Published in final edited form as:
J Immunol. 2014 March 1; 192(5): 2186–2194. doi:10.4049/jimmunol.1301875.

G-protein-coupled receptor kinase-6 interacts with Activator of G-protein signaling-3 to regulate CXCR2-mediated cellular functions

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Abstract

The interleukin-8 (IL-8/CXCL8) receptors, CXCR1 and CXCR2, couple to $G\alpha_i$ to induce leukocyte recruitment and activation at sites of inflammation. We have recently shown that CXCR1 couples predominantly to the G protein-coupled receptor (GPCR) kinase-2 (GRK2) whereas CXCR2 interacts with GRK6 to regulate cellular responses. In addition to GPCRs, GRKs have displayed a more diverse protein/protein interaction in cells. In this study we sought to identify GRK6 binding partner(s) that may influence CXCL8 activities, using RBL-2H3 cells stably expressing CXCR1 (RBL-CXCR1) or CXCR2 (RBL-CXCR2), as well as human and murine neutrophils. The data herein demonstrated that upon CXCR2 activation, GRK6 interacts with activator of G protein signaling 3 (AGS3) and $G\alpha_{i2}$ to form a GRK6/AGS3/$G\alpha_{i2}$ complex. This complex is time-dependent and peaked at 2-3 min post-activation. GTPγS pretreatment blocked GRK6/AGS3/$G\alpha_{i2}$ formation suggesting that this assembly depends on G protein activation. Surprisingly, CXCR2 activation induced AGS3 phosphorylation in a PKC-dependent but GRK6-independent fashion. Overexpression of AGS3 in RBL-CXCR2 significantly inhibited CXCL8-induced Ca^{2+} mobilization, phosphoinositide (PI) hydrolysis and chemotaxis. In contrast, shRNA inhibition of AGS3 enhanced CXCL8-induced Ca^{2+} mobilization, receptor resistance to desensitization and recycling to the cell surface with no effect in receptor internalization. Interestingly, RBL-CXCR2-AGS3$^{-/-}$ cells displayed a significant increase in CXCR2 expression in the cell surface, but decreased (extracellular signal-regulated kinases) ERK1/2 and P38 mitogen-activated protein kinase (MAPK) activation. Taken together, these results indicate that GRK6 complexes with AGS3-$G\alpha_{i2}$ to regulate CXCR2-mediated leukocyte functions at different levels including downstream effector activation, receptor trafficking and expression at the cell membrane.

Keywords

Activator of G protein signaling; Rat Basophilic Leukemia; Neutrophils; G protein-coupled receptor kinases; Signal transduction; Chemokine; Chemotaxis

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Footnotes

This work was supported by NIH Grants AI38910, CA156735, NIMHD P20 MD00175 and USAMRMC 07-1-0418 to R.M.R.; NIH Grant CA34590; Historically Black College and Universities Grant, Department of Veterans Affairs, to R.M.R and North Carolina Biotechnology Center (2009-BRG-1213 to TCL).

Introduction

Interleukin-8 (IL-8/CXCL8) is a classical member of the subfamily of N-terminal Glu-Leu-Arg (ELR) motif-containing CXC chemokines that induces neutrophil accumulation and activation at sites of inflammation. These functions are mediated via interaction of CXCL8 with two cell-surface receptors, CXCR1 and CXCR2 (1). CXCR1 and CXCR2 are members of the superfamily of seven transmembrane G-protein coupled receptors (GPCRs) that couple to heterotrimeric G-proteins ($G\alpha\beta\gamma$) to mediate cellular responses. Upon activation, both receptors couple to pertussis toxin (Ptx) sensitive $G\alpha_i$ proteins to activate phospholipase C (PLC), resulting in the generation of the intracellular messenger diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (2). In contrast, $G\beta\gamma$ subunits activate phosphotidyl inositol-3-kinase (PI3K), leading to phosphorylation of PI (4,5) diphosphate to form PI (3,4,5) tri-phosphate which activates many signal transduction pathways required for motility, growth, and gene expression.

Following CXCL8 activation, CXCR1 and CXCR2 become desensitized and internalized (3-4). Phosphorylation of GPCRs by G protein-coupled receptor kinases (GRKs) and recruitment of clathrin binding adaptor proteins to the cell membrane are prerequisites for receptor desensitization and internalization (5-6). Thus far, seven GRKs (GRK 1 to 7) have been identified and characterized (7). GRK1 and GRK7 are exclusively expressed in the visual system, in retinal rods and cones, respectively (8). GRK2, GRK3, GRK5 and GRK6 are expressed in most mammalian cell types, whereas GRK4 expression has been detected only in the testis, kidney and cerebellum (8-9). We have recently shown that CXCR1 couples predominantly to GRK2, whereas CXCR2 interacts with GRK6, to mediate and regulate cellular functions including chemotaxis, angiogenesis, tumor progression and metastasis (10).

In addition to the complex receptor/G protein/effector, several accessory proteins have been shown to act as receptor-independent G-protein signaling modulators (GPSM) (11). This group includes the activator of G protein signaling (AGS)-3 (GPSM1), AGS5 (GPSM2), AGS4 (GPSM3), AGS6 [Regulator of G protein Signaling (RGS)-12 (RGS12)], RGS14 and PCP2/L7) (11). AGS3 is a widely expressed protein that interacts with $G\alpha_i$ to promote $G\beta\gamma$ stimulated signals (12). It consists of seven tetratricopeptide repeats (TPR) in the amino terminus and four G protein regulator (GPR or GoLoco) motifs in the carboxyl terminus (13). The GPR motifs interact with $G\alpha_i$ and stabilize the inactive GDP bound conformation (14). A short form of AGS3 that lacks the tetratricopeptide rich region and possesses only three of the four GRP motifs was also identified in heart tissue (15).

Oner *et al.* (16-17) have recently reported that upon activation of the α2-adrenergic receptor or the μ-opioid receptor AGS3 and AGS4 associate with $G\alpha_i$ to form larger signaling complexes at the cell membrane to modulate cellular responses. Prompted by these findings and recent observations that GRKs are far more diverse in their protein interaction than GPCRs in cells (18-20), we sought in this study to identify binding partners of the CXCL8 receptors that may influence their distinct ability to mediate and regulate cellular functions. To that end, we used a co-immunoprecipitation screen in Rat Basophilic Leukemia (RBL-2H3) cells stably expressing CXCR1 (RBL-CXCR1) or CXCR2 (RBL-CXCR2) as well as murine and human neutrophils, with antibodies specific for GRK2, GRK6, $G\alpha_i$ and AGS3-6. The results herein demonstrated that upon activation by CXCL8, CXCR2, but not CXCR1, couples to the GRK6/AGS3/$G\alpha_{i2}$ complex to modulate cellular functions.

Materials and Methods

Materials

[^{32}P]Orthophosphate (8500-9120 Ci/mmol), [^{125}I]-CXCL8, and myo-[2-^{3}H] inositol (24.4 Ci/mmol) were purchased from Perkin Elmer. IL-8 (CXCL8) and CXCL1 were obtained from Peprotech (Rocky Hill, NJ). Indo-1 AM, Geneticin (G418) and all tissue culture reagents were purchased from Invitrogen (Gaithersburg, MD). Protein G-agarose and protease inhibitors were purchased from Roche (Indianapolis, IN). Anti-Human IL-8RA (CXCR1), IL-8RB (CXCR2) and rabbit anti-phospho-Akt and anti-Akt antibodies were purchased from BD Pharmingen (San Jose, CA). Anti-GRK6 was obtained from Millipore (Billerica, MA). Anti-AGS3, anti-$G\alpha_i$ and anti-$G\alpha_{i2}$ were purchased from Santa Cruz. Rabbit anti-ERK1/2, anti-phospho-ERK1/2, anti-P38, anti-phospho-P38, anti-NFκB p65 and anti-phospho-NFκB p65 antibodies were purchased from Cell Signaling (Beverly, MA). The shRNA targeting rat AGS3 (GPSM1) in pGFP-V-RS vector was purchased from Origene Technologies, Inc. (Rockville, MD). Phorbol 12-myristate 13-acetate (PMA), streptolysin O (SLO) and GTPγS were purchased from Sigma (St. Louis, MO). GFP-tagged rat AGS3 plasmid was a kind gift from Dr. Lanier. All other reagents were from commercial sources.

Cell culture and transfection

RBL-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (21). RBL-2H3 cells (1×10^7) were transfected by electroporation with 20 μg of pcDNA3 containing the receptor cDNAs, and Geneticin-resistant cells were cloned into single cells and conformed by (FACS) analysis (22). For overexpression of AGS3, RBL-CXCR2 and RBL-CXCR1 cells (1×10^7) were transiently transfected by electroporation with 20 μg of pcDNA3 containing GFP-AGS3 or GFP alone. Cells expressing GFP were sorted by FACS and used for further experiments. Protein expression level was determined by Western blotting using GFP and AGS3 specific antibodies. For shRNA mediated gene silencing, RBL-CXCR2 cells (1×10^7) were transfected by electroporation with 20 μg of HuSH-29 pGFP-V-RS containing shRNA for rat AGS3 or control plasmid. Puromycin-resistant cells were cloned into single cell by limiting dilution method. Levels of mRNA transcript and protein expression were monitored by Real-Time PCR and Western blotting.

FACS analysis

For flow cytometric analysis, RBL cells were detached by Versene treatment, washed with HEPES-buffered HBSS, and resuspended in the same medium. Cells ($1\text{-}5\times10^6$) were incubated with anti-CXCR2 antibody (1 μg/ml) in a total volume of 400 μl of HEPES-buffered HBSS for 60 min at 4°C. The cells were then washed and incubated with fluorescein (FITC)-anti-mouse IgG for 60 min at 4°C. Cells were then washed and analyzed for cell surface expression of the receptor on a Beckton Dickenson FACScan cytometer (4).

Immunoprecipitation and Immunoblotting

RBL-2H3 cells (2.5×10^6) expressing the receptors were sub-cultured for 24 hours in 60 mm dishes. The cells were stimulated with the indicated ligands for different time periods at 37°C. Reaction was stopped by adding ice-cold PBS and then cells were washed twice and lysed in radioimmunoassay buffer (RIPA) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. Cell lysates were cleared by centrifugation and then immunoprecipitated with specific antibodies against GRK6 and AGS3, and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and detected using anti-AGS3, anti-GRK6, anti-$G\alpha_i$ antibodies.

Radioligand binding assays and receptor internalization

For receptor internalization, RBL-2H3 cells were sub-cultured overnight in 24-well plates (5×10^5 cells/well) in growth medium. Cells were then rinsed with DMEM supplemented with 20 mM HEPES, pH 7.4, and 10 mg/ml BSA. Cells were treated with ^{125}I-labeled CXCL8 (100 nM) at 37°C for different time periods. Reactions were stopped by adding 1 ml of ice-cold PBS followed by centrifugation at 1,000 rpm for 2 min. The cells were then washed three times with ice-cold PBS and assayed for ^{125}I-CXCL8 binding (0.1 nM) as described previously (4). Nonspecific radioactivity bound was determined in the presence of 500 nM unlabeled CXCL8 (23).

Phosphoinositide hydrolysis and intracellular calcium measurement

RBL-2H3 cells were sub-cultured overnight in 96-well culture plates (50,000 cells/well) in inositol-free medium supplemented with 10% dialyzed fetal bovine serum and 1 μCi/ml [^{3}H] inositol. The generation of inositol phosphates was determined as reported (21, 24). For calcium mobilization, cells ($5\mu10^6$) were washed with HEPES-buffered saline and loaded with 1 μM Indo-1, AM in the presence of 1 μM pluronic acid for 30 min at room temperature. The cells were then washed with HEPES and resuspended in 1.5 ml of Siriganian buffer. Intracellular calcium increase in the presence or absence of ligands was measured as described previously (25).

Chemotaxis

RBL-2H3 cells (~50,000) were incubated at 37°C with different concentrations of CXCL8. Chemotaxis was assessed in 48-well microchemotaxis chambers using polyvinylpyrrolidone-free 8-μm pore size membranes, as described previously (4). The results are representative of three separate experiments.

Measurement of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) activity

To assess MAPK activity, RBL-CXCR2 cells (3×10^6) and RBL-CXCR2 cells in which AGS3 expression was stably suppressed (AGS3-shRNA) were washed three times with PBS and then resuspended in PBS containing CXCL8 (100 nM) for different periods of time at 37 °C. The reactions were stopped with ice-cold PBS; cells were collected by centrifugation; lysed with RIPA, and assayed for protein concentration as described previously (26). Equal amounts of protein (20 μg) from each sample were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibody against either total ERK1/2, phospho-ERK1/2 (pERK1/2), P38, phospho-P38 (pP38), Akt or phospho-Akt (pAkt). Detection was carried out with HRP-conjugated sheep anti-mouse antibody and by ECL. For NF-κB-p65 phosphorylation, cells were treated with or without CXCL8 as described above. Then, cells were washed, lysed, and immunoblotted with either anti-phospho-NFκB p65 or anti-NFκB p65 as previously described (27).

CXCR2 and AGS3 Phosphorylation

CXCR2 and AGS3 phosphorylation was performed as described previously (4, 23). RBL-CXCR2 cells (5×10^6) were incubated with [^{32}P]Orthophosphate (150 μCi/dish) for 90 min. Then labeled cells were stimulated with the indicated ligands for 5 minutes at 37°C. Cells were then washed with ice-cold PBS and solubilized in 1 ml of RIPA buffer. Cell lysates were immunoprecipitated with specific antibodies against CXCR2 or AGS3, analyzed by SDS electrophoresis and visualized by autoradiography.

Animals

All experiments were approved by and conformed to the guidelines of the Animal Care Committee of North Carolina Central University, Durham, NC. Animals were housed five per cage in a temperature-controlled room at 22 ± 5°C with an alternate 12 h light-dark cycle.

Isolation of human and murine neutrophils

Neutrophils were isolated from heparinized human blood on a multiple density gradient as described previously (28). The murine macrophages were isolated from the mouse peritoneal cavity as described previously (29). Briefly, zymosan was prepared in PBS to a final concentration of 1 mg/ml and 1.0 ml was injected into the peritoneum of control C57BL/6. Mice were euthanized by CO_2 asphyxiation and the peritoneal cavity was lavaged at 4 h post-injection with 8 ml of ice-cold RPMI 1640 containing 2% fetal bovine serum and 2 mM EDTA. Cells were collected by centrifugation, counted, stained with Diff-Quick to assess the percentage of neutrophils (30).

Statistical analyses

Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Differences between groups were determined by one-way ANOVA or Student *t* test (two-tailed), as appropriate. A p value <0.05 was considered statistically significant.

Results

CXCR2 interaction with GRK6 and AGS3

We have recently shown that CXCR2 couples to $G\alpha_{i2}$ and GRK6 to mediate and regulate cellular responses (10). In an attempt to identify potential AGS protein(s) that may associate with $G\alpha_{i2}$ and/or GRK6 to modulate CXCR2 functions, RBL-CXCR1 and RBL-CXCR2 cells were stimulated with CXCL8 (100 nM) for 2 min. Cell lysates were immunoprecipitated with anti-GRK6 and immunoblotted with specific antibodies against $G\alpha_{i2}$ and AGS3, 4, 5 and 6. As shown in Fig. 1, GRK6 co-immunoprecipitated with $G\alpha_i$ and AGS3 in CXCL8-treated RBL-CXCR2 cells (Fig. 1B), but not in CXCR1 expressing cells (Fig. 1A). To further confirm the specificity of the GRK6/AGS3 interaction, RBL-CXCR2 cell lysates were immunoprecipitated with anti-AGS3 and immunoblotted with GRK6 antibody. AGS3 association with GRK6 was also increased in CXCL8-stimulated cells (Fig. 1C). We next examined whether CXCR2 activation also induce GRK6/AGS3/$G\alpha_i$ association in leukocytes. To that end, zymosan-elicited peritoneal neutrophils from wild type C57Bl/6 mice were treated with or without CXCL1 (100 nM) and cell lysates were immunoprecipitated with anti-GRK6 and immunoblotted with AGS3 and $G\alpha_{i2}$ antibodies. As was the case in RBL-CXCR2 cells, pretreatment with CXCL1 (Fig. 1D) induced GRK6 association with AGS3 and $G\alpha_{i2}$.

We further assessed the association of GRK6 with AGS3-Gαi in human neutrophils purified from blood. As shown in Fig. 1E, upper 2 panels, cell lysates from CXCL8-stimulated cells immunoprecipitated with anti-GRK6 or anti-AGS3, and immunoblotted with either AGS3 or GRK6 antibodies showed a robust association of GRK6 with AGS3, relative to untreated cells. Cells lysates were also immunoblotted for AGS3, GRK6, CXCR1 and CXCR2 (Fig 1E, lower 4 panels) to ensure equal loading. Since CXCL8 activates both CXCR1 and CXCR2, and CXCR1 was shown to couple predominantly to GRK2 (10), cell lysates were also immunoprecipitated with anti-GRK2 and immunoblotted with AGS3. As shown in Fig. 1F, no association of GRK2 with AGS3 was detected.

Effect of $G\alpha_{i2}$ inhibition in CXCR2-induced GRK6/AGS3 interaction

To determine whether $G\alpha_{i2}$ activation is critical for the GRK6/AGS3 complex formation, RBL-CXCR2 cells were permeabilized with SLO (0.2 U/ml) for 30 min at 37°C with or without the non-hydrolysable GTP analogue, GTPγS (100 nM), and stimulated with CXCL8 (100 nM) for 2 min. Cell lysates were immunoprecipitated with anti-GRK6 and immunoblotted with AGS3 and $G\alpha_{i2}$ antibodies. GTPγS pretreatment prevented the association of GRK6 with AGS3 (Fig. 2A, lanes 3 vs. 4). Pertussis toxin (Ptx) pretreatment also inhibited CXCL8-induced GRK6/AGS3/$G\alpha_{i2}$ association (data not shown). Moreover, GRK6/AGS3/$G\alpha_{i2}$ complex formation was time-dependent and peaked at 2-3 min post CXCR2 activation (Fig. 2B). No GRK6/AGS3/$G\alpha_{i2}$ association was detected at 5 min.

Effect of AGS3 overexpression in CXCR2 mediated cellular responses

To determine the role of AGS3 in CXCR2-mediated cellular responses, a GFP-tagged AGS3 was transiently overexpressed in RBL-CXCR1 and RBL-CXCR2, and assayed for complex formation, receptor mediated phosphoinositide (PI) hydrolysis, intracellular Ca^{2+} mobilization and chemotaxis. As shown in Fig. 3A, upon activation by CXCL8 the GFP-AGS3 was co-immunopurified with GRK6 as well as the native AGS3 expressed in RBL-cells. No complex formation was detected in cells expressing GFP alone or RBL-CXCR1 cells expressing GFP-AGS3 (data not shown). Overexpression of GFP-AGS3 significantly inhibited (~80%) CXCL8-mediated PI hydrolysis in RBL-CXCR2 but showed no effect in RBL-CXCR1 (Fig. 3B). AGS3 overexpression also inhibited CXCR2 mediated chemotaxis (Fig. 3C) and intracellular Ca^{2+} mobilization (Fig. 3D) in response to CXCL8.

Inhibition of AGS3 expression in RBL-CXCR2 promoted receptor expression

To further assess the role of AGS3 in CXCR2 mediated cellular responses, the endogenous expression of AGS3 was knocked down in RBL-CXCR2 cells using a shRNA directed against AGS3. Puromycin resistant cells were analyzed by FACS analysis (Fig. 4A), real-time PCR (data not shown) and Western blotting (Fig. 4B). As shown in Fig. 4B, RBL-CXCR2 cells transfected with AGS3 shRNA exhibited a ~75% reduction in AGS3 expression as compared to cells transfected with control shRNA (Ctl-shRNA). Surprisingly, FACS analysis (Fig. 4A) and immunoblotting (Fig 4B, 2nd panel) showed a ~4 fold increase in CXCR2 expression in AGS3-shRNA cells relative to Ctl-shRNA cells (Fig. 4C). Suppression of AGS3 had no effect in GRK6 expression (Fig. 4B, 3rd panel & C). Ligand binding analysis using ^{125}I-CXCL8 further confirmed the increased receptor expression in AGS3-shRNA cell and also indicated that AGS3 depletion has no significant effect in CXCR2 affinity for ligand (Kd: 1.7±0.1 and 2.3±0.37, for Ctl-shRNA and AGS3-shRNA, respectively)

Effect of AGS3 knockdown in CXCR2-mediated cellular responses

To assess the role of AGS3 knockdown in CXCR2 we measured CXCL8-induced intracellular Ca^{2+} mobilization and chemotaxis. As shown in Fig. 5A, AGS3-shRNA and Ctl-shRNA exhibited dose-dependent CXCL8-induced Ca^{2+} mobilization which peaked at ~10 nM. Cells transfected with AGS3-shRNA showed a significantly higher increase in Ca^{2+} mobilization (B_{max}: 473.0 ± 29.63) as compared to Ctl-shRNA (B_{max}: 334.4 ± 23.63). Despite the increased receptor expression, no significant difference in CXCL8-mediated chemotaxis was shown in AGS3-shRNA cells relative to Ctl-shRNA (data not shown).

We next determined whether AGS3 inhibition affects receptor desensitization. Cells were first exposed to different concentrations of CXCL8 (0-100 nM) for 30 min and assayed for CXCL8 (10 nM) induced Ca^{2+} mobilization. As shown in Fig. 5B, AGS3 knockdown

increases CXCR2 resistance to desensitization (EC_{50}:~ 10 nM) relative to Ctl-shRNA cells (EC_{50}:~ 1 nM).

We further assessed the role of AGS3 knockdown in receptor internalization. AGS3-shRNA and Ctl-shRNA cells were pretreated with 100 nM CXCL8 for 0-30 min and assayed for ligand binding. As shown in Fig. 5C, AGS3 inhibition had no significant effect on CXCR2 internalization. Consistent with previously published data (31), both cell lines displayed ~95% internalization after ~5 min exposure to CXCL8. We also determined the effect of AGS3 inhibition in receptor recycling following CXCL8-induced internalization for 60 min. As shown in Fig. 5D, both AGS3-shRNA and Ctl-shRNA cells displayed time-dependent CXCR2 recovery to the cell surface (Fig 5D). AGS3-shRNA, however, showed a significant resistance to receptor recovery (~70% of total, after 120 min) relative to Ctl-shRNA cells (~100% after 120 min) (Fig. 5D).

Effect of AGS3 knockdown on CXCL8-induced MAPK activation

To determine the effect of AGS3 knockdown on MAP kinase activation we measured ERK1/2 and P38 phosphorylation in response to CXCL8. AGS3-shRNA and Ctl-shRNA cells exhibited time-dependent phosphorylation of both ERK1/2 (Fig. 6A and 6B) and P38 MAP kinase which peaked at ~2-5 min (Fig. 6A & 6C). Depletion of AGS3, however, resulted in a significant reduction (~60-80%) of CXCL8-mediated ERK1/2 and P38 phosphorylation (Fig. 6A, 6B & 6C). Inhibition of P38 activity was previously shown to correlate with an increase in Akt activation (32). We next determine whether AGS3 knockdown which inhibited P38 phosphorylation also promoted Akt activation. As shown in figure 6A and 6D, $AGS3^{-/-}$ displayed a significant increase in CXCL8-induced Akt phosphorylation relative to control cells.

CXCL8-induced AGS3 phosphorylation

To determine whether CXCL8 induces AGS3 phosphorylation, ^{32}P-labeled Ctl-shRNA, GRK6-shRNA and AGS3-shRNA cells were stimulated with CXCL8 (100 nM) for 5 min and cell lysates were immunoprecipitated with AGS3 and CXCR2 specific antibodies. As shown in Fig. 7A (lane 2) & 7C (lane 2), CXCL8-induced phosphorylation of both AGS3 and CXCR2 respectively. Inhibition of GRK6 (lanes 3 and 4), caused a marked increase in AGS3 phosphorylation (Fig. 7A, lane 4 versus lane 2, and 7B) but diminished CXCR2 phosphorylation (Fig. 7C, lane 4 versus lane 2, and 7D), as previously shown (10). AGS3 deficient cells (lanes 5 & 6) showed increase in CXCR2 phosphorylation (Fig. 7C, lane 6 versus lane 2, and 7D) whereas AGS3 phosphorylation was totally inhibited (Fig. 7A, lanes 5 & 6, and 7B).

We next assessed the role of protein kinase C (PKC) in AGS3 phosphorylation. RBL-CXCR2 cells were stimulated with CXCL8 or PMA in the presence and absence of the PKC inhibitor staurosporine. As shown in Fig. 8A, both CXCL8 and PMA induced AGS3 phosphorylation (lanes 3 & 5), although to a different extent (Fig. 8B). Pretreatment with staurosporine inhibited significantly AGS3 phosphorylation (lanes 4 & 6) suggesting that this process may be mediated via a PKC-dependent mechanism. As previously shown (3), staurosporine pretreatment inhibited partially CXCL8-, but totally PMA-induced CXCR2 phosphorylation (Fig. 8C, lanes 4 & 6, respectively; and 8D).

Discussion

CXCR1 and CXCR2 bind to CXCL8, thereby producing a group of equipotent responses in leukocytes (i.e. chemotaxis and exocytosis) (2), but their ability to generate other signals, including phospholipase D (PLD) activation, respiratory burst and cross-regulation are vastly different, indicating that the two receptors may play different physiological roles

during inflammation (33-35). Both receptors become desensitized and downregulated upon CXCL8 stimulation (4, 36). CXCR1 internalizes slowly (~45% after 60 min) but recovers rapidly, whereas CXCR2 internalizes rapidly (~95% after 5-10 min), but recovers slowly at the cell surface (3-4). Phosphorylation and internalization deficient mutants of CXCR2 were shown to activate PLD and mediate cross-regulatory signals as well as CXCR1 suggesting that differential phosphorylation between CXCR1 and CXCR2 may provide a molecular basis for their different rate of internalization as well as ability to generate cellular signals.

We have recently shown that upon activation by CXCL8, CXCR1 and CXCR2 couple to GRK2 and GRK6, respectively, to mediate and regulate cellular responses (10). This specificity, however, did not provide an explanation for the distinct signaling pattern of the two receptors. In addition to GPCRs, GRKs have been shown to bind and/or phosphorylate numerous novel non-GPCR protein molecules including cytoskeletal proteins (i.e. tubulin, erzin) (18, 37); transcription factors (i.e. NFκB and IκBα) (38-39); signaling proteins (i.e. synuclein and phosducin) (19, 40); and other membrane proteins such as the downstream regulatory element antagonist modulator (DREAM) (20). Such diversity in binding partners/substrates indicates that apart from their classical role of phosphorylating activated GPCRs, GRKs may play important roles in cellular functions. Indeed, the data obtained in this study have shown that upon CXCL8 activation, GRK6 but not GRK2 scaffolds with AGS3-$G\alpha_{i2}$ to modulate receptor functions. First, immunoprecipitation studies in RBL-CXCR2 using anti-GRK6 or anti-AGS3 antibody showed that both AGS3 and $G\alpha_i$ scaffold with GRK6 in cells activated with CXCL8 (Fig. 1B). Second, both human and murine neutrophils stimulated with CXCL1 or CXCL8, also revealed that CXCR2 mediates GRK6 association with AGS3-$G\alpha_{i2}$ (Fig. 1D & 1E). The formation of the GRK6/AGS3-$G\alpha_{i2}$ appeared to be specific and required receptor-induced G-protein activation. Supporting that contention is that pre-exposure of RBL-CXCR2 cells to GTPγS or pertussis toxin (Ptx), which prevents G protein activation, blocked the association of GRK6 with AGS3-$G\alpha_{i2}$ (Fig. 2, data not shown). In addition, immunoprecipitation of the cell lysates with AGS4, AGS5 or AGS6 specific antibodies followed by immunoblotting with anti-GRK6 displayed no association with GRK6 (data not shown).

Oner *et al.* (17) using bioluminescence resonance energy transfer (BRET) have recently shown that AGS4-$G\alpha_i$ couples directly to the α_{2A}-Adrenergic receptor (α_{2A}-AR) to modulate receptor/G-protein activation *in vivo*. The α_{2A}-AR/AGS4-Gαi complex formation was time-dependent and peaked at 2-3 min post-activation (16). Attempts to co-immunoprecipitate CXCR2 with AGS3, however, were not successful. A possible explanation could be that the co-immunoprecipitation technique used in this study is less sensitive than BRET, thereby, did not allow the detection of a CXCR2/GRK6/AGS3/$G\alpha_i$ complex. Contradictory to this explanation is the fact that this group, using the BRET system, has previously shown that the α_{2A}-AR and the μ-opioid receptor (μ-OR) induced AGS3-$G\alpha_i$ interaction but, as was the case for CXCR2, failed to report a direct receptor/AGS3-$G\alpha_i$ association (16). Another explanation could be that, upon CXCL8 engagement, GRK6 rapidly phosphorylates the receptor, thereby, causing its dissociation from the complex. Indeed, CXCR2 homologous phosphorylation and internalization peak at 2-5 minutes (Fig. 5C, Ref. 3), similar to that of the peak GRK6/AGS3/$G\alpha_{i2}$ complex formation (Fig. 2B).

To date, apart from being a guanine nucleotide dissociation inhibitor (GDI), little is known about the functional consequences of AGS3 in GPCR-mediated signal transduction and downregulation. Structural and mutagenesis studies have shown that AGS3 interacts with $G\alpha_i$ by virtue of its four G-protein regulatory (GPR) or the GoLOCO motifs, thereby stabilizing GDP-bound Gα and competes with Gβγ for interaction with Gα-GDP, the resultant being the dissociation of heterotrimeric G-proteins' subunits (41). In this study, a

dual approach was undertaken to fully assess the role of AGS3 in CXCR2 signaling by overexpressing and inhibiting its expression in RBL-CXCR2 cells. First, overexpression of AGS3 increased GRK6/AGS3/$G\alpha_i$ association but significantly inhibited CXCL8-induced PI hydrolysis, intracellular Ca^{2+} mobilization and chemotaxis (Fig 3A, B, D & C). Since one molecule of AGS3 can scaffold with up to four molecules of $G\alpha_i$ (16), it could be that the excess of AGS3 limits the pool of G protein thereby preventing receptor/G protein coupling and effector activation (42). Inhibition of AGS3 expression in RBL-CXCR2, in contrast, decreased GRK6/AGS3-$G\alpha_{i2}$ association (data not shown) but increased CXCR2 mediated-intracellular Ca^{2+} mobilization and receptor resistance to desensitization (Fig. 5A & 5B). Surprisingly, AGS3 inhibition also increased receptor expression on the cell surface (Fig. 4A). This suggests that the increased cellular responses could be due to the excess of receptors rather than a decrease in GRK6/AGS3/$G\alpha_{i2}$ association.

The mechanism underlying the increased expression of CXCR2 in RBL-AGS3$^{-/-}$ cells remains unclear. It was previously reported that AGS3 overexpression modulates the expression of several plasma membrane proteins including GPCRs and ion channels (43). mPins, a *Drosophila melanogaster* homologue of AGS3, was also shown to promote the membrane expression of the N-methyl-D-aspartate receptor (NMDAR) in neurons (44). Since these effects appeared to be mediated via regulation of protein trafficking from the cell surface to the endosomes we hypothesized that the increase in CXCR2 expression could be due to decreased internalization and/or rapid recycling of the receptor. As shown in Fig. 5, the rate of CXCR2 internalization was similar in both, control and AGS3 deficient cells. Recycling of the receptor after internalization, however, was significantly delayed in AGS3-depleted cells (~70% versus 100%, respectively, after 120 min, Fig. 5D). This suggests that the increased receptor expression in the cell surface is likely mediated through a mechanism different than AGS3 promoting a rapid Trans-Golgi-Network (TGN)-to-plasma membrane recycling of the internalized receptors as reported previously (43). Overexpression of CXCR2 in tumor cells has been associated with increased activity of several transcription factors including nuclear factor-κB (NF-κB) and hypoxia-inducible factor (HIF-1) (45). It could be that AGS3 deficiency enhances the activity of a CXCR2-associated transcription factor(s) thereby promoting receptor expression. Pretreatment of the cells with the NF-κB inhibitor BAY 11–7085 for 24 hours, however, showed no significant effect in CXCR2 expression in RBL-AGS3−/− cells relative to control (data not shown). In addition phosphorylation of the p65 subunit of NF-κB, which is associated with increased NF-κB activation and translocation to the nucleus (27), was similar in both control and AGS3 deficient cells (S1).

Interestingly, despite the increase in receptor cell surface expression, AGS3 deficient cells displayed a significant decrease in CXCR2-mediated ERK1/2 and P38 MAP kinase activation (Fig. 6). The reason for that inhibition remains unclear. It could be that the GRK6/AGS3-$G\alpha_{i2}$ complex is part of larger signaling complex that mediates MAP kinase activation. AGS3 inhibition could diminish the formation of this complex and, thereby, ERK1/2 and P38 phosphorylation (Fig. 6). Zhao et al (46) have shown that upon activation, CXCR2 recruits the low molecular weight G proteins Raf and Rac to the cell membrane to mediate MAP kinase activation. Thus, another explanation could be that inhibition of AGS3 prevented the translocation of these molecules and, thereby, receptor coupling to MAP kinase pathways. Supporting that contention is a recent report by Oner et al (47) showing that agonist-induced AGS3 translocation to the Golgi apparatus modulates protein secretion and/or endosome recycling events at the *trans*-Golgi network.

Apart of its classical role in GPCR phosphorylation and desensitization, GRK6 was shown to bind and/or phosphorylate other proteins including the low density lipoprotein receptor-related protein 6 (LRP6) which interacts with the Wnt/beta-catenin signaling cascade, the

Na^+/H^+ exchanger regulatory factor (NHERF), and DREAM which modulates the expression and trafficking of the of Kv4.2 potassium channel (20, 48-49). It was recently shown that LKB1, a serine/threonine kinase involved in cell polarity and development, interacts with the TPR domain of the AGS3 and phosphorylates specific residue in the GPR motifs thereby inhibiting AGS3 ability to scaffold $G\alpha_{i3}$-GDP (50). Thus, we postulated that GRK6 mediated phosphorylation of AGS3 could play a role in the rapid dissociation of the GRK6/AGS3-$G\alpha_{i2}$ complex. Indeed, CXCL8 induced phosphorylation of both CXCR2 and AGS3 in RBL-CXCR2 cells (Fig. 7A & 7C). Rather than GRK6, however, AGS3 appears to be phosphorylated via a protein kinase (PKC)-dependent mechanism. First, inhibition of GRK6 had no effect in AGS3 phosphorylation but diminished CXCR2 phosphorylation (10). Second, treatment of RBL-CXCR2 with the PKC activator PMA, induced AGS3 phosphorylation as well as CXCL8 (Fig. 8A). And, third, pre-exposure of the cells to the PKC inhibitor staurosporine blocked AGS3 phosphorylation (Fig. 8A). The role of PKC-mediated AGS3 phosphorylation remains unclear. However, it was recently shown that AGS3 rapid translocation to the Golgi Apparatus (GA) upon receptor activation is due to a single conserved residue (G90) in the TRP2 domain, and was independent of AGS3 phosphorylation (47).

In summary, the data herein indicate that AGS3 complexes with $G\alpha_{i2}$ and GRK6 to modulate CXCR2-mediated cellular functions including second messenger production, MAP kinase activation and chemotaxis. AGS3 knockdown decreased GRK6/AGS3-$G\alpha_{i2}$ association but increased receptor expression on the cell surface. Surprisingly, receptor recycling was delayed in AGS3-deficient cells, suggesting that the increased receptor expression on the cell surface is mediated via a different process. Several questions remained to be answered. 1) What is the role of GRK6 in the signaling complex? 2) Which TPR or GPR motif(s) does GRK6 interact with? 3) Does PKC mediated-phosphorylation of AGS3 diminish its GDI function thereby promoting the dissociation of the complex? AGS3 has been shown to block the sensitization of adenylyl cyclase following prolonged stimulation of the β2-adrenergic receptor (41); regulate motor sensitization and relapse to drug seeking behavior by promoting Gβγ-induced protein kinase A (PKA) activation (51); and modulate metabolic and cardiovascular functions. The findings herein provide new insights into the relevance of AGS3 in CXCR2 expression, activation and regulation; and further indicate that GPSM may play a critical role in chemokine-mediated inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Stephen M. Lanier and Dr. Joe Blumer for providing the GFP-tagged rat AGS3 plasmid construct. We are very thankful to Ms. Kimberly M. Malloy (JLC Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham NC) for technical support.

Abbreviations used in this paper

AGS3	Activator of G protein signaling 3
GRK	G protein coupled receptor kinase
GPCR	G protein-coupled receptor
CXCL8/IL-8	interleukin-8

CXCR2	IL-8 receptor B
ELR	Glu-Leu-Arg
MAPK	mitogen-activated protein kinase

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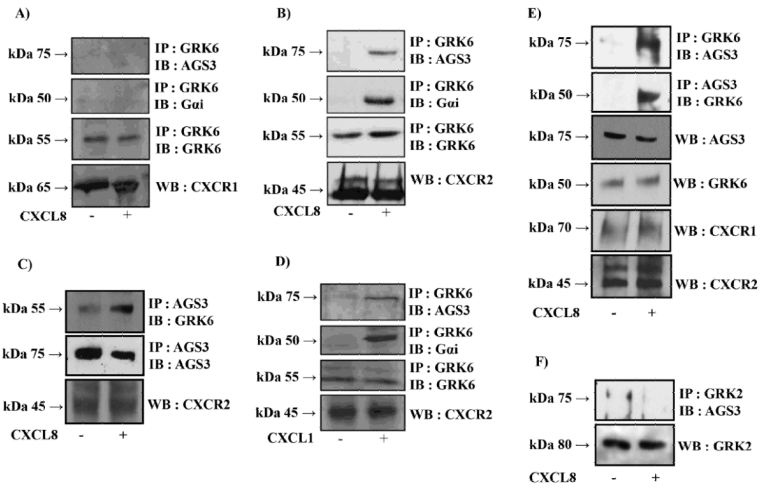

Figure 1. GRK6 complexes with AGS3 and $G\alpha_i$ in RBL-CXCR2, but not RBL-CXCR1 cells

RBL-CXCR1 **(A),** RBL-CXCR2 **(B & C),** murine **(D)** and human **(E & F)** neutrophils were treated with 100 nM CXCL8 **(A, B, C, E & F)** or CXCL1 **(D)** for 3 minutes at 37°C. Cell lysates were immunoprecipitated with GRK6 **(A, B, D & E),** AGS3 **(C & E)** or GRK2 **(F)** antibodies, resolved by SDS-PAGE and immunoblotted with anti-GRK6, anti-AGS3 and anti-$G\alpha_{i2}$ antibodies. For receptor expression, cell lysates (20 μg protein) were resolved by SDS-PAGE and immunoblotted with human CXCR1 **(A & E)** and CXCR2 **(B, C & E)** or murine CXCR2 **(D)** antibodies**.** The results shown are representative of one of at least three experiments.

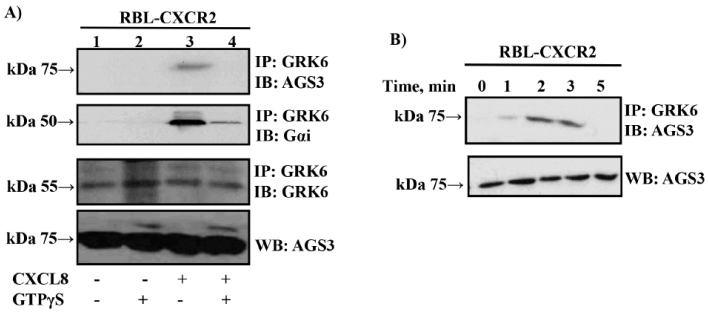

Figure 2. GTPγS inhibits CXCR2-induced GRK6/AGS3/Gα_{i2} association

A) RBL- CXCR2 cells (5×10^6) were permeabilized with streptolysin O in the presence (lanes 2 & 4) or absence (lanes 1 & 3) of GTPγS (100 nM) for 30 minutes at 37°C and stimulated with or without CXCL8 (100 nM) for 3 minutes. Cell lysates were immunoprecipitated as described in the legend of Fig. 1. As control, cell lysates (20 μg protein) were immunoblotted with anti-AGS3 antibody. **B)** RBL-CXCR2 cells were treated with CXCL8 (100 nM) for different period of times (0-5 min). Cell lysates were immunoprecipitated with anti-GRK6 antibody and immunoblotted with anti-AGS3 antibody. The results shown are representative of one of three experiments.

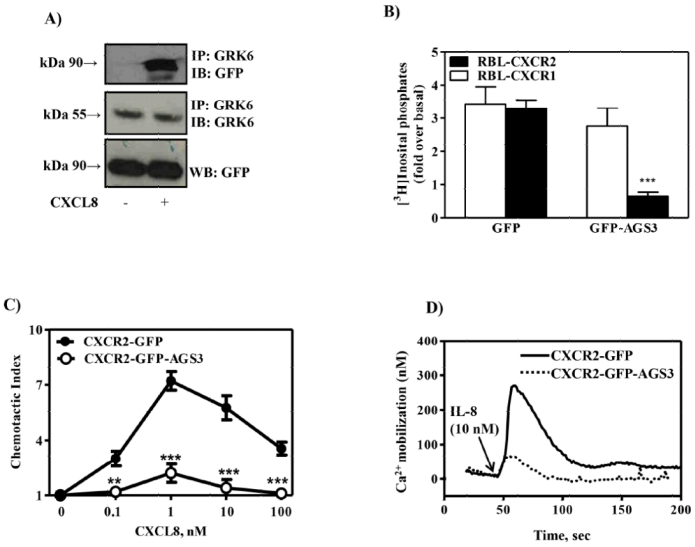

Figure 3. Functional consequences of AGS3 overexpression in RBL-CXCR1 and RBL-CXCR2 cells

A) RBL-CXCR2 cells (5×10^6) expressing either GFP alone or GFP-AGS3 were stimulated with or without CXCL8 (100 nM). After treatment, cell lysates were immunoprecipitated with GRK6 and immunoblotted with either GFP (upper panel) or GRK6 (middle panel) antibody. As control, cell lysates (20μg protein) were immunoblotted with anti-GFP antibody. The experiments were repeated twice. **B, C & D**) Cells were treated with CXCL8 and PI hydrolysis, chemotaxis and Ca^{2+} mobilization were measured as described in Materials and Methods. The experiment was repeated 4 times in triplicate. **P<0.01; *** P < 0.001, Student *t* test.

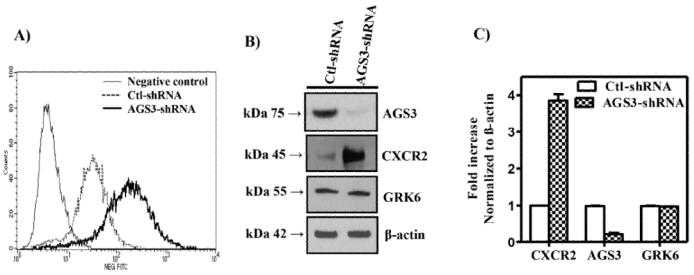

Figure 4. Inhibition of AGS3 promotes CXCR2 expression in RBL-CXCR2 cells

RBL-CXCR2 cells were transfected with scrambled shRNA control lentivirus (Ctl shRNA) or shRNA lentivirus specific for AGS3 (AGS3 shRNA). Puromycin-resistant cells were selected and single clones were generated and analyzed by FACS and immunoblotting. **A)** A representative histogram of FACS analysis showing surface expression of CXCR2 in RBL-2H3 cells after staining with CXCR2 specific antibody. B) Western blot analysis of AGS3, CXCR2 and GRK6 expression in Ctl shRNA and AGS3 shRNA cells. **C)** Band density was calculated by Image J software, normalized for β-actin expression and are the averages of three experiments.

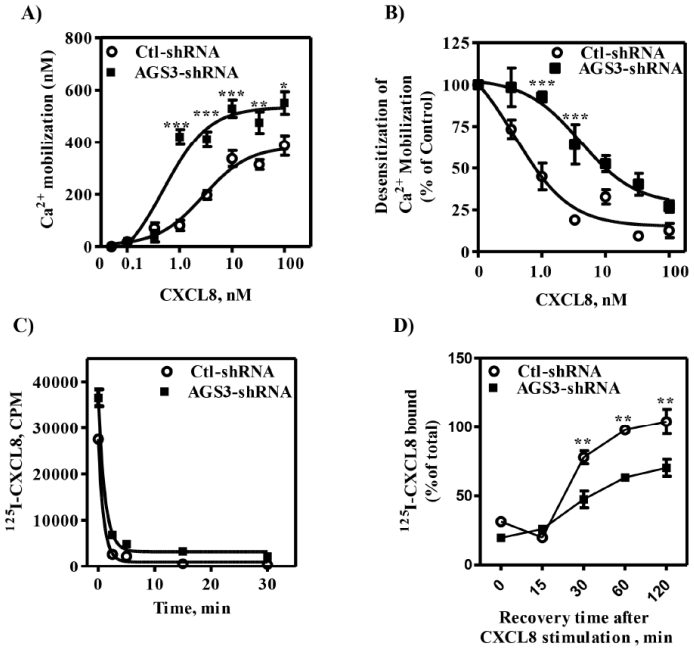

Figure 5. AGS3 knockdown increases Ca^{2+} mobilization and receptor resistance to desensitization but does not affect CXCR2 internalization

(A), RBL-CXCR2-control shRNA and RBL-CXCR2-AGS3 shRNA cells (5×10^6) were stimulated with different doses of CXCL8 and Ca^{2+} mobilization was measured. Data are the average of at least three traces. **(B)** For receptor desensitization, cells were loaded with Indo-1 AM in the presence or absence of different doses of CXCL8 for 30 min and assayed for CXCL8 (10 nM) induced intracellular Ca^{2+} mobilization. Desensitization was determined as the percentage of control, which is the peak of intracellular Ca^{2+} mobilization obtained in the absence of pretreatment. Data are the average of at least three traces. **(C)** For receptor internalization, cells (0.5×10^6 cells) were treated with CXCL8 (100 nM) for different periods of time and assayed for ^{125}I-CXCL8 binding. Data are represented as percentage of total ^{125}I-CXCL8 bound to control (untreated) cells.

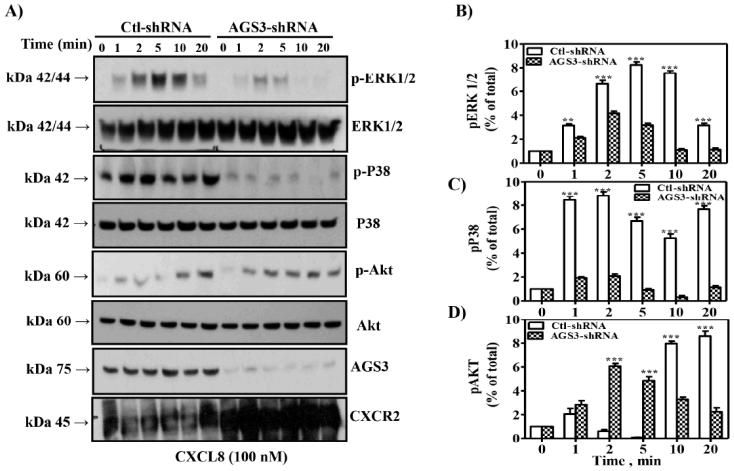

Figure 6. Effect of AGS3 knockdown in CXCR2-induced ERK1/2, P38 and Akt phosphorylation

(A) Control (Ctl-shRNA) and AGS3 deficient (AGS3-shRNA) RBL-CXCR2 cells were stimulated with CXCL8 (100 nM) for 0-20 min, and ERK1/2, P38 and Akt phosphorylation were determined by western blotting using anti-phospho-ERK1/2 (pERK1/2), P38 (pP38) and Akt (pAkt) antibodies, respectively. Total proteins were measured using ERK1/2, P38, Akt, AGS3 and CXCR2 antibodies. Band densities from the western blots were calculated by Image J software and represented as percentage of total ERK1/2 (**B)**, P38 (**C**) and Akt (**D**). Data shown are the averages of three experiments.

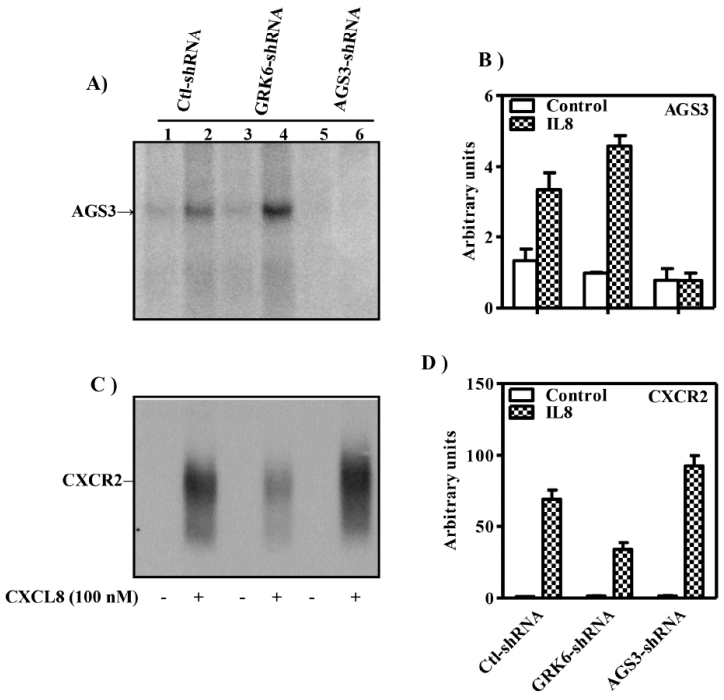

Figure 7. Effect of GRK6 knockdown on AGS3 phosphorylation

For receptor and AGS3 phosphorylation, ^{32}P-labeled (5×10^6) control shRNA (lanes 1 & 2), GRK6 shRNA (lanes 3 & 4) and AGS3 shRNA (lanes 5 & 6) were incubated for 5 min with (lanes 2, 4 & 6) or without (lanes 1, 3 & 5) 100 nM CXCL8. Cells lysates were immunoprecipitated with anti-AGS3 (A) followed by anti-CXCR2 (C) antibodies, analyzed by SDS-PAGE, and autoradiographed. The results shown are from a representative experiment that was repeated three times. **(B & D)** Band density of A & C, was quantified as mentioned in the legend of figure 4.

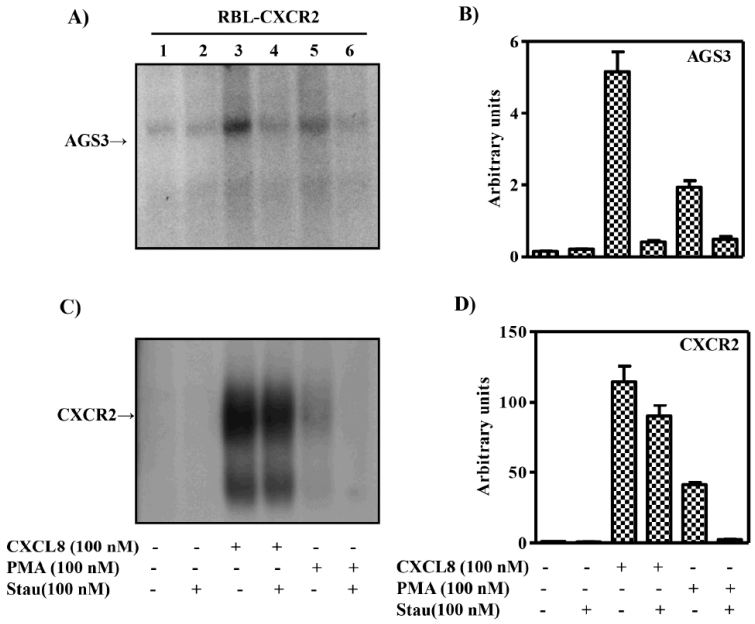

Figure 8. Effect of PKC inhibitor on AGS3 phosphorylation

(A & C) ^{32}P-labeled RBL-CXCR2 cells pretreated with (lanes 2, 4 & 6) or without (lanes 1, 3 & 5) staurosporine (100 nM) for 5 min were incubated for 5 min with or without stimulants as shown. Phosphorylation was measured as described above. **(B & D)** Band density of A & C, was quantified as mentioned in the legend of figure 4.