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14. ABSTRACT

Chimaerins are a family of intracellular receptors for the phorbol ester tumor promoters and the second messenger diacylglycerol. The discovery of chimaerins challenges the traditional view that protein kinase C is the only family of receptors for phorbol esters. This proposal was designed to investigate the biological functions of chimaerins. We found that (1) the mRNA levels of beta2-chimaerin, one of the most widely expressed chimaerin isoforms, were significantly lower in breast cancer cells and tissues than that in breast normal cells and tissues; (2) re-expression of beta2-chimaerin or its catalytical domain beta-GAP using an adenoviral gene delivery technique induced cell cycle arrest at G1 phase and subsequently inhibited breast cancer cell proliferation; (3) the effect of beta2-chimaerin on cell cycle progression and cell proliferation entirely depended on its Rac-GAP activity; (4) heregulin beta1 (HRG), an EGF-like growth factor and a mitogen for breast cancer cells, is a strong activator of Rac in breast cancer cells and promotes breast cancer cell proliferation through ErbB receptors/PI3K/Rac/Erk-dependent up-regulation of cyclin D1 and p21 expression; (5) expression of beta2-chimaerin inhibited HRG-induced Rac activation and impaired Rac-dependent responses including cell migration, Erk1/2 activation, cyclin D1 and p21 expression, and cell proliferation. These findings suggest that beta2-chimaerin may act as a tumor suppresso r.

15. SUBJECT TERMS
Chimaerin, cell proliferation, cell cycle, breast cancer, signal transduction

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Introduction

Chimaerins are novel non-kinase receptors for the phorbol ester tumor promoters and the second messenger diacylglycerol (DAG), consisting of four members: α1-, α2-, β1-, and β2-chimaerin (Kazanietz 2002). While their pharmacological properties of binding phorbol esters have been well characterized (Caloca *et al*., 1999; Kazanietz 2002), their biological functions remain largely unknown.

Studies have shown that β2-chimaerin possesses a C-terminal Rac-GAP (GTPase-activating protein) domain that accelerates the hydrolysis of GTP from the Rac GTPase, leading to its inactivation (Caloca *et al*., 2003; Canagarajah *et al*., 2004). Accumulating evidence indicates that Rac plays a critical role in the control of actin cytoskeleton reorganization, cell proliferation, cell cycle progression, gene expression and malignant transformation (Sahai and Marshall, 2002; Coleman *et al*., 2004). Moreover, recent studies also indicate that high level of Rac activation exists in breast cancer cells (Mira *et al*., 2000). Therefore, this proposal was designed to investigate if chimaerins inhibit Rac function and signaling in breast cancer cells, and thus they will impair those responses that depend on Rac, including cell migration, cell proliferation and cell cycle progression. The proposed research includes: (1) generating various wild-type and mutants of chimaerin-adenoviruses; (2) determining if chimaerins regulate cell proliferation and cell cycle progression in breast cancer cells; and (3) studying how chimaerins regulate Rac-dependent signaling pathways that regulate cell proliferation and cell cycle progression in breast cancer cells. This study was finished in the last three years.

Body

In the first and second year of my fellowship, as originally proposed and outlined in the approved Statement of Work, my research included making various adenoviruses (AdV) for chimaerins (task 1) and determining the effect of chimaerins on breast cancer cell proliferation and cell cycle progression (*Task 2*). The main findings have been published in *Journal of Biological Chemistry* (**280:** 24363-24370. 2005) (Please refer to the appended paper).

Heregulins are epidermal growth factor (EGF)-like growth factors and are often overexpressed in breast cancers, playing critical roles in breast cancer development and progression (Falls, 2003). The findings from my previous year studies indicated that heregulin β1 (HRG) is a strong activator of Rac in breast cancer cells. However, it is not known if Rac activation plays any role in HRG-triggered motogenic and mitogenic signal transduction that leads to cell migration and proliferation. Therefore, the research in my final year postdoc fellowship focused on signaling studies in breast cancer cells, investigating how chimaerins regulate signaling pathways that control breast cancer cell migration and proliferation induced by HRG stimulation (Specific Aim 3 in my original proposal).

As we found in our previous year studies that HRG is a strong activator of Rac in breast cancer cells, it is thus very appropriate to use HRG stimulation as a model for our signaling studies. As planned in *Task 3* in my original Statement of Work, I have done the following research work in the final year of my postdoc fellowship: (1) investigating the signaling pathways that lead to Rac activation by HRG in breast cancer cells; (2) determining the effects of β2-chimaerin on HRG-induced cytoskeleton reorganization and breast cancer cell migration; (3) investigating the effects of β2-chimaerin on HRG-induced breast cancer cell proliferation; (4) examining how β2-chimaerin regulates the mitogenic signal transduction of HRG in breast cancer cells; and (5) studying the mechanisms how β2-chimaerin regulates HRG-induced gene expression that promotes breast cancer cell proliferation (*Task 3*). The main findings have been published in *Molecular and Cellular Biology* (**26:** 831-842. 2006) (Please refer to the appended paper).

In addition to studies on *Task 3*, I also performed studies on how HRG regulates the expression of genes that promote breast cancer cell proliferation, and the effects of β2-chimaerin on HRG-induced gene expression and cell proliferation. In this part of study, we found that HRG promotes breast cancer cell proliferation through increasing the expression of cyclin D1 and p21. As shown in Fig. 1A, HRG stimulation increased the protein levels of cyclin D1 and p21, but did not affect the levels of p27 and p53. HRG also increased the mRNA levels of cyclin D1 and p21 (Fig. 1B). HRG-induced increase of cyclin D1 and p21 levels was completely abolished by inhibition of transcription or protein synthesis (Fig. 1C). Moreover, HRG-induced increase of cyclin D1 or p21 was required for cell proliferation as RNAi depletion of cyclin D1 or p21 impaired HRG-induced breast cancer cell proliferation (Fig. 1D and E).

Next, we determined if Rac play any role in HRG-induced gene expression and cell proliferation. We found that inactivation of Rac by expression of β2-chimaerin or a dominant negative Rac (N17Rac1) using the adenoviral gene delivery approach inhibited HRG-induced cyclin D1 and p21 expression (Fig. 2A and B), and cell proliferation (Fig. 2C). These findings were further confirmed by depletion of Rac using RNAi (Fig. 2D and E).

As we found that HRG promotes breast cancer cell proliferation mainly through Rac/Erk signaling, and inactivation of Rac by expression of β2-chimaerin inhibited Erk activation and subsequently impaired cell proliferation (Yang *et al*., Mol. Cell. Biol. **26:** 831-842. 2006. Please refer to the appended paper), we then examined the effect of Erk1/2 activity on HRG-induced gene expression. As shown in Fig. 3, inhibition of Erk1/2 activity using a specific inhibitor for MEK1 reduced the expression of cyclin D1 and p21 by HRG stimulation. Together, these results indicate that HRG promotes breast cancer cell proliferation through Rac/Erk signaling-regulated expression of cyclin D1 and p21, and β2-chimaerin

regulates breast cancer cell proliferation through modulating Rac/Erk signaling-mediated gene expression.

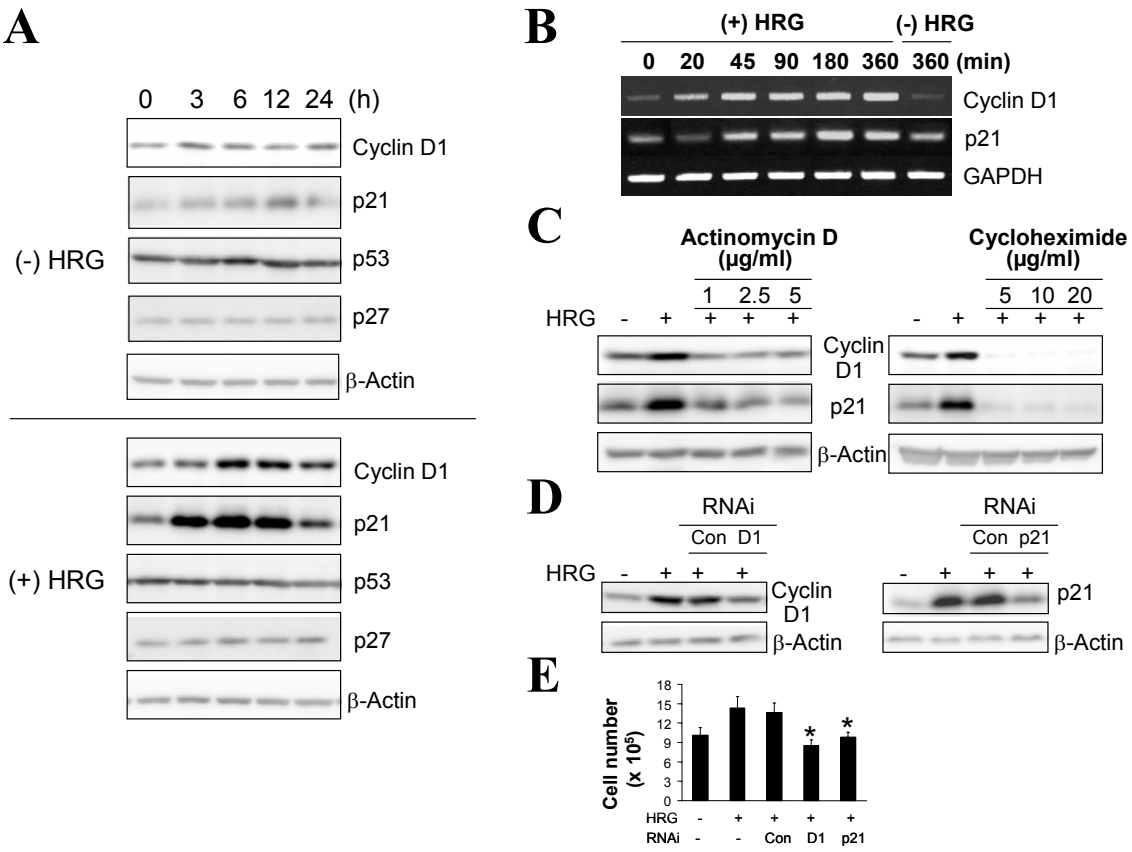


Fig. 1 HRG increases the expression of cyclin D1 and p21 in breast cancer T-47D cells. After 48 h serum starvation, cells were treated with HRG (10 ng/ml) as indicated in figures. *A*. Protein levels of cyclin D1, p21, p53, p27 and β-actin were determined by Western blot using specific antibodies. *B*. mRNA levels of cyclin D1 and p21 were determined by RT-PCR. *C*. Cells were first treated with actinomycin D or cycloheximide for 1 h, then stimulated with HRG for 6 h. The protein levels of cyclin D1 and p21 were determined by Western Blot. *D and E.* Specific siRNA oligoes for cyclin D1, p21 and control were transfected into T-47D cells using lipofectamine 2000 as described in the attached paper (MCB **26:** 831-842. 2006). The protein levels of cyclin D1 and p21 were determined 6 h after HRG stimulation and the cell numbers were counted using a hemacytometer 24 h after HRG treatment.

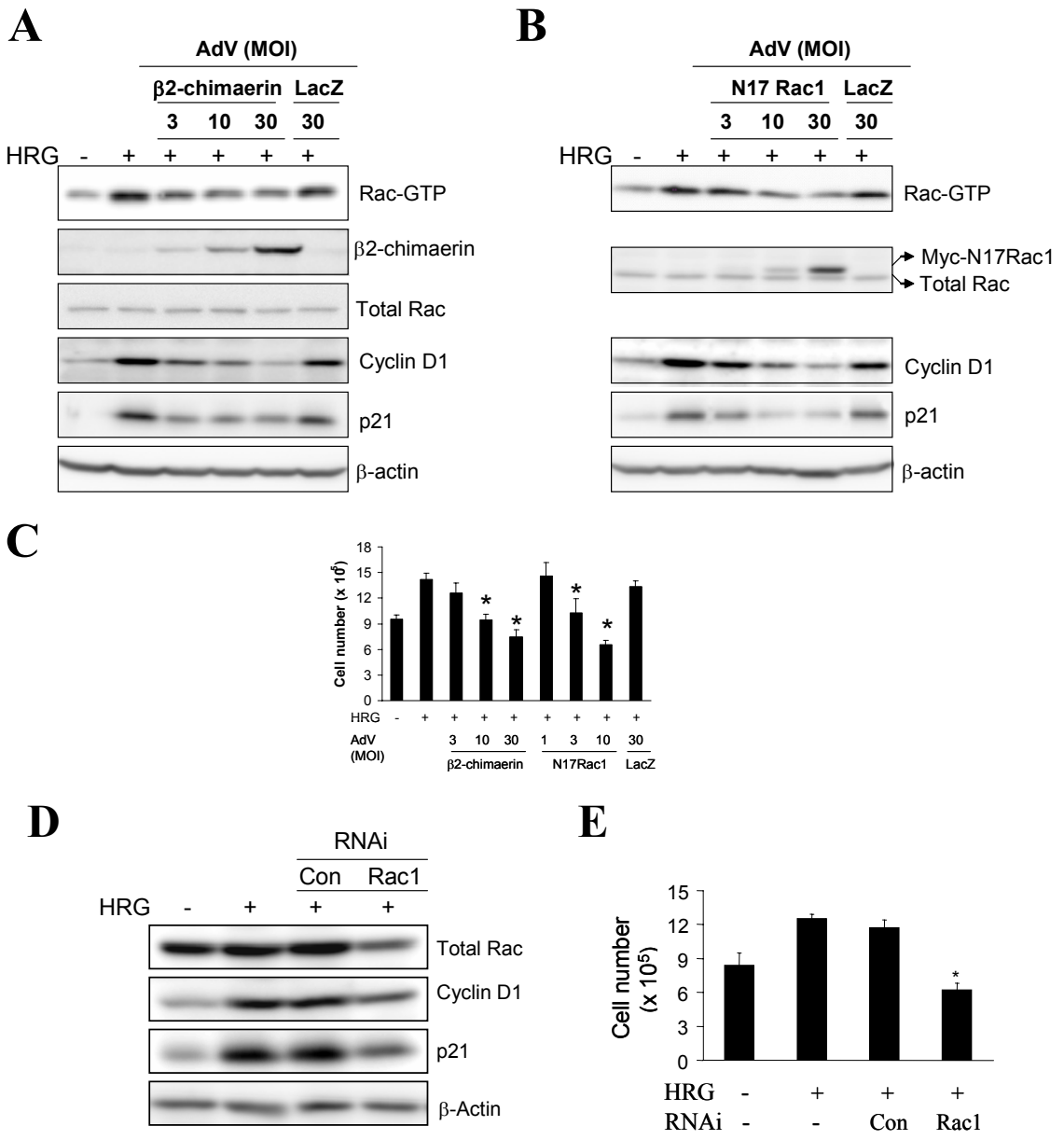


Fig. 2 Inactivation or depletion of Rac inhibits HRG-induced cyclin D1 and p21 expression and cell proliferation. *A, B and C.* After 24 h culture, T-47D cells were serum-starved for 8 h and then infected with different MOIs of adenoviruses for 16 h. Cells were washed and serum-starved for another 24 h before stimulation with HRG (10 ng/ml). Rac-GTP levels were measured using a pull-down assay 5 min after HRG stimulation; cyclin D1 and p21 protein levels were measured by Western blot 6 h after HRG treatment; and cell proliferation was determined 24 h after HRG stimulation. *D and E.* Specific siRNA oligoes for Rac1 and control were transfected into T-47D cells using lipofectamine 2000 as described in the attached paper (MCB **26:** 831-842. 2006). The protein levels of cyclin D1 and p21 were determined 6 h after HRG stimulation and the cell numbers were counted using a hemacytometer 24 h after HRG treatment.

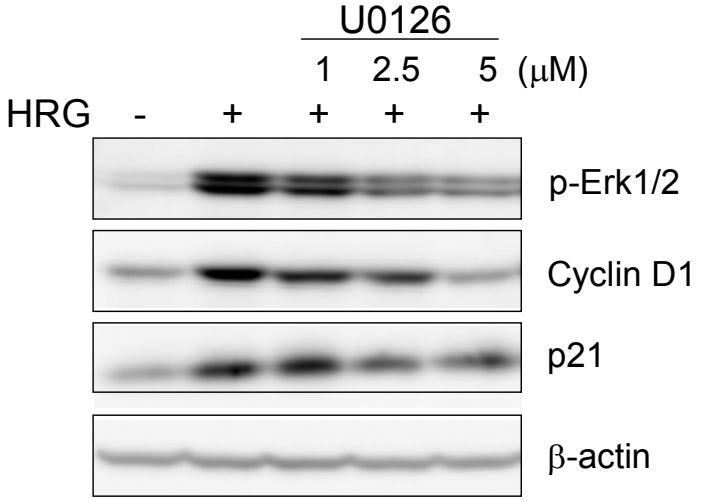
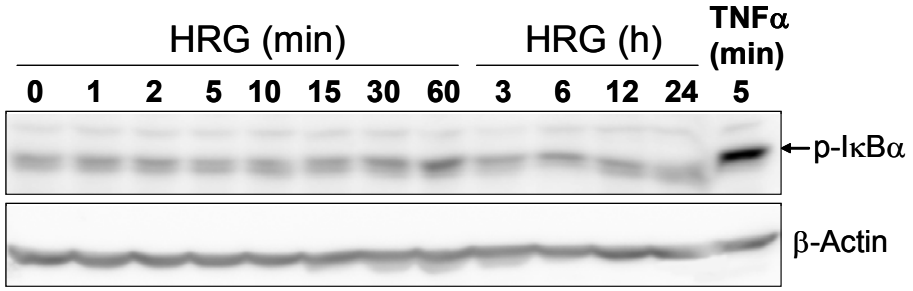


Fig. 3 Inhibition of Erk1/2 impairs HRG-induced expression of cyclin D1 and p21. After 48 h serum starvation, T-47D cells were first treated with a MEK1 inhibitor U0126 for 1 h as indicated in the figure, and then stimulated with HRG (10 ng/ml) for 6 h. The phosphorylation of Erk1/2 and the protein levels of cyclin D1, p21 and β-actin were determined by Western blot using specific antibodies.

We also explored the possible mechanisms how Rac/Erk signaling regulates gene expression. Previous studies have shown that Rac increases cyclin D1 expression through activation of NFκB (Joyce *et al*., 1999). We then examined the effect of HRG on NFκB activity. We found that HRG treatment did increase IκBα phosphorylation (a marker indicating NFκB activation) (Fig. 4A) and only slightly increased NFκB transcriptional activity using a luciferase reporter assay (Fig. 4B). Indeed, we used TNFα stimulation as a positive control and observed a strong activation of NFκB as indicated by the increase of IκBα phosphorylation and luciferase reporter activity (Fig. 4A and B). Moreover, inactivation of Rac by expressing β2-chimaerin or depletion of Rac by using RNAi did not significantly affect the NFκB luciferase reporter activity (Fig. 4B). It seems that HRG increases cyclin D1 and p21 expression through Rac/Erk signaling, but not through NFκB.

A



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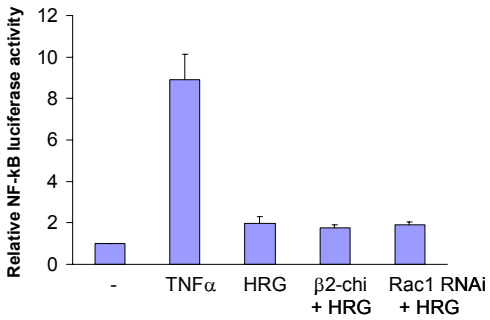

Fig. 4 HRG does not induce strong activation of NFκB. *A.* After 48 h serum starvation, T-47D cells were stimulated with HRG (10 ng/ml) or TNFα (10 ng/ml) as indicated in the figure. The phosphorylation of IκBα was determined by Western blot using a specific antibody. *B.* NFκB luciferase reporter plasmid and Renilla control plasmid were co-transfected into T-47D cells using lipofectamine 2000 as described before. In the experiments with β2-chimaerin or Rac1 RNAi, cells were co-transfected with NFκB luciferase reporter plasmid, Renilla control plasmid and β2-chimaerin plasmid or Rac1 RNAi oligoes. Cells were stimulated with HRG (10 ng/ml) 48 h after plasmid transfection. NFκB luciferase reporter activity was measured 24 h after HRG treatment using chemiluminometer.

Finally, as we demonstrated in our signaling studies that HRG activates Rac through the ErbB receptors/PI3K pathway (Yang *et al*., Mol. Cell. Biol. **26:** 831-842. 2006. Please refer to the appended paper), we then investigated if inactivation of one of the upstream ErbB receptors (for example EGFR) or PI3K affects the expression of cyclin D1 and p21. As shown in Fig. 5, inhibition of EGFR using a specific inhibitor AG1478 or inhibition of PI3K using the specific inhibitor Wortmannin impaired HRG-induced cyclin D1 and p21 expression.

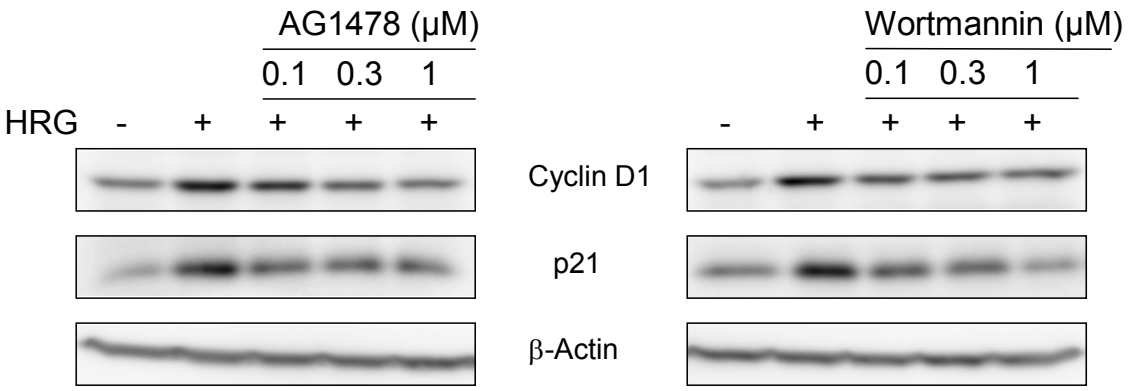

Fig. 5 Inhibition of EGFR or PI3K impairs HRG-induced expression of cyclin D1 and p21. After 48 h serum starvation, T-47D cells were first treated with specific inhibitors for EGFR (AG1478) or PI3K (Wortmannin) for 1 h as indicated in the figure, and then stimulated with HRG (10 ng/ml) for 6 h. The protein levels of cyclin D1, p21 and β-actin were determined by Western blot using specific antibodies.

In the final year of my training, under the supervision of my mentor Dr. Kazanietz, I have written and published one research paper entitled "Essential role for Rac in heregulin beta1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4" in *Molecular and Cellular Biology* (please see the appended paper MCB **26:** 831-842. 2006). Based on the data presented above, I am currently preparing another manuscript for publication.

Key Research Accomplishments

1. Various adenoviruses for full-length wild type chimaerins, GAP domains, GAP-inactive mutant and control LacZ gene have been generated, which provided important tools for studies on function of chimaerin isoforms.

2. It has been found that the β2-chimaerin mRNA expression levels in breast cancer cells and tissues are significantly lower than that in breast normal cells and tissues (please refer to the appended paper JBC **280:** 24363-24370. 2005).

3. Expression of β2-chimaerin inhibits Rac activation in breast cancer cells stimulated by serum or EGF (please refer to the appended paper JBC **280:** 24363-24370. 2005).

4. Expression of β2-chimaerin or its GAP-domain (β-GAP) induces MCF-7 cell cycle arrest and impairs breast cancer cell proliferation. The inhibition of β2-chimaerin on breast cancer cell growth is dependent on its Rac-GAP activity (please refer to the appended paper JBC **280:** 24363-24370. 2005).

5. Various stable cell lines expressing constitutively active Rac1 (V12Rac1), Cdc42 (V12Cdc42), and RhoA (V14RhoA) have been generated. Moreover, it has been found that expression of constitutively active Rac or Cdc42, but not RhoA, significantly rescued the inhibitory effect of β2-chimaerin on breast cancer cell proliferation (please refer to the appended paper JBC **280:** 24363-24370. 2005).

6. It has been found that β2-chimaerin inhibits breast cancer cell proliferation through inactivating Rac and decreasing Rb phosphorylation and cyclin D1 level, without affecting the levels of cyclin A, E, and CdK2 and 4 (please refer to the appended paper JBC **280:** 24363-24370. 2005).

7. It has been found that heregulin β1 (HRG) activates Rac in breast cancer cells and Rac activation by HRG depends on ErbB3, ErbB2 and EGFR, but is independent of ErbB4 (please refer to the appended paper MCB **26:** 831-842, 2006).

8. HRG-induced Rac activation is PI3K-dependent, but is Src kinase-independent. HRG activates Rac through ErbB receptors/PI3K signaling pathway (please refer to the appended paper MCB **26:** 831-842, 2006).

9. Rac activation by HRG promotes Erk1/2 MAPK activation, which in turn increases breast cancer cell proliferation. Inactivation of Rac by expression of β2-chimaerin impairs HRG-induced Erk1/2 MAPK activation, and subsequently inhibits cell proliferation (please refer to the appended paper MCB **26:** 831-842, 2006).

10. HRG increases breast cancer cell proliferation through up-regulating the expression of cyclin D1 and p21. Inactivation of Rac by expressing β2-chimaerin or depletion of Rac using RNAi impairs HRG-induced cyclin D1 and p21 expression, and subsequently inhibits cell proliferation (please refer to the results shown above).

11. Inhibition of Erk1/2 MAPK reduces HRG-induced cyclin D1 and p21 expression. Similarly, inhibition of upstream kinases such as EGFR or PI3K that are required for HRG-induced Rac and Erk1/2 activation, also impairs HRG-induced cyclin D1 and p21 expression (please refer to the results shown above).

12. HRG slightly increases NFκB luciferase reporter activity. Inactivation of Rac by expressing β2-chimaerin or depletion of Rac using RNAi does not affect NFκB activity (please refer to the results shown above).

Reportable Outcomes

1. Two published research papers and one review paper:

   Yang C, Liu Y, Lemmon MA, and Kazanietz MG. Essential role for Rac in heregulin beta1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4. *Molecular and Cellular Biology* **26**: 831-842. 2006.

   Yang C, Liu Y, Leskow FC, Weaver VM, Kazanietz MG MG. Rac-GAP-dependent inhibition of breast cancer cell proliferation by β2-chimerin. *Journal of Biological Chemistry* **280**: 24363-24370. 2005.

   Yang C, and Kazanietz MG. Divergence and complexities in DAG signaling: looking beyond PKC (review). *Trends in Pharmacological Sciences* **24**: 602-608. 2003.

2. Four conference poster presentations:

   Yang C, Liu Y, Lemmon MA, and Kazanietz MG. Heregulin β1 induces cyclin D1 and p21 expression and promotes breast cancer cell proliferation through ErbB receptor/Rac/Erk. *Proceedings of the 97th Annual Meeting of American Association for Cancer Research*, p868, April 1-5, 2006. Washington, DC.

   Yang C, Liu Y, and Kazanietz MG. Essential role for Rac1 in heregulin β1-induced mitogenic signaling in human breast cancer cells. *Proceedings of the 96th Annual Meeting of American Association for Cancer Research*, p868, April 16-20, 2005. Anaheim, CA.

   Yang C, Liu Y, and Kazanietz MG. Heregulin β1-induced Rac activation promotes breast cancer cell proliferation. *Proceedings of Era of Hope-05 Department of Defense Breast Cancer Research Program Meeting*, p223, June 8-11, 2005. Philadelphia, PA.

   Yang C, Liu Y, and Kazanietz MG. β2-chimaerin inhibits heregulin-induced activation of mitogen-activated protein kinases in human breast cancer cells. *Proceedings of 20th Annual Meeting on Oncogenes*, p133, June 16-20, 2004. Frederick, MD.

Conclusions

Based on the results obtained from my past three-year studies, I can make the following conclusions: (1) the mRNA expression level of β2-chimaerin, a specific Rac-GAP and one of the most widely-expressed chimaerin isoforms, is much lower in breast cancer cells and tissues than that in breast normal cells and tissues; (2) β2-chimaerin inhibits breast cancer cell proliferation and cell cycle progression through its Rac-GAP activity; (3) heregulin β1 (HRG), a EGF-like growth factor, is a strong activator of Rac in breast cancer cells and Rac activation by HRG in breast cancer cells depends on ErbB3, ErbB2 and EGFR, but is independent of ErbB4; (4) HRG-induced Rac activation is PI3K-dependent but Src kinase-independent. PI3K activation by HRG depends on ErbB receptors; (5) HRG-induced Rac activation plays critical roles in cytoskeleton reorganization, cell migration and proliferation; (6) HRG increases breast cancer cell proliferation through Rac/Erk-dependent up-regulating the expression of cyclin D1 and p21; (7) Inactivation of Rac by expressing β2-chimaerin or depletion of Rac using RNAi inhibits HRG-induced cell migration, cyclin D1 and p21 expression, and subsequently impairs cell proliferation; and (8) NFκB activity does not play a significant role in HRG-induced Rac/Erk mitogenic signaling. These findings suggest that (1) Rac activity is critically involved in breast carcinogenesis; and (2) β2-chimaerin may act as a tumor suppressor through modulating the small GTPase Rac activity and provide a strong rationale for interfering with Rac signaling in breast cancer treatment.

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Yang C, Liu Y, Leskow FC, Weaver VM, and Kazanietz MG. Rac-GAP-dependent inhibition of breast cancer cell proliferation by {beta}2-chimerin. *J .Biol. Chem*. **280:** 24363-24370. 2005.

Abbreviations

AdV, adenovirus;
EGF, epidermal growth factor;
Erk1/2, extracellular signal-regulated kinase1/2;
GAP, GTPase Activating Protein;
HRG, heregulin β1;
MAPK, mitogen-activated protein kinase;
MOI, multiplicities of infection;

Appendices

1. **Yang C**, Liu Y, Lemmon MA, and Kazanietz MG MG. Essential role for Rac in heregulin beta1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4. *Molecular and Cellular Biology* **26**: 831-842. 2006.

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Essential Role for Rac in Heregulin β1 Mitogenic Signaling: a Mechanism That Involves Epidermal Growth Factor Receptor and Is Independent of ErbB4

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Heregulins are a family of ligands for the ErbB3/ErbB4 receptors that play important roles in breast cancer cell proliferation and tumorigenesis. Limited information is available on the contribution of Rho GTPases to heregulin-mediated signaling. In breast cancer cells, heregulin β1 (HRG) causes a strong activation of Rac; however, it does so with striking differences in kinetics compared to epidermal growth factor, which signals through ErbB1 (epidermal growth factor receptor [EGFR]). Using specific ErbB receptor inhibitors and depletion of receptors by RNA interference (RNAi), we established that, surprisingly, activation of Rac by HRG is mediated not only by ErbB3 and ErbB2 but also by transactivation of EGFR, and it is independent of ErbB4. Similar receptor requirements are observed for HRG-induced actin cytoskeleton reorganization and mitogenic activity via extracellular signal-regulated kinase (ERK). HRG-induced Rac activation was phosphatidylinositol 3-kinase dependent and Src independent. Furthermore, inactivation of Rac by expression of the Rac GTPase-activating protein β2-chimerin inhibited HRG-induced ERK activation, mitogenicity, and migration in breast cancer cells. HRG mitogenic activity was also impaired by depletion of Rac1 using RNAi. Our studies established that Rac is a critical mediator of HRG mitogenic signaling in breast cancer cells and highlight additional levels of complexity for ErbB receptor coupling to downstream effectors that control aberrant proliferation and transformation.

The human ErbB/Her receptor family comprises four tyrosine kinase receptors (Her1/ErbB1 or epidermal growth factor receptor (EGFR), Her2/ErbB2, Her3/ErbB3, and Her4/ErbB4) that play important roles in the progression of various types of cancers, including breast, prostate, and colon cancer. It is well established that dysregulation of ErbB receptor signaling leads to enhanced cell proliferation, migration, and malignant transformation (22). Overexpression of ErbB2 is often associated with breast cancer progression, metastasis, and poor prognosis, and a blocking antibody for ErbB2 is widely used for breast cancer therapy. Overexpression of EGFR or ErbB3 is also correlated with reduced survival of breast cancer patients (35, 51, 54). In contrast, studies show that ErbB4 mediates antiproliferative and differentiation responses in breast cancer cells (42), and its expression is correlated with better survival in breast cancer patients (51). One of the features of ErbB receptors is their diverse coupling to signaling pathways that control mitogenicity as well as the progression and maintenance of the malignant phenotype. This is exemplified by the EGFR, which, upon binding of a specific ligand (such as epidermal growth factor [EGF] or transforming growth factor alpha), becomes activated by homodimerization and autophosphorylation and couples to multiple SH2 domain-containing adaptor molecules and effectors, including PLCγ, phosphatidylinositol 3-kinase (PI3K), Shc, and Grb2 (43). The four ErbB receptors differ in their pattern of phosphorylation sites (55) and thus couple to distinct (but overlapping) sets of downstream effectors. Diversity in ErbB signaling activation is further enhanced by combinatorial heterodimerization of the various receptors (55).

Heregulins (also called neuregulins) are a group of EGF-like ligands for the ErbB3 and ErbB4 receptors (13) and are often expressed in breast cancer tissues (11). Accumulating evidence indicates that heregulins increase breast cancer cell proliferation and promote tumorigenesis, aggressive and invasive phenotypes (3, 13). Moreover, blockade of heregulin expression inhibits tumorigenicity and metastasis of breast cancer cells (49). Heregulins activate PI3K-Akt and Erk mitogen-activated protein kinase (MAPK) in breast cancer cells (14, 34, 50), pathways that are critical in the mitogenic and tumorigenic effects of heregulins. The individual ErbB receptors and effectors responsible for MAPK activation by heregulins are a subject of intense investigation. Heregulins also promote marked changes in cytoskeleton reorganization accompanied by the formation of membrane ruffles, filopodia, and stress fibers, and they confer a motile phenotype (2). Thus, it is predictable that heregulin stimulation leads to the activation of Rho G proteins known to cause such phenotypic changes. Rac, one Rho family member, plays a major role in control of actin cytoskeleton but also controls cyclin expression, cell cycle progression, and malignant transformation (37, 39, 53). Some reports have shown Rac to be overexpressed or hyperactivated in breast cancer tissues (16, 44), and one Rac guanine nucleotide exchange factor (GEF) (Tiam1) is overexpressed in highly invasive breast tumors (1). Moreover, recent studies from our laboratory have demonstrated that inactivation of Rac by the Rac

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GTPase-activating protein (GAP) β2-chimerin inhibits breast cancer cell migration and proliferation, as well as actin cytoskeleton reorganization in response to growth factors (30, 53). The finding that Tiam1 activation by heregulin leads to a motile phenotype further points to Rac as a downstream player in heregulin signaling (1). However, while it is well established that EGF signaling activates Rho, Cdc42, and Rac, there is no direct evidence that heregulins activate Rho GTPases or of what the functional consequences of such activation might be. Given the complexities in ErbB receptor coupling to downstream effectors, one might expect differences in Rac regulation by heregulin relative to well-established paradigms, such as the EGFR- or PDGFR-mediated activation of Rho GTPases.

In this paper we explore the activation of Rac by heregulin β1 (HRG) in breast cancer cell lines. Our objectives in this study were threefold. First, we wanted to determine whether HRG indeed promotes Rac activation. Our results reveal that HRG is a strong activator of Rac and show that the time course of this activation is markedly different from that seen with EGF. Second, we determined which ErbB receptors are involved in Rac activation by HRG. Studies using a wide range of pharmacological and molecular approaches revealed that ErbB2, ErbB3, and EGFR (but not ErbB4) are required for HRG-induced activation of Rac. Last, we established a functional link between HRG-induced activation of Rac and mitogenic signaling.

MATERIALS AND METHODS

Materials. HRG was purchased from Lab Vision (Fremont, CA). EGF was obtained from Sigma (St. Louis, MO). Wortmannin and AG1478 were from LC Laboratories (Woburn, MA). PP2, U0126, and SP600125 were from Calbiochem (San Diego, CA). The EGFR blocking monoclonal antibody C225/cetuximab (Erbitux) was a generous gift from Kathryn M. Ferguson (University of Pennsylvania). Blocking antibodies for ErbB3 and ErbB4 were from Upstate Biotechnology (Lake Placid, NY).

Cell lines and cell culture. Human breast cancer cell lines MCF-7 and T-47D were purchased from ATCC. MCF-7-Tet-on cells were purchased from Clontech (Palo Alto, CA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and G418 (100 μg/ml) at 37°C in a humidified 5%- CO_2 atmosphere. Generation of MCF-7 V12Rac1 cells was described elsewhere (53).

Generation of adenoviruses. The generation of adenoviruses (AdVs) for β2-chimerin and LacZ (β2-chimerin-AdV and LacZ-AdV) was described elsewhere (53). Serum-starved (8 h) MCF-7 cells were infected with AdVs for 16 h. AdVs were then removed by extensive washing. Maximum expression was observed at 24 h and remained stable for at least three additional days. Experiments were performed 24 or 48 h after infection.

RNA interference (RNAi). Small interfering RNA (siRNA) duplexes were purchased from Dharmacon Research, Inc. (Chicago, IL). The target sequences were as follows: AACCCCGAGGGCAAATACAGC (EGFR), AAGGTGCTT GGATCTGGCGCT (ErbB2), AAGAGACAGAGCTAAGGAAGC (ErbB3), and AATCCAGTGGAGGAGAACCCT (ErbB4). The siRNA sequences for Rac1 were as follows: AAGGAGATTGGTGCTGTAAAA (Rac1-RNAi1) and AACCTTTGTACGCTTTGCTCA (Rac1-RNAi2). As a control, duplexes used were either a green fluorescent protein duplex (Dharmacon, Inc.) or a random control siRNA duplex (AACATCGCTGTAGCATCGTCT). siRNA duplexes (100 to 200 nM) were transfected using Oligofectamine (Invitrogen) in serum-free medium, and after 4 h the medium was supplemented with 10% FBS. Twenty hours later, cells were subjected to 48 h of serum starvation followed by various designated treatments.

Western blot. Cells were lysed using Tris-sodium dodecyl sulfate (SDS) as described by Yang et al. (53) and subjected to SDS-polyacrylamide gel electrophoresis (10 to 40 μg of protein/lane). The following antibodies were used: anti-Rac, anti-Cdc42, anti-ErbB3, and anti-ErbB4 (Upstate Biotechnology); anti-RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-hemagglutinin (anti-HA) tag, anti-phospho-tyrosine (p-Tyr-100), anti-EGFR, anti-phospho-EGFR (Tyr992), anti-Her2, anti-phospho-ErbB2 (Tyr1248), anti-phospho-ErbB3 (Tyr1289), anti-Src, anti-phospho-Src (Tyr416), anti-Akt, anti-phospho-Akt (Ser473), anti-Erk1/2, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-c-Jun N-terminal protein kinase (JNK), anti-phospho-JNK (Thr183/Tyr185), and anti-phospho-ATF2 (Thr71) (Cell Signaling Technology, Beverly, MA); anti-β2-chimerin (53); and anti-β-actin (Sigma).

Pull-down assays. After serum starvation (48 h), cells were stimulated with either HRG or EGF for different times. Rac GTP and Cdc42 GTP levels were determined with a pull-down assay using the p21-binding domain (PBD) of p21-activated kinase, as described previously (53), and using either anti-Rac or anti-Cdc42 antibodies for Western blot detection, respectively. RhoA-GTP levels were determined with a pull-down assay using the rhotekin binding domain (40) and an anti-RhoA antibody for Western blot detection.

IP assay. After 48 h of serum starvation, cells were stimulated with HRG (10 ng/ml) for different times. Cells were lysed at 4°C for 10 min in 500 μl of buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1:500) (Sigma). After preclearing with Gammabind G-Sepharose (Pharmacia Biotech) for 2 h at 4°C, the supernatant was used for immunoprecipitation (IP) using 4 μg of anti-ErbB4 polyclonal antibody (2 h, 4°C). Gammabind G-Sepharose (40 μl) was then added (overnight, 4°C). Samples were resolved in an 8% SDS-polyacrylamide gel and analyzed by Western blotting.

Phalloidin staining. After 48 h of serum starvation, cells cultured in cover slides were pretreated with various inhibitors or blocking antibodies for 1 h and then stimulated with either EGF (100 ng/ml, 5 min) or HRG (10 ng/ml, 10 min). Cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% formaldehyde in PBS for 10 min, and then permeabilized using 0.1% Triton X-100 in PBS for 3 min. Cells were stained with phalloidin (Molecule Probes) in PBS containing 1% bovine serum albumin (BSA) (20 min, room temperature) and then counterstained with 4′,6′-diamidino-2-phenylindole (1 μg/ml, 20 min, 4°C). Cells were visualized with a Nikon TE2000-U fluorescence microscope.

Cell migration. HRG-induced cell migration was determined using a Boyden Chamber (Neuro Probe, Inc., Gaithersburg, MD) according to instructions from the manufacturer. Briefly, after 8 h of serum starvation, cells were infected with AdVs at different multiplicities of infections (MOIs), washed, and serum starved for 24 h. Cells were then trypsinized and suspended in serum-free DMEM supplemented with 0.1% BSA. A polycarbonate filter with 12-μm pores (NeuroProbe) coated overnight with type IV collagen in cold PBS was placed on the lower-chamber wells filled with serum-free DMEM with or without HRG (20 ng/ml), and 2×10^5 cells were loaded into the upper-chamber wells. After incubation at 37°C in 5% CO_2 for 5 h, the upper side of the filter was wiped free of cells and the filter was fixed and stained with Wright Giemsa staining buffer (Sigma). Migrating cells were counted under a phase-contrast microscope. For each treatment, at least four randomly selected high-magnification fields (10×20) were counted. Each treatment was performed in quadruplicate. To evaluate the effect of Rac1 depletion on HRG-induced cell migration, siRNA duplexes (100 nM) were transfected into MCF-7 cells as described above. After 48 h of serum starvation, cells were trypsinized and suspended in serum-free DMEM supplemented with 0.1% BSA for the cell migration experiment, as described above. Parallel samples were used to determine Rac1 expression by Western blotting.

BrdU incorporation. 5-Bromo-2′-deoxyuridine (BrdU) incorporation was determined using flow cytometry (53) 24 h after incubation with HRG (10 ng/ml).

Statistical analysis. Data are presented as means ± standard deviations and were analyzed using either a Student *t* test or one-way analysis of variance with Scheffe's test. A P value of <0.05 was considered statistically significant.

RESULTS

Differential temporal activation of Rac by HRG and EGF in breast cancer cells. Two breast cancer cell lines, MCF-7 and T-47D, were used to examine if HRG activates Rac. Figure 1A shows that HRG triggered Rac activation in a dose-dependent manner. At 30 ng/ml, HRG caused 7.5-fold ± 0.6-fold and 8.6-fold ± 1.1-fold increases in Rac GTP levels in MCF-7 and T-47D cells, respectively (Fig. 1B). A time course analysis with MCF-7 cells revealed significant Rac activation at 2 min and maximum stimulation at 10 min. Rac GTP levels remained high (64% of maximum) 60 min after HRG stimulation (Fig. 1C and D). Sustained Rac activation by HRG was also ob-

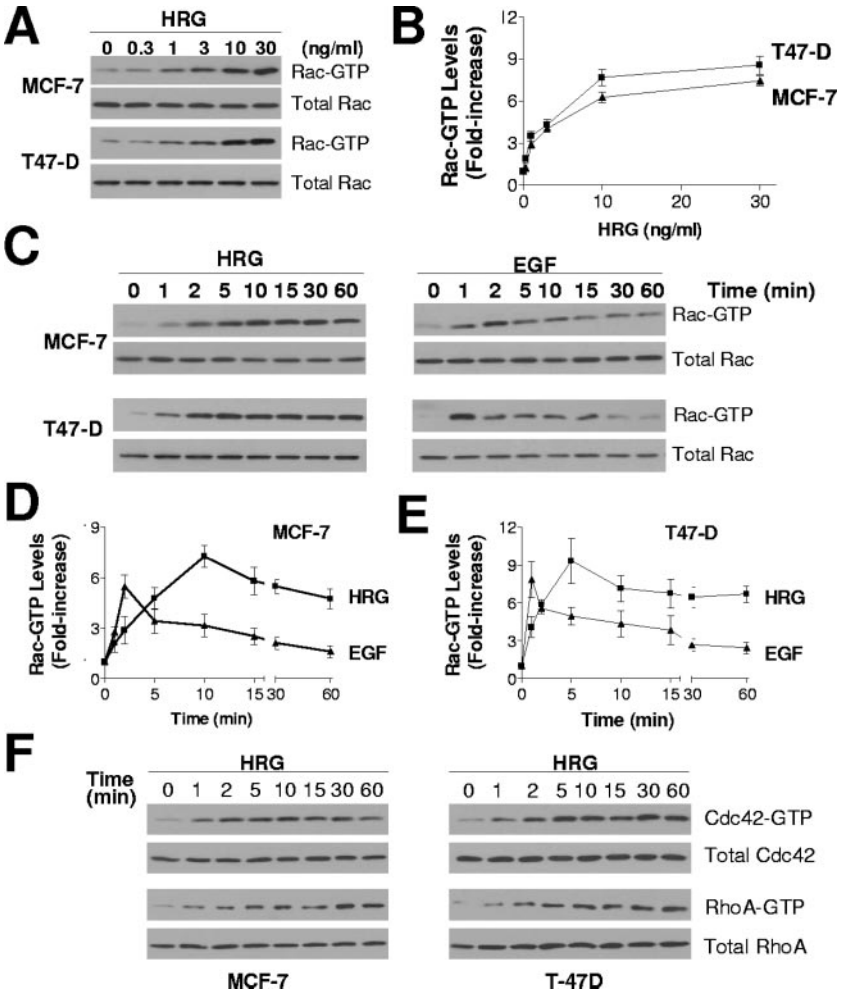

FIG. 1. HRG and EGF induce Rac/Cdc42/RhoA activation in MCF-7 and T-47D cells. (A) Dose-dependent activation of Rac by HRG. MCF-7 and T-47D cells were serum starved for 48 h and then stimulated with HRG (0 to 30 ng/ml) for 10 or 5 min, respectively. Rac GTP levels were determined using a PBD "pull-down" assay. (B) Densitometric analysis of Rac activation, normalized to the corresponding total Rac levels. Data are presented as means ± standard deviations ($n = 3$). (C) Time-dependent activation of Rac by HRG and EGF. Rac activation was determined in serum-starved cells after HRG (10 ng/ml) or EGF (100 ng/ml) treatment. (D and E) Densitometric analysis of time-dependent activation of Rac. Data are presented as means ± standard deviations ($n = 3$). (F) Cdc42 and RhoA activation by HRG (10 ng/ml) in MCF-7 and T-47D cells. Similar results were observed in three independent experiments.

served in T-47D cells. In this case, maximum Rac activation was observed at 5 min, and Rac GTP levels remained elevated (72% of maximum) 60 min after stimulation (Fig. 1C and E). A comparison with EGF revealed striking differences in the kinetics of Rac activation. EGF-induced maximum activation of Rac occurred at earlier times than with HRG (1 min in T-47D cells and 2 min in MCF-7 cells). The effect of EGF was not as persistent as that caused by HRG, with EGF-induced Rac GTP levels dropping by ~50% at 5 min and returning to near-basal levels at 30 to 60 min. The sustained activation of Rac by HRG was long lasting even after extensive washing with serum-free medium (data not shown). Thus, while both EGF and HRG strongly activate Rac in breast cancer cells, the activation by HRG is slightly slower and much more sustained. HRG also caused sustained activation of Cdc42 and RhoA (Fig. 1F).

ErbB3, ErbB2, and EGFR are all required for HRG-induced Rac activation. HRG is a specific ligand for ErbB3 and ErbB4, but it can also transactivate ErbB2 and/or EGFR through receptor heterodimerization (55). To define which ErbB receptors are involved in HRG-induced Rac activation, we used multiple approaches that include pharmacological inhibitors, ErbB receptor blocking antibodies, and RNAi for individual ErbB receptors. As shown in Fig. 2A and B, each ErbB receptor was successfully knocked down by >70% upon delivery of specific siRNA duplexes into T-47D cells. Interestingly, HRG-induced Rac activation was significantly blunted by depletion of ErbB3, ErbB2, or EGFR (Fig. 2A and C). RNAi depletion of either ErbB3 or ErbB2 led to a reduction in ErbB2 phosphorylation (activation), as expected if ErbB2/ErbB3 heterodimers form a major HRG receptor (9). More surprisingly, knock-down of ErbB2 as well as ErbB3 or EGFR reduced HRG-induced EGFR phosphorylation, whereas a reduction in EGFR expression only reduced the activation of EGFR, without obvious effect on ErbB2 phosphorylation. Remarkably, ErbB4 depletion had no effect on HRG-induced activation of

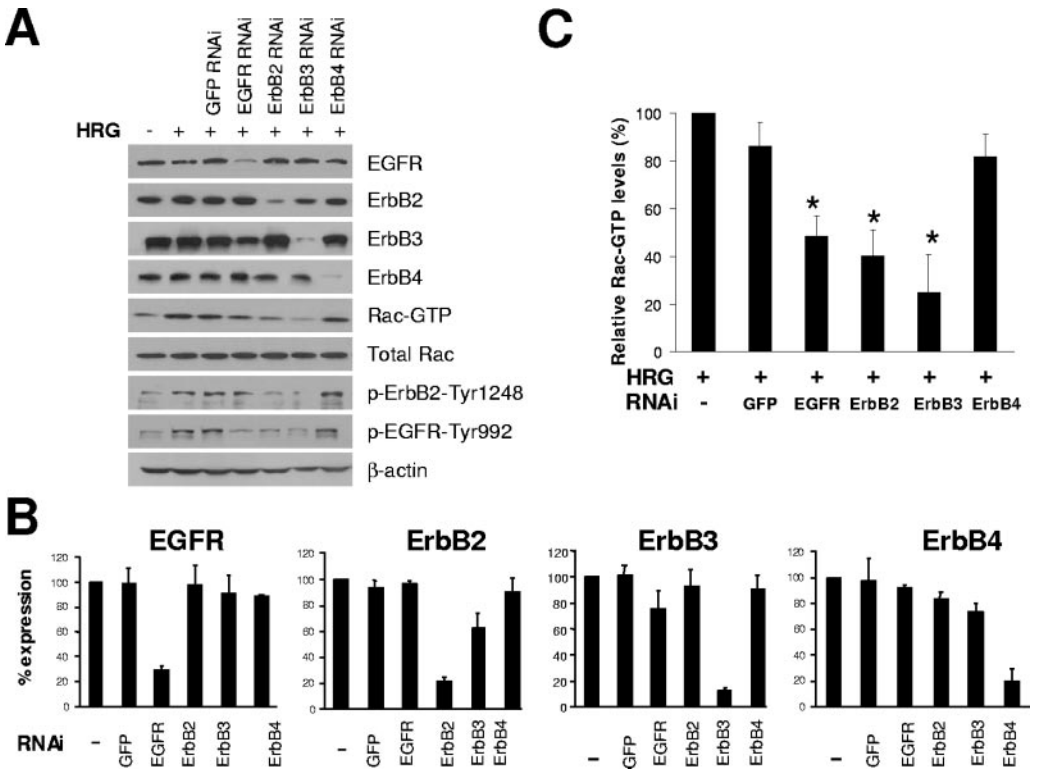

FIG. 2. Effect of siRNA knock-down individual ErbB receptors on Rac activation by HRG. (A) siRNA duplexes for each ErbB receptor were transfected into T-47D cells. Twenty-four hours later, cells were serum starved for 48 h, and Rac activation was determined after stimulation with HRG (10 ng/ml, 5 min). ErbB2 and EGFR phosphorylation after HRG (10 ng/ml, 10 min) were analyzed by Western blotting using specific anti-phospho-ErbB2-Tyr1248 or anti-phospho-EGFR-Tyr992 antibody. Similar results were observed in three independent experiments. (B and C) Densitometric analysis of the effect of individual ErbB receptor knock-down and its effect on HRG-induced Rac activation, respectively, shown as percentages of expression relative to that for control (nontransfected) cells. Data are presented as means ± standard deviations ($n = 3$). *, $P < 0.05$, compared to results with nontransfected HRG-stimulated cells.

Rac, ErbB2, or EGFR (Fig. 2A and C). Moreover, Rac activation by HRG could be blocked by an ErbB3 blocking antibody but not by an ErbB4 blocking antibody (data not shown). These results argue that ErbB2, ErbB3, and EGFR are all involved in HRG-induced Rac activation, whereas ErbB4 is not.

To further explore the EGFR requirement for HRG-induced Rac activation, we used the specific EGFR inhibitor AG1478. As shown in Fig. 3A, AG1478 impaired HRG-triggered Rac activation in a dose-dependent manner. Another EGFR tyrosine kinase inhibitor, Iressa (ZD1839), also completely blocked HRG-induced Rac activation (data not shown). Thus, inhibition of EGFR kinase activity blocks the ability of HRG to activate Rac. There are several possible explanations for this finding, which will be considered further in the Discussion.

Immunoblotting with antibodies against phosphorylated forms of the ErbB receptors showed that HRG treatment induces phosphorylation of all family members (Fig. 3C), so understanding its signaling consequences requires consideration of the entire ErbB network. Importantly, the kinetics of HRG-induced receptor phosphorylation matched that of Rac activation only for ErbB2, ErbB3, and EGFR. Despite not playing any clear role in HRG-induced Rac activation, ErbB4 became significantly autophosphorylated upon HRG treatment but with a time course that was significantly less sustained than that of any other ErbB receptor (Fig. 3C). Interestingly, the kinetics of EGFR phosphorylation following HRG treatment was strikingly different from that seen following EGF stimulation. Whereas EGF promotes rapid and transient EGFR phosphorylation, HRG treatment of cells leads to slower and more sustained phosphorylation of the EGFR (Fig. 3C). To exclude the possibility that EGF agonists are responsible for (indirect) EGFR activation by HRG (e.g., through an autocrine loop), we showed that cetuximab, an anti-EGFR antibody that blocks the ligand binding site on the receptor (29), could not prevent HRG-induced EGFR and Rac activation, even at concentrations that completely block EGFR and Rac activation by saturating levels of EGF (Fig. 3B).

HRG-induced Rac activation is Src independent and PI3K dependent. To understand the mechanism of Rac activation by HRG in breast cancer cells, we next assessed the roles of Src and PI3K, which are well-established effectors of the ErbB receptor network. Figure 4A shows that HRG triggers robust Src activation in T-47D cells, as determined using a specific anti-phospho-Src antibody. However, we find that this effect on Src is not required for Rac activation. Indeed, whereas HRG-induced Rac activation peaks at 5 min after HRG treatment (Fig. 1), the effect of HRG on Src activity is minimal at this time, peaking instead at 10 min after HRG addition. More-

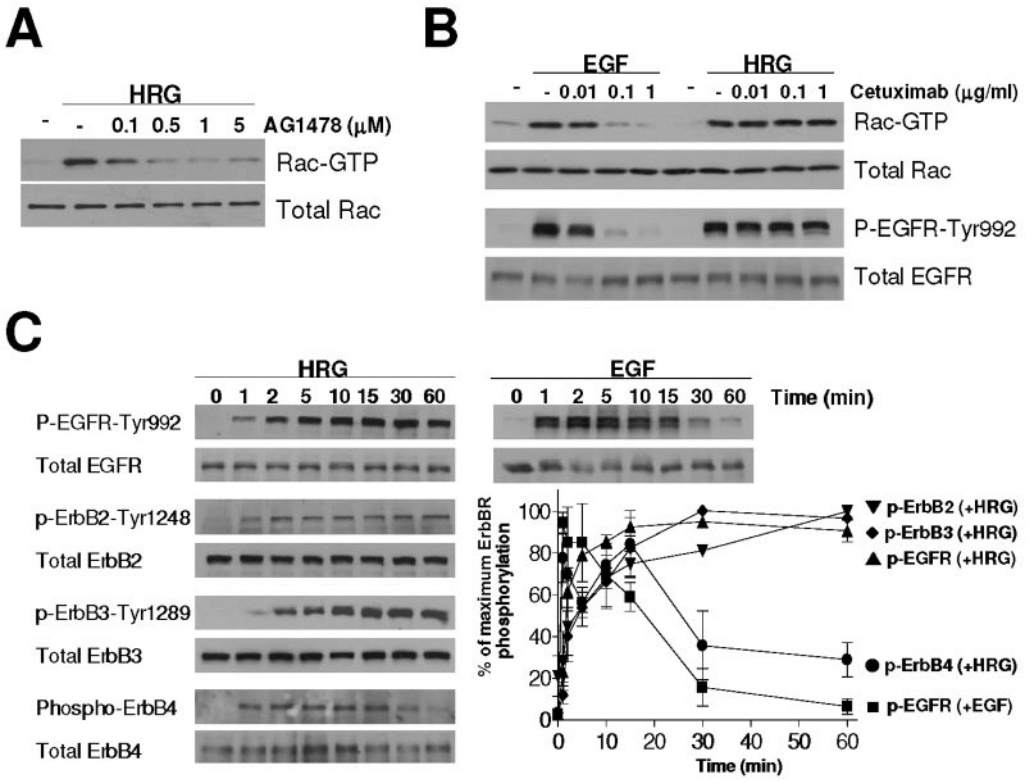

FIG. 3. Requirement of EGFR for HRG-induced Rac activation. (A) T-47D cells were serum starved for 48 h, incubated with different concentrations of AG1478 for 1 h, and then stimulated with HRG (10 ng/ml) for 5 min. Rac GTP levels were then assayed. (B) T-47D cells were serum starved for 48 h, pretreated with different concentrations of cetuximab for 1 h, and then stimulated with EGF (100 ng/ml for 1 min for the Rac GTP assay and 2 min for EGFR phosphorylation analysis) or HRG (10 ng/ml, 5 min for the Rac GTP assay and 10 min for EGFR phosphorylation analysis). Similar results were observed in three independent experiments. Panel C. After 48 h of serum starvation, T47D cells were treated with HRG (10 ng/ml) or EGF (100 ng/ml). Cell extracts were subjected to Western blot analysis using the indicated antibodies. ErbB4 phosphorylation was detected by IP. Inset. Densitometric analysis of ErbB receptor phosphorylation, expressed as a percentage of the maximum response in each case. Data are presented as means ± standard deviations ($n = 3$).

over, the Src inhibitor PP2 had no effect on HRG-induced Rac activation even at concentrations that completely block Src phosphorylation (Fig. 4B). On the other hand, the PI3K inhibitor wortmannin completely blocked the HRG-induced elevation in Rac GTP levels (Fig. 4C). In T-47D cells, HRG strongly activates Akt (a PI3K effector), an effect that persisted for at least 60 min (Fig. 4D). EGF strongly activates Akt, although the effect lasted for less than 60 min (data not shown). As shown in Fig. 4E, wortmannin abolished the activation of Akt by HRG. Interestingly, the EGFR tyrosine kinase inhibitor AG1478 also impaired Akt activation by HRG. Neither AG1478 nor wortmannin significantly affected phosphorylation of ErbB2; and wortmannin did not inhibit EGFR activation (Fig. 4E). The inhibitors of EGFR and PI3K also prevented HRG-triggered Akt and Rac activation in MCF-7 cells (data not shown). Together, these results indicate that HRG activation of Rac in breast cancer cells is dependent on PI3K and also requires EGFR kinase activity but is independent of Src.

Rac is required for HRG-induced activation of Erk1/2 and JNK. HRG activates MAPK cascades (14, 34), which are known effectors of Rac (12). As shown in Fig. 5A, HRG caused a marked activation of Erk1/2 and JNK, which was detectable within 2 to 5 min and remained sustained for at least 60 min in both MCF-7 and T-47D cells. On the other hand, EGF-induced activation of Erk1/2 and JNK was transient, peaking at 2 to 10 min and returning to basal levels within 30 to 60 min. The characteristic kinetics of Erk1/2 and JNK activation by each ligand strongly resembled the time course of Rac activation for that ligand shown in Fig. 1.

Activation of Erk1/2 and JNK by HRG was completely blocked by the EGFR kinase inhibitors AG1478 (Fig. 5B) and Iressa (data not shown), suggesting that the HRG-induced EGFR activation shown in Fig. 2A and 3B is required for this response. On the other hand, cetuximab did not affect HRG-induced activation of ERK, JNK, and Akt, although it completely impaired their activation by EGF (Fig. 5C). Wortmannin blocked Erk1/2 and JNK activation by HRG very efficiently (Fig. 5B), indicating that HRG-induced MAPK activation is also PI3K dependent. Similar results were observed with MCF-7 cells (data not shown).

To further investigate the role of Rac in HRG-induced MAPK activation, we employed β2-chimerin, a Rac-specific GAP that inactivates Rac both in cell-free systems and in cells, including breast cancer cells (7, 53). HA-tagged β2-chimerin was delivered into MCF-7 cells using an adenoviral approach. As shown in Fig. 6A (lanes 1 to 6), expression of β2-chimerin in MCF-7 cells inhibited Rac activation by HRG. The effect was proportional to the expression level of β2-chimerin

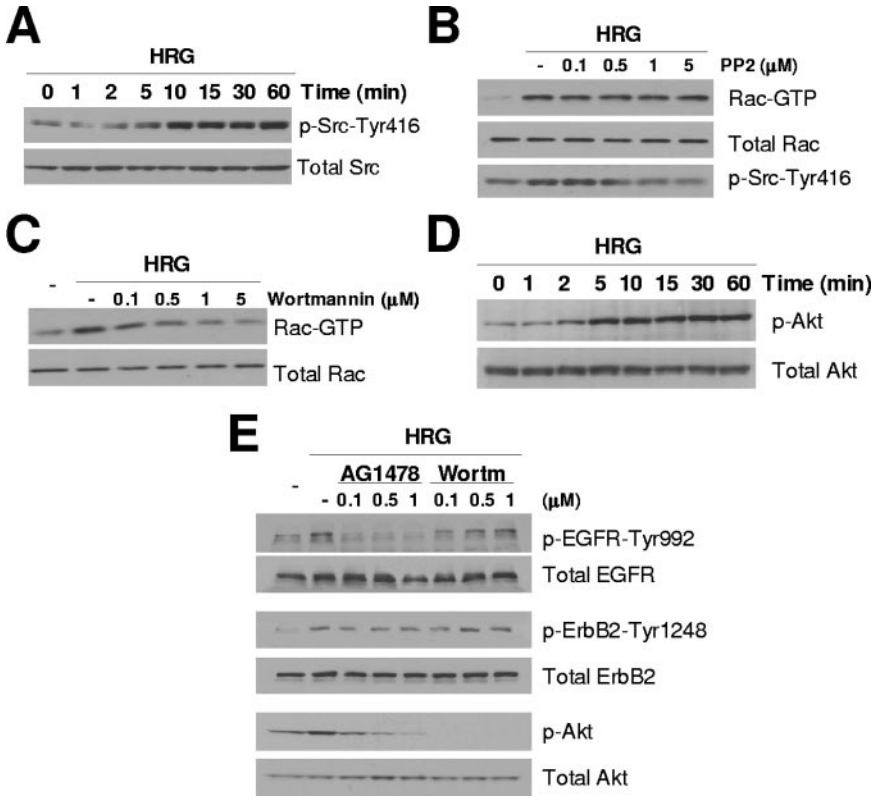

FIG. 4. HRG-induced Rac activation is Src independent and PI3K dependent. (A and D) Time course of Src and Akt activation by HRG. T-47D cells were serum starved for 48 h and then treated with HRG (10 ng/ml). Cell extracts were subjected to Western blot analysis using specific anti-phospho-Src and anti-phospho-Akt antibodies. (B and C) After 48 h of serum starvation, T-47D cells were treated either with PP2 (0 to 5 μM) or wortmannin (0 to 5 μM) for 1 h and stimulated with HRG (10 ng/ml) for 5 min, and Rac GTP levels were then determined. (E) Effect of AG1478 and wortmannin (Wortm) pretreatment on the activation of EGFR, ErbB2, and Akt. After 48 h of serum starvation, T-47D cells were treated with either AG1478 or wortmannin for 1 h and stimulated with HRG (10 ng/ml) for 10 min. Cell extracts were subjected to Western blotting. Similar results were observed in three independent experiments.

achieved by varying the MOIs of the β2-chimerin-AdV. A LacZ-AdV, on the other hand, was ineffective. Overexpressed β2-chimerin did not affect the levels of phospho-Akt induced by HRG treatment, arguing that PI3K/Akt activation by HRG occurs upstream of Rac. On the other hand, β2-chimerin significantly impaired HRG-induced activation of JNK and Erk1/2, arguing that these events are downstream of Rac activation. To confirm that the effects of β2-chimerin are Rac specific, we also assessed activation of Cdc42 and RhoA by HRG and found that these were unchanged even at high levels of β2-chimerin expression (Fig. 6B).

To further assess the Rac-MAPK link in our experimental model, we used MCF-7 cells stably expressing a constitutively active Rac1 mutant (HA-tagged V12Rac1). We hypothesized that cells retaining high levels of this active Rac mutant should be insensitive to the effect of the Rac GAP. Although we could readily detect V12Rac1 expression using an anti-HA antibody, total Rac levels remained basically unchanged, arguing that V12Rac1 was expressed at low levels compared with the endogenous wild-type protein, although it comprised the majority of the Rac GTP. As expected, HA-tagged Rac GTP levels remained high even after infection with the β2-chimerin-AdV (note that endogenous Rac GTP levels were reduced by β2-chimerin). Remarkably, expression of HA-tagged V12Rac1 largely overcame the inhibitory effect of β2-chimerin on HRG-induced Erk1/2 and JNK activation (Fig. 6A, lanes 7 to 12). Thus, Rac appears to be required for HRG-induced activation of Erk1/2 and JNK. An additional important observation is that Rac activation does not appear to be sufficient for Erk1/2 or JNK signaling, since levels of phospho-Erk or phospho-JNK were not elevated in cells expressing V12Rac1 in the absence of HRG treatment. Thus, our studies suggest that in breast cancer cells, Rac activation is necessary but not sufficient for the activation of these signaling pathways.

Rac dependence of HRG-induced cell migration and proliferation. HRG is known to cause morphological changes, including the formation of filopodia and membrane ruffles (2). When T47-D cells were treated with either HRG or EGF, characteristic ruffles were observed (Fig. 7A), which is consistent with the activation of Rac. Inhibition of EGFR with AG1478 impaired not only ruffle formation caused by EGF but also the effect of HRG. On the other hand, cetuximab inhibited only the morphological changes caused by EGF, without affecting the HRG effect. Consistent with our signaling studies, the effect of HRG was also blocked by the anti-ErbB3 blocking antibody but was not affected by the anti-ErbB4 blocking antibody or PP2. Wortmannin completely inhibited ruffle formation caused by EGF and HRG. Thus, these results parallel

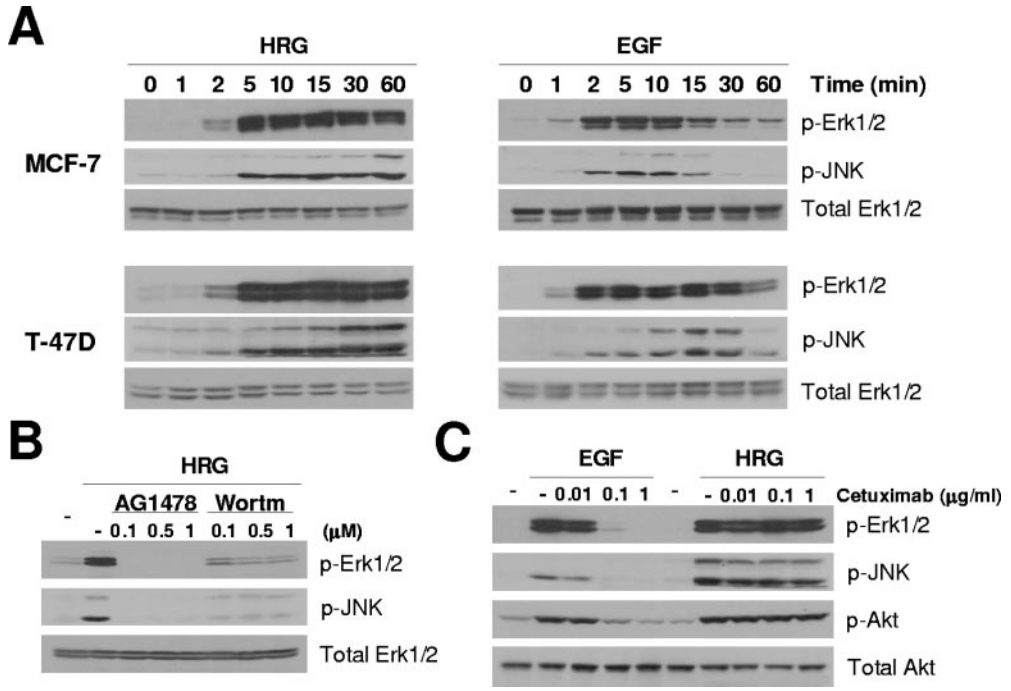

FIG. 5. Differential kinetics of Erk1/2 and JNK activation by HRG and EGF. (A) Cells were serum starved for 48 h and then treated with either HRG (10 ng/ml) or EGF (100 ng/ml). Cell extracts were subjected to Western blot analysis using the indicated antibodies. (B) Effect of AG1478 and wortmannin (Wortm) on HRG-induced Erk1/2 and JNK activation in T-47D cells. Cells were treated as described in the legend to Fig. 4E, and cell extracts were subjected to Western blot analysis. (C) T-47D cells were serum starved for 48 h, pretreated with different concentrations of cetuximab for 1 h, and then stimulated with EGF (100 ng/ml, 2 min) or HRG (10 ng/ml, 10 min). Cell extracts were subjected to Western blot analysis. Similar results were observed in three independent experiments.

those in which Rac GTP levels were determined. To further examine the biological significance of HRG-induced Rac activation, we also examined cell migration and proliferation. Consistent with its potent and sustained effect on Rac activation, HRG significantly enhanced MCF-7 cell migration, as determined with a Boyden chamber (Fig. 7B). Expression of the Rac GAP β2-chimerin significantly inhibited this effect. This inhibition was proportional to the expression levels of β2-chimerin. As expected, V12Rac1 significantly enhanced migration of MCF-7 cells. The expression of constitutively active Rac sig-

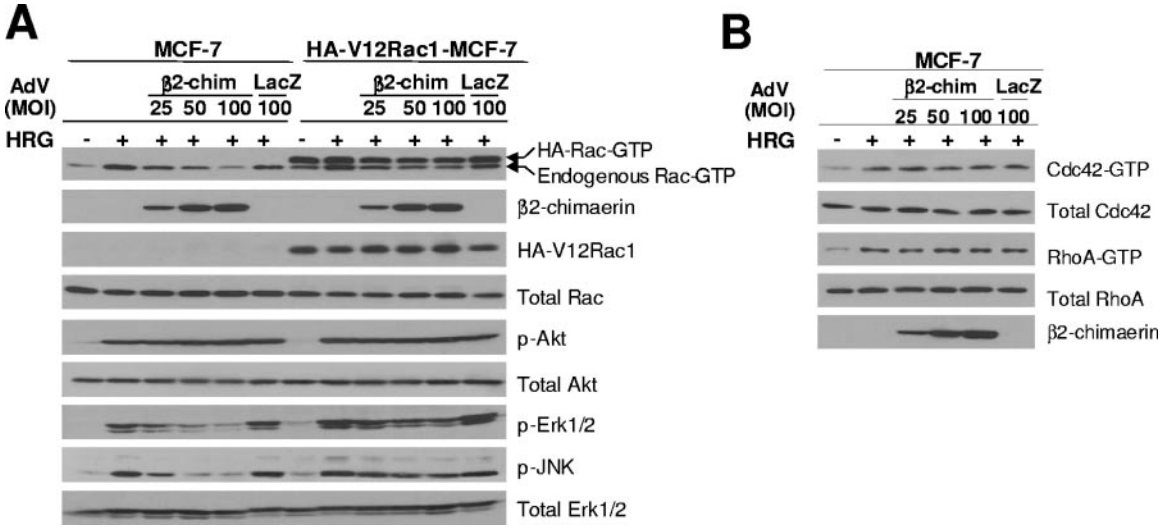

FIG. 6. Inhibition of β2-chimerin on HRG-induced Rac, Erk1/2, and JNK activation. (A) MCF-7 and HA-V12Rac1-MCF-7 cells were serum starved for 8 h and then infected with either HA-β2-chimerin-AdV (β2-chim) or LacZ-AdV (LacZ) for 16 h in serum-free DMEM. After extensive washing, cells were grown for 24 h in serum-free DMEM and then stimulated with HRG (10 ng/ml) for 10 min. Activation of Rac, Akt, Erk1/2, and JNK was then determined. Expression of HA-β2-chimerin and HA-V12Rac1 was examined by Western blotting using an anti-HA antibody. (B) MCF-7 cells were treated as described for panel A. Cdc42-GTP and RhoA-GTP levels were determined using pull-down assays.

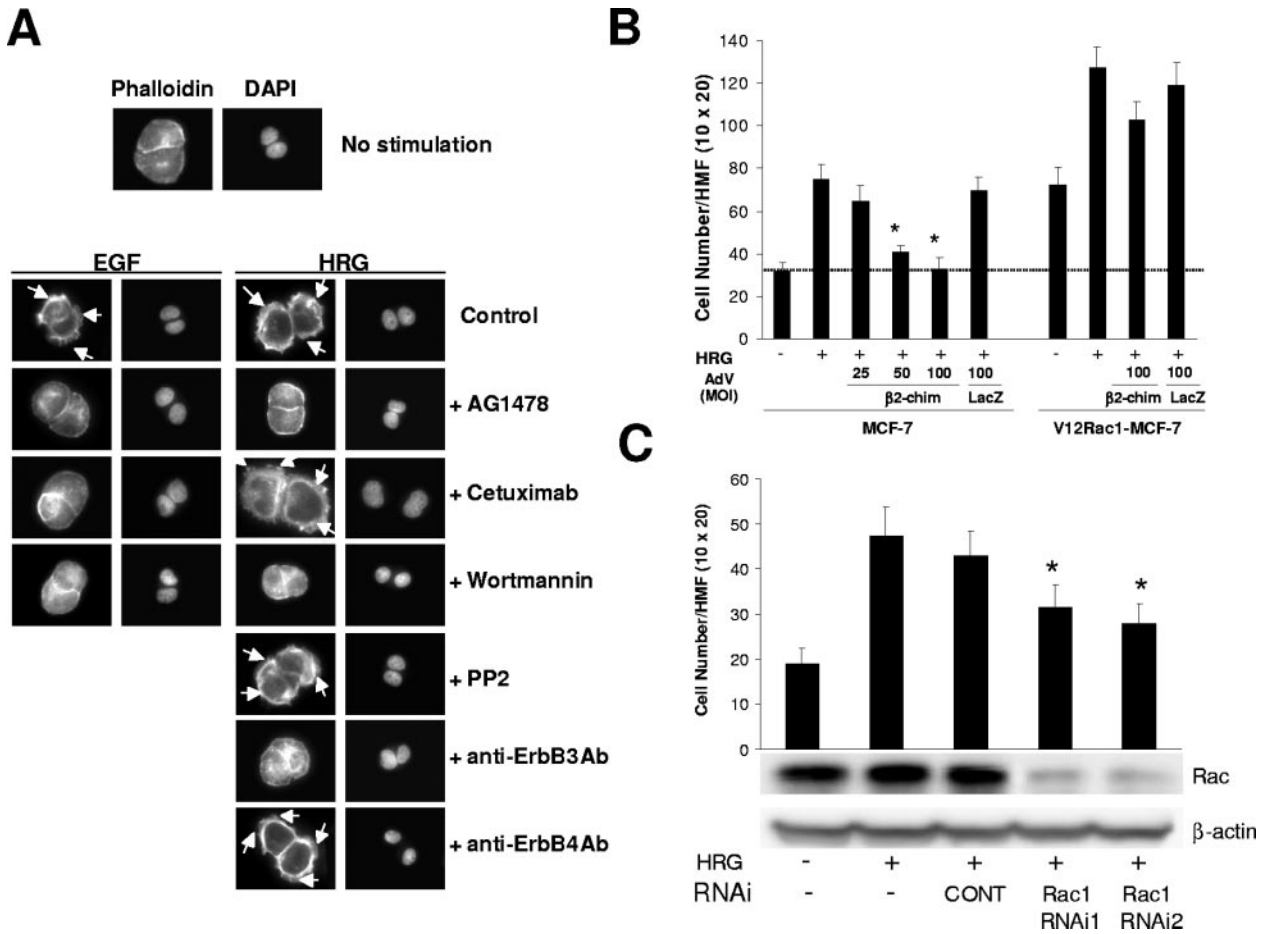

FIG. 7. β2-Chimerin inhibits HRG-induced MCF-7 cell migration. (A) Effects of inhibitors and blocking antibodies on EGF/HRG-induced ruffle formation. After 48 h of serum starvation, T-47D cells were treated with AG1478 (1 μM), Cetuximab (1 μg/ml), wortmannin (1 μM), PP2 (1 μM), ErbB3, or ErbB4 blocking antibody (10 μg/ml) for 1 h, stimulated with EGF (100 ng/ml, 5 min) or HRG (10 ng/ml, 10 min), and then stained with phalloidin. Ruffles are indicated by arrows. Similar results were observed in three independent experiments. (B) Effect of β2-chimerin on HRG-induced cell migration. Migration of MCF-7 or HA-V12Rac1-MCF-7 cells infected with HA-β2-chimerin-AdV or LacZ-AdV (see Fig. 6) was determined using a Boyden chamber. Data are presented as means ± standard deviations ($n = 4$). *, $P < 0.05$, compared to results for non-AdV-infected HRG-stimulated cells. (C) Effect of Rac1 depletion on HRG-induced cell migration. MCF-7 cells were transfected with siRNA duplexes for Rac1 (RNAi1 or RNAi2) or a control duplex (CONT), and cell migration was determined 72 h after transfection. Data are presented as means ± standard deviations ($n = 4$). *, $P < 0.05$, compared to results for nontransfected, HRG-stimulated cells. Rac expression is shown in a representative Western blot. HMF, high-magnification field.

nificantly overcame the inhibitory effect of β2-chimerin. Furthermore, HRG-induced cell migration was impaired by depletion of Rac1 using two different Rac1 siRNA duplexes (Fig. 7C).

Rac is involved in proliferation control through the regulation of G_1/S progression (37). We found that it plays an essential role in HRG stimulation of MCF-7 cell proliferation, as determined by BrdU incorporation (Fig. 8A). Expression of β2-chimerin dose-dependently inhibited the ability of HRG to stimulate proliferation. V12Rac1-expressing cells showed slightly higher levels of BrdU incorporation than control cells, and this was greatly further enhanced by treatment with HRG, in agreement with the finding in Fig. 6A that Rac activation is necessary but not sufficient for proliferative signaling. With V12Rac1 overexpression, β2-chimerin had no detectable inhibitory effect (Fig. 8A). The requirement for Rac in HRG-stimulated proliferation was further demonstrated using RNAi, with HRG-induced cell proliferation being significantly inhibited when Rac1 levels were depleted (Fig. 8B and C).

In order to determine the contribution of MAPKs in HRG-induced mitogenesis, we used a MEK1 inhibitor (U0126) and a JNK inhibitor (SP600125). Figure 8D shows that U0126 inhibited HRG-induced cell proliferation in T-47D cells by 72%, while SP600125 had little effect. The activity and selectivity of the inhibitors for each pathway was confirmed by their ability to inhibit Erk1/2 phosphorylation (U0126) or ATF2 phosphorylation (SP600125) in response to HRG (Fig. 8E).

Last, we examined the effect of ErbB receptor siRNA on the proliferative response of HRG in T-47D cells. This analysis revealed that the HRG effect was dependent on ErbB3, ErbB2, and EGFR (Fig. 8F), which are the receptors required for Rac activation (Fig. 2). Taken together, these results suggest a critical role for multiple ErbB receptors and Rac activation in HRG-stimulated cell proliferation.

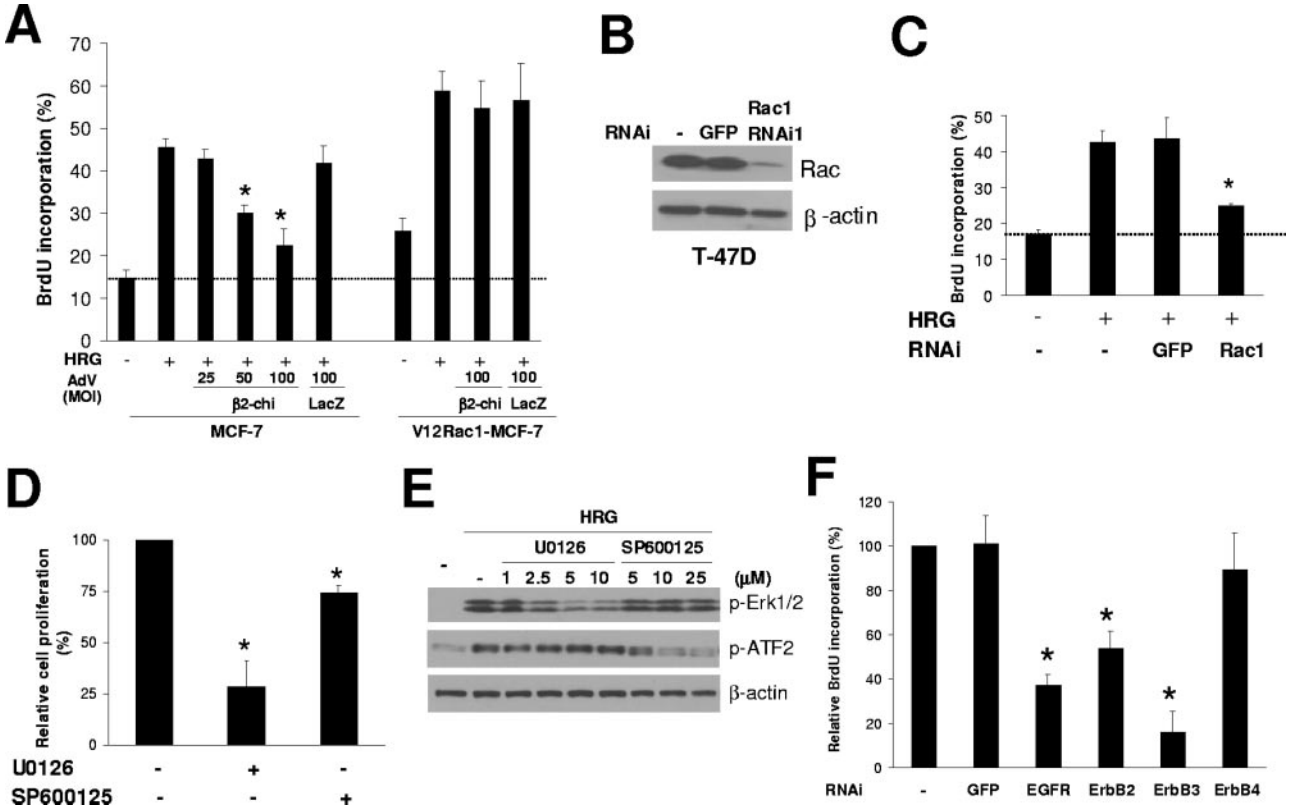

FIG. 8. Effect of β2-chimerin, MAPK inhibitors, and siRNA knock-down of Rac1 and individual ErbB receptors on MCF-7 and T-47D cell proliferation. (A) HA-β2-chimerin-AdV- or LacZ-AdV-infected MCF-7 or HA-V12Rac1-MCF-7 cells were stimulated with HRG (10 ng/ml) for 24 h, and then BrdU incorporation was determined. Data are presented as means ± standard deviations ($n = 3$). *, $P < 0.05$, compared to results for non-AdV-infected HRG-stimulated cells. (B, C, and F) siRNA duplexes for Rac1 or each ErbB receptor were transfected into T-47D cells. Twenty-four hours later, cells were serum starved for 48 h and stimulated with HRG (10 ng/ml). BrdU incorporation was determined 24 h later. Data are presented as means ± standard deviations ($n = 3$). *, $P < 0.05$, compared to results for nontransfected HRG-stimulated cells. Panel B shows a representative Western blot for Rac1 knock-down. (D) After 48 h of serum starvation, T-47D cells were incubated with U0126 (5 μM) or SP600125 (25 μM) for 1 h and then stimulated with HRG (10 ng/ml) in the presence of the inhibitors. BrdU incorporation was determined 48 h later. (E) Phospho-Erk and phospho-ATF2 were analyzed by Western blotting after treatment with HRG (10 ng/ml, 10 min) in the presence of UO126 and SP600125.

DISCUSSION

Our results reveal that HRG causes a strong and sustained activation of Rac and provide strong evidence that Rac is a critical component of the mitogenic and motile responses induced by HRG in breast cancer cells. While substantial evidence exists in support of a role for ErbB3 and ErbB2 as mediators of the mitogenic and oncogenic activities of heregulins, our results introduce a more complex paradigm, since HRG-mediated proliferation via Rac also depends on the EGFR (ErbB1) receptor. Remarkably, the ErbB4 receptor is not involved in Rac activation and mitogenicity, despite becoming significantly activated by HRG.

Multiple ErbB receptors mediate HRG-induced activation of Rac. It is generally believed that heterodimers formed by ErbB2 and ErbB3 constitute the primary receptor for HRG and elicit potent mitogenic and oncogenic signals (9). These two members of the ErbB receptor family are unique. ErbB2 has no known direct ligand, and ErbB3 appears to have a catalytically inactive or substantially impaired tyrosine kinase domain. Thus, ErbB2 and ErbB3 are thought to require heterodimerization for signaling activity. Our results using RNAi and blocking antibodies clearly establish a requirement for both ErbB2 and ErbB3 in HRG-induced Rac activation, as anticipated. On the other hand, ErbB4 was found to be dispensable for HRG-induced Rac activation and proliferation, despite being efficiently activated by HRG (as evidenced by its autophosphorylation). This finding is consistent with reports that ErbB4 has only a weak capacity to mediate proliferative signals or promotes antiproliferative responses to HRG (8, 42).

More surprisingly, our studies suggest an essential (rather than accessory) role for EGFR in HRG signaling in breast cancer cells. EGFR RNAi or inhibition of its tyrosine kinase activity with AG1478 (or Iressa) blocked the ability of HRG to activate Rac, Akt, Erk1/2, and JNK or to promote cell proliferation. The fact that HRG promotes robust tyrosine phosphorylation of EGFR (Fig. 3C) argues that this is a direct effect and that EGFR activation plays a key role in mediating the HRG response. Several other studies have demonstrated EGFR activation by HRG in cells expressing multiple ErbB receptors (10, 41, 56), although there are reports in which this was not seen, including some studies with T-47D cells (18). The mechanism of EGFR involvement in HRG signaling in our

studies is of interest. HRG treatment leads to phosphorylation of ErbB2, ErbB3, and EGFR, and each of these receptors plays an important role in Rac activation and cell proliferation. HRG is thought to stabilize (and activate) ErbB2/ErbB3 heterodimers. We initially hypothesized that EGFR activation could result from its heterodimerization with HRG-bound ErbB3. However, cetuximab had no effect at all on HRG-induced EGFR phosphorylation (Fig. 3B), despite the fact that this antibody prevents EGFR from adopting its dimerization (homo- or hetero-)-competent configuration (29). The failure of cetuximab to inhibit HRG-induced EGFR activation also rules out autocrine mechanisms, since this antibody directly occludes the ligand binding site in EGFR. Our results therefore suggest a mechanism for "transactivation" of EGFR in this system that differs from the view of ErbB receptor heterodimerization suggested by recent structural studies (5). It has alternatively been suggested that blockade of ErbB2/ErbB3 heterodimer signaling by EGFR kinase inhibitors can result from sequestration of ErbB2 in inactive EGFR/ErbB2 heterodimers and a consequent dominant-negative effect (33). We suggest that this mechanism is not relevant in our studies. The robust HRG-induced phosphorylation of EGFR (dependent on the presence of ErbB2 and ErbB3) argues for a positive role for EGFR in HRG signaling, as does the fact that RNAi knock-down of EGFR significantly impaired Rac activation and cell proliferation in response to HRG.

One possible mechanism for EGFR activation by HRG is that HRG-activated ErbB2 or ErbB3 forms "secondary" heterodimers with EGFR, as suggested by Gamett et al. (17), and these differ in structure from EGFR dimers observed crystallographically (so are unaffected by cetuximab). Alternatively, HRG could promote the formation of heterotetramers that include EGFR, ErbB2, and ErbB3, leading to phosphorylation of all three receptors through mechanisms that have yet to be defined (21, 43). A third possibility that is consistent with the failure of cetuximab to have an inhibitory effect is that EGFR becomes activated simply as a substrate for phosphorylation by activated ErbB2 in a manner similar to the JAK2-mediated EGFR phosphorylation promoted by growth hormone (52).

Differential kinetics of Rac activation by HRG and EGF. A particularly striking observation, illustrated in Fig. 1 and 3C, is that HRG stimulates the activation of EGFR and Rac with a different time course from that seen with EGF-induced activation. The sustained Rac activation seen with HRG tracks precisely with the time course of EGFR phosphorylation. By contrast, EGF causes a rapid and short-lived activation of Rac, an effect that we have also observed in other models, including COS-1, HeLa, and colon cancer cells (unpublished studies). The sustained Rac activation by HRG in breast cancer cells predicted a prominent role for this small GTPase in heregulin signaling. Indeed, interfering with Rac signaling, either by Rac RNAi or by the expression of the specific Rac GAP β2-chimerin, significantly impaired HRG-induced activation of Rac as well as Rac-dependent responses. β2-chimerin accelerates GTP hydrolysis from Rac, leading to its inactivation, and markedly impairs Rac-dependent signaling, motility, and proliferation in various cellular models including breast cancer cells (6, 30, 53).

The contrasting time courses of Rac activation in response to HRG and EGF stimulation could reflect the distinct internalization characteristics of different ErbB receptor dimers. EGFR that has been activated by EGF-induced homodimerization is rapidly internalized and targeted to the lysosome, and this explains the transient activation seen in Fig. 3B. By contrast, EGFR that has been activated through heterodimerization with other ErbB receptors (or possibly by other mechanisms) is internalized less efficiently (4) and/or is more readily recycled (28). These effects may be responsible for the different time courses for Rac activation seen in Fig. 1.

Rac as a mediator of HRG mitogenic signaling. The requirement of Rac and other Rho GTPases for mitogenic signaling has been defined in earlier studies (26, 37). Rac plays a crucial role in G_1/S transition through the control of cyclin D1 expression (24, 38). Growth factor-induced activation of cyclin D1 expression in breast cancer cells is dependent on Rac and is highly sensitive to the effect of the Rac GAP β2-chimerin (53). Here we observed that inhibition of Rac by β2-chimerin or Rac depletion using RNAi markedly inhibits BrdU incorporation in response to HRG, thus placing Rac as an essential mediator of HRG mitogenic signaling.

The inhibition of HRG-induced BrdU incorporation by the MEK-1 inhibitor UO126 supports the involvement of ERK activation in the HRG proliferative response, which is in agreement with previous studies (34, 50). While multiple ErbB receptors have been found to activate ERKs through the Ras-Raf pathway (31, 45), a distinctive aspect of our studies is that they underscore the absolute requirement of the EGFR in ERK activation in response to HRG. ERK activation by HRG was completely blocked by treatment with AG1478. While the mechanistic basis by which Rac modulates the activation of MAPK cascades is beyond the goals of these studies, our results using V12Rac1 (Fig. 6A) argue that in breast cancer cells Rac is required but is not sufficient for ERK (and JNK) activation. The fact that HRG treatment is needed even in V12Rac1-expressing cells to cause ERK activation and to promote the maximum proliferative response instead suggests a cooperative role for Rac signaling with inputs from the Ras cascade. Indeed, recent studies with smooth muscle cells show that the association of the Rac effector p21-activated kinase with ERK and Raf facilitates ERK signal transduction (47). Further studies would be required to understand the nature of those events in the context of HRG stimulation in breast cancer cells.

Recent studies have shown that Src is a mediator of Rac activation in response to various stimuli (25, 46). Consistent with other reports, we also observed Src activation by HRG, although it is delayed by comparison with Rac activation, and inhibition of Src by PP2 did not affect HRG-induced Rac activation. Thus, in this particular cellular context, Src is not involved in Rac activation by HRG. Instead, we found that Rac activation by HRG is PI3K dependent. ErbB receptor coupling to PI3K probably involves multiple mechanisms: while ErbB3 can recruit the p85 regulatory subunit of PI3K directly via an SH2-dependent mechanism (20, 36), PI3K activation by EGFR is primarily mediated by the docking protein Gab1 (32). A role for Gab proteins in ErbB3-mediated activation of PI3K has also been described recently (23). PI3K can activate Tiam1 and Vav Rac GEFs (15, 19), and activated Ras and Tiam1 can cooperate to activate Rac in a PI3K-independent manner (27). To our knowledge the activation of Rac GEFs by ErbB2 and

ErbB3 has not been reported. On the other hand, Vav exchange activity is stimulated in response to EGF through a PI3K-dependent mechanism (48), which would fit well with our paradigm of HRG-induced activation of Rac signaling via EGFR. Interestingly, Tiam1 becomes activated in response to HRG, leading to a motile phenotype (1). We determined that HRG not only caused Rac activation but also activated other Rho GTPases, such as Cdc42 and RhoA. Moreover, the PI3K inhibitor wortmannin also dose-dependently and efficiently inhibited Cdc42 activation by HRG (data not shown), suggesting that HRG is capable of activating multiple GEFs and/or GEFs with specificity for multiple Rho GTPases.

Final remarks. The results presented here establish that HRG is a strong activator of Rac in breast cancer cells and that there is an absolute requirement for Rac in HRG-induced breast cancer cell proliferation. With the exception of ErbB4, all of the ErbB family members are required for HRG-induced Rac activation. Indeed, the requirement of EGFR for HRG-induced Rac activation and Rac-mediated responses, including mitogenesis, argues for an additional level of receptor cross talk in HRG signaling. Given the implications of ErbB receptors in cancer progression, the identification of Rac as a key transducer of HRG mitogenic and motogenic signaling highlights a role for this Rho GTPase in breast tumorigenesis. Since inactivation of ErbB receptors represents a promising strategy for cancer treatment, including treatment for breast cancer, our results may have great implications for understanding the mechanistic basis of the action of targeted ErbB receptor therapy and for considering additional signaling pathways to target in combination.

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Rac-GAP-dependent Inhibition of Breast Cancer Cell Proliferation by β2-Chimerin*

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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 G_1 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 G_1/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.

Chimerins represent a family of four closely related GAPs[1] (GTPase-activating proteins) for small GTPases that were originally characterized as high affinity intracellular receptors for the second messenger diacylglycerol (DAG) and the phorbol ester tumor promoters (1–4). Structurally, chimerins possess a C1 domain highly homologous to those of PKC isozymes (the DAG/phorbol ester binding site) and a C-terminal GAP domain. The α2- and β2-chimerins also have a N-terminal Src homology 2 domain of unknown function, which is not present in the splice variants α1-(or *n*-) and β1-chimerins (5, 6). Very little information is available regarding the regulation, expression, and function of β2-chimerin or the other chimerin isoforms as well as their role in proliferation mechanisms and cancer progression. We have been focusing our attention on β2-chimerin, because there is emerging evidence that this isoform is directly regulated by phorbol esters (4, 7) as well as tyrosine-kinase receptors that couple to DAG generation.[2] Importantly, early studies in gliomas have suggested a potential role for β2-chimerin as a tumor suppressor (8) but its relevance in other cancer models is still unknown.

In vitro studies have shown that the C-terminal domain of chimerins is capable of accelerating GTP hydrolysis from the small GTPase Rac1 without affecting the activity of RhoA or Cdc42 GTPases (9, 10). Our recent studies in COS cells revealed that β2-chimerin decreases cellular Rac-GTP levels and inhibits the elevation of Rac-GTP levels caused by epidermal growth factor (EGF) (10, 11). Rac GTPase is known to act as a molecular switch, cycling between an active GTP-bound state (Rac-GTP) and an inactive GDP-bound state (Rac-GDP). This switch is regulated by three groups of molecules: 1) guanine nucleotide exchange factors, such as Vav and Tiam-1, that promote its conversion to the active GTP-bound form; 2) guanine nucleotide dissociation inhibitors; and 3) GAPs, which stimulate intrinsic GTPase activity, thus leading to Rac inactivation (12, 13). Active Rac interacts with various effectors to initiate downstream signaling events that control the dynamics of actin cytoskeleton reorganization, migration, adhesion, and gene expression (14–17). Rac and Rac-guanine nucleotide exchange factors play key roles in the control of various aspects of malignant transformation and the metastatic cascade in various models, including breast cancer cells (18–20). Several laboratories (21–25) have proposed a role for Rac in the control of mitogenesis through its ability to regulate G_1/S transition and cyclin D1 expression. Moreover, Rac and other members of the Rho GTPase family are overexpressed in human tumors such as in breast cancer (26, 27) and hyperactivation of Rac leading to a higher rate of cell proliferation has been found in cellular models of human breast cancer (28). Targeted expression of an activated Rac mutant in mammary epithelium causes mam-

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[1] The abbreviations used are: GAP, GTPase-activating protein; AdV, adenovirus; BrdUrd, 5-bromo-2′-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; DAG, diacylglycerol; EGF, epidermal growth factor; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PBD, p21 binding domain; PKC, protein kinase C; IDC, infiltrating ductal carcinoma; Q-PCR, quantitative real-time PCR; D1-RetroV, D1 T286A retrovirus; PBS, phosphate-buffered saline; Rb, retinoblastoma; m.o.i., multiplicity of infection(s); ANOVA, analysis of variance.

[2] H. Wang, C. Yang, F. Coluccio Leskow, J. Sun, B. Canagarajah, J. H. Hurley, and M. G. Kazanietz, submitted for publication.

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 G_1/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, Hs578T, and human breast immortalized non-malignant MCF-10A cells were purchased from ATCC and cultured as recommended by the provider. 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 and cultured in DMEM 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 Clinomix Biosciences, Inc. (Watervliet, NY). All 10 patients were diagnosed as infiltrating ductal carcinoma (IDC) 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 SuperScript™ 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′-TTGGAATAGGTATCATATGTG-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 ΔEIE-β2-chimerin adenoviral construct, a XhoI-MluI insert comprising the mutant ΔEIE-β2-chimerin (10) was ligated into pShuttle-CMV-HA. A similar strategy was used for the generation of an 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-HA (empty vector); pTRE-HA-V12Cdc42 (a kind gift from Dr. Margaret Chou, University of Pennsylvania); pTRE-HA-V12Rac1; and pTRE-HA-V14RhoA. These last two plasmids were generated by subcloning V12Rac1 (isolated from pcDNA3-V12Rac1) and V14RhoA (isolated from pXDR-HA-V14RhoA) into pTRE-HA, respectively. pcDNA3-V12 Rac1 and pXDR-HA-V14RhoA were generous gifts from Dr. Rick Assoian (University of Pennsylvania).

Generation of Cyclin D1 T286A Retrovirus (D1-RetroV)—D1-RetroV was generated by co-transfecting pMX-FLAG-D1 T286A (34) (a cyclin D1 mutant resistant to proteolysis degradation, 10 μg) and a helper plasmid (p-Helper, 5 μg) into 293T packaging cells (generous gifts from Dr. J. A. Diehl, University of Pennsylvania). Co-transfection was performed in a 10-cm dish (5×10^6 cells/dish) in DMEM supplemented with 10% FBS using Lipofectamine Plus (Invitrogen) following the instructions from the manufacturer. The medium was collected 48 h later, centrifuged ($1000 \times g$, 5 min), and filtered with a 0.24-μm filter. A control retrovirus using pMX empty vector was also generated (V-RetroV). For retroviral infections, MCF-7 cells cultured in 6-cm dishes were infected with 3 ml of either D1-RetroV or V-RetroV in the presence of Polybrene (10 μg/ml, Sigma) for 24 h.

Cell Proliferation and Cell Cycle Analysis—Cell proliferation was assessed by BrdUrd incorporation and by the MTS assay (CellTiter 96® Aqueous One solution cell proliferation assay, Promega). After overnight infection (16 h) with the different AdVs, cells were washed once with PBS and incubated in serum-free DMEM for 24 h and then cultured in DMEM supplemented with 10% FBS for another 24 h. BrdUrd (Sigma) was then added into the medium for 30 min (final concentration: 0.2 mM). Cells were then collected by trypsinization, washed with PBS, and fixed with 70% ethanol for BrdUrd incorporation analysis using flow cytometry (35). For the MTS assay, after overnight adenoviral infection in 10-cm dishes, cells were collected using trypsin, counted, and then seeded onto 96-well plates (1×10^4 cells/well in 100 μl of DMEM supplemented with 10% FBS). MTS was added after 24, 48, or 72 h, and absorbance was measured at 490 nm (36). For cell cycle analysis, after overnight (16 h) adenoviral infection, cells were washed once with PBS, incubated in serum-free DMEM for 24 h, and then cultured in DMEM supplemented with 10% FBS for another 24 h. Cells were then collected by trypsinization and analyzed using flow cytometry as previously described (37).

Western Blot—15 μg of proteins were used for Western blot analysis (38). The following antibodies were used: anti-β2-chimerin (10, 11); anti-Rac; anti-Cdc42; anti-cyclins A, D1, and E (Upstate Biotechnology); anti-pRb (BD Transduction Laboratories); anti-HA tag (Cell Signaling); and anti-β-actin (Sigma).

Rac-GTP and Cdc42-GTP Pull-down Assays—After overnight infection (16 h) with different AdVs, cells were incubated in serum-free DMEM for 24 h and then stimulated with EGF (100 ng/ml, 1 min). Alternatively, after adenoviral infection, cells were cultured in 10% FBS DMEM for an additional 24-h period. Rac-GTP and Cdc42-GTP levels were determined with a "pull-down" assay using the PBD (p21-binding domain) of p21-activated kinase, as previously described (10, 11), and using either anti-Rac or anti-Cdc42 antibodies for detection, respectively.

Statistical Analysis—Data were analyzed using either a Student's *t* test or one-way analysis of variance (ANOVA) with Scheffe's test. A *p* value of <0.05 was considered statistically significant.

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. 1*A*). The results were confirmed by a quantitative analysis using Q-PCR. Indeed, β2-chimerin mRNA was not detected in MCF-7 and Hs578T 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. 1*B*). 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 (T4-2) (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. 1*C*). Together, these results reveal a significant reduction of β2-chimerin expression in human breast cancer.

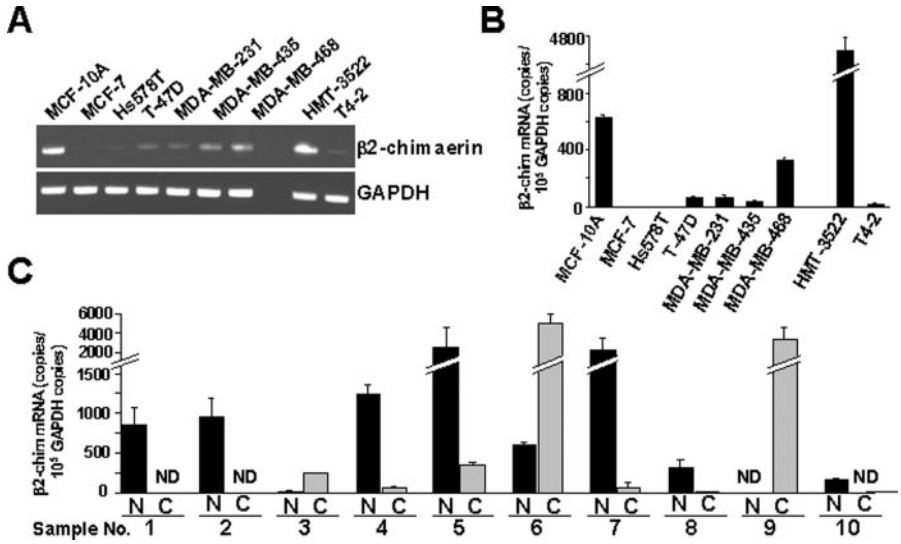

FIG. 1. **β2-Chimerin transcript levels in human breast cancer cells and tissues.** *Panel A*, β2-chimerin mRNA levels in non-malignant and cancer human breast cell lines, as determined by PCR. A representative experiment is shown. Similar results were obtained in two additional experiments. *Panel B*, analysis of β2-chimerin mRNA levels in non-malignant and cancer human breast cell lines using Q-PCR. *Panel C*, Q-PCR analysis of β2-chimerin mRNA levels in 10 pairs of human breast cancer tissue RNA (*C*) and corresponding matched normal breast tissue RNA (*N*). Q-PCR results are presented as mean ± S.D. ($n = 3$). *ND*, not detected.

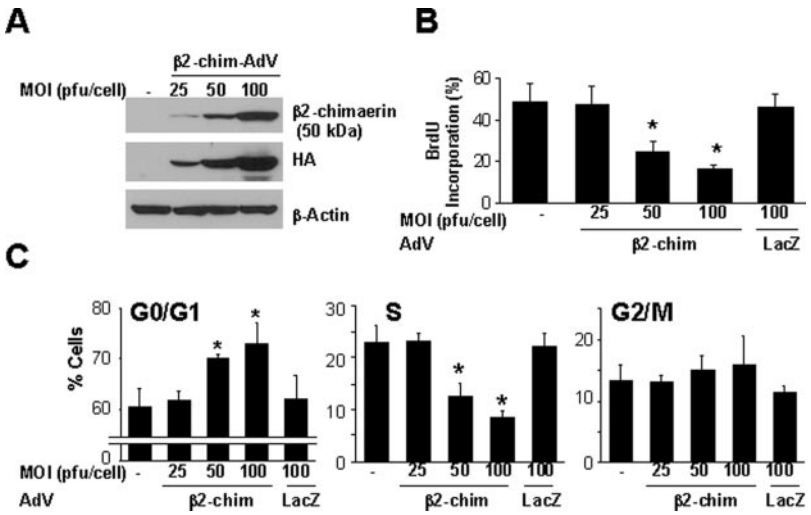

FIG. 2. **β2-Chimerin inhibits BrdUrd incorporation and G_1/S transition in MCF-7 cells.** *Panel A*, MCF-7 cells were infected with different m.o.i. of β2-chimerin-AdV or LacZ-AdV, as indicated in the figure, and the expression of β2-chimerin was assessed by Western blot 48 h after infection using either anti-HA or anti-β2-chimerin antibodies. *Panel B*, BrdUrd incorporation was determined in cells infected with either β2-chimerin-AdV or LacZ-AdV using flow cytometry, as described under "Experimental Procedures." *Panel C*, MCF-7 cells were infected with the 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.

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. 2*A*). 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. 2*B*).

To begin elucidating the mechanisms involved in the β2-chimerin effect, we examined cell cycle progression (Fig. 2*C*). Flow cytometry analysis revealed a significantly higher percentage of cells in G_0/G_1 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_2/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 RacGAP 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. 3*A*). 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 was generated. Upon delivery into MCF-7 cells, ΔEIE-β2-chimerin-AdV was unable to inhibit BrdUrd incorporation (Fig. 3, *D* and *E*).

β2-Chimerin Reduces Cyclin D1 Expression and Inhibits pRb Phosphorylation—Because expression of β2-chimerin in MCF-7 cells leads to G_1/S arrest, we next assessed the effect of β2-chimerin on pRb phosphorylation. The expression of β2-chimerin dose-dependently reduced pRb phosphorylation (Fig. 4*A*). Notably, β2-chimerin significantly inhibited the expression of cyclin D1. β2-Chimerin also reduced the expression of cyclin A and caused a slight increase in cyclin E levels. On the other hand, cells infected with control LacZ-AdV (100 m.o.i.) showed no obvious alterations in cyclin expression and pRb phosphorylation. Infection of MCF-7 cells with β2-chimerin-AdV did not cause any significant changes on the expression of cyclin-dependent kinases 2, 4, and 6 (data not shown). Infection of MCF-7 cells with the β-GAP-AdV also led to a reduction in cyclin D1 levels and pRb phosphorylation (Fig. 4*A*). However,

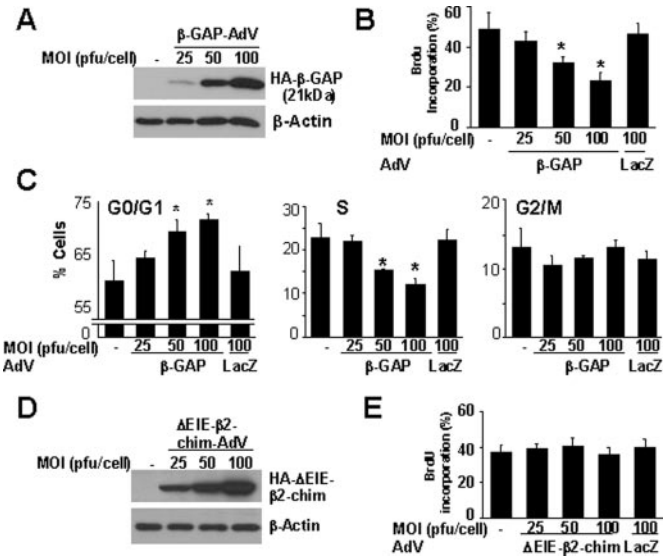

FIG. 3. **The anti-proliferative effect of β2-chimerin depends on a functional β-GAP domain.** Expression of β-GAP or ΔEIE-β2-chimerin was determined by Western blot 48 h after infection of MCF-7 cells with increasing m.o.i. of either β-GAP-AdV (*panel A*) or ΔEIE-β2-chimerin-AdV (*panel D*). *Panels 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.

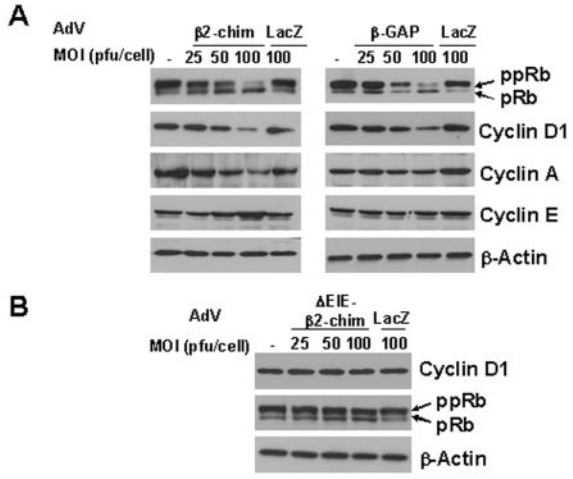

FIG. 4. **β2-Chimerin reduces cyclin D1 expression and inhibits pRb phosphorylation.** MCF-7 cells were infected with increasing m.o.i. of AdVs for β2-chimerin, β-GAP (*panel A*), or ΔEIE-β2-chimerin (*panel B*). A LacZ-AdV (100 m.o.i.) was used as a control. After 48 h, cell extracts were prepared and 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. 4*B*). Taken together, these results suggest that the inhibition of G_1/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 *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 3.3 ± 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

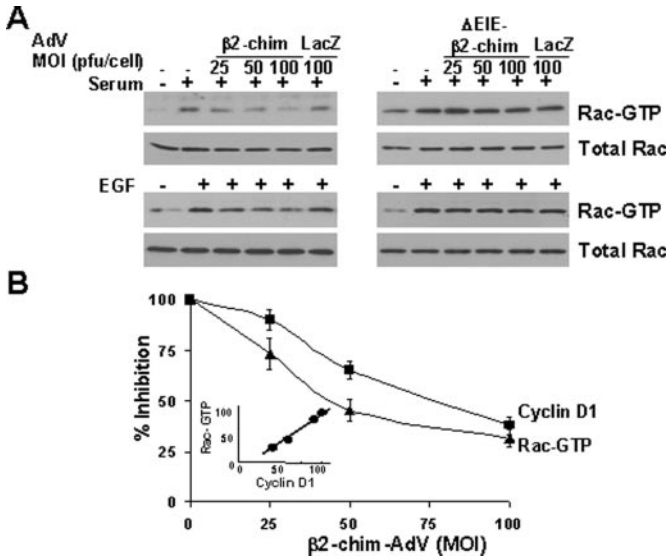

FIG. 5. **Effect of β2-chimerin on Rac-GTP levels in MCF-7 cells.** *Panel A*, cells were infected with different AdVs at the m.o.i. indicated in the figure. After serum starvation (24 h), cells were either incubated with 10% FBS DMEM for 24 h or stimulated with EGF (100 ng/ml, 1 min). Rac-GTP levels were determined using a PBD pull-down assay, as described under "Experimental Procedures." *Panel B*, densitometric analysis of inhibitory effect of β2-chimerin on serum-induced Rac activation normalized to the corresponding total Rac levels in each case. The panel also shows the densitometric analysis of cyclin D1 inhibition by the β2-chimerin-AdV from Fig. 4. *Inset* shows the correlation between the inhibition of Rac-GTP and cyclin D1 levels at the different m.o.i. of the β2-chimerin-AdV (expressed as percentage of control cells).

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. 5*A*). A densitometric analysis of the β2-chimerin effect on serum-induced activation of Rac is presented in Fig. 5*B*. 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. 6*A*). 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. 6*B*).

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. 7*A*). These cells show higher levels of Rac-GTP than control (vector-transfected) cells (Fig. 7*D*). Interestingly, whereas β2-chimerin markedly reduced proliferation in control MCF-7 cells, HA-V12Rac1-MCF-7 cells were insensitive to β2-chimerin (Fig. 7*B*). 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. 7*A*).

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. 7*A*). 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 Rb dephosphorylation (Fig. 7, *A* and *B*). Unexpectedly, in cells expressing active Cdc42,

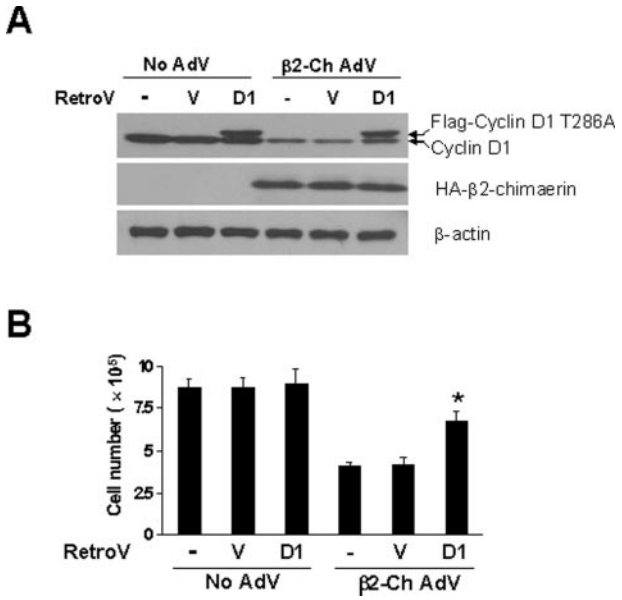

FIG. 6. **Ectopic expression of cyclin D1 rescues the anti-proliferative effect of β2-chimerin.** MCF-7 cells were infected with either a FLAG-cyclin D1 T286A retrovirus (D1-RetroV) (*D1*) or a control retrovirus (V-RetroV) (*V*) for 24 h. After extensive washings with PBS, cells were serum-starved for 8 h and then infected with HA-β2-chimerin-AdV (100 m.o.i.) for 16 h. After 24-h 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 A*). Cell proliferation was determined by hemacytometer counting (*panel B*). Data are presented as mean ± S.D. ($n = 3$). *, $p < 0.05$ compared with cells infected with HA-β2-chimerin-AdV without retroviral infection or with control retroviral infection.

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. 7*C*). 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. 8*A*). Remark-

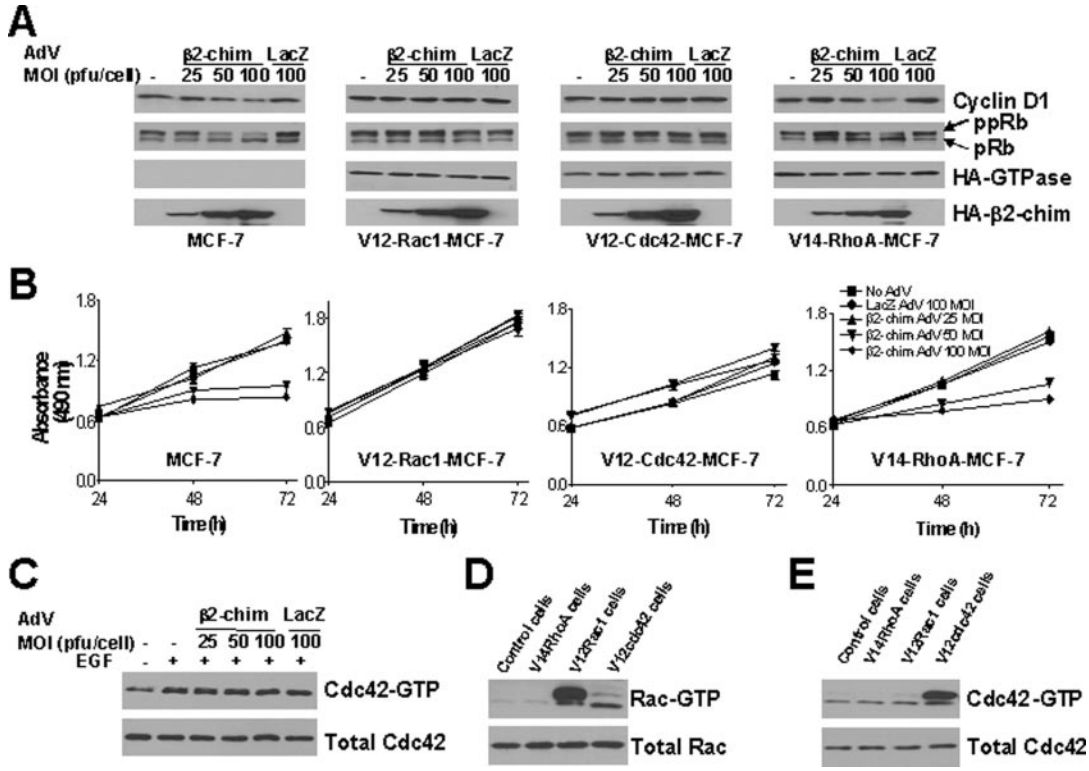

FIG. 7. **Effect of β2-chimerin in MCF-7 cells stably expressing constitutively active GTPases.** *Panel A*, generation of MCF-7 cell lines expressing HA-tagged V12Rac1, V12Cdc42, and V14RhoA. Western blots were performed in cell lines stably expressing active GTPases and control cells (transfected with empty vector) that had been infected with the β2-chimerin AdV for 48 h. *Panel B*, analysis of cell proliferation in the various cell lines using an MTS assay, as described under "Experimental Procedures." Data are presented as mean ± S.D. ($n = 8$). ■, control (no AdV); ●, LacZ-AdV (100 m.o.i.); ▲, β2-chimerin-AdV (25 m.o.i.); ▼, β2-chimerin-AdV (50 m.o.i.); ♦, β2-chimerin-AdV (100 m.o.i.). *Panel C*, lack of effect of β2-chimerin on Cdc42 activity. Control MCF-7 cells were infected with different m.o.i. of β2-chimerin-AdV, and upon serum starvation for 24 h, they were treated with EGF (100 ng/ml, 1 min). Cdc42-GTP levels were measured using a pull-down assay. *Panels D* and *E*, determination of Rac-GTP and Cdc42-GTP levels in MCF-7 cell lines growing in 10% FBS DMEM. Similar results were obtained in three independent experiments.

ably, even if I130A-β2-chimerin was expressed at very low levels, it caused a 51% reduction in Rac-GTP levels (Fig. 8*A*). Moreover, I130A-β2-chimerin markedly reduced cyclin D1 levels (Fig. 8*B*) and impaired cell proliferation (Fig. 8*C*).

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 impairs G_1/S cell cycle progression by reducing cyclin D1 levels and Rb phosphorylation. Inhibition of proliferation by β2-chimerin in MCF-7 cells is dependent on the β2-chimerin GAP activity, and indeed, a functionally active GAP domain is required for the anti-mitogenic effect. Our results suggest that Rac activity is critical for G_1/S progression in breast cancer MCF-7 cells.

Analysis of β2-chimerin mRNA levels revealed that breast cancer cells have significantly lower levels than non-malignant cells. This effect is particularly striking when we compare the non-malignant HMT3522 cell line with its malignant derivate T4-2 cell line. Moreover, studies using matched pairs of RNA samples from breast cancer patients revealed that β2-chimerin is significantly down-regulated in 70% of tumor samples. Members of the Rho GTPase family such as Rac and Rho are overexpressed in human tumors, particularly in breast cancer (26, 27). Rac activity was found to be elevated in transformed cells, as recently described in v-Src-transformed fibroblasts, and inhibition of Rac function using dominant-negative Rac mutants dramatically reduced the ability of v-*src* to transform NIH 3T3 cells (39). Small GTPase hyperactivation may be the consequence of enhanced upstream inputs and/or reduced activity of GAPs, as suggested by Mira and co-workers (28) in breast cancer cell models. Various mechanisms can account for the elevated upstream inputs including receptor hyperactivation and/or enhanced activation of Rac-guanine nucleotide exchange factors, such as Tiam1 and Vav (20, 40). On the other hand, the relative contribution of the down-regulation of Rho GAPs in cancer progression and their potential roles as tumor suppressors has not been extensively studied. For example, a recent study (41) has found that the Rho/Cdc42 GAP DLC2 is

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. ($n = 3$).

Cells	β2-Chimerin mRNA (copies/10^5 GAPDH copies)
HMT-3522	4527 ± 862
MCF-10A	625 ± 16
MCF-7	3.3 ± 0.2
MCF-7 + I130A-β2-chimerin-AdV	5902 ± 1414

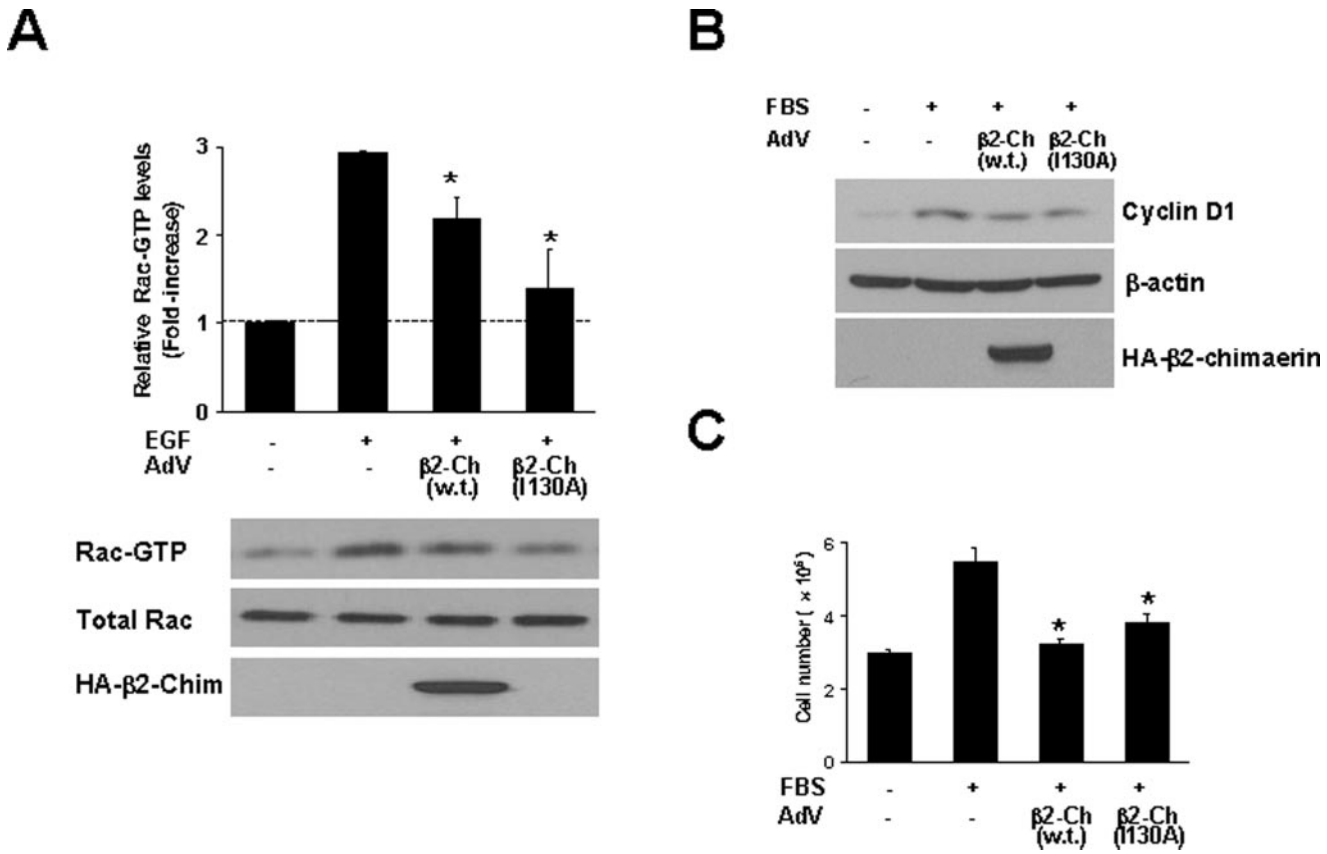

FIG. 8. **The active mutant I130A-β2-chimerin inhibits Rac-GTP levels, cyclin D1 expression, and proliferation.** *Panel A*, after 8 h of serum starvation, cells were infected for 16 h with I130A-β2-chimerin-AdV (*β2-Ch(I130A)*) (25 m.o.i.). Wild-type β2-chimerin-AdV (*β2-Ch-AdV(w.t.)*) (100 m.o.i.) 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 carcinoma. 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.[3] 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 I130A-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-GAP activity in these effects. β2-Chimerin also impairs heregulin β1-induced Rac activation and proliferation in breast cancer cells.[4] 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 β2-chimerin RNAi in HeLa cells leads to a significant potentiation of EGF-induced Rac activation.[2] Our results also emphasize the importance of Rac in cell cycle control, as previously reported using constitutively active and dominant-negative Rac1 mutants (42–44). A dominant-negative N17Rac1 mutant impairs serum-induced DNA synthesis in fibroblasts and has been reported to causes cell growth arrest in G_1 (21) or G_2/M (45). Although the specificity of dominant-negative Rac mutants may be questioned, our experiments revealed that the inhibition of Rac activity with a specific Rac-GAP leads to G_1/S arrest in MCF-7 breast cancer cells, an effect that strongly correlates with the reduction in cyclin D1 and pRb phosphorylation. Rac regulates cyclin D1 expression, probably at multiple levels, depending on the experimental condition and cell type. For example, dominant-negative and constitutively active forms of Rac1 regulate cyclin D1 promoter activity in smooth muscle cells through Rac-dependent generation of reactive oxygen species (24). A role for the NF-κB pathway downstream of Rac has also been described in NIH 3T3 cells (23). Regulation of the cyclin D1 messenger by Rac at a translational level has also been reported (21, 25). Activated Rac is capable of enhancing pRb phosphorylation and E2F-mediated transcription of genes required for S phase entry and DNA replication (46). It has also been proposed that Rac integrates signals from specific integrins and growth factors to promote the synthesis of cyclin D1 and tumor cell survival (24, 25, 47). The insensitivity of V12Rac1-expressing MCF-7 cells to β2-chimerin-induced reduction in cyclin D1 levels and Rb phosphorylation, as well as the rescue of the β2-chimerin effect by ectopic expression of cyclin D1, further supports the Rac-cyclin D1-G_1/S progression link.

An emerging paradigm is that β2-chimerin can be regulated by cell surface receptors, as it is well known for Ras-GAPs (48). Receptors coupled to the generation of the lipid second messenger DAG, such as the EGF receptor, control the activity of β2-chimerin both by positional and allosteric mechanisms,[2] which substantiates the concept of DAG divergence via the activation of "non-PKC" pathways. These DAG-regulated mechanisms, as well as the selectivity of β2-chimerin for the Rac GTPase, have been further validated by the recently solved three-dimensional structure of this Rac-GAP (33). Our hypothesis is that, in the context of receptors such as the EGF or platelet-derived growth factor receptor, β2-chimerins represent a DAG-regulated negative loop that self-limits Rac activation and Rac-mediated responses. Our focus now is to elucidate the molecular basis of such lipid regulation, which will provide further insight into the receptor-mediated control of chimerin function and G_1/S cell cycle progression in breast cancer and other diseases.

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[3] C. Yang and M. G. Kazanietz, unpublished observations.

[4] C. Yang and M. G. Kazanietz, manuscript in preparation.

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Divergence and complexities in DAG signaling: looking beyond PKC

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For many years protein kinase C (PKC) has been the subject of extensive studies as a molecular target for the treatment of cancer and other diseases. To better define the role of PKC isozymes in the control of cell proliferation, survival and transformation, the examination of PKC-mediated signal transduction pathways by isozyme-specific intervention has become essential. However, issues related to the selectivity of activators and inhibitors of PKC isozymes, in addition to convoluted cross-talks between phorbol ester-regulated pathways, have greatly complicated our understanding of PKC-mediated responses. An additional level of complexity is provided by the fact diacylglycerol (DAG) signals can be transduced by phorbol ester receptors other than PKC. These receptors include chimaerins, RasGRPs, MUNC13s, PKD (PKCμ) and DAG kinases β and γ. Thus, it is conceivable that some of the effects that were originally attributed to PKC isozymes in response to phorbol esters might be mediated by PKC-independent pathways. A key issue for the design of novel therapeutic strategies that target PKC isozymes is a comprehensive analysis of isozyme-specific signal transduction pathways in different cell types and the development of pharmacological and molecular tools that can distinguish between the various PKC and 'non-PKC' phorbol ester receptors.

Diacylglycerol (DAG) is one of the key lipid second messengers that are generated transiently following the stimulation of seven-transmembrane and tyrosine-kinase receptors. Binding of DAG to the C1 domains of protein kinase C (PKC) isozymes of the classic (cPKCα, βI, βII and γ) and novel (nPKCδ, ϵ, η and θ) families results in their activation [1,2]. Phorbol esters, natural compounds that bind to C1 domains of PKC isozymes and thus mimic DAG action, affect cell proliferation, differentiation, survival and transformation. The elucidation of the intracellular receptors for DAG and phorbol esters and the regulation of these compounds, in addition to the dissection of the various signaling pathways activated by DAG, are essential to validate these molecules as pharmacological targets.

The complexities: numerous targets and imperfect tools

In recent years, significant progress in the elucidation of DAG signaling mediated by individual PKC isozymes has been achieved largely as a result of the development of isozyme-specific tools that modulate kinase activity or expression. These tools include: (i) PKC isozyme-specific activators and inhibitors [3,4]; (ii) overexpression of wild-type, constitutive active and dominant-negative PKCs [4,5]; (iii) isozyme-specific ribozymes and antisense oligonucleotides [6,7]; and (iv) siRNA (short interfering double-stranded RNA) [8]. In addition, PKC isozyme-specific knockout animal models have been generated [9,10], which have enabled the assessment of the roles of individual PKCs in signaling and biology. However, issues of selectivity still exist for many of these pharmacological and molecular tools. For example, inhibitors that target the ATP-binding sites in PKCs might not be totally specific. Furthermore, kinase-inactive PKC mutants and PKC regulatory domains have been widely used as dominant-negative inhibitors, but the high homology among PKC isozymes and the lack of knowledge about the intracellular targets of these mutants make this approach questionable. Indeed, dominant-negative PKCδ is capable of inhibiting both PKCα and PKCϵ in COS-1 cells [11].

The cellular effects elicited by phorbol esters have often been attributed exclusively to the activation of PKC. However, this is not true because PKC isozymes are not the only 'receptors' for DAG and phorbol esters [12–16]. At least five additional families of DAG and phorbol ester receptors exist: PKD kinases, chimaerin Rac GTPase-activating proteins (GAPs), RasGRP (Ras guanyl nucleotide-releasing protein) Ras and Rap1 exchange factors, MUNC13 scaffolding proteins and DAG kinases (DGK) β and γ. Like PKCs, all of these molecules have C1 domains that bind DAG and phorbol esters in a phospholipid-dependent fashion [14]. Therefore, some DAG signals could be mediated by molecules other than PKCs, which could have important implications for the design of therapeutic strategies aimed at modulating PKC activity.

PKC-mediated signal transduction

PKC actively participates in a diversity of signaling pathways that control cell proliferation, differentiation, survival, transformation and apoptosis (Figure 1). PKC-mediated signal transduction is isozyme-, cell type-, and stimulation-specific. Although the reasons for such diversity are not totally understood, a key factor is the unique compartmentalization of each PKC isozyme. Specificity is provided by PKC-interacting proteins, such

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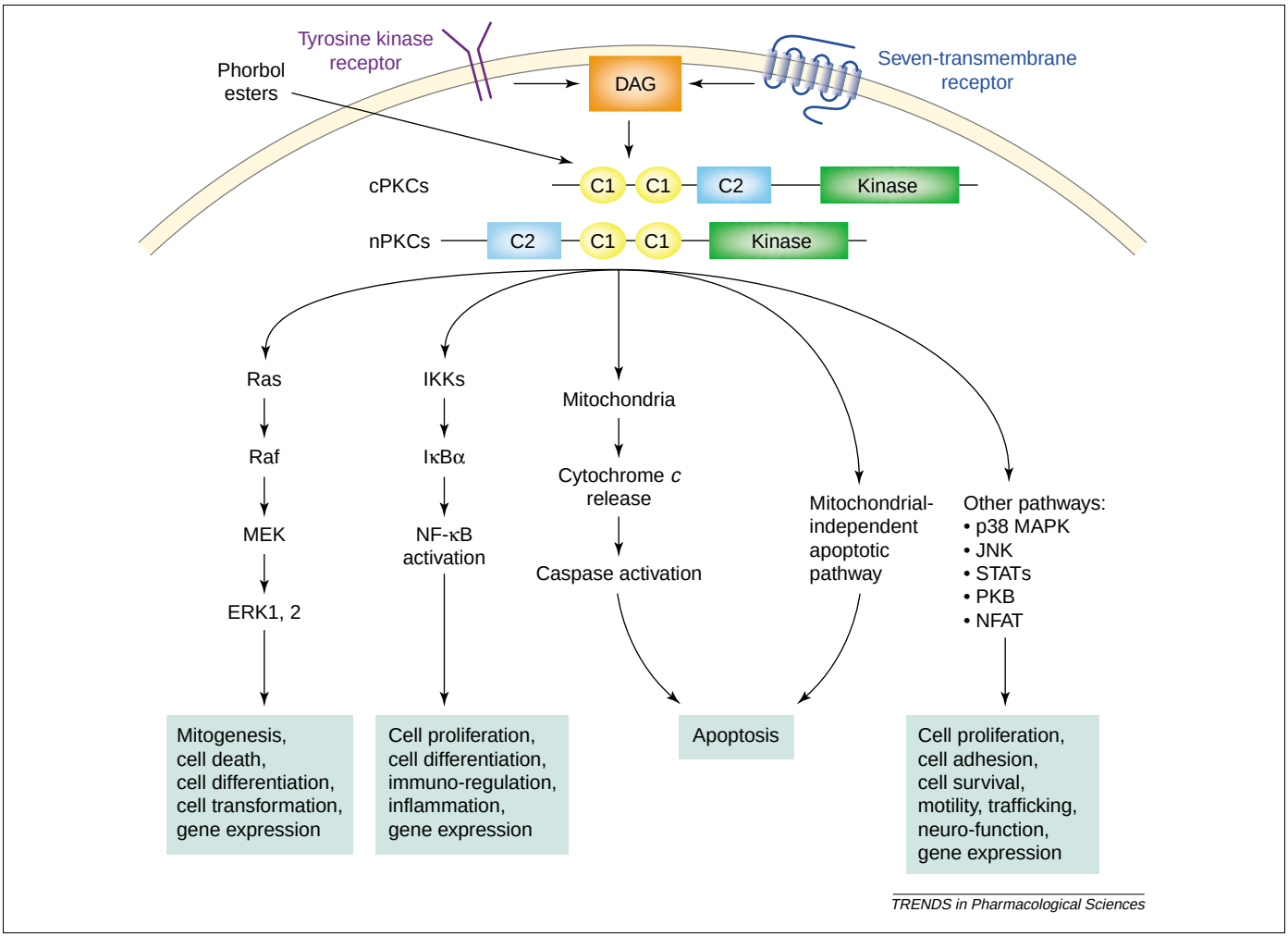

Figure 1. Regulation of signaling pathways by diacylglycerol (DAG)-responsive protein kinase C isozymes (PKCs). The lipid second messenger DAG is transiently generated following the activation of tyrosine kinase and seven-transmembrane receptors and binds to the C1 domains of classic PKC (cPKC) and novel PKC (nPKC) isozymes. C2 domains play a key role in membrane phospholipid recognition, and in the case of the cPKCs it is the Ca^{2+}-binding domain. Upon activation, cPKCs and nPKCs modulate various signaling pathways, including pathways that control proliferation and apoptosis. The differential signaling regulation by discrete PKC isozymes is strongly dependent on the stimuli and cell type, and might involve unique associations with isozyme-specific intracellular partners. Abbreviations: JNK, c-JUN N-terminal kinase; ERK, extracellular signal-regulated kinase; IκB, inhibitor of κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; PKB, protein kinase B; STAT, signal transducer and activator of transcription.

as RACKs (receptors for activated C kinases), STICKs (substrates that interact with C kinases) and other proteins. Targeting PKC isozymes to a particular intracellular compartment might confer access to specific substrates, thus promoting isozyme-specific signal transduction [17,18]. Issues of isozyme localization could explain the diversity of PKC function, which might result in either overlapping or opposite effects mediated by individual PKCs, such as those observed in cell proliferation. Deciphering the complexities of PKC signaling is required for rationalizing the use of PKC modulators as therapeutic agents.

PKC and the Raf–MEK–ERK cascade

The Raf–MEK (mitogen-activated protein kinase kinase)–ERK (extracellular signal-regulated kinase) cascade is widely implicated in cell proliferation and differentiation. PKC isozymes actively participate in the modulation of this cascade; however, the functional outcome is both PKC-isozyme-specific and cell-type-specific. For example, upon stimulation of NIH3T3 cells with phorbol 12-myristate-13-acetate (PMA), PKCα is activated, which in turn, activates Raf-1 kinase, turning on the ERK cascade and promoting proliferation [19]. In human rhabdomyosarcoma cells, however, PKCα-mediated activation of ERK, c-JUN N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) results in growth arrest and myogenic differentiation [20]. Another relevant example of such complexity was demonstrated in L6 skeletal muscle cells, in which insulin-activated PKCβ but not PKCα stimulates ERK activity and DNA synthesis [21]. PKCβ-mediated Raf–MEK–ERK signaling upon insulin receptor stimulation is independent of Ras [21]. Furthermore, although exposure of endothelial cells to shear stress results in the activation of PKCα and PKCε, only activated PKCε contributes to ERK signaling [22]. These are only a few of the many studies in which a single PKC isozyme-mediated activation of the ERK cascade and its functional outcome depends on the cellular context.

Although it is generally believed that PKCδ is a growth-inhibitory or pro-apoptotic PKC (see later), studies have

revealed that PKCδ is capable of activating the ERK signaling pathway, regulating cell proliferation and cell differentiation. PKCδ activates ERK in a manner that is independent of Ras and dependent on Raf in COS and NIH3T3 cells [23] whereas MCF-7 breast cancer cell proliferation and airway squamous cell differentiation are regulated by PKCδ-mediated, Ras-dependent modulation of ERK [24,25]. Opposite effects have also been observed for PKCε. In most cell systems, PKCε-mediated activation of ERK signaling promotes cell proliferation and survival. For example, PKCε activates ERK and enhances proliferation in leukotriene D_4-treated intestinal epithelial cells [26]. Activation of PKCε protects against myocardial ischemic injury via activation of ERK and JNK [27,28]. Studies using PKCε transgenic mice revealed that PKCε, ERKs, JNKs and p38 MAPK colocalize with cardiac mitochondria. The formation of a mitochondrial PKCε–ERK complex appears to play a role in PKCε-mediated cardioprotection through inactivation of the pro-apoptotic protein Bad [29]. In some instances, however, PKCε-mediated activation of ERK promotes cell death. PKCε participates in ultraviolet B-induced apoptosis in mouse epidermal JB6 cells and in β-adrenoceptor-induced apoptosis in adult rat ventricular myocytes through activation of ERKs [30]. Moreover, in NIH3T3 cells PKCε mediates radiation-induced cell death through Ras–Raf1–ERK1,2 [31].

PKC and the activation of NF-κB and AP-1: lessons from knockout mice

Transcriptional control through nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) is crucial in the control of inflammation, cell survival and malignant transformation. Generally, NF-κB activation results from the phosphorylation and degradation of an inhibitor of κB (IκB), a process mediated by upstream IκB kinases (IKKs). The signaling events leading to PKC-mediated activation of NF-κB and AP-1 have been actively investigated in PKC knockout animal models [32] (see later). Two independent studies using knockout mice revealed that PKCβ controls NF-κB activation in B cells through selective activation of IKKα in response to B-cell receptor stimulation [33,34]. In T cells, however, the NF-κB pathway is mainly regulated by the novel PKCθ isozyme. PKCθ mediates T-cell receptor activation of NF-κB and AP-1, which then bind to the promoter of the gene encoding interleukin 2 (IL-2) and increase IL-2 expression [35,36]. The PKCθ effect involves the activation of IKKβ [37]. Although *in vitro* studies have suggested that the SEK1 (stress-activated protein kinase kinase)–JNK pathway might contribute to AP-1 activation by phosphorylating c-JUN [38–40], subsequent studies using PKCθ knockout mice showed that T-cell receptor CD28 stimulation still induces JNK activation but no AP-1 activation [9], suggesting the existence of alternative signaling pathways that mediate AP-1 activation by PKCθ. Interestingly, a recent study revealed that NF-κB activation was only partially reduced in PKCθ knockout mice, but the nuclear factor of activated T cells (NFAT) transactivation was primarily abrogated, showing that PKCθ plays a crucial and non-redundant role in T-cell receptor-induced NFAT activation [41]. Taken together, these findings suggest that PKCθ could represent a molecular target for the modulation of T-cell activation and survival, which might reveal attractive strategies for the treatment of autoimmune diseases and lymphoma.

PKC and apoptotic signaling

Activation of certain PKC isozymes could inhibit proliferation or even induce apoptosis in some cell types. PKCδ seems to be the main isozyme involved in apoptotic signaling that is initiated by various extracellular stimuli [42]. Therefore, PKCδ or its downstream effectors could be exploited as potential therapeutic targets for proliferative diseases. PKCδ translocates to mitochondria in response to PMA in HeLa cells and keratinocytes [43,44]. Activated PKCδ decreases mitochondrial membrane potential, resulting in the release of cytochrome *c*, activation of caspases and apoptotic cell death. Moreover, PKCδ can be cleaved by caspase-3, and the release of a PKCδ catalytic fragment can further amplify the apoptotic signal [45,46]. By contrast, our recent studies using LNCaP prostate cancer cells revealed that activation of either PKCδ or PKCα triggers apoptosis, but the effect does not involve their proteolytic cleavage [4,47]. In this context, activation of ERK by PKC inhibits apoptotic signaling, whereas the p38 MAPK pathway is required for PKC-induced apoptosis [48]. Nuclear translocation of PKCδ can mediate DNA-damage-induced apoptosis [49], suggesting that the players involved in PKCδ-triggered apoptosis could depend on the cellular context and stimulation. Consistent with the widely observed pro-apoptotic properties of PKCδ, PKCδ knockout mice have increased B-cell numbers [10,50], suggesting an indispensable function for PKCδ as a negative regulator of B-cell proliferation. However, the involvement of PKCδ in apoptotic signaling is not a universal phenomenon. Indeed, overexpression of PKCδ is also capable of inhibiting cell proliferation or regulating differentiation in other cellular models [51,52]. The reported ability of PKCδ to activate the ERK cascade and to promote proliferation in MCF-7 cells [24], to enhance cell survival and chemotherapeutic resistance in non-small lung cancer cells [53], and to increase breast tumor cell anchorage-independent growth [54] is an indication that no dogmas should be applied and that the cellular context is highly relevant. Without a thorough understanding of the intracellular targets of individual PKCs in different cell types, the use of pharmacological or molecular therapeutic approaches based on PKC modulation would be inadequate, as discussed in recent reviews [55,56].

Signal transduction mediated by 'non-PKC' DAG and phorbol ester receptors

A second major issue of complexity in DAG signaling has been the discovery of non-PKC phorbol ester receptors. DAG can transduce signals through molecules other than PKC isozymes (Figure 2), which demonstrates the non-redundancy and divergence of DAG signaling. Because most of the 'non-PKC' DAG receptors regulate key molecules in proliferation and malignant transformation, elucidation of their function and signaling-mediated

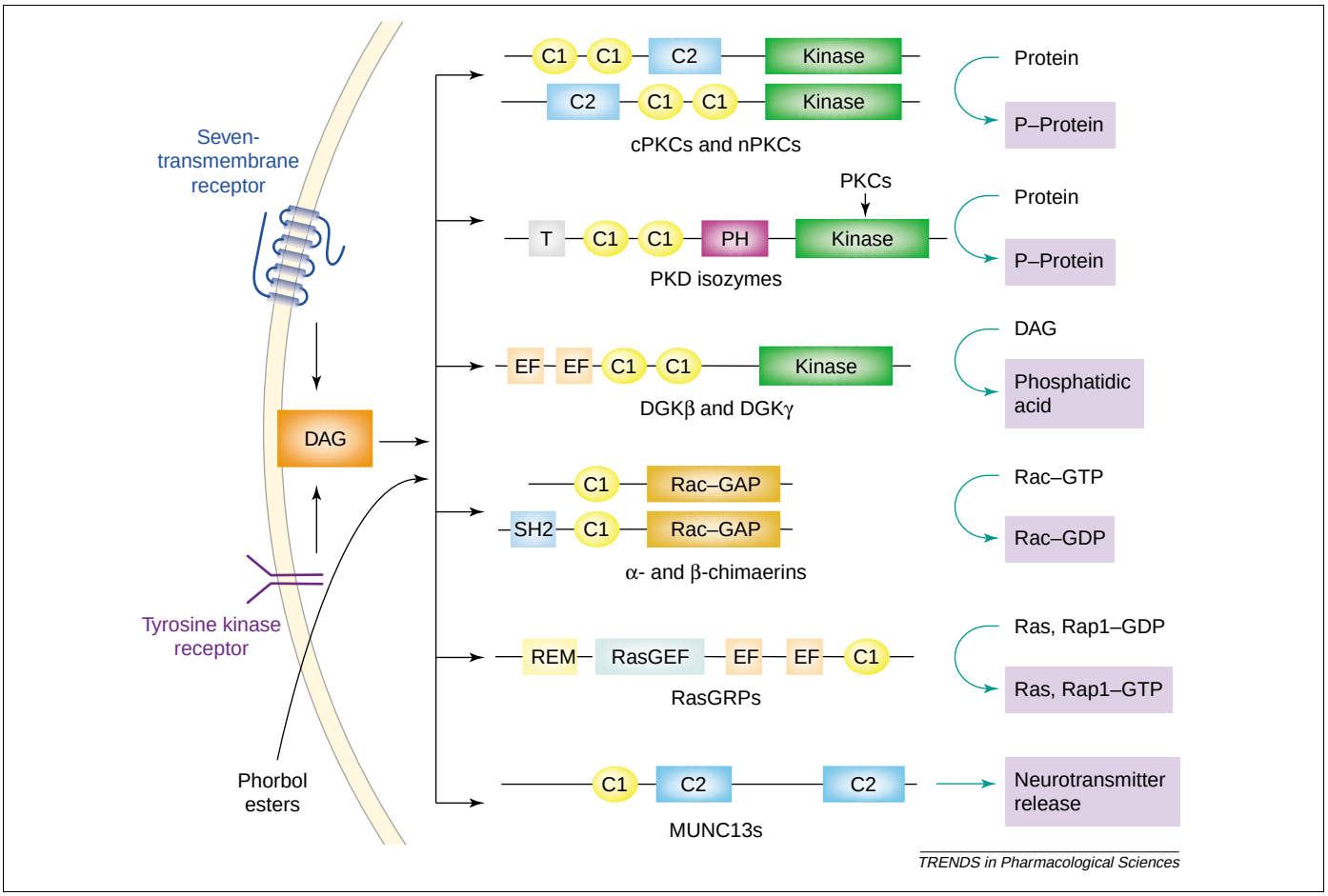

Figure 2. Divergence in diacylglycerol (DAG) signaling: the DAG and phorbol ester receptor family. Stimulation of membrane receptors that generate DAG leads to the activation of various proteins that possess DAG- and phorbol ester-responsive C1 domains. Non-redundant DAG signaling involves the activation of at least six different families of DAG and phorbol ester receptors, which include the protein kinase C [PKC; both classic (c) and novel (n)] and PKD kinases and the 'non-kinase' phorbol ester receptors [diacylglycerol kinases (DGKs), chimaerins, RasGRPs (Ras guanyl nucleotide-releasing proteins) and MUNC13s]. PKD isozymes are highly related to PKCs but they have a totally different pattern of substrate specificity. PKD isozymes are downstream effectors of PKC isozymes and have a transmembrane domain (T) and a pleckstrin homology (PH) domain. DGKβ and DGKγ also bind phorbol esters with high affinity. They have EF hands in their structure, and a kinase domain that is responsible for the phosphorylation of DAG to generate phosphatidic acid. DGKs might be key molecules in a negative feedback aimed at turning off DAG signaling. The inactivation of Rac by chimaerins [through the Rac–GAP (GTPase activating protein) domain] and the activation of Ras and Rap1 by RasGRPs [through the REM (Ras exchange motif) and GEF (guanine nucleotide exchange factor) domains] represent a novel link between DAG signaling and the regulation of small GTPases. MUNC13 isozymes are scaffolding proteins that have two C2 domains (or three in the case of MUNC13–1) and play a key role in phorbol ester-mediated exocytosis. Abbreviation: SH2, Src homology domain 2.

events will provide us with opportunities for identifying additional novel pharmacological targets.

Chimaerins and the regulation of Rac signaling via DAG

Chimaerins were the first family of non-PKC phorbol ester receptors to be discovered [57,58]. Structurally, the chimaerins resemble a 'chimaera' between the C1 domain of PKC isozymes and the GAP domain of the breakpoint cluster region protein (BCR), which is involved in chronic myelogenous leukemia. There are at least four chimaerin isoforms (i.e. α1-, α2-, β1- and β2-chimaerin) [14–16]. All four chimaerins have a single DAG- and phorbol ester-responsive C1 domain and a C-terminal GAP domain that promotes GTP hydrolysis from the small GTPase Rac, leading to Rac inactivation [58]. The structural features of chimaerins have been described in previous reviews [14–16]. Although the DAG and phorbol ester binding properties of chimaerins, their redistribution and their interaction with other proteins in response to phorbol ester stimulation have been actively investigated [59–62], the chimaerin-mediated signal transduction pathways and the biological functions of chimaerins have yet to be clarified. Rac, the target for chimaerins, controls important cellular signals involved in actin cytoskeleton organization, cell-cycle progression, malignant transformation, adhesion and migration [63,64]. We have recently shown that β2-chimaerin impairs migration and inhibits the aggressiveness of metastatic mouse mammary carcinoma cells, probably through inhibition of Rac activity [65]. β2-chimaerin also impairs epidermal growth factor (EGF)-mediated activation of Rac [58]. The expression levels of β2-chimaerin are high in low-grade astrocytoma compared with malignant gliomas, suggesting that decreased expression of β2-chimaerin might be a feature of cancer progression [66]. β2-Chimaerin associates with trafficking molecules such as Tmp21-I (p23) [62]. It has also been observed that α1-chimaerin markedly affects cell adhesion and promotes important changes in cell morphology [67,68]. Taken together, it is now timely to examine the potential role of chimaerins as targets for cancer therapeutics.

RasGRPs: DAG talks to Ras

RasGRPs comprise four members (RasGRP1–4) that each contains a single C1 domain at their C-termini [69]. RasGRP1 and RasGRP3 bind DAG and phorbol esters with high affinity through their C1 domains [70,71]. Although no study has, as yet, shown directly that RasGRP4 binds DAG and phorbol esters, RasGRP4 translocates to membranes in response to phorbol ester stimulation [72]. RasGRPs are highly expressed in brain, thymus, spleen and bone marrow, with the exception of RasGRP4, which is not expressed in the brain or in lymphoid cells [72]. As a consequence of DAG generation or phorbol ester treatment, RasGRP1 translocates to the membrane where it complexes with Ras, thereby promoting GTP loading onto Ras and the activation of the ERK cascade [73–75]. Therefore, RasGRP1 could serve as a direct link between receptors coupled to DAG generation and Ras signaling activation. RasGRP1 null mice display an impaired activation of ERK and defective thymocyte proliferation in response to phorbol esters or anti-CD3 stimulation [76]. A yet unexplored scenario is the potential contribution of RasGRP isozymes to phorbol ester-induced tumor promotion. Interestingly, a recent study showed that oncogenic Vav mediates Ras stimulation by direct activation of RasGRP1 [77]. Pharmacological agents that target RasGRPs might be effective inhibitors of the Ras pathway, particularly in those situations in which RasGRPs or its upstream activators (such as Vav) are dysregulated, and therefore they might represent potential agents for the treatment of cancer and other diseases.

More DAG receptors: MUNC13s, PKD and DGKs

MUNC13s consist of three mammalian homolog (MUNC13–1, –2, and –3) of the *Caenorhabditis elegans* UNC-13 protein [78]. Structurally, MUNC13s have a N-terminal C2 domain (in MUNC13–1 and MUNC13–2), a central C1–C2 tandem domain and a C-terminal C2 domain [13]. MUNC13s are expressed mainly in brain and specifically localized to presynaptic terminals [79–81]. MUNC13 proteins play important roles in modulating neurotransmitter release in response to DAG stimulation, as confirmed using a knock-in animal model [80,82]. Importantly, phorbol ester-induced augmentation of transmitter release seems to be mediated by MUNC13s and not by PKCs [13].

The PKD family is composed of three members: PKD (also called PKCμ), PKD2 and PKD3 (also called PKCν) [83]. PKD has two C1 domains, a pleckstrin homology (PH) domain and a catalytic or kinase domain [83]. PKD acts as a downstream effector of PKCs, including PKCε, PKCη, PKCθ and PKCδ [84–87], and has been implicated in the regulation of ERK and JNK signaling and cell proliferation [85]. A recent study by the Toker's laboratory showed that PKD activates NF-κB through the activation of IKKβ and degradation of IκB, promoting cell survival in response to oxidative stress [88]. PKD is also involved in apoptotic signaling in human U-937 myeloid leukemia cells in response to genotoxic agents. Although the generation of a PKD catalytic domain resulting from caspase-3 cleavage is not sufficient to trigger apoptosis, it sensitizes cells to apoptosis induced by diverse anticancer agents [89]. The potential of PKD as a therapeutic target has not been extensively explored.

Diacylglycerol kinases (DGKs) represent a family of nine related isozymes encoded by separate genes [90]. Although some DGK isotypes are regulated by small GTPase RhoA and might play a role in cytoskeletal reorganization, other DGK isotypes are found in the nucleus and regulate cell-cycle progression [91]. All nine DGK isozymes contain C1 domains; however, only C1 domains in DGKβ and DGKγ bind phorbol esters with high affinity [92,93]. DGKγ phosphorylates DAG to generate phosphatidic acid, and thus it decreases DAG levels. It is possible that DAG-regulated DGKs might serve as negative feedback molecules that turn off DAG signaling upon receptor activation.

Concluding remarks

In the past decade the development of pharmacological and molecular tools that modulate specifically PKC isozyme expression or activity have been central to shed light on the PKC isozyme-specific regulation of signal transduction pathways. However, the complex regulation and the cell and stimuli dependency of the pathways controlled by PKC isozymes, in addition to the existence of non-PKC DAG and phorbol ester targets could make any potential interventional strategies targeting PKCs more difficult than initially predicted. A clear understanding of PKC isozyme-mediated and cell-specific signal transduction and function is required to validate PKC as a therapeutic target. Moreover, it is possible that cross-talks among the different DAG receptors might exist, as recently reported for PKC and RasGRP1 [94].

It is imperative to design powerful new tools that are not only PKC isozyme-specific but also discriminative to non-PKC phorbol ester receptors. A question still remains: is it possible to design C1 domain-specific tools to modulate the activity of each specific DAG receptor? The high homology between DAG-responsive C1 domains suggests that this is not going to be an easy task, and that modulators that target other regions or affect intracellular localization such as RACK-derived peptides [3], might be the key to selectivity. It is conceivable that drugs designed to target the C1 domain of PKCs, such as the bryostatins or phorbol esters (both in clinical trials for various types of cancers), might also modulate other pathways through PKC-independent routes. Indeed, bryostatins bind to C1 domains of chimaerins and RasGRPs with nanomolar affinities [59,70]. Based on current knowledge of the structural properties of individual C1 domains and differences observed in their ligand-binding properties [14], small molecules that could discriminate among various C1 domains might be developed. Surprisingly, ligands that have similar *in vitro* affinities for PKC isozymes still present unique translocation properties, thus conferring isozyme-specific responses. This has been observed with bryostatin 1 [95] and the novel DAG lactones [4]. Decoding the molecular events that specifically regulate the various DAG receptors and the molecular mechanisms that control their intracellular compartmentalization represent a future challenge.

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Heregulin β1 induces cyclin D1 and p21 expression and promotes breast cancer cell proliferation through ErbB receptor/Rac/Erk

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The human ErbB receptor family comprises 4 tyrosine kinase receptors (ErbB1 or EGFR, ErbB2, ErbB3, and ErbB4). It is well established that dysregulation of ErbB receptor signaling plays important roles in the progression of various types of cancers, including breast, prostate and colon cancer. Heregulins (also called neuregulins) are a group of EGF-like ligands for the ErbB3 and ErbB4 receptors and are often expressed in breast cancer tissues. In our previous studies, we found that heregulin β1 (HRG) is a potent activator of Rac in breast cancer cells. The Rac activation by HRG is impaired by RNAi depletion of ErbB2 and ErbB3 but not by ErbB4, and it also involves the transactivation of EGFR. In this study, we further determined how Rac activation by HRG mediates breast cancer T-47D cell proliferation. We found that expression of β2-chimaerin, a Rac-GAP, or a dominant negative Rac (N17Rac1) dose-dependently inhibited HRG-induced Rac activation in T-47D cells. Rac inhibition with β2-chimaerin and N17Rac1, or Rac1 depletion using RNAi, dose-dependently inhibited HRG-induced cyclin D1 mRNA and protein expression and cell proliferation. Rac inhibition also impaired HRG-induced activation of Erk1/2. Inhibition of MEK with U0126 significantly reduced cyclin D1 induction and proliferation by HRG. HRG also induced rapid mRNA and protein expression of p21 while it promotes cell proliferation. Rac inactivation or depletion, or MEK inhibition impaired HRG-induced p21 up-regulation. Moreover, RNAi depletion of p21 impaired HRG-induced cell proliferation. RNAi depletion of ErbB3, ErbB2, or EGFR, but not ErbB4, also impaired HRG-induced cell proliferation. Together, these results indicate that heregulin β1 promotes breast cancer cell proliferation through ErbB receptor/Rac/Erk-dependent cyclin D1 and suggests a paradoxical role for p21 in proliferation. (The U.S. Army Medical Research and Material Command under DAMD 17-03-1-0469 supported this work.)

Essential role for Rac1 in Heregulin β1-induced Mitogenic Signaling in Human Breast Cancer Cells

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The ErbB family of tyrosine kinase receptors comprises 4 members (EGFR or ErbB1, Her2 or ErbB2, ErbB3 and ErbB4) which play important roles in the progression of various types of cancers, including breast, prostate and colon cancer. Heregulin β1 (HRG) belongs to the family of neuregulins, a group of peptide ligands for the ErbB3 and ErbB4 receptors. How HRG causes the activation of PI3K-Akt and MAPKs to control cell survival and proliferation is not fully understood. We explored whether Rho GTPases play critical roles in HRG-triggered mitogenic signaling. Using a PBD pull down approach, we determined that HRG activates Rac1 in a dose- and time-dependent manner in MCF-7 and T-47D breast cancer cells. HRG-induced Rac1 activation showed a striking different kinetics from EGF-triggered activation of Rac1 in these two cell lines. While EGF-induced Rac1 activation peaked at 1-2 min and returned to basal within 15-30 min, HRG-triggered activation of Rac1 peaked at 5-10 min and still remained high 60 min after stimulation. HRG also caused sustained activation of Cdc42 and RhoA with a similar time-course. By using pharmacological inhibitors, specific blocking antibodies and small interference RNA (siRNA) for individual ErbB receptors, it was determined that the activation of Rac1 by HRG is mediated by ErbB2 and ErbB3 receptors, and that there was an essential requirement for the EGFR in the HRG effect. HRG-induced Rac1 activation was dependent on PI3K but not on Src, as it was impaired by wortmannin but not by PP2. The kinetics of HRG- and EGF-induced Rac1 activation strongly correlated with that for the activation of Erk1/2, JNK and p38 MAPKs. Inactivation of Rac1 by adenoviral delivery of beta2-chimaerin, a Rac-GAP, impaired HRG-induced activation of MAPKs. Moreover, beta2-chimaerin, which did not affect Cdc42 and RhoA activation, also inhibited HRG-induced breast cancer cell proliferation. On the other hand, expression of a constitutively active Rac mutant (V12Rac1) in MCF-7 cells rescued the inhibitory effect of beta2-chimaerin on cell proliferation. These results suggest that Rac1 is an important mediator of HRG mitogenic signaling in breast cancer cells and highlight the complexity in HRG responses via activation of multiple tyrosine-kinase receptors (The U.S. Army Medical Research and Material Command under DAMD 17-03-1-0469 supported this work.).

HEREGULIN β1-INDUCED RAC ACTIVATION PROMOTES BREAST CANCER CELL PROLIFERATION

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Heregulin β1 (HRG) belongs to the family of neuregulins, a group of epidermal growth factor (EGF)-like peptide ligands for the ErbB3 and ErbB4 receptors. It has been found that HRG1 is overexpressed in many kinds of human cancers including breast cancer, and that it promotes breast cancer cell proliferation. The ErbB family of tyrosine kinase receptors comprises 4 members (EGFR or ErbB1, Her2 or ErbB2, ErbB3 and ErbB4) which play important roles in the progression of various types of cancers including breast cancer. How HRG causes the activation of PI3K-Akt and mitogen-activated protein kinases (MAPKs) to control breast cancer cell survival and proliferation is not fully understood. We explored whether Rho GTPases play critical roles in HRG-triggered mitogenic signaling.

Recombinant HRG and two human breast cancer cell lines MCF-7 and T-47D were used for this study. Using a PBD pull down approach, we found that HRG activates Rac1 in a dose- and time-dependent manner in MCF-7 and T-47D breast cancer cells. HRG-induced Rac1 activation showed a striking different kinetics from EGF-triggered activation of Rac1 in these two cell lines. While EGF-induced Rac1 activation peaked at 1-2 min and returned to basal within 15-30 min, HRG-triggered activation of Rac1 peaked at 5-10 min and still remained high 60 min after stimulation. HRG also caused sustained activation of Cdc42 and RhoA with a similar time-course. By using pharmacological inhibitors, specific blocking antibodies and small interference RNA (siRNA) for individual ErbB receptors, it was determined that the activation of Rac1 by HRG is mediated by ErbB2 and ErbB3 receptors, and that there was an essential requirement for the EGFR in the HRG effect. HRG-induced Rac1 activation was dependent on PI3K but not on Src, as it was impaired by wortmannin but not by PP2. The kinetics of HRG- and EGF-induced Rac1 activation strongly correlated with that for the activation of Erk1/2, JNK and p38 MAPKs. Inactivation of Rac1 by adenoviral delivery of beta2-chimaerin, a specific Rac-GAP, impaired HRG-induced activation of MAPKs. Moreover, beta2-chimaerin, which did not affect Cdc42 and RhoA activation, also inhibited HRG-induced breast cancer cell proliferation. On the other hand, expression of a constitutively active Rac mutant (V12Rac1) in MCF-7 cells rescued the inhibitory effect of beta2-chimaerin on cell proliferation.

We conclude that HRG-induced Rac activation is an important mediator of HRG mitogenic signaling in breast cancer cells. These results also indicate that approaches aimed at targeting Rac signaling could open new avenues for breast cancer therapeutics.

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β2-Chimaerin Inhibits Heregulin-induced Activation of Mitogen-Activated Protein Kinases in Human Breast Cancer Cells

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β2-Chimaerin is a "non-protein kinase C (PKC)" phorbol ester receptor. Previous studies have shown that phorbol ester stimulation induces intracellular β2-chimaerin redistribution and interaction with other proteins. Its biological function, however, remains largely unknown. Heregulin is a specific ligand for ErbB3 (Her3) and ErbB4 (Her4) receptors that are overexpressed in human breast cancers. Heregulin signaling is considered to play an important role in breast carcinogenesis. Our preliminary study showed that the expression of β2-chimaerin in human breast cancer cells is greatly lower than that in human normal breast cells. The present study is designed to investigate the effect of β2-chimaerin on heregulin-induced signal transduction and cell proliferation in human breast cancer MCF-7 and T-47 D cells. The overexpression of β2-chimaerin in breast cancer cells was achieved by adenoviral gene delivery. The results showed that treatment of breast cancer cells with heregulin-β1 resulted in the activation of AKT, extracellular signal-regulated kinase (Erk), c-Jun NH(2)-terminal kinase (JNK) and cell proliferation in the absence of serum. Heregulin-induced AKT, Erk and JNK activation and cell growth were dose- and time-dependent. Inhibition of phosphatidylinositol 3'-kinase (PI-3-K) activity with wortmannin completely blocked heregulin-induced AKT, Erk and JNK activation and cell growth. Overexpression of β2-chimaerin by adenoviral infection dose-dependently inhibited heregulin-triggered Erk and JNK, but not AKT activation. Moreover, overexpression of β2-chimaerin also does-dependently suppressed heregulin-induced cell proliferation as determined by BrdU incorporation. These results suggest that β2-chimaerin impairs heregulin-initiated mitogenic signaling downstream of PI-3-K through inhibition of Erk and JNK activity. (The U.S. Army Medical Research and Material Command under DAMD 17-03-1-0469 supported this work.)