β2-Chimerin is a member of the “non-protein kinase C” intracellular receptors for the second messenger diacylglycerol and the phorbol esters that is yet poorly characterized, particularly in the context of signaling pathways involved in proliferation and cancer progression. β2-Chimerin possesses a C-terminal Rac-GAP (GTPase-activating protein) domain that accelerates the hydrolysis of GTP from the Rac GTPase, leading to its inactivation. We found that β2-chimerin messenger levels are significantly down-regulated in human breast cancer cell lines as well as in breast tumors. Adenoviral delivery of β2-chimerin into MCF-7 breast cancer cells leads to inhibition of proliferation and G1 cell cycle arrest. Mechanistic studies show that the effect involves the reduction in Rac-GTP levels, cyclin D1 expression, and retinoblastoma dephosphorylation. Studies using the mutated forms of β2-chimerin revealed that these effects were entirely dependent on its C-terminal GAP domain and Rac-GAP activity. Moreover, MCF-7 cells stably expressing active Rac (V12Rac1) but not RhoA (V14RhoA) were insensitive to β2-chimerin-induced inhibition of proliferation and cell cycle progression. The modulation of G1/S progression by β2-chimerin not only implies an essential role for Rac in breast cancer cell proliferation but also raises the intriguing possibility that diacylglycerol-regulated non-protein kinase C pathways can negatively impact proliferation mechanisms controlled by Rho GTPases.
mary gland lesions (29). In addition, there is strong evidence that Rac effectors such as p21-activated kinase 1 are dysregulated in breast cancer cells (30). Collectively, these findings suggest critical implications of Rac in tumorigenesis, particularly in models of breast cancer.

In this paper, we investigated the expression of the DAG/phorbol ester receptor β2-chimerin in breast cancer and its role in proliferation. We have found that β2-chimerin mRNA levels are strikingly reduced in breast cancer cell lines and tissues. By means of adenoviral delivery into MCF-7 breast cancer cells, we have found that β2-chimerin, but not the mutated forms lacking Rac-GAP activity, causes a significant impairment in G1/S cell cycle progression due to a reduction in the expression levels of cyclin D1. The effect of β2-chimerin is strictly dependent on its ability to inhibit Rac function via the C-terminal GAP domain, suggesting the possibility that Rac-mediated control of cell proliferation is modulated by DAG-regulated pathways.

EXPERIMENTAL PROCEDURES

Human Breast Non-malignant and Cancer Cell Lines—Human breast cancer cell lines MCF-7, T-47D, MDA-MB231, MDA-MB-435, MDA-MB-468, and 957ST, and human breast immortalized non-malignant MCF-10A cells were purchased from ATCC and cultured according to the recommended protocol. The human non-malignant breast cell line HMT-3522 and its malignant derivative T4-2 were cultured as previously described (31). MCF-7-Tet-On cells were purchased from Clontech in 6-well plates growing in serum-free DMEM in MEM supplemented with 10% FBS, 2 mM glutamine, and 100 μg/ml G418.

Examination of β2-Chimerin mRNA Levels in Human Breast Cells and Tissues—For tissue RNA, 10 pairs of high quality human breast cancer tissue total RNA and matched-normal tissue total RNA (from the same patient) were purchased from Cloninom Biosciences, Inc. (Watervliet, NY). All 10 patients were diagnosed as infiltrating ductal carcinoma at different stages including two Stage I IDCs (samples 1–2), two Stage II IDCs (samples 3–4), two Stage III IDCs (samples 5–6), and four Stage IV IDCs (samples 7–10). Total RNA was prepared using TRIzol and reversibly transcripted using SuperScriptTM II Reverse Transcriptase (Invitrogen). β2-Chimerin mRNA levels were determined either by standard PCR (30 cycles) using the following primers: 5′-TGATCTCAAGAGGATCAAGAA-3′ (forward) and 5′-GTG-GAAATAGGTATCATATGTG-3′ (reverse), which specifically amplify a 297-bp fragment of β2-chimerin. Primers used for real-time PCR (Q-PCR) are described elsewhere (32). The real-time PCR reactions were plated in triplicate and performed in 384-well plates using the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization (32).

Generation of Adenoviruses (AdVs)—AdVs were generated with the AdEasy™ adenoviral vector system (Stratagene). Generation of the β2-chimerin and β-GAP AdVs was described elsewhere (10, 32). For the generation of the AdV for the mutant I130A-β2-chimerin (33). A control LacZ-AdV was generated from pShuttle-CMV-LacZ (provided by the kit) and therefore has the same backbone as the β2-chimerin AdVs. For adenoviral infections, MCF-7 cells in 6-well plates growing in serum-free DMEM were infected with various AdVs for 16 h. AdVs were removed after extensive washing, and experiments were performed 48 h later.

Generation of Stable Cell Lines Expressing Constitutively Active Small GTPases—Stable cell lines expressing active mutants of Rac1, Cdc42, or RhoA were generated upon transfection of MCF-7-Tet-On cells using FuGENE 6 (Roche Applied Science) followed by G418 hygromycin selection. The following plasmids were used: pTRE-β2-chimerin and β-GAP AdVs AdV was described elsewhere (10, 32). For the generation of the AdV for the mutant I130A-β2-chimerin (33). A control LacZ-AdV was generated from pShuttle-CMV-LacZ (provided by the kit) and therefore has the same backbone as the β2-chimerin AdVs. For adenoviral infections, MCF-7 cells in 6-well plates growing in serum-free DMEM were infected with various AdVs for 16 h. AdVs were removed after extensive washing, and experiments were performed 48 h later.

Results

Reduced Expression of β2-Chimerin in Human Breast Cancer Cells and Tissues—The expression of β2-chimerin in normal and breast cancer cells is unknown. Using standard PCR analysis, we found high levels of β2-chimerin mRNA in non-malignant immortalized MCF-10A cells. On the other hand, in all of the cancer cell lines examined, the β2-chimerin transcript was barely detected or dramatically reduced (Fig. 1A). The results were confirmed by a quantitative analysis using Q-PCR. Indeed, β2-chimerin mRNA was not detected in MCF-7 and 957ST cells and it was very low in T-47D, MDA-MB-231, and MDA-MB-435 cells. Only MDA-MB-468 cells showed significant levels of β2-chimerin transcript, although much lower than MCF-10A cells (Fig. 1B). Similarly, whereas β2-chimerin mRNA was readily detected in the non-malignant breast cell line HMT3522 (31), it was barely detectable in its malignant derivative (T-42) (Fig. 1, A and B). We next examined β2-chimerin mRNA levels in a small sample of human breast cancer tissues and their corresponding matched-normal tissues (from the same patient). It was found that the expression of β2-chimerin mRNA in normal tissues was highly variable. However, among the 10 patients, the β2-chimerin transcript was significantly lower in the cancer tissues of 7 patients (Fig. 1C). Together, these results reveal a significant reduction of β2-chimerin expression in human breast cancer.
Ectopic Expression of β2-Chimerin Inhibits Proliferation of MCF-7 Cells—Because Rac is known to control proliferation and there is evidence for Rac hyperactivation in breast cancer models, we examined how β2-chimerin affects human breast cancer cell proliferation. HA-tagged β2-chimerin was introduced into MCF-7 cells using an adenoviral gene delivery approach. Upon infection of MCF-7 cells with different m.o.i. of the β2-chimerin-AdV, HA-β2-chimerin was readily detected (Fig. 2A). Interestingly, β2-chimerin dose-dependently reduced BrdUrd incorporation in MCF-7 cells. On the other hand, infection with a control AdV (LacZ-AdV, 100 m.o.i.) did not change BrdUrd incorporation significantly (Fig. 2B).

To begin elucidating the mechanisms involved in the β2-chimerin effect, we examined cell cycle progression (Fig. 2C). Flow cytometry analysis revealed a significantly higher percentage of cells in G<sub>0</sub>/G<sub>1</sub> phase upon infection with the β2-chimerin-AdV compared with control (non-infected) or LacZ-AdV-infected MCF-7 cells. The effect was proportional to the m.o.i. used for infection. A concomitant decrease in the percentage of cells in S phase was observed, but no significant changes were observed in the number of cells in G/M phase.

The Anti-proliferative Effect of β2-Chimerin Is Dependent on a Functional β-GAP Domain—To examine whether the inhibitory effect on cell proliferation depends on β2-chimerin Rac-GAP activity, we expressed the C-terminal catalytic region of β2-chimerin (β-GAP domain) using an AdV. Infection of MCF-7 cells with β-GAP-AdV resulted in a m.o.i.-dependent increase in the expression of the β-GAP domain (Fig. 3A). Expression of β-GAP caused similar effects on cell proliferation and cell cycle progression as those observed with full-length β2-chimerin (Fig. 3, B and C). We then took advantage of the β2-chimerin mutant ΔEIE-β2-chimerin (deletion in positions 298–300 in the β-GAP domain), which is unable to accelerate GTP hydrolysis from Rac (10). An AdV for the GAP-inactive ΔEIE-β2-chimerin-AdV at different m.o.i. Cell cycle analysis was carried out 48 h later using flow cytometry. Data are presented as mean ± S.D. (n = 3). *, p < 0.05 compared with control cells.
FIG. 3. The anti-proliferative effect of β2-chimerin depends on a functional β-GAP domain. Expression of β-GAP or ΔEIE-β2-chimerin was determined by Western blot 48 h after infection of MCF-7 cells with increasing m.o.i. of either β-GAP-AdV (panel A) or ΔEIE-β2-chimerin-AdV (panel D). Panels B and E, BrdUrd incorporation was determined in cells infected with either β-GAP-AdV (panel B) or ΔEIE-β2-chimerin-AdV (panel E) using flow cytometry, as described under “Experimental Procedures.” Panel C, MCF-7 cells were infected with the β-GAP-AdV at different m.o.i. Cell cycle analysis was determined 48 h later using flow cytometry. Data are presented as the mean ± S.D. (n = 3). *, p < 0.05 compared with control cells.

FIG. 4. β2-Chimerin reduces cyclin D1 expression and inhibits pRb phosphorylation. MCF-7 cells were infected with increasing m.o.i. of AdVs for β2-chimerin, β-GAP (panel A), or ΔEIE-β2-chimerin (panel B). A LacZ-AdV (100 m.o.i.) was used as a control. After 48 h, cell extracts were prepared and subjected to Western blot analysis for pRb and cyclins. Similar results were observed in three independent experiments.

The Rac-GAP inactive mutant, ΔEIE-β2-chimerin, did not impair cyclin D1 expression or pRb phosphorylation (Fig. 4B). Taken together, these results suggest that the inhibition of G1/S transition by β2-chimerin via its β-GAP domain involves the reduction of cyclin D1 and pRb phosphorylation levels.

Inhibition of Rac by β2-Chimerin in MCF-7 Cells—β2-Chimerin has specificity for the Rac GTPase both in vitro GAP assays and in COS-1 cells but does not affect RhoA or Cdc42 activity (10, 11). EGF (100 ng/ml, 1 min) caused a 3.5 ± 0.5-fold (n = 3) increase in Rac-GTP levels in MCF-7 cells, which was significantly impaired by the expression of β2-chimerin. β2-Chimerin also reduced Rac-GTP levels in MCF-7 cells growing in 10% serum. The effect was proportional to the m.o.i. used for infection, and it was not observed with the GAP-inactive mutant, ΔEIE-β2-chimerin (Fig. 5A). A densitometric analysis of the β2-chimerin effect on serum-induced activation of Rac is presented in Fig. 5B. A striking correlation was observed between the inhibitory effect of β2-chimerin on Rac activity and the reduction in cyclin D1 levels by different m.o.i. of the β2-chimerin-AdV (r = 0.95).

Ectopic Expression of Cyclin D1 Rescues the Anti-proliferative Effect of β2-Chimerin—To further explore the link between Rac and cyclin D1 in our experimental model, we expressed cyclin D1 using a retroviral approach (Fig. 6A). Interestingly, the ectopic expression of cyclin D1 using the D1-RetroV significantly rescued the anti-proliferative effect of β2-chimerin, whereas the control retrovirus (V-RetroV) did not (Fig. 6B).

MCF-7 Cells Expressing Constitutively Active Rac1 Are Insensitive to β2-Chimerin—We reasoned that the expression of a constitutively active Rac mutant in MCF-7 cells should impair the effects of β2-chimerin on cell proliferation and cell cycle progression. A MCF-7 cell line stably expressing active V12Rac1 was generated (HA-V12Rac1-MCF-7) (Fig. 7A). These cells show higher levels of Rac-GTP than control (vector-transfected) cells (Fig. 7D). Interestingly, whereas β2-chimerin markedly reduced proliferation in control MCF-7 cells, HA-V12Rac1-MCF-7 cells were insensitive to β2-chimerin (Fig. 7B). Consistent with these results, adenoviral delivery of β2-chimerin into HA-V12Rac1-MCF-7 cells did not reduce cyclin D1 levels or cause pRb dephosphorylation (Fig. 7A).

We then determined whether the expression of other active Rho-GTPases could rescue the effect of β2-chimerin. MCF-7 cell lines stably expressing constitutively active Cdc42 (V12Cdc42) or RhoA (V14RhoA) were generated (Fig. 7A). Similar to control MCF-7 cells, HA-V14RhoA-MCF-7 cells were highly sensitive to β2-chimerin for the inhibition of cell proliferation, reduction of cyclin D1, and pRb dephosphorylation (Fig. 7, A and B). Unexpectedly, in cells expressing active Cdc42,
adenoviral delivery of β2-chimerin was unable to inhibit cell proliferation, cyclin D1 expression, and pRb phosphorylation (Fig. 7, A and B). To further examine the mechanisms involved in the protective effect of V12Cdc42, we determined Cdc42-GTP levels in response to EGF (100 μg/ml, 1 min). A 3.1 ± 0.6-fold (n = 3) increase in Cdc42-GTP levels was observed in response to the growth factor, which was not affected by the β2-chimerin-AdV, even at the highest m.o.i. used (100 plaque-forming units/cell) (Fig. 7C). Interestingly, we found that basal Rac-GTP levels were elevated in HA-V12Cdc42-MCF-7 cells (Fig. 7, D and E), which probably explain the protective effect of active Cdc42 on β2-chimerin-induced inhibition of cyclin D1 levels, pRb phosphorylation, and cell proliferation.

A Hyperactive β2-Chimerin Mutant Is a Potent Inhibitor of Cyclin D1 Expression and Proliferation—Based on structural predictions gained from the recently solved structure of β2-chimerin, we generated an AdV encoding for a β2-chimerin mutant locked in the constitutively active conformation. This mutant, I130A-β2-chimerin, was shown to have constitutive Rac-GAP activity when expressed in COS-1 cells by bypassing lipid activation (33). An AdV encoding for I130A-β2-chimerin was generated and used to infect MCF-7 cells. We optimized conditions to achieve similar low levels of expression as those observed in the non-malignant breast cell line HMT3522 cells (as detected by Q-PCR, Table I). In this case, we used a lower m.o.i. and shorter expression times (16 h instead of 40 h) and the levels of the mutant I130A-β2-chimerin in MCF-7 cells were well below the detection levels using Western blot. Under these experimental conditions, the wild-type β2-chimerin still showed very high levels of expression by Western blot and caused a ~25% reduction in Rac-GTP levels (Fig. 8A). Remark-
ably, even if I130A-β2-chimerin was expressed at very low levels, it caused a 51% reduction in Rac-GTP levels (Fig. 8A). Moreover, I130A-β2-chimerin markedly reduced cyclin D1 levels (Fig. 8B) and impaired cell proliferation (Fig. 8C).

**DISCUSSION**

Understanding the functional properties of Rac-GAPs is relevant, because Rac is a key player in the process of malignant transformation and metastasis (12, 18, 30). The two most relevant findings in the present study are that β2-chimerin expression is down-regulated in breast cancer and that the expression of this Rac-GAP in MCF-7 breast cancer cells impair G1/S cell cycle progression by reducing cyclin D1 levels and Rb phosphorylation. Inhibition of proliferation by β2-chimerin in

| Cells                  | β2-Chimerin mRNA (copies/10^6 GAPDH copies) |
|------------------------|--------------------------------------------|
| HMT-3522               | 4527 ± 862                                  |
| MCF-10A                | 625 ± 16                                    |
| MCF-7                  | 3.3 ± 0.2                                   |
| MCF-7 + I130A-β2-chimerin-AdV | 5902 ± 1414                               |

**TABLE I**

Comparison of β2-chimerin mRNA levels in breast cells

The β2-chimerin mRNA levels were determined by Q-PCR as described under “Experimental Procedures.” For I130A-β2-chimerin adenoviral infection, MCF-7 cells were serum-starved for 8 h and infected with I130A-β2-chimerin-AdV (25 m.o.i.) for 16 h. Cells were then harvested using TRIzol reagent for total RNA extraction and Q-PCR analysis as described under “Experimental Procedures.” Data are presented as mean ± S.E. (n = 3).

![Graph A](image1)

**Panel A**

The active mutant I130A-β2-chimerin inhibits Rac-GTP levels, cyclin D1 expression, and proliferation. Panel A, after 8 h of serum starvation, cells were infected for 16 h with I130A-β2-chimerin-AdV (β2-Ch (I130A)) (25 m.o.i.). Wild-type β2-chimerin-AdV (β2-Ch (w.t.)) (100 m.o.i.) was used as a control. Cells were then stimulated with EGF (100 ng/ml, 1 min). Rac-GTP levels were determined using a PBD pull-down assay, as described under “Experimental Procedures,” Densitometric analysis of the inhibitory effect of β2-chimerin on EGF-induced Rac activation was normalized to the corresponding total Rac levels in each case. Data are presented as mean ± S.D. (n = 3). *, p < 0.05 compared with cells stimulated with EGF or without β2-chimerin-AdV infection. Panels B and C, MCF-7 cells were infected as described in panel A. After 24 h of serum starvation, cells were stimulated with 10% FBS for 24 h. The expression of cyclin D1 and HA-β2-chimerin was determined by Western blot (panel B). Cell proliferation was examined by counting cell number using a hemacytometer (panel C). Data are presented as mean ± S.D. (n = 3). *, p < 0.05 compared with cells stimulated with 10% FBS without HA-β2-chimerin-AdV infection.
significantly underexpressed in 18% human hepatocellular carcinomas. It is conceivable that the down-regulation of β2-chimerin in breast cancer cells may contribute, at least in part, to the progression of the disease. Early studies in glioma models have identified β2-chimerin as a gene that is significantly down-regulated in high-grade gliomas compared with normal brain and low-grade astrocytomas (8). Down-regulation of β2-chimerin in advanced stages of the disease could contribute to the enhanced proliferation and metastatic dissemination of glioma cells due to dysregulation of Rac activity. Along the same lines, we have recently found using tissue microarrays that β2-chimerin expression is reduced by ~60% in benign duodenal adenomas and ~80% in duodenal adenocarcinomas when compared with normal tissues. β-GAP significantly inhibits cell migration as well as tumor growth, invasiveness, and metastatic dissemination in vivo (32), suggesting that specific inhibition of Rac by β2-chimerin may impinge on various steps of malignant transformation. Although more extensive studies would be required to establish whether this Rac-GAP may serve as a prognostic marker, this body of evidence suggests that down-regulation of β2-chimerin expression may contribute to breast cancer progression. This may also be relevant in tissues that express high levels of β2-chimerin, including brain, pancreas, and intestine.

Adenoviral delivery of β2-chimerin, β-GAP, or II10A-β2-chimerin, but not ΔEIE-β2-chimerin, significantly impairs proliferation and elevations in Rac-GTP levels in MCF-7 breast cancer cells, suggesting an essential role for chimerin Rac activity in these effects. β2-Chimerin also impairs heregin β1-induced Rac activation and proliferation in breast cancer cells. This highlights the potential relevance of β2-chimerin as a general negative regulator of growth factor-mediated mitogenic responses. Moreover, we have recently observed that the inhibition of Rac activity with specific Rac-GAP significantly in...
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