

# Regulation of vascular smooth muscle proliferation and migration by $\beta 2$ -chimaerin, a non-protein kinase C phorbol ester receptor

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**Abstract.** The proliferation and migration of vascular smooth muscle cells (SMC) are important aspects of atherogenesis. Activated growth factor signaling in injured vessels subsequently promotes a number of intracellular events resulting in the phenotypic modulation of SMC. Here, we investigated the role of  $\beta 2$ -chimaerin, a non-protein kinase C phorbol ester receptor with Rac-GTPase-activating protein activity, in growth factor-stimulated SMC. The endogenous expression of  $\beta 2$ -chimaerin was detected in cultured human SMC by reverse transcription-polymerase chain reaction and immunohistochemistry. Next, the overexpression of HA-tagged wild-type human  $\beta 2$ -chimaerin was attempted using cultured rat SMC with a recombinant adenovirus (Adv- $\beta 2$ -Chim). Adv-LZ encoding  $\beta$ -galactosidase (LacZ) was used as the control. The proliferation of SMC stimulated by platelet-derived growth factor (PDGF-BB, 10 ng/ml), as measured by cell-counting and 5-bromo-2'deoxyuridine incorporation assay, was suppressed by infection with Adv- $\beta 2$ -Chim (50-200 MOI), but not with control viruses. PDGF-induced SMC migration was inhibited by approximately 25% after infection with Adv- $\beta 2$ -Chim (200 MOI) using a modified Boyden's chamber assay with a fibronectin-coated membrane. Confocal microscopy revealed that PDGF stimulation altered the sub-cellular localization of  $\beta 2$ -chimaerin. The administration of 12-O-tetradecanoyl phorbol 13-acetate also induced changes in the sub-cellular localization of  $\beta 2$ -chimaerin, which was not affected by a presence of the PKC inhibitor (GF109203X). Finally, PDGF-induced Rac1 activation was found to be inhibited in the Adv- $\beta 2$ -Chim-infected cells. Thus, we demonstrated that  $\beta 2$ -chimaerin regulates the proliferation and migration of SMC

downstream of growth factor signaling pathway via the regulation of Rac1 activity. The signaling mediated by  $\beta 2$ -chimaerin may play a role in the regulation of SMC phenotypes, thereby implicating human atherogenesis.

## Introduction

Vascular smooth muscle cells (SMC) change their proliferation and migration properties in the response to vascular injury, which mediates atherogenesis and vascular remodeling (1,2). It has been widely accepted that the activated cytokine-growth factor network regulates SMC phenotypes (3,4). The majority of soluble SMC mitogens, as well as exposure of the vessels to mechanical or oxidative stresses can activate various intracellular molecules such as G proteins, mitogen-activated protein kinase (MAPK), phosphoinositide 3-OH kinase (PI3K), and protein kinase C (PKC) (5-7). However, the precise intracellular signaling mechanism regulating SMC phenotypes is not fully understood.

Platelet-derived growth factor (PDGF) is known to be one of the critical mitogens and chemoattractants of SMC that mediates hyperplasia and vascular remodeling in the development of cardiovascular diseases (8,9). The binding of PDGF causes PDGF-receptor (PDGF-R) dimerization and the subsequent activation of multiple signaling cascades (5,8). The activation of Ras and the MAPK cascade plays an important role in PDGF-induced DNA synthesis and proliferation (8). The activation of PI3K is involved in growth factor-mediated SMC proliferation and cell survival via the activation of Akt (a serine/threonine kinase) (10). Rac1, a member of the Rho family of small GTPases, is also a critical downstream mediator of PI3K and it regulates the reorganization of the actin cytoskeleton as well as cell migration and proliferation (11-14). The activation of PDGF-R also leads to the activation of phospholipase C- $\gamma$  (PLC- $\gamma$ ), resulting in the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>), which in turn induces intracellular Ca<sup>2+</sup> release (15). Protein kinase C (PKC), a family of serine-threonine kinases, has been identified as a cellular receptor for DAG and the phorbol ester tumor promoters (16). The activation of some PKC isozymes is known to be involved in the control of many cellular mechanisms downstream of DAG generation (17,18).

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The isozymes of PKCs are grouped into three subclasses by their regulatory properties. Conventional (classical) PKCs (cPKCs) include PKC  $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ , which are activated by  $\text{Ca}^{2+}$  and/or by DAG and phorbol esters. The novel PKCs (nPKCs) include PKC  $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\eta$  are activated DAG and/or phorbol esters, but are  $\text{Ca}^{2+}$  independent. PKC $\xi$  and PKC $\iota$  are atypical PKCs, which are unresponsive to  $\text{Ca}^{2+}$  and DAG and/or phorbol esters (16). PKC isozymes have been implicated in several cellular responses (e.g. proliferation, migration, hypertrophy, and apoptosis) that are important in the development of various diseases (17,18). For many years, it was believed that cPKCs and nPKCs were only receptors for DAG and/or phorbol esters. However, additional DAG and/or phorbol ester receptors have been identified such as: mammalian  $\alpha$ - and  $\beta$ -chimaerins, Ras-GRP, and *Caenorhabditis elegans* Unc-13/Munc13 (16,19-21). Thus, it could be anticipated that the PDGF-mediated cellular responses are potentially regulated by these novel 'non-kinase' DAG/phorbol ester receptors, an issue that has not been explored in SMC.

The first cloned member of the chimaerins is  $\alpha$ 1-chimaerin (n-chimaerin), which is a 38 kDa protein that is highly expressed in the brain and which resembles a 'chimera' between the regulatory domain of PKC and Bcr (the product of breakpoint cluster region gene involved in Philadelphia chromosome translocation in chronic myelogenous leukemia) (16,22). Several chimaerin isoforms ( $\alpha$ 1- or n-,  $\alpha$ 2-,  $\beta$ 1-, and  $\beta$ 2-chimaerins) have been isolated to date, and different isoforms are derived from alternative splicings of the  $\alpha$ - and  $\beta$ -chimaerin genes (16,19). All chimaerin isoforms have a single  $\text{NH}_2$ -terminal C1 domain (the DAG/phorbol ester-binding site) and a COOH-terminal GTPase-activating protein (GAP) domain responsible for Rac inactivation (23-27). A recent study showed that  $\beta$ 2-chimaerin accelerates the hydrolysis of GTP from Rac1, without affecting GTP hydrolysis from Cdc42 or RhoA (27). Analysis of the crystal structure of  $\beta$ 2-chimaerin revealed that DAG binding to the C1 domain triggers the cooperative dissociation of four regions of the molecule (i.e. the  $\text{NH}_2$ -terminus, SH2 domain, Rac-GAP domain, and the linker between the SH2 and C1 domains), thus resulting in the activation of the enzyme (28). The  $\beta$ 2-chimaerin gene (CHN2) is mapped to chromosome 7p15.2, the expression of which has been demonstrated in a variety of human tissues (29). Down-regulation of  $\beta$ 2-chimaerin expression has been shown in high-grade human gliomas and breast cancers (29-31). Adenoviral delivery of wild-type  $\beta$ 2-chimaerin was found to inhibit proliferation of human MCF-7, breast cancer cells, along with a reduction in Rac-GTP levels (30). Forced expression of the catalytic domain of  $\beta$ 2-chimaerin (the  $\beta$ -GAP domain) in F3II, sarcomatoid mammary carcinoma cells, has been shown to result in a reduction of metastatic ability of the cells in a mouse model (31).

In the present study, we tested the hypothesis that  $\beta$ 2-chimaerin is involved in the cellular response of mitogen-stimulated SMC. Endogenous expression of  $\beta$ 2-chimaerin was shown in human SMC. Adenoviral delivery of wild-type  $\beta$ 2-chimaerin resulted in inhibition of Rac1 activity in SMC and in a reduction of the proliferation rate and migratory properties in PDGF-stimulated rat SMC. We speculate that  $\beta$ 2-chimaerin may play a role in the regulation of SMC

responses in response to activated cytokines and growth factors in vascular lesions.

## Materials and methods

**Cell culture of SMC and proliferation assay.** Human neonatal aortic SMC (MS27W), rat aortic SMC (RASMC), and human glioma cells (U251MG) were obtained as previously described (32-34). MS27W and RASMC passage 5-10 times were used in the experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Filtron Pty. Ltd., Brooklyn, Australia) together with antibiotics (100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin), incubated at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$ -95% air atmosphere. All media and supplements were purchased from Life Technologies (Grand Island, NY) unless otherwise noted. Human recombinant PDGF-BB was purchased from Pepro-Tech (Rocky Hill, NJ). Phorbol-12-myristate-13-acetate (TPA) was purchased from Calbiochem (La Jolla, CA). A PKC-specific inhibitor, GF109203X [2-[1-3-(Dimethylaminopropyl) indol-3-yl]-3-(1*H*-indol-3-yl)maleimide], was purchased from LC Laboratories (Woburn, MA). For the assessment of cell proliferation, cells were seeded on a 6-well dish and cultured for 24 h in serum-free media before PDGF stimulation. Cell numbers were then determined using a hemocytometer after trypan blue exclusion staining. Alternatively, cells were seeded on 96-well plates, and 5-bromo-2'-deoxyuridine (BrdU) incorporation was determined using a cell proliferation ELISA kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. Absorbance at 450-690 nm was measured using a scanning multiwell spectrophotometer (Eimax A-4, Fujirebio, Japan).

**Adenovirus-mediated in vitro gene transfer.** The generation of the replication-deficient human adenovirus vector expressing haemagglutinin (HA)-tagged full-length  $\beta$ 2-chimaerin (Adv- $\beta$ 2-Chim) under the control of the CMV promoter, was described elsewhere (30,31). Adv-LZ, which encodes  $\beta$ -galactosidase (LacZ) was used as a control vector. The adenoviral vectors were amplified and titrated in HEK293 cells (American Tissue Culture Collection, Bethesda, MD) using standard techniques (35). The transfection efficiency was evaluated by *in situ* X-Gal staining, and infection with 200 MOI (multiplicity of infection) of Adv-LZ resulted in 80-100% of RASMC testing positive. For adenoviral infections, cells were incubated with the corresponding vectors at the indicated MOI for 2 h in DMEM containing 2% FCS. Then, serum-contained media were added  $\leq 5\%$  of the final serum concentration and the cells were cultured for further 20 h.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and was then incubated with DNase I (Life Technologies), to avoid the contamination of the genomic DNA. Extracted RNA (1  $\mu\text{g}$ ) was exposed to MnLV reverse transcriptase in the presence of 20 U of RNase inhibitor, 2.5 mM of random hexamers, dNTP (2 mM each), 2.5 mM  $\text{MgCl}_2$ , and 1X RCR buffer II (RNA PCR kit; Perkin Elmer, Norwalk, CT) in a total volume of 20  $\mu\text{l}$ . The reaction was allowed to continue for 30 min at

42°C, and then subjected to PCR in the presence of human  $\beta$ 2-chimaerin-specific primers (forward primer: 5'-ACATgAAgAACACACAgCgg-3'; reverse primer: 5'-TgCATATgTCTACCACCATgg-3'), 2 mM  $MgCl_2$ , and 2.5 U of Gold Taq Polymerase (Perkin Elmer) in a total volume of 100  $\mu$ l. For the internal controls, amplification with  $\beta$ -actin primers (Research Genetics, Huntsville, AL) was also carried out. The temperature profile of the PCR was an initial denaturing step at 95°C for 3 min, followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 57°C for 1 min, and extension at 72°C for 1 min in the case of both  $\beta$ 2-chimaerin and  $\beta$ -actin. PCR product (10  $\mu$ l) were applied to 1% agarose gel, electrophoresed, and visualized with a UV transilluminator.

**Immunohistochemistry.** Cells were grown on a glass coverslip at a density of  $2 \times 10^4/cm^2$  and were further incubated under the serum-free condition 24 h prior to stimulation with 1  $\mu$ M of TPA or 10 ng/ml of PDGF. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 10 min, and permeabilized with 0.2% Triton X-100. For the detection of endogenous  $\beta$ 2-chimaerin expression, cells were incubated with a 1:250 dilution of monoclonal  $\beta$ 2-chimaerin antibody (26) for 1 h, followed by incubation with a 1:1000 dilution of FITC-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA). For the detection of exogenously expressed HA-tagged  $\beta$ 2-chimaerin, the cells were incubated with a mouse monoclonal anti-HA antibody (1:1000 dilution, Covance, Berkeley, CA). After counterstaining with 0.1  $\mu$ g/ml of propidium iodide (Sigma, St. Louis, MO), the specimens were observed with an Olympus fluorescence microscope (Tokyo, Japan) or a confocal microscope (Model C1, Nikon, Tokyo, Japan).

**Western blot analysis.** Total cell extracts were prepared with Nonidet P (NP)-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP-40). The protein concentrations were determined with the use of commercially available protein assay reagent (Bio-Rad Laboratories). Proteins (15  $\mu$ g for each lane) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Bioscience, Buckinghamshire, UK), followed by probing with a mouse monoclonal anti-HA antibody (1:1000 dilution, Covance) for the detection of adenovirally expressed HA-tagged  $\beta$ -chimaerin. Horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (Bio-Rad Laboratories) was used as a second antibody. Detection was performed with enhanced chemiluminescence (Amersham Bioscience).

**Migration assay.** Migration ability of RASMC was assayed according to Boyden's chamber method, which was modified by the use of microchemotaxis chambers and polycarbonate filters (Corning, Corning, NY) with a pore size of 5.0  $\mu$ m (36). The filters were coated with 20  $\mu$ g/ml fibronectin (Sigma) and were placed between the chambers. Cells were trypsinized and suspended at a concentration of  $5.0 \times 10^5$  cells/ml in DMEM supplemented with 10% FBS. The RASMC suspension (50  $\mu$ l) was placed in the upper chamber, and DMEM containing 10 ng/ml of human recombinant PDGF-BB was placed in the lower chamber. Both chambers were incubated for 6 h and then

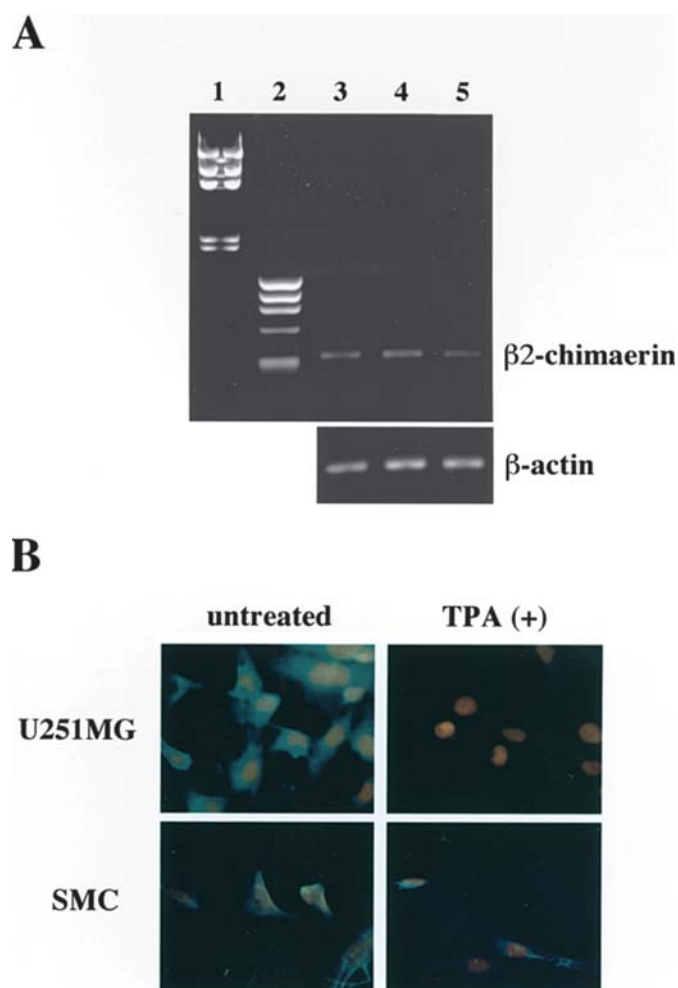


Figure 1. Endogenous  $\beta$ 2-chimaerin expression in human SMC. (A) RT-PCR for  $\beta$ 2-chimaerin. Total RNA (1  $\mu$ g) was isolated and then subjected to RT-PCR. Lane 1:  $\lambda$ DNA-HindIII digest. Lane 2:  $\phi$ X174-Hae III digest, DNA molecular weight markers (Takara Shuzo, Co., Ltd., Otsu, Japan). Lane 3: MS27W, human SMC cultured with serum-free media. Lane 4: MS27W cultured with 10% FBS-containing media. Lane 5: U251MG, human glioma cells. For the internal controls, amplification with  $\beta$ -actin primers was also tested. (B) Immunohistochemistry for  $\beta$ 2-chimaerin. MS27W and U251MG cells were cultured on coverslips and indirect immunohistochemical staining for  $\beta$ 2-chimaerin was performed. The cells were also treated with 1  $\mu$ M of TPA for 5 min (right panels).

the filter was removed. Cells, which migrated from the upper side to the lower side of the filter, were stained with Diff-Quick staining solution (Sysmex, Kobe, Japan), and the number of cells was counted under a microscope. Migration activity was expressed as the mean number of cells that had migrated per high-power field (hpf).

**Determination of Rac-GTP levels.** Rac activation levels were measured by selective affinity precipitation of Rac-GTP with the immobilized p21-binding domain (PBD) of p21/Cdc42/Rac1-activated kinase1 (PAK1), using a commercially available pull-down assay kit (Upstate, Charlottesville, VA) (37). Briefly, the cells were lysed in a buffer containing 8  $\mu$ g of GST-PBD, 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM  $MgCl_2$ , 150 mM NaCl, 0.5% (v/v) NP-40, 5 mM  $\beta$ -glycerophosphate, and proteinase inhibitors [5  $\mu$ g/ml 4-(2-aminoethyl)benzenesulphonyl fluoride, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin and



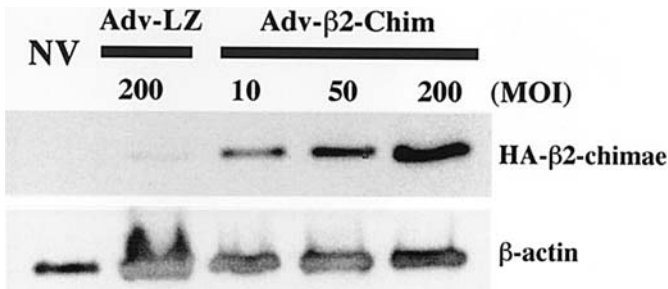


Figure 2. Overexpression of haemagglutinin (HA)-tagged full-length  $\beta$ 2-chimaerin in RASMC. The cells were infected with 200 MOI of Adv-LZ or with 10-200 MOI of Adv- $\beta$ 2-Chim, otherwise mock-infected (NV). The cell lysates were collected at 48 h after infection, and then analyzed by Western blotting. The blots were probed with an antibody against HA-tag, and then reprobed with an antibody against  $\beta$ -actin.

1  $\mu$ g/ml pepstatin A]. Lysates were centrifuged at 14,000  $\times$  for 10 min at 4°C and were then incubated with glutathione-Sepharose 4B beads for 1 h. After extensive washing, beads were boiled in loading buffer, and the samples were resolved on 12% SDS-PAGE gels, transferred onto nitrocellulose membranes, and analyzed by Western blotting using a panisofom-specific anti-Rac1 antibody. Densitometric analysis was performed with NIH image-analyzing software.

**Statistical analysis.** Experimental groups were compared by analysis of variance (ANOVA), and, when appropriate, with Scheffe's test for multiple comparisons. The data are expressed as the mean  $\pm$  SD. A level of  $P < 0.05$  was considered as statistically significant.

## Results

**Expression of endogenous  $\beta$ 2-chimaerin in human SMC and exogenous  $\beta$ 2-chimaerin in RASMC.** We first examined by RT-PCR the endogenous expression of  $\beta$ 2-chimaerin in cultured human SMC.  $\beta$ 2-chimaerin transcript was detected both in serum-stimulated and starved SMC in RNA levels. Consistently with the results of a previous report (29), weak expression of  $\beta$ 2-chimaerin was detected in U251MG, human glioma cells (Fig. 1A). By conventional immunohistochemistry, cytoplasmic staining of  $\beta$ 2-chimaerin was detected in human SMC, as well as in glioma cells and 30 min incubation with 1  $\mu$ M TPA resulted in a reduction in the cytoplasmic signal of  $\beta$ 2-chimaerin in both cell types (Fig. 1B). To further investigate the role of  $\beta$ 2-chimaerin in the growth factor response of SMC, we attempted the overexpression of  $\beta$ 2-chimaerin in cultured RASMC. HA-tagged full-length  $\beta$ 2-chimaerin was successfully expressed by infection with Adv- $\beta$ 2-Chim in a dose-dependent fashion (Fig. 2). In agreement with our previous study (35), cells infected with 200 MOI of Adv-LZ, a control vector, exhibited no changes in terms of either cell proliferation or morphology (data not shown).

**Proliferation of mitogen-stimulated RASMC expressing exogenous  $\beta$ 2-chimaerin.** For the assessment of cell proliferation, RASMC infected with 50-200 MOI of adenovirus vectors were cultured in DMEM supplemented with or without 10 ng/ml of PDGF for 48 h. Under the unstimulated condition,

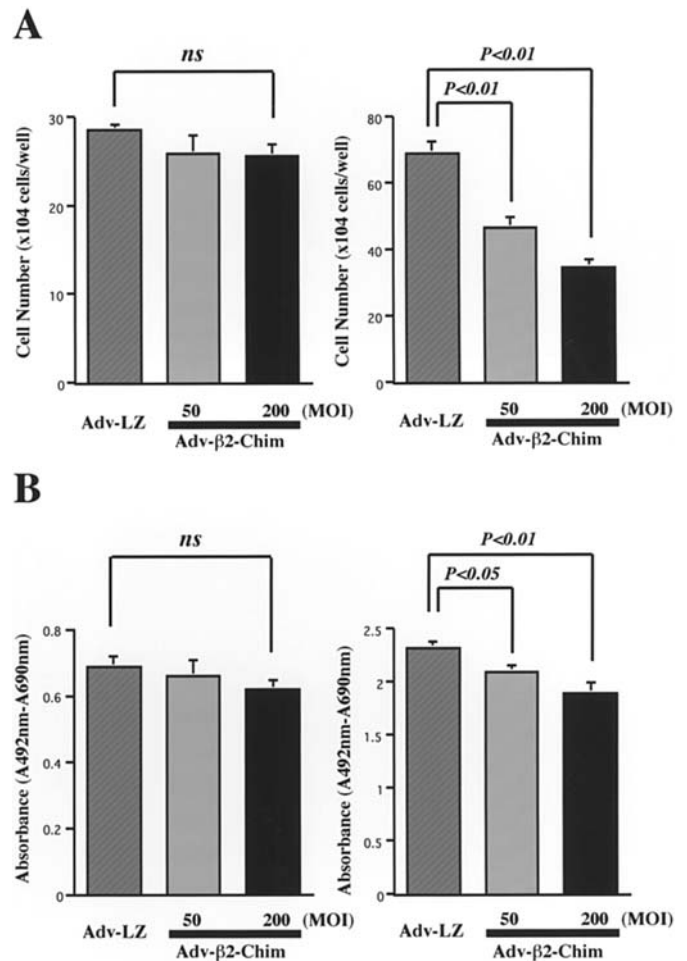


Figure 3. The effect of the exogenous expression of  $\beta$ 2-chimaerin on the proliferation of RASMC. (A) Hand-counting assay. RASMC were infected with 200 MOI of Adv-LZ or with 50-200 MOI of Adv- $\beta$ 2-Chim. The cells were further incubated under serum-free conditions (left panel) or in media containing 10 ng/ml of PDGF (right panel) for 48 h, and then hand-counted. The data are presented as the mean  $\pm$  SD ( $n=4$ ). ns; not significant. P-values are indicated. (B) BrdU incorporation assay. RASMC were infected with adenovirus vectors as described above. The cells were further incubated under serum-free conditions (left panel) or media containing 10 ng/ml of PDGF (right panel) for 24 h, and then incubated with BrdU-labeling solution for an additional 4 h. The data are presented as the mean  $\pm$  SD ( $n=6$ ). ns; not significant. P-values are indicated.

the cell number was unchanged; however, PDGF-induced RASMC proliferation was found to be reduced by Adv- $\beta$ 2-Chim in a dose-dependent fashion (Fig. 3A). In the BrdU incorporation assay, cells were cultured in DMEM supplemented with or without 10 ng/ml of PDGF for 24 h and then subsequently incubated with BrdU-labeling solution for an additional 4 h. We previously reported observing maximal S-phase entry 24 h after PDGF stimulation of quiescent SMC (38). Consistently with the data of cell counting, BrdU incorporation assay showed a significant reduction of PDGF-induced RASMC proliferation when  $\beta$ 2-chimaerin was expressed by adenoviral means (Fig. 3B).

**Migration of mitogen-stimulated RASMC expressing exogenous  $\beta$ 2-chimaerin.** PDGF-induced RASMC migration was assayed by using a modified Boyden's chamber method. The number of migrated Adv-LZ-infected cells remained

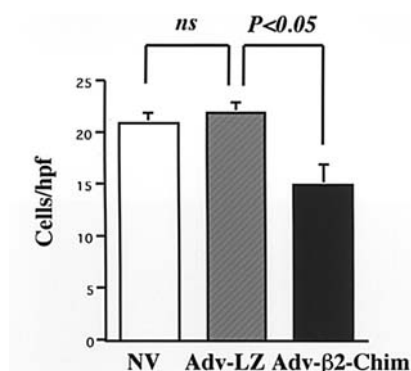


Figure 4. The effect of the exogenous expression of  $\beta 2$ -chimaerin on the migration of RASMC. The cells were infected with 200 MOI of Adv-LZ or Adv- $\beta 2$ -Chim, otherwise mock-infected (NV). PDGF (10 ng/ml)-induced migration of RASMC was assayed by modified Boyden's chamber method, as described in Materials and methods. The migration activity was expressed as the mean number of cells that had migrated per high-power field (hpf). The data are presented as the mean  $\pm$  SD (n=3). ns; not significant. P-values are indicated.

unaltered, in comparison with mock-infected cells. PDGF-induced RASMC migration was reduced in Adv- $\beta 2$ -Chim-infected cells by 19-26% ( $P<0.05$ ) (Fig. 4).

**Subcellular localization of  $\beta 2$ -chimaerin in mitogen-stimulated RASMC.** TPA-induced redistribution of  $\beta 2$ -chimaerin has been reported, suggesting a close association between subcellular localization and molecular function (26). Thus, we evaluated the subcellular localization of adenovirally transfected HA-tagged  $\beta 2$ -chimaerin in RASMC by confocal microscopy. In the unstimulated cells, HA-tagged  $\beta 2$ -chimaerin was distributed throughout the cytoplasm. Upon PDGF stimulation, a significant fraction of HA-tagged  $\beta 2$ -chimaerin distributed to the plasma membrane with weak perinuclear staining in a time-dependent manner (Fig. 5A). Changes in the subcellular localization of HA-tagged  $\beta 2$ -chimaerin were also observed upon TPA (1  $\mu$ M, 10 min)-stimulation. Pre-treatment of 5  $\mu$ M of a PKC inhibitor, GF109203X, was not found to affect the subcellular localization of HA-tagged  $\beta 2$ -chimaerin in TPA-stimulated cells (Fig. 5B).

**Rac-GTP levels in mitogen-stimulated RASMC expressing exogenous  $\beta 2$ -chimaerin.** We investigated whether  $\beta 2$ -chimaerin regulates Rac-GTP levels in RASMC or not. To avoid the potential influence of PKC activity on Rac-GTP levels, cells were pre-treated with 5  $\mu$ M of a PKC inhibitor, GF109203X, 30 min before PDGF stimulation. Ten minutes after stimulation with 10 ng/ml of PDGF, Rac-GTP levels were elevated by approximately 30% in Adv-LZ-infected cells. On the other hand, the Rac-GTP levels were down-regulated by about approximately 80% in Adv- $\beta 2$ -Chim-infected cells, irrespective of PDGF stimulation (Fig. 6).

## Discussion

The elucidation of the biological functions of chimaerins is important, because their target, the Rac1-GTPase, regulates various cellular events such as proliferation and migration (11-14,39). Although accumulated lines of evidence suggest

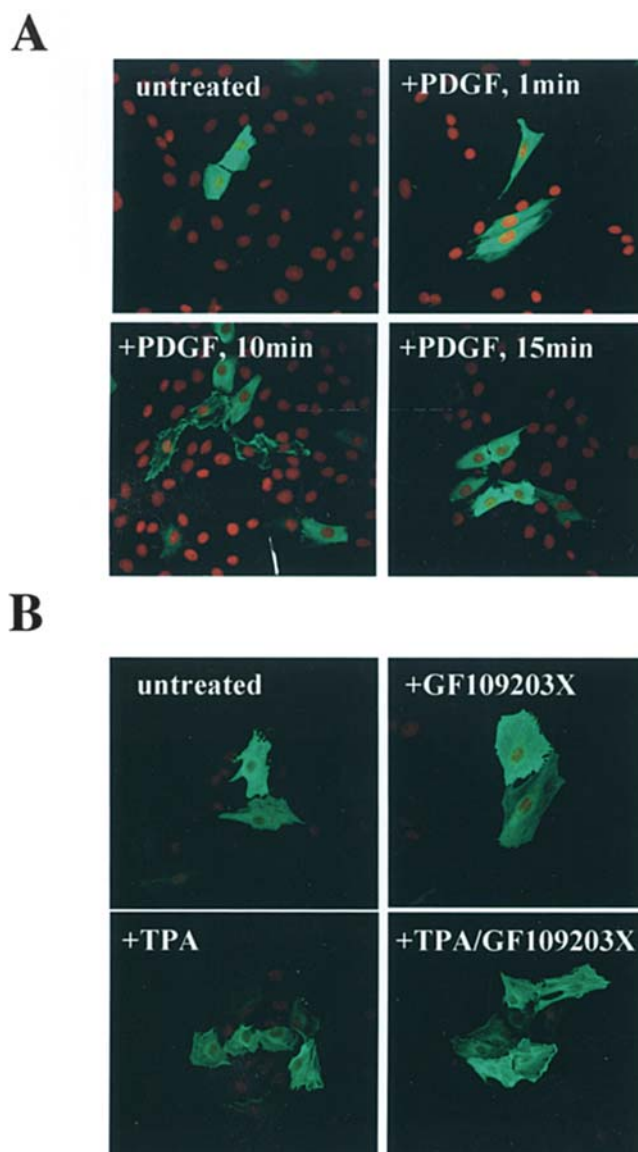


Figure 5. Subcellular localization of  $\beta 2$ -chimaerin in mitogen-stimulated RASMC. (A) Time-dependent effects induced by PDGF. The cells were infected with 200 MOI of Adv- $\beta 2$ -Chim (expressing HA-tagged  $\beta 2$ -chimaerin) and were then stimulated with 10 ng/ml of PDGF for the indicated periods of time. Immunohistochemical staining with an anti-HA-tag antibody was performed, and the specimens were observed by confocal microscopy. (B) TPA-induced effects and the influence of PKC inhibitor. RASMC were infected with Adv- $\beta 2$ -Chim as described above. The cells were then stimulated with 1  $\mu$ M of TPA for 10 min with or without 5  $\mu$ M of PKC inhibitor, GF109203X. Immunohistochemical staining and observation were performed as described above.

that chimaerins act as a non-protein kinase C phorbol ester/DAG receptor with Rac-GAP activity (16,19-28), there is very limited information regarding their roles in the context of development and diseases. In the present study, we demonstrated for the first time that endogenous  $\beta 2$ -chimaerin is expressed in human SMC at both the RNA and protein levels (Fig. 1). The changes observed in the subcellular localization of  $\beta 2$ -chimaerin in TPA-stimulated SMC implicated that this molecule is a non-protein kinase C phorbol ester receptor in SMC, as reported in other cell types (26). As the expression of  $\beta 2$ -chimaerin was observed in either serum-stimulated or

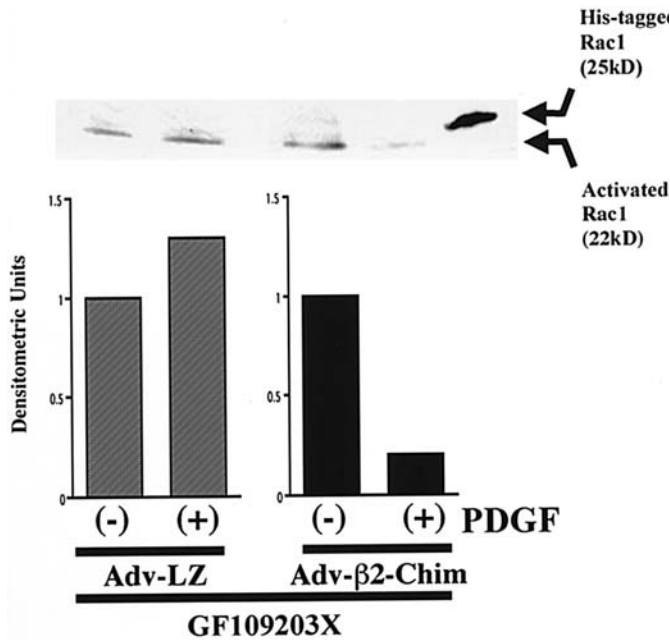


Figure 6. Rac-GTP levels in exogenous  $\beta$ 2-chimaerin-expressing RASMC. The cells were infected with 200 MOI of Adv-LZ or Adv- $\beta$ 2-Chim. After 30 min of incubation with 5  $\mu$ M of GF109203X, the cells were stimulated with 10 ng/ml of PDGF for 10 min. Rac-GTP levels were assayed with a pull-down assay kit as described in Materials and methods. Representative results of SDS-PAGE and the data from the densitometric analysis are shown (n=2).

serum-starved SMC, it is thought that  $\beta$ 2-chimaerin may function irrespective of SMC phenotypes (33). Moreover, our preliminary data of immunohistochemical study using human aortic frozen sections showed that  $\beta$ 2-chimaerin-expressing cells were localized in neointimal fibroproliferative lesions, thus suggesting a potential role of  $\beta$ 2-chimaerin in human atherogenesis (data not shown).

To further investigate the role of  $\beta$ 2-chimaerin in the growth factor response of SMC, we attempted overexpression of HA-tagged full-length  $\beta$ 2-chimaerin in cultured RASMC. Proliferation of PDGF-stimulated cells was dose-dependently inhibited by the exogenous expression of  $\beta$ 2-chimaerin (Fig. 3). It has consistently been reported that the overexpression of  $\beta$ 2-chimaerin leads to growth suppression in human breast cancer cells (30). We found that overexpression of  $\beta$ 2-chimaerin did not cause a reduction in cell number in quiescent SMC (Fig. 3A). This suggests that activation of  $\beta$ 2-chimaerin would occur only in the context of receptor stimulation, such as the activation of tyrosine-kinase receptors coupled to DAG generation. Migration of PDGF-stimulated cells was significantly inhibited by exogenous expression of  $\beta$ 2-chimaerin (Fig. 4). It has been reported that overexpression of the catalytic domain (the  $\beta$ -GAP domain) of  $\beta$ 2-chimaerin reduced metastatic potential in the mouse transplanted tumor of human breast cancer cells (31). Thus,  $\beta$ 2-chimaerin may be an important regulatory molecule of the cell motility in normal, as well as in transformed cells, and this may have great implications for the metastatic dissemination of cancer cells.

Activation of cPKCs and nPKCs requires binding of a membrane-embedded ligand, DAG, to their C1 domain, and the energy of this interaction facilitates the access of PKCs to substrates and triggers downstream signaling (40). Activation

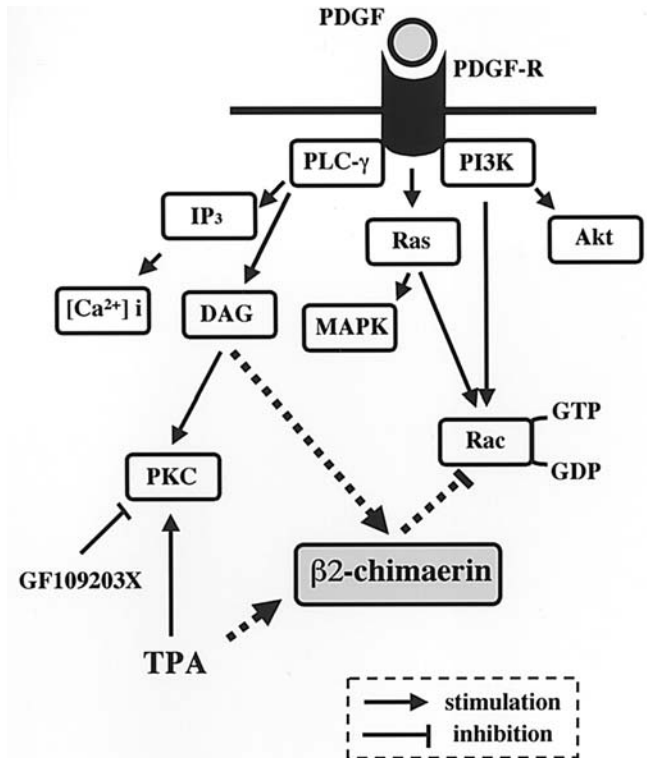


Figure 7. Hypothetical pathways of  $\beta$ 2-chimaerin-mediated signaling downstream of PDGF stimulation in SMC. Known pathways described in the literature are indicated by solid lines, and hypothetical pathways proposed on the basis of the present study are indicated by dotted lines. DAG and TPA potentially activate not only PKC but also  $\beta$ 2-chimaerin, a novel Rac-GAP protein, resulting in the negative regulation of Rac activity and the inhibition of proliferation and migration of SMC.

of PKCs by phorbol esters is also associated with the translocation from cytosolic to particulate fractions (41). Chimaerins possess a single C1 domain with high homology to those present in PKCs (25-27). It has been demonstrated that  $\beta$ 2-chimaerin translocates from the cytosolic to the particulate fraction as a consequence of treatment with TPA or DAG analogs, and this redistribution is thought to be a key event in the regulation of Rac activity (25-27). In the present study, we also observed changes in the subcellular distribution of  $\beta$ 2-chimaerin in TPA- and PDGF-stimulated cells (Figs. 1 and 5). Upon PDGF stimulation, HA-tagged  $\beta$ 2-chimaerin appeared to be re-distributed to the plasma membrane (Fig. 5A). TPA-induced re-distribution of  $\beta$ 2-chimaerin was not affected by the PKC inhibitor, GF109203X (Fig. 5B), which may further support the notion that TPA directly activates  $\beta$ 2-chimaerin (25,26). It has also been reported that TPA induces the perinuclear localization of  $\beta$ 2-chimaerin, and in particular, Golgi localization in COS cells (26). A chimaerin-interacting (or anchoring) protein, Tmp21-I (p23), has been identified, which localizes in the *cis*-Golgi network and is involved in intracellular vesicular trafficking (42). In the present study, we observed only weak perinuclear staining of  $\beta$ 2-chimaerin in TPA- and PDGF-stimulated SMC (Figs. 1 and 5B), suggesting a differential regulation in different cell lines. Further studies are needed to understand the biological significance of the subcellular localization and whether it relates to different interacting proteins or specific generation of lipid pools in different cell contexts.



In agreement with the findings of previous studies (11-13), PDGF up-regulated Rac-GTP levels in the control conditions. Marked down-regulation of PDGF-induced Rac1 activation was observed in RASMC after forced expression of  $\beta 2$ -chimaerin, in the presence of a PKC inhibitor (Fig. 6). We also performed similar experiments in the absence of the PKC inhibitor. Under the latter condition, PDGF slightly up-regulated Rac1 activity, irrespective of the overexpression of  $\beta 2$ -chimaerin (data not shown). As the TPA-induced translocation of  $\beta 2$ -chimaerin was not affected by the PKC inhibitor (Fig. 5B), it is expected that PKCs exert an influence on Rac1 activity, but not on  $\beta 2$ -chimaerin activation. It has been reported that TPA has a dual effect on Rac-GTP levels in COS-1 cells transfected with a plasmid expressing full-length  $\beta 2$ -chimaerin. TPA is known to increase Rac-GTP levels in the absence of a PKC inhibitor, whereas TPA markedly reduces Rac-GTP levels in the presence of a PKC inhibitor and thus potentiates  $\beta 2$ -chimaerin activity (27). Therefore, it could be anticipated that PKCs, as well as  $\beta 2$ -chimaerin, play an important role in the regulation of Rac-GTP levels in SMC.

Downstream of activated PDGF-R, it has been reported that Rac-GTP levels are mainly regulated by PI3K (11,14). In some cells, oncogenic Ras also drives both the Rac and MAPK pathways, which co-operate to cause malignant transformation (43,44). Our data may implicate the presence of signaling cross-talk between PI3K-/Ras-Rac pathways and the PLC $\gamma$ -DAG-chimaerin pathway downstream of PDGF-R. The former pathways act as positive regulators, whereas the latter appears to act as a down-modulator of Rac activity (Fig. 7). Further studies are required to understand the complex signaling pathways involved in the regulation of Rac activity, which have not been fully elucidated in SMC.

In conclusion, this is the first demonstration that  $\beta 2$ -chimaerin is expressed in cultured human smooth muscle cells.  $\beta 2$ -chimaerin may impact on PDGF-induced proliferation and migration, and Rac-GTP levels. Thus, it is likely that  $\beta 2$ -chimaerin signaling may play a role in the regulation of SMC phenotypes and therefore have implications in human atherogenesis.

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