Molecular Pharmacology Fast Forward. Published on August 14, 2007 as DOI: 10.1124/mol.107.036566
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MOL #36566

INVOLVEMENT OF HSP-90 IN CB2 CANNABINOID RECEPTOR-MEDIATED CELL MIGRATION ----A NEW ROLE OF HSP-90 IN MIGRATION SIGNALING OF A G PROTEIN-COUPLED RECEPTOR

Fang He, Zhuan-Hong Qiao, Jian Cai, William Pierce, Da-Cheng He, Zhao-Hui Song

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA (H.F., Z-H.Q., J.C., W.P., Z-H.S.)

Institute of Cell Biology, College of Life Science, Beijing Normal University, Beijing 100875, P. R. China (H.F., D-C.H.)

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Running title: Hsp90 and CB2-mediated cell migration

Address correspondence to: Zhao-Hui Song, Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, Tel. 502 852-5160; Fax. 502 852-7868; E-Mail: zhsong@louisville.edu

The number of text pages: 33

The number of tables: 1

The number of figures: 14

The number of references: 39

The number of words in the Abstract: 250

The number of words in the Introduction: 547

The number of words in the Discussion: 1195

Abbreviations: CB2, cannabinoid receptor subtype 2; GPCR, G protein-coupled receptor; HEK293, human embryonic kidney epithelial cell line; 2-AG, 2-arachidonoylglycerol; SR144528, N-[(1S)-endo-1,3,3-trimethyl-bicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; PTX, pertussis toxin; GA, geldanamycin.

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ABSTRACT

The endocannabinoid 2-arachidonoylglycerol (2-AG) enhances cell migration through the CB2 receptor. In this study, using an immunoprecipitation and mass spectrometry based proteomic approach; we first identified Hsp90, a chaperone protein with novel signaling functions, as a CB2 interacting protein. The CB2/Hsp90 interaction was confirmed in HEK293 cells expressing transfected CB2 and in differentiated HL-60 cells expressing endogenous CB2, by co-immunoprecipitation and western-blot experiments, as well as by treatment with geldanamycin (GA), a specific Hsp90 inhibitor. Disruption of the CB2/Hsp90 interaction by treatment with GA or reducing Hsp90 levels with specific short interfering RNAs markedly inhibited 2-AG-induced cell migration, demonstrating that Hsp90 is crucial for 2-AG-induced cell migration. 2-AG treatment resulted in a CB2-mediated stimulation of Rac1 activity, and treatment with GA blocked 2AG-induced activation of Rac1. Importantly, expression of the dominant negative form of Rac1 reduced 2-AG-induced cell migration. These data demonstrate that 2-AG-induced activation of Rac1 is essential for 2-AG-induced cell migration, and the CB2/Hsp90 interaction is needed for 2-AG-induced activation of Rac1. Furthermore, 2-AG-induced Rac1 activation was sensitive to pertussis toxin treatment, hence involving G_i proteins. In addition, treatment with GA significantly inhibited the CB2/$G_{\alpha i2}$ interaction. Collectively, our data indicate that Hsp90 may serve as scaffold to keep the CB2 receptor and its signaling components, including $G_{\alpha i2}$, in close proximity, thus facilitating CB2-mediated signaling to cell migration through the G_i-Rac1 pathway. By demonstrating that Hsp90 is essential for CB2-mediated signaling to cell migration, this study reveals a novel role of Hsp90 in the signaling events mediated by a G protein-coupled receptor.

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INTRODUCTION

G protein coupled receptors (GPCRs) are of vital importance for cellular functions and they are important targets for drug discovery [Gao and Jacobson, 2006]. Two G-protein-coupled receptors have been identified as cannabinoid receptors and are referred to as CB1 and CB2 [Matsuda et al., 1990; Munro et al., 1993]. CB1 is predominantly expressed in the brain, whereas CB2 is mainly expressed on cells of immune system [Buckley et al., 1998; Matsuda et al., 1990; Munro et al., 1993; Pertwee, 1997]. In addition to psychotropic effects mediated by the CB1, many studies have described a cannabinoid-induced modulation of immune functions, which could be attributed to the CB2 [Buckley et al., 2006; Lunn et al., 2006; Pertwee, 2005; Sugiura et al., 2004].

2-arachidonoylglycerol (2-AG) is an endogenous ligand for the cannabinoid receptor [Mechoulam et al., 1995; Sugiura et al., 1995]. 2-AG binds to both CB1 and CB2, and exhibits a variety of cannabimimetic activities in vitro and in vivo [Kogan and Mechoulam, 2006; Mechoulam et al., 1995; Sugiura et al., 1995; Sugiura and Waku, 2000]. Evidences are accumulating that 2-AG is a physiologically essential molecule, which acts both as a novel type of neuromodulator, and as an important immunodulator [Kogan and Mechoulam, 2006; Sugiura et al., 2004].

Cell migration is of paramount importance in physiological activities of, for example, leukocyte–mediated immune surveillance, as well as in pathological processes such as leukemia metastasis. In recently years, endocannabinoid 2-AG has been reported to enhance the migration of a variety of cell types, e.g., normal leukocytes, as well as differentiated and undifferentiated leukemia cells, through the CB2 receptor [Jorda et al., 2003; Jorda et al., 2002; Kishimoto et al., 2003; Oka et al., 2004]. Despite the important roles that CB2 receptors play in the migration of normal leukocytes and leukemia cells, the identification of molecular mechanisms underlying CB2 receptor mediated cell migration is still in its early stage.

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Recent evidences have strongly suggested that GPCRs interact with a wide variety of proteins in addition to G proteins, which can participate in the trafficking, signaling, fine-tuning and allosteric regulation of GPCRs [Bockaert et al., 2004; Brady and Limbird, 2002; Tilakaratne and Sexton, 2005]. In this study, to better define the CB2 receptor complex involved in cell migration, we first used a co-immunoprecipitation and mass spectrometry based proteomic approach to study the CB2 receptor interacting proteins. We then focused on Hsp90, one of the CB2 receptor interacting proteins that we identified using the proteomic technology.

Hsp90 is a highly conserved protein. Its role as a molecular chaperone in preventing protein aggregation and in promoting refolding of denatured proteins has been well studied [Bohen et al., 1995; Gaestel, 2006; Pratt, 1997; Rutherford and Zuker, 1994]. More recently, interest in Hsp90 has expanded to include an apparently mechanistically distinct function. By forming hetero-complexes with its substrates, Hsp90 has been shown to play a key role as scaffolding site for various signaling events under non-stress conditions [Bohen et al., 1995; Gaestel, 2006; Pratt, 1997; Rutherford and Zuker, 1994].

In this study, we reveal that there are interactions between Hsp90 and CB2 receptors in HEK293 cells expressing transfected CB2 receptors as well in differentiated HL-60 cells expressing endogenous CB2 receptors. Furthermore, we demonstrate that Hsp90 is involved in the CB2 receptor signaling and CB2 receptor-mediated cell migration.

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MATERIALS AND METHODS

Materials. The following primary antibodies were used in our experiments: mouse monoclonal anti-FLAG M2 antibody (Sigma, St. Louis, MO), rabbit polyclonal anti-CB2 antibody (Cayman Chemicals, Ann Arbor, MI), mouse monoclonal anti-Hsp90 antibody (BD, Franklin Lakes, NJ), rabbit anti-mouse IgG (Chemicon, Temecula, CA), His-Tag® monoclonal antibody (EMD Bioscience, Madison, WI), mouse monoclonal anti-HA antibody (Roche, Indianapolis, IN), rabbit polyclonal anti-β-actin antibody (Cell Signaling, Danvers, MA), rabbit polyclonal anti-Rac antibodies (Cytoskeleton, Denver, CO). The (HRP)-conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham (Piscataway, NJ). 2-arachidonoylglycerol (2-AG) was obtained from Tocris (Ellisville, MO). SR144528 was from National Institute of Drug Abuse (Bethesda, MD). Geldanamycin was from Biomol (Swedesboro, NJ). pIRESneo vector was from BD (Franklin Lakes, NJ). The Plasmid encoding human dominant-negative Rac-1 proteins was obtained from University of Missouri-Rolla cDNA Resource Center (Rolla, MO). Hsp90 siRNA and scrambled siRNA were obtained from Santa Cruz Biotechnology, INC (Santa Cruz, CA). DMEM, RPMI-1640, and EDTA-trypsin were from Fisher (Pittsburgh, PA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). HEK293 and HL-60 cells were from ATCC (Manassas, VA).

Cell culture. Transfected HEK293 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM L-glutamine and 500 µg/ml geneticin. HL-60 promyelocytic leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 mM L-glutamine. Cultures were maintained at 37^{o}C in an atmosphere of 95% air and 5% CO_2. To differentiate HL-60 cells into neutrophil-like cells, 250µL of DMSO was added to 2×10^6 cells in 20ml of RPMI-1640 medium, followed by an incubation of 5 days at 37^{o}C in the CO_2 incubator without changing the medium.

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DNA constructs and transfection. To facilitate co-immunoprecipitation, a FLAG sequence was inserted into N-terminal of human CB2 receptor contained in mammalian expression vector pIRESneo. 16μg plasmid was transfected into HEK293 cells at a confluence of 80% in 10cm culture plate using Lipofectamine 2000. Transfected cells were selected in culture medium containing 500 μg/mL of geneticin, and cell lines stably expressing FLAG-CB2 receptor were established according to a method developed previously[Song and Bonner, 1996; Song et al., 1999]. In siRNA knockdown experiments, 2 μg of Hsp90 SiRNA or scrambled SiRNA was transiently transfected into HEK293 cells stably expressing FLAG-CB2 receptor at a confluence of 80% or differentiated HL-60 cells at a density of 2X 10^6 cells/ml, using Lipofectamine 2000. Transient transfection of cells with the dominant negative Rac1 plasmid (16μg) was performed using Lipofectamine 2000, according to manufacturer's instructions. Subsequent assays were performed at 24 h after transfection.

Membrane preparation and solubilization. For membrane preparations, cells were washed twice with cold PBS, scraped off the tissue culture plates and collected by centrifugation at 1000g for 5 min at 4°C. Subsequently, the cells were homogenized in membrane buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, and 2.5 mM EDTA, pH 7.4) with a Polytron homogenizer. After the homogenate was centrifuged at 32,000 g for 20 min at 4°C, the pellet was resuspended in membrane buffer containing 1% dodecylmaltoside and incubated with rotation for 2 hours at 4°C. The solubilized samples were then centrifuged for 20 min at 100,000g for 20 min at 4°C, and the protein content in the supernatant was determined using a Broadford protein assay kit from Bio-Rad (Hercules, CA).

Immunoprecipitation. For co-immunoprecipitation experiments, 500μg proteins from solubilized membrane preparations were incubated with 20μg primary antibody and 80μl protein G-Sepharose beads. After overnight incubation at 4°C, the resins were washed with 10 volumes of solubilization buffer with increasing concentrations of NaCl and proteins were eluted from beads in sample loading buffer.

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In-gel digestion and MALDI-TOF-MS. Samples containing immunoprecipitated receptor complex were separated by SDS-PAGE. The Coomassie-stained gel pieces were destained by 3 cycles of dehydration with 50% acetonitrile(ACN) in 25mM NH_4HCO_3 pH8.4 and hydration with 25mM NH_4HCO_3. The destaind protein bands were dried in a vacuum centrifuge. The protein bands were reduced with 10mM dithiothreitol (DTT) in 25mM NH_4HCO_3 for 45 min at 56^oC. For alkylation of proteins, the DTT solution was replaced by 55mM iodoacetic acid in 25mM NH_4HCO_3 and incubated for 30 min in the dark. The gel pieces were then washed alternatively with 25mM NH_4HCO_3 and 25mM NH_4HCO_3 /50% ACN three times and completely dried in a vacuum centrifuge. The gel pieces were incubated with trypsin in 25mM NH_4HCO_3 at 37^oC for 16 h. The digestion peptide fragments were mixed with equal amount of matrix solution (α-cyano-cinnamic acid in 50% ACN/0.1% TFA), and loaded onto target plate. Mass spectra were obtained using a TofSpec 2E MALDI-TOF mass spectrometer (Micromass, Manchester, UK). Proteins were identified based on matching the MS data with the calculated mass values of peptide fragments using Mascot.

Western Blot Analysis. Samples were incubated with 2x Laemmli buffer under reducing conditions at room temperature for 20 min, and proteins were resolved on a 10% SDS-polyacrylamide gel using an Invitrogen minigel electrophoresis system. Proteins were transferred onto a nitrocellulose membrane for immunodetection with indicated antibodies. The nitrocellulose membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 20 mM Tris, 137 mM NaCl, and 0.1% Tween 20, pH 7.6) for 1 h and then incubated overnight at 4°C with the primary antibody. Thereafter, the membranes were washed twice for 10 min each time with TBS-T buffer and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were then washed three times with TBS-T buffer for 10 min each time, and the antibody-recognized protein bands were visualized using an enhanced chemiluminescence kit from Amersham (Piscataway, NJ).

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Migration Assay. The migration of differentiated HL-60 cells was assayed using the Transwell inserts (pore size, 5 μm) and 24-well culture plates (Corning Costar, Cambridge, MA). Briefly, 2-AG or vehicle (0.01% DMSO in RPMI-1640 medium) was added to 0.6 ml of the RPMI-1640 medium containing 0.1% bovine serum albumin in the well of the culture plate (the lower compartment). Subsequently, 1.0 x 10^6 cells were washed several times, suspended in 0.1 ml of RPMI 1640 medium containing 0.1% bovine serum albumin and transferred to the Transwell insert (the upper compartment). After incubation at 37^{o}C for 4 h in a cell culture incubator, the number of cells that migrated from the upper to the lower compartment was counted using a hemocytometer. For adherent transfected HEK293 cells, Transwell inserts (pore size, 8 μm) were first coated overnight at 4°C with 5μg/ml fibronectin. Cells were harvested from the culture dish into a 50-ml centrifuge tube using PBS-EDTA. Subsequently, the cells were washed with DMEM supplemented with 0.2% BSA, centrifuged at 500g, and cell pellets were resuspended in the same medium with a final cell concentration of 1.0 × 10^6 cell/ml. 2-AG or vehicle (0.01% DMSO in DMEM) was added to 0.6 ml DMEM supplemented with 0.2% BSA in the well of the culture plate (the lower compartment). The 0.1 ml of transfected HEK293 cells (containing 1.0 x 10^5 cells) were transferred to the Transwell inserts (the upper compartment). After incubation at 37^{o}C for 4 h in cell culture incubator, the Transwell insert was stained with a HEMA 3 staining kit (Fisher Scientific Inc., Houston, TX). Five randomly chosen microscopy fields were photographed. Migrated cells in each filed were quantified by counting using a scorer and the Northern Eclipse software. Results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Three independent experiments were performed. In each experiment, triplicate was performed for each condition.

Rac1-GTPase Pull-down Assay. Rac1 activation assay kit was purchased from Cytoskeleton and assays were performed according to attached protocols. Briefly, transfected HEK293 cells with a confluent of 90% in 10 cm culture plate or 1.0 × 10^7 differentiated HL-60 cells were serum-starved for

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18h. Then the cells (pretreated with geldanamycin or vehicle: 0.01% DMSO in DMEM or RPMI-1640 medium) were stimulated with 500 nM 2-AG or vehicle (0.01% DMSO in DMEM or RPMI-1640 medium) and immediately lysed with lysis buffer supplied in the kit and centrifuged at 14,000g for 3 min. A portion of each supernatant was diluted in SDS-PAGE sample buffer for detection of total (both GTP- and GDP-bound) Rac1 GTPases. The remaining supernatant fractions were incubated with 20μl PAK-PBD-agraose for 1 h at 4^{o}C, followed by washing twice with lysis buffer and wash buffer. Proteins bound to the beads were eluted in 30μl 2×SDS-PAGE sample buffer, and subjected to Western blot analysis.

Data Analysis. The data presented in figures represent mean ± S.E.. The figures were generated with the use of GraphPad Prism software. One-way ANOVA with Newman-Keuls post-test was used to compare the data of different treatment groups. The level of significance was chosen as $P < 0.01$.

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RESULTS

Identification of Hsp90 as a CB2 receptor interacting protein by proteomic analysis. A proteomic strategy combining immunoprecipitation and mass spectrometry was used in this study to identify CB2 receptor interacting proteins. To facilitate the immunoprecipitation of CB2 receptor protein complex, we introduced a FLAG tag sequence at the N-terminal of the human CB2 receptor. In a previous study, we have shown that the FLAG tag sequence does not affect the functions of human CB2 cannabinoid receptor[Zhang et al., 2007].

Using an anti-FLAG antibody, CB2 receptor complex was immunoprecipitated from HEK293 cells stably expressing FLAG-CB2. HEK293 cells transfected with an empty vector were used as a negative control and subject to the same immunoprecipitation procedure. As shown in lane 4 of Fig. 1, when the immunoprecipitated receptor complex was separated by SDS-PAGE and stained with Coomassie Blue, bands ranging in sizes from 37 to 100 kDa were apparent. In contrast, most of these bands were not present in the negative control sample (lane 3 of Fig. 1). These experiments were repeated three times and similar protein bands profiles were obtained. Bands that appeared only in immunoprecipitates of HEK293 cells stably expressing FLAG-CB2 were excised, subject to trypsin digestion, and identified by MALDI-TOF-MS. Among the protein bands identified unambiguously by MALDI-TOF-MS were CB2 receptor at about 40kDa and Hsp90 at about 90kDa. As shown in Table 1, 8 peptides obtained from tryptic digestion of the 90kDa band matched with calculated molecular weight for the tryptic peptides of Hsp90.

Verification of the interaction between Hsp90 and CB2 receptor. The association of Hsp90 with CB2 receptor was confirmed by co-immunoprecipitation and immunoblotting experiments. Firstly, CB2 receptor complex was pulled down from HEK293 cells stably expressing FLAG-CB2 with an anti-CB2 antibody and immunoblotted with the anti-Hsp90 antibody. As shown in Fig. 2A upper panels, the Hsp90 band was detected in membranes prepared from HEK293 cells stably expressing FLAG-CB2, as

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well as HEK293 cells transfected with an empty vector. However, after immuoprecipitation, Hsp90 band was detected only in immunoprecipitates of HEK293 cells expressing FLAG-CB2 receptor, and not in HEK293 cells transfected with the empty vector. This result obtained using anti-CB2 antibody ruled out the possibility that the interaction of Hsp90 with CB2 might be caused by the presence of FLAG epitope, and indicated that the interaction between Hsp90 and CB2 receptor was indeed specific. Secondly, to further verify the interaction of Hsp90 with CB2 receptor, co-immunoprecipitation and immunoblotting experiments were carried out in a reverse order, i.e., anti-Hsp90 antibody was used for immunoprecipitation to see if CB2 receptor could be co-immunoprecipitated. As shown in Fig. 2A lower panels, CB2 band was detected in membrane preparations and immunoprecipitates of HEK293 cells stably expressing FLAG-CB2, but not in membranes and immunoprecipitates prepared from HEK 293 cells transfected with the empty vector. These reverse co-immunoprecipitation and immunoblotting experiments provided further evidence for the physical association of Hsp90 with CB2 receptor.

Furthermore, differentiated HL-60 cells were used to test whether the interaction between Hsp90 and CB2 receptor exists in native cells that express endogenous CB2 receptor. As shown in Fig. 2B, both CB2 receptor and Hsp90 proteins were detected in membrane preparations of differentiated HL-60 cells. Using the anti-CB2 antibody, Hsp90 was co-immunoprecipitated with the endogenous CB2 receptor. In contrast, the signal was absent when membrane fractions were immunoprecipitated with a non-relevant antibody (rabbit anti-mouse IgG) (Fig 2B upper panel). Moreover, using the anti-Hsp90 antibody, endogenous CB2 receptor was co-immunoprecipitated with Hsp90 from the solubilized membrane preparations. However, the band was not detected when membrane fractions were immunoprecipitated with a non-relevant antibody (His-Tag® monoclonal antibody, Fig 2B lower panel). These data from differentiated HL-60 cells clearly demonstrated that the specific interaction between Hsp90 and CB2 receptor exists in native cells expressing endogenous CB2 receptor. It needs to be noted that two bands were detected in the Western blot with the CB2 antibody (Fig 2A and 2B, lower panels). These are likely

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corresponding to glycosylated and non-glycosylated forms of CB2 receptors, as we have demonstrated previously that CB2 receptors exist in glycosylated and non-glycosylated forms [Feng et al., 2002].

The effect of geldanamycin on the interaction between Hsp90 and CB2 receptor. Geldanamycin, a small molecule that specifically binds to the ATP-binding pocket in Hsp90, but not other chaperone proteins, alters the tertiary structure of Hsp90, and prevents Hsp90 from interacting with target proteins[Stebbins et al., 1997]. In this study, the effect on geldanamycin on the interaction between Hsp90 and CB2 receptor was investigated. In HEK293 cells stably expressing FLAG-CB2, geldanamycin treatment did not significantly affect the amount of CB2 receptor and Hsp90 in membrane fractions (Fig. 3A, panel 3, 4). However, treatment with geldanamycin markedly reduced the amount of Hsp90 pulled down by anti-CB2 antibody in co-immunoprecipitation and immunoblotting studies. In addition, similar results were obtained with differentiated HL-60 cells, i.e. geldanamycin treatment significantly reduced the amount of Hsp90 pulled down by anti-CB2 antibody (Fig. 3B). These data further demonstrate that the interaction of Hsp90 with CB2 receptor is specific.

The effect of geldanamycin on 2-AG-induced cell migration. 2-AG, an endogenous cannabinoid agonist for both CB1 and CB2 receptors, has been shown to cause cell migration in a number of cell types. In this study, we first tested whether 2-AG can induce the migration of HEK293 cells stably expressing FLAG-CB2, and whether the 2-AG-induced cell migration is mediated by CB2 receptors. As shown in Fig. 4A, addition of 2-AG to the lower chamber of the Transwell cell migration system resulted in a marked enhancement of cell migration from the upper chamber to the underside of the porous membrane. In the presence of 2-AG, the total number of cells migrated (499.17 ± 45.8, mean ± S.E.) was about three times that of cells migrated in the absence of 2-AG (142.38 ±14.9, mean ± S.E.). SR144528, a selective CB2 antagonist, was used to examine whether CB2 receptor is involved in 2-AG-induced cell migration. Pretreatment of cells with SR144528 markedly reduced the cell migration induced by 2-AG (Fig. 4A). However, as shown in Fig 4A, SR144528 alone had no significant effect on the cell migration.

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Moreover, 2-AG did not induce the migration of HEK293 cells transfected with an empty vector. These data demonstrate that 2-AG-induced cell migration was mediated via CB2 receptors stably expressed in these cells.

Differentiated HL-60 cells are valid models for analyzing human leukocyte migration and chemotaxis[Hauert et al., 2002; Xu et al., 2003]. However, the effects of 2-AG on the migration of differentiated neutrophil-like HL-60 cells have not been examined with the Transwell (Boyden chamber) system. To study the down stream signaling pathways of CB2 receptor-mediated migration and the involvement of Hsp90, in this study, we first wanted to test whether 2-AG could induce migration of HL-60 cells differentiated into neutrophil-like cells and whether CB2 receptor is involved. As shown in Fig. 4B, adding 500 nM 2-AG to the lower chamber of the Transwell cell migration system resulted in a marked enhancement of cell migration from the upper chamber to the lower chamber. In the presence of 2-AG, the total number of cells migrated was about 7.5 times that of cells migrated in the absence of 2-AG. In addition, pretreatment of cells with 1 μM SR144528 markedly reduced the 2-AG-induced enhancement of cell migration, whereas SR144528 alone did not affect cell migration (Fig. 4B).

Having shown that geldanamycin can inhibit the interaction of Hsp90 with CB2 receptor, we next examined the possible role of Hsp90 in 2-AG-induced cell migration using geldanamycin. As shown in Fig. 5A and 5B, pretreatment of HEK 293 cells stably expressing FLAG-CB2 and differentiated HL-60 cells expressing endogenous CB2 with geldanamycin reduced 2-AG-induced cell migration whereas GA alone had no significant effect on the cell migration. This inhibitory effect of geldanamycin was concentration-dependent in both types of cells. Collectively, these data on the inhibitory effect of geldanamycin on 2-AG-induced, CB2 receptor-mediated migration of HEK293 and differentiated HL-60 cells suggest that the interaction between Hsp90 and CB2 receptor is crucial for 2-AG-induced cell migration.

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The effect of Hsp90 siRNA on 2-AG-induced cell migration. To examine the effect of decreasing Hsp90 levels on 2-AG-induced cell migration, Hsp90 SiRNA was transfected into HEK293 cells stably expressing FLAG-CB2, as well as differentiated HL-60 cells that expressing endogenous CB2 receptor. In cells that were transfected with Hsp90 SiRNA, the expression levels of Hsp90 were significantly decreased (upper panels of Fig. 6A and 6B). Correspondingly, 2-AG induced cell migration was decreased significantly as a result of Hsp90 knockdown by SiRNA (lower panels of Fig. 6A and 6B). In contrast, Hsp90 SiRNA by itself had no significant effect on the cell migration. These data further indicate that Hsp90 plays an important role in 2-AG-induced, CB2 receptor mediated cell migration.

The effect of geldanamycin on 2-AG-induced Rac1-GTPase activity. The small GTP-binding protein Rac1 plays a central role in the regulation of the actin-based cytoskeleton and cell movement [Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004; Ridley, 2001], and Rac1 activation is mediated by G_i protein. Since CB2 receptor is G_i protein coupled receptor, we investigated whether Rac1 is involved in the 2-AG induced cell migration. As shown in Fig. 7A, in HEK293 cells stably expressing FLAG-CB2, by a pull-down assay we observed that stimulation with 2-AG provoked the rapid accumulation of GTP-bound Rac1, which reached a maximum at ~5 min. Similar results were also obtained in differentiated HL-60 cells expressing endogenous CB2 receptor (Fig. 7B), i.e. treatment with 2-AG led to the increase in Rac1 activity, which reached a plateau at about 5 minutes of 2-AG stimulation. Pretreatment with SR144528 markedly inhibited the activation of Rac1 induced by 2-AG (Fig. 8), indicating that the modulation of Rac1 activity by 2-AG is mediated by CB2 receptor. However, as shown in Fig 8, SR144528 alone had no significant effect on either GTP-bound Rac1 or total Rac1 levels.

Since we have shown that the interaction of Hsp90 with CB2 plays an important role in 2-AG-induced cell migration, we wondered whether this interaction is also important for 2-AG-induced enhancement of Rac1 activity. This question was answered again by using geldanamycin. As shown in

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Fig. 9, pretreatment of HEK293 cells stably expressing FLAG-CB2 as well as differentiated HL-60 cells expressing endogenous CB2 with geldanamycin blocked the 2-AG-induced Rac1 activity in both types of cells. However, geldanamycin alone had no significant effect on either GTP-bound Rac1 or total Rac1 levels. These data indicate that by interacting with CB2 receptor, Hsp90 plays an important role in regulating 2-AG-induced increase of Rac1 activity.

The effect of dominant-negative form of Rac1 on 2-AG-induced cell migration. After finding out that 2-AG stimulates Rac1 activity and the interaction of Hsp90 with CB2 is important for this process, the next question we asked was whether Rac1 is required for 2-AG-induced cell migration. To answer this question, the dominant-negative form of Rac1 was used. Fig. 10 demonstrates that 3×HARac1T17N, the dominant-negative form of Rac1, was expressed 24 hr after transfection into HEK293 cells stably expressing FLAG-CB2, as well as differentiated HL-60 cells expressing endogenous CB2. Importantly, expression of the dominant negative form of Rac1 markedly reduced 2-AG-induced cell migration in both types of cells (Fig. 10A and 10B). However, expression of the dominant negative form of Rac1 by itself had no significant effect on the cell migration. These data directly demonstrate that Rac1 plays important roles in 2-AG-induced cell migration.

Involvement of G_i in 2-AG induced cell migration and Rac1-GTPase activity. Pertussis toxin (PTX), which ADP ribosylates $G_{\alpha i}$, thereby uncoupling it from receptor activation [Ui and Katada, 1990], was used to investigate the role of G_i in the process of cell migration stimulated by 2-AG. Treatment with PTX inhibited 2-AG induced migration of both types of cells (Fig. 11A and 11B), whereas PTX alone had no significant effect on the cell migration. These data confirmed that G_i participates in the migratory response initiated by CB2. Since Rac1 plays important roles in 2-AG-induced cell migration, we then investigated whether G_i protein is involved in the 2-AG induced Rac1 activation. As shown in Fig. 12, pretreatment of two types of cells with PTX attenuated the 2-AG induced Rac1-GTPase activity.

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However, PTX alone had no significant effect GTP-bound Rac1 or total Rac1 levels. These data indicate that 2-AG induces Rac1 activation through PTX-sensitive G proteins of the G_i class.

The effect of geldanamycin on the interaction between $G_{\alpha i2}$ and CB2 receptor. Since we have shown that the interaction of Hsp90 with CB2 receptor plays an important role in 2-AG-induced cell migration and Rac1 activation, the next question is how Hsp90 modulate 2-AG-induced migration response through interaction with CB2 receptor. It is well known that CB2 receptor coupled to G_i protein, and our data in Fig. 11 and 12 clearly showed that PTX sensitive G protein of the G_i family is involved in the migratatory response mediated by CB2 receptor. A recent paper have indicated that Hsp90 protein binds to $G_{\alpha i2}$ [Falsone et al., 2005]. Based on this previous finding and our data in Fig. 11 and 12, we hypothesized that Hsp90 might act as an adaptor between CB2 receptor and $G_{\alpha i2}$, thereby regulating Rac1 activation and cell migration. To test this hypothesis, we used geldanamycin to address the role of Hsp90 in the maintenance of CB2/$G_{\alpha i2}$ coupling. In HEK293 cells stably expressing FLAG-CB2, geldanamycin pretreatment did not significantly affect the amount of $G_{\alpha i2}$ (Fig. 13A). However, treatment with geldanamycin markedly reduced the amount of $G_{\alpha i2}$ pulled down by the anti-FLAG antibody in co-immunoprecipitation and immunoblotting studies in a time-dependent manner. In addition, similar results were obtained with differentiated HL-60 cells, i.e. geldanamycin treatment significantly reduced the amount of $G_{\alpha i2}$ pulled down by the anti-CB2 antibody (Fig. 13B). These data indicate that Hsp90 might play an important role in keeping the physical coupling between CB2 receptor and its signaling component $G_{\alpha i2}$.

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DISCUSSION

CB2 is expressed predominantly in cells of the immune system, and may be involved in a wide range of physiological phenomenon related to immunity[Buckley et al., 2006; Lunn et al., 2006; Pertwee, 2005; Sugiura et al., 2004]. Previous studies have shown that endocannabinoid 2-arachidonoylglycerol (2-AG) induces the migration of several types of immune cells, such as macrophages, monocytes, and eosinophils through a CB2 receptor-dependent mechanism, thereby participating in inflammatory reactions and immune responses[Kishimoto et al., 2003; Oka et al., 2004]. In addition, CB2 is aberrantly over-expressed in a high percentage of human acute myeloid leukemia cells[Alberich Jorda et al., 2004]. Similar to metastasis of other primary tumors, leukemia cells spread throughout the body and invade into secondary tissues. 2-AG has been shown to induce the migration of leukemia cells and has been suggested to play an important role in leukemia metastasis[Alberich Jorda et al., 2004; Jorda et al., 2003; Jorda et al., 2002].

Many cellular events depend on the regulated protein-protein interactions. Discovering novel protein interactions should facilitate the understanding of the molecular mechanisms underlying biological processes. In the current study, we first identified Hsp90 as a possible constitutive interacting protein of CB2 receptor by immunoprecipitation and mass spectrometry. The interaction between Hsp90 and CB2 receptor in FLAG-CB2 transfected HEK293 cells was found to be specific since Hsp90 was not immunoprecipitated with either anti-FLAG or anti-CB2 antibodies in HEK293 cells transfected with an empty vector. The interaction was also seen in differentiated HL-60 cells, which express endogenous CB2 receptors. The rationales for us to choose HL-60 cells were: 1. It is a human promyelocytic leukemia cell line, from where CB2 receptor was originally cloned[Munro et al., 1993]; 2. Differentiated HL-60 cells are established models widely used for studying the mechanisms of leukocyte migration[Hauert et al., 2002; Xu et al., 2003].

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Geldanamycin, a small molecule that specifically binds the ATP-binding pocket in Hsp90 (but not other chaperones), alters the tertiary structure of Hsp90, thus preventing Hsp90 from interacting with target proteins[Stebbins et al., 1997]. In this study, pretreatment of cells with geldanamycin inhibited the interaction of Hsp90 with CB2. These results indicate that the interaction between Hsp90 and CB2 is specific and exist in physiological conditions.

In this study, we have confirmed that 2-AG can induce migration of HEK293 cells stably expressing CB2 receptor, as well as HL-60 cells differentiated into neutrophil-like cells. Furthermore, we have demonstrated that CB2 antagonist SR144528 can block 2-AG-induced migration of both types of cells. More importantly, we have shown that pretreatment with geldanamycin markedly reduced the 2-AG-induced, CB2 receptor-mediated migration of HEK293 as well as differentiated HL-60 cells. Furthermore, in Hsp90 knocking-down experiments performed with Hsp90 siRNA, we have shown that decreasing the cellular expression of Hsp90 reduced 2-AG-induced migration. These data clearly demonstrate that the interaction between Hsp90 and CB2 is essential for 2-AG-induced, CB2 receptor-mediated cell migration.

How can Hsp90 play a role in the 2-AG-induced, CB2 receptor-mediated cell migration? Before answering this question, we must first understand the signaling pathways of 2-AG-induced cell migration. Rho-families of small GTPases are well-known, key regulators in cell migration[Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004; Ridley, 2001]. Previous reports have found that the activation of different component of Rho-families depends upon different signaling pathways, i.e. the activation of RhoA involves $G_{12/13}$-mediated pathway whereas the activation of Rac depends on G_i-mediated pathway[Xu et al., 2003]. It is well known that CB2 receptor is a G_i protein coupled receptor. As a first step in elucidating the molecular mechanism by which 2-AG induces cell migration, we studied the effects of 2-AG on Rac1 activity. Our data demonstrate that 2-AG induced a time-dependent enhancement of Rac1 activity, and this enhancement was inhibited by CB2 antagonist SR144528,

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suggesting that Rac1-GTPases may be involved in 2-AG-induced cell migration. Our hypothesis that Rac1-GTPases may be involved in 2-AG-induced cell migration is further supported by our data demonstrating that 2-AG-induced cell migration was marked inhibited by dominant-negative form of Rac1. To our knowledge, this study is the first to directly establish the involvement of Rac1 in cannabinoid-induced cell migration using the dominant-negative form of the Rho-families of small GTPases. These data are consistent with a recent report on the effect of the dominant negative form of Rac1 on fMLP-induced cell migration[Pestonjamasp et al., 2006]. In the current study, we have found that pretreatment with PTX inhibited the cell migration induced by 2-AG in both types of cells. These data confirm that G_i participates in the migratory response mediated by CB2 receptor. Furthermore, pretreatment of both two types of cells with PTX attenuated the 2-AG induced Rac1 GTPase activity, again confirming the involvement of G_i protein. Taken together, our experimental data support the hypothesis that 2-AG-induced; CB2 receptor-mediated cell migration involves Rac1 activation through PTX-sensitive G proteins of the G_i class.

In this study, we have found that pretreatment of cells with geldanamycin inhibited 2-AG-induced activation of Rac1. In addition, we have shown that pretreatment of cells with geldanamycin also decreased 2-AG-induced cell migration. A previous study has reported the effects of geldanamycin on PAR-1 stimulated, thrombin receptor-mediated changes in cell morphology and cytoskeleton[Pai et al., 2001]. To our knowledge, the current study is the first to demonstrate a modulatory effect of geldanamycin on ligand-induced, GPCR-mediated cell migration. These data on the modulatory effects of geldanamycin on 2-AG-induced Rac1 activity, and 2-AG-induced cell migration, support the hypothesis that Hsp90 regulates 2-AG-induced cell migration by modulating 2-AG-induced Rac1 activity.

How might Hsp90 modulate 2-AG-induced Rac1 activity? The current study indicated that Hsp90 acts upstream of Rac1 since geldanamycin blocked 2-AG induced activation of Rac1. In a previous publication on Hsp90 interactome analysis, it has been shown that Hsp90 can interact with $G_{\alpha i2}$ [Falsone

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et al., 2005]. Using PTX, in this study we also confirmed the involvement of G_i protein in 2-AG induced, CB2 receptor mediated Rac1 activation and cell migration. Therefore, we hypothesized that Hsp90 may regulate CB2-mediated Rac1 activation through modulation of the coupling between CB2 receptor and $G_{\alpha i2}$. This hypothesis is supported directly by our experimental data demonstrating that treatment with geldanamycin decreased the physical coupling of CB2 receptor with $G_{\alpha i2}$. Therefore, it is likely that Hsp90 serves as a scaffold to keep the CB2 receptor and its signaling components, including $G_{\alpha i2}$, in close proximity, thus ensure proper signaling transduction. To our knowledge, this is the first time that Hsp90 has been shown to play a critical role in the coupling of a GPCR to its G protein. However, it is also possible that Hsp90 may be necessary to stabilize receptor on the cell surface.

Comparing with other heat shock proteins, Hsp90 is distinguished by its specific interactions with various signaling proteins and its important role in signal transduction under non-stress conditions. Our studies add support to this premise by demonstrating that geldanamycin not only inhibited the interaction between CB2 receptor and Hsp90, but also but also prevented the CB2/$G_{\alpha i2}$ coupling, the activation of Rac1 and the subsequent cell migration induced by 2-AG.

In conclusion, as outlined in Fig. 14, this study demonstrates that Hsp90 is essential for CB2-mediated cell migration, and reveals a novel role of Hsp90 in the signaling events mediated by a G protein-coupled receptor.

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FOOTNOTES

This work is supported in part by NIH grants DA11551 and EY13632.

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FIGURE LEGENDS

Fig. 1. Identification of Hsp90 as a CB2 receptor interacting protein by proteomic analysis.

Protein staining demonstrating immunoprecipitation of the CB2 receptor protein complex from HEK293 cells stably expressing FLAG-CB2. Lanes 1 and 2 represent membrane preparations from HEK293 cells stably transfected with an empty expression vector and HEK293 cells stably expressing FLAG-CB2, respectively. Lanes 3 and 4 represent immunoprecipitates from HEK293 cells stably transfected with an empty expression vector and HEK293 cells stably expressing FLAG-CB2, respectively. The position of CB2 receptor and Hsp90 identified by mass spectrometry is indicated. The experiment was repeated three times, with similar results.

Fig. 2. Verification of the interaction between Hsp90 and CB2 receptor.

A. Confirmation of Hsp90 interaction with CB2 receptor in HEK293 cells stably expressing FLAG-CB2 by co-immunoprecipitation. Upper panel: membranes prepared from HEK293 cells stably expressing FLAG-CB2 and HEK293 cells stably transfected with an empty expression vector were subject to immunoprecipitation with the anti-CB2 antibody, and were then immunoblotted with the anti-Hsp90 antibody. Lower panel: membranes were subject to immunoprecipitation with the anti-Hsp90 antibody, and were then immunoblotted with the anti-CB2 antibody. B. Confirmation of Hsp90 interaction with CB2 receptor in differentiated HL-60 cells expressing endogenous CB2 by co-immunoprecipitation. Upper panel: membranes prepared from differentiated HL-60 cells were subject to immunoprecipitation with the anti-CB2 antibody, and were immunoblotted with the anti-Hsp90 antibody. Lower panel: membranes were subject to immunoprecipitation with the anti-Hsp90 antibody, and were then immunoblotted with the anti-CB2 antibody. Control experiments were performed using nonrelevant IgGs (lane 3). The experiment was repeated three times, with similar results.

Fig. 3. The effect of geldanamycin on the interaction between Hsp90 and CB2 receptor.

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A. The effect of geldanamycin on the interaction between Hsp90 and CB2 receptor in HEK293 cells stably expressing FLAG-CB2. Cells were treated with 10 μM geldanamycin (GA) for 2 hours. Membranes were prepared, subjected to co-immunoprecipitation with anti-CB2 antibody, and the samples were immunoblotted with the anti-Hsp90 (panel 1) the anti-CB2 antibodies (panel 2). B. The effect of geldanamycin on the interaction between Hsp90 and CB2 receptor in differentiated HL-60 cells expressing endogenous CB2. Cells were treated with 10 μM geldanamycin (GA) for 2 hours. Membranes were prepared; subject to co-immunoprecipitation with the anti-CB2 antibody, and the samples were immunoblotted with the anti-Hsp90 (panel 1) the anti-CB2 antibodies (panel 2). The experiment was repeated three times, with similar results.

Fig. 4. 2-AG-induced, CB2 receptor-mediated cell migration.

A. 2-AG-induced migration of HEK293 cells stably expressing FLAG-CB2. 500 nM 2-AG or vehicle was added to the lower compartments and cells were added to the upper compartments of the Transwell cell migration system. The system was then incubated for 4 hours at 37^{o}C and the cells migrated to the underside of the porous membrane were determined as described in the Materials and Methods. For antagonist treatment, 1μM SR144528 (SR2) was used. Results are presented as migration index, which is the ratio of cells migrated in the presence of 2-AG versus cells migrated in the presence of vehicle. Data shown are mean ±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by Newman-Keuls post test). B. 2-AG-induced migration of differentiated HL-60 cells expressing endogenous CB2. 500 nM 2-AG or vehicle was added to the lower compartments and cells were added to the upper compartments of the Transwell cell migration system. The system was then incubated for 4 hours at 37^{o}C and the cells migrated to the lower compartments were determined as described in the Materials and Methods. For antagonist treatment, 1μM SR144528 (SR2) was used. Results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Data

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shown are mean ±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by Newman-Keuls post test).

Fig. 5. **The effect of geldanamycin on 2-AG-induced cell migration.**

A. The effect of geldanamycin on 2-AG-induced migration of HEK293 cells stably expressing FLAG-CB2. Cells were pretreated with 10 μM of geldanamycin for 2 hours before detaching from the plate and then subject to migration assays using the Transwell cell migration system as described in the Materials and Methods. Results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Data shown are mean ±S.E. of three independent experiments, each performed in triplicates. B. The effect of geldanamycin on 2-AG-induced migration of differentiated HL-60 cells expressing endogenous CB2. Cells were pretreated with 10 μ M of geldanamycin for 2 hours and then subject to migration assays using the Transwell cell migration system as described in the Materials and Methods. Data shown are mean±S.E. of three independent experiments, each performed in triplicates.

Fig. 6. The effect of Hsp90 siRNA on 2-AG-induced cell migration.

A. The effect of Hsp90 siRNA on 2-AG-induced migration of HEK293 cells stably expressing FLAG-CB2. Cells were transfected with 2 μg of Hsp90 siRNA or a scrambled control RNA mixture for 24 hours and then subject to immunoblotting (upper panel) or migration assays (lower panel) as described in the Materials and Methods. Data shown for immunoblotting are representative for three independent experiments. Data shown for migration assays are mean±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by post test). B. The effect of Hsp90 siRNA on 2-AG-induced migration of differentiated HL-60 cells expressing endogenous CB2. Cells were transfected with 2 μg of Hsp90 siRNA or a scrambled control RNA mixture for 24 hours and then subject to immunoblotting (upper panel) or

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migration assays (lower panel) as described in the Materials and Methods. Data shown for immunoblotting are representative for three independent experiments. Migration results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Data shown for migration assays are mean±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by Newman-Keuls post test).

Fig. 7. Time course of 2-AG-induced Rac1-GTPase activity.

Cells were serum-starved for 18 hours and then stimulated with 500 nM of 2-AG for the indicated times. Rac1-GTP in HEK293 cells stably expressing FLAG-CB2 (A) and differentiated HL-60 cells (B) was assessed by PAK-PBD-GST pull down assays, respectively. Blots shown are representative of 4 independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 band in the corresponding samples (n=4).

Fig. 8. The effect of SR144528 (SR2) on 2-AG-induced Rac1-GTPase activity.

Cells were serum-starved for 18 hours and pretreated with 1μM SR144528 or vehicle at 37 ^{o}C incubator for 30min, followed by stimulation with 500 nM of 2-AG for 5 minutes, and then the Rac1-GTP levels in HEK293 cells stably expressing FLAG-CB2 (A) and differentiated HL-60 cells (B) were measured by PAK-PBD-GST pull down assay, respectively. Blots shown are representative of 4 independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 band in the corresponding samples (n=4).

Fig. 9. The effect of geldanamycin on 2-AG-induced Rac1-GTPase activity.

Cells were pretreated with vehicle or 10μM of geldanamycin for 2h, followed by stimulation with 500 nM of 2-AG for 5 minutes, and then the Rac1-GTP levels in HEK293 cells stably expressing FLAG-CB2 (A) and differentiated HL-60 cells (B) were measured by PAK-PBD-GST pull down assay, respectively.

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Blots shown are representative of 4 independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 band in the corresponding samples (n=4).

Fig. 10. The effect of the dominant-negative form of Rac1 on 2-AG-induced migration.

Cells were transiently transfected with the dominant-negative form of Rac1 plasmid (3× HA Rac1-T17N,). At 24h after transfection, the expression of the dominant-negative form of Rac1, and its effects on 2-AG-induced the migration of HEK293 cells stably expressing FLAG-CB2 (A) and differentiated HL-60 cells (B) were assayed. Data shown for immunoblotting are representative for three independent experiments. Migration results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Data shown for migration assays are mean±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by Newman-Keuls post test).

Fig. 11. The effects of pertussis toxin (PTX) on 2-AG induced cell migration.

HEK293 cells stably expressing FLAG-CB2 (A) or differentiated HL-60 cells expressing endogenous CB2 (B) were incubated with 100ng/ml PTX for 18h, and then subject to migration assays using the Transwell cell migration system as described in the Materials and Methods. Migration results are presented as migration index, which is the ratio of cells migrated in the presence of drug versus cells migrated in the presence of vehicle. Data shown are Mean ±S.E. of three independent experiments, each performed in triplicates. ** Indicates significant differences ($p<0.01$) between experimental groups (ANOVA followed by Newman-Keuls post test).

Fig. 12. The effects of pertussis toxin (PTX) on 2-AG induced Rac1 GTPase activity.

Cells were cultured overnight in serum-free growth medium with 100ng/ml PTX and stimulated with 500 nM of 2-AG for 5 minutes. The Rac1-GTP levels from HEK293 cells stably expressing FLAG-CB2 (A) and differentiated HL-60 cells expressing endogenous CB2 (B) were measured by PAK-PBD-GST pull

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down assays. Blots shown are representative of 4 independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 band in the corresponding samples (n=4).

Fig. 13. Time course of the effect of geldanamycin on the coupling of CB2 receptor with Gαi2.

(A) HEK293 cells stably expressing FLAG-CB2. (B) Differentiated HL-60 cells expressing endogenous CB2. Cells were pretreated with 10 μM geldanamycin (GA) for indicated time and stimulated with 500 nM of 2-AG for 5 minutes. Membranes were prepared, subjected to co-immunoprecipitation with the anti-CB2 antibody, and the samples were immunoblotted with the anti-Gαi2 and the anti-CB2 antibodies. The experiment was repeated twice, with similar results.

Fig. 14. Working model concerning the involvement of Hsp90 in CB2 receptor mediated cell migration. Hsp90 may function as a scaffold orienting Gαi2 to interact with the CB2 receptor or stabilizing CB2 receptor on the cell surface, thus facilitating CB2-mediated signaling to cell migration through the Gi-Rac1 pathway.

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Table 1. Identification of Hsp90 protein by MALDI-TOF-MS.

Comparison of the molecular weights obtained from MALDI-TOF-MS for the tryptic digests of the 90kDa protein and the calculated molecular weights for the tryptic peptides of Hsp90.

Start-End	$(M+H)^+$ (observed) (Da)	$(M+H)^+$ (calcualted) (Da)	Delta
73-82	1193.6527	1193.6404	0.0123
205-219	1807.9727	1807.9508	0.0219
292-306	1846.7327	1846.7897	-0.057
320-330	1347.6627	1347.6571	0.0056
379-392	1512.7827	1512.7783	0.0044
439-449	1296.6527	1296.6534	-0.0007
625-639	1781.9127	1781.9424	-0.0297
686-719	3531.6227	3531.6375	-0.0148

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Fig. 1

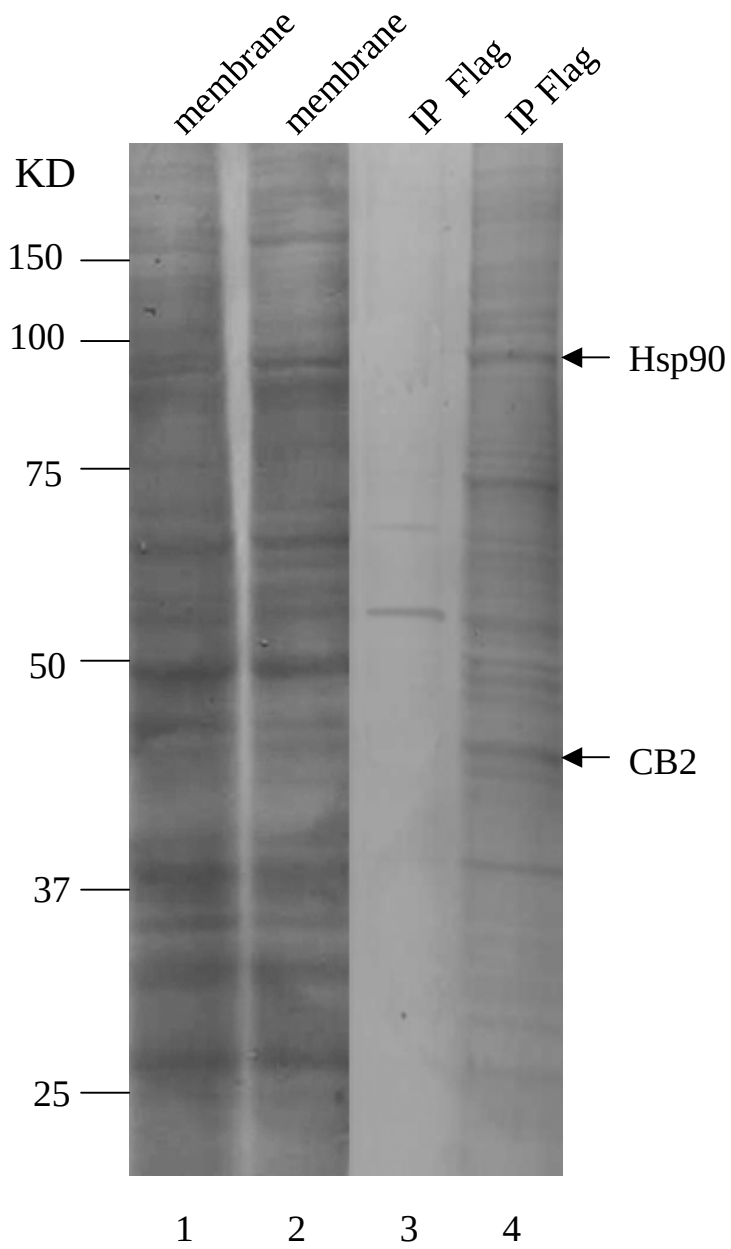

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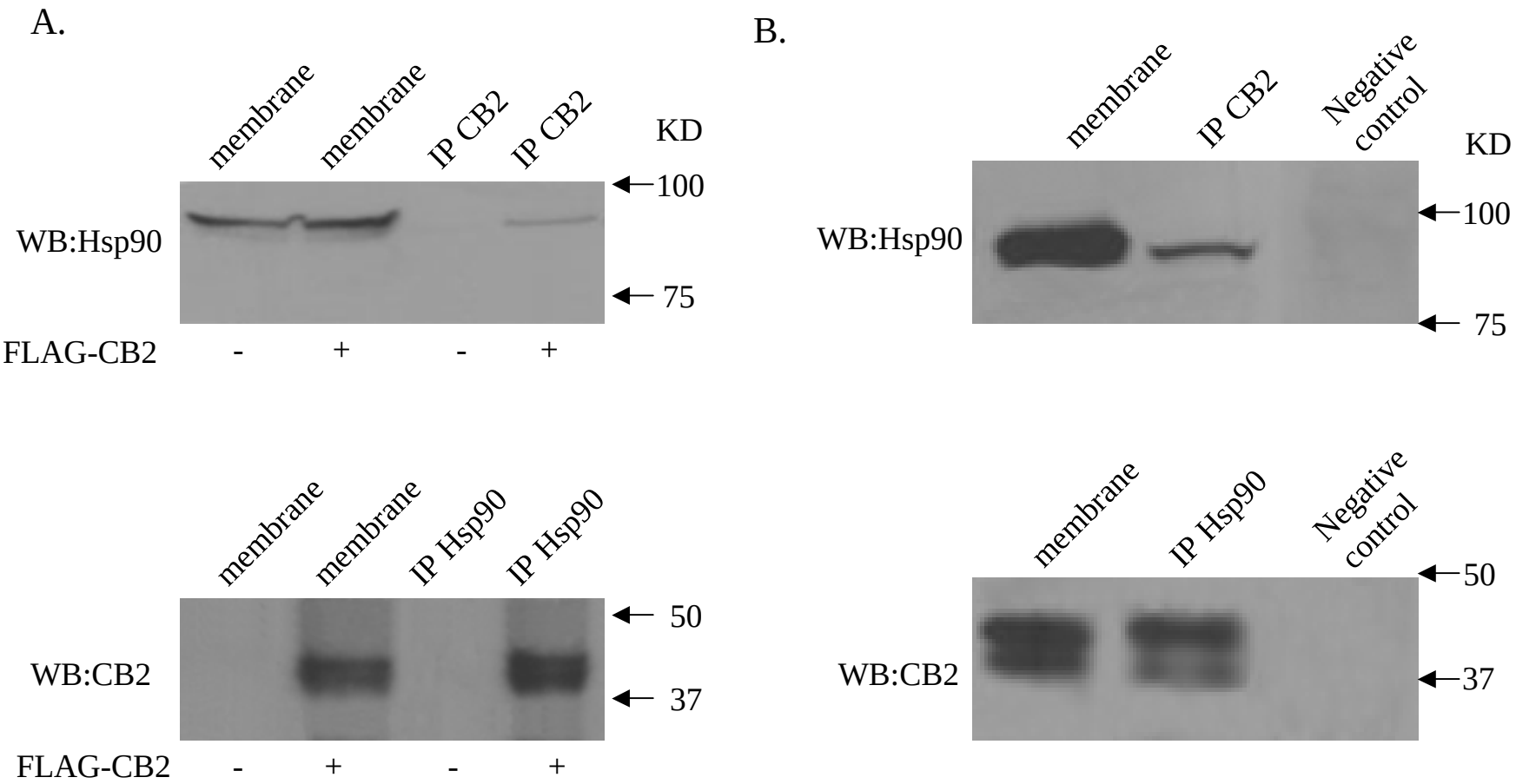
Fig. 2



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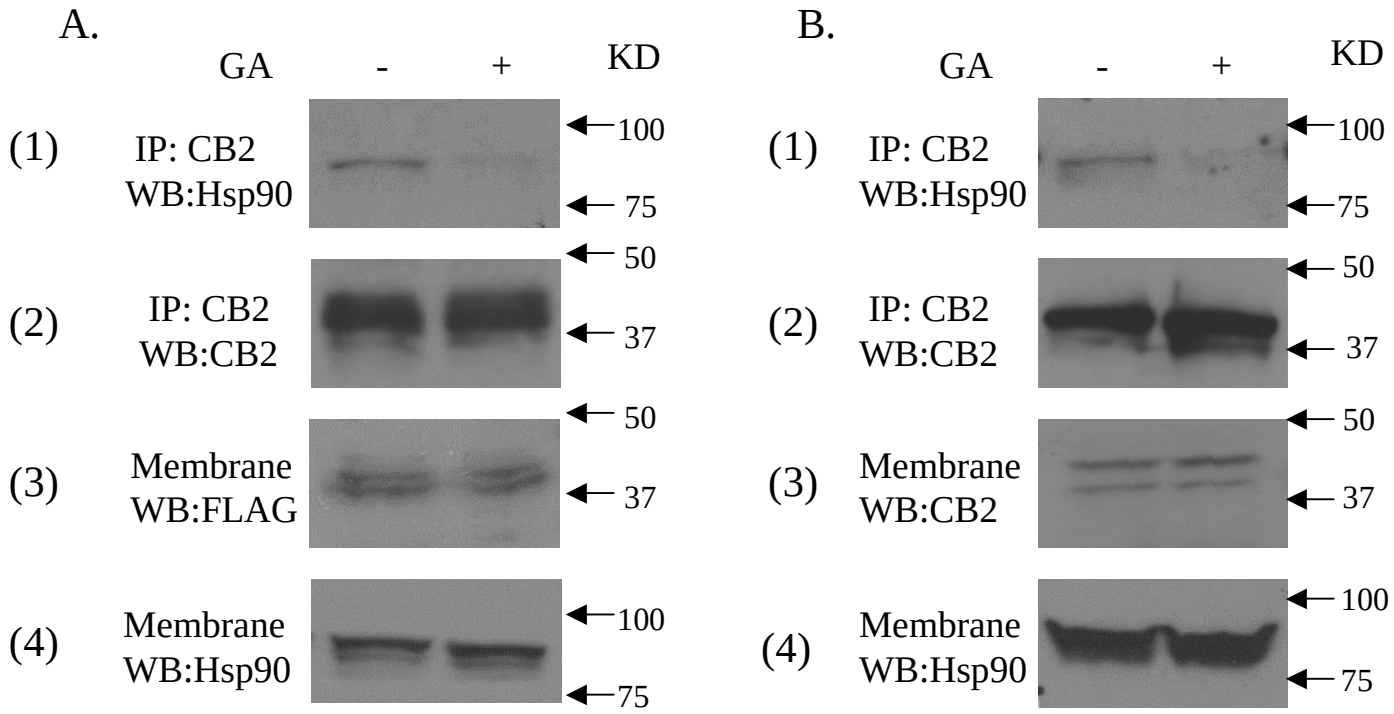

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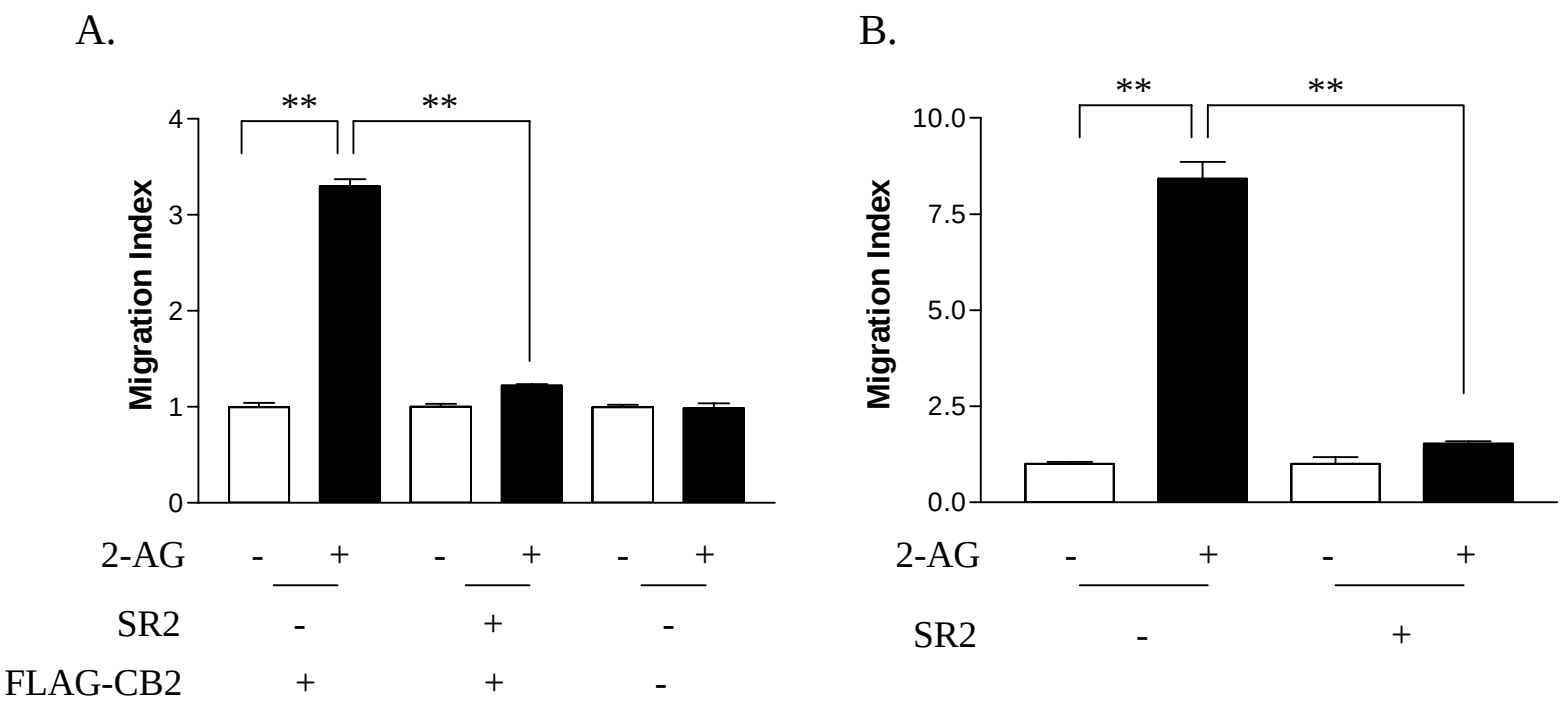

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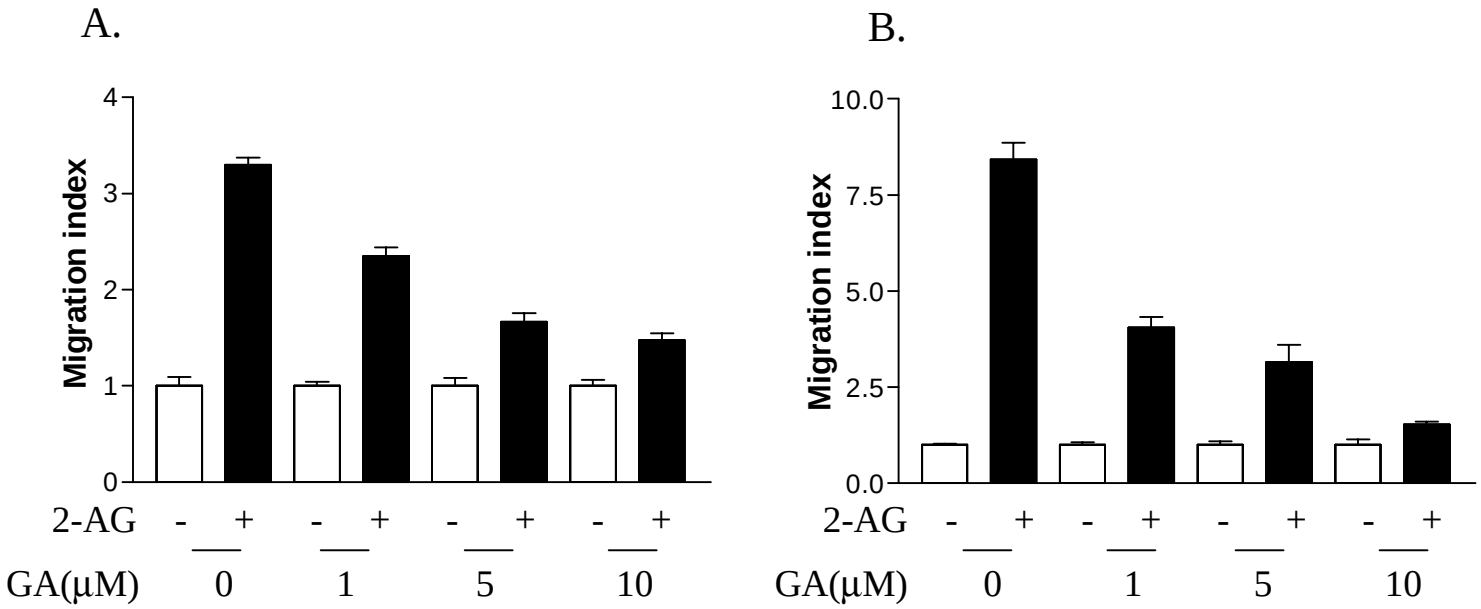

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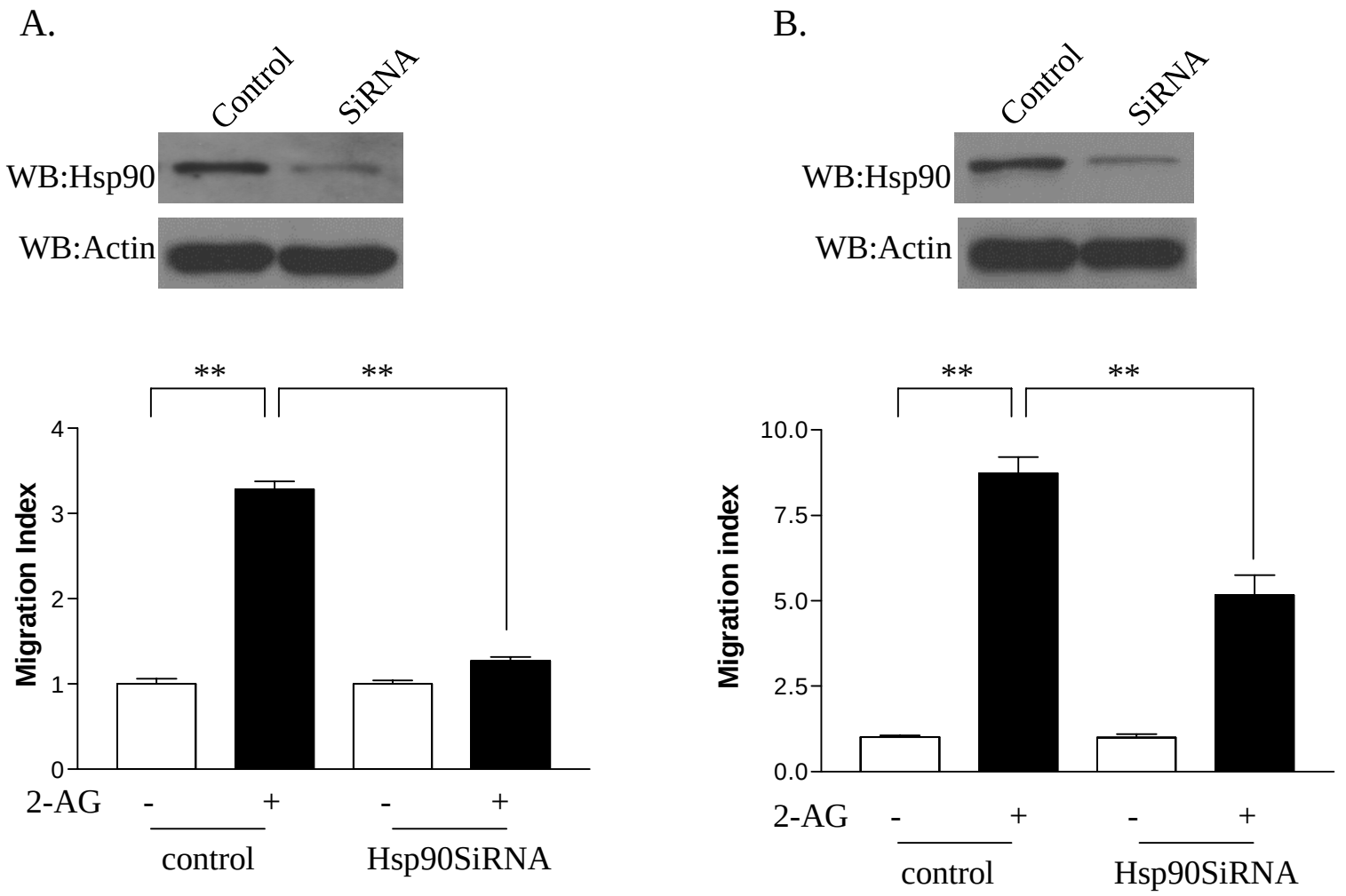

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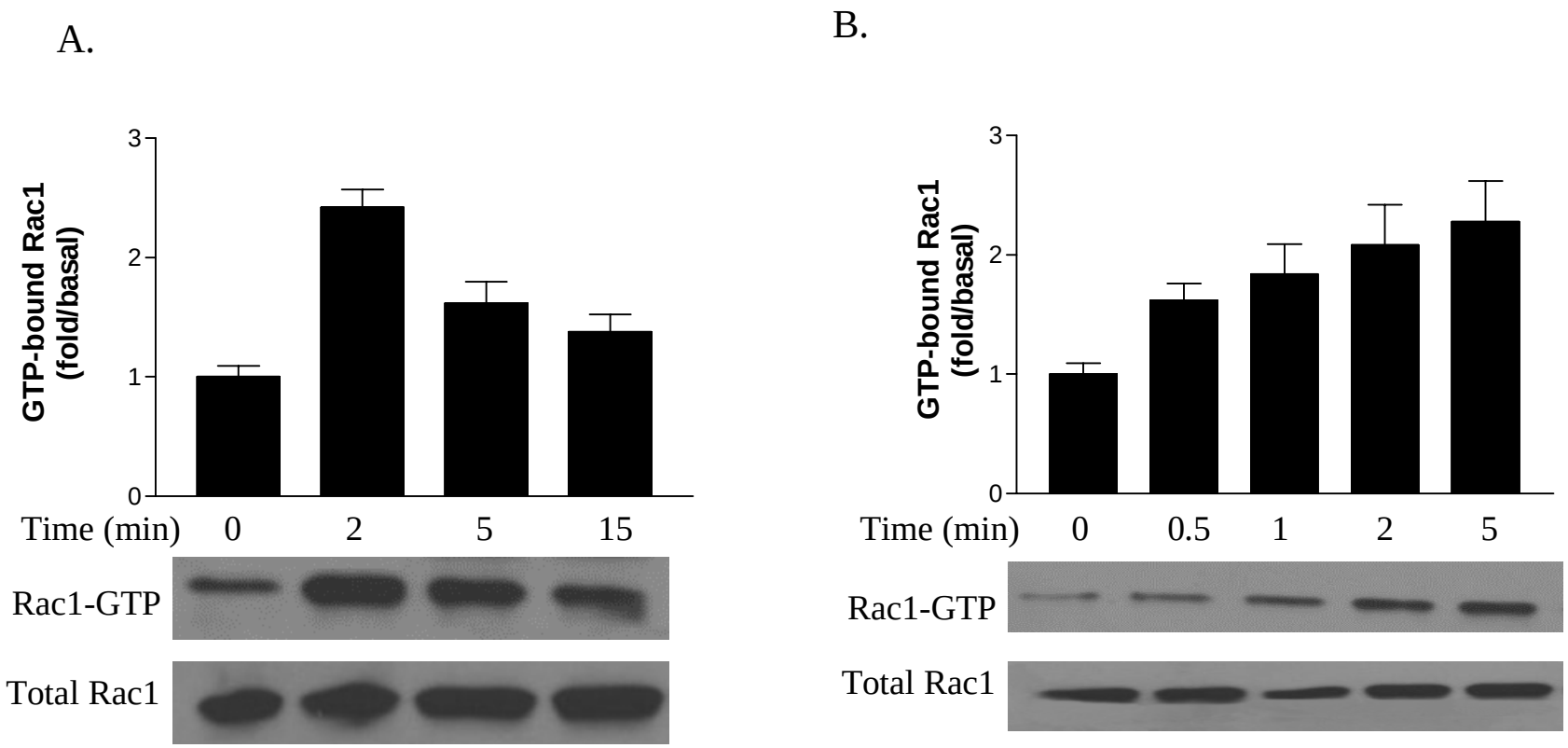
Fig. 7



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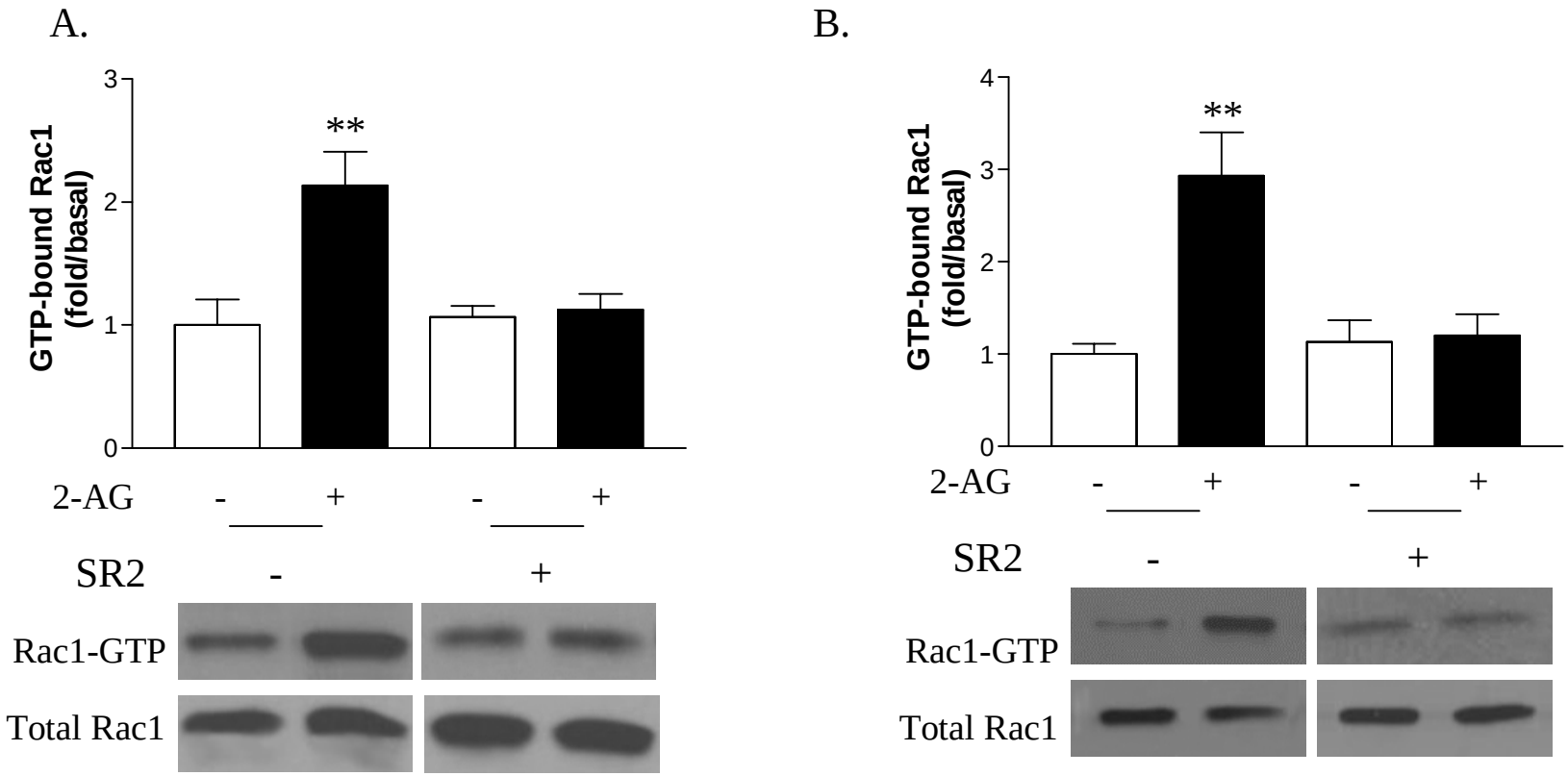

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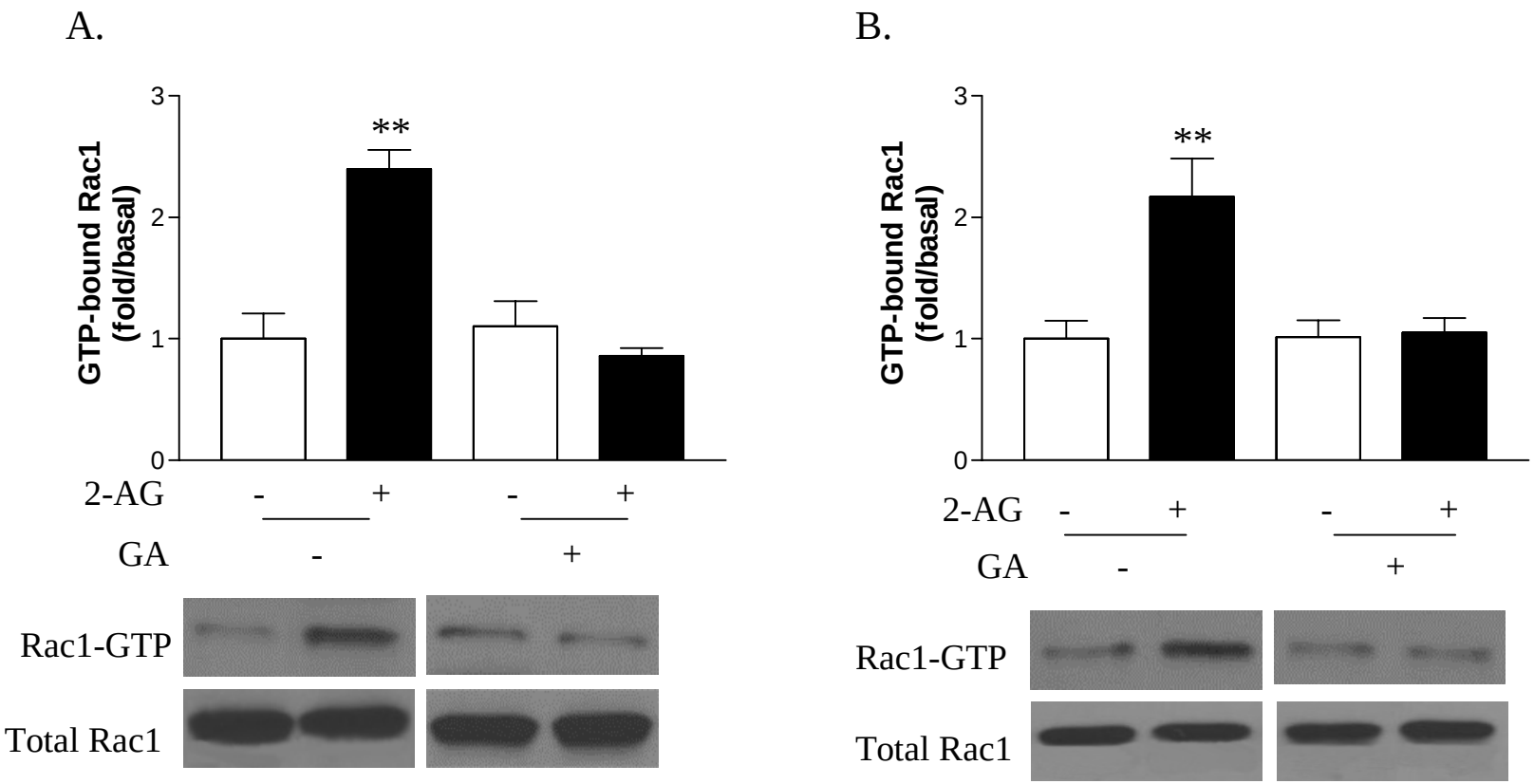

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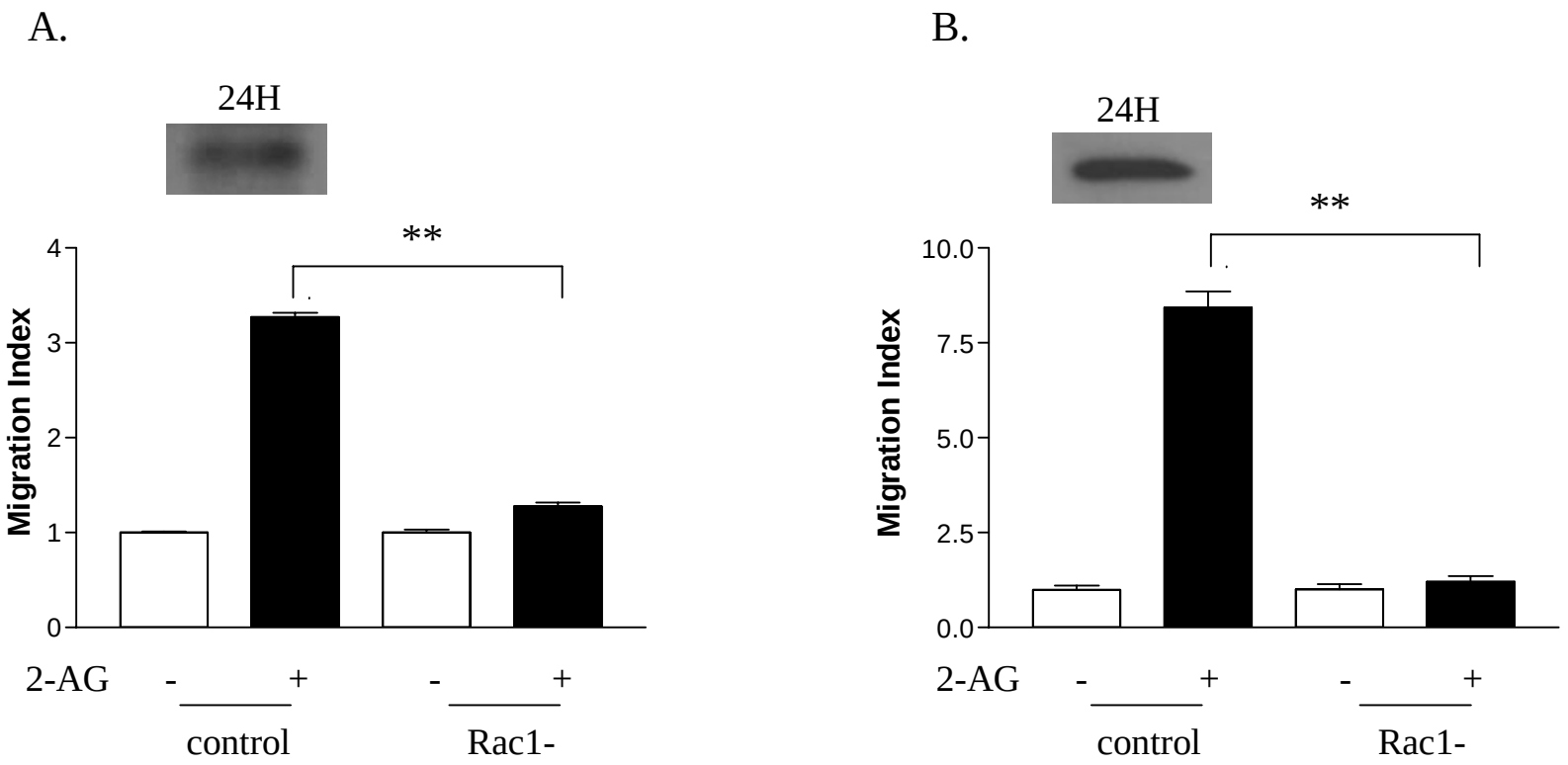
Fig. 10



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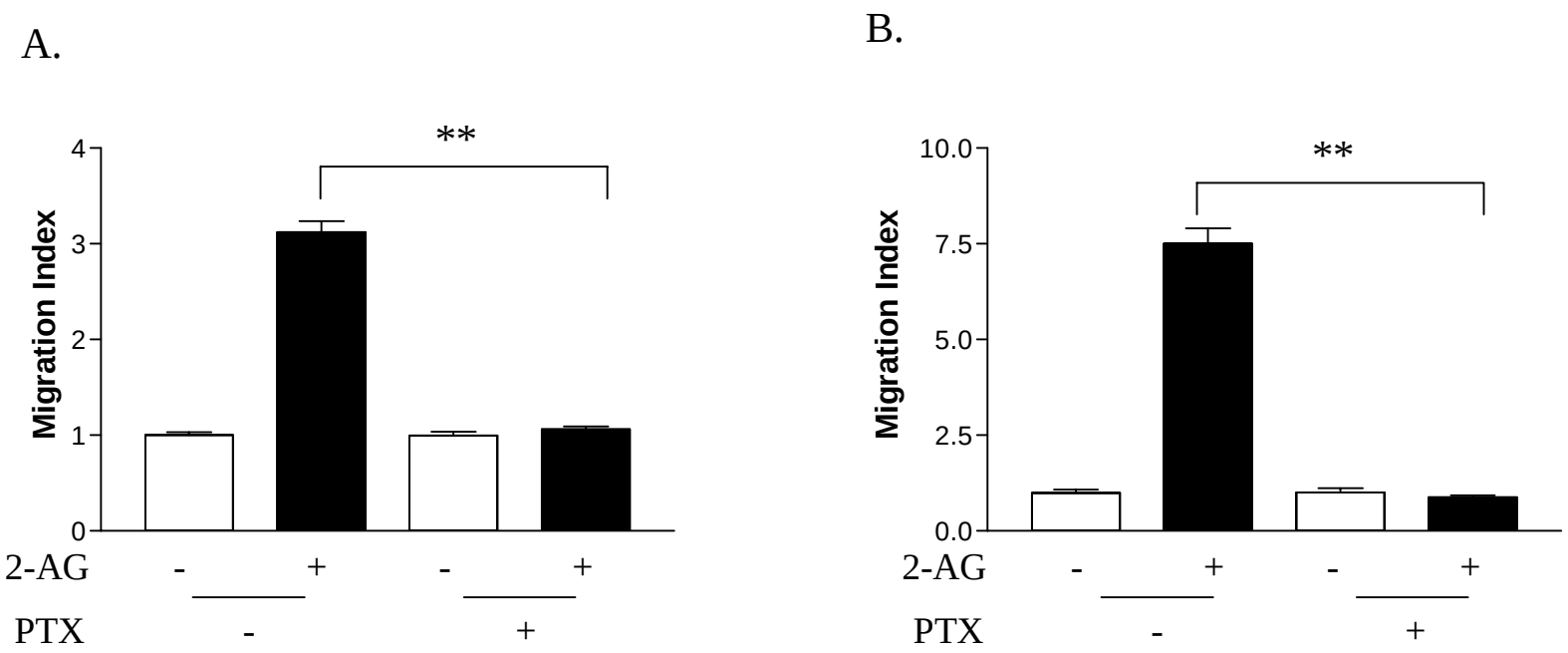

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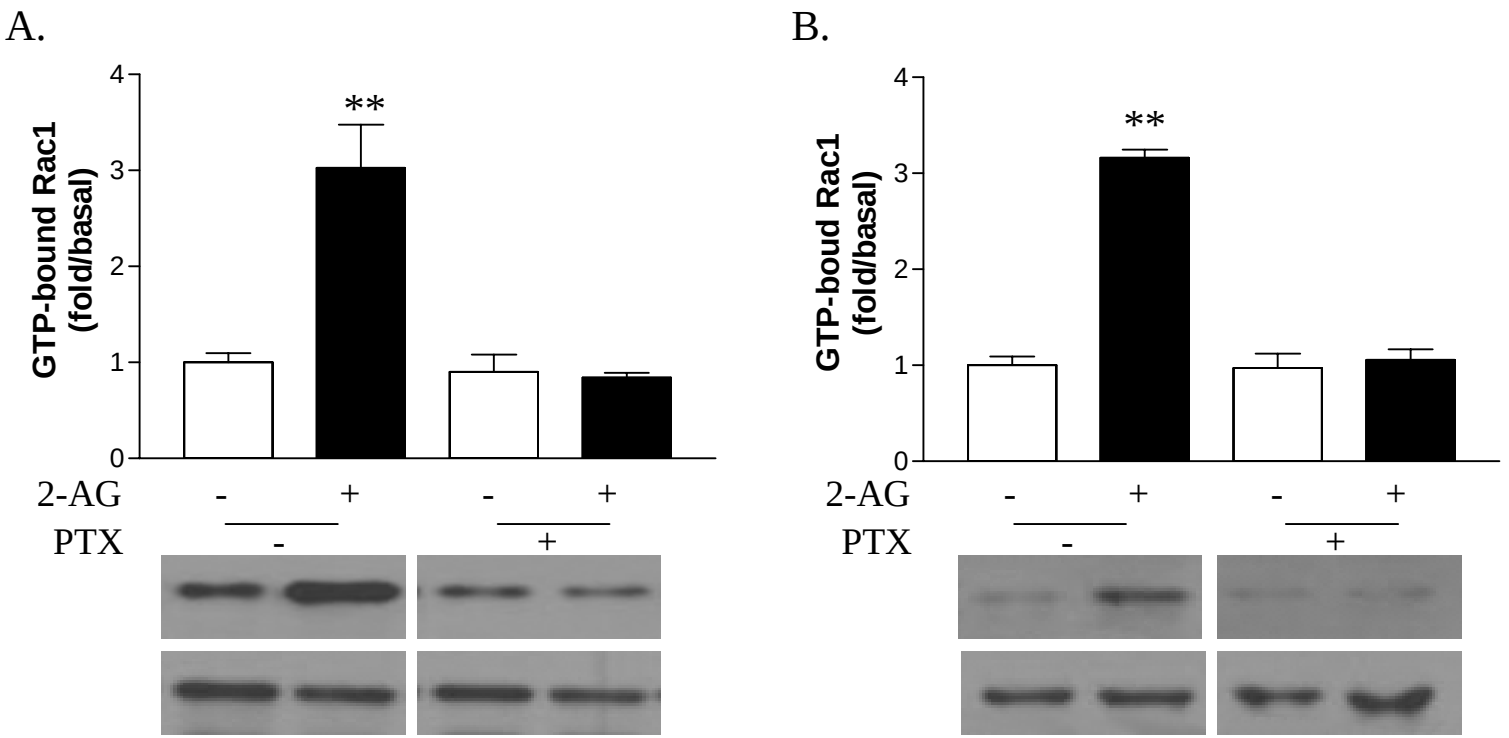

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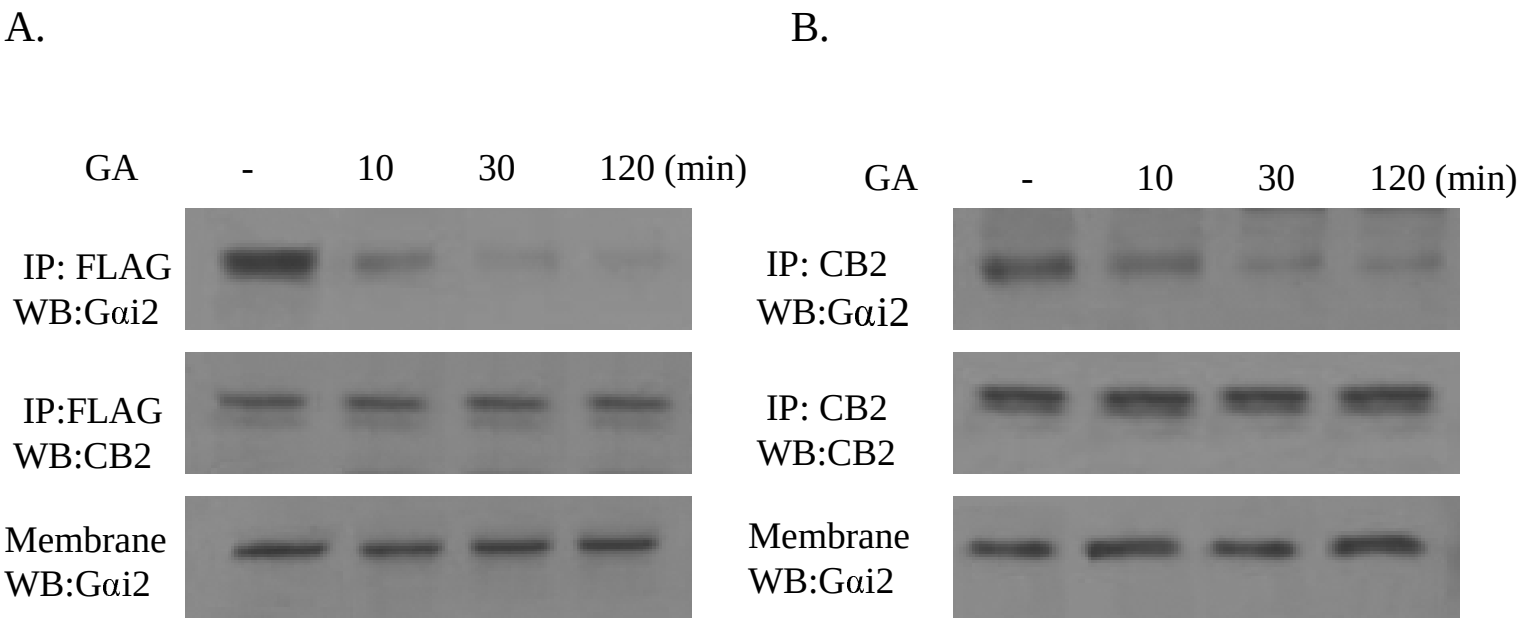

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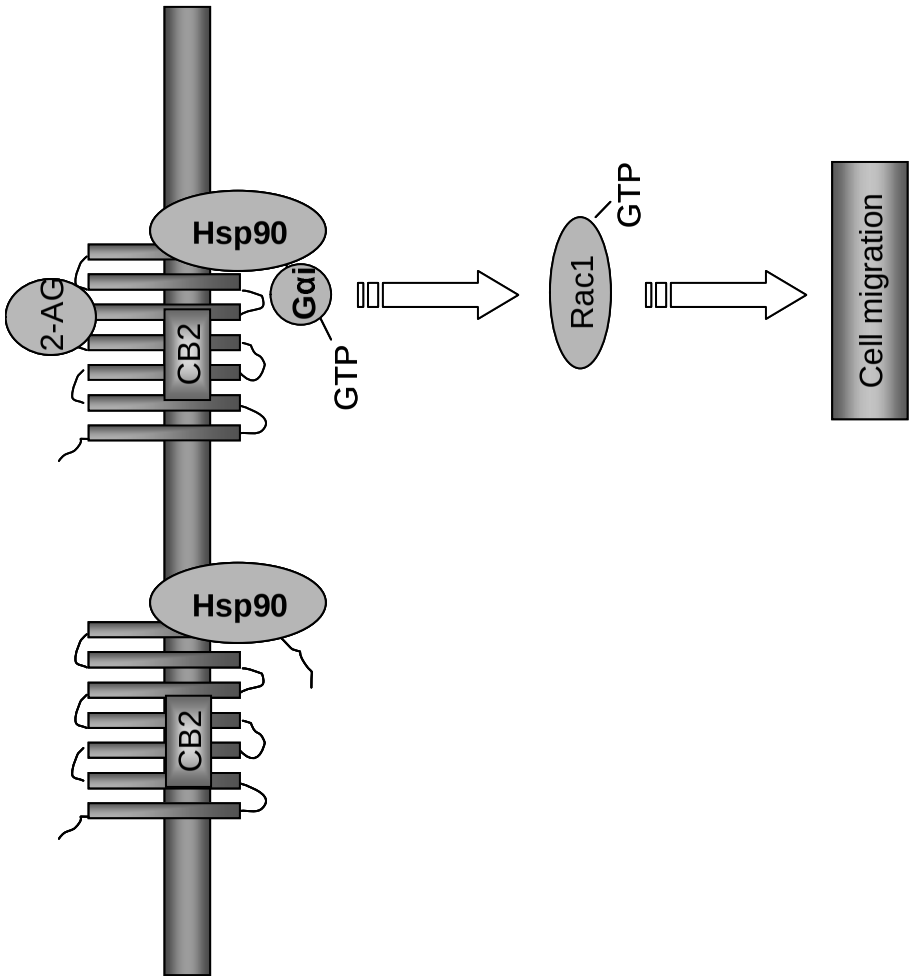

Fig.14

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