SPANDIDOS PUBLICATIONS

Regulation of vascular smooth muscle proliferation and migration by ß2-chimaerin, a non-protein kinase C phorbol ester receptor

MITSUHIDE MAEDA[1,2], SEIYA KATO[2], SHINTRO FUKUSHIMA[1,2], UTAKO KANEYUKI[2], TERUHIKO FUJII[3], MARCELO G. KAZANIETZ[4], KOICHI OSHIMA[2] and MINORU SHIGEMORI[1]

Departments of [1]Neurosurgery and [2]Pathology, and [3]Department of Surgery and Research Center for Innovate Cancer Therapy, Kurume University School of Medicine, Kurume, Japan; [4]Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Received November 4, 2005; Accepted December 19, 2005

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 ß2-chimaerin, a non-protein kinase C phorbol ester receptor with Rac-GTPase-activating protein activity, in growth factor-stimulated SMC. The endogenous expression of ß2-chimaerin was detected in cultured human SMC by reverse transcription-polymerase chain reaction and immunohistochemistry. Next, the overexpression of HA-tagged wild-type human ß2-chimaerin was attempted using cultured rat SMC with a recombinant adenovirus (Adv-ß2-Chim). Adv-LZ encoding ß-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-ß2-Chim (50-200 MOI), but not with control viruses. PDGF-induced SMC migration was inhibited by approximately 25% after infection with Adv-ß2-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 ß2-chimaerin. The administration of 12-O-tetradecanoyl phorbol 13-acetate also induced changes in the sub-cellular localization of ß2-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-ß2-Chim-infected cells. Thus, we demonstrated that ß2-chimaerin regulates the proliferation and migration of SMC downstream of growth factor signaling pathway via the regulation of Rac1 activity. The signaling mediated by ß2-chimaerin may play a role in the regulation of SMC phenotypes, thereby implicating human atherogenesis.

Correspondence to: Dr Seiya Kato, Department of Pathology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
E-mail: seikato@med.kurume-u.ac.jp

Key words: smooth muscle cells, ß2-chimaerin, rac, proliferation, migration

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-γ (PLC-γ), resulting in the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which in turn induces intracellular Ca^{2+} release (15). Protein kinase C (PKC), a family of serine-threonin 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).

The isozymes of PKCs are grouped into three subclasses by their regulatory properties. Conventional (classical) PKCs (cPKCs) include PKC α, ßI, ßII, and γ, which are activated by Ca^{2+} and/or by DAG and phorbol esters. The novel PKCs (nPKCs) include PKC δ, ε, θ, and η are activated DAG and/or phorbol esters, but are Ca^{2+} independent. PKCξ and PKCι are atypical PKCs, which are unresponsive to 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 α- and ß-chimaerins, Ras-GRP, and *Caenorhabditid 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 α1-chimaerin (n-chimaerin), which is a 38 kDa protein that is highly expressed in the brain and which resembles a 'chimera' between the regulatory domein of PKC and Bcr (the product of breakpoint cluster region gene involved in Philadelphia chromosome translocation in chronic myelogenic leukemia) (16,22). Several chimaerin isoforms (α1- or n-, α2-, ß1-, and ß2-chimaerins) have been isolated to date, and different isoforms are derived from alternative splicings of the α- and ß-chimaerin genes (16,19). All chimaerin isoforms have a single 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 ß2-chimaerin accelerates the hydrolysis of GTP from Rac1, without affecting GTP hydrolysis from Cdc42 or RhoA (27). Analysis of the crystal structure of ß2-chimaerin revealed that DAG binding to the C1 domain triggers the cooperative dissociation of four regions of the molecule (i.e. the 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 ß2-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 ß2-chimaerin expression has been shown in high-grade human gliomas and breast cancers (29-31). Adenoviral delivery of wild-type ß2-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 ß2-chimaerin (the ß-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 ß2-chimaerin is involved in the cellular response of mitogen-stimulated SMC. Endogenous expression of ß2-chimaerin was shown in human SMC. Adenoviral delivery of wild-type ß2-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 ß2-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 μg/ml of streptomycin), incubated at 37˚C in a humidified 5% 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 (Woburum, 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 ß2-chimaerin (Adv-ß2-Chim) under the control of the CMV promoter, was described elsewhere (30,31). Adv-LZ, which encodes ß-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 ≤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 μg) 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 $MgCl_2$, and 1X RCR buffer II (RNA PCR kit; Perkin Elmer, Norwalk, CT) in a total volume of 20 μl. The reaction was allowed to continue for 30 min at

l then subjected to PCR in the presence of human erin-specific primers (forward primer: 5'-ACATgAA gAACACACAgCgg-3': reverse primer: 5'-TgCATATgTCT ACCACCATgg-3'), 2 mM $MgCl_2$, and 2.5 U of Gold Taq Polymerase (Perkin Elmer) in a total volume of 100 μl. For the internal controls, amplification with ß-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 ß2-chimaerin and ß-actin. PCR product (10 μl) 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 $2x10^4/cm^2$ and were further incubated under the serum-free condition 24 h prior to stimulation with 1 μM 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 ß2-chimaerin expression, cells were incubated with a 1:250 dilution of monoclonal ß2-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 ß2-chimaerin, the cells were incubated with a mouse monoclonal anti-HA antybody (1:1000 dilution, Covance, Berkeley, CA). After counterstaining with 0.1 μg/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 μg 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 ß-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 μm (36). The filters were coated with 20 μg/ml fibronectin (Sigma) and were placed between the chambers. Cells were trypsinized and suspended at a concentration of $5.0x10^5$ cells/ml in DMEM supplemented with 10% FBS. The RASMC suspension (50 μl) 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

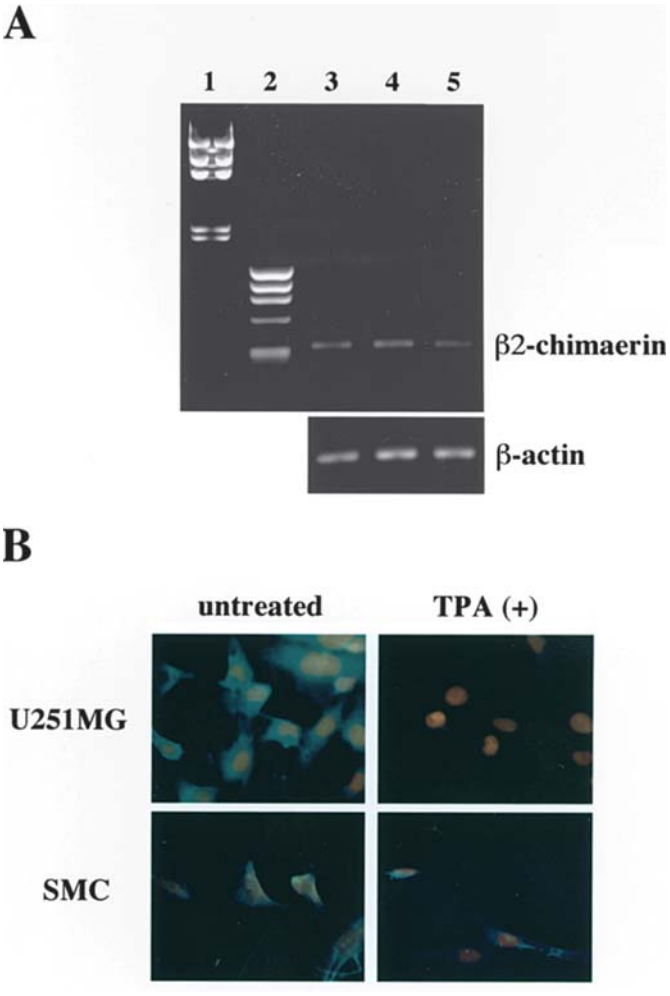

Figure 1. Endogenous ß2-chimaerin expression in human SMC. (A) RT-PCR for ß2-chimaerin. Total RNA (1 μg) was isolated and then subjected to RT-PCR. Lane 1: λDNA-HindIII digest. Lane 2: ϕX174-Hae III digest, DNA molecular weight markers (Takara Shuzo, Co., Ltd., Otsu, Japan). Lane 3: MS27W, human SMC 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 ß-actin primers was also tested. (B) Immunohistochemistry for ß2-chimaerin. MS27W and U251MG cells were cultured on coverslips and indirect immunohistochemical staining for ß2-chimaerin was performed. The cells were also treated with 1 μM 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 containg 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) NP-40, 5 mM ß-glycerophosphate, and proteinase inhibitors [5 μg/ml 4-(2-aminoethyl)benzene-sulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin and

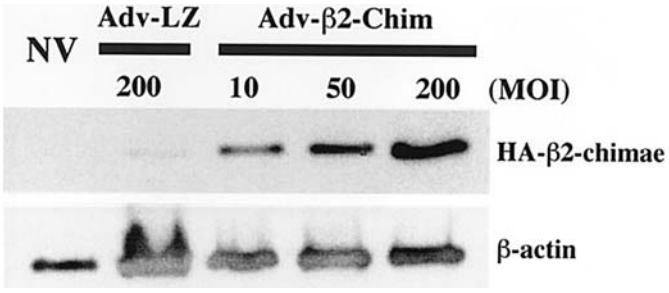

Figure 2. Overexpression of haemagglutinin (HA)-tagged full-length ß2-chimaerin in RASMC. The cells were infected with 200 MOI of Adv-LZ or with 10-200 MOI of Adv-ß2-Chim, otherwise mock-infected (NV). The cell lysates were collected at 48 h after infection, and then analyzed by Western blotting. The blots were probed with an antibody against HA-tag, and then reprobed with an antibody against ß-actin.

1 μg/ml pepstatin A]. Lysates were centrifuged at 14,000 x 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 panisoform-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 ± SD. A level of P<0.05 was considered as statistically significant.

Results

Expression of endogenous ß2-chimaerin in human SMC and exogenous ß2-chimaerin in RASMC. We first examined by RT-PCR the endogenous expression of ß2-chimaerin in cultured human SMC. ß2-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 ß2-chimaerin was detected in U251MG, human glioma cells (Fig. 1A). By conventional immunohistochemistry, cytoplasmic staining of ß2-chimaerin was detected in human SMC, as well as in glioma cells and 30 min incubation with 1 μM TPA resulted in a reduction in the cytoplasmic signal of ß2-chimaerin in both cell types (Fig. 1B). To further investigate the role of ß2-chimaerin in the growth factor response of SMC, we attempted the overexpression of ß2-chimaerin in cultured RASMC. HA-tagged full-length ß2-chimaerin was successfully expressed by infection with Adv-ß2-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 ß2-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,

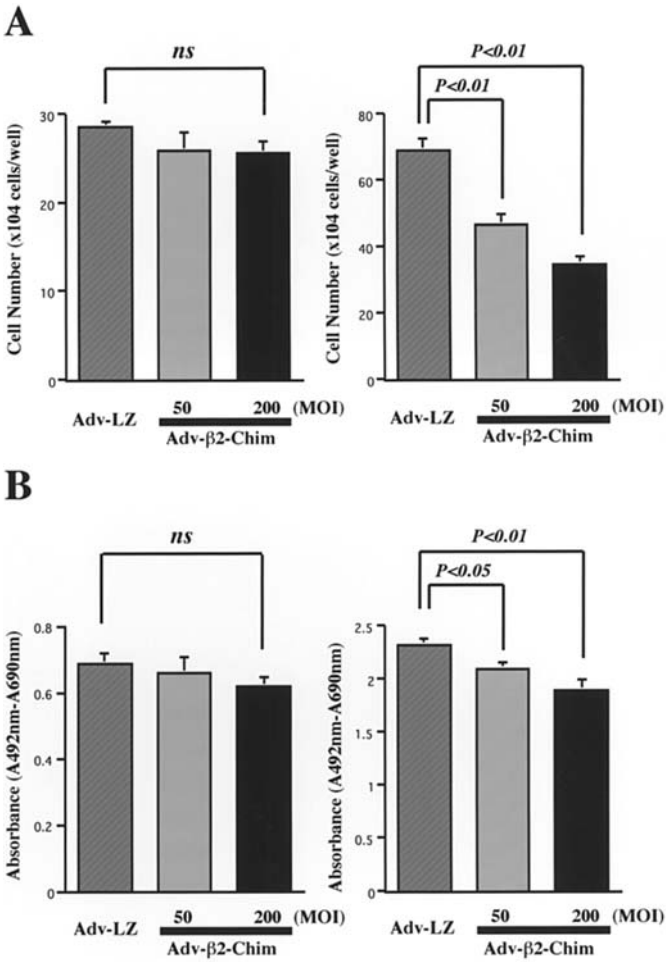

Figure 3. The effect of the exogenous expression of ß2-chimaerin on the proliferation of RASMC. (A) Hand-counting assay. RASMC were infected with 200 MOI of Adv-LZ or with 50-200 MOI of Adv-ß2-Chim. The cells were further incubated under serum-free conditions (left panel) or in media containing 10 ng/ml of PDGF (right panel) for 48 h, and then hand-counted. The data are presented as the mean ± 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 ± 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-ß2-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-phasic 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 ß2-chimaerin was expressed by adenoviral means (Fig. 3B).

Migration of mitogen-stimulated RASMC expressing exogenous ß2-chimaerin. PDGF-induced RASMC migration was assayed by using a modified Boyden's chamber method. The number of migrated Adv-LZ-infected cells remained

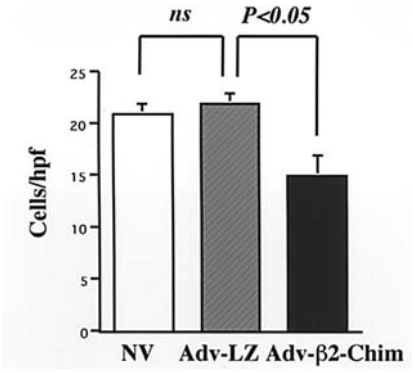

Figure 4. The effect of the exogenous expression of ß2-chimaerin on the migration of RASMC. The cells were infected with 200 MOI of Adv-LZ or Adv-ß2-Chim, otherwise mock-infected (NV). PDGF (10 ng/ml)-induced migration of RASMC was assayed by modified Boyden's chamber method, as described in Materials 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 ± 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-ß2-Chim-infected cells by 19-26% (P<0.05) (Fig. 4).

Subcellular localization of ß2-chimaerin in mitogen-stimulated RASMC. TPA-induced redistribution of ß2-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 ß2-chimaerin in RASMC by confocal microscopy. In the unstimulated cells, HA-tagged ß2-chimaerin was distributed throughout the cytoplasm. Upon PDGF stimulation, a significant fraction of HA-tagged ß2-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 ß2-chimaerin were also observed upon TPA (1 μM, 10 min)-stimulation. Pre-treatment of 5 μM of a PKC inhibitor, GF109203X, was not found to affect the subcellular localization of HA-tagged ß2-chimaerin in TPA-stimulated cells (Fig. 5B).

Rac-GTP levels in mitogen-stimulated RASMC expressing exogenous ß2-chimaerin. We investigated whether ß2-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 μM of a PKC inhibitor, GF109203X, 30 min before PDGF stimulation. Ten minutes after stimulation with 10 ng/ml of PDGF, Rac-GPT levels were elevated by approximately 30% in Adv-LZ-infected cells. On the other hand, the Rac-GPT levels were down-regulated by about approximately 80% in Adv-ß2-Chim-infected cells, irrespective of PDGF stimulation (Fig. 6).

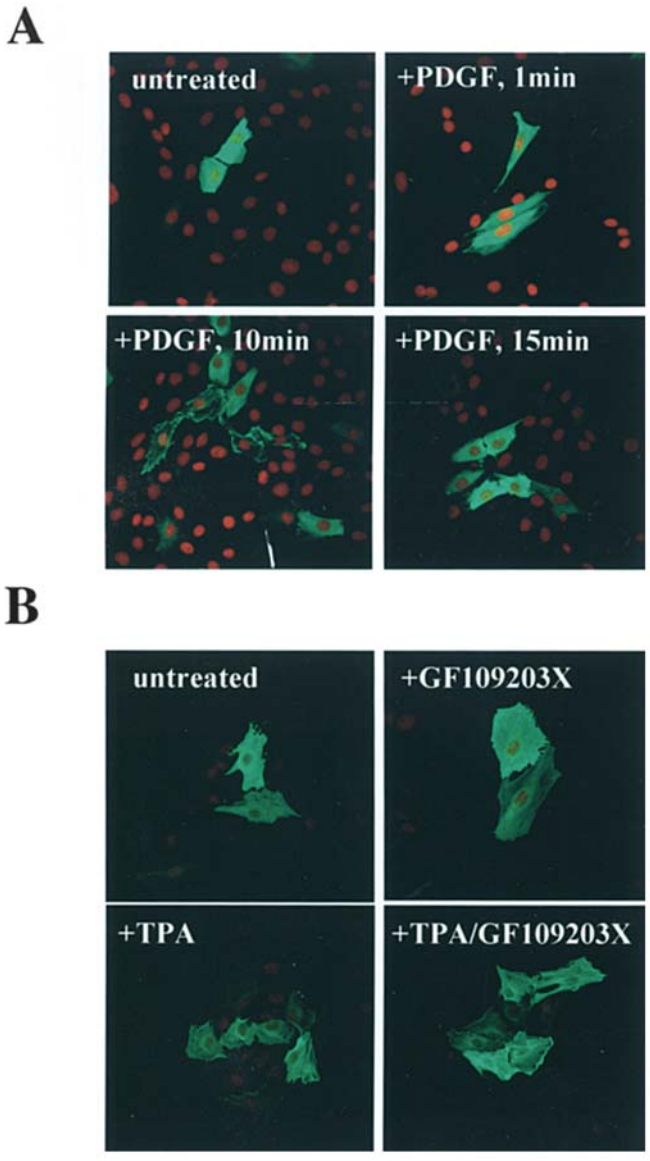

Figure 5. Subcellular localization of ß2-chimaerin in mitogen-stimulated RASMC. (A) Time-dependent effects induced by PDGF. The cells were infected with 200 MOI of Adv-ß2-Chim (expressing HA-tagged ß2-chimaerin) and were then stimulated with 10 ng/ml of PDGF for the indicated periods of time. Immunohistochemical 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-ß2-Chim as described above. The cells were then stimulated with 1 μM of TPA for 10 min with or without 5 μM of PKC inhibitor, GF109203X. Immunohistochemical staining and observation were performed as described above.

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 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 ß2-chimaerin is expressed in human SMC at both the RNA and protein levels (Fig. 1). The changes observed in the subcellular localization of ß2-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 ß2-chimaerin was observed in either serum-stimulated or

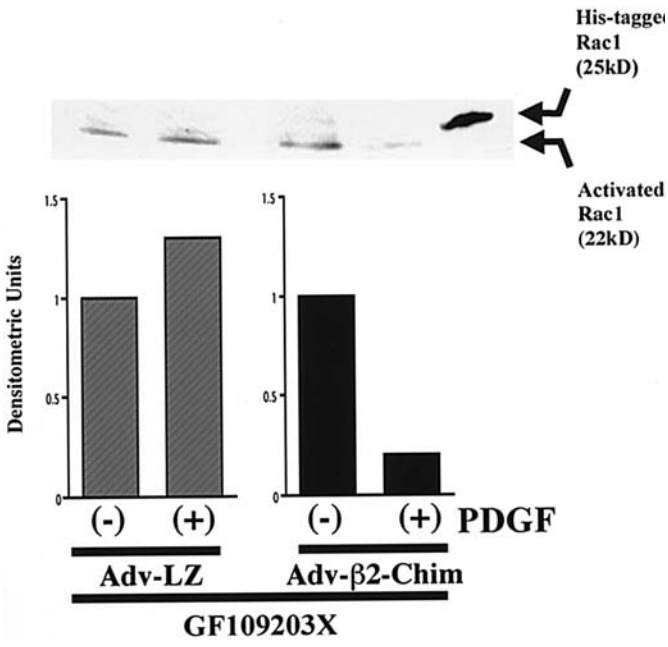

Figure 6. Rac-GTP levels in exogenous ß2-chimaerin-expressing RASMC. The cells were infected with 200 MOI of Adv-LZ or Adv-ß2-Chim. After 30 min of incubation with 5 μM of GF109203X, the cells were stimulated with 10 ng/ml of PDGF for 10 min. Rac-GTP levels were assayed with a pull-down assay kit as described in Materials and methods. Representative results of SDS-PAGE and the data from the densitometric analysis are shown (n=2).

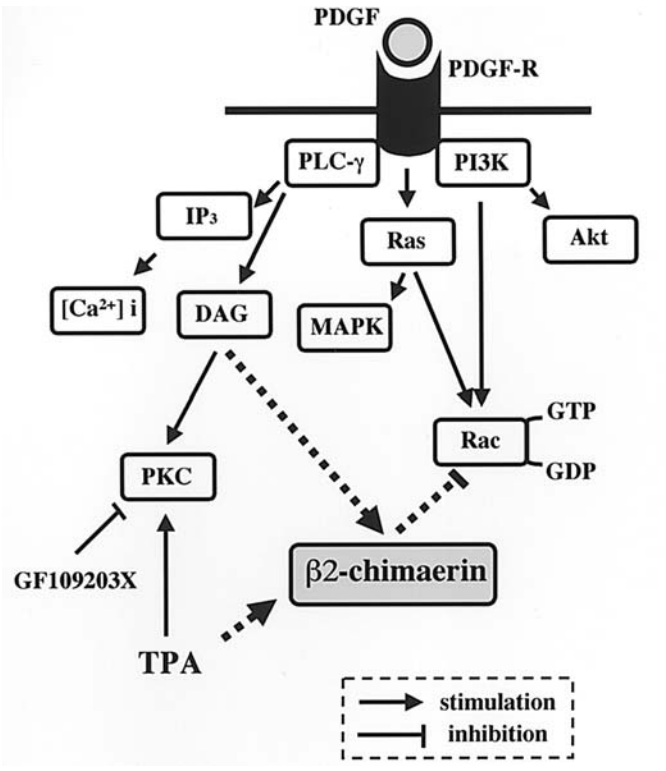

Figure 7. Hypothetical pathways of ß2-chimaerin-mediated signaling downstream of PDGF stimulation in SMC. Known pathways described in the literature are indicated by solid lines, and hypothetical pathways proposed on the basis of the present study are indicated by dotted lines. DAG and TPA potentially activate not only PKC but also ß2-chimaerin, a novel Rac-GAP protein, resulting in the negative regulation of Rac activity and the inhibition of proliferation and migration of SMC.

serum-starved SMC, it is thought that ß2-chimaerin may function irrespective of SMC phenotypes (33). Moreover, our preliminary data of immunohistochemical study using human aortic frozen sections showed that ß2-chimaerin-expressing cells were localized in neointimal fibroproliferative lesions, thus suggesting a potential role of ß2-chimaerin in human atherogenesis (data not shown).

To further investigate the role of ß2-chimaerin in the growth factor response of SMC, we attempted overexpression of HA-tagged full-length ß2-chimaerin in cultured RASMC. Proliferation of PDGF-stimulated cells was dose-dependently inhibited by the exogenous expression of ß2-chimaerin (Fig. 3). It has consistently been reported that the overexpression of ß2-chimaerin leads to growth suppression in human breast cancer cells (30). We found that overexpression of ß2-chimaerin did not cause a reduction in cell number in quiescent SMC (Fig. 3A). This suggests that activation of ß2-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 ß2-chimaerin (Fig. 4). It has been reported that overexpression of the catalytic domain (the ß-GAP domain) of ß2-chimaerin reduced metastatic potential in the mouse transplanted tumor of human breast cancer cells (31). Thus, ß2-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 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 ß2-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 ß2-chimaerin in TPA- and PDGF-stimulated cells (Figs. 1 and 5). Upon PDGF stimulation, HA-tagged ß2-chimaerin appeared to be re-distributed to the plasma membrane (Fig. 5A). TPA-induced re-distribution of ß2-chimaerin was not affected by the PKC inhibitor, GF109203X (Fig. 5B), which may further support the notion that TPA directly activates ß2-chimaerin (25,26). It has also been reported that TPA induces the perinuclear localization of ß2-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 ß2-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.

agreement with the findings of previous studies (11-13), PDGF-regulated Rac-GTP levels in the control conditions. Marked down-regulation of PDGF-induced Rac1 activation was observed in RASMC after forced expression of ß2-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 ß2-chimaerin (data not shown). As the TPA-induced translocation of ß2-chimaerin was not affected by the PKC inhibitor (Fig. 5B), it is expected that PKCs exert an influence on Rac1 activity, but not on ß2-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 ß2-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 ß2-chimaerin activity (27). Therefore, it could be anticipated that PKCs, as well as ß2-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 PLCγ-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 ß2-chimaerin is expressed in cultured human smooth muscle cells. ß2-chimaerin may impact on PDGF-induced proliferation and migration, and Rac-GTP levels. Thus, it is likely that ß2-chimaerin signaling may play a role in the regulation of SMC phenotypes and therefore have implications in human atherogenesis.

Acknowledgements

This study was supported in part by a grant-in-aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 13832007) and by a grant from the Ishibashi Foundation for the Promotion of Science. M.G.K. is supported by grants from NIH.

References

1. Chamley-Campbell JH, Champbell GR and Ross R: Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. J Cell Biol 89: 379-383, 1981.
2. Thyberg J, Hedin U, Sjölund M, Palmberg L and Bottger BA: Regulation of differentiated properties and proliferation of arterial smooth muscle cells. Arteriosclerosis 10: 966-990, 1990.
3. Ross R: Atherosclerosis - An inflammatory disease. N Engl J Med 340: 115-126, 1999.
4. Chabrier PE: Growth factors and vascular wall. Int Angiol 15: 100-103, 1996.
5. Berk BC: Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 81: 999-1030, 2001.
6. Zou Y, Hu Y, Metzler B and Xu Q: Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells (Review). Int J Mol Med 1: 827-834, 1998.
7. Blanc A, Pandey NR and Srivastava AK: Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H_2O_2 in vascular smooth muscle cells: potential involvement in vascular disease (Review). Int J Mol Med 11: 229-234, 2003.
8. Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG and Ross R: Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. Ann NY Acad Sci 766: 416-430, 1995.
9. Raines EW: PDGF and cardiovascular disease. Cytokine Growth Factor Rev 15: 237-254, 2004.
10. Miyamoto T and Fox JC: Autocrine signaling through Ras prevents apoptosis in vascular smooth muscle cells *in vitro*. J Biol Chem 275: 2825-2830, 2000.
11. Welch HC, Coadwell WJ, Stephens LR and Hawkins PT: Phosphoinositide 3-kinase-dependent activation of Rac. FEBS Lett 546: 93-97, 2003.
12. Moore KA, Sethi R, Doanes AM, Johnson TM, Pracyk JB, Kirby M, Irani K, Goldschmidt-Clermont PJ and Finkel T: Rac1 is required for cell proliferation and G2/M progression. Biochem J 326: 17-20, 1997.
13. Anand-Apte B, Zetter BR, Viswanathan A, Qiu RG, Chen J, Ruggieri R and Symons M: 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, 1997.
14. Kjoller L and Hall A: Signaling to Rho GTPases. Exp Cell Res 253: 166-179, 1999.
15. Rhee SG: Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem 70: 281-312, 2001.
16. Ron D and Kazanietz MG: New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J 13: 1658-1676, 1999.
17. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA and Messing RO: Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 279: L429-L438, 2000.
18. Rask-Madsen C and King GL: Proatherosclerotic mechanisms involving protein kinase C in diabetes and insulin resistance. Arterioscler Thromb Vasc Biol 25: 487-496, 2005.
19. Kazanietz MG: Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. Mol Carcinog 28: 5-11, 2000.
20. Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ and Stone JC: RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 280: 1082-1086, 1998.
21. Maruyama IN and Brenner S: A phorbol ester/diacylglycerol-binding protein encoded by the unc-13 gene of *Caenorhabditis elegans*. Proc Natl Acad Sci USA 88: 5729-5733, 1991.
22. Hall C, Monfries C, Smith P, Lim HH, Kozma R, Ahmed S, Vanniasingham V, Leung T and Lim L: Novel human brain cDNA encoding a 34,000 Mr 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, 1990.
23. Areces LB, Kazanietz MG and Blumberg PM: Close similarity of baculovirus-expressed n-chimaerin and protein kinase C alpha as phorbol ester receptors. J Biol Chem 269: 19553-19558, 1994.
24. Caloca MJ, Fernandez N, Lewin NE, Ching D, Modali R, Blumberg PM and Kazanietz MG: Beta2-chimaerin is a high affinity receptor for the phorbol ester tumor promoters. J Biol Chem 272: 26488-26496, 1997.
25. Caloca MJ, Garcia-Bermejo ML, Blumberg PM, Lewin NE, Kremmer E, Mischak H, Wang S, Nacro K, Bienfait B, Marquez VE and Kazanietz MG: beta2-chimaerin is a novel target for diacylglycerol: binding properties and changes in subcellular localization mediated by ligand binding to its C1 domain. Proc Natl Acad Sci USA 96: 11854-11859, 1999.
26. Caloca MJ, Wang H, Delemos A, Wang S and Kazanietz MG: Phorbol esters and related analogs regulate the subcellular localization of beta2-chimaerin, a non-protein kinase C phorbol ester receptor. J Biol Chem 276: 18303-18312, 2001.
27. Caloca MJ, Wang H and Kazanietz MG: Characterization of the Rac-GAP (Rac-GTPase-activating protein) activity of beta2-chimaerin, a 'non-protein kinase C' phorbol ester receptor. Biochem J 375: 313-321, 2003.

28. Canagarajah B, Leskow FC, Ho JY, Mischak H, Saidi LF, Kazanietz MG and Hurley JH: Structural mechanism for lipid activation of the Rac-specific GAP, beta2-chimaerin. Cell 119: 407-418, 2004.
29. Yuan S, Miller DW, Barnett GH, Hahn JF and Williams BR: Identification and characterization of human beta2-chimaerin: association with malignant transformation in astrocytoma. Cancer Res 55: 3456-3461, 1995.
30. Yang C, Liu Y, Leskow FC, Weaver VM and Kazanietz MG: Rac-GAP-dependent inhibition of breast cancer cell proliferation by ß2-chimerin. J Biol Chem 280: 24363-24370, 2005.
31. Menna PL, Skilton G, Leskow FC, Alonso DF, Gomez DE and Kazanietz MG: Inhibition of aggressiveness of metastatic mouse mammary carcinoma cells by the beta2-chimaerin GAP domain. Cancer Res 63: 2284-2291, 2003.
32. Yamaguchi M, Kato S, Fujii T, Miyagi N and Morimatsu M: Expression of the cyclin-dependent kinase inhibitor, p21Waf-1/Cip-1/Sdi-1, in human vascular smooth muscle cells in the proliferating state. Heart Vessels 13: 246-255, 1998.
33. Kato S, Shanlay JR and Fox JC: Serum stimulation, cell-cell interactions, and extracellular matrix independently influence smooth muscle cell phenotype *in vitro*. Am J Pathol 149: 687-697, 1996.
34. Miyagi N, Kato S, Terasaki M, Shigemori M and Morimatsu M: Fibroblast growth factor-2 and -9 regulate proliferation and production of matrix metalloproteinases in human gliomas. Int J Oncol 12: 1085-1090, 1998.
35. Kato S, Yamaguchi M, Fujii T, Miyagi N, Terasaki M, Hamada T, Sugita Y and Morimatsu M: Overexpression of p21Waf-1 in vascular smooth muscle cells: Regulation of proliferation, differentiation, and cell size. Exp Mol Pathol 66: 39-52, 1999.
36. Koyama N, Harada K, Yamamoto A, Morisaki N, Saito Y and Yoshida S: Purification and characterization of an autocrine migration factor for vascular smooth muscle cells (SMC), SMC-derived migration factor. J Biol Chem 268: 13301-13308, 1993.
37. Benard V, Bohl BP and Bokoch GM: Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem 274: 13198-13204, 1999.
38. Wada Y, Kato S, Okamoto K, Izumaru S, Aoyagi S and Morimatsu M: Diltiazem, a calcium antagonist, inhibits matrix metalloproteinase-1 (tissue collagenase) production and collagenolytic activity in human vascular smooth muscle cells. Int J Mol Med 8: 561-566, 2001.
39. Schmitz AA, Govek EE, Bottner B and van Aelst L: Rho GTPases: signaling, migration, and invasion. Exp Cell Res 261: 1-12, 2000.
40. Newton AC: Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J 370: 361-371, 2003.
41. Newton AC: Regulation of protein kinase C. Curr Opin Cell Biol 9: 161-167, 1997.
42. Wang H and Kazanietz MG: 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, 2002.
43. Qiu RG, Chen J, Kirn D, McCormick F and Symons M: An essential role for Rac in Ras transformation. Nature 374: 457-459, 1995.
44. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS and Der CJ: Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol 15: 6443-6453, 1995.