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Phospholipase Cγ/diacylglycerol-dependent activation of β2-chimaerin restricts EGF-induced Rac signaling

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Although receptor-mediated regulation of small G-proteins and the cytoskeleton is intensively studied, the mechanisms for attenuation of these signals are poorly understood. In this study, we have identified the Rac-GAP β2-chimaerin as an effector of the epidermal growth factor receptor (EGFR) via coupling to phospholipase Cγ (PLCγ) and generation of the lipid second messenger diacylglycerol (DAG). EGF redistributes β2-chimaerin to promote its association with the small GTPase Rac1 at the plasma membrane, as determined by FRET. This relocalization and association with Rac1 were impaired by disruption of the β2-chimaerin C1 domain as well as by PLCγ1 RNAi, thus defining β2-chimaerin as a novel DAG effector. On the other hand, GAP-deficient β2-chimaerin mutants show enhanced translocation and sustained Rac1 association in the FRET assays. Remarkably, RNAi depletion of β2-chimaerin significantly extended the duration of Rac activation by EGF, suggesting that β2-chimaerin serves as a mechanism that self-limits Rac activity in response to EGFR activation. Our results represent the first direct evidence of divergence in DAG signaling downstream of a tyrosine-kinase receptor via a PKC-independent mechanism.

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Introduction

The epidermal growth factor receptor (EGFR) represents a paradigmatic tyrosine kinase that activates a wide array of signaling pathways controlling proliferation, differentiation, survival, and motility, including the Ras-ERK, PI3K-Akt, PLC-PKC, and STAT pathways. Such diversity is conferred by coupling to multiple effectors and adaptor molecules that associate with the receptor upon ligand binding, a mechanism that is mediated by specific phosphotyrosine motifs and distinctive recruitments via protein–protein interactions (Carpenter, 2000; Schlessinger, 2002; Gschwind *et al*, 2004). It is well established that the stimulation of the EGFR leads to Rac activation to cause the characteristic actin cytoskeleton rearrangements, ruffle formation, and stimulation of cell motility (Azuma *et al*, 1998; Menard and Mattingly, 2003; Kurokawa *et al*, 2004). EGF-induced activation of Rac is mediated by Rac GEFs (guanine exchange factors), such as Vav isoforms (Moores *et al*, 2000; Tamas *et al*, 2003).

One of the key effectors activated by the EGFR is the phospholipase C (PLC) pathway. PLCγ, the first identified partner for the EGFR, is recruited to the receptor by binding to the phosphorylated Tyr 992 docking site in the cytoplasmic receptor tail (Nishibe *et al*, 1990; Rotin *et al*, 1992). Activation of PLCγ causes the breakdown of PIP_2 into IP_3 and the lipid second messenger diacylglycerol (DAG). DAG is present at very low levels in the plasma membrane and it is transiently elevated upon activation of EGFR or other tyrosine kinase receptors. DAG is known to activate both classical and novel protein kinase C isozymes (cPKCs and nPKCs), a mechanism mediated by lipid binding to the C1 (cysteine-rich) domain, a stretch of 50 amino acids duplicated in tandem in DAG-responsive PKCs (Newton, 1997). While the prevailing view is that DAG signals mainly via PKCs and the related PKD kinases, this linear concept of DAG-PKC activation upon receptor stimulation is in stark contrast with the diversity of putative intracellular DAG targets. Indeed, C1 domains are present in a large number of molecules in databases, although only a few have the stringent structural requirements for binding of DAG or its related natural analogs, the phorbol esters (Yang and Kazanietz, 2003; Brose *et al*, 2004).

β2-Chimaerin is a member of the chimaerin family of Rho GAPs (GTPase-activating proteins) that also comprises β1-, α1- (or '*n*-'), and α2-chimaerins, alternative spliced variants from the β (CHN2) and α (CHN1) chimaerin genes. A single C1 domain is present in all chimaerin isoforms, which, like those in nPKCs and cPKCs, is capable of binding phorbol esters and other DAG-mimetics with low nanomolar affinity in the presence of acidic phospholipids (Caloca *et al*, 1999). Modeling studies have indeed predicted similar lipid-binding modes for β2-chimaerin and PKC C1 domains (Caloca *et al*, 2001). An N-terminal SH2 domain that presumably interacts with phosphotyrosine proteins is also present in α2- and β2-chimaerin. The C-terminal chimaerin GAP domain is structurally homologous to other Rho GAP domains, such as those in p190RhoGAP, p50RhoGAP, and BCR. Experiments *in vitro* have established that β2-chimaerin accelerates GTP hydrolysis from the small G-protein Rac1, leading to the conversion to the inactive GDP-bound form of Rac (Caloca *et al*, 2003a). Rac1 plays a fundamental role in the control of

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actin cytoskeleton reorganization, migration, adhesion, transcription, and cell cycle progression (Bar-Sagi and Hall, 2000; Ridley, 2001). It is therefore conceivable that chimaerins could play important negative regulatory functions in the context of Rac-mediated responses.

It is not known whether chimaerins can be regulated by tyrosine kinase receptors coupled to DAG generation, a well-establish paradigm for the PKCs, as well as the potential functional consequences of such regulation. A scenario that can be envisaged is that DAG operates as a positional signal and/or promotes the allosteric activation of β2-chimaerin GAP activity. The presence of a single C1 domain in chimaerins together with major structural differences with PKCs regarding membrane-targeting motifs predicts distinctive regulatory mechanisms, as anticipated by our recent structural analysis (Canagarajah *et al*, 2004). In this context, one previously unappreciated scenario is that the activation of the PLCγ-DAG axis by receptor stimulation directly activates this Rac-GAP and attenuates Rac signaling.

In this paper, we define β2-chimaerin as a novel effector of the EGFR. EGF causes the peripheral redistribution of β2-chimaerin, which results in its rapid association with Rac1 and the subsequent inactivation of the small G-protein. This positional regulation is entirely dependent on receptor-activation of PLCγ1 and direct binding of DAG to the β2-chimaerin C1 domain. Our studies have major implications for receptor-mediated signaling, as they not only uncover a novel role for the lipid second messenger DAG via a PKC-independent pathway but also identify a lipid-regulated loop that self-limits the magnitude of the Rac response upon receptor activation.

Results

EGF causes the intracellular redistribution of β2-chimaerin: essential role for the C1 domain

The presence of a phorbol ester-responsive C1 domain in β2-chimaerin was strongly suggestive of a spatial regulation upon receptor-stimulated generation of DAG. Proof-of-principle in a 'nonphysiological' context has been previously established using synthetic phorbol esters (Caloca *et al*, 2001) and is supported by our recent 3-D structural analysis (Canagarajah *et al*, 2004). However, the relevance of these findings in the context of cell surface receptor activation still remains undefined. We chose the EGFR as a paradigm since it couples to DAG generation via PLCγ activation, and in addition it activates Rac, the putative target for chimaerins (Caloca *et al*, 2003a). Plasmids encoding for GFP-tagged β2-chimaerins (wild type or mutants, depicted in Figure 1A) were transfected into HeLa cells and their localization assessed by confocal microscopy. GFP-β2-chimaerin shows a characteristic cytoplasmic localization in HeLa cells. PMA (phorbol 12-myristate 13-acetate) caused an appreciable redistribution of β2-chimaerin to the cell periphery and the perinuclear region. EGF caused a time-dependent redistribution to the cell periphery, although not as intense as that caused by PMA (Figure 1C; see also Figure 2A in COS-7 cells). Membrane translocation can be better appreciated by colocalization with the membrane marker RFP-CAAX (Figure 1D). Translocation of endogenous β2-chimaerin by EGF was also observed (Supplementary Figure S1). We speculated that Rac-GTP generated by receptor stimulation might contribute to the peripheral localization of β2-chimaerin, and that the limited relocalization of β2-chimaerin by EGF could be related to the reduction in Rac-GTP levels caused by β2-chimaerin Rac-GAP activity upon peripheral translocation. We therefore assessed EGF-mediated translocation of the GAP-deficient mutant ΔEIE-β2-chimaerin (with a three amino-acid deletion in positions 298–300; Caloca *et al*, 2003a). This mutant showed a much more pronounced translocation in response to EGF than wild-type β2-chimaerin. In response to EGF, ΔEIE-β2-chimaerin showed a substantial redistribution to the periphery, including membrane ruffles, as well as to the perinuclear region (Figure 1C). A similar effect was observed with the GAP-deficient mutant ΔYRV-β2-chimaerin, with deletions in residues 310–312 in the GAP domain (data not shown).

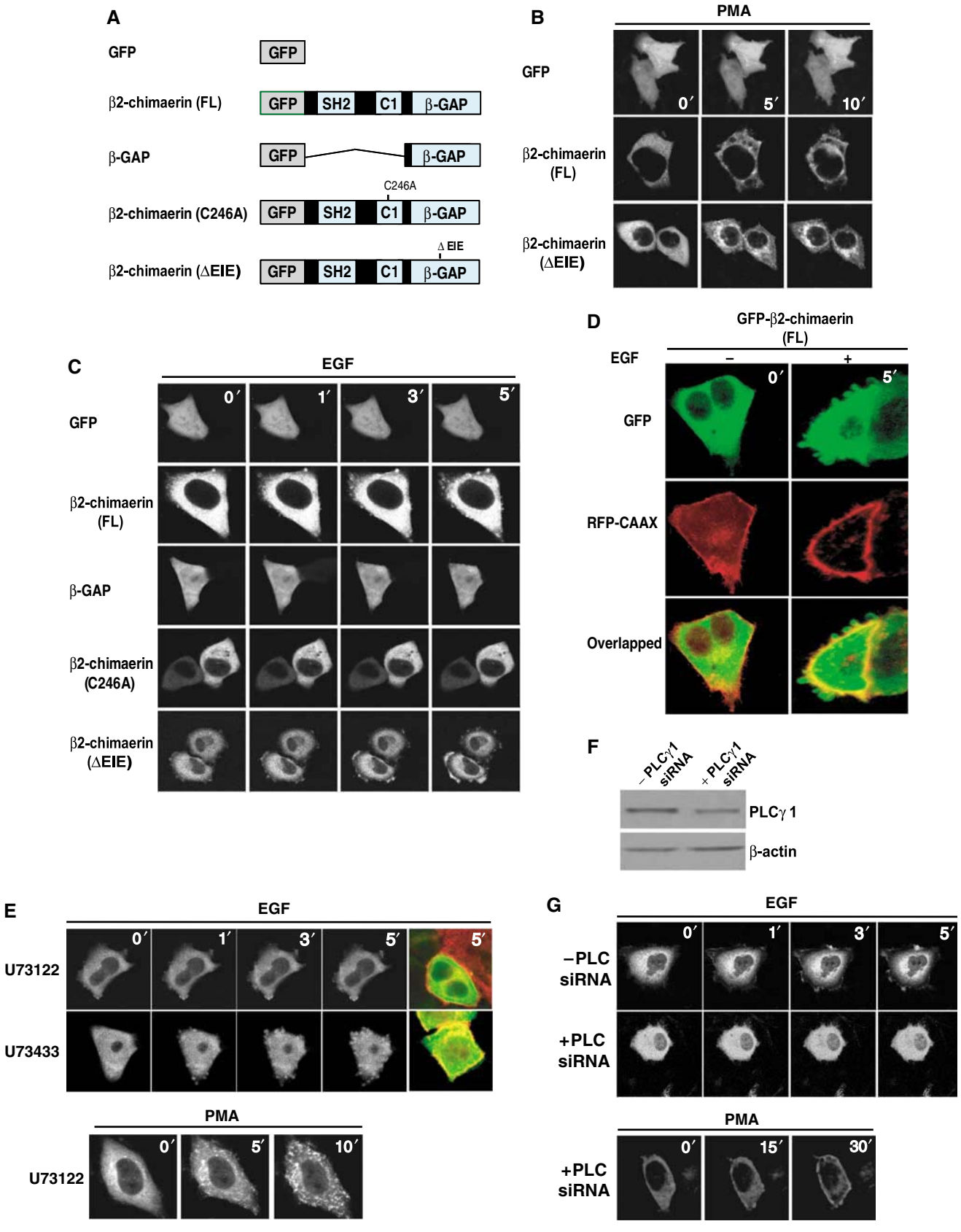
We hypothesized that the C1 domain in β2-chimaerin was essential for translocation by EGF. A C1 domain point-mutant, C246A-β2-chimaerin, which lost the ability to bind phorbol esters in radioligand binding assays (Caloca *et al*, 1999), localized in the cytoplasm in nonstimulated cells and was unable to translocate in response to EGF (Figure 1C), in agreement with our previous reports using PMA (Caloca *et al*, 2001; Wang and Kazanietz, 2002). A mutant comprising the C-terminal GAP domain, which lacks the C1 domain (β-GAP), showed cytoplasmic and also some nuclear staining, but no changes in localization were observed upon EGF (Figure 1C) or PMA treatment (Caloca *et al*, 2001). To determine the role of endogenously generated DAG, we first used the PLC inhibitor U73122. U73122, but not its inactive analog U73433, significantly impaired EGF-induced translocation of ΔEIE-β2-chimaerin (Figure 1E). U73122 also inhibit EGF-induced translocation of PKCα or an isolated PKCα C1 domain fused to GFP (data not shown). As expected, U73122 did not affect translocation by exogenously added PMA. The PKC inhibitor GF109203X did not affect β2-chimaerin translocation by either EGF (data not shown) or PMA (Caloca *et al*, 1999).

As DAG generation by EGFR involves coupling to PLCγ1, we knocked down the DAG-generating enzyme using RNAi. We achieved ~70% depletion upon delivery of a specific PLCγ1 RNAi duplex into HeLa cells (Figure 1F). Under these conditions, GFP-ΔEIE-β2-chimaerin translocation in response to EGF was significantly impaired (Figure 1G). Although at a slower rate, GFP-ΔEIE-β2-chimaerin still translocated efficiently in response to exogenously added PMA in PLCγ1-depleted cells. Thus, EGFR activation leads to the spatial redistribution of β2-chimaerin via a PLCγ-DAG pathway.

Analysis of β2-chimaerin association using FRET

HA-β2-chimaerin and various forms of GST-fused Rac1 mutants were coexpressed in COS-1 cells, and their association was determined using a GST pull-down approach. β2-Chimaerin strongly associates with active G12V-Rac1. On the other hand, binding to either the inactive GST-T17N-Rac1 mutant, GST-Rac1 (wild-type) or GST alone was barely detected (Supplementary Figure S2A). Unlike G12V-Rac1, G12V/S189A-Rac1, an activated mutant with impaired geranylgeranylation and membrane targeting, failed to bind to β2-chimaerin in the pull-down assays (Supplementary Figure S2B). Thus, β2-chimaerin preferentially associates with activated (GTP-bound), membrane-bound Rac1. β2-

Chimaerin also co-immunoprecipitates with an active Rac1 mutant (Supplementary Figure S2C). We hypothesized that peripheral redistribution of chimaerins serves as a means to promote its association with its target, Rac. Measuring the association of DAG effectors to membrane targets using standard approaches, such as co-IPs, have proven difficult,



mainly due to the transient nature of DAG generation. To circumvent this limitation in our analysis of the β2-chimaerin–Rac interaction in response to EGF, we developed a fluorescence resonance energy transfer (FRET) approach (Figure 2A). Constructs encoding for Rac1 (wild type) fused to CFP as well as for β2-chimaerin (wild type and mutants) fused to YFP were generated. FRET was determined in peripheral sections of COS-7 cells at different times after EGF treatment. Peripheral translocation of β2-chimaerin was readily detected in these cells (Figure 2A). When cells coexpressing CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) were subject to EGF treatment, significant FRET was detected in the cell periphery (Figure 2B). The effect of EGF was dose dependent. At 30 ng/ml EGF, maximum FRET was generally observed at ~1–3 min, which returned to basal levels at ~5 min (Figure 2C). Several controls were performed to validate these results. First, FRET was not detected in cytoplasmic regions of cells expressing CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) in response to EGF (data not shown). Second, EGF did not cause FRET in cells expressing CFP and YFP alone. Third, cells expressing only CFP-Rac1 showed no FRET in response to EGF. Lastly, no FRET was detected upon EGF treatment in cells coexpressing CFP-Rac1 and

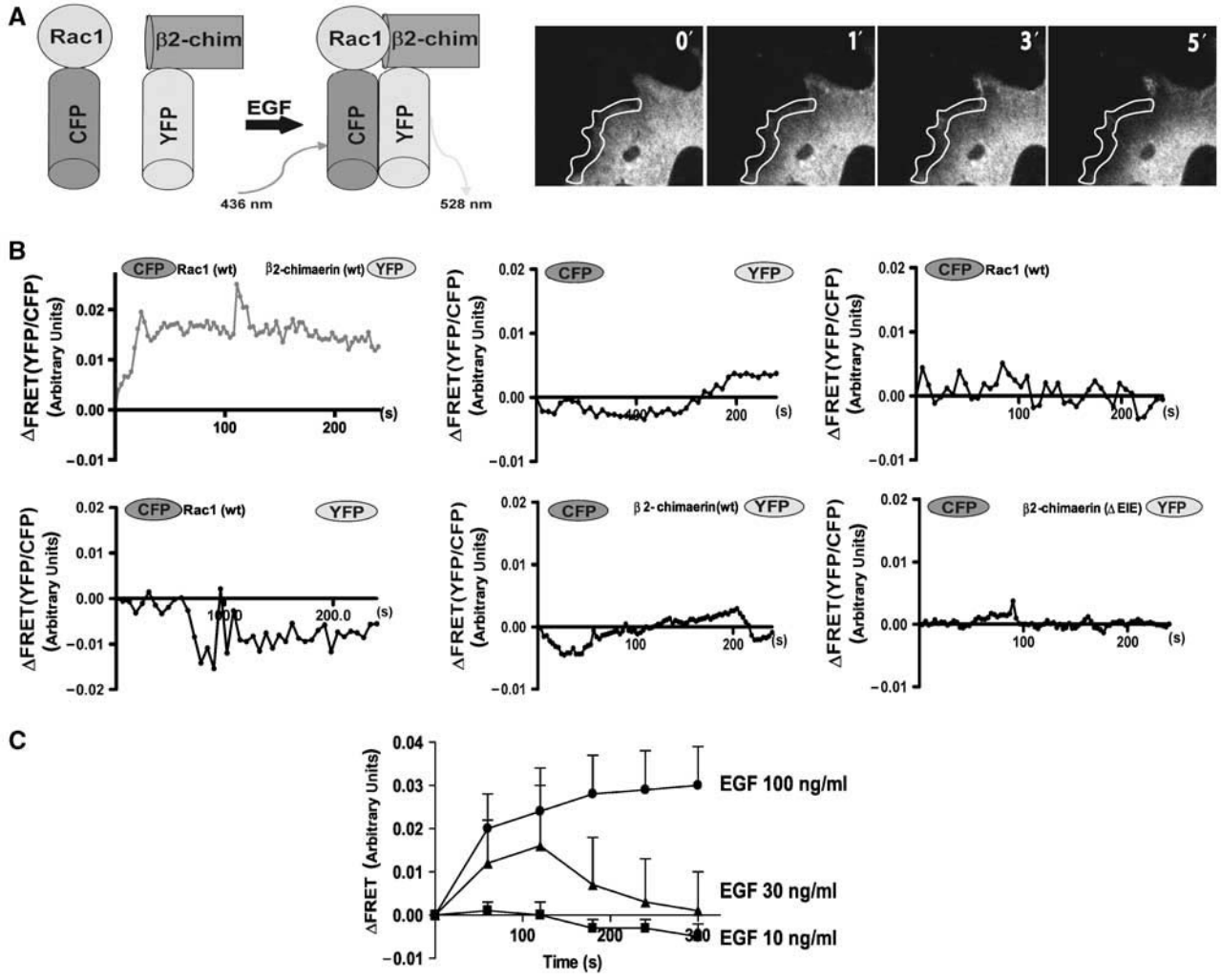


Figure 2 Analysis of β2-chimaerin–Rac1 interaction by FRET. (**A**) Schematic representation of the FRET assay to assess Rac1-β2-chimaerin association. A typical cell section selected for analysis and the peripheral translocation of YFP-β2-chimaerin in serum-starved (20 h) COS-7 cells in response to EGF (100 ng/ml) is shown. (**B**) FRET was measured every 6 s after EGF treatment (100 ng/ml). Representative experiments in cells expressing various CFP and YFP constructs are shown. Each experiment was repeated at least three times. (**C**) Effect of different concentrations of EGF in cells expressing CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type). Data are expressed as mean ± s.e. of nine cells.

Figure 1 EGF induces translocation of β2-chimaerin in a PLCγ/DAG-dependent manner. (**A**) Schematic representation of the GFP-fused constructs used in these experiments. (**B**, **C**, **E**, **G**) Localization of the various GFP-fused proteins in HeLa cells was assessed 48 h after transfection. Cells were treated with either PMA (1 μM) or EGF (100 ng/ml) for the times indicated in the figure. U73122 or U73433 (10 μM) were added 30 min before and during the incubation with EGF or PMA. In the case of EGF treatment, cells were previously serum-starved for 20 h. Cells were monitored by time-lapse confocal microscopy for the times indicated in the figure. Similar results were observed in at least three different experiments. Similar results have been observed in at least more than 10 individual cells. Colocalization of GFP-β2-chimaerin (green) and the membrane marker RFP-CAAX (red) in response to EGF (100 ng/ml) is also shown in (E). (**D**) Colocalization of GFP-β2-chimaerin (green) and the membrane marker RFP-CAAX (red) in response to EGF (100 ng/ml). (**F**) Inhibition of PLCγ1 expression in HeLa cells 48 h after transfecting the dsRNA.

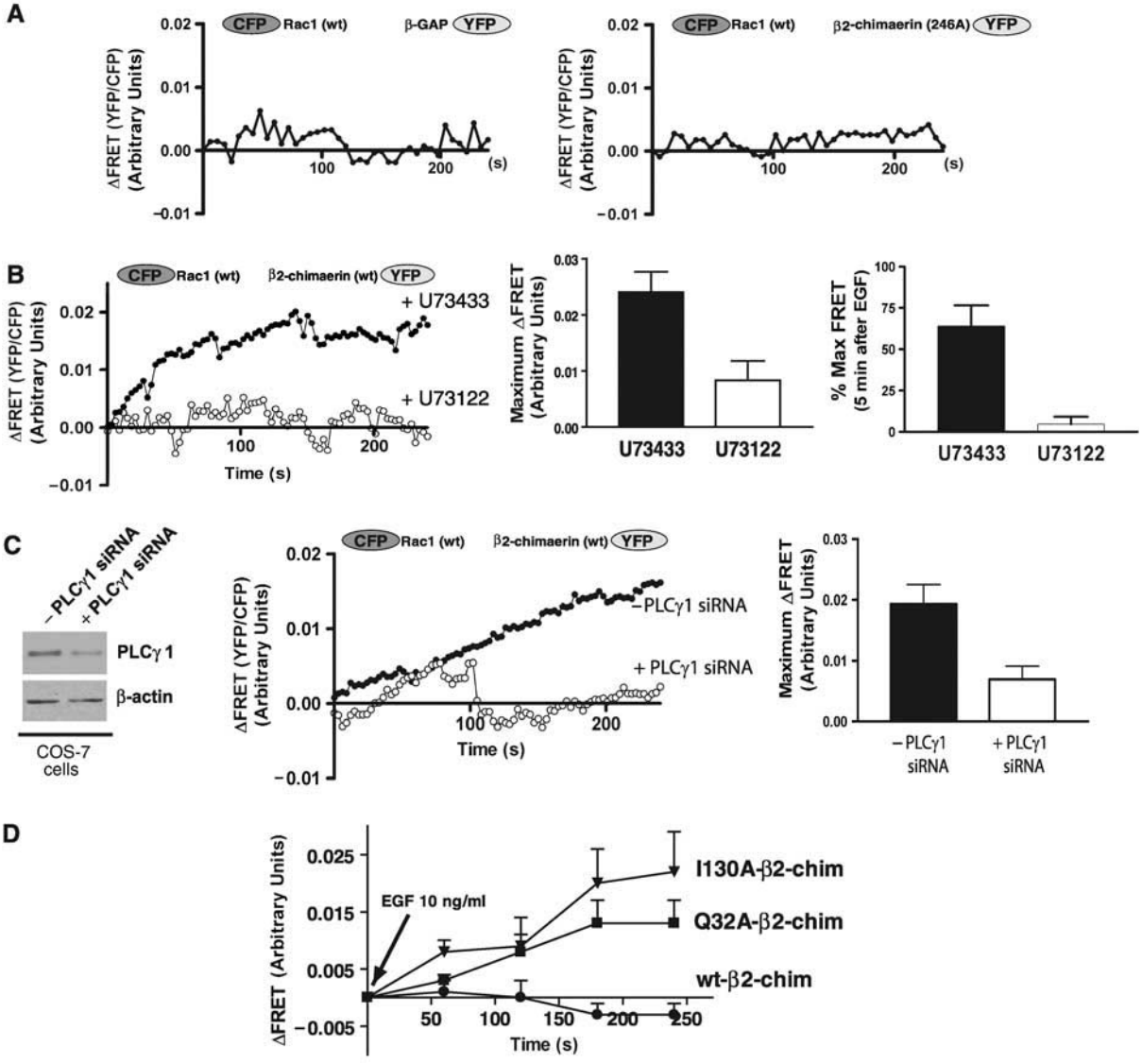


Figure 3 The β2-chimaerin C1 domain and DAG generation are required for the β2-chimaerin-Rac1 association in response to EGF. (**A**) Representative experiment showing the absence of FRET in response to EGF (100 ng/ml) in COS-7 cells coexpressing CFP-Rac1 (wild type) and YFP-β-GAP or YFP-C246A-β2-chimaerin. Each experiment was carried out at least three times. (**B**) FRET in cells coexpressing CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) was determined in response to EGF (300 ng/ml), either in the presence of the PLC inhibitor U73122 or its inactive analog U73433 (10 μM, added 30 min before and during EGF treatment). Left panel, representative experiment. FRET was measured every 3 s. Central panel, quantitative analysis of maximum FRET. Right panel, quantitative analysis of FRET 5 min after EGF treatment, expressed as the percentage of maximum obtained in each case. Data are expressed as mean ± s.e. of six cells. (**C**) FRET in PLCγ1 knocked-down COS-7 cells that coexpress CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) was determined in response to EGF (300 ng/ml). Left panel, knock down of PLCγ1 in COS-7 cells. Expression was assessed by Western blot 48 h after dsRNA transfection. Central panel, a representative experiment using 300 ng/ml EGF is shown. Right panel, quantitative analysis of maximum FRET. Data are expressed as mean ± s.e. of 10 cells. (**D**) Enhanced FRET in cells coexpressing CFP-Rac1 (wild type) and C1 domain exposed β2-chimaerin mutants fused with YFP in response to 10 ng/ml EGF. Data are expressed as mean ± s.e. of 10 cells.

YFP alone, or in cells coexpressing CFP alone and either wild type or ΔEIE-YFP-β2-chimaerin (Figure 2B). Thus, β2-chimaerin and Rac1 associate in the cell periphery upon activation of the EGFR.

β2-Chimaerin–Rac association by EGF is PLCγ-DAG dependent

We then investigated the role of DAG generation and the C1 domain in the β2-chimaerin–Rac1 interaction. DAG-unresponsive constructs YFP-C246-β2-chimaerin and YFP-β-GAP were generated. None of these mutants caused FRET in response to EGF when coexpressed with CFP-Rac1 (wild type), suggesting a strict dependence on the β2-chimaerin C1 domain for the β2-chimaerin–Rac1 association upon EGFR stimulation (Figure 3A). The involvement of DAG generation was then assessed using the PLC inhibitor U73122 and PLCγ1 RNAi. When cells expressing CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) were treated with EGF (300 ng/ml) in the presence of U73122 (10 μM), FRET was substantially lower than that observed with the inactive analog U73433 (Figure 3B). Likewise, PLCγ1 depletion in COS-7 cells significantly reduced the ability of CFP-Rac1 (wild type) and YFP-β2-chimaerin (wild type) to associate in response to EGF (Figure 3C).

Our recent structural analysis revealed that β2-chimaerin undergoes a conformational change upon activation that exposes the C1 domain. Mutated forms of β2-chimaerin with destabilized intramolecular interactions have been generated, thereby locking the molecule in a conformation in which the C1 domain exposed. We used two prototype mutants (Q32A- and I130A-β2-chimaerin, which are ~20- and 80-fold more sensitive to phorbol ester-induced translocation, respectively (Canagarajah *et al*, 2004). The prediction is that these mutants should be hypersensitive in the FRET assay in response to EGF. We used a low EGF concentration (10 ng/ml) that caused minimal FRET for wild-type YFP-β2-chimaerin. Remarkably, under this limiting experimental condition, significant FRET was observed for YFP-fused Q32A- and I130A-β2-chimaerin mutants (Figure 3D). Similarly, enhanced FRET was observed upon deletion of the N-terminal SH2 domain (YFP-C1-GAP-β2-chimaerin, aa 145–466) (data not shown), which exposes the C1 domain (Canagarajah *et al*, 2004).

A Rac-GAP-deficient β2-chimaerin mutant exhibits prolonged FRET in response to EGF

Next, we investigated whether the inactivation of β2-chimaerin Rac-GAP activity affects FRET. As Rac-GAP inactive mutants show enhanced translocation in response to EGF (see Figure 1), we predicted that this should result in sustained and/or increased FRET levels. In pull-down assays, ΔEIE-β2-chimaerin strongly associates with active Rac (Supplementary Figure S3). The construct YFP-ΔEIE-β2-chimaerin was generated and coexpressed together with CFP-Rac1 (wild type). FRET in response to EGF (30 ng/ml) in cells expressing the Rac-GAP inactive mutant was significantly higher than that observed with wild-type β2-chimaerin (Figure 4A). Remarkably, in cells coexpressing ΔEIE-β2-chimaerin and wild-type Rac1, FRET remained high even at longer time points (Figure 4A and B).

Given the ability of phorbol esters to cause both Rac activation (Menard and Mattingly, 2003; Caloca *et al*, 2003a) and β2-chimaerin peripheral redistribution, we examined whether FRET could be observed in response to PMA. Significant FRET between wild-type β2-chimaerin and Rac1 was detected in response to PMA, an effect that was quite high and sustained at concentrations greater than 30 nM (data not shown). Under limiting experimental conditions that would confer a lower and reversible FRET response (PMA 10 nM), the Rac-GAP inactive mutant ΔEIE-β2-chimaerin showed a much higher and prolonged FRET than wild-type β2-chimaerin. Indeed, at 400 s post PMA stimulation, FRET in cells coexpressing wild-type β2-chimaerin and wild-type Rac1 had returned to basal levels, but it still remained high in cells coexpressing the GAP-inactive mutant YFP-ΔEIE-β2-chimaerin and wild-type Rac1 (Figure 4C and D).

Active Rac redistributes β2-chimaerin in a DAG-dependent manner

Several recent reports have shown that Rac activation can stimulate PLC (Kanazawa *et al*, 2002; Illenberger *et al*, 2003; Snyder *et al*, 2003; Zugaza *et al*, 2004). Thus, we reasoned that activated Rac, via DAG generation, could influence β2-chimaerin localization. HeLa cells were cotransfected with plasmids encoding for HA-tagged-G12V-Rac1 together with different GFP-fused vectors for β2-chimaerin (wild type and mutants), and localization was assessed in nonstimulated, serum-starved cells. While GFP-β2-chimaerin did not show a significant degree of colocalization with wild-type Rac1 under serum-free conditions, it colocalized with constitutively

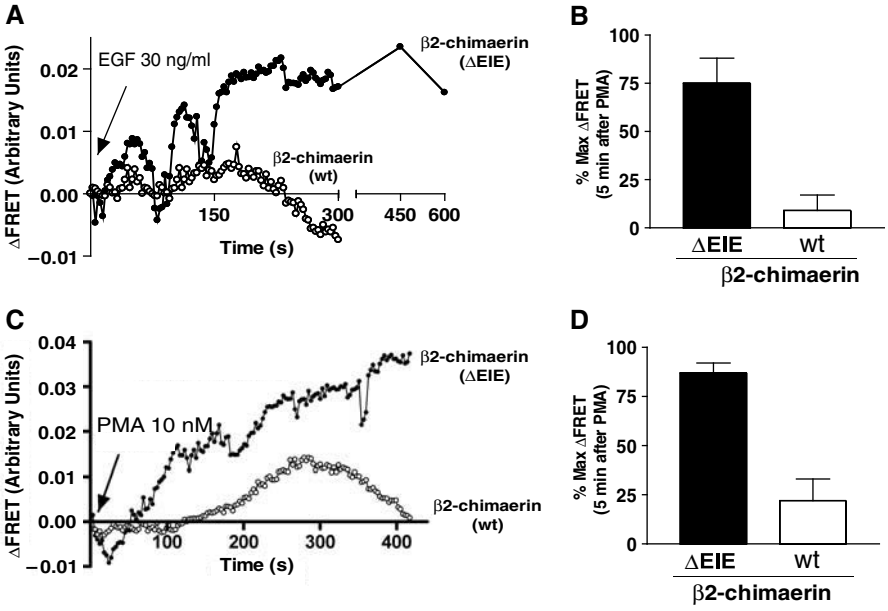


Figure 4 Enhanced Rac association of the Rac-GAP-deficient mutant ΔEIE-β2-chimaerin. (**A**) FRET in cells coexpressing YFP-ΔEIE-β2-chimaerin and CFP-Rac1 (wild type) after 30 ng/ml EGF treatment, measured every 3 s. A representative experiment comparing wild type and ΔEIE-β2-chimaerin is shown. (**B**) Quantitative analysis of FRET from (A) at 5 min. Data are expressed as mean ± s.e. of six cells. (**C**) FRET in cells coexpressing YFP-ΔEIE-β2-chimaerin and CFP-Rac1 (wild type) after 10 nM PMA treatment, measured every 3 s. A representative experiment comparing wild type and ΔEIE-β2-chimaerin is shown. (**D**) Quantitative analysis of FRET from (C) at 5 min. Data are expressed as mean ± s.e. of six cells.

active G12V-Rac1 in peripheral ruffles (Figure 5A). A GAP domain-deleted mutant comprising the N-terminal region of β2-chimaerin (N-β2-chimaerin, aa 1–291, which includes the C1 domain) showed a marked colocalization with HA-G12V-Rac1 in membrane ruffles. However, disruption of the C1 domain in this truncated mutant (N, C246A-β2-chimaerin) resulted in the loss of colocalization. Thus, despite the absence of stimulation, wild-type β2-chimaerin or mutants with an intact C1 domain localize to the periphery in G12V-Rac1-expressing cells.

To determine whether the G12V-Rac1 effect involves DAG generation, we used several approaches. First, we used a PKC C1 region fused to GFP as a DAG sensor, as initially described by Oancea *et al* (1998). While GFP-C1-PKCα is localized in the cytoplasm under basal conditions, a significant degree of peripheral colocalization with the activated small G-protein was observed in cells expressing G12V-Rac1 (Figure 5B). Second, we used GFP-fused PKCα (full length). In control, nonstimulated cells, GFP-PKCα is essentially cytoplasmic. Like the isolated C1 domain, full-length PKCα colocalizes with G12V-Rac1 in the periphery. The effect was independent of PKC activation via DAG, as it was not affected by the PKC inhibitor GF109203X (data not shown). Third, we used the PLC inhibitor U73122 (Figure 5C). We reasoned that if Rac activation leads to DAG generation via PLC, the PLC inhibitor should disrupt β2-chimaerin relocalization. As a matter of

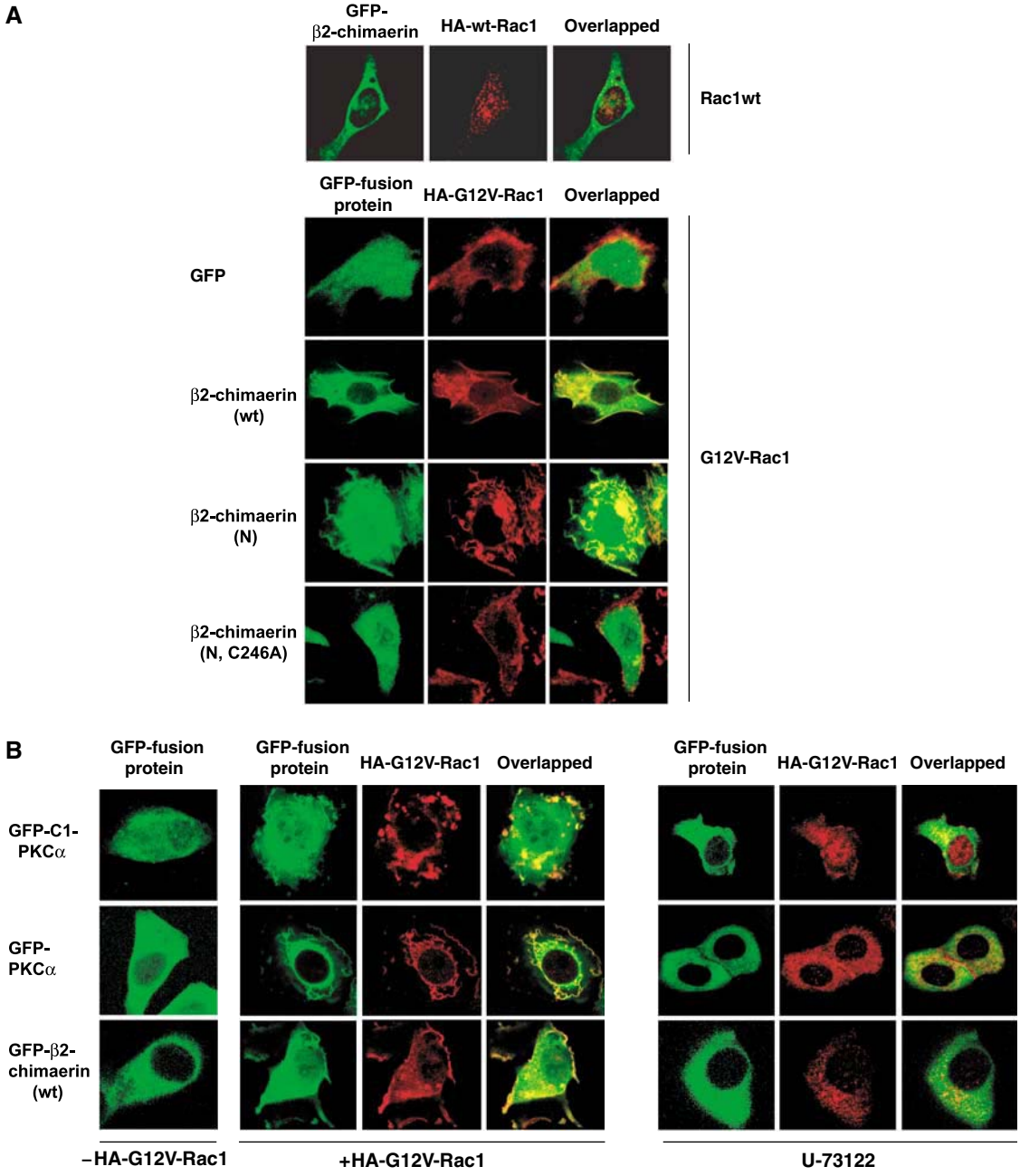


Figure 5 Redistribution of β2-chimaerin by activated Rac1 in PLC/DAG-dependent manner. HeLa cells were cotransfected with plasmids encoding for various forms of HA-tagged Rac1 and GFP-fusion proteins. After 48 h, cells were serum starved for 20 h and fixed. Cells were stained with a mouse anti-HA antibody followed by donkey anti-mouse-IgG conjugated to Cy3. (**A**) Localization of GFP-β2-chimaerin (wild type or mutants). (**B**) Localization of GFP-PKCα and GFP-PKCα C1 domain. U73122 (10 μM) was added for 60 min.

fact, treatment of serum-starved HeLa cells with U73122 reversed the peripheral localization of β2-chimaerin in G12V-Rac1-expressing cells. Some internal colocalization could be detected in the overlapped images. Translocation of GFP-ΔEIE-β2-chimaerin in response to EGF is still detectable when Rac function is inhibited with a dominant-negative Rac mutant, T17N-Rac1 (Supplementary Figure S4), which as expected impairs ruffle formation. This suggests that DAG generated via direct coupling of EGFR to PLCγ is still the dominant pathway in triggering β2-chimaerin translocation. It might be possible that indirect DAG generation via Rac-PLC contributes to sustain the association of β2-chimaerin to membranes.

β2-Chimaerin inhibition of EGF-mediated responses and the effect of β2-chimaerin depletion

Limited information is available on the cellular effects of β2-chimaerin. EGF causes a rapid and transient activation of Rac, as determined using a PBD pull-down assay. Expression of β2-chimaerin using an adenovirus (HA-β2-chim AdV) significantly impaired the elevation in Rac-GTP levels caused by EGF. A control AdV, LacZ-AdV, which has the same backbone as β2-chim-AdV, was ineffective. The effect was proportional to the expression levels of β2-chimaerin (Supplementary Figure S5). EGF also caused significant activation of RhoA and to a lesser extent Cdc42 in HeLa cells. As predicted from *in vitro* studies (Caloca *et al*, 2003a), elevations in RhoA-GTP and Cdc42-GTP levels in response to EGF were not affected by overexpression of β2-chimaerin (Figure 6A and Supplementary Figure S5). Expression of HA-β2-chimaerin did not seem to affect EGFR function, as judged by its lack of effect on EGFR autophosphorylation, including phosphorylation on Tyr 992, the PLCγ docking site (Figure 6B).

Ectopic expression of β2-chimaerin also caused striking effects on Rac-dependent functional responses. Actin cytoskeleton reorganization in response to EGF was markedly impaired in cells that ectopically express GFP-fused β2-chimaerin, an active C-terminal β2-chimaerin GAP domain (β-GAP), C246A-β2-chimaerin (Figure 6C), or the active mutant GFP-Q32A-β2-chimaerin (data not shown). On the other hand, the Rac-GAP-deficient mutant ΔEIE-β2-chimaerin had no effect on actin cytoskeleton reorganization (Figure 6C). β2-Chimaerin also affects cell motility (Figure 6D). Migration of MCF-7 breast cancer cells in response to EGF was drastically reduced in cells infected with the β2-chim-AdV. Maximum inhibition was observed at an MOI of 100 PFU/cell. On the other hand, LacZ-AdV (100 MOI) did not affect migration. In MCF-7 cells, EGF (100 ng/ml, 1 min) caused a 3.3 ± 0.5-fold increase in Rac1-GTP levels relative to non-stimulated cells ($n = 3$), which was reduced by 55 and 70% upon infection with 50 and 100 MOI β2-chim AdV, respectively (data not shown). To further assess the role of β2-chimaerin on migration, we generated a stable MCF-7 cell line that expresses active Rac (G12V-Rac1). As expected, these cells have high levels of basal Rac1-GTP compared to control (vector-transfected) cells (Figure 6D). Consistent with the well-established role of Rac1 in migration, G12V-Rac1-expressing MCF-7 cells showed a ~4-fold increase in migration under basal conditions. EGF did not cause any additive effect. In marked contrast to the effect observed in control MCF-7 cells, adenoviral delivery of β2-chimaerin (50–100 MOI) into G12V-Rac1-MCF-7 cells did not impair migration (Figure 6E) or the constitutively elevated Rac1-GTP levels (data not shown). Expression of β2-chimaerin upon infection with the β2-chim-AdV was similar in both control and G12V-Rac1-MCF-7 cells (data not shown). Thus, β2-chimaerin inhibits Rac activity and Rac-mediated cellular effects upon EGFR activation.

Last, we studied the effect of β2-chimaerin depletion on Rac activation by EGF. Depletion of β2-chimaerin by delivery of a specific RNAi duplex into HEK 293 cells (which express this chimaerin isoform) significantly extended the duration of the Rac activation response caused by EGF. Indeed, while at 5 min after EGF stimulation, Rac1-GTP levels returned almost to basal in control cells, they remained elevated in β2-chimaerin-depleted cells (Figure 6F). In HeLa cells, which express both α2- and β2-chimaerins, a remarkable potentiation of Rac activation by EGF was observed upon depletion of both chimaerin isoforms (Supplementary Figure S6). Taken together, these results support a model in which chimaerins serve as modulators of Rac activation in response to EGF.

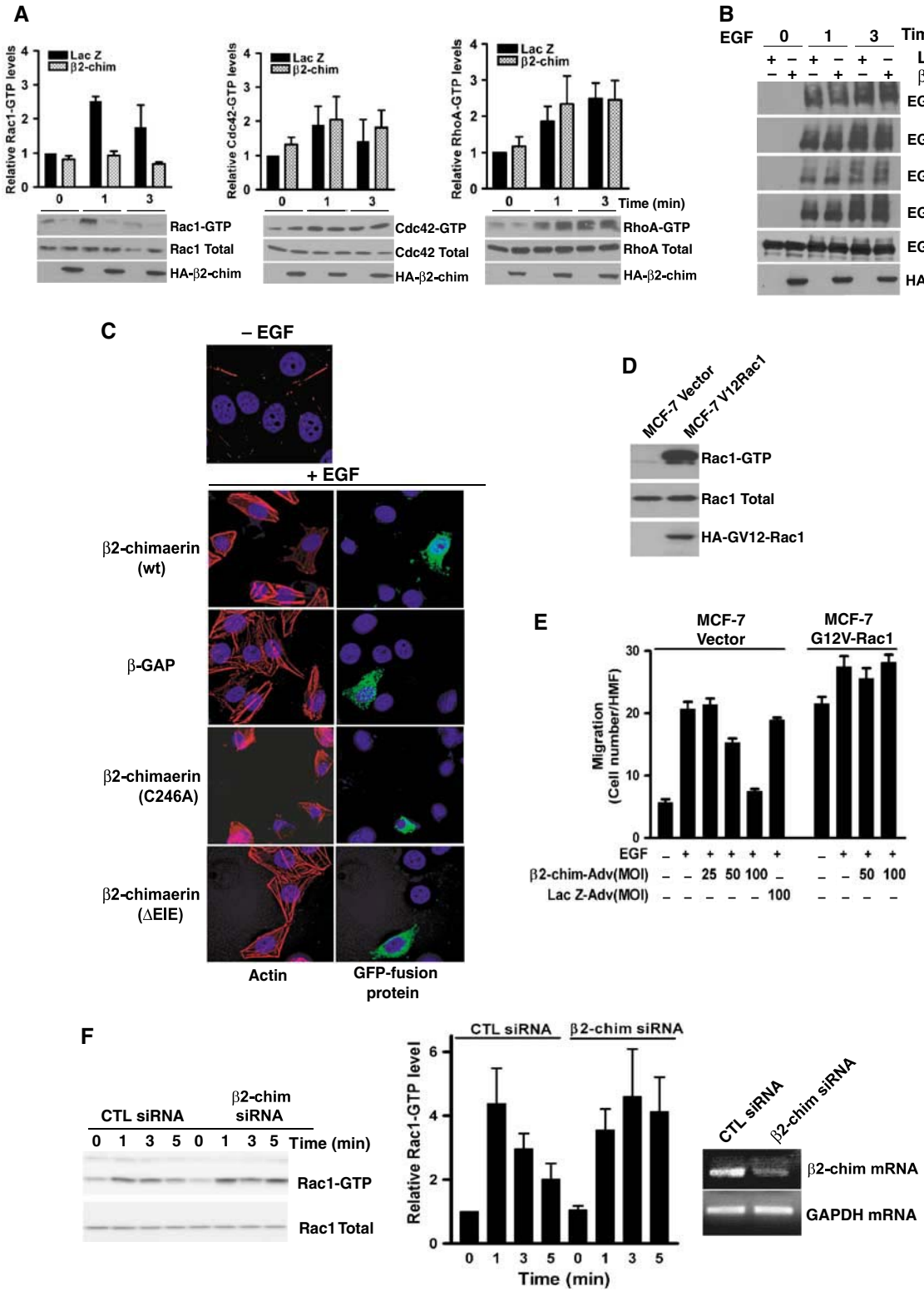
Discussion

The results presented in this paper provide the first evidence for a receptor-mediated pathway for the inactivation of the Rac small G-protein via DAG. Although the role of DAG as a PKC activator is firmly established, our results reveal a previously unappreciated role for this lipid second messenger as a self-limiting regulator of Rac signaling upon activation of a receptor tyrosine kinase. β2-Chimaerin is a target of DAG generated upon EGFR stimulation, and therefore it exemplifies a distinctive paradigm of a Rac-GAP regulated by cell surface receptors in a PLCγ-dependent manner. A model summarizing these concepts is presented in Figure 7.

β2-Chimaerin: a DAG-regulated, Rac-specific GAP

Like PKCs, β2-chimaerin is subject to positional regulation by receptors coupled to DAG generation. We postulate that a major purpose for this redistribution is to facilitate its association with Rac at peripheral sites. The interaction will occur preferentially when Rac is in its active, GTP-bound state. The impaired targeting of β2-chimaerin mutants lacking a functional C1 domain suggests an absolute requirement for the DAG-binding motif. Biochemical evidence and structural predictions from the recently solved structure of β2-chimaerin also support a model for lipid-mediated allosteric activation of Rac-GAP activity upon C1 domain ligand binding, which involves an extensive cooperative dissociation of intramolecular interactions. β2-Chimaerin exists in an active state (open conformation) and an inactive state (closed conformation) in which the C1 and GAP domains are not fully exposed (Canagarajah *et al*, 2004). As acidic phospholipids enhance β2-chimaerin Rac-GAP activity (Caloca *et al*, 2003a), the prediction is that its association with membrane lipids competes with intramolecular interactions, thus causing a major conformational change that removes steric inhibitions and exposes the Rac-GAP active site (Canagarajah *et al*, 2004). The dynamics of the conformational change may be substantially influenced by the nature of the stimuli. The short-lived nature of DAG may represent a major factor in the reversibility of the activation status of β2-chimaerin and its transient redistribution upon EGFR stimulation, and this may explain why complete translocation is not observed in this case. The

long-lasting phorbol esters, on the other hand, cause a more sustained β2-chimaerin translocation. Experiments using the active state mutants further support this concept. Indeed, I130A-β2-chimaerin is ~80-fold more sensitive to PMA-induced translocation (Canagarajah *et al*, 2004) and highly sensitive to EGF in the FRET assays. It is not known at the present time whether other levels of regulation, such as phosphorylation or binding to specific partners, control this conformational switch, as described for other GAPs (Takai *et al*, 2001). β2-Chimaerin is subject to serine phosphorylation (unpublished results), suggesting additional regulatory mechanisms for activation. It is possible that translocation depends on the phosphorylation status, and that only a fraction of the protein is available for translocation and activation.



While there is significant redundancy in the specificity of Rho GAPs, at least *in vitro* (Takai *et al*, 2001), β2-chimaerin possesses specificity towards Rac, and it does not accelerate GTP hydrolysis from either RhoA or Cdc42 in *in vitro* GAP assays (Caloca *et al*, 2003a). This conclusion supports the predictions from the 3-D structure of β2-chimaerin (Canagarajah *et al*, 2004), which shows unfavorable steric and electrostatic interactions with RhoA or Cdc42 that negate catalysis towards these GTPases. β2-Chimaerin also inhibits Rac-mediated responses in cells, including actin cytoskeleton reorganization and migration. The inability of β2-chimaerin to inhibit motility of cells expressing constitutively active G12V-Rac1 further support this conclusion. Interestingly, β2-chimaerin was found to inhibit invasive and metastatic properties of murine breast cancer cells (Menna *et al*, 2003), responses in which Rac plays a prominent role. Moreover, adenoviral delivery of β2-chimaerin into MCF-7 breast cancer cells causes G1 arrest due to a reduction of cyclin D1 levels and Rb dephosphorylation. These results fit well with the established role of Rac in the control of cyclin D1 expression and cell cycle progression (Ridley, 2001). These effects cannot be observed in MCF-7-G12V-Rac1 cells but still occur in cells expressing an active form of RhoA (Yang *et al*, 2005).

Role of the GAP domain and Rac-induced generation of DAG

The peripheral localization of β2-chimaerin in cells expressing G12V-Rac1 is noteworthy. More strikingly, this localization was impaired by PLC inhibition. This suggests that Rac, via activation of a PLC-DAG axis, may contribute to the positional regulation of β2-chimaerin by sustaining DAG production. Several reports have recently suggested a potential link between the activation of Rac and DAG signaling. For example, active Rac, but not other Rho GTPases, bind to and activate PLCβ and PLCε (Illenberger *et al*, 2003; Snyder *et al*, 2003), causing sustained DAG generation. An interesting report by Caloca *et al* (2003b) showed that in T cells both the Rac-GEF Vav and constitutively active Rac lead to the activation of the phorbol ester receptor RasGRP1 (a Ras-GEF) in a PLC-dependent manner, which supports a novel hierarchical role for Rac upstream of DAG generation and Ras activation. Further support for this hypothesis is provided by the observed peripheral localization of DAG sensors (PKCα or its C1 domain) in G12V-Rac1-expressing cells (Figure 5B), which suggests localized DAG generation by activated Rac. These results suggest that Rac-mediated activation of PLC may represent a relevant factor in regulating the spatial distribution of β2-chimaerin and other DAG receptors through their C1 domains. This pathway does not seem to be dominant, since a dominant-negative Rac mutant does not impair translocation. We speculate that the Rac-PLC-DAG pathway participates in regulating temporal association of chimaerins. PLCγ may also influence actin rearrangement, since loss of PLC function results in impaired ruffle formation (Kurokawa *et al*, 2004; see also Figure 5), suggesting complex regulatory mechanisms.

Our results show that the Rac-GAP activity of β2-chimaerin also influences its own spatial and temporal redistribution. Indeed, upon EGFR stimulation, the Rac-GAP-deficient mutant ΔEIE-β2-chimaerin shows a much more prolonged translocation and sustained association with Rac1 compared to wild-type β2-chimaerin, as revealed by FRET. Thus, Rac-GTP possibly contributes to the membrane stabilization of β2-chimaerin. This may be achieved both by DAG generation via Rac, as discussed above, and by direct association to the small G-protein, as suggested by the preferential binding of β2-chimaerin to membrane-associated, active Rac. A likely scenario is that membrane anchoring of β2-chimaerin is reversed when it causes GTP hydrolysis from Rac, both as a consequence of a decline in Rac-GTP and in DAG levels. Thus, translocation of β2-chimaerin triggers the signal that causes its own dissociation from the membrane, which makes this mechanism highly dynamic. Other factors that might potentially control the magnitude of the Rac-GAP response include receptor internalization and the involvement of lipid microdomains.

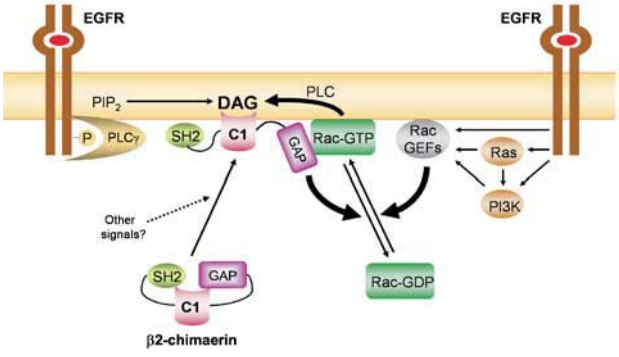


Figure 7 Model for EGFR-mediated regulation of β2-chimaerin.

Figure 6 β2-Chimaerin inhibits EGF-induced activation of Rac1 and Rac-mediated responses. (**A**) HeLa cells were infected with a recombinant HA-β2-chim-AdV or a LacZ-AdV (MOI 10 PFU/cell, 16 h). Cells were serum-starved for 20 h and then treated with EGF (100 ng/ml) for the times indicated in the figure. Active Rac1, Cdc42, and RhoA were measured using pull-down assays. Lower panels, representative experiments. Expression of HA-tagged β2-chimaerin was assessed using an anti-HA antibody. Upper panels, densitometric analysis of active GTPase levels, normalized in each case to the total immunoreactivity in cell lysates. Results are expressed as % of control (LacZ-AdV, without EGF), and represent the mean ± s.e. ($n = 3$). (**B**) Analysis of EGFR phosphorylation by Western blot using antibodies that recognized various phosphotyrosine sites. (**C**) Inhibition of actin reorganization by β2-chimaerin. HeLa cells transfected with vectors encoding for various GFP-β2-chimaerin forms (green cells) were serum-starved for 20 h and then treated with EGF (100 ng/ml, 10 min). Cells were fixed, stained with rhodamine–phaloidin for actin staining (red), and DAPI (blue), and analyzed by microscopy. Similar results have been observed in at least 10 cells. (**D**) Rac1-GTP levels in MCF-7 cells stably expressing G12V-Rac1. (**E**) Migration assay. Control (vector-transfected) MCF-7 cells and G12V-Rac1-MCF-7 cells were infected overnight in serum-free medium with either β2-chim-AdV or LacZ-AdV at different MOIs. AdVs were removed 20 h later by extensive washing in serum-free medium. Migration was assessed in response to EGF (50 ng/ml) using a Boyden chamber for a period of 5 h. Values corresponding to cell number per HMF are expressed as the mean ± s.e. of four independent experiments. (**F**) At 48 h after transfection with dsRNA for β2-chimaerin or control duplex (*CTL*), HEK 293 cells were stimulated with EGF (100 ng/ml) for different times, and Rac-GTP levels were determined using the PBD pull-down assay. Left panel, representative experiment. Middle panel, densitometric analysis of Rac-GTP levels normalized to total Rac in each case ($n = 3$). Results were expressed as fold-increase relative to control in the absence of EGF. Right panel, RT–PCR for β2-chimaerin in HEK 293 cells.

Role of β2-chimaerin in EGFR signaling

Rac activation by tyrosine-kinase receptors is regulated mainly by GEFs, which are subjected to multiple levels of regulation, including tyrosine phosphorylation. Activation of Rac-GEFs via EGFR involves the PI3K and Ras arms (Schmidt and Hall, 2002; Mertens *et al*, 2003). More recently, a role for Sos-1 as a dual, Abl-regulated Ras/Rac GEF has been described (Sini *et al*, 2004). Thus, the EGFR is capable of activating both Rac-GEFs and Rac-GAPs. The PLCγ branch of the EGFR signaling pathway is responsible for Rac inactivation and makes the activation of the small G-protein self-limiting, as also supported by our chimaerin depletion studies using RNAi. In that regard, this is analogous to p120Ras-GAP, although this Ras negative switch is mediated by binding of the Ras-GAP SH2 domain to the growth factor receptor. Whether in addition to the C1 domain the N-terminal SH2 domain in β2-chimaerin contributes to positional regulation is currently under investigation. It is likely that a similar paradigm may exist for other tyrosine-kinase receptors which couple both to PLCγ and Rac activation via GEFs, such as the PDGF receptor (Moores *et al*, 2000). Although the temporal sequence of events in the context of different tyrosine-kinase receptors requires further investigation, β2-chimaerin or other chimaerin isoforms may represent a common regulatory mechanism to turn off Rac activity for receptors coupled to both PLCγ and Rac-GEFs.

Concluding remarks

In summary, our results have established that the Rac-GAP β2-chimaerin is a component of the EGFR-PLCγ-DAG pathway, and implicate the lipid second messenger DAG as the trigger of a negative switch that serves for the attenuation of Rac1 activity. The β2-chimaerin C1 domain has a fundamental role in positioning this Rac-GAP in proximity to its target, Rac1, at the plasma membrane. Our studies also have major implications for the pathogenesis of cancer. β2-Chimaerin is downregulated in glioma (Yuan *et al*, 1995) and breast cancer (Yang *et al*, 2005). As hyperactivation of the EGFR pathway and dysregulation of Rac or Rac activators/effectors is implicated in the development and progression of many types of cancers, it is likely that the chimaerin Rac-GAPs could represent an important regulatory component of malignant transformation and invasion.

Materials and methods

Materials

EGF was purchased from Roche Molecular Biochemicals. PMA and GF 109203X were purchased from LC Laboratories (Woburn, MA). Cell culture reagents were obtained from Invitrogen Life Technologies. Reagents for the expression and purification of GST-fusion proteins were from Amersham Biosciences, Inc. U73122 and U73433 were purchased from Sigma.

Cell culture and transfections

HeLa, HEK 293, COS-7, COS-1, and Neuro-2a cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Transfection of plasmids (1–2 μg) was carried out with FuGENE-6 (COS cells) or ExtremeGENE (HeLa cells). Generation of G12V-Rac1 MFC-7 cells is described elsewhere (Yang *et al*, 2005).

Plasmid constructions and site-directed mutagenesis

Generation of the various vectors encoding for β2-chimaerin GFP-fusion proteins was described elsewhere (Wang and Kazanietz, 2002). Vectors for β2-chimaerin YFP-fusion proteins were generated by ligation in-frame of the corresponding inserts into *Xho*I–*Bam*HI sites of pEYFP-N1 (Clontech). A cDNA encoding wild-type Rac1 was inserted in-frame into *Eco*RI–*Bam*HI sites of pECFP-C1 (Clontech). Generation of β2-chimaerin mutants C246A and ΔEIE (298–300) is described elsewhere (Caloca *et al*, 2003a). A pEGFP vector encoding for GFP-PKCα was a kind gift of Dr Peter M Blumberg (NIH). A construct encompassing the C1a-C1b region (aa 1–168) in PKCα was generated by PCR and subcloned into *Eco*RI and *Bam*HI sites of pEGFP-C2 (Clontech).

RNAi

The following target sequences were used: AAGACGATGGACCTC CCCTTC (PLCγ1), AGCTGCCGAGTACATTTCA (β2-chimaerin), and AACATCGCTGTAGCATCGTCT (control duplex). dsRNAs were purchased from Dharmacon (Lafayette, CO), and were transfected alone (50 or 100 nM) into HeLa or HEK 293 cells using Oligofectamine (Invitrogen Life Technologies). Maximum inhibition of expression was achieved ~48 h after transfection. Protein depletion was assessed by Western blot unless levels were below limit of detection with the β2-chimaerin antibody. In that case (HEK 293 cells), RT–PCR was performed. Total RNA and cDNA preparation was carried out as described before (Yang *et al*, 2005). The following PCR primers were used: GCTGCCGAGTACA TTTCAAAAA and CTCATAATTGAAGTGGTTGTCG (β2-chimaerin); and TGAAGGTCGGAGTCAACGGATTT and GATGGGATTTCCATTG ATGACAAGC (GAPDH).

Western blots

The following antibodies were used for Western blots: anti-β2-chimaerin (1:1000, Wang and Kazanietz, 2002), anti-HA (1:3000, Covance), anti-phospho-EGF (pTyr 845, 992, 1045, and 1068, 1:1000, Cell Signaling), anti-Rac (1:3000, Upstate), anti-Cdc42 (1:3000, Upstate), and anti-RhoA (1:3000, Santa Cruz Biotechnology), anti-PLCγ1 (Upstate), anti-AU5 (1:3000, Covance). Immunoreactive bands were visualized by chemiluminescence. Densitometry analysis was performed using AlphaImager™ system (Alpha Innotech).

Adenoviral constructs and infections

Generation of a recombinant HA-tagged β2-chimaerin AdV using the AdEasy System (Stratagene) is described elsewhere (Menna *et al*, 2003). A control LacZ-AdV using the same parent vector was also generated. For infection, 2×10^5 cells were seeded onto six-well plates in DMEM with 5% FBS. At 2 h before infection, cells were incubated in serum-free DMEM. Cells were then infected with the corresponding AdVs for 24 h at different MOIs and then serum starved for 20 h before EGF treatment.

Affinity-precipitation of GTP-bound Rac, Cdc42, and Rho

GTP-Rac1 and GTP-Cdc42 levels were determined using a PBD 'pull-down' assay, as previously described (Wang and Kazanietz, 2002), followed by the detection of the active GTPase with either anti-Rac1 or anti-Cdc42 antibodies. For the determination of GTP-RhoA, a rhotekin 'pull-down' assay was used (Ren *et al*, 1999). Briefly, HeLa cells were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Cell lysates were cleared by centrifugation (13 000 *g*, 10 min, 4°C), and then incubated with GST-RBD beads (25 μg) for 60 min at 4°C. The pulled-down GTP-RhoA in the beads was detected by Western blot using an anti-RhoA antibody. In all cases, the levels of active GTPases were normalized to the total levels in cell lysates.

Co-precipitations and co-immunoprecipitations

COS-1 cells at ~50% confluence were cotransfected with pEBG vectors encoding for Rac (wild type or mutants, kind gifts from Dr Margaret M Chou, UPenn). After 24 h, cells were infected with β2-chim-AdV, as described above. In some experiments, cotransfection with pEGFP-ΔEIE-β2-chimaerin was carried out instead of the adenoviral infection. After 24 h, cells were washed twice with cold PBS and lysed (30 min at 4°C) in 300 μl of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5%

deoxycholate, 0.1% SDS, and protease inhibitors (5 μg/ml AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin A). Glutathione-Sepharose 4B beads (20 μl) were added to the lysate and incubated overnight at 4°C. The beads were extensively washed in lysis buffer, boiled, and analyzed by Western blot. Co-immunoprecipitation assays are described elsewhere (Wang and Kazanietz, 2002).

Localization studies and immunofluorescence

Cells transfected with plasmids encoding for the different GFP-fused proteins were grown in glass-bottomed culture dishes (MatTek, Ashland, MA) and cultured for at least 16 h before being subject to various treatments. Living cells were visualized with a confocal laser scanning fluorescence microscope (LSM 410 or 510; Carl Zeiss). All experiments were performed at 37°C. Immunofluorescence using an anti-HA antibody is described elsewhere (Wang and Kazanietz, 2002). In some experiments, the membrane marker RFP-CAAX (kind gift of Dr Margaret M Chou, UPenn) was used in co-transfections.

Migration assays

Cells were cultured in six-well plates for 24 h, and then serum-starved for 8 h. Cells were then infected with the different AdVs (16 h), washed, and suspended in serum-free DMEM with 0.1% BSA. Migration was determined using a Boyden chamber (1×10^5 cells/well, 37°C, 5 h). For each treatment, at least four randomly selected high magnification fields (HMF, 10×20) were counted. Each treatment was performed in quadruplicate.

FRET

COS-7 cells were transfected with CFP- and YFP-based vectors, and imaged 48 h after transfection. Optical recordings were performed using a Nikon Eclipse TE2000 inverted microscope with a q-imaging Exi digital cooled camera (1360×1036 pixels, Burnaby, Canada). A dual-view emission beamsplitter (Optical Insights, Santa Fe, New Mexico) was used to display two wavelengths simultaneously on the CCD camera chip. Recordings were done using Northern Eclipse 6.0 software (Empix Imaging Inc., Cheektowaga, NY). A $\times 40$ planar objective (Nikon) was used for all recordings. For FRET measurements, the excitation filter 436/10, emission filters 480/30 (cyan) and 535/40 (yellow), and 505 dichroic mirror (all from Chroma Technology, Brattleboro, VT) were used. Exposures of 500–1000 ms were taken every 3 or 6 s after the addition of either EGF or PMA, and the CFP and YFP image stacks were converted to ratio stacks using IonWaveN for data analysis in EXCEL. Image analysis, including regional measurements and background substraction, was performed using IonWaveN software (Empix Imaging Inc.). Cells with similar level of basal fluorescence, within a 30% range, were used in the experiments. Results were expressed as changes in YFP/CFP ratio (ΔFRET), in arbitrary units.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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