize the high complexity of DAG signalling due to the activation of PKC-independent pathways, and cast doubts regarding the selectivity of phorbol esters and DAG analogues as selective PKC activators.

Key words: β 2-chimaerin, diacylglycerol (DAG), phorbol ester, protein kinase C (PKC), Rac-GTPase-activating protein (Rac-GAP), Rho GTPase.

INTRODUCTION

The phorbol ester tumour promoters are the most widely used compounds for the activation of PKC (protein kinase C), a family of related serine/threonine kinases that have important roles in the control of cell proliferation, cell death, differentiation and malignant transformation [1–4]. Although it is widely accepted that PKC isoenzymes are the main receptors for the phorbol ester tumour promoters, an important concept that has emerged in the last years is that additional proteins that lack the archetypical kinase domain are also capable of binding phorbol esters with high affinity. The novel phorbol ester/DAG (diacylglycerol) receptors include RasGRPs (guanyl nucleotide-releasing proteins, which are exchange factors for Ras and Rap1), Munc13 isoforms (a family of proteins involved in exocytosis) and chimaerins (a family of 'chimaeric' proteins between the regulatory domain of PKC and BCR, the breakpoint cluster region protein involved in the translocation of Philadelphia chromosome in chronic myelogenous leukaemia) [5–7]. The common feature among all phorbol ester receptors is the presence of the C1 domain, a 50–51 amino acid motif that is duplicated in tandem in classical PKCs (cPKCs), novel PKCs (nPKCs) and the related kinase PKC μ /PKD (protein kinase D). This domain has been defined unequivocally as the phorbol ester/DAG binding site in PKC isoenzymes by means of mutagenesis and structural analysis. The novel 'non-PKC' phorbol ester receptors, unlike cPKCs and nPKCs, possess only a single copy of the C1 domain [1,3,7–9]. The discovery of novel receptors for the phorbol esters raised the provocative hypothesis

that multiple signalling pathways can be regulated by DAG, an issue that has been largely ignored. Importantly, the discovery of novel high affinity phorbol ester receptors challenges the use of the phorbol esters and related compounds as selective PKC activators.

Chimaerins comprise a family of four isoenzymes (α 1- or ' n ', α 2-, β 1- and β 2-chimaerins), which are splice variants of the α - and β -chimaerin genes. The main structural difference between the spliced variants is a putative SH2 domain at the N-terminus of α 2- and β 2-chimaerins [10–12]. Sequence alignment of the C1 domain of chimaerin isoforms with known phorbol ester receptors reveals approx. 40 % identity with the C1 domains in PKCs. The C1 domains in α - and β -chimaerins are almost identical (94 % identity). *In vitro* pharmacological studies have firmly established that chimaerins are phorbol ester receptors. Using [³H]PDBu (phorbol 12,13-dibutyrate) as a radioligand, Blumberg and co-workers [13] have shown that α 1-chimaerin binds this radioligand with high affinity, and remarkable similarities in ligand-binding properties and cofactor requirements for binding have been found for α 1-chimaerin and PKC α . A thorough pharmacological characterization of the phorbol ester binding properties of β 2-chimaerin in our laboratory has revealed striking similarities with PKCs. Indeed, the K_d of β 2-chimaerin for [³H]PDBu is in the low nanomolar range, and only minor differences in ligand recognition were found relative to PKCs [14]. As with PKCs, binding of phorbol esters to β 2-chimaerin is phospholipid-dependent, and acidic phospholipids such as phosphatidylserine (PS) are the most efficient cofactors for supporting ligand binding to this novel phorbol ester receptor. Remarkably, phorbol esters promote the

Abbreviations used: BCR, breakpoint cluster region; DAG, diacylglycerol; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating protein; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; PBD, p21-binding domain; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; cPKC, conventional or classical PKC; nPKC, novel PKC; PS, phosphatidylserine.

¹ These authors contributed equally to this paper.

² Present address: Centro de Investigación del Cáncer, University of Salamanca-CSIC, Campus Unamuno, E-37007 Salamanca, Spain.

³ To whom correspondence should be addressed (e-mail marcelo@spirit.gcrp.upenn.edu).

redistribution (translocation) of $\beta 2$ -chimaerin from cytosol to membranes, as it had previously been established for phorbol-ester-responsive PKC isoenzymes. Translocation requires binding of the phorbol ester to the $\beta 2$ -chimaerin C1 domain, and mutation of the essential cysteine residue at position 246 within the C1 domain impairs translocation [15].

The C-terminal domain of chimaerin isoforms is highly similar to the GAP (GTPase-activating protein) domain for small GTPases found in BCR [16]. Indeed, it has been previously established that $\alpha 1$ -chimaerin has GAP activity for Rac1, a small GTP-binding protein that is critical for numerous cellular functions, including actin cytoskeleton organization, gene expression, cell-cycle progression, Ras-mediated transformation and invasion [17–19]. Despite the extensive characterization of $\beta 2$ -chimaerin as a phorbol ester receptor, and unlike the PKC isoenzymes, only limited data are available regarding its regulation and cellular function. We have determined that $\beta 2$ -chimaerin has Rac-GAP activity, hence it accelerates the hydrolysis of GTP from Rac1. However, an important question that arises based on the previously described findings is whether or not phospholipids and phorbol esters would be able to regulate its activity. The accepted model for the regulation of PKC activity involves its allosteric activation upon phospholipid-dependent phorbol ester binding, leading to the activation of its catalytic (kinase) activity. Whether or not a similar regulatory mechanism operates for the Rac-GAP activity of $\beta 2$ -chimaerin is still unknown. The results presented in the present paper reveal novel aspects of the regulation of $\beta 2$ -chimaerin activity both *in vitro* and in cells. Importantly, we observed that $\beta 2$ -chimaerin activity is regulated by phospholipids. In addition, we provide strong evidence that $\beta 2$ -chimaerin associates with Rac in the plasma membrane and that it possesses Rac-GAP activity in cells.

MATERIALS AND METHODS

Materials

PMA and GF 109203X were purchased from LC Laboratories (Woburn, MA, U.S.A.). Phospholipids were obtained from Sigma. EGF (epidermal growth factor) was purchased from Roche Molecular Biochemicals. Cell culture reagents and media were obtained from Life Technologies. Reagents for expression and purification of recombinant GST (glutathione S-transferase)-fusion proteins were purchased from Amersham Biosciences.

Cell culture

COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

Expression and purification of recombinant $\beta 2$ -chimaerin from Sf9 (*Spodoptera frugiperda*) insect cells

$\beta 2$ -Chimaerin was expressed in Sf9 cells as a GST-fused protein and purified with glutathione-Sepharose 4B beads. The GST partner was cleaved with thrombin, and thrombin was then removed using benzamidine-Sepharose 6B beads (Amersham Biosciences). The purity of recombinant $\beta 2$ -chimaerin was > 90%, as assessed by Coomassie Blue staining. A detailed description of the expression and purification procedures has been described elsewhere [14].

Generation of $\beta 2$ -chimaerin expression vectors

A 1.4 kb *XhoI*-*MluI* fragment comprising the full-length $\beta 2$ -chimaerin was ligated into the mammalian expression vector pCR3 ϵ to generate pCR3 ϵ - $\beta 2$ -chimaerin, as we have previously described elsewhere [14]. To generate a GFP (green fluorescent protein) construct for $\beta 2$ -chimaerin (pEGFP- $\beta 2$ -chimaerin), a 1.4 kb *EcoRI*-*EcoRI* fragment was isolated from pCRII- $\beta 2$ -chimaerin [14,15] and was subcloned in-frame into the GFP plasmid pEGFP-C3 (Clontech). Deletion mutants of $\beta 2$ -chimaerin were generated by PCR using pEGFP- $\beta 2$ -chimaerin as a template. The following oligonucleotides were used (*EcoRI* and *Sall* restriction sites are underlined): 5'-CATGAATTCA-TGCGTCTCCTCTCC-3' and 3'-AGATGTCGACGGCAGTC-ATTGGGAAC-5' (amino acids 1–262), for pEGFP- $\beta 2$ -N-C1; 5'-GAGGAATTCCACAACCTTTAAGGTCC-3' and 3'-AGATGTCGACGGCAGTCATTGGGAAC-5' (amino acids 213–262), for pEGFP- $\beta 2$ -C1; 5'-GAGGAATTCCACAACCTTTAAGGTCC-3' and 3'-GCGCGTCGACATTAGAATAAAACGTCTTCG-5' (amino acids 213–466), for pEGFP- $\beta 2$ -C1-GAP. The PCR products were ligated into pCRII using the TA cloning kit (Invitrogen). The corresponding *EcoRI* and *Sall* fragments were isolated and subcloned into the GFP plasmid pEGFP-C2; (Clontech). The construct pEGFP- $\beta 2$ -GAP was generated by PCR from pAcG2T- $\beta 2$ -chimaerin [14] using the following oligonucleotides: 5'-CGCACGCGTGAATAAAACGTCTTCG-TTTTCTATTAA-3' and 3'-AGCCTCCAGATGGTGGTAGACA-TATGCATTCGGGAA-5'. The PCR product was ligated into pCRII, and a fragment comprising the GAP domain (amino acids 291–466) was isolated by digestion with *EcoRI* and ligated in frame into pEGFP-C2 to generate pEGFP- $\beta 2$ -GAP.

To construct pEGFP- $\alpha 1$ -chimaerin, the full-length cDNA for $\alpha 1$ -chimaerin [13] was used as a template, and *EcoRI* and *XhoI* sites were created by PCR (restriction sites are underlined) using the following oligonucleotides: 5'-CCGGAATTCATGC-CATCCAAAGAGTCTTGGTC-3' and 3'-CCGCTCGAGCTAA-AATAAAATGTCTTC-5'. The insert was ligated in frame into pEGFP-C2. $\beta 1$ -Chimaerin was isolated from human testis cDNA (Clontech) by PCR using the following oligonucleotides: 5'-GTCAGGCTCGAGGGATCCATGCTTTGCACGTCTCCCGT-C-3' (*XhoI* site underlined) and 3'-CGCACGCTGAAACA-GAACATCTTCGTTTTCTATTAA-5'. The $\beta 1$ -chimaerin cDNA was subcloned into pCRII vector, and a *XhoI*-*EcoRI* fragment from that plasmid was subcloned into pEGFP-C3 to generate pEGFP- $\beta 1$ -chimaerin. In all cases, constructs were sequenced by the dideoxy chain termination method.

Site-directed mutagenesis

The generation of mutant C246A (Cys²⁴⁶ → Ala) was described elsewhere [15]. A mutant in which amino acids 298–300 within the GAP domain have been deleted (Δ EIE- $\beta 2$ -chimaerin) was generated using the Unique Site Elimination (USE) mutagenesis system from Amersham Biosciences, pCR3 ϵ - $\beta 2$ -chimaerin was used as a template. The primer 5'-GGTAGACATATGCATTCCGG-GCAAGAGGATTAAATCG-3' was used to generate the deletion. The deletion was confirmed by sequencing.

Determination of GAP activity *in vitro*

Recombinant purified Rac1, Cdc42 or RhoA were first incubated with [γ -³²P]GTP (60 μ Ci/nmol; Amersham Biosciences) at 30 °C for 10 min in loading buffer [50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.1 mM DTT (dithiothreitol) and 0.5 μ M MgCl₂].

The loading reaction was stopped by the addition of MgCl_2 (10 mM final concentration). Purified β 2-chimaerin (expressed in Sf9 cells) was then incubated with the loaded GTPases in reaction buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM DTT, 10 mM MgCl_2 , 1 mg/ml BSA and 1 mM GTP) at 15 °C, and GTPase activity was determined in filters using the method described by Garret et al. [20]. To determine the effect of phospholipids on Rac-GAP activity, purified β 2-chimaerin was pre-incubated with 100 $\mu\text{g/ml}$ phospholipids [various proportions of PS, with the remaining lipid being neutral PC (phosphatidylcholine)] for 45 min at 30 °C in reaction buffer, before adding the loaded Rac1. Phospholipid vesicles were prepared by sonication, as previously described [21]. To determine the effect of PMA on the GAP activity, purified β 2-chimaerin was pre-incubated with PMA (100 nM) with or without 100 $\mu\text{g/ml}$ phospholipids for 30–45 min at 30 °C in reaction buffer, before adding the loaded Rac1. Experiments were performed in duplicate. In general, duplicate determinations differed by <10 %.

To assess Rac-GAP activity in immunoprecipitates, COS-1 cells were transfected with different β 2-chimaerin-encoding plasmids (in the ϵ -tagged vector pCR3 ϵ , [14]), and 60 h later, cells were lysed in RIPA buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate and 0.1 % (w/v) SDS, with protease inhibitors (5 mg/ml 4-(2-aminoethyl)benzenesulphonyl fluoride, 5 mg/ml leupeptin, 5 mg/ml aprotinin and 1 mg/ml pepstatin A)] and subjected to immunoprecipitation using the anti- ϵ -tag antibody. Rac-GAP activity in the immunoprecipitates was then determined as described below.

Determination of Rac-GTP levels in cells

COS-1 cells were transfected with different chimaerin plasmids using FuGENE6 (Roche Molecular Biochemicals), following the manufacturer's instructions. In some experiments, cells were kept in growing medium for 48 h and then Rac-GTP levels were determined. In another set of experiments, cells were serum-starved 24 h after transfection for an additional 24 h, and were then treated with EGF (100 ng/ml, 10 min). Rac-GTP levels were determined using a pull-down assay to isolate Rac-GTP by binding to the PBD (p21-binding domain) of PAK1 (p21/Cdc42/Rac1-activated kinase 1) [22]. Cells were lysed in a buffer containing 8 μg of GST-PBD, 20 mM Tris/HCl, pH 7.5, 1 mM DTT, 5 mM MgCl_2 , 150 mM NaCl, 0.5 % (v/v) Nonidet P40, 5 mM β -glycerophosphate and protease inhibitors [5 $\mu\text{g/ml}$ 4-(2-aminoethyl)benzenesulphonyl fluoride, 5 $\mu\text{g/ml}$ leupeptin, 5 $\mu\text{g/ml}$ aprotinin and 1 $\mu\text{g/ml}$ pepstatin A]. Lysates were centrifuged at 14 000 g for 10 min at 4 °C and were then incubated with glutathione-Sepharose 4B beads for 1 h at 4 °C. After extensive washing, the beads were boiled in loading buffer. The samples were resolved on a SDS/(12 %) polyacrylamide gel, transferred on to nitrocellulose membranes and analysed by Western blot using an anti-Rac1 antibody.

Western blot analysis

Membranes were blocked with 5 % (w/v) non-fat dried milk in PBS, and were subsequently immunostained with any of the following antibodies: anti- β 2-chimaerin monoclonal antibody (1:1000) [15], anti- ϵ -tag antibody [14], or anti-Rac monoclonal antibody (1:1000; Upstate Biotechnologies, Lake Placid, NY, U.S.A.). Membranes were then washed three times with 0.1 % (v/v) Tween 20 in PBS and were incubated with either anti-mouse (1:3000; Bio-Rad) or anti-rat antibody (1:3000; Jackson

Immunoresearch Laboratories, West Grove, PA, U.S.A.) conjugated to horseradish peroxidase. Bands were visualized by the ECL[®] (enhanced chemiluminescence) Western blotting detection system (Amersham Biosciences).

Co-localization studies

COS-1 cells were co-transfected with pEGFP vectors for chimaerin isoforms (or pEGFP control) and activated Rac (V12Rac)-HA (haemagglutinin) (1 μg of each plasmid) using FuGENE6. At 60 h after transfection, cells were fixed and stained with a mouse anti-HA antibody (1:1000; BabCO, Richmond, CA, U.S.A.) and a donkey anti-mouse Cy3-conjugated secondary antibody (1:1500; Jackson Immunoresearch Laboratories). Slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and were 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.

RESULTS

Rac-GAP activity of β 2-chimaerin

It has been previously demonstrated that α 1-(n)-chimaerin has Rac-GAP activity in *in vitro* assays, and hence it accelerates the hydrolysis of GTP from Rac, leading to its inactivation [23]. The GAP domain in α 1-chimaerin is located at the C-terminal region, and it shares approx. 80 % identity with the GAP domain in β -chimaerins. We have previously shown that β 2-chimaerin has Rac-GAP activity [15]. In order to assess its specificity for small GTPases, we assessed the effect of β 2-chimaerin on GTP hydrolysis *in vitro* from Rac1, Cdc42, and RhoA. In these assays, we used human β 2-chimaerin expressed in Sf9 insect cells after infection with a β 2-chimaerin baculovirus. As shown in Figure 1, β 2-chimaerin accelerates the hydrolysis of GTP from Rac1 in a concentration-dependent manner. However, β 2-chimaerin does not affect GTP hydrolysis from Cdc42 or RhoA.

Effect of phospholipids and phorbol esters on β 2-chimaerin Rac-GAP activity

Acidic phospholipids are required as cofactors for the activation of PKC isoenzymes [1–4]. To explore whether phospholipids could also modulate β 2-chimaerin activity, GTP hydrolysis from Rac1 was determined *in vitro* in the presence of phospholipid vesicles of different compositions. In the first set of experiments, we used phospholipid vesicles (100 $\mu\text{g/ml}$) with different proportions of PS, with the remaining phospholipid being neutral (PC). We used a limited concentration of β 2-chimaerin (0.5 ng/ml) that by itself produced only 20–30 % acceleration in Rac-GAP activity. In this way, we have a dynamic range which allows us to measure further stimulation, since higher concentrations of β 2-chimaerin would have led to a rapid hydrolysis of GTP even at very short times. Using this experimental approach, we found that PS accelerates GTP hydrolysis in the presence of β 2-chimaerin. The effect was proportional to the concentration of PS in the vesicle (Figure 2A). We then tested the effect of different phospholipids on the Rac-GAP activity of β 2-chimaerin. As shown in Figure 2(B), while neutral phospholipids PC or phosphatidylethanolamine vesicles were unable to accelerate GTP hydrolysis, acidic phospholipids [PS and phosphatidic acid (PA)] accelerated GTP hydrolysis from Rac1 in the presence of β 2-chimaerin. In order to determine that

Figure 1 Rac-GAP activity of $\beta 2$ -chimaerin

Purified Rac1, Cdc42 or RhoA were labelled with [γ - 32 P]GTP, and GTP hydrolysis was evaluated in the presence of purified recombinant $\beta 2$ -chimaerin, as described by Garrett et al. [20]. ●, control; ■, $\beta 2$ -chimaerin (0.2 ng/ml); ▲, $\beta 2$ -chimaerin (1 ng/ml); ▼, $\beta 2$ -chimaerin (5 ng/ml). Similar results were observed in three independent experiments.

Figure 2 Effect of phospholipids on the Rac-GAP activity of $\beta 2$ -chimaerin

Rac-GAP activity was evaluated in the presence of vesicles containing the indicated phospholipid, and the remaining was neutral PC. The total concentration of phospholipid was 100 μ g/ml, and the concentration of $\beta 2$ -chimaerin was 0.5 ng/ml. (A) Effect of PS: ●, control in the absence of $\beta 2$ -chimaerin; ○, PS (0 %); ■, PS (25 %); □, PS (50 %); ▲, PS (75 %). (B) Effect of different phospholipids. Black bars, lipid alone; white bars, phospholipid + $\beta 2$ -chimaerin (0.5 ng/ μ l). (C) Effect of PMA (100 nM) added to vesicles of different phospholipid compositions. Similar results were observed in three independent experiments.

PS and PA were acting on $\beta 2$ -chimaerin rather than having a direct effect on Rac1, control experiments were performed in the absence of $\beta 2$ -chimaerin. As shown in Figure 2(B), acidic phospholipids did not promote GTP hydrolysis from Rac1 in the absence of $\beta 2$ -chimaerin.

We then evaluated the effect of PMA on $\beta 2$ -chimaerin Rac-GAP activity. The effect was assessed in the presence of phospholipid vesicles having different proportions of PS (0 %, 20 % and 100 %), with the remaining phospholipid being PC. Under none of those conditions PMA was able to accelerate GTP hydrolysis from Rac (Figure 2C). Thus these results show that PMA does not stimulate the catalytic activity of $\beta 2$ -chimaerin *in vitro*.

Analysis of Rac-GTP levels in cells

In the next set of experiments, we explored whether or not $\beta 2$ -chimaerin and other chimaerin isoforms regulate Rac-GTP levels in cells. We used mammalian expression vectors for $\alpha 1$ -, $\beta 1$ - and $\beta 2$ -chimaerin, as well as for a series of truncated mutants. A schematic representation of these constructs is depicted in Figure 3(A). Upon transfection into COS-1 cells, the levels of active Rac (Rac-GTP) were determined using a pull-down assay [22]. As shown in Figure 3(B), and in agreement with our recent observation with $\beta 2$ -chimaerin [24], all chimaerin isoforms tested were able to decrease Rac-GTP levels. Expression of the GAP domain from either α - or β -chimaerins, or a $\beta 2$ -chimaerin C1 domain mutant with an intact GAP domain (C246A- $\beta 2$ -chimaerin), also resulted in a significant decrease in Rac-GTP levels. Likewise, expression of a $\beta 2$ -chimaerin mutant comprising the C1 domain and the β -GAP domain markedly decreased Rac-GTP levels. On the other hand, expression of mutants comprising the N-terminal regions of the different chimaerin isoforms (with the corresponding GAP domain deleted) did not affect Rac-GTP levels. As expected, expression of the $\beta 2$ -chimaerin C1 domain alone was also without effect. A densitometric analysis showing the effect of the different chimaerin isoforms and mutants is presented in Figure 3(C). These results clearly demonstrate that chimaerin isoforms decrease Rac-GTP levels through their C-terminal GAP domain.

$\beta 2$ -Chimaerin inhibits EGF-induced activation of Rac

To analyse the Rac-GAP effects of $\beta 2$ -chimaerin in the context of receptor activation, we chose as a paradigm the determination of Rac-GTP levels upon activation of EGF receptors. It has been established that EGF and other growth factors activate Rac1,

Figure 3 Effects of chimaerins on Rac-GTP levels in COS-1 cells

COS-1 cells growing in Dulbecco's modified Eagle's medium supplemented with 10% FBS were transfected with different chimaerin isoforms or mutants in pEGFP vector. **(A)** Schematic representation of the different constructs used in the experiments. **(B)** Rac-GTP levels were determined 60 h later as described in the Materials and methods section. The expression levels of total Rac1 and GFP-fusion proteins were monitored by Western blot using anti-Rac1 and anti-GFP antibodies, respectively. **(C)** Densitometric analysis of Rac-GTP levels, expressed as percentages of the values observed in control (vector-transfected) cells. Results are means $\pm$ S.E.M. of three independent experiments.

thus leading to an increase in GTP-bound Rac [25–27]. COS-1 cells were transfected with either pCR3 ϵ - β 2-chimaerin or empty vector (pCR3 ϵ) and were then stimulated with EGF. Rac-GTP levels were then determined with the PBD pull-down assay. Whereas EGF increased Rac-GTP levels markedly in control (empty vector-transfected) cells, the expression of β 2-chimaerin markedly impaired the EGF effect (Figure 4A). Expression of the β -GAP domain alone using the plasmid pCR3 ϵ - β -GAP also inhibited EGF-induced elevation of Rac-GTP levels (Figure 4B). To investigate the role of the GAP domain of β 2-chimaerin further, we generated a mutant in which conserved amino acids 298–300 within the GAP domain were deleted (Δ EIE). As previously shown for α 1-chimaerin [23], the Δ EIE- β 2-chimaerin mutant was devoid of Rac-GAP activity in *in vitro* assays (Figure 4C). Interestingly, this GAP inactive mutant did not impair the EGF response (Figure 4D). Similar results were observed with other GAP-inactive mutants (P394G- β 2-chimaerin, replacement of conserved Pro³⁹⁴ with glycine; Δ YRV- β 2-chimaerin, deletion of conserved amino acids 310–312; results not shown). Taken together, these results confirmed the essential role of the β 2-chimaerin GAP domain in GTP hydrolysis from Rac.

PMA decreases Rac-GTP levels in a PKC-independent manner

In the next set of experiments, we explored the effect of a phorbol ester, PMA, on cellular Rac-GTP levels. COS-1 cells were treated with increasing concentrations of PMA (1 nM–10 μ M). As shown in Figure 5(A), a biphasic effect for the PMA effect was observed: at low concentrations, PMA increased Rac-GTP levels slightly, and at higher concentrations, Rac-GTP levels dropped markedly. Densitometric analysis reveals a 49% increase in Rac-GTP levels at 10 nM PMA (Figure 5B). Since PMA is known to exert effects through the activation of phorbol-ester-responsive PKC isoenzymes, we performed similar experiments in the presence of the pan-PKC inhibitor GF 109203X (bisindolylmaleimide I; 5 μ M), which is capable of inhibiting all phorbol-ester-responsive PKCs, including those in COS-1 cells [24,28]. To confirm that we have fully inhibited PKC in our COS-1 model, we assessed PMA-induced activation of ERK1/2 (extracellular-signal-regulated kinase 1/2), a well-characterized PKC-mediated response, and observed that in our experimental condition, GF 109203X totally abolished PMA-induced stimulation of ERK1/2, even at lower concentrations than

Figure 4 Effect of chimaerins on EGF-stimulated Rac-GTP levels in COS-1 cells

(A) COS-1 cells were transfected with 1 μ g of a plasmid encoding for full-length β 2-chimaerin (pCR3 ϵ - β 2-chimaerin, F) or empty vector (pCR3 ϵ , V). After 48 h, cells were serum-starved overnight and then treated with EGF (100 ng/ml, 10 min). Rac-GTP levels were then determined using the pull-down assay, as described in the Materials and methods section. Total Rac1 and β 2-chimaerin (ϵ -tagged) expression levels were monitored by Western blot. (B) Experiments similar to those described in (A) were performed, but using a mammalian expression vector for the β 2-chimaerin GAP domain (β -GAP). Almost identical results were observed in three independent experiments. (C) Lack of Rac-GAP activity of Δ EIE- β 2-chimaerin mutant. COS-1 cells were transfected with 1 μ g of pCR3 ϵ - β 2-chimaerin (F), pCR3 ϵ - Δ EIE β 2-chimaerin (Δ EIE) or empty vector (pCR3 ϵ , V). After 48 h, cells were lysed and subjected to immunoprecipitation using an anti- ϵ -tag antibody. Rac-GAP activity was measured in the immunoprecipitates, as described in the Materials and methods section. (D) Rac-GTP levels were determined upon stimulation with EGF (100 ng/ml, 10 min) in COS-1 cells transfected with 1 μ g of either pCR3 ϵ - Δ EIE β 2-chimaerin (Δ EIE) or empty vector (pCR3 ϵ , V) using the pull-down assay.

those that we used in the Rac-GTP pull-down assays (results not shown). Remarkably, when PKC was fully inhibited, PMA induced a significant decrease in Rac-GTP levels (Figure 5A). Indeed, a concentration-response analysis for the PMA effect revealed that Rac-GTP levels were decreased by 22% at 10 nM PMA, and the effect reaches a plateau at 100 nM (40% inhibition), as revealed by densitometry (Figure 5B). We then investigated whether or not the expression of β 2-chimaerin affected the PMA response in the presence of the PKC inhibitor. Figure 5(C) shows that in cells transfected with a mammalian expression vector for β 2-chimaerin (pCR ϵ - β 2-chimaerin), the PMA effect was even more pronounced, and indeed Rac-GTP levels were almost undetected (Figure 5C). Thus phorbol esters can potentiate the Rac-GAP effect of β 2-chimaerin in a cellular context. These experiments also provide strong evidence that phorbol esters exert effects independently of PKC activation. Co-immunoprecipitation experiments in COS-1 cells revealed β 2-chimaerin co-immunoprecipitates with an activated form of Rac (Rac-QL) (results not shown).

Co-localization of β 2-chimaerin and V12Rac1

To explore further the effect of chimaerins on Rac1, we next performed co-localization experiments. As an approach, we co-

expressed GFP-chimaerins and an HA-tagged form of activated Rac1 (V12Rac1) in COS-1 cells. As previously described by others [29,30], the active form of Rac1 has a prominent localization in membrane ruffles. Interestingly, a remarkable pattern of co-localization for β 2-chimaerins and HA-V12Rac was observed in plasma membrane ruffles, as judged from the yellow colour in the overlapped images. Co-localization with HA-V12Rac was also observed for α 1- and β 1-chimaerins (Figure 6). Thus these results strongly suggest that the activated form of Rac1 is a target for chimaerin isoforms.

DISCUSSION

In the present paper, we show that human β 2-chimaerin expressed in Sf9 cells accelerates GTP hydrolysis *in vitro* from Rac1, but not Cdc42 or RhoA, in a concentration- and time-dependent manner. Basal Rac-GAP activity is observed even in the absence of phospholipid cofactors, suggesting that the enzyme has a certain degree of constitutive activity. We believe that this is unrelated to the presence of a proteolytic release of the GAP domain, since our preparation is highly pure and we do not detect any obvious proteolytic fragments in our β 2-chimaerin preparation purified from Sf9 cells. Interestingly, in our *in vitro* assays, the Rac-GAP activity of β 2-chimaerin is enhanced by acidic phospholipid (PS

Figure 5 Effect of PMA on Rac-GTP levels in COS-1 cells

(A) COS-1 cells were treated with different concentrations of PMA for 30 min either in the absence or presence of the PKC inhibitor GF 109203X (5 μ M, 30 min before and during PMA stimulation). Rac-GTP levels were determined using the pull-down assay. (B) Densitometric analysis of Rac-GTP levels, expressed as percentages of the values before PMA stimulation. Results are means $\pm$ S.E.M. of three independent experiments. (C) COS-1 cells were transfected with either 1 μ g of pCR3 ϵ - β 2-chimaerin or empty vector (pCR3 ϵ). After 60 h, cells were treated for 1 h with 1 μ M PMA or vehicle in the presence of GF 109203X (5 μ M). Rac-GTP levels were determined using the pull-down assay. A representative experiment is shown together with a densitometric analysis of three independent experiments.

and PA). Thus, as in the case of PKC isoenzymes, membrane phospholipids have an important role in enzyme activation. Indeed, acidic phospholipids are an absolute requirement for the activation of most PKC isoenzymes [1–4]. A main difference, however, is that phorbol esters do not cause activation of β 2-chimaerin Rac-GAP activity in our *in vitro* assays, suggesting that important differences exist between chimaerins and PKC isoenzymes in terms of their molecular activation.

β 2-Chimaerin is a high-affinity receptor for the phorbol ester, and binding occurs at the C1 domain. This domain presents high similarity to those in PKC isoenzymes, and we have previously reported that phorbol ester binding to the β 2-chimaerin C1 domain is phospholipid-dependent [14]. Similar phospholipid requirements for binding were observed for β 2-chimaerin and PKC δ in [3 H]PDBu-binding assays [14]. Ahmed et al. [23] have reported that phorbol esters stimulate the Rac-GAP activity of α 1-chimaerin, although this stimulation is relatively small. In cells, however, we observed that PMA potentiates the Rac-GAP activity of β 2-chimaerin, suggesting that phorbol esters (or their physiological analogue, DAG) may play a role in positional regulation by promoting the access of chimaerins to membranes. We hypothesize that whereas DAG could have a key role in membrane anchoring, acidic phospholipids probably facilitate the allosteric activation of β 2-chimaerin in the membrane. A similar anchoring role for DAG or phorbol esters has been postulated for novel phorbol ester receptors of the RasGRP and Munc13 family [9,31–35].

In order to elucidate further the functional properties of β 2-chimaerin and related chimaerin isoforms in a cellular context, we examined their Rac-GAP properties using a Rac-GTP pull-down assay. We used different chimaerin isoforms comprising either the N-terminal region (where the GAP domain has been deleted) or the GAP domains alone. Our results show clearly that the GAP

domain is required for the reduction of cellular Rac-GTP levels, and, as expected, the N-terminal region of chimaerins has no GAP activity. The mutant Δ EIE- β 2-chimaerin, which is GAP-inactive, confirmed further the essential role of the C-terminal GAP domain in Rac-GTP hydrolysis, as previously observed in α 1-chimaerin [23]. We are currently examining a series of β 2-chimaerin mutants that probably have impaired binding to Rac, and, in this way, we may be able to dissociate between Rac binding and Rac-GAP activity. This will allow for a comprehensive dissection of the structural elements responsible for β 2-chimaerin activity and pursuit of an extensive analysis of Rac-signalling regulation by this yet poorly explored family of Rac-GAPs.

Our studies show that GFP-chimaerins co-localize with an activated form of Rac1 in the plasma membrane, and that β 2-chimaerin co-immunoprecipitates with activated Rac. Thus we predict that chimaerins bind to the GTP-bound, active form of Rac. Furthermore, an inactive Rac mutant (N17Rac1) associates very weakly with β 2-chimaerin (H. Wang and M. G. Kazanietz, unpublished work). The model that we favour is that chimaerins translocate to the plasma membrane through the C1 domain upon receptor activation (for example after EGF receptor activation, which is coupled to DAG generation). Receptor activation also results in the loading of GTP on to Rac1 [25,26]. Upon redistribution to the plasma membrane, chimaerin isoforms associate with the activated form of Rac and promote GTP hydrolysis, which leads to Rac inactivation. Thus, in this context, chimaerins probably have a pivotal role as a ‘switch off’ mechanism for Rac. One may expect that if such a balance is impaired, it could lead to Rac hyperactivation. This may be relevant in some cancer models. For example, Yuan et al. [36] showed that highly invasive neuroblastoma cells have a pronounced down-regulation in the expression levels of β 2-chimaerin, resulting in Rac hyperactivation. It might also be possible that decreased Rac-GAP activity

Figure 6 Co-localization of chimaerin isoforms and activated Rac1

COS-1 cells were co-transfected with pEGFP vectors for $\alpha 1$ -, $\beta 1$ - or $\beta 2$ -chimaerin, and a mammalian expression vector for HA-Rac (HA-V12Rac1). After 60 h, cells were fixed with 4% (w/v) paraformaldehyde and were processed for immunofluorescence using an anti-HA antibody. HA-V12Rac is visualized as red after staining with Cy3-conjugated donkey anti-mouse secondary antibody. Slides were analysed by confocal microscopy. Similar results were observed in at least three independent experiments.

contributes to the Rac hyperactivation observed in other models, such as in breast cancer cells, as suggested by Mira et al. [37]. Importantly, Rac guanine nucleotide-exchange factors (Rac-GEFs), such as Tiam1 or Vav1, also lead to Rac hyperactivation, thereby contributing to the malignant and invasive phenotype [38–40]. We believe that, in this context, chimaerin isoforms may have important roles in Rac-mediated regulation of the actin cytoskeleton, which are important for cell spreading, migration and invasion. In support of this hypothesis, we have recently shown that expression of the GAP domain of $\beta 2$ -chimaerin in F3II murine carcinoma cells leads to a substantial reduction in motility and migration [41]. Moreover, β -GAP-expressing F3II cells have a marked decrease in their invasive and metastatic potential in nude mice, which is consistent with an impairment of Rac function [41].

In summary, our results show that $\beta 2$ -chimaerin and other chimaerin isoenzymes are negative regulators of Rac activity. These phorbol ester/DAG-regulated Rac-GAPs may have critical roles in the control of Rac-mediated signalling and function. These findings have important implications because they challenge the generally held perception that all phorbol ester/DAG signalling proceeds through the activation of PKC isoenzymes. The discovery of novel high-affinity phorbol ester receptors unrelated to

PKC isoenzymes challenges the use of phorbol esters and related compounds as selective PKC activators, and strongly emphasizes the concept that multiple signalling pathways can be regulated by DAG and phorbol esters.

This work was supported by grant R01-CA74197 (National Institutes of Health) and RPG 97-092-06-CNE (American Cancer Society).

REFERENCES

- 1 Jaken, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168–173
- 2 Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332**, 281–292
- 3 Ron, D. and Kazanietz, M. G. (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* **13**, 1658–1676
- 4 Newton, A. C. (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* **101**, 2353–2364
- 5 Hall, C., Monfries, C., Smith, C., Lim, P., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T. and Lim, L. (1990) Novel human brain cDNA encoding a 34,000 M_r protein *n*-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. *J. Mol. Biol.* **211**, 11–16

- 6 Kazanietz, M. G. (2000) Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. *Mol. Carcinogenesis* **28**, 5–11
- 7 Kazanietz, M. G., Caloca, M. J., Garcia-Bermejo, M. L., Reilly, M., Eroles, P., Fujii, T. and Wang, H. (2000) Pharmacology of the receptors for the phorbol ester tumor promoters: multiple receptors with different biochemical properties. *Biochem. Pharmacol.* **60**, 1417–1424
- 8 Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M. and Nishizuka, Y. (1997) Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* **6**, 477–480
- 9 Kazanietz, M. G. (2002) Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol. Pharmacol.* **61**, 1–9
- 10 Hall, C., Sin, W. C., Teo, M., Michael, G. J., Smith, P., Dong, J. M., Lim, H. H., Manser, E., Spurr, N. K., Jones, T. A. and Lim, L. (1993) $\alpha 2$ -Chimerin, an SH2-containing GTPase-activating protein for the ras-related protein p21^{rac} derived by alternate splicing of the human *n*-chimerin gene, is selectively expressed in brain regions and testes. *Mol. Cell. Biol.* **13**, 4986–4998
- 11 Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P. and Lim, L. (1990) Human brain *n*-chimaerin cDNA encodes a novel phorbol ester receptor. *Biochem. J.* **272**, 767–773
- 12 Leung, T., How, B.-E., Manser, E. and Lim, L. (1994) Cerebellar $\beta 2$ -chimaerin, a GTPase-activating protein for p21 ras-related rac is specifically expressed in granule cells and has a unique N-terminal SH2 domain. *J. Biol. Chem.* **269**, 12888–12892
- 13 Arces, L. B., Kazanietz, M. G. and Blumberg, P. M. (1994) Close similarity of baculovirus-expressed *n*-chimaerin and protein kinase C α as phorbol ester receptors. *J. Biol. Chem.* **269**, 19553–19558
- 14 Caloca, M. J., Fernandez, M. N., Lewin, N. E., Ching, D., Modali, R., Blumberg, P. M. and Kazanietz, M. G. (1997) $\beta 2$ -Chimaerin is a high affinity receptor for the phorbol ester tumor promoters. *J. Biol. Chem.* **272**, 26488–26496
- 15 Caloca, M. J., Wang, H., Delemos, A., Wang, S. and Kazanietz, M. G. (2001) Phorbol esters and related analogues regulate the subcellular localization of $\beta 2$ -chimaerin, a non-protein kinase C phorbol ester receptor. *J. Biol. Chem.* **276**, 18303–18312
- 16 Diekmann, D., Brill, S., Garret, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. (1991) Bcr encodes a GTPase-activating protein for p21^{rac}. *Nature (London)* **351**, 400–402
- 17 Ridley, A. J. (2001) Rho family proteins: coordinating cell responses. *Trends Cell Biol.* **11**, 471–477
- 18 Qiu, R. G., Chen, J., Kirn, D., McCormick, F. and Symons, M. (1995) An essential role for Rac in Ras transformation. *Nature (London)* **374**, 457–459
- 19 Olson, M. F., Ashworth, A. and Hall, A. (1995) An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* **269**, 1270–1272
- 20 Garrett, M. D., Self, A. J., Van Oers, C. and Hall, A. (1989) Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J. Biol. Chem.* **264**, 10–13
- 21 Kazanietz, M. G., Krausz, K. W. and Blumberg, P. M. (1992) Differential irreversible insertion of protein kinase C into phospholipid vesicles by phorbol esters and related activators. *J. Biol. Chem.* **267**, 20878–20886
- 22 Benard, V., Bohl, B. P. and Bokoch, G. M. (1999) Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* **274**, 13198–13204
- 23 Ahmed, S., Lee, J., Wen, L.-P., Zhao, Z., Ho, J., Best, A., Kozma, R. and Lim, L. (1994) Breakpoint cluster region gene product-related domain of *n*-chimaerin: discrimination between Rac-binding and GTPase-activating residues by mutational analysis. *J. Biol. Chem.* **269**, 17642–17648
- 24 Wang, H. and Kazanietz, M. G. (2002) 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. *J. Biol. Chem.* **277**, 4541–4550
- 25 Kim, B. C., Yi, J. Y., Yi, S. J., Shin, I. C., Ha, K. S., Jhun, B. H., Hwang, S. B. and Kim, J. H. (1998) Rac GTPase activity is essential for EGF-induced mitogenesis. *Mol. Cells* **8**, 90–95
- 26 Chiariello, M., Marinissen, M. J. and Gutkind, J. S. (2001) Regulation of c-myc expression by PDGF through Rho GTPases. *Nat. Cell Biol.* **3**, 580–586
- 27 Anand-Apte, B., Zetter, B. R., Viswanathan, A., Qiu, R. G., Chen, J., Ruggieri, R. and Symons, M. (1997) Platelet-derived growth factor and fibronectin-stimulated migration are differentially regulated by the Rac and extracellular signal-regulated kinase pathways. *J. Biol. Chem.* **272**, 30688–30692
- 28 Fujii, T., Garcia-Bermejo, M. L., Bernabo, J. L., Caamano, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S. H. and Kazanietz, M. G. (2000) Involvement of protein kinase C δ (PKC δ) in phorbol ester-induced apoptosis in LNCaP prostate cancer cells: lack of proteolytic cleavage of PKC δ . *J. Biol. Chem.* **275**, 7574–7582
- 29 Zhang, Q., Calafat, J., Janssen, H. and Greenberg, S. (1999) ARF6 is required for growth factor- and rac-mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. *Mol. Cell. Biol.* **19**, 8158–8168
- 30 Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410
- 31 Ebinu, J. O., Bortoff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J. and Stone, J. C. (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280**, 1082–1086
- 32 Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J. and Kay, R. J. (1998) Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* **18**, 6995–7008
- 33 Lorenzo, P. S., Kung, J. W., Bortoff, D. A., Garfield, S. H., Stone, J. C. and Blumberg, P. M. (2001) Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* **61**, 943–949
- 34 Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Rettig, J. and Brose, N. (1998) Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21**, 123–136
- 35 Betz, A., Thakur, P., Junge, H. J., Ashery, U., Rhee, J. S., Scheuss, V., Rosenmund, C., Rettig, J. and Brose, N. (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. *Neuron* **30**, 183–196
- 36 Yuan, S., Miller, D. W., Barnett, G. H., Hahn, J. F. and Williams, B. R. (1995) Identification and characterization of human $\beta 2$ -chimaerin: association with malignant transformation in astrocytoma. *Cancer Res.* **55**, 3456–3461
- 37 Mira, J. P., Benard, V., Groffen, J., Sanders, L. C. and Knaus, U. G. (2000) Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 185–189
- 38 van Leeuwen, F. N., van der Kammen, R. A., Habets, G. G. and Collard, J. G. (1995) Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. *Oncogene* **11**, 2215–2221
- 39 Stam, J. C., Michiels, F., van der Kammen, R. A., Moolenaar, W. H. and Collard, J. G. (1998) Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling. *EMBO J.* **17**, 4066–4074
- 40 Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S. and Bustelo, X. R. (1997) Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature (London)* **385**, 169–172
- 41 Lorenzano Menna, P., Skilton, G., Coluccio Leskow, F., Alonso, D. F., Gomez, D. E. and Kazanietz, M. G. (2003) Inhibition of aggressiveness of metastatic mouse mammary carcinoma cells by the $\beta 2$ -chimaerin GAP domain. *Cancer Res.* **63**, 2284–2291

Received 19 May 2003/15 July 2003; accepted 23 July 2003

Published as BJ Immediate Publication 23 July 2003, DOI 10.1042/BJ20030727