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Diacylglycerol kinase γ interacts with and activates β2-chimaerin, a Rac-specific GAP, in response to epidermal growth factor

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Abstract Diacylglycerol kinase (DGK)γ was shown to act as an upstream suppressor of Rac1. Here we report that, in COS7 cells stimulated with epidermal growth factor (EGF), DGKγ specifically interacts and co-localizes at the plasma membrane with β2-chimaerin, a GTPase-activating protein (GAP) for Rac. Moreover, DGKγ enhanced EGF-dependent translocation of β2-chimaerin to the plasma membrane. Interestingly, DGKγ markedly augmented EGF-dependent GAP activity of β2-chimaerin through its catalytic action. These results indicate that DGKγ is a novel regulator of β2-chimaerin, and thus suggest that β2-chimaerin is an effector molecule, linking DGKγ functionally with Rac1.

Keywords: Diacylglycerol kinase; Chimaerin; GTPase-activating protein; Epidermal growth factor; Diacylglycerol; Phosphatidic acid

1. Introduction

It is well recognized that a variety of lipid second messengers in low abundance carry out specific tasks for a wide range of biological processes in eukaryotic cells. The cellular concentrations of such signaling lipids must be strictly regulated by the action of metabolic enzymes. Diacylglycerol (DAG) kinase (DGK) phosphorylates DAG to yield phosphatidic acid (PA) [1–3]. DAG is an established activator of conventional and novel protein kinase Cs (PKCs), Unc-13 and Ras guanyl nucleotide-releasing protein [4,5]. PA has also been reported to regulate a number of signaling proteins such as phosphatidylinositol-4-phosphate 5-kinase (PIP5K), Ras GTPase-activating protein, Raf-1 kinase and atypical PKC [2,3,6,7]. In this context, it is of interest to note that chimaerin, a Rac-specific GTPase activating protein (GAP), is activated by both DAG [4,5,8] and PA [9,10].

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Abbreviations: DGK, diacylglycerol kinase; DAG, diacylglycerol; PA, phosphatidic acid; PKC, protein kinase C; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; EGF, epidermal growth factor; GAP, GTPase-activating protein; GFP, green fluorescent protein; EGFP, enhanced GFP; ECFP, enhanced cyan fluorescent protein; PDGF, platelet-derived growth factor; RT, reverse transcriptase; GST, glutathione *S*-transferase; PBD, p21-binding domain; PAK, p21 activated kinase; siRNA, small interfering RNA; WT, wild-type

Mammalian DGK is known to exist as a large protein family consisting of 10 isozymes classified into five subtypes according to their structural features [1–3]. The type I DGKs consisting of α, β, and γ-isozymes contain two sets of Ca^{2+}-binding EF-hand motifs at their N-termini. The tissue- and cell-dependent expression patterns detected distinctively for these isozymes suggest that, even belonging to the same subfamily, each member exerts differentiated functions in particular types of cells. Moreover, we already reported that the EF-hand motifs of the type I DGKs have properties distinct from each other with respect to affinities for Ca^{2+} and to Ca^{2+}-induced conformational changes [11].

Rac1 is a member of the Rho small GTPase family together with RhoA and Cdc42. The active form of Rac1 interacts with various effectors to initiate downstream signaling events that control cell morphology, actin dynamics, migration, metastasis, gene expression, apoptosis and cell cycle [12,13]. 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 the three groups of effector molecules: guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors, and GAPs.

While there is a significant redundancy in the specificities of Rho GAPs at least in vitro, chimaerin possesses specificity toward Rac, and it does not accelerate GTP hydrolysis for either RhoA or Cdc42 in vitro and in vivo [4,5,14]. Chimaerin comprises a family of four isoforms (α1- or 'n'-, α2-, β1- and β2-chimaerins), which are splice variants of the α- and β-chimaerin genes. The common structural feature among all chimaerins is, in addition to the GAP domain, the presence of the C1 domain that was first found in PKC [4,5]. Although these chimaerin Rac-GAPs are activated in response to several cell stimuli [4,5,14], their activation mechanisms are not fully understood.

We previously reported that DGKγ acts as an upstream suppressor of Rac1, but not Cdc42 [15]. However, effector molecules that mediate the function of DGKγ remain unclear. In the present study, we report that DGKγ specifically interacts with and activates β2-chimaerin in response to cell stimulation with epidermal growth factor (EGF) and propose that β2-chimaerin is an effector molecule operating between DGKγ and Rac1.

2. Materials and methods

2.1. Materials

Anti-DGKγ polyclonal antibody was generated as described previously [16]. Other antibodies were obtained from commercial

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sources as follows: anti-FLAG M2 (Sigma-Aldrich, St. Louis, MO), anti-c-myc (9E10, Roche, Indianapolis, IN), anti-green fluorescent protein (GFP) (B-2, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFP polyclonal antibody (Takara-Clontech, Tokyo, Japan), and anti-Rac1 (23A8, Upstate Biotechnology, Lake Placid, NY). Platelet-derived growth factor (PDGF)-BB was from Sigma-Aldrich. EGF was from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Plasmid constructs

The cDNA coding for human DGKγ [17] was amplified by PCR and inserted into the *Hin*d III–*Xho* I site of pCMV-Tag3B (Stratagene, La Jolla, CA) for N-terminal c-myc tagging. pEGFP (enhanced GFP)-DGKγ, pEGFP-DGKγ-G494D (a kinase-dead mutant) and p3 × FLAG-DGKγ were generated as described previously [18]. To generate p3 × FLAG-α1-, α2-, and β2-chimaerins, α1-, α2- and β2-chimaerin cDNAs were amplified by PCR from human brain QUICK-clone cDNA (Takara-Clontech) using the following primers: α1-chimaerin, 5′-CCGAATTCAATGAAACTGGTTCTCCAAAGTC-3′ and 5′-CCGTCGACTTAAAATAAATGTCTTCGTTTTTGATAAGC-3′; α2-chimaerin, 5′-TTCGAATTCTATGGCCCTGACCCTGTTTG-3′ and 5′-TACCGTCGACTTAAAATAAAATGTCTTCGTTTTTG-3′; β2-chimaerin, 5′-CCGAATTCGATGGCAGCGTCCAGCAACTCC-3′ and 5′-CCGTCGACGGATTAGAATAAAACGTCTTCGTTTC-3′. The PCR fragments of α1-, α2- and β2-chimaerins were digested with *Eco*RI and *Sal*I and inserted into p3 × FLAG-CMV-7.1 (Sigma-Aldrich). To construct pECFP (enhanced cyan fluorescent protein)-β2-chimaerin and pDsRed-monomer-β2-chimaerin, an *Eco*RI–*Sal*I cut fragment of p3 × FLAG-β2-chimaerin was inserted into pECFP-C1 and pDsRed-monomer-C1 (Takara-Clontech), respectively. The authenticity of the constructs was confirmed by DNA sequencing.

2.3. Cell culture and transfections

NIH3T3 and COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. NIH3T3 cells ($\sim 1 \times 10^6$ cells per 60-mm dish) were transfected with 10 nM RNA oligonucleotides (see Section 2.4) using HiPerFect (QIAGEN, Tokyo) according to the instructions from the manufacturer. After 48 h, cells were serum-starved for 5 h in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and stimulated with 50 ng/ml PDGF-BB for 5 min. COS7 cells ($\sim 1 \times 10^6$ cells per 60-mm dish) were transiently transfected with plasmids (1 μg) using Effectene (QIAGEN) according to the instructions from the manufacturer. After 24 h, cells were serum-starved for 5–15 h in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and stimulated with 100 ng/ml EGF for 2–10 min.

2.4. RNA interference and reverse transcriptase (RT)-PCR

To silence the expression of mouse DGKγ (GenBank Accession No. NM138650) and β2-chimaerin (GenBank Accession No. AK131641), the following RNA oligonucleotides (iGENE Therapeutics, Tsukuba, Japan) were used: DGKγ, sense: 5′-UGUGUGAAAACAAACUCCAAAGCCAAG-3′, antisense: 5′-UGGCUUUGGAGUUUGUUUUCACACAAU-3′; β2-chimaerin, sense: 5′-UGAGCAGGUCUAAAAACGAAUCAAGAG-3′, antisense: 5′-CUUGAUUCGUUUUUAGACCUGCUCAAU-3′; EGFP (control), sense: 5′-ACGGCAUCAAGGUGAACUUCAAGAUAG-3′, antisense: 5′-AUCUUGAAGUUCACCUUGAUGCCGUAU-3′. The annealed oligonucleotide duplex, small interfering RNA (siRNA), was transfected as described above (see Section 2.3).

Isolation of total RNA and reverse-transcription were performed as described previously [15]. The synthesized cDNA was amplified with TaKaRa Ex Taq (Takara). PCR conditions were as follows: 94 °C for 1 min; 25 cycles (β-actin) or 35 cycles (DGKγ and β2-chimaerin) at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and 72 °C for 3 min. Sequences of PCR primers were as follows: DGKγ, 5′-TCAAGAAGCCCACTTACTGCAAC-3′ and 5′-CATTTGCGGTGAAACGTCATC-3′; β2-chimaerin, 5′-CTGCTGAGTACATCGCGAAAATGAC-3′ and 5′-ATGTGTGCACCTTGAAGTTGTGTG-3′; β-actin, 5′-GGGAAATCGTGCGTGACAT-3′ and 5′-ACATCTGCTGGAAGGTGGACAG-3′. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

2.5. Affinity precipitation of activated Rac1

NIH3T3 cells were transfected with siRNA targeting EGFP (control), DGKγ or β2-chimaerin. After 48 h of transfection, cells were serum-starved for 5 h and stimulated with 50 ng/ml PDGF-BB for 5 min. COS7 cells were transfected with p3 × FLAG-β2-chimaerin and either pEGFP, pEGFP-DGKγ-WT (wild-type) or pEGFP-DGKγ-G494D. After 24 h, cells were serum-starved for 5 h and incubated with or without 100 ng/ml EGF for 10 min. NIH3T3 and COS7 cells were lysed in 500 μl of ice-cold buffer A (25 mM Tris–HCl, pH7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 1 mM dithiothreitol) containing 5% glycerol, protease inhibitor mixture (Roche) and 5 μg of glutathione *S*-transferase (GST)-fused p21-binding domain (PBD) of p21 activated kinase 1 (PAK) (GST-PAK-PBD, Cytoskeleton, Denver, CO). After centrifugation at $12000 \times g$ for 5 min at 4 °C, the supernatant was incubated with 20 μl of Glutathione Sepharose 4B (GE-Healthcare Bio-Sciences, Piscataway, NJ) for 30 min at 4 °C. The beads were washed three times with 500 μl of ice-cold buffer A and then boiled in SDS sample buffer. Rac1 associated with GST-PAK-PBD and total Rac1 in cell lysates were detected with anti-Rac1 antibody by Western blotting.

2.6. Other procedures

Immunoprecipitation and Western blotting were performed as described previously [15].

Fluorescence microscopy was carried out as described previously [15].

3. Results and discussion

3.1. DGKγ selectively interacts with β2-chimaerin in response to EGF

We have previously demonstrated using a DGK inhibitor and kinase-dead- and constitutively active-mutants of DGKγ that DGKγ acts as an upstream suppressor of Rac1, suppressing lamellipodium/membrane ruffle formation in NIH3T3 fibroblasts stimulated with PDGF [15]. To validate this more convincingly, we silenced DGKγ expression in NIH3T3 cells by transfecting siRNA that specifically targets DGKγ mRNA. Successful silencing was confirmed by RT-PCR 48 h after transfection (Fig. 1A). The siRNA-mediated knockdown of DGKγ significantly enhanced the PDGF-dependent increase of the Rac1-GTP level in comparison with the control transfection (Fig. 1B), further indicating that DGKγ serves as a Rac1 suppressor. However, the molecular mechanism underlying the regulation of Rac1 by DGKγ remains unknown. In this context, β2-chimaerin, a Rac-specific GAP [4,5], was recently reported to regulate growth factor (EGF)-induced Rac1 activity using HeLa and COS7 cells overexpressing β2-chimaerin [14]. We therefore investigated whether the knockdown of β2-chimaerin, which is expressed in NIH3T3 cells (Fig. 1A), affects Rac1 activity. The β2-chimaerin-specific siRNA successfully depleted the β2-chimaerin expression (Fig. 1A) and, moreover, markedly augmented the PDGF-dependent increase of the Rac1-GTP level (Fig. 1B). The siRNAs for DGKγ and β2-chimaerin failed to reciprocally decrease the mRNA levels of β2-chimaerin and DGKγ, respectively (data not shown). Thus, these results indicate that both DGKγ and β2-chimaerin are critically involved in the regulation of Rac1 activity in NIH3T3 cells.

Since it was already reported that β2-chimaerin is activated in vitro by PA [10], the reaction product of DGK, we hypothesized that this GAP is a candidate molecule for linking functionally DGKγ with Rac1. First, we attempted to determine whether DGKγ associates with a signaling complex containing the chimaerin isoform in NIH3T3 cells. For this purpose,