

Inhibition of Aggressiveness of Metastatic Mouse Mammary Carcinoma Cells by the β 2-Chimaerin GAP Domain¹

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ABSTRACT

The biological and functional properties of β 2-chimaerin, a novel phorbol ester/diacylglycerol receptor unrelated to protein kinase C isozymes, are largely unknown. It has previously been established that β 2-chimaerin accelerates the hydrolysis rate of GTP from Rac1 *in vitro*, leading to the inactivation of this GTPase, which plays important roles in the control of actin cytoskeleton organization, proliferation, motility, and invasiveness. To explore the potential role of β 2-chimaerin in invasion and metastasis, we generated stable transfectants for its catalytic domain (the β -GAP domain) in F3II murine mammary carcinoma cells. Reduced Rac-GTP levels were observed upon stimulation with epidermal growth factor in the β -GAP clones compared with control cells. Moreover, a marked alteration in actin polymerization in response to epidermal growth factor was observed in the β -GAP clones, suggesting impairment of Rac-dependent responses. The β -GAP transfectants also evidenced slower growth rates and a striking reduction in their migratory properties. Adenoviral delivery of the β -GAP domain into F3II cells also led to reduced proliferative and migratory responses. Importantly, significant differences were found between β -GAP transfectants and control cells regarding their tumorigenic and metastatic properties after s.c. inoculation in syngeneic BALB/c mice. Tumors originating from β -GAP transfectants showed a significantly lower growth rate and reduced invasive ability; in addition, a lower incidence of spontaneous lung metastases was observed. Our results indicate that β 2-chimaerin impairs key steps in the metastatic cascade and provide evidence for a rational modulation of the Rac signaling pathway in cancer treatment.

INTRODUCTION

Although PKC⁴ is generally viewed as the only family of intracellular receptors for the phorbol esters, recent findings from several laboratories have established the existence of novel, “non-kinase” receptors for the phorbol esters, including the chimaerins, RasGRP, and Unc-13/Munc13 (1–5). Like PKC isozymes, these proteins bind with high affinity to phorbol esters and DAG, a lipid second messenger generated upon activation of seven transmembrane and tyrosine kinase receptors. Among this group of novel phorbol ester receptors, the chimaerins may have important implications in carcinogenesis because they regulate the activity of Rac1, a small GTP-binding protein of the Ras superfamily that plays a key role in the regulation of cytoskeleton organization, gene transcription, cell cycle, adhesion,

migration, and metastasis (6–9). Indeed, it has been shown that chimaerins have Rac-GAP (GTPase-activating protein) activity, leading to acceleration of GTP hydrolysis from Rac1 and its subsequent inactivation (10–13).

Several chimaerin isoforms have been isolated to date (α 1- or *n*-, α 2-, β 1-, and β 2-chimaerin). In all cases, chimaerin isoforms have a single NH₂-terminal C1 domain (the DAG/phorbol ester binding site) and a COOH-terminal GAP domain responsible for Rac inactivation (4, 5). Early work from the Blumberg’s laboratory established that α 1-chimaerin binds phorbol esters with an affinity similar to those of PKC isozymes (14). More recently, we have established that β 2-chimaerin is also a high-affinity receptor for phorbol esters *in vitro* as well as in cellular models (15, 16). Interestingly, phorbol esters promote the association of β 2-chimaerin to Rac1 in COS-1 cells, which leads to a reduction in the amount of GTP bound to Rac1 (14). The discovery of the chimaerins has challenged the traditional view that DAG signaling occurs only through activation of PKC isozymes. To date, despite the biochemical characterization of chimaerins as phorbol ester/DAG receptors, very limited information is available on their biological properties and functional roles in cellular models.

It is well established that Rac1 plays an important regulatory role in oncogenic transformation and in the metastatic cascade. Rac1 is necessary for Ras-induced transformation and is a key player in the control of actin cytoskeleton rearrangements, thus regulating essential cellular functions such as spreading and migration (8, 9, 17, 18). Numerous studies have implicated Rac1 and its activators (the Rac exchange factors, or RacGEFs) as key regulators of the metastatic cascade. Moreover, Rac exchange factors have oncogenic properties in numerous cell types, and Rac hyperactivation has been observed in numerous types of cancers, including breast cancer (19–23).

The aim of the present study was to determine whether β 2-chimaerin modulates tumor cell proliferation and invasion. We used as a model F3II cells, highly aggressive mammary carcinoma cells that have been extensively characterized in terms of their invasive properties (24–27). Breast cancer mortality is strongly related to the ability of tumor cells to invade and metastasize. Tumor cell invasion and metastasis involve adherence to the basement membrane, secretion of proteolytic enzymes, and cell migration into blood vessels or lymphatic tissue, followed by extravasation at distant sites. It is predictable that inhibition of Rac function by chimaerins will affect functions that depend on actin reorganization, such as cell motility. Thus the hypothesis we wished to explore is whether β 2-chimaerin, by interfering with the Rac pathway, will affect invasion and metastasis. Despite the critical role of Rac1 and related small GTP-binding proteins in carcinogenesis and the relevance of Rac signaling in the sequential steps of the metastatic cascade, very limited information is available on how chimaerins regulate such functions. As an approach we generated F3II cell lines that stably expressed the catalytic domain of β 2-chimaerin (the β -GAP domain) and analyzed their *in vitro* behavior as well as their aggressiveness in syngeneic BALB/c mice. Because the β 2-chimaerin GAP domain is constitutively active and therefore not subject to regulation by phorbol esters/DAG (11, 28), it represents an ideal approach to explore the biological effects of these

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⁴ The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; AdV, adenovirus; MOI, multiplicities of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGF, epidermal growth factor.

novel Rac-GAP proteins. Our results revealed that ectopic expression of β -GAP domain has profound effects on the tumorigenic potential of F3II mammary cancer cells, as well as it markedly reduced their invasive and metastatic properties.

MATERIALS AND METHODS

Tumor Cell Line and Culture Conditions. The sarcomatoid mammary carcinoma cell line F3II is a highly invasive and metastatic variant established from a clone of a spontaneous, hormone-independent BALB/c mouse mammary tumor (24). F3II cells were cultured in MEM 41500 (Life Technologies, Inc., Gaithersburg, MD), supplemented with heat-inactivated 5% FBS, 2 mM glutamine, and 80 μ g/ml gentamicin at 37°C in a 5% CO₂ atmosphere. Stock cell cultures were routinely subcultured twice a week by trypsinization, using standard procedures.

Vector Construction and Transfection. The β -GAP domain was isolated by PCR from the parental vector pCR3 ϵ - β 2 (15) with use of the following primers (*Xho*I and *Mlu*I restriction sites are underlined): 5'-GTCAGGCTC-GAGATGGTGGTAGACATATGCATTCGGGAA-3' and 5'-GTGAGGAC-GCGTGAATAAAACGTCTCTCGTTTCTATTAA-3'. The fragment was subcloned into *Xho*I and *Mlu*I restriction sites in pCR3 ϵ , an epitope-tagged (ϵ -tag) Neo^R mammalian expression vector (15). The resulting plasmid (pCR3 ϵ - β -GAP) was transfected into semiconfluent F3II mammary carcinoma cells with use of Lipofectamine (Life Technologies) according to the manufacturer's instructions. Control F3II cells were transfected with pCR3 ϵ (empty vector). Forty eight hours later, transfected tumor cells were transferred to complete culture medium containing 400 μ g/ml G418 (Life Technologies). After 30 days of culture, colonies resistant to G418 were selected by limiting dilution. G418-resistant clones were then expanded, and the expression of β -GAP domain was determined by quantitative RT-PCR.

Assessment of Expression of β 2-Chimaerin GAP Domain by Real-Time RT-PCR. Total RNA from control and β -GAP-transfected cells was extracted using a guanidinium isothiocyanate method. To eliminate potential genomic DNA contamination, 1 μ g of each sample was treated with amplification-grade DNase I (Life Technologies). The samples were used for real-time RT-PCR in a Prism 7700 Sequence Detection System (TaqMan; Applied Biosystems). The principles of this technique have been described elsewhere (29–31). Reverse transcription of RNA was performed with random primers (First Strand cDNA Synthesis kit; Life Technologies). The β -GAP TaqMan system consisted of the amplification primers β -GAP (F) (5'-AGACTTACCCATCCCTGTCATCA-3') and β -GAP (R) (5'-CAGCATCAGCACTTCATGGAC-3') and the dual-labeled fluorescent TaqMan probe 5'-(6-carboxyfluorescein)-TTTATAGATG CAGCAAAAATCTCCAATGCA-(6-carboxytetramethylrhodamine)-3', which were designed for the exon-intron boundary. Normalization of the cDNA load for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was performed, using 5'-CCCCTTCA-TTGACCTCAACTA-3' and 5'-CGCTCCTGGAAGATGGTGAT-3' primers. In this last case, SYBR Green PCR Master Mix (Applied Biosystems) was used.

To generate a calibration curve, we first constructed a standard plasmid in which the corresponding fragment to be amplified was subcloned into pGEM-T vector (Promega); we then used five serial dilutions of the reference plasmid to construct the calibration curve. The concentration of the reference plasmid was measured spectrophotometrically and converted into number of copies/ μ l according to the formula: number of copies/ μ l = g/ μ l \times (mol/660 g) \times (1/n) \times 6.023 \times 10²³, where 660 mol/g is the average molecular weight of a nucleotide pair, *n* is the number of nucleotide pairs in the plasmid, and 6.023 \times 10²³ is Avogadro's number (number of copies/mol). The number of copies in each sample was calculated from the calibration curve, which was run in triplicate. Every PCR experiment also included control samples without template. For the experiments using standard RT-PCR, the β -GAP forward and reverse primers described above were used, and primers for actin (5'-ACATGTGCCATCTACGAGG-3' and 5'-AGGGGCCGGACTCGTCATACT-3') were used as control.

Generation of β -GAP-AdV and AdV Infections. The AdV vector pShuttle-CMV-HA was generated by inserting a linker into the *Not*I and *Xho*I sites of pShuttle-CMV (Stratagene, La Jolla, CA). This linker includes a 5' HA tag followed by *Xho*I and *Mlu*I sites and stop codons. The β -GAP domain was

isolated from pCR3 ϵ (12, 15) and subcloned into the *Xho*I and *Mlu*I restriction sites of pShuttle-CMV-HA. β -GAP-AdV was generated using the AdEasy Adenoviral Vector System (Stratagene), according to the manufacturer's instructions. Using a similar strategy, we generated an AdV for full-length β 2-chimaerin (β 2-chim-AdV). The AdV construct for β 2-chim-AdV was generated by inserting the full-length β 2-chimaerin cDNA into *Xho*I-*Mlu*I sites of pShuttle-CMV-HA. A control LacZ-AdV was generated from pShuttle-CMV-LacZ; it therefore has the same backbone as β -GAP-AdV and β 2-chim-AdV. AdVs were amplified and titrated in HEK293 cells by standard techniques. For infection of F3II cells, 2 \times 10⁵ cells were seeded on 6-well plates in MEM with 5% FBS. Two h before infection, cells were incubated in serum-free MEM. Cells were then infected with the corresponding AdVs at different MOI. Two h after infection, 5% FBS was added, and cells were cultured for 20 h. Cells were then collected, using trypsin/EDTA, counted in a hemocytometer, seeded in 96-well plates (10³ cells/well), and cultured in MEM containing 5% FBS for several days. At different times after infection, cell proliferation or cell migration was determined (see below). Expression of the β -GAP domain and full-length β 2-chimaerin was monitored by Western blotting with a mouse monoclonal anti-HA antibody (1:1000 dilution; Babco, Richmond, CA).

In Vitro Growth and Cell Morphology. F3II cells (1 \times 10⁵) were plated on 35-mm Petri dishes in MEM containing 5% FBS, and each day cells were removed using trypsin/EDTA with vigorous pipetting. Cell numbers were quantified by hemocytometer counting, and doubling times in the logarithmic growth phase and saturation densities were calculated. In parallel experiments, 2.5 \times 10⁴ cells were plated onto 24-well plates, and cell growth was monitored every 24 h by the MTT assay (32). To document the morphology of transfected tumor cells, we photographed semiconfluent F3II monolayers in a phase contrast microscope (Olympus, Tokyo, Japan).

Migration Assay. Cell migration was measured by an *in vitro* wound assay, as described previously (25). Briefly, confluent monolayers were wounded by scratching 0.5-mm lines with a plastic tip. After washing with PBS, tumor cells were incubated for 12 h in MEM containing 10% FBS. Monolayers were then fixed and stained with methylene blue, and the number of cells that had advanced into the cell-free space was counted in three different areas on each line at \times 100 magnification, using a 0.36 mm² reticle.

Adhesion Assay. Adhesion was measured as described previously (33). Briefly, wells from 96-well plates were coated with 1 μ g of fibronectin. F3II cells were harvested with an enzyme-free cell dissociation buffer (Life Technologies) and seeded at a concentration of 4 \times 10⁴ cells/well in MEM containing 5% FBS. After a 60-min incubation at 37°C, cells were washed with PBS, and nonadherent cells were removed by aspiration. Adherent cells were stained with 0.5% crystal violet diluted in 20% methanol and rinsed with distilled water. The dye in the stained cells was solubilized by adding 1% SDS, and the absorbance was measured at 595 nm.

Actin Staining. Cells grown on glass coverslips were incubated for 24 h in serum-free medium; stimulated with EGF (100 ng/ml) for 15 min and then fixed in 4% formaldehyde in PBS. Cells were permeabilized with 0.5% NP-40 in PBS, blocked with 5% BSA, and then incubated for 1 h with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR). Images were recorded in a fluorescence microscope (Nikon, Tokyo, Japan).

In Vivo Assays. Adult female BALB/c inbred mice were inoculated with either control or transfected tumor cells in the subcutis of the right flank (2 \times 10⁵ cells in 0.2 ml of serum-free medium). The time of appearance of local tumors was monitored by palpation, and tumor size was then measured with a caliper twice a week. After 60 days, mice were sacrificed. Tumors were removed and fixed in 10% formalin, and specimens were processed for routine histopathology. To investigate the presence of spontaneous metastasis, lungs were fixed in Bouin's solution, and the number of surface lung nodules was determined under a dissecting microscope, as described previously (24).

RESULTS

Reduced Levels of β 2-Chimaerin in F3II Mammary Carcinoma Cells. In the first set of experiments, we evaluated the expression of β 2-chimaerin in untransfected F3II cells by RT-PCR and compared it with the levels in NMuMG, an epithelial cell line derived from normal murine mammary gland. We designed a set of primers for the ampli-

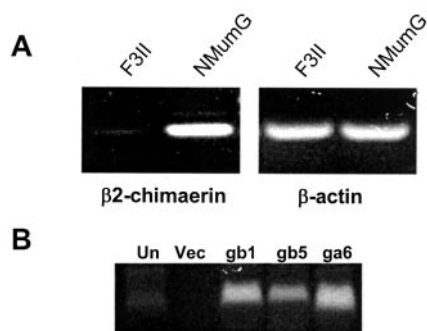


Fig. 1. A, RT-PCR analysis of β2-chimaerin in murine F3II mammary carcinoma cells and normal murine mammary NMuMG cells. A fragment comprising 297 bp was selected for detection. For β-actin, a 640-bp fragment was amplified. B, RT-PCR analysis of β-GAP in untransfected F3II cells (Un), F3II cells transfected with the empty vector pCR3ε (Vec), and stable F3II clones (gb1, gb5, and ga6). Primers for the RT-PCR analysis of the β-GAP region are described in "Materials and Methods."

Table 1 Characteristics of β-GAP F3II transfectants

Cell lines were generated by clonal selection with G418. Table 1 shows the mRNA expression level assessed by real-time RT-PCR and the growth properties of the different clones. The average number of mRNA copies for the β-GAP domain has been standardized in each case per 10⁵ copies of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase). For the determination of cell growth in culture, 1 × 10⁵ cells were plated on 35-mm plates. Cell number was determined, after trypsinization, by hemocytometer count, and doubling times and maximum saturation densities were calculated from the growth curve.

Cell line	β-GAP mRNA (copies)	Doubling time ^a (h)	Saturation density ^a (10 ⁵ cells/cm ²)
F3II (untransfected)	5	17.7 ± 2.6	1.9 ± 0.1
pCR3ε (vector control)	ND ^c	24.9 ± 2.6	2.3 ± 0.1
gb1	1001	33.3 ± 3.4 ^d	2.4 ± 0.1
gb5	1550	32.4 ± 1.4 ^e	2.1 ± 0.1
ga6	915	27.6 ± 2.0	2.2 ± 0.2

^a Mean ± SE of three determinations. An additional experiment gave similar results.

^c ND, not determined.

^{d,e} Compared with control vector (one-way ANOVA contrasted with Tukey-Kramer multiple-comparisons test); ^d P < 0.01; ^e P < 0.05.

fication of the β-GAP region (amino acids 251–440), the catalytic domain of β2-chimaerin. Whereas the β2-chimaerin transcript was readily detected in NMuMG cells, only negligible levels were detected in F3II mammary carcinoma cells. Similar levels of actin transcript were observed in the two cell lines (Fig. 1A).

Generation of β-GAP Transfectants in F3II Mammary Carcinoma Cells. As an approach to investigate whether β2-chimaerin regulates the tumorigenic and invasive properties of F3II cells, we

generated stable transfectants for the catalytic β-GAP domain, the region in β2-chimaerin that is responsible for the acceleration of GTP hydrolysis from Rac1. The plasmid pCR3ε-β-GAP was transfected into F3II cells, and >20 G418-resistant clones were selected. Initial analysis of these clones failed to reveal expression of the β-GAP domain, as determined by Western blotting with an anti-ε-tag antibody (15) or an anti-β2-chimaerin antibody (12). This did not come as a surprise because our previous experience indicated that stable clones for chimaerin isoforms were difficult to generate in many cell lines, and in most cases our functional studies have to rely on transient expression methodologies (12, 16).

Thorough investigation of this problem revealed that only those clones that express low levels of the β-GAP domain survive the selection process and that a negative selection occurred when high levels of chimaerins were expressed. However, higher levels of transcript were detected in most clones by RT-PCR. Because transient expression would be only partially useful for many of our experiments, we decided to select clones that expressed high levels of the β-GAP transcript. This approach proved to be useful because severe phenotypic changes were observed in these β-GAP clones relative to control (vector-transfected) cells (see below). Three independent β-GAP clones were chosen for additional experiments (gb1, gb5, and ga6). As shown in Fig. 1B, the levels of the transcript detected in these F3II clones were significantly increased relative to untransfected and empty vector (pCR3ε)-transfected cells. Quantitative real-time RT-PCR analysis revealed that β-GAP clones had ~200–300-fold increase over endogenous β2-chimaerin levels (Table 1). Vector-transfected and untransfected F3II cells grew in monolayer cultures that were composed mostly of epithelioid polyhedric cells and some fusiform cells. Stable β-GAP transfectants were composed of spindle and polyhedric cells and frequently grew in a whorl-like pattern (data not shown).

Assessment of Growth Properties in β-GAP Transfectants. We then investigated the growth properties of β-GAP transfectants in culture. As shown in Table 1, clones gb1, gb5, and ga6 had slower growth rates with respect to control (untransfected) cells or vector-transfected cells, as evidenced by a significant increase in doubling time. Saturation densities were similar, with a mean of ~2 × 10⁵ cells/cm². Cell proliferation was also evaluated with a MTT assay, and as shown in Fig. 2A, we observed a significant reduction in growth rate in clones gb1, gb5, and ga6 relative to control cells. To further confirm that the β-GAP domain impairs proliferation, we also used a transient expression strategy. As an approach we generated a

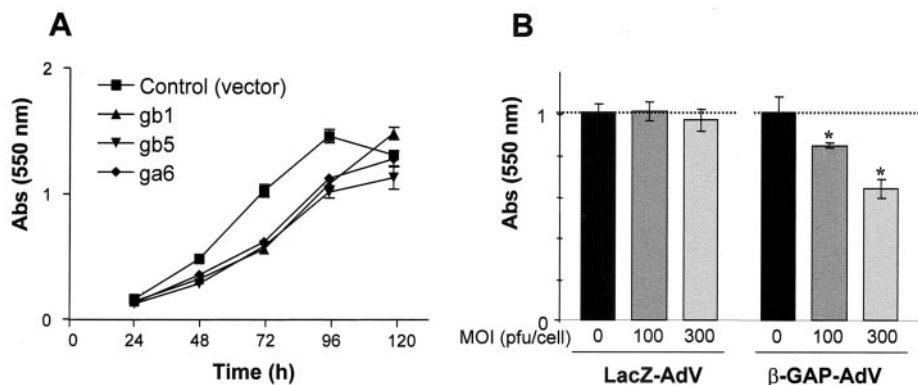
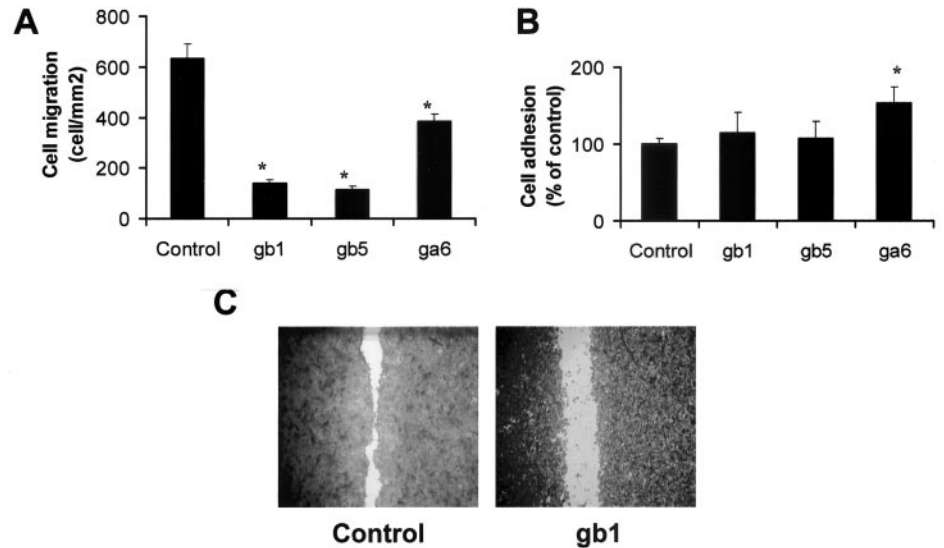


Fig. 2. In vitro growth. A, control (vector-transfected) cells and β-GAP domain transfectants (gb1, gb5, and ga6). We plated 2.5 × 10⁴ cells on 24-well plates, and cell growth was monitored with the MTT assay. Each point represents the mean of four independent samples. Bars, SE. Statistical analysis yielded P < 0.01 (control versus each of the clones) at 48, 72, and 96 h (one-way ANOVA contrasted with Tukey-Kramer multiple-comparisons test). Two additional experiments gave similar results. B, cells were infected with different MOI of either β-GAP-AdV or LacZ-AdV and then seeded in 12-well plates or 96-well plates (10³ cells/well). Cell growth was measured with the MTT assay. Each column represents the mean of eight independent samples; bars, SE. Values are expressed as absorbance (Abs) at 550 nm relative to the control (noninfected) 3 days after infection with the corresponding AdV. Similar results were observed at early time points. Three additional experiments gave similar results. *, P < 0.005 versus control (Mann-Whitney test). pfu, plaque-forming units.

Fig. 3. Migratory and adhesive properties of control (vector-transfected) and β -GAP transfectants. *A*, cell migration in the wound assay, performed as described in "Materials and Methods." The data show the number of cells that migrated per mm². Results are expressed as the mean \pm SE (*bars*) of three independent experiments. *, $P < 0.001$. *B*, cell adhesion on fibronectin-coated surfaces, expressed as percentage of control cells. Adhesion was determined as described in "Materials and Methods." Results are expressed as the mean \pm SE (*bars*) of three independent experiments. *, $P < 0.001$, ANOVA. *C*, representative experiment showing the reduced migratory properties of the β -GAP-transfectant gb1.



recombinant AdV for the β -GAP domain (HA-tagged- β -GAP-AdV). Upon infection of F3II cells with this AdV, expression of β -GAP could be readily detected by Western blot analysis using an HA-tag antibody (see below and Fig. 4*B*).

When we analyzed cell proliferation, we observed a significant reduction in cell number upon infection with β -GAP-AdV. Similar results were observed upon AdV expression of full-length β 2-chimaerin (data not shown). On the other hand, a LacZ-AdV, which was generated under identical experimental conditions (with the same viral backbone), did not produce any noticeable effect (Fig. 2*B*). In agreement with our previous experiments in COS-1 cells transiently transfected with β 2-chimaerin (12, 13), F3II cells expressing β -GAP had reduced levels of Rac-GTP and an impaired response to stimulation with EGF (data not shown), a well-established paradigm of Rac activation in cellular models (34). Thus, despite the low levels of expression of the β -GAP domain in F3II cells, a significant effect on Rac-GTP levels was observed, suggesting that Rac-dependent responses could be impaired in these clones.

Tumor Cell Migration, Adhesion, and Proteolysis. We next explored whether the β -GAP domain affects adhesion, proteolysis, and migration, the three sequential steps in the invasion process. To study the migratory properties of the different cell lines, we used a wound assay. As shown in Fig. 3*A*, we observed a dramatic reduction in the migratory properties of the three β -GAP transfectants, particularly in clones gb1 and gb5, which showed reductions of 78% and 82% in cell migration, respectively. Clone ga6 also showed a significant reduction in migration compared with control cells, although the effect was not as pronounced as in the gb1 and gb5 clones (~40% reduction). A representative experiment showing the reduced migratory properties of the β -GAP clone gb1 is presented in Fig. 3*C*.

Evaluation of adhesive properties in fibronectin-coated dishes, on the other hand, showed no reduction in adhesion for the β -GAP transfectants. Moreover, we observed an unexpected moderate increase in the adhesive capacity of clone ga6 on fibronectin coatings (Fig. 3*B*). Because proteolytic activity is linked to cytoskeleton organization and dynamics, we explored whether secretion of tumor proteases was also affected. In the conditions analyzed, however, β -GAP transfectants showed similar levels of plasminogen activator and metalloproteinase activities in conditioned medium with respect to control cells (data not shown).

To further confirm the effects observed with the β -GAP clones on migration and to rule out any potential misleading results with the

stable clones, we transiently expressed the β -GAP domain, using AdV delivery, as described below. Upon infection with the β -GAP-AdV, we observed a marked inhibition in the migratory properties of F3II cells (measured at 12 h). Reduced migration was observed even at short times (4 h). The reduced migratory phenotype was associated with a marked inhibition in the formation of membrane pseudopods (data not shown). Results similar to those observed with the β -GAP-AdV were observed upon expression of full-length β 2-chimaerin when we used a β 2-chimaerin-AdV (β 2-chim-AdV). Under similar experimental conditions, the LacZ-AdV had no noticeable effect on migration (Fig. 4).

Actin Cytoskeleton Reorganization. Because Rac1 plays an essential role in actin cytoskeleton reorganization as a result of growth factor stimulation, we speculated that an impaired response might be observed in β -GAP clones. Changes in actin polymerization upon EGF stimulation were examined using rhodamine-labeled phalloidin staining. Actin reorganization induced by growth factors, including EGF, is a Rac-dependent mechanism (6, 7, 35). As reported in other cell types (35), the addition of EGF to control (vector-transfected) F3II cells caused an increase in polymerized actin localized in the plasma membrane. On the other hand, this response was markedly impaired in β -GAP transfectants. A representative experiment for the gb1 clone is shown in Fig. 5.

Reduced Tumorigenicity in β -GAP Transfectants. The results obtained to this point prompted us to investigate the tumorigenic properties of β -GAP-expressing cells. F3II cells (control and β -GAP-transfected) were injected s.c. into BALB/c mice, and tumor growth was evaluated for a period of 30 days. Although no significant differences were observed in tumor latency or tumor incidence (Table 2), those tumors originating from β -GAP-transfected cells showed a significantly lower growth rate compared with those originating from control (vector-transfected) cells (Fig. 6*A*). s.c. tumors originating from both control (vector-transfected) and untransfected F3II mammary tumor cells were diagnosed as highly aggressive sarcomatoid carcinomas, confirming previous data (24). Tumor cells grew by invading the muscular and adipose layers of the subcutis, the dermis, and dermal papillae, causing necrosis in the epidermal layer and visible ulceration on top of the tumors. On the other hand, tumors originating from β -GAP clones grew by filling the subcutis, without signs of active invasion of the dermis. Some tumors from the β -GAP clones were surrounded by a capsule-like deposition of connective tissue (Fig. 6*B*). In addition, expression of the β -GAP domain dras-

A

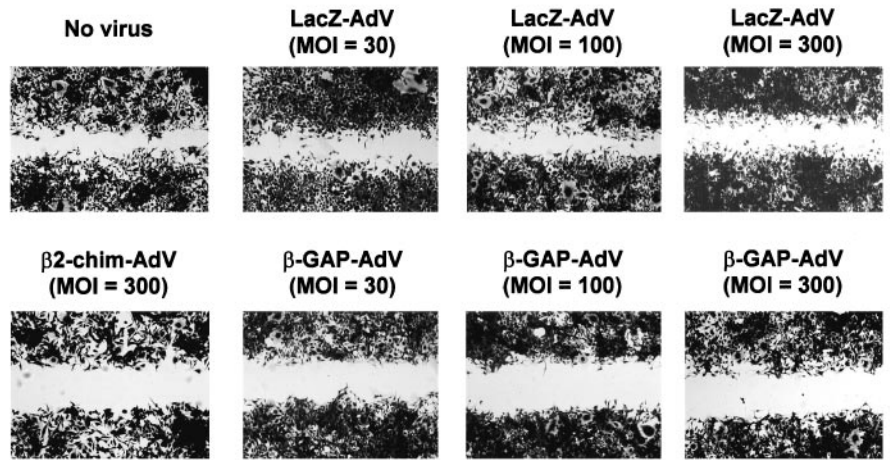


Fig. 4. Inhibition of migration upon AdV delivery of β -GAP into F3II cells. F3II cells were seeded in 60-mm dishes at \sim 80% confluence and then infected with β -GAP-AdV, β 2-chimaerin-AdV (β 2-*chim-AdV*), or LacZ-AdV at the MOI indicated. *A*, wounds were made 24 h after infection, when cells were $>$ 90% confluent, and migration was assessed 12 h later. A representative experiment is shown. Three additional experiments gave similar results. *B*, Western blot analysis of F3II-infected cells, using an anti-HA antibody. Molecular masses are 23 and 50 kDa, respectively, for HA- β -GAP and HA- β 2-chimaerin.

B



tically reduced the metastatic ability of F3II cells. Indeed, we observed a marked reduction in the incidence and number of lung metastases in animals inoculated with gb1, gb5, or ga6 clones (Table 2; Fig. 6C). Therefore, β -GAP-transfected cells have an impaired tumorigenic and metastatic potential in BALB/c mice.

DISCUSSION

The elucidation of the biological functions of the chimaerins is critical because their target, the Rac1 GTPase, controls numerous processes associated with proliferation, malignant transformation, and invasion. We investigated how the ectopic expression of the catalytic

domain of β 2-chimaerin, the β -GAP domain, affects the tumorigenic and metastatic properties of F3II mammary carcinoma cells. Use of *in vitro* GTP hydrolysis assays has established that chimaerins (including β 2-chimaerin) accelerate the hydrolysis of GTP from Rac1 without affecting the function of other related small GTP-binding proteins, including Rho and Cdc42 (10–13, 36). Thus, based on their *in vitro* properties, chimaerins were categorized as Rac-GAP proteins. In this study, we show for the first time that the expression of the catalytic domain of β 2-chimaerin markedly affects proliferation, invasion, and metastasis, effects for which a fundamental role has been ascribed to Rac1. It is evident that despite the low levels of expression of the

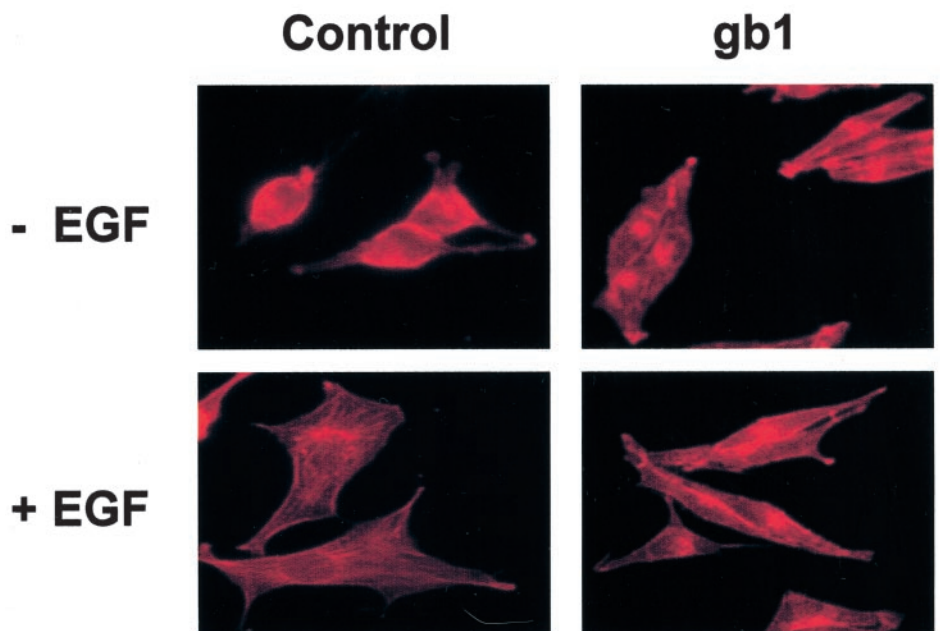


Fig. 5. Actin polymerization in β -GAP-transfected cells. Cells were seeded on glass coverslips, and after starvation, they were stimulated with EGF (100 ng/ml) for 15 min. Actin was stained with rhodamine-labeled phalloidin and photographed in a fluorescence microscope at \times 400 magnification. Shown is a representative experiment using control (vector-transfected) cells and the gb1 clone. Similar results were observed with the gb5 and ga6 clones. Two additional experiments gave similar results.

Table 2 Summary of tumorigenic properties of β-GAP F3II transfectants

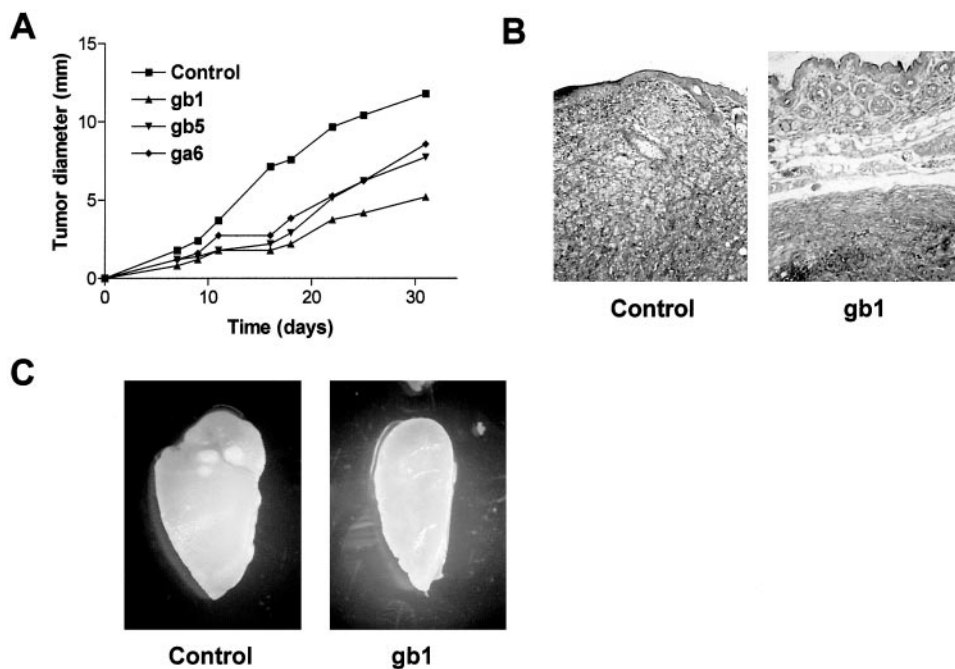
Results are representative of two independent experiments.

Cell line	Tumor latency (days ± SE)	Tumor incidence, positive/total (%)	Tumor growth rate (mm/day ± SE)	Incidence of lung metastases, positive/total (%)	No. of lung metastases (nodules ± SE)
F3II (untransfected)	7 ± 2	9/9 (100%)	0.44 ± 0.05	7/9 (78%)	6 ± 2
pCR3e (vector control)	6 ± 1	8/8 (100%)	0.40 ± 0.02	6/8 (75%)	8 ± 3
gb1	6 ± 1	8/10 (80%)	0.16 ± 0.04 ^a	1/10 (10%)	0 ± 0 ^c
gb5	6 ± 1	5/5 (100%)	0.30 ± 0.04	2/5 (40%)	2 ± 1 ^d
ga6	6 ± 1	5/5 (100%)	0.29 ± 0.02 ^b	3/5 (60%)	1 ± 1 ^e

^{a,b} One-way ANOVA contrasted with Dunnett multiple comparisons test: ^a *P* < 0.05; ^b *P* < 0.01.

^{c-e} Compared with control (*t* test with Welch correction): ^c *P* < 0.001; ^d *P* < 0.05; ^e *P* < 0.01.

Fig. 6. Reduced tumor growth and invasion of β-GAP-transfected cells. *A*, *in vivo* tumor growth of control (vector-transfected) cells and β-GAP domain transfectants (*gb1*, *gb5*, and *ga6*) in syngeneic BALB/c mice. Five mice received s.c. inoculations containing 2 × 10⁵ cells, and tumor growth was evaluated for 30 days. A second experiment gave similar results. *B*, representative micrographs of paraffin sections from s.c. F3II mammary carcinoma tumors. *Left*, tumor from control (vector-transfected) cells. *Right*, tumor from β-GAP-transfected cells (clone *gb1*). H&E staining; magnification, ×100. *C*, reduced number of metastases in lungs of animals receiving inoculations of clone *gb1*.



β-GAP domain, marked phenotypic changes could be observed. From this study, as well as from unpublished studies from our laboratory,⁵ we can conclude that a negative selection occurs for those clones with high levels of expression of β2-chimaerin after transfection of mammalian expression vectors. We speculate that this may be the consequence of impaired proliferative mechanisms or other essential functions that depend on an intact Rac function. This is not surprising because the normal expression levels of β2-chimaerin in most cell lines are low, and in many cases (including F3II and other cancer cells), the protein is barely detected or undetected by Western blot.

The reduced growth rate in culture observed for the β-GAP clones and upon transient expression of the β-GAP domain after AdV delivery suggests a role for β2-chimaerin in the control of cell proliferation. An essential role in cell cycle progression has been established for Rac1. Microinjection of an activated form of Rac1 into fibroblasts stimulates DNA synthesis and cell cycle progression through G₁, whereas a dominant-negative form of Rac1 (N17Rac1) blocks serum-induced DNA synthesis (37). AdV delivery of a dominant-negative Rac1 mutant into fibroblasts also produces cell growth arrest (38). Moreover, Rac1 plays an essential role in Ras malignant transformation, and overexpression of a constitutively activated form of Rac1 causes malignant transformation in fibroblasts (17, 18). Rac1 also regulates the Ras/Raf/extracellular signal-regulated kinase cascade, which is critical for cell proliferation (39, 40). We have recently

observed that AdV delivery of full-length β2-chimaerin or its β-GAP domain into MCF-7 breast cancer cells leads to G₁-S arrest, which is associated with a significant reduction in cyclin D1 levels.⁶ Because Rac stimulates cyclin D1 transcription (41, 42), one potential mechanism is that β2-chimaerin serves as a negative regulator of this pathway. The reduction in proliferation rate caused by the β-GAP domain supports the involvement of Rac1 in F3II cell growth, thus reinforcing the concept that interference with Rac1 signaling leads to significant reductions in proliferation.

During recent years, a role for Rac in migration has also been established. Cell migration involves a dynamic and coordinated regulation of the actin cytoskeleton architecture. Initiation of migration requires rapid reorganization of actin to the cell edge, protrusion of lamellipodium, and formation of membrane ruffles. The involvement of Rac1 in these complex processes has been extensively documented. Although activated forms of Rac1 lead to the formation of lamellipodia and membrane ruffles, dominant-negative Rac1 mutants impair actin cytoskeleton reorganization upon activation of different receptor types (6–8, 19). Along the same lines, expression of a dominant-negative Rac1 mutant inhibits migration in several cell types (43, 44). Noninvasive T-lymphoma cells expressing either activated Rac1 or Rac exchange factors that promote GDP/GTP exchange (such as Tiam1) become invasive, suggesting an important role for the Tiam1-Rac signaling pathway in the regulation of invasion and metastasis

⁵ M. J. Caloca and M. G. Kazanietz, unpublished observations.

⁶ C. Yang and M. G. Kazanietz, unpublished observations.

(19, 21, 45, 46). Interestingly, Tiam1 also promotes migration and invasion of breast cancer cells, an indication that Rac signaling plays a pivotal role in the control of cytoskeleton function and motility in breast tumor cell progression (47). Downstream effectors of Rac1, such as PAK1, are also critical for cell migration, probably by coordinating leading edge adhesion formation and polarized cell movement (48). The striking reduction in the migratory properties of β -GAP transfectants is an indication of a crucial role of Rac signaling in such processes. In agreement with our results, expression of the recently identified Rac-GAP CdGAP or Rac-GAPs from lower organisms markedly interferes with actin polymerization and cell motility (49, 50). Moreover, the GAP domain of the related α 1(*n*)-chimaerin isoform inhibits cytoskeletal responses to formyl-methionyl-leucyl-phenylalanine and colony-stimulating factor-1 (51). Although in this report we did not explore potential differences between chimaerin isoforms in cell migration, the high degree of homology between the GAP domains of α - and β -chimaerins suggests that they may have similar activities. This is under investigation at present in our laboratory.

Important observations in this report are that tumors originating from the β -GAP F3II clones showed a significantly lower growth rate and that the incidence of spontaneous lung metastases in mice was also lower. This may be the consequence of reduced proliferation and/or impaired migratory properties conferred by the β -GAP domain. Thus, inactivation of the Rac pathway markedly affects the dissemination of cancer cells *in vivo*. An interesting report by Yuan *et al.* (52) showed that the expression levels of β 2-chimaerin in gliomas vary during the progression of the disease. Using a differential display approach, these researchers identified β 2-chimaerin as a gene differentially expressed in normal brain and low-grade astrocytoma compared with glioblastoma tissues. In these high-grade gliomas, β 2-chimaerin expression is down-regulated, which may lead to enhanced Rac signaling and favor the invasive phenotype. Although we have observed that expression of β 2-chimaerin in F3II murine mammary carcinoma cells is substantially lower than in normal mammary epithelial cells, it remains to be determined whether this phenomenon also applies to human breast cancer and to other types of cancers. In support of this concept, recent experimental evidence revealed that Rac- and Rac-dependent signaling is hyperactivated in highly proliferative human breast cancer-derived cell lines and tumor tissues (22). It remains to be explored whether this is a consequence of changes in the function/expression of Rac-GAP proteins.

In summary, our results show that β -GAP, the catalytic domain of β 2-chimaerin, has profound effects on the growth of F3II cells and that it reduces the aggressiveness of this mammary carcinoma cell line. The inhibition of invasiveness in cells expressing β -GAP strongly suggests that chimaerins impair key steps involved in tumor progression. Understanding the regulation and function of the novel chimaerin DAG/phorbol ester receptors with Rac-GAP activity may provide insight into the signaling pathways that control malignant transformation and metastasis and reveal novel targets for cancer therapy.

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