doi: 10.4248/IJOS.09059

ORIGINAL SCIENTIFIC ARTICLES

CXCL12/SDF-1α Activates NF-κB and Promotes Oral Cancer Invasion through the Carma3/Bcl10/Malt1 Complex

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Abstract

Aasia O. Rehman, Cun-yu Wang. CXCL12/SDF-1α Activates NF-κB and Promotes Oral Cancer Invasion through the Carma3/Bcl10/Malt1 Complex. *International Journal of Oral Science*, 1(3): 105–118, 2009

Aim To determine how SDF-1α/CXCR4 activates nuclear factor-kappa B (NF-κB) and promotes oral squamous cell carcinoma (OSCC) invasion.

Methodology A lentivirus-based knockdown approach was utilized to deplete gene expression. NF-κB activation was evaluated by Western blot analysis and electrophoretic mobility shift (EMSA).

Results We show that the activation of NF-κB by CXCR4 occurs through the Carma3/Bcl10/Malt1 (CBM) complex in OSCC. We found that loss of components of the CBM complex in HNSCC can inhibit SDF-1α induced phosphorylation and degradation of IκBα, while TNFα induced IKK activation remains unchanged. Further, we identified a role for novel and atypical, but not classical, PKCs in activating IKK through CXCR4. Importantly, inhibition of the CBM complex leads to a significant decrease in SDF-1α mediated invasion of OSCC.

Conclusion The CBM complex plays a critical role in CXCR4-induced NF-κB activation in OSCC. Targeting molecular components of the NF-κB signaling pathway may provide an important therapeutic opportunity in controlling the progression and metastasis of OSCC mediated by SDF-1α.

Keywords CXCR4, NF-κB, head and neck cancer, invasion, signal transduction

Document code: A **CLC number:** Q257 Received Jul. 30, 2009; Revision accepted Sep. 1, 2009

Introduction

CXCR4 is a 7-transmembrane G protein coupled receptor (GPCR) that is expressed on peripheral blood lymphocytes, monocytes, pre-B cells, dendritic cells, $CD34^+$ progenitor cells, and endothelial cells (Kucia *et al.*, 2004). The chemokine ligand of CXCR4, CXCL12/stromal cell-derived factor-1α (SDF-1α) is a potent chemotactic factor for the various cell types that express CXCR4. Cells expressing CXCR4 are able to migrate towards SDF-1α gradients to elicit a variety of biological responses such as angiogenesis, inflammation, and lymphocyte maturation. CXCR4 also serves as a co-receptor for T-cell tropic HIV and has a crucial role in HIV infection and pathogenesis (Feng *et al.*, 1996). Further, both CXCR4 and SDF-1α are critical during embryogenesis, as knockout mice show identical phenotypes and prove embryonic lethal due to severe defects in hematopoiesis, vascular development, and cardiogenesis (Nagasawa *et al.*, 1996; Ma *et al.*, 1998; Zou *et al.*, 1998).

CXCR4 is also overexpressed in more than 20 kinds of malignancies including OSCC and is known to contribute to the metastatic spread of cancer (Zlotnik, 2006). OSCC is the sixth most common cancer worldwide with a five-year survival rate of less than 50% (Parkin *et al.*, 2005).

Treatment options have not significantly improved in decades and can often be debilitating due to the location of the cancer in patients presenting advanced stage disease. The prognosis of OSCC remains poor due to local invasion of soft tissues, a high rate of cervical lymph node metastasis, and difficulties in controlling locoregional recurrence. CXCR4 has been found to be elevated in metastatic OSCC tissue compared to normal and non-metastatic counterparts (Delilbasi *et al*., 2004). Tissues to which tumor cells selectively metastasize, such as the lymph nodes, bone, lungs, and liver, are often the most abundant source of SDF-1α (Muller *et al*., 2001). A significant association between CXCR4 expression and lymph node metastasis has been reported in OSCC (Almofti *et al*., 2004; Uchida *et al*., 2004; Ishikawa *et al*., 2006). However, the molecular mechanisms associated with CXCR4-mediated OSCC metastasis remains poorly understood.

We previously demonstrated that SDF-1α can activate NF-κB signaling through CXCR4 and induce OSCC invasion (Rehman and Wang, 2008). The NF-κB pathway has a well-documented role in innate immunity, inflammation, apoptosis, and tumorigenesis. The NF-κB family of transcription factors consist of five family members [RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2)] which function as homo- or hetero-dimers. In unstimulated cells, the NF-κB proteins are bound to inhibitory molecules called IκBs (inhibitors of κBs) and maintained in an inactive state in the cytoplasm. When cells are stimulated by cytokines, growth factors, infectious agents, or stress stimuli, the IKK complex (which consists of catalytically active IKKα and IKKβ and the regulatory molecule IKKγ) is activated. This leads to the direct phosphorylation of IκB proteins on two conserved serine residues, allowing for polyubiquitination and rapid degradation of IκB. NF-κB is subsequently liberated, allowing it to move freely into the nucleus to activate gene transcription (Perkins, 2007).

The precise mechanism by which IKK is activated and regulated by various stimuli remains unclear and until recently it was unknown how GPCRs can activate NF-κB signaling. Several groups reported the involvement of the Carma3/Bcl10/Malt1 (CBM) complex in the activation of IKK by GPCRs (Grabiner *et al*., 2007; Klemm *et al*., 2007; McAllister-Lucas *et al*., 2007; Wang *et al*., 2007; Mahanivong *et al*., 2008; Martin *et al*., 2009). The CBM complex was initially found to function downstream of antigen-receptor signaling in B and T lymphocytes. Bcl10, an adaptor protein, and Malt1, a paracaspase, are both involved in separate translocations in a subset of MALT lymphomas (Dierlamm *et al*., 1999; Zhang *et al*., 1999). Bcl10 and Malt1 interact constitutively through their caspase recruitment domains (CARD) to cooperatively activate NF-κB signaling (Lucas *et al*., 2001). Carma1, a homolog of Carma3, expressed only in immune cells, is a member of the MAGUK family of scaffolding molecules and serves to bridge the interactions of Bcl10, Malt1, and NF-κB activating molecules. MAGUK molecules typically contain multiple protein-protein interaction domains to facilitate the binding of transmembrane molecules to intracellular signaling molecules and components of the cytoskeleton. After antigen receptor stimulation, protein kinase C (PKC) isoforms are activated in a phospholipase dependent manner, which in turn leads to direct phosphorylation of Carma1 in its linker region resulting in conformational changes to allow binding of Bcl10 and Malt1 (Matsumoto *et al*., 2005; Sommer *et al*., 2005). The culmination of protein interactions, possibly involving Tak1 protein kinase and Traf6 ubiquitin ligase, leads to activation of the IKK complex resulting in NF-κB transcriptional activity (Sun *et al*., 2004; Wang *et al*., 2004; Shinohara *et al*., 2005).

In this study, we investigated whether the CBM complex might be involved in SDF-1α mediated activation of NF-κB. Using a lentivirus-based knockdown approach we found that SDF-1α mediated phosphorylation and degradation of IκBα is dependent on Carma3, Bcl10 and Malt1 in OSCC. Inhibition of PKCθ and PKCζ (but not the classical PKCα or PKCβ isozymes), blocked IKK activation. Importantly, knockdown of components of the CBM complex significantly interfered with SDF-1α mediated invasion of OSCC. These results suggest that disrupting the interactions with the CBM complex may be beneficial in preventing NF-κB dependent invasion and metastasis of OSCC mediated by SDF-1α.

Materials and methods

Cell culture and reagents

TB2-T4 OSCC cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin.

Antibodies were obtained from the following companies: p-IκBα (ser32/36), p-MARCKS (ser 152/156), Cell Signaling; IκBα, Bcl10, Malt1, Santa Cruz; α-tubulin, Sigma-Aldrich. Recombinant human SDF-1α was purchased from R&D Systems (USA). Myristoylated pseudosubstrate peptide inhibitor against PKCζ was purchased from Biomol International. Pertussis Toxin, Bisindolylmaleimide Ⅰ, Go6976, and PKCθ inhibitors were purchased from Calbiochem (USA).

Lentivirus-based stable gene silencing

For short hairpin RNA mediated knockdown experiments, the lentivirus construct pLKO.1 was used to silence expression of Carma3, Bcl10, and Malt1. The shRNA sequences used (Ngo *et al*., 2006) were as follows:

Carma3 sense,
5'-GATCTACCCCATCGTCATCCA-3';
Carma3 antisense,
5'-TGGATGACGATGGGGTAGATC-3' (17);
Bcl10 sense,
5'-GATGAAGTGCTGAAACTTAGA-3';
Bcl10 antisense,
5'-TCTAAGTTTCAGCACTTCATC-3' (29);
Malt1 sense,
5'-GTCACAGAATTGAGTGATTTC-3';
Malt1 antisense,
5'-GAAATCACTCAATTCTGTGAC-3'.

Sequences were inserted into the AgeⅠ-EcoRⅠ sites of pLKO.1. Positive clones were sequence verified. 293T cells were transfected with either pLKO.1-shRNA (3 μg) or pLKO.1-scramble control (3 μg) along with packaging plasmids pCMV-dR8.2 dvpr (2.25 μg) and pCMV-VSVG (0.75 μg) using lipofectamine 2000 (Invitrogen). Virus-containing media was collected 48 hours post-transfection, spun down, filtered using a 0.45 μm filter, and stored at -70℃ until ready for use. TB2-T4 cells were infected with virus in the presence of 4 $\mu g \cdot mL^{-1}$ of polybrene. Forty-eight hours post-transduction, cells were selected with 2 $\mu g \cdot mL^{-1}$ of puromycin for 7–10 days. Surviving colonies were pooled and maintained indefinitely with 1 $\mu g \cdot mL^{-1}$ of puromycin. Knockdown of protein expression was confirmed by Western blot analysis for Bcl10 and Malt1 or real-time PCR for Carma3.

Quantitative real-time PCR

Total RNA from TB2T4 scramble control and TB2T4 Carma3 shRNA cells was extracted using Trizol reagent (Invitrogen, USA). RNA (1 μg) was reverse transcribed to produce cDNA using Oligo-dT primer and the Superscript Ⅲ Reverse Transcription Kit (Invitrogen). Primer sequences were as follows:

GAPDH
5'-ATCATCCCTGCCTCTACTGG-3',
5'-GTCAAGTCCACCACTGACAC-3';
Carma3
5'-CCTGCTGGACATCCTAGAGC-3',
5'-TGGTCTCGCTCCTTCTCAAT-3'.

Real-time PCR was performed using iQ SYBR Green Supermix (BioRad, USA) on an iCycler iQ real-time PCR detection system (BioRad).

Western blotting

Cells were grown to 70% confluency, rinsed with PBS, serum-starved overnight, and treated with 50 $ng \cdot mL^{-1}$ of SDF-1α for the indicated time points. Cells were rinsed with and scraped in cold PBS, spun down, and cell pellets were lysed with Cell Lytic Solution (Sigma, USA) containing phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. Proteins were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (BioRad) using a semi-dry transfer apparatus (BioRad). Membranes were blocked for one hour at room temperature with 5% non-fat dry milk (BioRad) in TBS-T and incubated with primary antibody overnight at 4℃. Membranes were washed with TBS-T and incubated with horseradish peroxidase conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence system (Pierce, USA).

EMSA

Cells were treated with SDF-1α for the indicated times, rinsed with PBS, and pelleted. Cytoplasmic proteins were isolated by lysing the cell membrane using buffer containing 10 mmol·L^{-1} Hepes, pH 7.6, 60 mmol·L^{-1} KCl, 1 mmol·L^{-1} EDTA, 0.075% NP-40, 1 mmol·L^{-1} DTT, 1 mmol·L^{-1} PMSF, and protease inhibitor cocktail. Cytoplasmic protein lysate were removed after centrifugation and nuclear extract was prepared by lysing the nuclear pellet in buffer containing 20 mmol·L^{-1} Tris, pH 8.0, 420 mmol·L^{-1} NaCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.2 mmol·L^{-1} EDTA, 25% glycerol, 0.5 mmol·L^{-1} PMSF, and protease inhibitor cocktail. Ten μg of nuclear extract was incubated with 2 μg poly (dI-dC) (Amersham), DNA binding buffer (250 mmol·L^{-1} NaCl, 50 mmol·L^{-1} Tris, 50% glycerol, 5 mmol·L^{-1} DTT, 2.5 mmol·L^{-1} EDTA), and approximately 10-15 thousand cpm of αP^{32}-CTP labeled NF-κB consensus sequence. Extracts were separated on a 5% non-denaturing polyacrylamide gel and subjected to autoradiography at -80℃. Supershift analysis for NF-κB was conducted using an antibody against p65.

Invasion assay

Invasion assay was performed according to the manufacturer's protocol (BD Biosciences, USA). Briefly, 100,000 cells were seeded on top of growth factor reduced matrigel-coated membranes containing 8-μm pores in serum free DMEM. The lower chamber contained DMEM plus 0.1% FBS with or without 50 ng·mL^{-1} SDF-1α. After 48 hours, cells on the top of the membrane that had not migrated were gently removed with a cotton swab and cells that had migrated through the membrane were stained with Protocol™ Hema3 kit and counted under the microscope.

Results

SDF-1α induces NF-κB activation in OSCC through $G\alpha_i$

We demonstrated that SDF-1α can activate the NF-κB signaling pathway in OSCC. When stimulated with the appropriate factors, the IKK complex is activated and phosphorylates IκB inhibitor molecules targeting them for proteasomal destruction. Degradation of IκBα frees NF-κB molecules from inhibition in the cytoplasm and allows them to enter the nucleus to trigger gene expression. In this study we used TB2-T4 cells, an oral squamous cell carcinoma cell line. Varying concentrations of SDF-1α were used to stimulate TB2-T4 cells and to assess activation of IKK by analyzing phosphorylation of IκBα and the subsequent degradation of IκBα by Western blot (Figure 1A). We observed a dose-dependent increase of IκBα phospohorylation and degradation. Furthermore, time course treatment with 50 ng·mL^{-1} of SDF-1α showed induction of IκBα phosphorylation as early as 5 minutes and a robust response at 30 minutes, while degradation of IκBα was most obvious at 60 minutes (Figure 1B). EMSA analysis further demonstrated that p65 translocation to the nucleus was strongly induced at 30 minutes and maintained through 60-minute treatment with SDF-1α (Figure 1C).

CXCR4 is coupled to heterotrimeric G proteins consisting of α, β and γ subunits. There are four families of the α subunits: α_i, α_s, α_q, and $\alpha_{12/13}$. CXCR4 is most commonly known to couple with $G\alpha_i$, however, it is also known to signal through other α subunits as well (Busillo *et al*., 2007; Dorsam *et al*., 2007). GPCRs are often able to elicit signaling pathways in G protein independent manners as well, for example by dimerizing to other receptors, binding directly to scaffolding molecules, non-receptors kinases, or guanine exchange factors (Andreeva *et al*., 2007; Sun *et al*., 2007). Pertussis toxin is a well-known inhibitor of $G\alpha_i$ and specifically blocks the coupling of the heterotrimeric G proteins to the receptor thereby inhibiting downstream signal activation. Pertussis toxin was utilized to determine if SDF-1α mediated IKK activation is dependent on the heterotrimeric G proteins or if IKK may be activated in a G protein independent manner. TB2-T4 cells were pretreated with various concentrations of pertussis toxin and then stimulated with 50 ng·mL^{-1} of SDF-1α for 30 minutes (Figure 1D). Western blot was performed to assess IκBα phosphorylation. Concentrations as low as 2 ng·mL^{-1} of pertussis toxin were able to completely block phosphorylation of IκBα. This suggests that

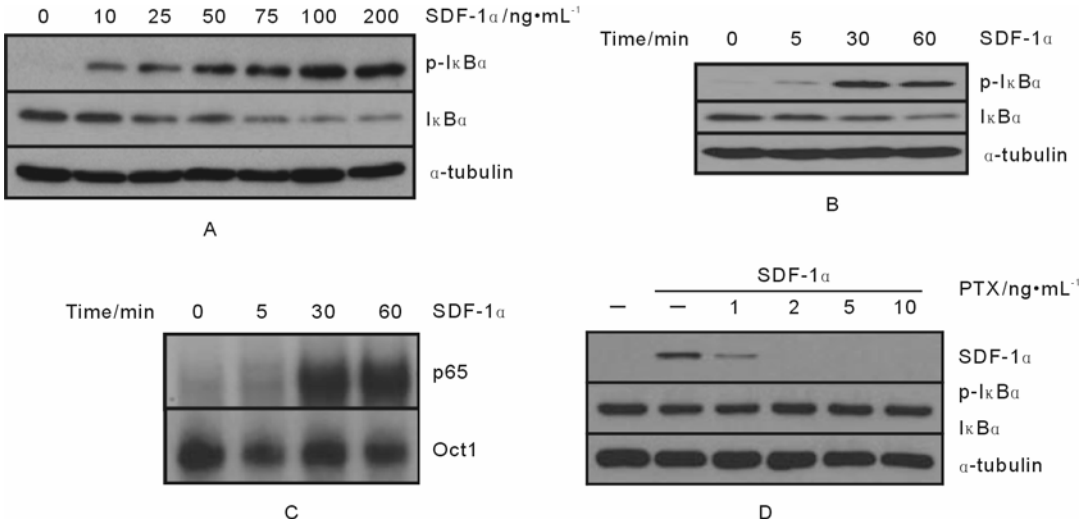

Figure 1 SDF-1α activates NF-κB in OSCC through $G\alpha_i$

(A): SDF-1α activates IκBα phosphorylation and degradation in a dose dependent manner in TB2-T4 cells. TB2-T4 cells were serum-starved overnight and stimulated with various concentrations of SDF-1α ranging from 10–200 ng·mL^{-1} for 30 minutes. Whole cell lysates were prepared and 50 μg of protein were separated on a 10% SDS-PAGE and subjected to Western blot analysis using antibodies specific for phospho-IκBα, IκBα, and α-tubulin. Proteins were visualized by enhanced chemoluminescence. (B): Time course treatment of TB2-T4 cells with SDF-1α. TB2-T4 cells were serum-starved overnight and stimulated the next day with 50 ng·mL^{-1} of SDF-1α for the indicated times. Whole cell lysates were prepared and 50 μg of protein were subjected Western Blot analysis using the indicated antibodies. (C): SDF-1α induces nuclear translocation of p65 in OSCC. TB2-T4 cells were serum-starved and treated with 50 ng·mL^{-1} of SDF-1α for the times indicated. Nuclear proteins were extracted and 10 μg were incubated with P^{32}-labeled DNA probe containing an NF-κB consensus sequence for 30 minutes at room temperature. EMSA was performed using a non-denaturing 5% polyacrylamide gel. The gel was dried and subjected to autoradiography for visualization. Oct-1 served as a loading control. (D): SDF-1α mediated IKK activity is pertussis toxin sensitive in OSCC. TB2-T4 cells were serum-starved and treated with concentrations ranging from 1–10 ng·mL^{-1} of pertussis toxin (PTX) overnight. The following day cells were stimulated with 50 ng·mL^{-1} of SDF-1α for 30 minutes. Whole cell extracts were prepared and 50 μg of protein were run on a 10% SDS-PAGE. Western blot was performed with indicated antibodies.

CXCR4 activates the IKK complex specifically through $G\alpha_i$.

Carma3/Bcl10/Malt1 are required for SDF-1α-mediated IκBα phosphorylation and degradation

The Carma1/Bcl10/Malt1 (CBM) complex has been extensively studied in the context of antigen-receptor mediated activation of NF-κB signaling in lymphocytes. Whereas OSCC cells do not express Carma1, Carma3 is detected in OSCC cells. We sought to determine if the CBM complex also has a role in mediating SDF-1α signaling to NF-κB. In order to investigate this, we generated TB2-T4 cells stably expressing short-hairpin RNAs (shRNA) against Carma3, Bcl10, and Malt1 by using a lentivirus-based approach. After infection of TB2-T4 cells with virus and antibiotic selection, gene expression levels were assessed by either Western blot for Bcl10 and Malt1, or quantitative real-time PCR for Carma3. Compared to TB2-T4 scramble control cells, Carma3 expression was blocked by 87% in TB2-T4 cells expressing Carma3 shRNA as confirmed by real-time analysis (Figure 2A).

Similarly, expression of both Bcl10 and Malt1 were also significantly inhibited using shRNA (Figures 2B, 2C) as shown by Western blot analysis. Next we investigated whether Carma3, Bcl10, and Malt1 were required for SDF-1α mediated IKK activation. Stable cell lines were serum starved overnight and then stimulated with SDF-1α for the indicated times. Western blot showed a significant decrease in phosphorylation and degradation of IκBα in Carma3, Bcl10, and Malt1 knockdown cells when compared to TB2-T4 scramble control cells (Figures 2D–2F). This suggests that the CBM

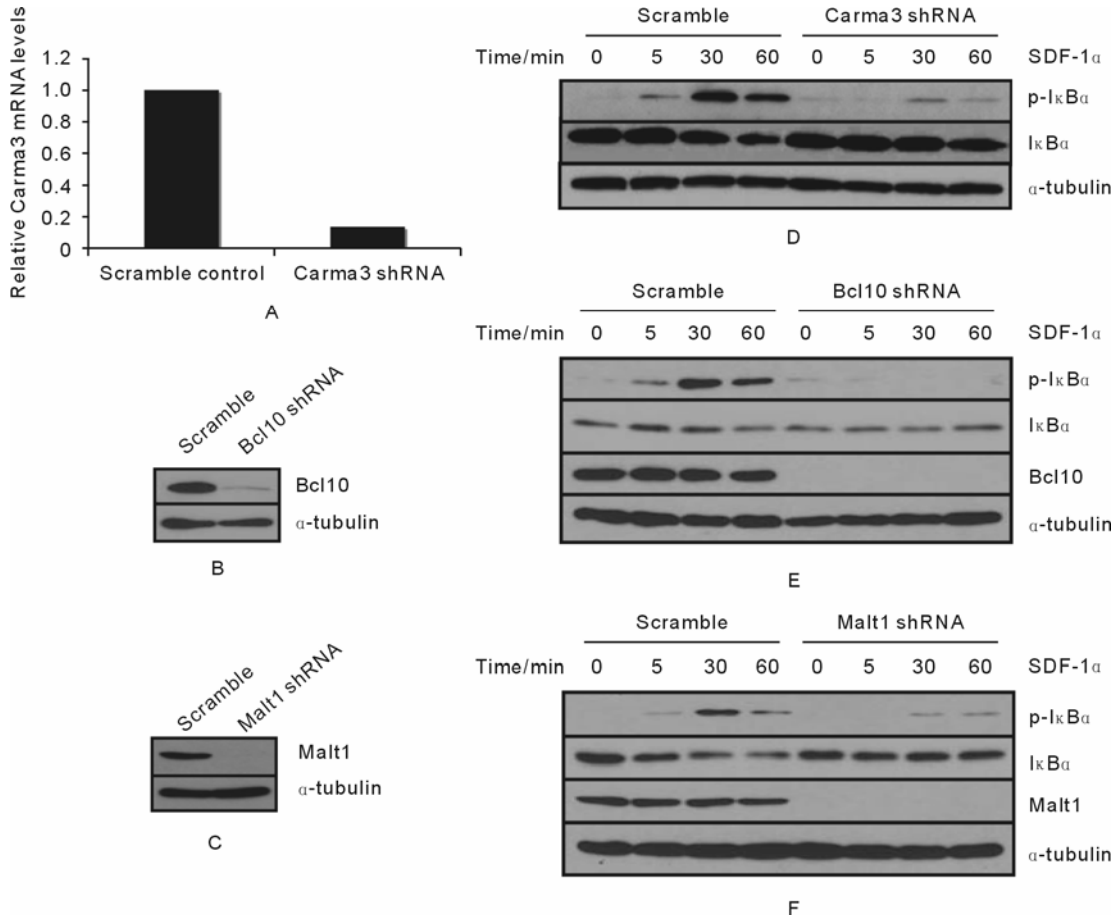

Figure 2 SDF-1α mediated IKK activation is dependent on the CBM complex in OSCC

(A–C): Stable knockdown confirmation of Carma3, Bcl10, and Malt1 using short hairpin RNA. shRNAs for Carma3, Bcl10, and Malt1 and a scramble control were cloned into a lentiviral expression vector. Lentivirus encoding these shRNAs were used to infect TB2-T4 cells to produce stable knockdown cell lines. (A): Total RNA was extracted from TB2-T4 scramble control and TB2-T4 Carma3 knockdown cells, reverse transcribed, and real-time PCR was performed using primers for Carma3 and GAPDH. Carma3 mRNA expression levels were normalized against GAPDH levels. Data are the average of triplicates from a representative experiment. (B, C): Whole cell lysates were prepared from TB2-T4 scramble control, TB2-T4 Bcl10 knockdown, and TB2-T4 Malt1 knockdown cells and 50 μg of protein were run on a 10% SDS-PAGE and subjected to Western blot analysis using the antibodies indicated. α-Tubulin served as a loading control. (D–F): Knockdown of Carma3, Bcl10, and Malt1 block SDF-1α mediated IκBα phosphorylation and degradation in TB2-T4 cells. Stable knockdown cells were serum-starved overnight and treated with 50 ng·mL^{-1} of SDF-1α for the indicated times. Whole cell lysates were prepared and 50 μg of protein were run on a 10% SDS-PAGE. Western blot analysis was performed using the indicated antibodies.

complex likely plays a role in activating IKK through the CXCR4 G protein coupled receptor.

Carma3/Bcl10/Malt1 are not required for TNFα-mediated IκBα phosphorylation and degradation

While it was observed that SDF-1α mediated IKK activation was inhibited in Carma3, Bcl10, and Malt1 knockdown cell lines we wondered whether the activity of the CBM complex was specific to G protein coupled receptors in OSCC or if it may also be involved in TNF receptor signaling to IKK as well. TB2-T4 knockdown cell lines were treated with 10 ng·mL^{-1} of TNFα for the indicated times and Western blotting was performed to assess activation of IKK. We observed that TNFα mediated IκBα phosphorylation and degradation in Carma3, Bcl10, and Malt1 knockdown cell lines remained unchanged compared to

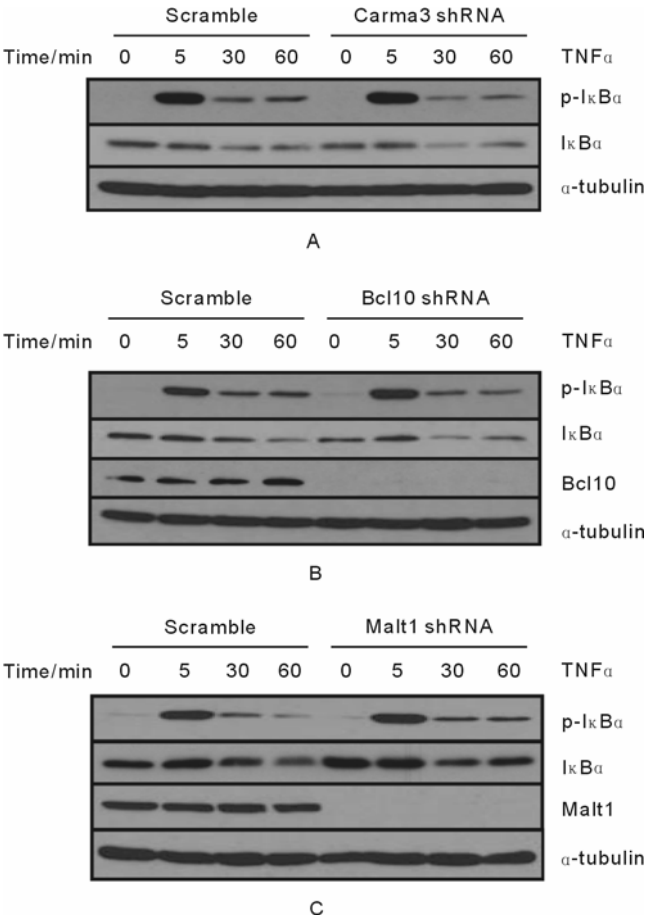

Figure 3 TNFα mediated activation of IKK is not dependent on the CBM complex in OSCC

(A–C): Carma3, Bcl10, and Malt1 are not required for TNFα mediated IκBα phosphorylation or degradation. TB2-T4 scramble control and TB2-T4 Carma3, TB2-T4 Bcl10, or TB2-T4 Malt1 stable knockdown cell lines were treated with 10 ng·mL^{-1} of TNFα for the indicated times. Whole cell lysates were prepared and 50 μg of protein were loaded into a 10% SDS-PAGE. Western blot analysis was performed using the indicated antibodies.

scramble control cells (Figures 3A–3C). Thus, the CBM complex is likely not involved in TNFα mediated IKK activation in OSCC, suggesting that this complex might specifically function with G protein coupled receptors to activate NF-κB.

SDF-1α mediated IKK activation is PKC dependent

Several PKC isoforms have been shown to control events upstream of IKK activation. PKCβ and PKCθ directly phosphorylate Carma1 in B and T cells, respectively, allowing for recruitment of Bcl10 and Malt1 to Carma1 (Sommer *et al*., 2005; Shinohara *et al*., 2005). In addition, PKCα has also recently been shown to function with Ras to control LPA induced NF-κB acitivation (Mahnivong *et al*., 2008). To determine if PKC is involved in SDF-1α mediated IKK activation, Bisindolylmaleimide Ⅰ (BIMI), an inhibitor of both the classical and novel PKC isoforms, was tested. Further, since classical PKCα and β have been shown to function upstream of Carma3 and Carma1, respectively, Go9676, an inhibitor of classical PKCα and β was also used. Serum starved TB2-T4 cells were pretreated for one hour with 10 μmol·L^{-1} of BIMI or Go6976 and then stimulated with 50 ng·mL^{-1} of SDF-1α for the times indicated (Figure 4A).

Western blot was performed to assess activation of IKK. While phosphorylation of MARCKS, a known substrate of PKC, was blocked, phospho-

rylation and degradation of IκBα was either minimally changed using BIMI or increased using Go6976. This suggests that classical PKCs are not involved in SDF-1α mediated IKK activation, while novel PKCs may be involved. Next we used specific inhibitors to further explore the possible roles of PKCθ in SDF-1α mediated IKK activation and also PKCζ since it has been demonstrated to be involved in SDF-1α mediated migration of $CD34^+$ progenitor cells (Petit *et al*., 2005). Pre-treatment with 10 μmol·L^{-1} of myristoylated pseudosubstrate peptide inhibitors against PKCθ and PKCζ followed by stimulation with 50 ng·mL^{-1} of SDF-1α and Western blot analysis, showed a significant reduction of IκBα phosphorylation, suggesting that both novel PKCθ and atypical PKCζ are involved in SDF-1α mediated activation of IKK (Figure 4B).

SDF-1α-mediated invasion is Carma3/Bcl10 /Malt1 dependent

We and others have reported the necessity of NF-κB signaling in the invasion and metastasis of various malignancies (Duffey *et al*., 1999; Huber *et al*., 2004; Park *et al*., 2007). Previously we showed that SDF-1α mediated invasion of OSCC requires NF-κB signaling (Rehman and Wang, 2008). Thus, we wondered whether the CBM complex might play a role in SDF-1α mediated invasion of TB2-T4 cells. To test this, we used an *in vitro* chamber assay with Matrigel coated membranes to mimic the metastatic potential of tumor cells *in vivo*. When stimulated with SDF-1α, TB2-T4 scramble control cells showed a significant increase in invasion compared to untreated scramble control

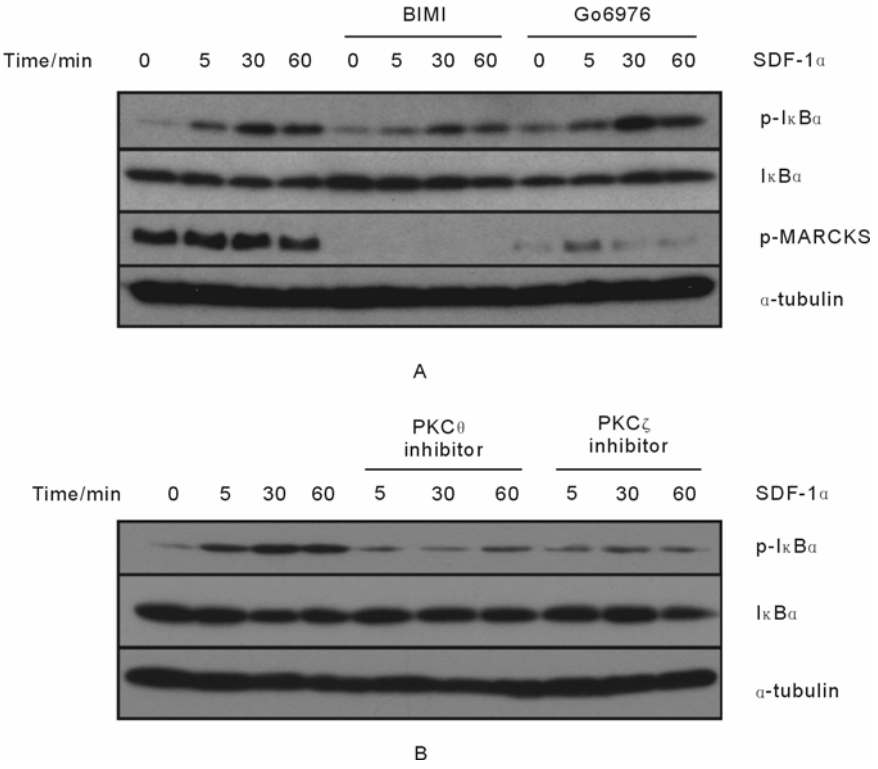

Figure 4 SDF-1α mediated IKK activation requires non-classical PKC isoforms in OSCC

(A): SDF-1α mediated IκBα phosphorylation and degradation do not depend on classical PKC isozymes. TB2-T4 cells were serum starved overnight, then pre-treated for one hour with 10 μmol·L^{-1} of BIMI or Go6976, and then stimulated with 50 ng·mL^{-1} of SDF-1α for the times indicated. Whole protein extracts were prepared and 50 μg of protein were run on a 10% SDS-PAGE. Western blotting was performed using the indicated antibodies. (B): SDF-1α mediated IκBα phosphorylation and degradation require novel PKCθ and atypical PKCζ. TB2-T4 cells were serum starved overnight, pre-treated with 10 μmol·L^{-1} of myristoylated pseudosubstrate peptide inhibitors of PKCθ or PKCζ for one hour, and then treated with 50 ng·mL^{-1} of SDF-1α. Western blotting was performed as described.

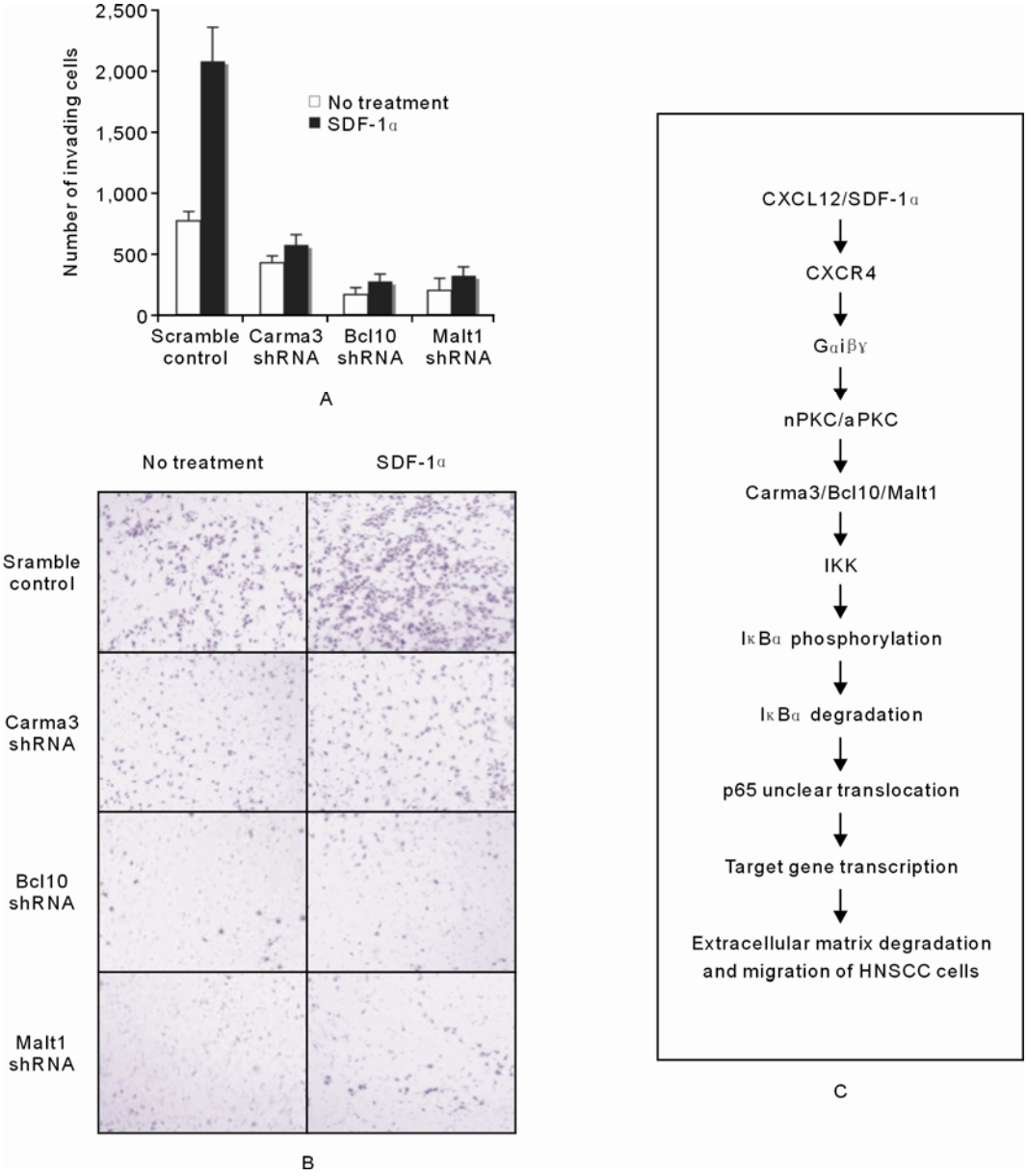

Figure 5 SDF-1α mediated invasion of OSCC requires the CBM complex

(A): SDF-1α induces OSCC invasion and knockdown of CBM complex blocks SDF-1α induced invasion. Medium with 0.1% fetal bovine serum with or without 50 ng·mL^{-1} of SDF-1α was added to the lower well of the invasion chamber. TB2-T4 stable knockdown cells were seeded in the upper well of the invasion chamber in serum free medium (100,000 cells per chamber). After 48 hours, cells that had invaded through the Matrigel coated membranes were stained with hematoxylin and eosin and then counted under the microscope. Data are an average of triplicates ± standard deviation. Invasion assay was repeated at least three times. (B): Invasive OSCC staining by hematoxylin and eosin. (C): Proposed model of SDF-1α/CXCR4 mediated activation of NF-κB signaling in OSCC. Pathway includes possible activators of the IKK complex culminating in the activation of NF-κB and resulting in OSCC invasion.

after 48 hours (Figures 5A–5B). Carma3, Bcl10, and Malt1 knockdown cell lines all showed a marked reduction of SDF-1α mediated invasion of TB2-T4 cells. These results suggest that the CBM complex is involved in SDF-1α mediated OSCC invasion.

Discussion

Chemokine receptors belong to the superfamily of G protein coupled receptors and have a broad role in normal and pathophysiological functions. The role of CXCR4 and its chemokine ligand SDF-1α, in normal developmental processes is recapitulated in cancer progression. For example, SDF-1α supports the proliferation of pre-B lymphocytes in bone marrow stromal cell compartments just as it contributes to proliferation of malignant cells in the primary or secondary tumor. Tumor fibroblasts in breast cancer and OSCC have been shown to secrete SDF-1α, thus supporting the growth of tumor tissue in a paracrine manner (Orimo *et al*., 2005; Daly *et al*., 2008). CXCR4 is also expressed on endothelial cells and is critical for normal vascular development of the embryo and also plays a role in tumor angiogenesis by recruiting endothelial cells into the tumor microenvironment. The more unique role of SDF-1α is seen in its ability to induce homing of hematopoietic progenitor cells to stromal cell niches within the bone marrow and to orchestrate naive lymphocyte trafficking to lymphoid tissues, just as it is able to direct migration of tumor cells (Kunkel *et al*., 2002). Multiple organs such as lymph nodes, bone, lung, and liver constitutively express SDF-1α, thus it is believed that tumor cells employ the CXCR4 receptor to selectively migrate to these locations in order to establish metastases (Muller *et al*., 2001). Establishment of metastases in the new host tissue is further supported by the ability of SDF-1α to activate cell survival and proliferation signals and by enhancing invasive characteristics, by upregulating expression of proteolytic enzymes and cell adhesion molecules.

NF-κB has been shown to have an important role in tumor metastasis as it can regulate the expression of various genes involved in the metastatic process (Sethi *et al*., 2008). Consitutive activation of NF-κB is also observed in many human tumors including OSCC (Tamatani *et al*., 2001; Jackson-Bernitsas *et al*., 2007; Sethi *et al*., 2008). We previously investigated the role of the NF-κB signaling pathway in OSCC and found that SDF-1α can activate NF-κB specifically through the CXCR4 receptor to induce invasion of OSCC. However, the molecular components involved in activating NF-κB signaling through the CXCR4 receptor remained unclear. In this study we describe a possible mechanism by which SDF-1α can activate NF-κB by examining elements upstream of the IKK complex (summarized in Figure 5C). Recent studies have provided strong evidence that GPCR agonists such as LPA, ET-1, AngⅡ, and IL8/CXCL8 can activate NF-κB by utilizing the CBM complex (Klemm *et al*., 2007; McAllister-Lucas *et al*., 2007). The Carma1-containing CBM complex was originally characterized as playing an important role in adaptive immunity by functioning downstream of T cell receptor after stimulation by antigen-presenting cells at the immunological synapse. Among a myriad of signaling molecules activated by TCR, activation of specific PKC isozymes is required for a proper immune response. PKC dependent activation of the CBM complex results in recruitment of Traf6 ubiquitin ligase to lipid raft domains to activate the IKK complex and induce NF-κB target gene transcription in order to control T cell proliferation and activation. Similarities between TCR, LPA, and AngⅡ signaling to NF-κB *via* PKC activation led to the discovery of the involvement of the CBM complex downstream of GPCRs. In this study we found that knockdown of Carma3, Bcl10, and Malt1 using RNA interference can significantly impair SDF-1α/CXCR4 mediated phosphorylation and degradation of IκBα in OSCC. In agreement with other studies, TNFα mediated NF-κB signaling remained unchanged with or without knockdown of CBM components.

Further, we found that SDF-1α mediated IKK activation is pertussis toxin sensitive and occurs primarily through the $G\alpha_i$ family of G proteins, which is coupled to CXCR4. Interestingly, a previous study showed that NF-κB is activated by the $G\alpha_q$ family of heterotrimeric G proteins specifically through Carma3 (Grabiner *et al*., 2007), so it is likely that depending on the receptor type

different G proteins can be utilized to activate NF-κB. It is thought that when $G\alpha_i$ is activated the release of Gβγ subunits is primarily responsible for downstream activity such as cell migration (Neptune *et al*., 1997; Arai *et al*, 1997). The βγ subunits have been found to interact directly with scaffolding molecules (Sun *et al*., 2007), so it will be of interest to determine if the heterotrimeric G proteins are able to directly bind to Carma3 perhaps facilitating interactions between the CBM complex and other IKK activating molecules.

The AGC family of serine threonine kinases includes the protein kinase C subfamily. PKCs are further divided into three subtypes: the classical calcium and diacyglycerol (DAG) dependent PKCs (α, βⅠ, βⅡ, γ); the novel DAG dependent PKCs (δ, ε, η, θ); and the atypical calcium and DAG independent PKCs (ζ, λ/ι). Currently, it is thought that several PKC isozymes are able to control the activation of NF-κB signaling by the CBM complex. Direct phosphorylation of Carma1 by PKCθ or PKCβ results in conformational changes that allows recruitment and binding of Bcl10 and Malt1 (Matsumoto *et al*., 2005). PKCθ deficient T cells show impaired NF-κB signaling and T cell activation in response to antigen stimulation. We show that inhibition of PKCθ can block SDF-1α mediated IκBα phosphorylation. Interestingly, inhibition of PKCs α and β using the Go6976 inhibitor slightly enhanced SDF-1α mediated IκBα phosphorylation, suggesting that classical PKCs might negatively regulate the intensity and duration of CXCR4 mediated NF-κB activation. PKCα has been previously shown to control GPCR mediated transactivation of EGFR and blocking PKCα with inhibitors or siRNA can enhance EGFR activity (Shah *et al*., 2005; Santiskulvong *et al*., 2007). Thus, it is plausible that classical PKC isozymes may be involved in dampening SDF-1α mediated IKK activity, however, the mechanism by which this might occur is unclear.

Since PKCζ is critical for SDF-1α mediated proliferation, adhesion, and migration of $CD34^+$ progenitor cells (Petti *et al*., 2005), we also tested the involvement of this atypical PKC in activation of IKK through CXCR4 and found that phosphorylation of IκBα was blocked by treatment with a pseudosubstrate peptide inhibitor. While PKCζ is known to activate NF-κB by phosphorylation of p65 (Duran *et al*., 2003), our data suggests that PKCζ may also have an additional role upstream of IKK. To date, it is unclear if PKC isozymes can directly phosphorylate Carma3 within its linker region or elsewhere to induce CBM complex formation and subsequent activation of the IKK signalosome. Studies have shown that ectopic expression of Carma3 can rescue TCR mediated NF-κB signaling in Carma1 deficient T cells, illustrating that these molecules might play identical roles in different systems and therefore, be activated in very similar manners (Matsumoto *et al*., 2005).

Importantly, we have previously shown that SDF-1α can induce NF-κB dependent invasion of OSCC. In this study we found that knockdown of Carma3, Bcl10, or Malt1 can significantly interfere with SDF-1α mediated invasion of OSCC. Klemm *et al*. have shown that LPA induced invasion of ovarian cancer cells requires Carma3 (Klemm *et al*., 2007). Interestingly, a recent study showed high levels of Bcl10 expression in oral squamous cell carcinoma tumor tissue compared to normal oral mucosa and that Bcl10 expression was significantly correlated with larger tumor size, lymph node metastasis, more advanced clinical stages, and locoregional recurrence, suggesting that Bcl10 expression levels might predict an unfavorable prognosis for individuals with OSCC (Chang *et al*., 2008). Thus, enhanced Bcl10 expression in oral cancers in combination with CXCR4 overexpression, might contribute to constitutive activation of NF-κB signaling and increased ability to invade and metastasize.

Conclusion

In conclusion, our data suggest that the G protein coupled receptor CXCR4 can activate NF-κB signaling through the CBM complex to induce invasion of OSCC. Targeting these molecules may provide a novel therapeutic oppurtunity in preventing or reducing the occurrence of OSCC metastasis mediated by SDF-1α.

Acknowledgements

This work was supported by the grants DE13848 and DE15964 from National Institute of Dental and Craniofacial Research, National Institute of Health, USA.

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