Molecular Pharmacology Fast Forward. Published on February 6, 2009 as doi:10.1124/mol.108.054270

MOL #54270

KiSS1 Metastasis Suppressor Gene Product Induces Suppression of Tyrosine Kinase Receptor Signaling to Akt, TNF Family Ligand Expression, and Apoptosis

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Molecular Pharmacology Fast Forward. Published on February 6, 2009 as DOI: 10.1124/mol.108.054270
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Running title: Effect of KiSS1 on apoptosis, Akt and TNF family ligands

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Number of text pages: 39

Number of figures: 7 (+ 3 supplemental figures)

Number of references: 40

Number of words in abstract: 238

Number of words in introduction: 689

Number of words in discussion: 1084

Abbreviations used: BSA: bovine serum albumin; DIC: differential interference contrast, DMEM: Dulbecco's modification of Eagle Medium; EGF: epidermal growth factor; EGFR: EGF receptor; ERK: extracellular signal regulated kinase; FBS: fetal bovine serum; GPCR: G protein-coupled receptor; JNK: c-jun N-terminal kinase; Kp: kisspeptin; MAPK: mitogen activated protein kinase; PI: propidium iodide; PI3K: phosphatidylinositol-3 kinase; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C; PVDF: polyvinylidene fluoride; RTK: receptor tyrosine kinase; SDF-1: stromal derived factor 1; TNF-α: tumor necrosis factor alpha.

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Abstract

The powerful metastasis suppressor function of *KiSS1* gene products has been demonstrated both in clinical studies and in experimental models but its mechanism is still incompletely understood. Studies on the anti-metastatic function of KiSS1 and GPR54 largely focused on the autocrine inhibition of cell motility, despite experimental evidence of an alternative post-migratory effect. We showed previously that the activation of its cognate receptor GPR54 by Kp-10 suppressed the capacity of the pro-metastatic chemokine receptor CXCR4 to induce chemotaxis in response to SDF-1 and abolished the activation of Akt by CXCR4. We demonstrate here that activation of GPR54 can also abolish the activation of Akt by the tyrosine kinase receptors for EGF and insulin. The signaling of GPR54 was sufficient to trigger apoptosis in epithelial and lymphoid cell lines. Surprisingly, this phenomenon depended largely on the activation of ERK rather than the inhibition of Akt. Activation of GPR54 resulted in the ERK-dependent expression of TNF-α and FasL in a lymphoid cell line, the latter being the main trigger of apoptosis. These data provide novel mechanisms relevant to a potential autocrine metastasis suppression effect of KiSS1 on GPR54-positive tumor cells. More importantly, they also establish an experimental basis for a paracrine mode of action by which kisspeptins suppress the metastatic potential of tumor cells lacking expression of the receptor as observed in several animal models of metastasis. The action on stromal cells significantly broadens the clinical relevance of this metastasis suppressor.

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Introduction

Metastasis is a complex process that requires tumor cells to acquire multiple characteristics that their normal and non-metastatic counterparts lack (Gupta and Massague, 2006). Biologic selection experiments have revealed genes associated with the metastatic phenotype to common target organs (Kang *et al.*, 2003; Minn *et al.*, 2005), including the chemokine receptor CXCR4 that can direct tumor cells to home into organs expressing its ligand stromal cell derived factor 1 (SDF-1, CXCL12). An antithetical group of genes encodes molecules that specifically inhibit the spread of cancer cells but not tumorigenicity by targeting multiple steps of the metastatic process (Stafford *et al.*, 2008). One of these metastasis suppressors, KiSS1, was initially identified in experiments in which introduction of chromosome 6 in metastatic melanoma cells suppressed metastasis to the lungs and lymph nodes (Lee *et al.*, 1996; Welch *et al.*, 1994). The gene was later localized to chromosome 1q32 suggesting that genes located on chromosome 6 regulate its expression (Shirasaki *et al.*, 2001; West *et al.*, 1998), a fact that was confirmed more recently (Goldberg *et al.*, 2003; Mitchell *et al.*, 2007).

Besides melanoma, the metastasis suppressing function of KiSS1 has been demonstrated in breast cancer (Lee and Welch, 1997b) and ovarian carcinoma models (Jiang *et al.*, 2005). Additionally, in clinical studies, the loss of KiSS1 expression has been linked to poor prognosis in several malignancies, including ovarian cancer (Hata *et al.*, 2007; Prentice *et al.*, 2007), melanoma (Martins *et al.*, 2008; Shirasaki *et al.*, 2001) and carcinomas of the stomach (Dhar *et al.*, 2004), urinary bladder (Sanchez-Carbayo *et al.*, 2003) and esophagus (Ikeguchi *et al.*, 2004).

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The KiSS1 protein is proteolitically processed to polypeptides ranging from 54 to 10 amino acids known as kisspeptins (Kp) which are secreted (Bilban et al., 2004; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). A G-protein coupled receptor (GPCR), GPR54 (also known as AXOR12 and hOT7T175) has been identified as the receptor for kisspeptins (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Kp binding to GPR54 activates Gαq and results in inhibition of chemotaxis to FBS, activation of ERK MAPK, formation of stress fibers, phosphorylation of focal adhesion complexes, decreased matrix metalloproteinase activity and reduced cell proliferation in receptor transfectants (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001). We have shown previously that activation of GPR54 by Kp-10 abrogated cellular responses to SDF-1, including chemotaxis, calcium mobilization and activation of Akt/protein kinase B by CXCR4. (Navenot *et al.*, 2005).

The PI3 kinase (PI3K) – Akt pathway plays a major role in several key cellular processes including survival and apoptosis, glucose metabolism, cell cycle progression and gene expression (Manning and Cantley, 2007). Both GPCRs and receptor tyrosine kinases (RTKs) can activate Akt, although through different isoforms of PI3K (Carpenter and Cantley, 1996). Since we previously demonstrated that GPR54 signaling did not activate Akt and induced a negative cross-talk with CXCR4 resulting in the inactivation of Akt, we investigated whether activation of GPR54 could also suppress RTK signaling through Akt, whether cell viability would be compromised and what signaling mechanism would be involved.

The nature of the cells expressing Kp and GPR54 still remains to be determined. In fact, if transcripts of GPR54 have been shown to be increased in some primary cancer

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cells relatively to normal tissues (Ohtaki et al., 2001), several of the cell lines shown to have decreased metastatic behavior when expressing Kp did not express GPR54 (Becker et al., 2005; Lee and Welch, 1997a; Lee and Welch, 1997b; Nash et al., 2007). This suggests that the anti-metastatic effect of Kp at least in some malignancies, may target non-tumor cells in the microenvironment rather than the tumor cells themselves in an autocrine loop. This hypothesis is supported by the fact that a number of normal organs and cells types (such as peripheral blood lymphocytes) have been shown to express transcripts of GPR54 (Kotani et al., 2001; Ohtaki et al., 2001). We elected to use HEK-293 and Jurkat cells transfected with GPR54 to investigate the signaling events resulting from GPR54 activation that could be relevant to a paracrine as well as an autocrine mode of action of Kp.

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Materials and Methods

Cell lines and reagents. The human cell line HEK-293T was modified to express GPR54 with an-N-terminal Myc epitope tag by transfection with a pcDNA3.1 construct (Invitrogen, Carlsbad, CA) as previously described (Navenot *et al.*, 2005). Jurkat cells were transfected with a Myc-tagged GPR54 in a pME vector (a gift from Dr Makio Iwashima, Medical College of Georgia). After selection either in G418 (293) or puromycin (Jurkat), transfected cells were selected for expression of the Myc tag by magnetic sorting (Miltenyi Biotec, Auburn, CA) using the 9E10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Kp-10 (YNWNSFGLRF-NH2) was synthesized at the Kyoto University, Japan. Inhibitors of PKA (4-cyano-3-methylisoquinoline), PKC (Bis-indolylmaleimide-1), PLC (U73122), MEK1/2 (UO126 and PD98059), PI3K (LY294002 and wortmannin), p38 MAPK (SB202190 and SB203580) and JNK (SP600125, JNK inhibitor II) were from Calbiochem (La Jolla, CA). All antibodies used for western blots were from Cell Signaling Technology (Danvers, MA). The antibody to CXCR4 12G5 was a gift from Dr. James Hoxie, University of Pennsylvania, Philadelphia, PA.

Activation of ERK1/2 and Akt by western blot: 293 and 293-GPR54 cells (2×10^5) were seeded in 35 mm dishes and grown for 24 hours in complete growth medium (DMEM containing 10% FBS) before an overnight starvation (DMEM containing 0.25% BSA). After stimulation for 5 min at 37°C with Kp-10 (100 nM), Epidermal Growth Factor (EGF, 10 ng/mL, PeproTech, Rocky Hill, NJ) or insulin (100 nM, Sigma, St Louis, MO), the cells were washed with PBS before being solubilized in 200 µL of sample buffer containing SDS and dithiothreitol. Jurkat and Jurkat-GPR54 cells (1×10^6)

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in serum-free medium (RPMI 1640 containing 0.25% BSA) were exposed to Kp-10 (100 nM) or the PI3K inhibitors LY294002 (50 μM) or wortmannin (100 nM) before being washed in PBS. Whole cell lysates were prepared in SDS sample buffer for 5 min at 100°C. The relative amount of proteins in the samples was determined after SDS-PAGE and Coomassie blue staining of 10 μL of each sample and analysis with a LAS-3000 digital imaging system (Fuji, Stamford, CT). Identical amounts of proteins of all samples were then analyzed by western blotting, the PVDF membranes (GE Healthcare, Piscataway, NJ) being incubated with antibodies specific for total ERK1/2 (#9102), phosho-ERK1/2 (#9106), total Akt (#9272), phospho-Akt Ser^{473} (#4058) and phospho-Akt Thr^{308} (#4056). After incubation with the appropriate horseradish peroxidase-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by the ECL Plus substrate (GE Healthcare), blots were analyzed with the LAS-3000 system and the intensity of the specific bands was quantified using the Multi Gauge software (Fuji).

Analysis of apoptosis by western blot: 293 cells were seeded at 5 x 10^4 cells / 35 mm dish in complete growth medium. After 24 hours, the medium was replaced with serum-free medium containing 0.25% BSA. For inhibition experiments, the medium contained one of the following inhibitors of: PKA (10 μM), PKC (1 μM), PLC (2 μM), PI3K (LY294002, 50 μM, or wortmannin, 100 nM), MEK1/2 (UO126, 10 μM, or PD98059, 50 μM) p38 MAPK (SB202190, 10 μM, or SB203580, 20 μM), JNK MAPK (10 μM). After 1 hour at 37°C, Kp-10 (100 nM) was added and the cells were grown for another 48-72 hours. The cells in the supernatant were collected and washed in ice-cold-PBS. The cells adhering to the dishes were also washed in ice-cold PBS. Detached and adherent cells

were pooled for each sample and solubilized in SDS reducing sample buffer for 5 min at 100°C. Samples were processed for western blotting as described above. Blots were incubated with antibodies to caspase 9 (#9502), cleaved caspase 9 (#9501), caspase 7 (#9492), cleaved caspase 7 (#9491), caspase 3 (#9662), cleaved caspase 3 (#9661), caspase 8 (#9746, detects both uncleaved and cleaved fragments, including the 18 kDa catalytically active fragment), PARP (#9442) and cleaved PARP (#9541). Incubations and detection were conducted as described above.

Measurement of cell viability: 293-GPR54 cells were seeded in triplicates in 12-well plates (2 x 10^4 cells / well) and treated as described above. Cell morphology was assessed from pictures of representative fields from an inverted microscope (TE-2000E, Nikon, Melville, NY) equipped with a 10 x phase contrast objective and a CCD camera (CoolSnap HQ, Roper, Pleasanton, CA), using Metamorph (Molecular Devices, Sunnyvale, CA). Supernatants were collected and transferred to 5 mL tubes. Adherent cells in the plates were washed with PBS, detached with trypsin and resuspended in their medium supplemented with propidium iodide (PI, Sigma) to a final concentration of 5 μg /mL. Live cells were counted for 1 minute by flow cytometry (LSR II, Becton-Dickinson, San Jose, CA) based on their morphology (forward vs. side scatter) and their capacity to exclude PI. For Jurkat cells, the protocol was similar except that cells were resuspended in complete growth medium and exposed to Kp-10 for 16-30 hours before being resuspended in their own medium supplemented with PI and counted by flow cytometry.

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Staining with annexin-V and detection of mitochondrial potential: Expression of phosphatidyl-serine on the cell surface was detected with annexin-V, combined, in Jurkat cells, with a measurement of the loss of mitochondrial potential. For 293 cells, cells were grown on glass-bottom 35 mm dishes (MatTek, Ashland, MA) and treated as described above. After 48 h of exposure to Kp-10, cells were washed in annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) and incubated with 5 μL of annexin-V AlexaFluor 488 (Molecular Probes, Invitrogen, Carlsbad, CA) for 15 min at room temperature. Cells were then washed twice in binding buffer, covered with buffer containing 10 μg/mL Hoechst 33342 (Molecular Probes) and observed with an inverted microscope equipped with a 60 x objective. Images of representative fields were captured for differential interference contrast (DIC), fluorescence of annexin-V and fluorescence of Hoechst. Jurkat cells were resuspended at 5 x 10^5 cells/mL in complete growth medium and exposed to Kp-10, LY294002 or staurosporine for 12 hours at 37°C. The mitochondrial membrane potential indicator 1,1′,3,3,3′,3′-hexamethylindodicarbo-cyanine iodide ($DiIC_1(5)$, Molecular Probes) was added for 30 min at 37°C at a final concentration of 50 nM. After 1 wash in PBS, cells were resuspended in 100 μL of annexin-V binding buffer and 5 μL of annexin-V Alexa Fluor 488 for 15 min at room temperature. Cells were finally diluted in 400 μL of binding buffer with 5 μg/mL PI and analyzed by flow cytometry.

Measurement of secreted TNF-α by ELISA: Jurkat cells in complete medium were placed in 24-well plates at 1 x 10^6 cells/mL and exposed to Kp-10 or SDF-1 (100 nM) for intervals ranging from 30 min to 6 h. Cells were pre-incubated with MAPK inhibitors for

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1 h before addition of Kp-10 when indicated. Samples were collected in 1.5 mL microtubes, centrifuged at low speed (2 min at 1,500 rpm) to remove cells, then at high speed (5 min at 13,000 rpm) to remove any debris. 293-GPR54 cells were plated in complete medium in 35 mm dishes (2.5 x 10^5 cells/dish) and grown for 24 h. Medium was then replaced with 1 mL of serum-free medium and the cells were exposed or not to Kp-10 for 6h (duplicates for each condition). Supernatants were collected and centrifuged (5 min at 13,000 rpm) to remove debris and frozen at -80°C until use. ELISA for TNF-α was performed using the Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Detection of FasL on the cell surface: Jurkat-GPR54 cells (5 x 10^5) were exposed to Kp-10 (100 nM) in complete medium for 2, 4 or 6 h at 37°C. Cells were then washed in cold PBS containing 0.1% BSA and incubated on ice for 1 h with 10 μg/mL of control mouse IgG1 or mouse monoclonal antibody to human FasL (MAB126, R&D Systems). After 2 washes in PBS-BSA, cells were incubated with an R-phycoerythrin-labeled secondary antibody at 10 μg/mL (Jackson ImmunoResearch) for 1 h on ice. After 2 washes, cells were analyzed by flow cytometry.

Inhibition of apoptosis with neutralizing antibodies to TNF-α, FasL and TRAIL: Jurkat-GPR54 and 293-GPR54 cells were processed for induction of apoptosis as described above. Neutralizing antibodies to TNF-α (mouse monoclonal antibody MAB210 and goat polyclonal antibody AF-210-NA, both from R&D) were pooled and added to Jurkat-GPR54 at concentrations ranging from 5 ng/mL to 5 μg/mL and from 1

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ng/mL to 1 μg/mL, respectively. Kp-10 (100 nM) was then added and the cells were incubated for 16 h at 37°C. Live cells were counted by flow cytometry as indicated above. 293-GPR54 cells were incubated for 48 h with the highest concentrations of antibodies only and apoptosis was assessed by microscopy based on morphological changes. Inhibition of apoptosis was also studied with neutralizing antibodies specific for FasL (concentrations from 0.1 to 10 μg/mL, MAB126, mouse IgG2b, R&D) and TRAIL (concentrations from 0.05 to 5 μg/mL, goat IgG, AF375, R&D). Mouse IgG2b at 10 μg/mL (Southern Biotechnology, Birmingham, AL) was used as a control.

Statistical analyses: Averages of quantitative data were compared by Student's t test. Two-tailed p values are expressed as * $p<0.05$%, ** $p<0.01$, *** $p<0.001$.

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Results

The activation of GPR54 abolishes the activation of Akt by EGFR and the insulin receptor

The consequences of GPR54 signaling were investigated in 293-GPR54 and Jurkat-GPR54 obtained by stable transfection. Limited rounds of selection of GPR54-positive cells by magnetic sorting were performed in order to keep the expression of the receptor to a physiologic level and avoid the bias of an oligoclonal selection. Immunofluorescence and flow cytometric analysis show that the expression level of GPR54 obtained in both 293 and Jurkat cells is similar to the endogenous expression of CXCR4 in Jurkat cells (Fig. 1).

The exposure of 293-GPR54 cells to EGF or insulin resulted in a strong phosphorylation of Akt (Fig. 2A and 2B). Kp-10 induced phosphorylation of ERK, but not of Akt, in 293-GPR54 cells. In addition, basal phosphorylation of Akt was reduced (Fig 2). Pre-exposure to Kp-10 abolished Akt phosphorylation in response to EGF or insulin. (Fig. 2) Thus, the negative cross-talk from GPR54 signaling also affects Akt activation by RTK. These effects of Kp-10 were reproduced by the PI3K inhibitor LY294002 and were independent of the order of addition of the ligands (not shown). Exposure of parental 293 cells to Kp-10 had no effect on ERK and Akt phosphorylation (Fig. 2).

Kp-10 mediates GPR54-dependent cell death in 293 and Jurkat cells

Cell rounding consistent with an apoptotic phenotype was detected after 24 h of exposure of 293-GPR54 cells to Kp-10 (Fig. 3A). The phenomenon intensified after 48

and 72 h, at which point the number of live cells present among GPR54-positive cells was significantly reduced compared with control cells, which remained unaffected by Kp-10 (Fig. 3B). Increasing concentrations of FBS up to 10% only partially reduced the effect (Fig. 3A). 293 and 293-GPR54 cells exhibited identical morphologies in the absence of Kp-10, irrespectively of the FBS concentration (not shown). Similarly, the number of live cells after 16 to 30 h was severely reduced in Jurkat-GPR54 cells, but not in control cells (Fig. 3C). Cell death was proportional to the dose of Kp-10 between 3 and 100 nM (Fig. 3D).

Activation of GPR54 triggers apoptosis

Exposure of Jurkat-GPR54 cells to Kp-10 resulted into the emergence of a population with a reduced mitochondrial potential but no annexin-V staining (cells in early apoptosis) and a large increase of the annexin-V positive population (mid to late apoptosis) compared to the untreated control (Fig. 4A), indicative of the involvement of the mitochondrial pathway of apoptosis. The PI3K inhibitor LY294002 reproduced this effect. In control Jurkat cells, only LY294002 and staurosporine, but not Kp-10, could trigger apoptosis (Fig. 4A). Dose response of Kp-10 (Fig. 4B) and kinetics experiments (Fig. 4C) with annexin-V staining confirmed that apoptosis increased between 3 and 100 nM of Kp-10 and from 6 h to 24 h of treatment. In 293-GPR54, most fields were devoid of apoptotic cells in unstimulated samples, but a large fraction of the cells treated with Kp-10 for 48 to 72 h were positive for annexin-V (Fig. 4D), suggesting that apoptosis was also responsible for cell death in that cell line.

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In both 293-GPR54 and Jurkat-GPR54, addition of Kp-10 resulted in the activation of caspases and the resulting cleavage of PARP (Fig. 4E and F). This indicates that the activation of GPR54 alone had the capacity to trigger a complete array of pro-apoptotic signals. Similar treatment had no effect on control cells (data not shown). Interestingly, caspase 8 appeared to be extensively cleaved to its active 18 kDa fragment in Jurkat-GPR54, suggesting the involvement of the extrinsic pathway, but not in 293-GPR54, in which only a very small amount of intermediate fragments (p41 and p43) and no p18 could be detected (Fig. 4E).

Inhibition of Akt activity alone is not sufficient for GPR54-mediated apoptosis

We sought to establish a connection between loss of phosphorylation of Akt and apoptosis by first testing whether the pharmacological inhibition of PI3K resulted in apoptosis. Whereas Jurkat-GPR54 cells were equally sensitive to the treatment with the PI3K inhibitor LY294002 and Kp-10 (Figure 4A), treatment of 293-GPR54 cells with LY294002 or wortmannin resulted into a reduced growth rate, but not apoptosis (Fig. 5A). Furthermore, pre-treatment with PI3K inhibitors did not alter the capacity of Kp-10 to trigger apoptosis (Fig. 5A). This strongly suggests that inhibition of Akt is not the main mechanism for induction of apoptosis by GPR54. Additional evidence came from experiments performed in Jurkat cells which have high basal phospho-Akt resulting from a constitutive defect in expression of the lipid phosphatases PTEN and SHIP-1. Stimulation of Jurkat-GPR54 cells with Kp-10 did not decrease Akt phosphorylation, although ERK was phosphorylated (Fig. 5B and C). A parallel treatment with LY294002 or wortmannin resulted into a rapid decrease in Akt phosphorylation, mediated by PP2A,

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which demonstrates the short half-life of phospho-Akt. This confirms that the negative cross-talk between activated GPR54 and Akt is not the only mechanism by which this GPCR triggers apoptosis.

ERK MAPK plays an essential role in GPR54-mediated apoptosis

We and others have previously observed that the activation of GPR54 resulted in the phosphorylation of ERK, p38 and JNK MAPK. Since p38 and JNK activation can contribute to apoptosis, we tested whether the inhibition of MAPK could influence the capacity of GPR54 to trigger apoptosis in Jurkat and 293 cells. Because this receptor is known to signal through Gq, a direct activator of phospholipase C (PLC), we also determined whether PLC and PKC inhibitors reduced apoptosis. Neither inhibition of PLC nor PKC had any effect on Kp-10-mediated apoptosis or suppression of Akt phosphorylation in 293-GPR54 (data not shown). Prolonged inhibition of JNK itself induced extensive cell death in 293 cells during the course of the experiment. Cell death was also important in Jurkat-GPR54 cells with JNK inhibition alone. Decreasing the concentration of inhibitor to reduce toxicity showed that Kp-10 could further decrease the number of live cells, indicating that activation of JNK by GPR54 did not seem required for GPR-54-mediated apoptosis (Supplemental Fig. 1A). Inhibition of p38 had no effect on apoptosis in 293-GPR54 (Supplemental Fig. 1B and C) but elicited a partial inhibitory effect in Jurkat-GPR54 (Supplemental Fig. 1D). In contrast, inhibition of the MEK-ERK pathway significantly reduced the apoptotic effect of Kp-10 as assessed by cell morphology (data not shown) and cell survival (Fig. 6A and B), as well as the induction of cleavage of caspases and PARP in 293-GPR54 and Jurkat-GPR54 (Fig. 6C and D).

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Thus the activation of ERK appears to be a major component of the pro-apoptotic signaling of GPR54. We confirmed that the role of ERK in GPR54-mediated apoptosis was independent of the capacity of the receptor to suppress Akt activity by showing that a treatment with a MEK inhibitor that inhibits both apoptosis and ERK1/2 phosphorylation did not later the ability of Kp-10 to abrogate basal and EGF-induced phosphorylation of Akt in 293-GPR54 (Supplemental Fig. 2).

Activation of GPR54 induces ERK-dependent expression of pro-inflammatory and pro-apoptotic ligands in Jurkat cells

Because we noted that Kp-10 induced extensive activation of caspase 8 in Jurkat-GPR54 cells, we investigated the possible role of the expression of death receptor ligands, TNF-α, TRAIL and FasL, in Kp-10-induced apoptosis. Exposure of Jurkat-GPR54 cells to Kp-10 revealed a rapid and abundant secretion of TNF-α into culture supernatants (Fig. 7A). In contrast, exposure of Jurkat-GPR54 cells to SDF-1 and parental Jurkat cells to Kp-10 had no effect on TNF-α secretion (Fig. 7A). The amount of TNF-α secreted by 293-GPR54 cells exposed to Kp-10 was 12 pg/mL, which is at the lower limit of detection of the assay. Inhibition of the ERK pathway by UO126 significantly reduced the secretion of TNF-α, from 77% to 89% in 3 independent experiments (Fig. 7B). Inhibition of p38 and JNK had more limited effects (Fig. 7B). Since the activation of TNF-α receptors can induce either pro-apoptotic or pro-survival signals, the role of TNF-α secretion in apoptosis in this system was determined with neutralizing antibodies. Neutralization of TNF-α had no effect on the induction of apoptosis by Kp-10 (Supplemental Fig. 3). Similarly, neutralization of TRAIL did not

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inhibit the effect of Kp-10 on cell viability in Jurkat-GPR54 and 293-GPR54 cells (not shown). In contrast, neutralization of FasL inhibited apoptosis in Jurkat-GPR54 cells exposed to Kp-10 in a dose-dependent fashion (Fig. 7C). The maximum efficacy was similar to that obtained by inhibition of MEK. Neutralization of FasL also decreased the cleavage of PARP and caspases in Jurkat-GPR54 (Fig. 7D and E) but did not reduce apoptosis in 293-GPR54 cells (not shown). Since the induction of apoptosis is most effective with membrane-bound FasL, expression of FasL on the surface of Jurkat-GPR54 cells was determined after stimulation with Kp-10. FasL was detected at 2 hours after addition of Kp-10, was maximum at 4 hours and started to decline at 6 hours (Fig. 7F), at which time apoptosis could be detected, as shown in Figure 4C. Pre-treatment with UO126 iinhibited the up-regulation of FasL (Fig. 7F), indicating that ERK regulates GPR54-induced apoptosis in Jurkat cells by modulating the expression of FasL.

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Discussion

Among the approximately 23 genes classified so far as metastasis suppressors (Stafford *et al.*, 2008), KiSS1 is the only ligand of a GPCR. Although several effects of the expression of KiSS1 and the activation of GPR54 by Kps have been described *in vitro*, the mechanism(s) for the anti-metastatic action of KiSS1 and the exact nature of the cells expressing the ligand and its receptor are still largely unknown. We provide here evidence that Kp signaling through GPR54 induces functional negative crosstalk with mechanisms involved in metastatic cell homing and survival and can induce the secretion of soluble factors capable of altering the support to tumor cells from the microenvironment.

Whereas multiple GPCRs contribute to tumor growth, angiogenesis and metastasis (Dorsam and Gutkind, 2007), Kps and GPR54 exert opposing functions that suppress migration and survival. We previously reported the crosstalk between GPR54 and the chemokine receptor CXCR4 evidenced by the suppression of migration and Akt phosphorylation induced by SDF-1 (Navenot *et al.*, 2005). Here we demonstrate that GPR54 efficiently suppressed the activation of Akt by RTKs, iincluding EGFR involved in the proliferation of multiple tumors types. This raised the possibility that Kps could exert a broad spectrum negative regulation of this pathway. Other Gq-coupled GPCRs, like the angiotensin II receptor (ATIIR1), have been shown to partially reproduce that effect (Folli *et al.*, 1997), but this effect is not universal for Gq-coupled receptors.

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Previous studies reported that cell lines transfected with GPR54 and exposed to Kps exhibited reduced cell growth (Becker *et al.*, 2005; Hori *et al.*, 2001; Kotani *et al.*, 2001), but not apoptosis (Kotani *et al.*, 2001). Exposure of mammary carcinoma cells MDA-MB-435S programmed to express GPR54 to Kp-10 resulted in nuclear condensation consistent with apoptosis (Becker *et al.*, 2005). We show here that Kp-10 induced pro-apoptotic signals and cell death in two common cell lines programmed to express GPR54, but not in the parental cells. Although it is unclear what expression level of GPR54 is necessary, apoptosis was triggered by concentrations of Kp-10 in the low nanomolar range, which corresponds to the EC50 for ligand binding. Additionally, the conditioned medium of tumor cells programmed to secrete the 54 residue Kp designated metastin (Nash *et al.*, 2007) was capable of inducing apoptosis with an efficiency similar to Kp-10 (data not shown).

The inhibition of Akt did not appear to play a major role in GPR54-mediated apoptosis despite the pro-survival function of this kinase. Since it is central to multiple essential signaling pathways, negative regulation of Akt could position Kp-10 as an agent to sensitize tumor cells or stromal cells in the microenvironment to chemotherapeutic agents. The major role the ERK1/2 pathway played in Kp-10 mediated apoptosis seems surprising in regard of the largely recognized role of this pathway in cancer cell survival and proliferation (Roberts and Der, 2007; Sebolt-Leopold, 2008; Sebolt-Leopold and Herrera, 2004; Shaul and Seger, 2007) and contradicts the finding that upregulation of a subset of genes involved in apoptosis induced by GPR54 activation was suppressed by inhibition of PKC and PLC but not ERK (Becker *et al.*, 2005). In Jurkat cells, ERK

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activation was necessary for the expression of FasL and TNF-α. Several studies have shown the role of ERK in TNF-α up-regulation (Dumitru *et al.*, 2000; Hacker and Karin, 2006; Skinner *et al.*, 2008). Based on kinetics and neutralization data, we can conclude that the activation of ERK by GPR54 led to the expression of FasL which, in turn, bound Fas on the cell surface to assemble a Death-Inducing Signaling Complex and activate caspase 8. This mechanism is reminiscent of the one underlying activation-induced cell death of T cells (van den Brink et al., 1999; Zhu et al., 1999) in which activation of ERK controls the expression of FasL. To our knowledge, this is the first description of such a mechanism triggered by a GPCR. The effector(s) of ERK signaling in Kp-10-mediated apoptosis of 293-GPR54 cells were not identified. It can be postulated that, either the activation of ERK occurs in conjunction with other critical signaling events, or GPR54 activates ERK in a specific fashion. For instance, it may induce the assembly of a specific signaling complex not reproduced by other receptors, such as the complexes constituted after recruitment of β-arrestins (Kolch, 2005; Lefkowitz and Whalen, 2004; Luttrell et al., 2001).

KiSS1 being a metastasis suppressor and not a tumor suppressor, it may not induce apoptotic death of tumor cells at the primary site. Rather, secreted Kps could target subpopulations of GPR54-expressing metastatic cancer cells in an autocrine fashion by inhibiting their motility and/or compromising their survival by activating pro-apoptotic signals and blocking pro-survival signals from RTKs. In addition, induction of FasL expression by Fas-negative tumor cells was reported to promote tumor rejection *in vivo* in mice, induce inflammation, recruit neutrophils and favor tumor-specific immunity

(Seino et al., 1997; Shimizu et al., 1999; Shimizu et al., 2005). Besides this potential autocrine model, the observation that the activation of GPR54 could promote the expression of ligands like TNF-α and FasL also suggests the possibility of a paracrine mode of action for Kp-10 in which Kps secreted by tumor cells would activate GPR54 expressed by cells present in the microenvironment and stimulate them to express membrane-bound or soluble pro-inflammatory mediators. These soluble factors could then in turn target the metastatic tumor cells either directly or indirectly by altering components of their microenvironment (such as carcinoma-associated fibroblasts) and depriving them from a nurturing support. The combined effects of kisspeptins on tumor cells and stromal cells (disruption of cell recruitment, survival, response to local growth factors) could ultimately prevent the formation of a functional supportive niche for metastatic cells. Several lines of evidence provide direct support for that model. First, we have demonstrated that endogenously produced and secreted Kps exert a potent anti-metastatic effect on the human melanoma cell line C8161.9 despite the absence of GPR54 expression or response to Kp-10 *in vitro* (Nash *et al.*, 2007). Similarly, it was shown that the human breast cancer cell line MDA-MB-435, which also lacks GPR54 expression, lost its metastatic potential when programmed to express KiSS1 (Lee and Welch, 1997b). However, an *in vitro* response to Kp-10 was achieved only after GPR54 transfection (Becker *et al.*, 2005). The nature of the GPR54-positive cells present in the microenvironment in that model remains to be determined. Whether the mechanism of action of Kps is predominantly autocrine or paracrine is likely to be tumor-specific. The data presented here provides new mechanistic insights into the remarkable efficiency of

Molecular Pharmacology Fast Forward. Published on February 6, 2009 as DOI: 10.1124/mol.108.054270
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KiSS1 by demonstrating effects that are most relevant to isolated metastatic cells (micrometastasis) and could contribute to the phenomenon of dormancy.

Acknowledgements

The authors are grateful for the technical assistance provided by Tanya North (Medical College of Georgia).

References

Becker JA, Mirjolet JF, Bernard J, Burgeon E, Simons MJ, Vassart G, Parmentier M and Libert F (2005) Activation of GPR54 promotes cell cycle arrest and apoptosis of human tumor cells through a specific transcriptional program not shared by other Gq-coupled receptors. *Biochem Biophys Res Commun* **326**(3):677-686.

Carpenter CL and Cantley LC (1996) Phosphoinositide kinases. *Curr Opin Cell Biol* **8**(2):153-158.

Dhar DK, Naora H, Kubota H, Maruyama R, Yoshimura H, Tonomoto Y, Tachibana M, Ono T, Otani H and Nagasue N (2004) Downregulation of KiSS-1 expression is responsible for tumor invasion and worse prognosis in gastric carcinoma. *Int J Cancer* **111**(6):868-872.

Dorsam RT and Gutkind JS (2007) G-protein-coupled receptors and cancer. *Nat Rev Cancer* **7**(2):79-94.

Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G and Tsichlis PN (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* **103**(7):1071-1083.

Folli F, Kahn CR, Hansen H, Bouchie JL and Feener EP (1997) Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *J Clin Invest* **100**(9):2158-2169.

Goldberg SF, Miele ME, Hatta N, Takata M, Paquette-Straub C, Freedman LP and Welch DR (2003) Melanoma metastasis suppression by chromosome 6: evidence for a pathway regulated by CRSP3 and TXNIP. *Cancer Res* **63**(2):432-440.

Gupta GP and Massague J (2006) Cancer metastasis: building a framework. *Cell* **127**(4):679-695.

Hacker H and Karin M (2006) Regulation and function of IKK and IKK-related kinases. *Sci STKE* **2006**(357):re13.

Hata K, Dhar DK, Watanabe Y, Nakai H and Hoshiai H (2007) Expression of metastin and a G-protein-coupled receptor (AXOR12) in epithelial ovarian cancer. *Eur J Cancer* **43**(9):1452-1459.

Hori A, Honda S, Asada M, Ohtaki T, Oda K, Watanabe T, Shintani Y, Yamada T, Suenaga M, Kitada C, Onda H, Kurokawa T, Nishimura O and Fujino M (2001) Metastin suppresses the motility and growth of CHO cells transfected with its receptor. *Biochem Biophys Res Commun* **286**(5):958-963.

Ikeguchi M, Yamaguchi K and Kaibara N (2004) Clinical significance of the loss of KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in esophageal squamous cell carcinoma. *Clin Cancer Res* **10**(4):1379-1383.

Jiang Y, Berk M, Singh LS, Tan H, Yin L, Powell CT and Xu Y (2005) KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. *Clin Exp Metastasis* **22**(5):369-376.

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA and Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3**(6):537-549.

Kolch W (2005) Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* **6**(11):827-837.

Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G and Parmentier M (2001) The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* **276**(37):34631-34636.

Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE and Welch DR (1996) KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst* **88**(23):1731-1737.

Lee JH and Welch DR (1997b) Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. *Cancer Res* **57**(12):2384-2387.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* **98**(5):2449-2454.

Manning BD and Cantley LC (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**(7):1261-1274.

Martins CM, Fernandes BF, Antecka E, Di Cesare S, Mansure JJ, Marshall JC and Burnier MN, Jr. (2008) Expression of the metastasis suppressor gene KISS1 in uveal melanoma. *Eye* **22**(5):707-711.

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL and Massague J (2005) Genes that mediate breast cancer metastasis to lung. *Nature* **436**(7050):518-524.

Mitchell DC, Stafford LJ, Li D, Bar-Eli M and Liu M (2007) Transcriptional regulation of KiSS-1 gene expression in metastatic melanoma by specificity protein-1 and its coactivator DRIP-130. *Oncogene* **26**(12):1739-1747.

Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL and Harrison DC (2001) AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol Chem* **276**(31):28969-28975.

Nash KT, Phadke PA, Navenot JM, Hurst DR, Accavitti-Loper MA, Sztul E, Vaidya KS, Frost AR, Kappes JC, Peiper SC and Welch DR (2007) Requirement of KISS1 secretion for multiple organ metastasis suppression and maintenance of tumor dormancy. *J Natl Cancer Inst* **99**(4):309-321.

Navenot JM, Wang Z, Chopin M, Fujii N and Peiper SC (2005) Kisspeptin-10-induced signaling of GPR54 negatively regulates chemotactic responses mediated by CXCR4: a potential mechanism for the metastasis suppressor activity of kisspeptins. *Cancer Res* **65**(22):10450-10456.

Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O and Fujino M (2001)

Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **411**(6837):613-617.

Prentice LM, Klausen C, Kalloger S, Kobel M, McKinney S, Santos JL, Kenney C, Mehl E, Gilks CB, Leung P, Swenerton K, Huntsman DG and Aparicio SA (2007) Kisspeptin and GPR54 immunoreactivity in a cohort of 518 patients defines favourable prognosis and clear cell subtype in ovarian carcinoma. *BMC Med* **5**:33.

Roberts PJ and Der CJ (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**(22):3291-3310.

Sanchez-Carbayo M, Capodieci P and Cordon-Cardo C (2003) Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. *Am J Pathol* **162**(2):609-617.

Sebolt-Leopold JS (2008) Advances in the Development of Cancer Therapeutics Directed against the RAS-Mitogen-Activated Protein Kinase Pathway. *Clin Cancer Res* **14**(12):3651-3656.

Seino K, Kayagaki N, Okumura K and Yagita H (1997) Antitumor effect of locally produced CD95 ligand. *Nat Med* **3**(2):165-170.

Shimizu M, Fontana A, Takeda Y, Yagita H, Yoshimoto T and Matsuzawa A (1999) Induction of antitumor immunity with Fas/APO-1 ligand (CD95L)-transfected neuroblastoma neuro-2a cells. *J Immunol* **162**(12):7350-7357.

Shirasaki F, Takata M, Hatta N and Takehara K (2001) Loss of expression of the metastasis suppressor gene KiSS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23. *Cancer Res* **61**(20):7422-7425.

Skinner SJ, Deleault KM, Fecteau R and Brooks SA (2008) Extracellular signal-regulated kinase regulation of tumor necrosis factor-alpha mRNA nucleocytoplasmic transport requires TAP-NxT1 binding and the AU-rich element. *J Biol Chem* **283**(6):3191-3199.

Stafford LJ, Vaidya KS and Welch DR (2008) Metastasis suppressors genes in cancer. *Int J Biochem Cell Biol* **40**(5):874-891.

van den Brink MR, Kapeller R, Pratt JC, Chang JH and Burakoff SJ (1999) The extracellular signal-regulated kinase pathway is required for activation-induced cell death of T cells. *J Biol Chem* **274**(16):11178-11185.

Welch DR, Chen P, Miele ME, McGary CT, Bower JM, Stanbridge EJ and Weissman BE (1994) Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. *Oncogene* **9**(1):255-262.

West A, Vojta PJ, Welch DR and Weissman BE (1998) Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1). *Genomics* **54**(1):145-148.

Zhu L, Yu X, Akatsuka Y, Cooper JA and Anasetti C (1999) Role of mitogen-activated protein kinases in activation-induced apoptosis of T cells. *Immunology* **97**(1):26-35.

Footnotes:

Funding for this project was provided by the Georgia Cancer Coalition (S.C. Peiper).

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Molecular Pharmacology Fast Forward. Published on February 6, 2009 as DOI: 10.1124/mol.108.054270
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Legends for figures

Figure 1

Expression of GPR54 and CXCR4 by flow cytometry. 293-GPR54 and Jurkat-GPR54 were stained with a saturating concentration of anti-Myc antibody 9E10 and PE-labeled secondary antibody to measure the surface expression of GPR54. The expression level of endogenous CXCR4 was similarly measured on Jurkat-GPR54 by incubating the cells with a saturating concentration of the anti-CXCR4 antibody.

Figure 2

Activation of GPR54 abolishes the activation of Akt by RTKs EGFR and insulin receptor. A. HEK-293 cells transfected with GPR54 or CXCR4 (GPR54-negative) were stimulated with Kp-10 100 nM for 7 min, EGF 10 ng/mL or insulin 100 nM for 5 min. For combined stimulations, cells were first exposed to Kp-10 for 2 min before addition of EGF or insulin for an additional 5 min. Whole cell SDS lysates were analyzed by western blots with antibodies to total Akt, phospho-Akt Ser473 and Thr308 and phospho-ERK1/2. The results are representative of at least 4 independent experiments. B. Quantitative analysis of experiment in Fig. 2A.

Figure 3

Kp-10 induces GPR54-dependent cell death in 293 and Jurkat cells. A. 293-GPR54 cells were plated for 24 h in complete growth medium. The medium was then replaced with serum-free medium or medium containing from 2% FBS, complemented or not with 100 nM of Kp-10. After 48 h, cells were observed by phase contrast microscopy (10x

magnification). B. 293 cells were plated in 12-well plates for 24 h then exposed to 100 nM of Kp-10 in DMEM-BSA. After 72 h, cells were detached and live cells, defined by morphological gating and PI exclusion, were counted by flow cytometry. Graphs show average cell count (+/- SD) of 3 identical samples for each condition (** $p<0.01$ for Kp-10 vs. control). The results are representative of 3 independent experiments. C. Jurkat or Jurkat-GPR54 cells were incubated with 100 nM Kp-10 for 24 h. The cells were then resuspended and live cells were counted by flow cytometry for 1 min after addition of PI. Graphs show the number (average +/- SD) of live cells in 4 identical samples for each condition obtained in 1 experiment representative of 3 independent experiments. D. Dose response of Kp-10 in Jurkat-GPR54. Cells were exposed to concentration of Kp-10 ranging from 0.1 to 200 nM for 24 h. Live cells were then counted by flow cytometry. Results are representative of 2 independent experiments.

Figure 4

Kp-10 induces apoptosis in Jurkat and 293 cells expressing GPR54. A. Kp-10 induced loss of mitochondrial potential and expression of phosphatidyl serine on the plasma membrane of Jurkat-GPR54. Cells were exposed to 100 nM Kp-10, 50 mM LY294002 or 100 nM staurosporine for 16 h before being stained with DiIC5 for mitochondrial membrane potential and annexin-V AlexaFluor 488 for expression of PS before being analyzed by flow cytometry. Early apoptotic cells had decreased mitochondrial membrane potential but remained largely negative for annexin-V staining (bottom left quadrant). Cells in later stage apoptosis had both decreased mitochondrial potential and positive staining with annexin-V (top left quadrant). B-C. Dose response of

Kp-10 (B) and time course of apoptosis (C) determined by annexin-V staining in Jurkat-GPR54. For the dose response, cells were incubated with the indicated concentrations of Kp-10 for 16 h. The percentage of apoptotic cells (defined by annexin-V positivity and PI exclusion) over the entire cell population is indicated. For the time course, Jurkat-GPR54 and control Jurkat cells were incubated with 100 nM of Kp-10 for the indicated times. The results are representative of 2 independent experiments. D. Induction of apoptosis in 293-GPR54 cells after 48 h of incubation with 100 nM Kp-10. Cells were then stained with annexin-V AlexaFluor 488 (green) and Hoechst 33342 (blue) and analyzed by fluorescence microscopy and DIC. The results are representative of 2 independent experiments performed in duplicate. E. Activation of caspases by Kp-10 in 293 and Jurkat cells transfected with GPR54. For 293 cells (samples 1-4), cells were plated for 24 h before being exposed to 100 nM of Kp-10 for 48 h. For Jurkat cells (samples 5-8), cells in complete growth medium were treated with 100 nM of Kp-10 for 16 h. Duplicate samples were prepared for each experimental condition. Samples were analyzed by western blots with antibodies specific for whole proteins or cleaved (active) fragments of caspase 9, caspase 7, caspase 3 and PARP, as well as an antibody to caspase 8 capable of binding the whole protein as well as the intermediate fragments p43 and p41 and the active fragment p18. The results are representative of 3 independent experiments. F. Quantitative analysis of experiment in Fig 4E. Statistically significant differences between control and Kp-10-treated samples are indicated.

Molecular Pharmacology Fast Forward. Published on February 6, 2009 as DOI: 10.1124/mol.108.054270
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Figure 5

Induction of apoptosis by Kp-10 does not correlate with Akt inhibition. A. 293-GPR54 cells resisted chemical inhibition of PI3K by LY294002 and wortmannin but were sensitive to treatment with Kp-10. Cells were plated and grown for 24 h in complete medium before being pre-treated with LY294002 (50 μM) or wortmannin (10 μM) in DMEM-BSA for 1 hour. Kp-10 (100 nM) was then added for 48 hours. Representative fields observed by phase contrast are shown. The results are representative of 2 independent experiments. B. Kp-10 does not reduce the high constitutive phosphorylation of Akt in Jurkat-GPR54. Cells were treated for the indicated time with LY294002 (50 μM), wortmannin (100 nM), Kp-10 (100 nM) or vehicle. Whole cell lysates were analyzed by western blotting for phospho-Akt Ser473 or phospho-ERK1/2. The results are representative of 2 independent experiments. C. Quantitative analysis of experiment in Fig. 5B.

Figure 6

Inhibition of the MEK-ERK pathway inhibits Kp-10-induced apoptosis in 293 and Jurkat cells. A. Inhibition of MEK inhibits apoptosis by Kp-10 in 293-GPR54. 293-GPR54 were pre-treated with the MEK inhibitors UO126 (10 μM) for 1 h before addition of Kp-10 (100 nM) for 72 hours and counting by flow cytometry. Results represent the average +/- SD of duplicate samples for each condition in one experiment representative of 4 independent experiments. B. Inhibition of MEK significantly reduced the induction of apoptosis of Jurkat-GPR54 cells by Kp-10. Cells were pre-treated with UO126 (10 μM) before addition of Kp-10 (100 nM) for 24 h. Live cells were counted by flow

cytometry. Graphs represent the results (average +/- SD) of triplicate samples of one experiment representative of 3 independent experiments. C. 293-GPR54 cells (samples 1-4) were pre-treated with UO126 (10 μM) for 1 h before being exposed to 100 nM of Kp-10 for 48 h. Whole cell lysates were analyzed by western blotting for cleavage of caspases and PARP (arrow: uncleaved PARP). Jurkat-GPR54 cells (samples 5-8) were pre-treated with UO126 for 1 h before addition of Kp-10 for 24 h. Whole cell SDS lysates were analyzed by western blotting. The results are representative of 2 independent experiments. D. Quantitative analysis of experiment in Fig 6C for 293-GPR54 cells (top panel) and Jurkat-GPR54 cells (bottom panel).

Figure 7

Activation of GPR54 promotes ERK-dependent expression of TNF-α and FasL and FasL-mediated apoptosis in Jurkat-GPR54 cells. A. Kp-10 induces expression of TNF-α by Jurkat-GPR54. Cells were exposed to 100 nM of Kp-10 or SDF-1 (S) for the indicated times (from 0.5 to 6 h), then supernatant was collected and analyzed for TNF-α content by ELISA. Data representative of 3 identical experiments in which each histogram represents duplicate samples. B. Inhibition of TNF-α secretion by inhibitors of MEK (UO126), p38 (SB) and JNK. Cells were pre-treated with each inhibitor (10 μM) for 1 h before being exposed to 100 nM of Kp-10 for 4 h. Data representative of 3 experiments performed with duplicate samples for each condition. C-D. Neutralization of FasL inhibits apoptosis by Kp-10 in Jurkat-GPR54 similarly to MEK inhibition. A neutralizing mouse monoclonal antibody was added to the culture medium at the indicated concentration before addition of Kp-10 (100 nM). After 16 h of incubation, live

cells were counted by flow cytometry (C). Data representative of 5 experiments performed with duplicate samples for each condition. For assessment of caspase activation (D), cells were treated with UO126 (10 μM) or a neutralizing antibody to FasL (10 μg/mL) before addition of Kp-10 (100 nM) for 16 h. Samples were analyzed by western blotting with the indicated antibodies. The results are representative of 2 independent experiments. E. Quantitative analysis of experiment in D. F. Membrane expression of FasL is promoted by Kp-10 and prevented by ERK inhibition. Jurkat-GPR54, with (bottom panel) or without (top panel) pre-treatment with MEK inhibitor UO126, were stimulated with 100 nM Kp-10 for the indicated time before being stained with an antibody specific for FasL as described in Material and Methods. Cell fluorescence representative of FasL expression was analyzed by flow cytometry. The results are representative of 2 independent experiments.

Figure 1

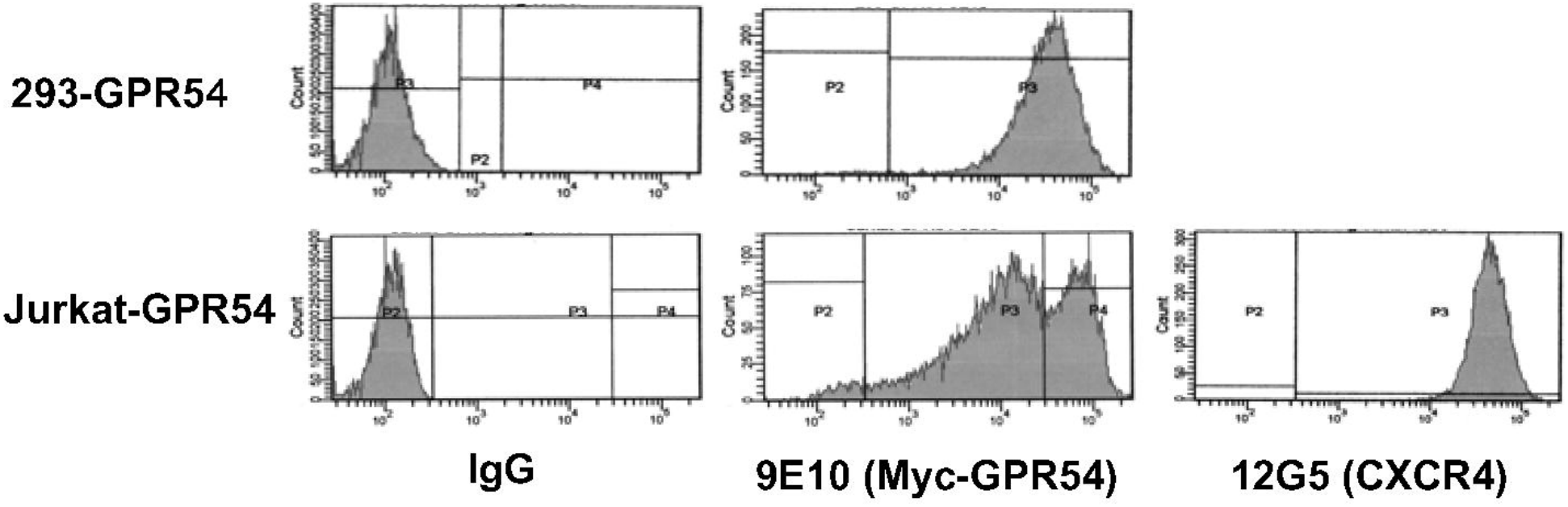

293-GPR54
Jurkat-GPR54
Count
P2
P3
P4
IgG
9E10 (Myc-GPR54)
12G5 (CXCR4)

Figure 2

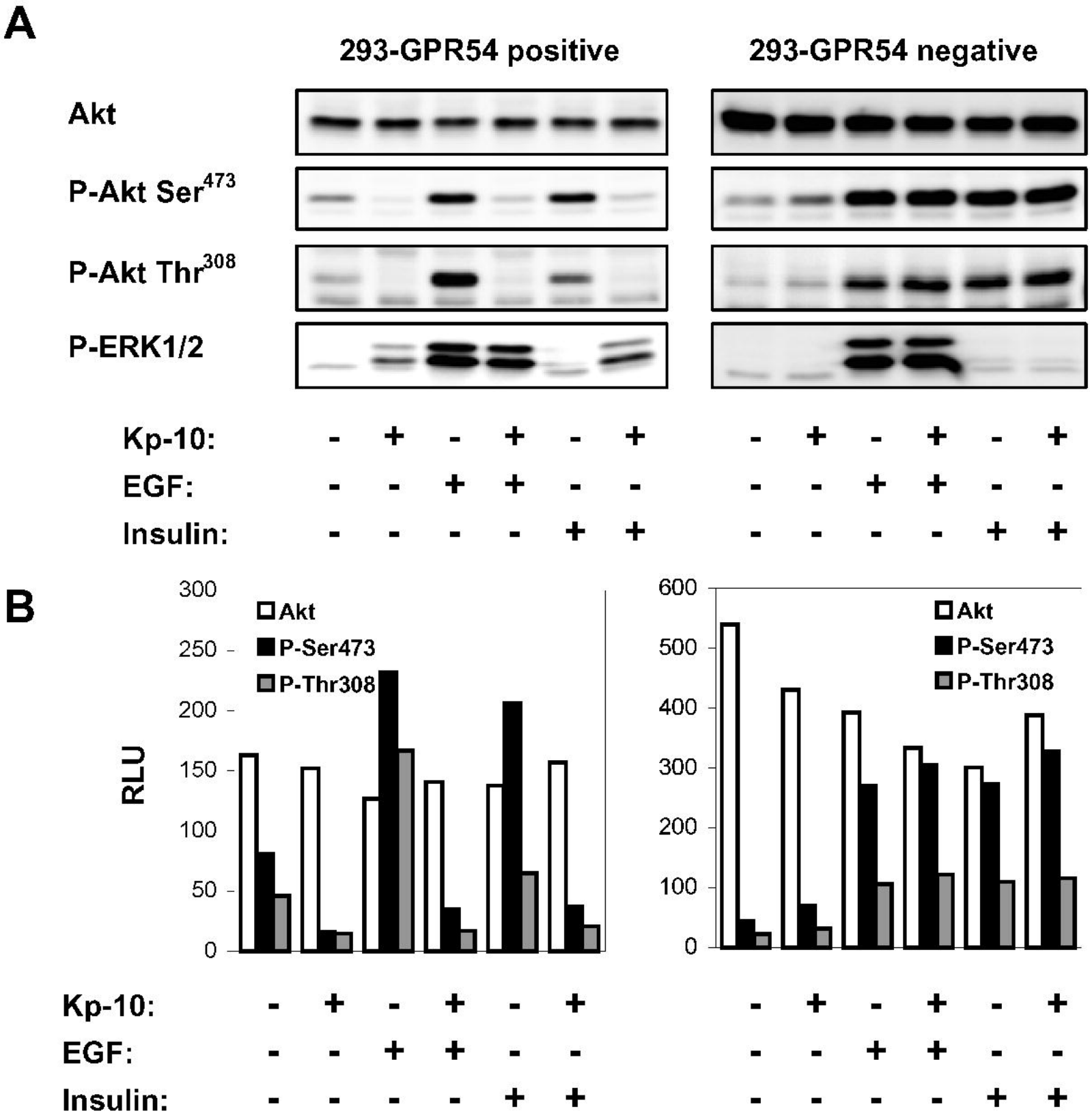

A
293-GPR54 positive
293-GPR54 negative
Akt
P-Akt Ser473
P-Akt Thr308
P-ERK1/2
Kp-10: - + - + - + - + - + - +
EGF: - - + + - - - - + + - -
Insulin: - - - - + + - - - - + +
B
RLU
Akt
P-Ser473
P-Thr308
300
250
200
150
100
50
0
600
500
400
300
200
100
0
Kp-10: - + - + - + - + - + - +
EGF: - - + + - - - - + + - -
Insulin: - - - - + + - - - - + +

Figure 3

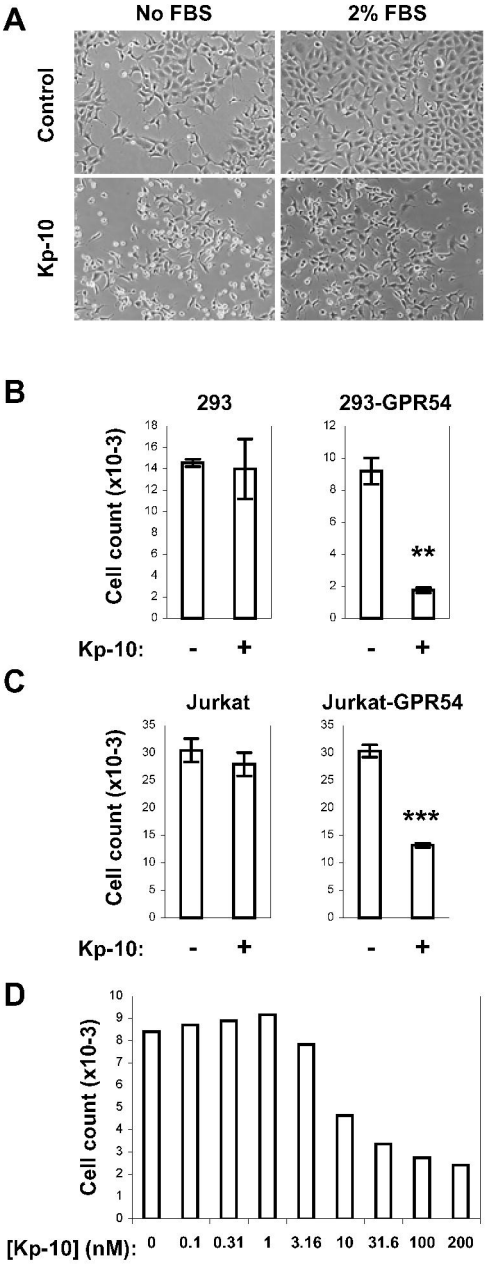

A
No FBS
2% FBS
Control
Kp-10
B
293
293-GPR54
Cell count (x10-3)
**
Kp-10:
- +
C
Jurkat
Jurkat-GPR54
Cell count (x10-3)
***
Kp-10:
- +
D
Cell count (x10-3)
[Kp-10] (nM):
0 0.1 0.31 1 3.16 10 31.6 100 200

Figure 4

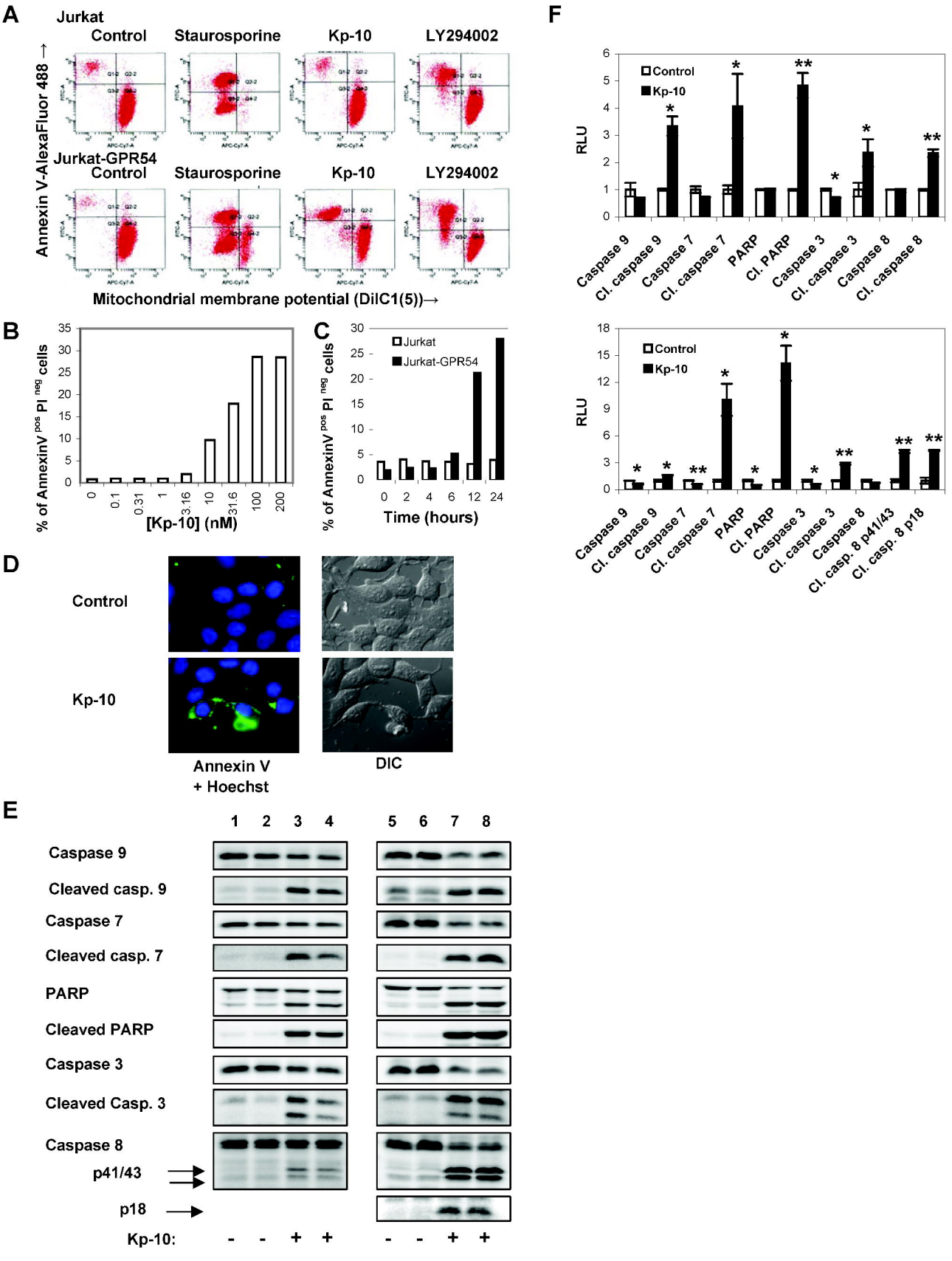

A
Jurkat
Control
Staurosporine
Kp-10
LY294002
Jurkat-GPR54
Annexin V-AlexaFluor 488 →
Mitochondrial membrane potential (DilC1(5))→
B
% of AnnexinV pos PI neg cells
[Kp-10] (nM)
C
Jurkat
Jurkat-GPR54
Time (hours)
D
Control
Kp-10
Annexin V
+ Hoechst
DIC
E
Caspase 9
Cleaved casp. 9
Caspase 7
Cleaved casp. 7
PARP
Cleaved PARP
Caspase 3
Cleaved Casp. 3
Caspase 8
p41/43
p18
Kp-10:
F
RLU
Control
Kp-10
Caspase 9
Cl. caspase 9
Caspase 7
Cl. caspase 7
PARP
Cl. PARP
Caspase 3
Cl. caspase 3
Caspase 8
Cl. caspase 8
Cl. casp. 8 p41/43
Cl. casp. 8 p18

Figure 5

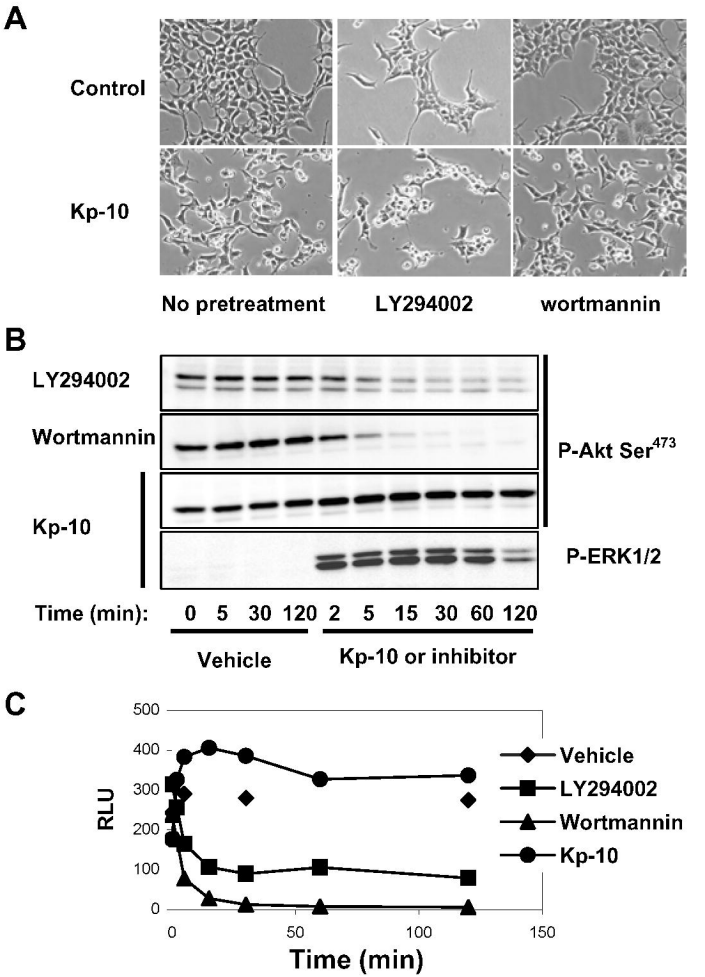

A
Control
Kp-10
No pretreatment
LY294002
wortmannin
B
LY294002
Wortmannin
Kp-10
P-Akt Ser473
P-ERK1/2
Time (min): 0 5 30 120 2 5 15 30 60 120
Vehicle
Kp-10 or inhibitor
C
RLU
500
400
300
200
100
0
0
50
100
150
Time (min)
Vehicle
LY294002
Wortmannin
Kp-10

Figure 6

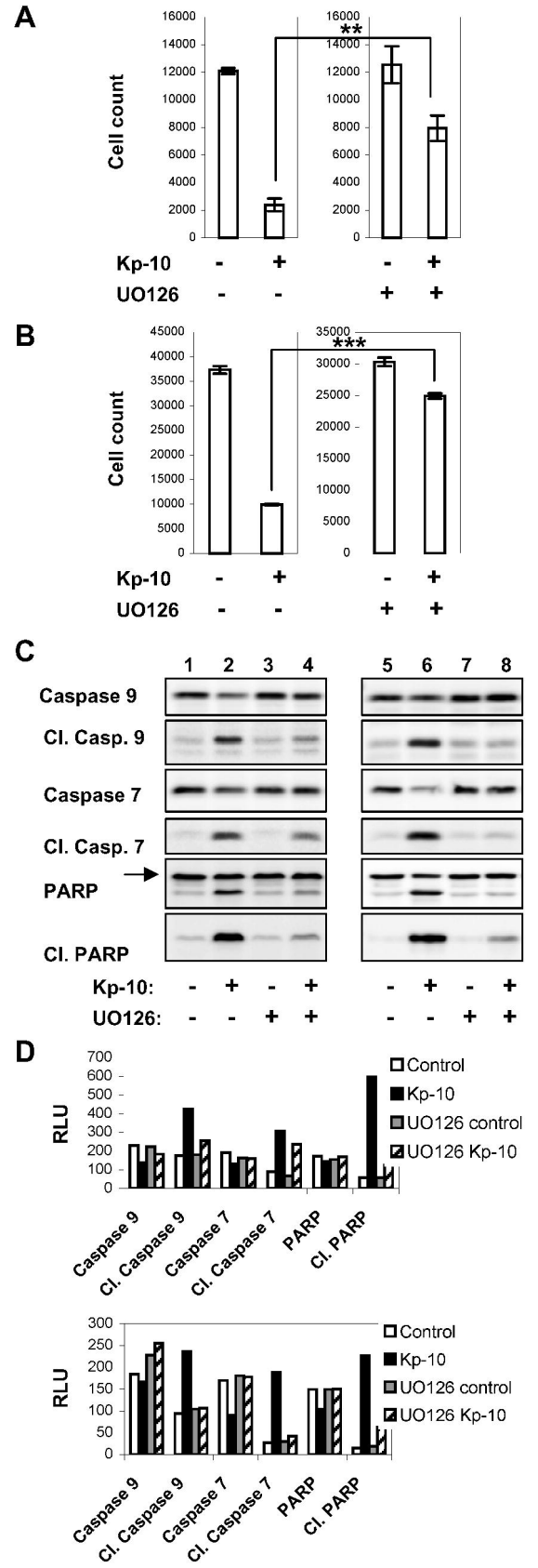
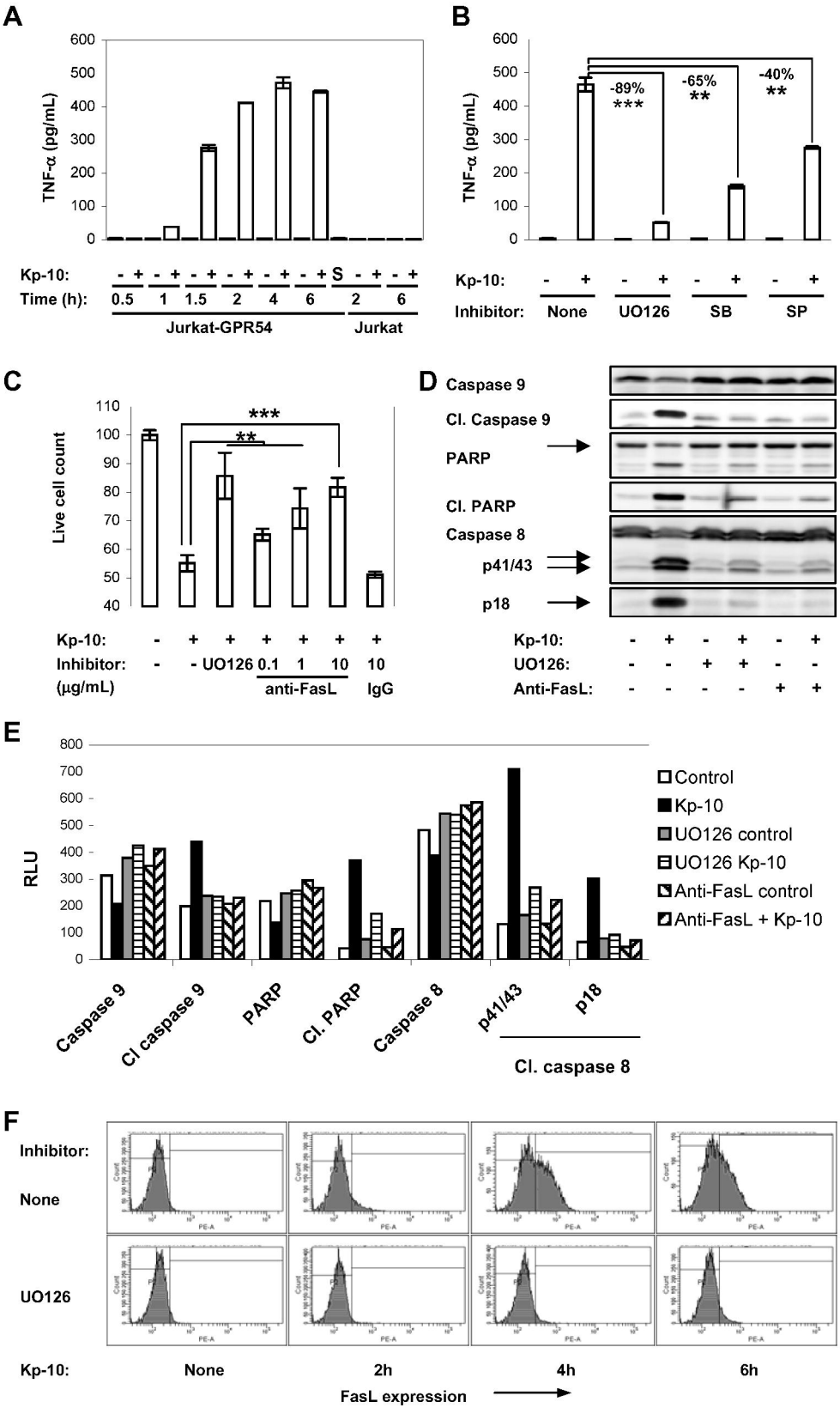

A
Cell count
16000
14000
12000
10000
8000
6000
4000
2000
0
**
Kp-10 - + - +
UO126 - - + +
B
Cell count
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
***
Kp-10 - + - +
UO126 - - + +
C
1 2 3 4 5 6 7 8
Caspase 9
Cl. Casp. 9
Caspase 7
Cl. Casp. 7
PARP
Cl. PARP
Kp-10: - + - + - + - +
UO126: - - + + - - + +
D
RLU
700
600
500
400
300
200
100
0
Control
Kp-10
UO126 control
UO126 Kp-10
Caspase 9
Cl. Caspase 9
Caspase 7
Cl. Caspase 7
PARP
Cl. PARP
RLU
300
250
200
150
100
50
0
Control
Kp-10
UO126 control
UO126 Kp-10
Caspase 9
Cl. Caspase 9
Caspase 7
Cl. Caspase 7
PARP
Cl. PARP

Figure 7


A
TNF-α (pg/mL)
600
500
400
300
200
100
0
Kp-10: - + - + - + - + - + - + S - + - +
Time (h): 0.5 1 1.5 2 4 6 2 6
Jurkat-GPR54
Jurkat
B
TNF-α (pg/mL)
-89%
***
-65%
**
-40%
**
Kp-10: - + - + - + - +
Inhibitor: None UO126 SB SP
C
Live cell count
110
100
90
80
70
60
50
40
***
**
Kp-10: - + + + + + +
Inhibitor: - - UO126 0.1 1 10 10
(µg/mL) anti-FasL IgG
D
Caspase 9
Cl. Caspase 9
PARP
Cl. PARP
Caspase 8
p41/43
p18
Kp-10: - + - + - +
UO126: - - + + - -
Anti-FasL: - - - - + +
E
RLU
800
700
600
500
400
300
200
100
0
Control
Kp-10
UO126 control
UO126 Kp-10
Anti-FasL control
Anti-FasL + Kp-10
Caspase 9
Cl caspase 9
PARP
Cl. PARP
Caspase 8
p41/43
p18
Cl. caspase 8
F
Inhibitor:
None
UO126
Kp-10: None 2h 4h 6h
FasL expression