Proline326 in the C-terminus of murine CX3CR1 prevents G-protein and PI3-Kinase dependent stimulation of Akt and ERK in CHO cells.

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Non-standard abbreviations and chemical makeup of compounds:

CHO	chinese hamster ovary
Akt/PKB	protein kinase B
PI3K	phosphatidylinositol 3-kinase
FKN	fractalkine
FKN-CD	fractalkine-chemokine domain
IGF-1	insulin-like growth factor 1
FBS	fetal bovine serum
MAPK	mitogen activated protein kinase
ERK	extracellular regulated kinase
HIV	human immunodeficiency virus
AIDS	acquired immune deficiency syndrome
TM	transmembrane
LY294002	[2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]

Abstract

Naturally occurring single nucleotide polymorphisms (SNPs) have been identified in human CX3CR1, the chemokine receptor for fractalkine (FKN/CX3CL1). Individuals carrying the I249/M280 variant of CX3CR1 have a lower risk of cardiovascular disease as compared to those homozygous for the common variant (V249/T280). The precise molecular basis for this phenotype is unclear although differences in FKN binding, adhesive properties, and signaling efficiency between the CX3CR1 variants have been reported. FKN binding to CX3CR1 leads to an increase in intracellular calcium, actin rearrangement, and activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways. Regulation of these signaling pathways underlies the known roles for FKN in cell survival, proliferation, and migration. In the present study, we demonstrate that FKN stimulates phosphorylation of Akt/PKB in chinese hamster ovary (CHO) cells individually expressing the naturally occurring variants of human CX3CR1-, as well as rat CX3CR1-, but not in murine CX3CR1-expressing cells. Substitution of Pro326 in the C-terminus of murine CX3CR1, with Ser (residue found in the analogous position of human CX3CR1) produced a mutant receptor that mimicked the human receptor in its ability to stimulate the phosphorylation of both Akt and ERK in a time, PI3K, and pertussis toxin-sensitive G-protein dependent manner. These results identify a critical structural determinant of CX3CR1 important for activation of downstream signaling pathways.

Introduction

Chemokine receptors stimulate signal transduction pathways that regulate cytoskeletal reorganization and integrin activation leading to increased adhesion and the migration of cells. Activation of CX3CR1 by its sole chemokine ligand, FKN (CX3CL1), results in the stimulation of PI3K, MAPK, and Akt/PKB signaling cascades (Cambien et al., 2001; Kansra et al., 2001; Maciejewski-Lenoir et al., 1999; Meucci et al., 1998). The phosphorylation and activation of Akt has been implicated in mechanisms associated with cell survival by controlling the activity of caspases and decreasing levels of pro-apoptotic peptides. FKN regulation of this pathway has been demonstrated in monocytes (Cambien et al., 2001), microglial cells (Maciejewski-Lenoir et al., 1999), neuronal cells (Meucci et al., 1998) and recombinant CX3CR1 expressing CHO cells (Kansra et al., 2001). Stimulation of rat microglial cells with FKN increases levels of anti-apoptotic proteins, down regulates pro-apoptotic pathways (Boehme et al., 2000), and protects cells against death receptor induced apoptosis. FKN-induced activation of the PI3K pathway also plays a role in neuronal survival (Meucci et al., 2000). Collectively, these data support a role for FKN and its receptor in cell survival and proliferation.

Phenotypes associated with CX3CR1 polymorphisms have provided clues into the function of this receptor. Faure *et al*. originally reported single nucleotide polymorphisms (SNP) in the genes encoding CX3CR1 in humans (Faure et al., 2000). These polymorphisms cause codon changes from valine to isoleucine at position 249 and from threonine to methionine at position 280. In this original study individuals from a French cohort of HIV infected patients, identified as homozygous for the I249/M280 variant of CX3CR1, were found to progress more quickly to AIDS when compared to individuals

carrying other haplotypes of the receptor (Faure et al., 2000). However, no such association was found in North American cohorts of HIV infected patients (McDermott et al., 2000). More recently, it has been shown that individuals heterozygous for the I249 variant of CX3CR1 showed a decreased risk for acute coronary events (Moatti et al., 2001), as well as a reduction in the prevalence and severity of coronary artery disease (McDermott et al., 2001; McDermott et al., 2003). A positive association between the CX3CR1-I249/M280 haplotype and brain infarction has been identified (Lavergne et al., 2005). On the other hand, a reduced risk of internal carotid artery occlusive disease is associated with the M280 polymorphism (Ghilardi et al., 2004) while none of the CX3CR1 polymorphisms appear to be associated with either peripheral artery disease (Gugl et al., 2003) or ischemic cerebrovascular disease (Hattori et al., 2005). The underlying mechanisms by which these mutations produce these various effects remains unclear. Studies have shown that the I249/M280 variant has reduced affinity for FKN, primarily as a consequence of a slower rate of ligand association (Faure et al., 2000; McDermott et al., 2003). However, it was recently shown that the variant receptors bound FKN similarly, yet the I249/M280 CX3CR1 variant had increased adhesive properties (Daoudi et al., 2004). These latter results contrast the reduced adhesive capacity of the I249/M280 variant observed by McDermott et al. (McDermott et al., 2003)

To date, no information is available regarding the activation of PI3-kinase dependent signaling pathways by the different human CX3CR1 variants. To this end, we investigated the ability of the human variant forms of CX3CR1 as well as the known rodent (murine and rat) orthologs to activate PI3K dependent signaling pathways in CHO cells. Our results show that the human variant forms of CX3CR1 are not impaired in their

ability to activate PI3K dependent signaling. However, the murine ortholog of CX3CR1 was unable to activate this pathway. Furthermore, we identify Pro at position 326 in the C-terminus of the murine receptor as a key determinant of the inability of this receptor ortholog to stimulate the phosphorylation of Akt and ERK in a G-protein and PI3K dependent manner.

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Methods

Generation of CHO cells expressing human, rat, and murine CX3CR1: human variants, human/murine chimers, and murine CX3CR1 site-directed mutants.

Using sequential site-directed, oligonucleotide-based mutagenesis, the DNA sequence encoding the amino acid Val (V) at position 249 was mutated to encode Ile (I) and the DNA sequence encoding the amino acid Thr (T) at position 280 was mutated to encode Met (M) in huCX3CR1/pcDNA3.1. These mutations were created independently and sequentially to produce three variant receptors: CX3CR1-I249/T280, -V249/M280, and -I249/M280. Following PCR, DNA was transformed by electroporation into *E. Coli* JM-109 cells. Plasmid DNA was isolated from random ampicillin resistant bacterial colonies. The entire protein coding sequences of each mutated form of the receptor were subjected to DNA sequencing to confirm the fidelity of the DNA polymerase (*Pfu*). The following complementary oligonucleotides were used to create CX3CR1-I249: forward (5'- TGG ACA CCC TAC AAT ATT ATG ATT TTC CTG GAG -3') and reverse (5'- CTC CAG GAA AAT CAT AAT ATT GTA GGG TGT CCA -3'). The following complementary oligonucleotides were used to create CX3CR1-M280: forward (5'- CTC AGT GTG ACT GAG ATG GTT GCA TTT AGC CAT -3') and reverse (5'- ATG GCT AAA TGC AAC CAT CTC AGT CAC ACT GAG -3').

The *Kpn*I fragments containing the N-terminal portions (up to and including the third transmembrane (TM) domain) of human and murine CX3CR1 were isolated and used to replace the corresponding domains in human and murine CX3CR1 to produce Hu(TM1-3)/Mur(TM4-7) and Mur(TM1-3)/Hu(TM4-7). Following ligation, *E. Coli* JM-109 cells

were transformed with plasmid DNA by electroporation. DNA was isolated from random ampicillin resistant bacterial colonies and subjected to DNA sequence analysis.

The DNA sequence encoding the amino acid His (H) at position 318 of murine CX3CR1 was mutated to encode Arg (R) and the DNA sequence encoding the amino acid Pro (P) at position 326 was mutated to encode Ser (S) to generate murCX3CR1(R318) and murCX3CR1(S326), respectively. The following complementary oligonucleotides were used to create murCX3CR1(R318): forward (5'- TGG ACA CCC TAC AAT ATT ATG ATT TTC CTG GAG -3') and reverse (5'- CTC CAG GAA AAT CAT AAT ATT GTA GGG TGT CCA -3'). The following complementary oligonucleotides were used to create murCX3CR1(S326): forward (5'- CTC AGT GTG ACT GAG ATG GTT GCA TTT AGC CAT -3') and reverse (5'- ATG GCT AAA TGC AAC CAT CTC AGT CAC ACT GAG -3'). Following PCR, DNA was transformed by electroporation into *E. Coli* JM-109 cells. Plasmid DNA was isolated from random ampicillin resistant bacterial colonies. The entire protein coding sequences of each mutated form of the receptor were subjected to DNA sequence analysis.

Chinese hamster ovary (CHO) cells, cultured in Ham's F12/10%FBS supplemented with penicillin and streptomycin, were transfected with plasmid DNA and LipofectAMINE according to the manufacturer's suggested protocol. Clonal cell lines resistant to G418 were isolated and propagated. All phenomena associated with each specific receptor were characterized in multiple (at least two), independently generated clonal cell lines in order to rule out artifacts associated from characterizing a single clonal cell line.

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Whole Cell Radioligand Binding Analysis.

Procedures for radioligand binding analysis and radiolabeling of chemokines were described in detail in previously published protocols (Davis et al., 2003). CHO cells stably expressing CX3CR1 were seeded at a density of ~100,000 cells/well into 12-well cell culture plates (Corning, Corning, NY) with HAMS F-12 medium containing 10% FBS and 1% Pen/Strep. A concentration of either 0.2 nM or 0.5 nM ^{125}I-FKN-CD (100-200 Ci/mmol) was used in each of the competition binding experiments. Non-specific binding was determined in the presence of 100 nM unlabeled FKN-CD. Mean total and non-specific cpms ($\pm$ S.E.M.) for the cell lines analyzed at a concentration of 0.5 nM ^{125}I-FKN-CD were: huCX3CR1-V249/T280 (common variant): 10,338 $\pm$ 504 (total), 1294 $\pm$ 99 (non-specific); huCX3CR1-I249/T280: 9538 $\pm$ 442 (total), 1347 $\pm$ 70 (non-specific); huCX3CR1-V249/M280: 10,478 $\pm$ 310 (total), 2119 $\pm$ 197 (non-specific); huCX3CR1-I249/M280: 9346 $\pm$ 360 (total), 1314 $\pm$ 110 (non-specific). Mean total and non-specific cpms ($\pm$ S.E.M.) for the cell lines analyzed at a concentration of 0.2 nM ^{125}I-FKN-CD were: human CX3CR1 (common variant): 3260 $\pm$ 141 (total), 548 $\pm$ 43 (non-specific); rat CX3CR1: 2463 $\pm$ 47 (total), 480 $\pm$ 35 (non-specfic); murine CX3CR1: 2838 $\pm$ 248 (total); 483 $\pm$ 43 (non-specific); hu(TM1-3)/mur(TM4-7) CX3CR1: 3848 $\pm$ 481 (total); 1322 $\pm$ 101 (non-specific); mur(TM1-3)/hu(TM4-7): 3116 $\pm$ 313 (total), 1336 $\pm$ 68 (non-specific); murine CX3CR1-R318: 2757 $\pm$ 181 (total), 574 $\pm$ 33 (non-specifc); murine CX3CR1-P326: 3484 $\pm$ 403 (total), 556 $\pm$ 30 (non-specific). Experimentally determined numbers of receptor binding sites were in the range of 30,000 – 75,000 sites/cell for all receptor-expressing cell lines.

Western Blot Analysis of FKN-Stimulated Akt Phosphorylation.

CX3CR1-expressing CHO cells were plated into 12-well cell culture dishes and allowed to grow 48 hrs to 70-90% confluency. Just prior to stimulation, cells were incubated in serum free HAMS F-12 medium for 2 hrs. Cells were then incubated in serum free HAMS F-12 containing various concentrations of FKN in a total reaction volume of 200 µl per well. Following stimulation, medium was aspirated, the plates were placed directly on ice, and then each well was washed one time with ice cold PBS. Cells to be analyzed for Phos-Akt were collected in 100 µl of 1X Laemmli sample buffer containing 3% BME and sonicated 5-10 sec on ice to shear DNA and reduce sample viscosity. Cells to be analyzed for Phos-ERK were washed with ice-cold PBS and collected with lysis buffer (PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 100 µg/mL PMSF, 20 µg/mL Aprotinin, 1 mM Na orthovanadate, 1 mM DTT, pH 7.4). A 4X Laemmli sample buffer containing 12% BME was added and sonicated 5-10 sec on ice. Phos-Akt and Phos-ERK samples were boiled for 5 min and centrifuged at 10,000 X g for 1 min. At this point, 50-60 µl of sample was immediately subjected to SDS-PAGE and Western blot analysis. Membranes were blocked on an orbital shaker for 1 hr in TBS-T-5% milk (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and subsequently incubated overnight at 4° C with either anti-Phos-Akt or anti-Phos-ERK antibody (Cell Signaling Technology, Beverley, MA) at a 1:1,000 dilution. The membranes were washed 3 X 10 min in TBS-T and then incubated in goat anti-rabbit IgG/HRP secondary antibody (for Phos-Akt, Cell Signaling Technology, Beverley, MA) 1:2,000 dilution or sheep anti-mouse IgG/HRP (for Phos-ERK; Invitrogen, Carlsbad, CA) at a 1:5,000 dilution in TBS-T 5% milk at RT for 1 hr. Membranes were washed vigorously 3 X 5 min in TBS-T, then incubated with Pierce

Supersignal chemiluminescent substrate (Pierce, Rockford, IL) for 1 min. Membranes were then exposed onto Amersham ECL film (Amersham International, Buckinghamshire, GB). To strip anti-Phos-Akt antibody, PVDF membranes were incubated in stripping buffer (25 mM Glycine-HCl, pH 2.0, 1% SDS) for 30 min at 65°C. To strip anti-Phos-ERK antibodies, nitrocellulose membranes were incubated in 0.2 M NaOH for 2 min. Membranes were then washed 2 X 10 min in TBS-T, blocked in TBS-T 5% milk for 1 hr at RT, and incubated subsequently with either anti-Akt or anti-ERK antibody (Cell Signaling, Minneapolis, MN) at a 1:2,000 dilution overnight at 4° C. After washing in TBS-T, membranes were incubated in goat anti-rabbit IgG/HRP secondary antibody at a 1:2,000 dilution for 1 hr at RT. Membranes were washed vigorously 3 X 5 min in TBS-T, and then developed as described above.

Data Analysis

Graphic and statistical analyses were performed using Prism and InStat from Graph Pad Software (San Diego, CA).

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Results

FKN-induced Akt phosphorylation in human variant and rodent CX3CR1 expressing CHO cells

Competition binding analysis of CHO cells stably expressing the known variants of human CX3CR1 indicated that each receptor variant bound FKN with similar affinities (Figure 1A). More notable, FKN stimulated the phosphorylation of Akt (Phos-Akt) in the various cell lines in a comparable time-dependent manner (Figure 1B). Figure 1B-E shows representative Western blots and the bar graphs summarize results (n=3) indicating that FKN increased levels of Phos-Akt as early as two minutes. The increase in Phos-Akt peaked between five and ten minutes, and persisted for at least twenty minutes after the addition of FKN. The results indicate that the amino acid changes in the receptor do not impact the ability of FKN to induce time-dependent changes in Phos-Akt in CHO cells expressing the known CX3CR1 variants.

CHO cells stably expressing rat and murine CX3CR1 were engineered and characterized by competition binding analysis. FKN competed with ^{125}I-huFKN-CD (0.2 nM) for binding to human (common variant, V249/T280), rat, and murine CX3CR1 with equivalent affinities. These data are consistent with original studies documenting human FKN specifically binds cells expressing either the murine, or the rat receptor orthologs (Combadiere et al., 1998; Harrison et al., 1998). Human FKN stimulated Phos-Akt in human- and ratCX3CR1-expressing CHO cells (Figure 2B), while no stimulation was seen in CHO cells stably expressing the murine receptor. The lack of stimulation was evident in multiple, independently generated clonal murCX3CR1-CHO cell cultures with receptor

densities that ranged from 30,000 - 75,000 sites per cell, which ruled out potential artifacts inherent to a single stable CHO cell line.

Human FKN has been shown to induce chemotaxis and calcium transients in HEK293 cells expressing murine CX3CR1 (Combadiere et al., 1998). To determine whether the lack of FKN-induced Phos-Akt in the CHO cell was due to the characteristics of the ligand or of the receptor, murine FKN was tested for its ability to activate both human and murine CX3CR1. Murine FKN stimulated Phos-Akt in human but not in murine CX3CR1-expressing CHO cells. These data indicate that the inability of FKN to stimulate Phos-Akt in the murine CX3CR1-expressing cells is an intrinsic property of the receptor and not an inability of the human ligand to activate the murine receptor.

FKN stimulates Phos-Akt in CHO cells expressing a chimeric CX3CR1 containing the human C-terminal amino acids sequences

In order to identify the region of murine CX3CR1 responsible for the signaling defect, two chimeric forms of CX3CR1 were generated by taking advantage of a common *Kpn*I restriction endonuclease digestion site in the DNA sequences encoding the human and murine forms of the receptor. The N-terminal region up to and including TM three were replaced to form two novel chimeric receptors. These chimeric receptors were termed Hu(TM1-3)/Mur(TM4-7) and Mur(TM1-3)/Hu(TM4-7). FKN competed for ^{125}I-huFKN-CD (0.2 nM) and bound human, murine, Hu(TM1-3)/Mur(TM4-7) and Mur(TM1-3)/Hu(TM4-7) with similar affinities (Figure 3A) indicating that the ligand binding pocket of the chimeras remained intact. These chimeric forms of CX3CR1 were evaluated for FKN stimulation of Phos-Akt in the receptor-expressing CHO cells. Figure 3B shows that

FKN stimulated Phos-Akt only in cells expressing the Mur(TM1-3)/Hu(TM4-7) chimeric CX3CR1. These data indicate that the replacement of the C-terminal half of the murine receptor with the corresponding region of the human receptor imparted onto the murine receptor an ability to stimulate Phos-Akt in the CHO cell and suggested that intracellular loop 3 and/or the C-terminus determined whether or not this receptor activated this downstream signaling event.

A single mutation in the murine C-terminus rescues FKN stimulated phosphorylation of Akt in CHO cells that is dependent on PI3Kinase and pertussis toxin sensitive G-protein(s)

The importance of amino acid residues contained in the intracellular C-termini of chemokine receptors with regards to cell-surface expression and/or coupling to signal transduction pathways is well documented (Alkhatib et al., 1996; Arai et al., 1997; Gosling et al., 1997). Figure 4A depicts an amino acid alignment of the C-terminal regions of human, rat, and murine CX3CR1. While these C-terminal sequences contain many identical amino acids, two specific residues that are similar or identical in the C-termini of human and rat CX3CR1 yet differ in the analogous positions of the murine receptor are apparent; no such similarities and differences exist in the third intracellular loops of human, rat, and murine CX3CR1. To assess the specific role of the His at position 318 and Pro at position 326 in receptor activation, these amino acid were mutated to Arg, and Ser, respectively, to generate two mutant murine receptors (termed murCX3CR1-R318 and murCX3CR1-S326). Stable CHO cell lines expressing each of the mutants were generated and evaluated for FKN binding and stimulation of the phosphorylation of Akt and ERK.

FKN competed for binding of ^{125}I-huFKN-CD (0.2 nM) to each of the receptors with equivalent affinities (Figure 4B), although FKN competition curves for human CX3CR1 and murCX3CR1-S326 were slightly steeper, i.e. occurred over a narrower range of FKN concentrations.

Human FKN stimulated, to the same extent, Phos-Akt and Phos-ERK in CHO cells expressing human CX3CR1 or murCX3CR1-S326. No stimulation of Akt or ERK was evident in cells expressing either murine CX3CR1, or murCX3CR1-R318 (Figure 5A). Insulin-like growth factor I (IGF-I) is a known PI3K-dependent stimulant of Phos-Akt and Phos-ERK in CHO cells (Banno et al., 2003; Okamoto et al., 2000). IGF-I stimulated Phos-Akt in CHO cells expressing human, murine, muCX3CR1-S326, and muCX3CR1-R318 forms of the receptor (Figure 5B), which indicated that each stable CHO cell line had fully functional PI3K dependent signaling to Akt.

The kinetics of Phos-Akt stimulation by FKN was compared in each of the receptor expressing cell lines. Over the thirty-minute time frame examined, only the human CX3CR1 and murCX3CR1-S326 mutant receptors stimulated the phosphorylation of Akt. Figure 6 shows that the time dependent activation of Akt by FKN in the human CX3CR1 and muCX3CR1-S326 expressing cells are similar. These results indicated that the mutation of the single amino acid within the intracellular domain of the murine receptor C-terminus imparted an ability to activate Akt in a time-dependent manner comparable to what was observed in the human CX3CR1-expressing cells.

In CX3CR1-expressing CHO cells, MAPK signaling events are inhibited by the PI3K specific inhibitor, LY294002 (Kansra et al., 2001). Furthermore, chemokine receptors couple to downstream signaling events via pertussis toxin sensitive G-proteins.

Pretreatment of human CX3CR1 and murCX3CR1-S326 cells with LY294002, an inhibitor of PI3K, or with pertussis toxin, an inhibitor of Gi/o proteins abrogated FKN-mediated Phos-Akt activation, suggesting that those pathways require an upstream activation of both PI3K and Gi/o proteins (Figure 7). More important, these data indicate that restoration of signaling in the murine CX3CR1 mutant expressing cells, as a consequence of the Pro to Ser change, occurs thru a Gi/o protein and PI3K dependent pathway that is utilized by human CX3CR1.

Discussion

Two common single SNPs within the receptor for the human chemokine FKN (CX3CR1) gene have been identified (Faure et al., 2000). In the present study, we demonstrate that these receptor variants do not display any differences in FKN dependent stimulation of Akt phosphorylation in CHO cells. Furthermore, we have shown that human and rat, but not murine CX3CR1 couple to PI3K dependent signaling to Akt and MAPK pathways in the CHO cell. The lack of regulation of these pathways by the murine receptor is a direct consequence of a Pro at position 326 in the receptor C-terminus. Mutation of this residue to Ser (corresponding residue in human CX3CR1) rescued FKN stimulated phosphorylation of Akt and ERK in a PI3K and G-protein dependent manner.

Human CX3CR1 polymorphisms have provided evidence for specific roles for this receptor in the kinetics of progression to AIDS in some HIV-infected populations. In addition, certain CX3CR1 variants have been linked to the prevalence and severity of coronary artery disease as well as a decreased risk for acute coronary events (McDermott et al. 2001; Moatti et al., 2001). The underlying mechanism accounting for the cardiovascular phenomena was suggested from *in vitro* studies directed at characterizing the ligand binding and adhesive properties of the CX3CR1 variants. A slight reduction in ligand affinity and ability to stimulate a calcium response, as well as defective FKN dependent adhesive activity *in vitro* (McDermott et al., 2003) was identified for the CX3CR1-I249/M280 variant. These findings are consistent with original reports that associated impaired FKN/CX3CR1 function with reduced risk of cardiovascular disease in humans. Dissimilar results were reported more recently in a study demonstrating that cells

containing the CX3CR1-I249/M280 variant have enhanced adhesive activity (in both transfected HEK cells and peripheral blood cells) and a slightly increased FKN-stimulated Phos-ERK (Daoudi et al., 2004). The authors of this latter study suggest that the disparate results are a consequence of the nature of the ligand used in the assay, i.e. soluble or membrane-bound FKN. Our analysis indicates that the amino acid changes associated with the CX3CR1 polymorphisms do not affect the ability of the receptor to couple to PI3K sensitive signaling pathways in the CHO cell. CHO cells expressing the most common receptor (V249/T280) and the other receptor variants bound FKN with similar affinities and stimulated to the same extent, the phosphorylation of Akt. Thus, phenotypes associated with a specific CX3CR1 variant are probably not a consequence of differential regulation of this signaling pathway.

Murine CX3CR1 binds human FKN-CD and is capable of inducing calcium transients in HEK293 cells expressing the receptor (Combadiere et al., 1998). While these characteristics of murine CX3CR1 are analogous to the human ortholog, other properties of the murine FKN/CX3CR1 axis differ from its human counterpart. For instance, murine FKN does not promote firm adhesion of CX3CR1 expressing cells under conditions mimicking physiological blood flow (Haskell et al., 2000). The experiments described in this study demonstrate that the murine receptor does not stimulate the phosphorylation of Akt in the CHO cell. This signaling defect is due to an intrinsic characteristic of the murine receptor, and not an inability of the ligand to bind or to activate the receptor. Analysis of two human and murine chimeric receptors identified the C-terminus of murine CX3CR1 as the region of the receptor responsible for the deficient signaling properties. Further

analysis identified Pro326 in murine CX3CR1 as the specific structural determinant of the functional defect. Human CX3CR1 contains a Ser while rat CX3CR1 contains a Thr in the analogous position. These Ser and Thr residues are possible phosphorylation sites in the C-terminus of the receptor. Similar to many GPCRs, ligand binding induces the rapid phosphorylation of chemokine receptors on C-terminal residues by G protein-coupled receptor kinases (GRK) and protein kinase C (Aramori et al., 1997; Kraft et al., 2001; Oppermann et al., 1999; Vila-Coro et al., 1999). It is possible that the C-terminus is directly involved in the coupling of CX3CR1 to PI3K in the CHO cell. This impaired coupling seen in the murine receptor could be due to two possibilities; 1) the Pro residue alters the confirmation of the C-terminus in a way that prevents other regions of the receptor, i.e. intracellular loops, from activating downstream elements, or 2) the phosphorylation of the Ser residue is required to couple to this signaling pathway. Further experimentation will be required to provide support for either of these models.

What is the potential significance of these differences in signaling between human and murine CX3CR1? Major dissimilarities in cellular expression patterns of CX3CR1 in humans and mice are known. In the human lymphoid system, cytotoxic effector CD4+, CD8+, and NK cells have been shown to express CX3CR1 (Nishimura et al., 2002). However, CX3CR1 expression is limited to monocytes, NK, and dendritic cells in mice (Jung et al., 2000). The exact nature of this differential expression pattern in the mouse is poorly understood. Nonetheless, the results reported in this study offer up an intriguing hypothesis. The sensitivity of GPCR- and RTK-mediated mitogenic signaling to PI3K inhibitors like wortmannin or LY294002 emphasizes the role of PI3K in the regulation of

cellular growth (van Biesen et al., 1996). The phosphorylation of Akt (PKB) and ERK1,2 (p44/p42 MAPK) lie downstream of CX3CR1 activation of the PI3K pathway in CHO cells (Kansra et al., 2001). In addition calcium signaling events are inhibited by the PI3K specific inhibitor, LY294002, in human CX3CR1-expressing CHO cells (Kansra et al., 2001). CX3CR1 activation of the PI3K pathway has been shown to play a role in cell survival and FKN-induced chemotaxis (Kansra et al., 2001; Boehme et al. 2000; Meucci et al., 2000). Furthermore, there is some evidence that Akt activation can promote T cell survival based on studies of transgenic mice expressing a constitutively active membrane targeted gag-Akt construct under the control of a T cell specific promoter (Jones et al., 2000). As PI3K inhibitors, which prevent Akt activation, cause T cells to arrest in the G_0/early G_1 phase of the cell cycle (Brennan et al., 1997), it remains a possibility that a subset of T cells in the mouse may depend on an appropriate CX3CR1 signaling event(s) in order to survive. Our results indicate that murine CX3CR1 is deficient in coupling to Akt in a PI3K dependent manner and, as such, cells expressing this receptor may not get a critical FKN dependent survival signal. Thus, the T cell populations expressing CX3CR1 would disappear.

Multiple mechanisms couple G-protein couple receptor (GPCR) activation with the PI3K/Akt and ERK/MAPK cascades. The dependence of activation of both Akt and MAPKs in CHO cells is in contrast to what is seen in MonoMac6 cells where PI3K does not control ERK, JNK or p38 activation (Cambien et al., 2001). While FKN induced changes in intracellular calcium in murine CX3CR1-expressing HEK293 cells (Combadiere et al., 1998) an examination of murine CX3CR1 signaling to ERK and Akt in

the HEK293 cells will be required to determine if regulation of this specific pathway is also defective in another heterologous expression system. More important, the observations reported herein require extending studies to native cells and tissues. This is particularly relevant in order to draw meaningful comparisons between the function of the FKN/CX3CR1 axis in humans and rodents.

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Footnotes

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Legends for Figures

Figure 1: FKN stimulates time dependent phosphorylation of Akt in CHO cells expressing CX3CR1 variants. **A**) Competition binding of increasing concentrations of huFKN with ^{125}I-huFKN-CD (0.5 nM) to CHO cells stably expressing CX3CR1-V249/T280 (squares), CX3CR1-I249/T280 (diamonds), CX3CR1-V249/M280 (circles), or CX3CR1-I249/M280 (triangles). Calculated IC_{50} values (mean $\pm$ S.E.M., nM): V249/T280 (1.83 $\pm$ 0.83); I249/T280 (1.94 $\pm$ 0.70); V249/M280 (2.52 $\pm$ 0.86); I249/M280 (1.73 $\pm$ 0.64). **B**-**E**) Time dependent stimulation of the phosphorylation of Akt by 30 nM huFKN-CD is plotted as a function of relative Phos-Akt staining normalized to total-Akt staining determined by densitometry (means ± S.E.M.). **B**) CX3CR1-V249/T280 (common variant); **C**) CX3CR1-I249/T280 (single mutation); **D**) CX3CR1-V249/M280 (single mutation); **E**) CX3CR1-I249/M280 (double mutation). Representative Western blot analyses are shown while bar graphs depict results (mean $\pm$ S.E.M.) of three or four independent experiments.

Figure 2: FKN does not activate murine CX3CR1 in CHO cells. **A**) Competition binding of ^{125}I-huFKN-CD (0.2 nM) to CHO cells stably expressing human (filled circles), rat (filled squares), or murine (open circles) CX3CR1 in the presence of increasing concentrations of huFKN. Results are expressed as a mean ± S.E.M. from three independent experiments performed in triplicate. Calculated IC_{50} values (mean $\pm$ S.E.M., nM): human CX3CR1 (0.98 $\pm$ 0.56); rat CX3CR1 (1.3 $\pm$ 0.19); murine CX3CR1 (0.95 $\pm$ 0.19). **B**) Stimulation of Phos-Akt by 30 nM huFKN in CHO cells stably expressing rat, human, or murine CX3CR1. Autoradiographs of Western blot analysis are shown as a representative result of two independent experiments. **C**) Stimulation of Phos-Akt by

either human or murine FKN (20 and 50 nM) in CHO cells stably expressing human or murine CX3CR1. Autoradiographs of Western blot analysis are shown and are representative of two experiments.

Figure 3: FKN activates a chimeric CX3CR1 containing the human C-terminal amino acid sequences. **A**) Competitive binding of ^{125}I-huFKN-CD (0.2 nM) to CHO cells stably expressing human CX3CR1 (circles), murine CX3CR1 (diamonds), Hu(TM1-3)/Mur(TM4-7) (squares), or Mur(TM1-3)/Hu(TM4-7) (triangles) in the presence of increasing concentrations of huFKN. Results are expressed as a mean ± S.E.M. from three independent experiments performed in triplicate. Calculated IC_{50} values (mean $\pm$ S.E.M., nM): human CX3CR1 (0.56 $\pm$ 0.05); murine CX3CR1 (0.39 $\pm$ 0.05); Hu(TM1-3)/Mur(TM4-7) CX3CR1 (0.54 $\pm$ 0.03); Mur(TM1-3)/Hu(TM4-7) CX3CR1 (0.30 $\pm$ 0.09). **B**) Stimulation of Phos-Akt by 30 nM huFKN in CHO cells stably expressing either Hu(TM1-3)/Mur(TM4-7), or Mur(TM1-3)/Hu(TM4-7) CX3CR1 chimeric receptors. Autoradiographs of Western blot analysis are shown as a representative result of two independent experiments.

Figure 4: FKN binds murine CX3CR1-R318 and -S326 mutants. **A**) Sequence alignment of the C-termini of rat, human, and murine CX3CR1. Alignment was achieved using the PILEUP algorithm of GCG. Amino acid identities (|), similarities (:), and gaps (-) are noted. A rectangular box over each amino acid indicates the residues in the murine receptor mutated in this study. **B**) Competitive binding of ^{125}I-huFKN-CD (0.2 nM) to CHO cells stably expressing human CX3CR1 (filled circles), murine CX3CR1 (filled

squares), murCX3CR1-R318 (open squares), and murCX3CR1-S326 (open circles) in the presence of increasing concentrations of huFKN. Results are expressed as a mean ± S.E.M. from three independent experiments performed in triplicate. Calculated IC_{50} values (mean ± S.E.M., nM): human CX3CR1 (0.62 ± 0.15); murine CX3CR1 (0.48 ± 0.48); murCX3CR1-R318 (0.41 ± 0.36); murCX3CR1-S326 (0.64 ± 0.11).

Figure 5: Murine CX3CR1 Ser326 stimulates Phos-Akt and Phos-ERK in response to FKN. **A**) HuFKN (5 or 20 nM) stimulation of Phos-Akt and Phos-ERK in CHO cells stably expressing human CX3CR1 (Human WT), murine CX3CR1 (Murine WT), murine CX3CR1-S326 (Murine S326), and murine CX3CR1-R318 (Murine R318). Autoradiographs of Western blot analysis are shown as a result of two independently conducted experiments. **B**) Stimulation of receptor-expressing CHO cells by 100 ng/ml IGF-I or 20 nM human FKN. Results are representative of two independent experiments.

Figure 6: Time-dependent stimulation of Phos-Akt in human, murine, and mutant murCX3CR1 expressing cells by human FKN. Stimulation of Phos-Akt by 30 nM huFKN in CHO cells stably expressing **A**) human CX3CR1, **B**) murine CX3CR1, **C**) murine CX3CR1-S326, and **D**) murine CX3CR1-R318. Shown are representative autoradiographs of Western blot analysis as well as bar graphs that depict mean ± S.E.M. of results obtained from three independent experiments.

Figure 7: Human and MurCX3CR1-S326 stimulate phosphorylation of Akt in a PI3K and pertussis toxin sensitive G-protein dependent manner. FKN stimulated Phos-Akt in human

and murCX3CR1-S326 was inhibited by LY294002 (25 μM, 2 hr pretreatment) or Pertussis Toxin (PTX: 100 ng/ml, 12 hr pretreatment). Autoradiographs of Western blot analysis are shown as a representative result of two independent experiments.

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Figure 1

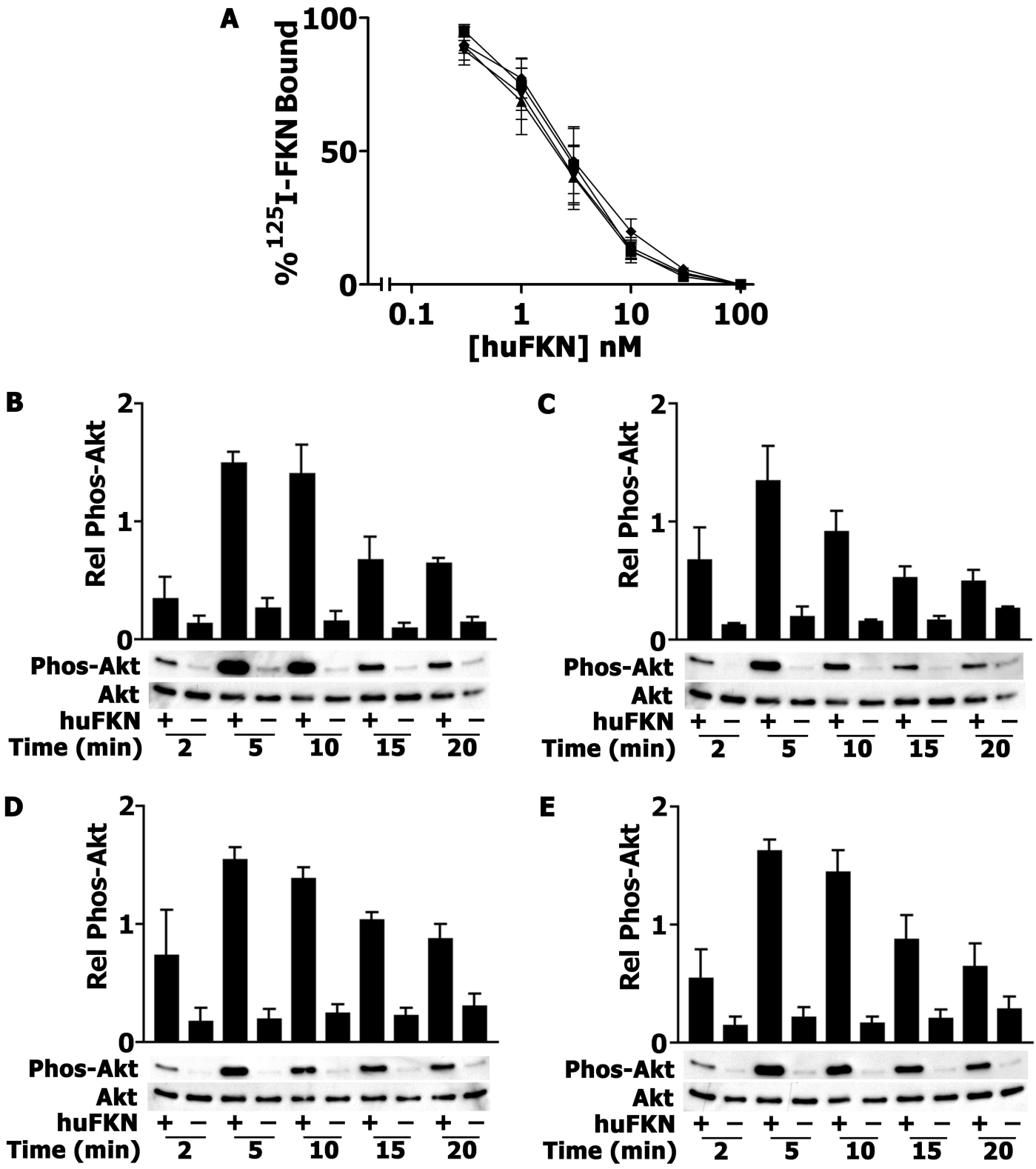

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Figure 2

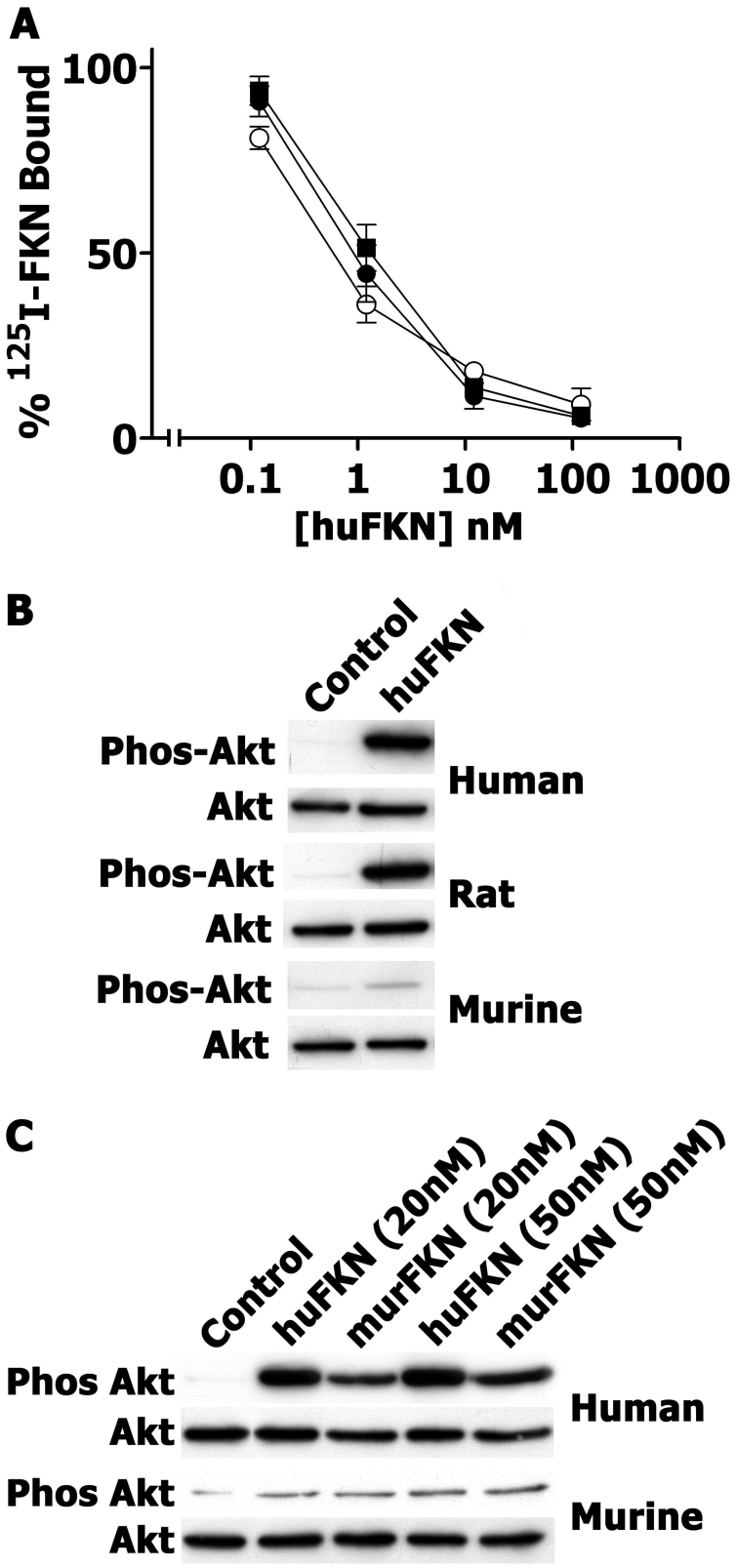

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Figure 3

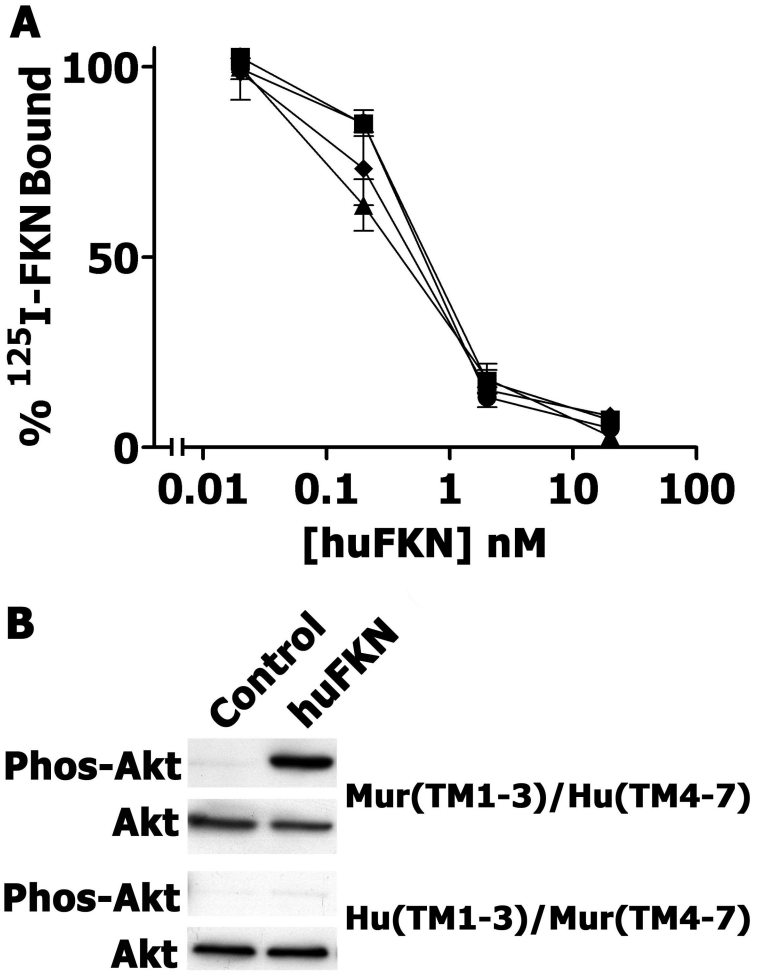

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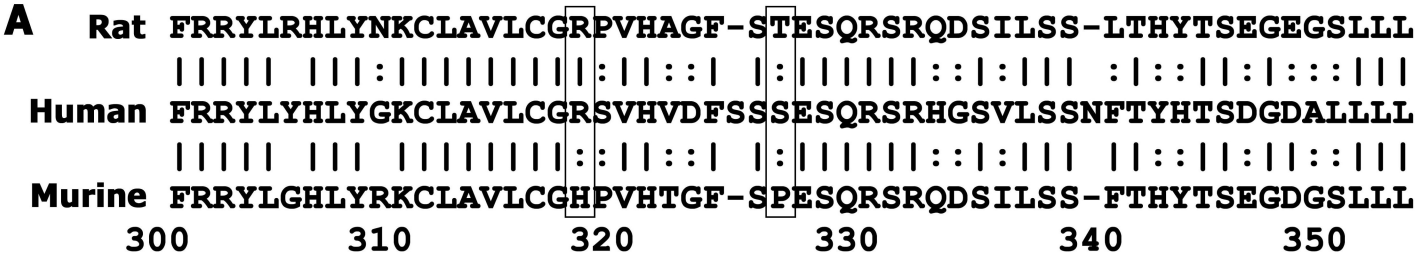
Figure 4



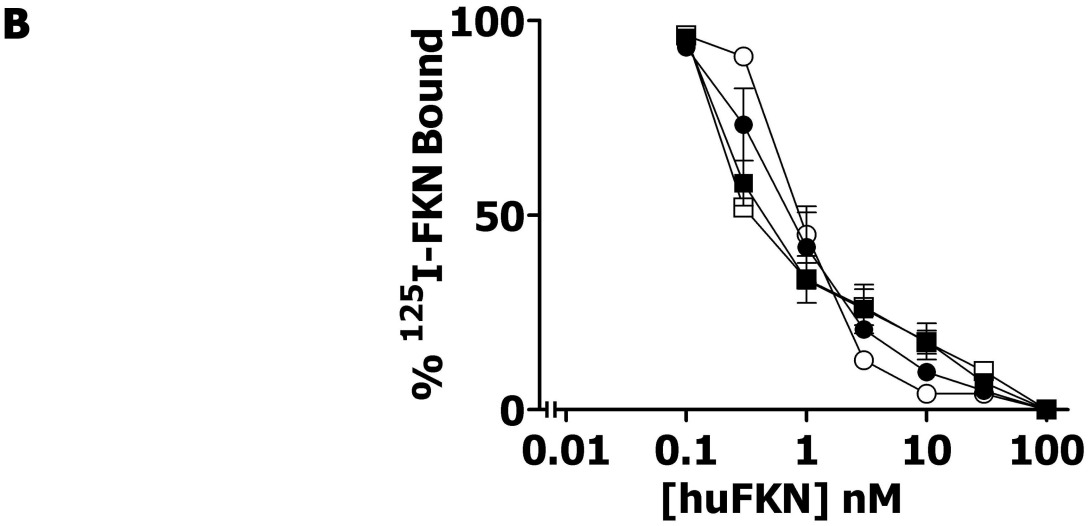


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Figure 5

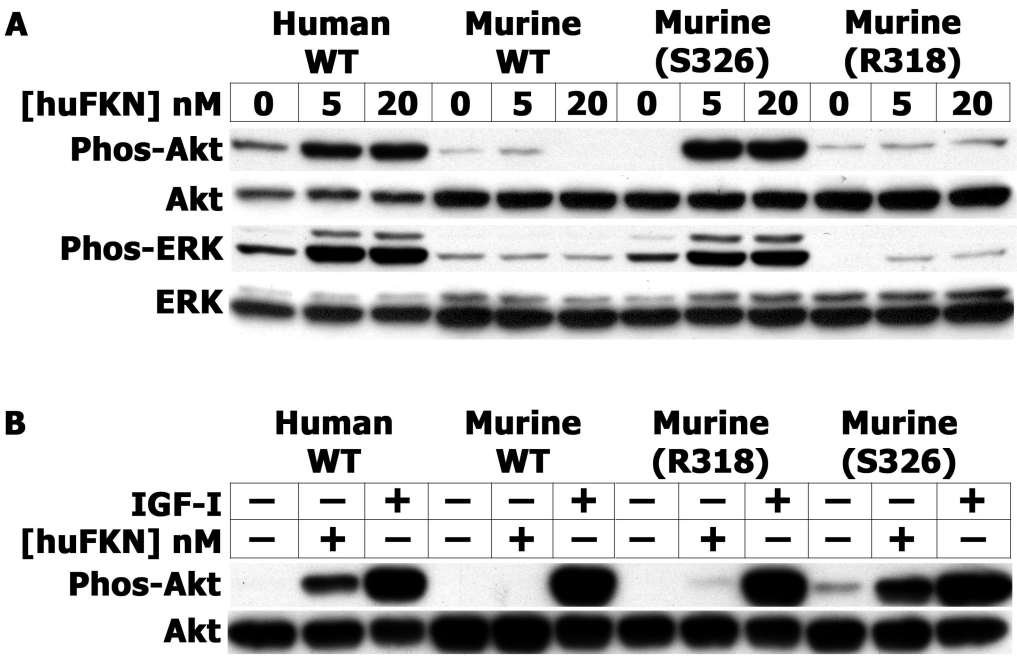

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Figure 6

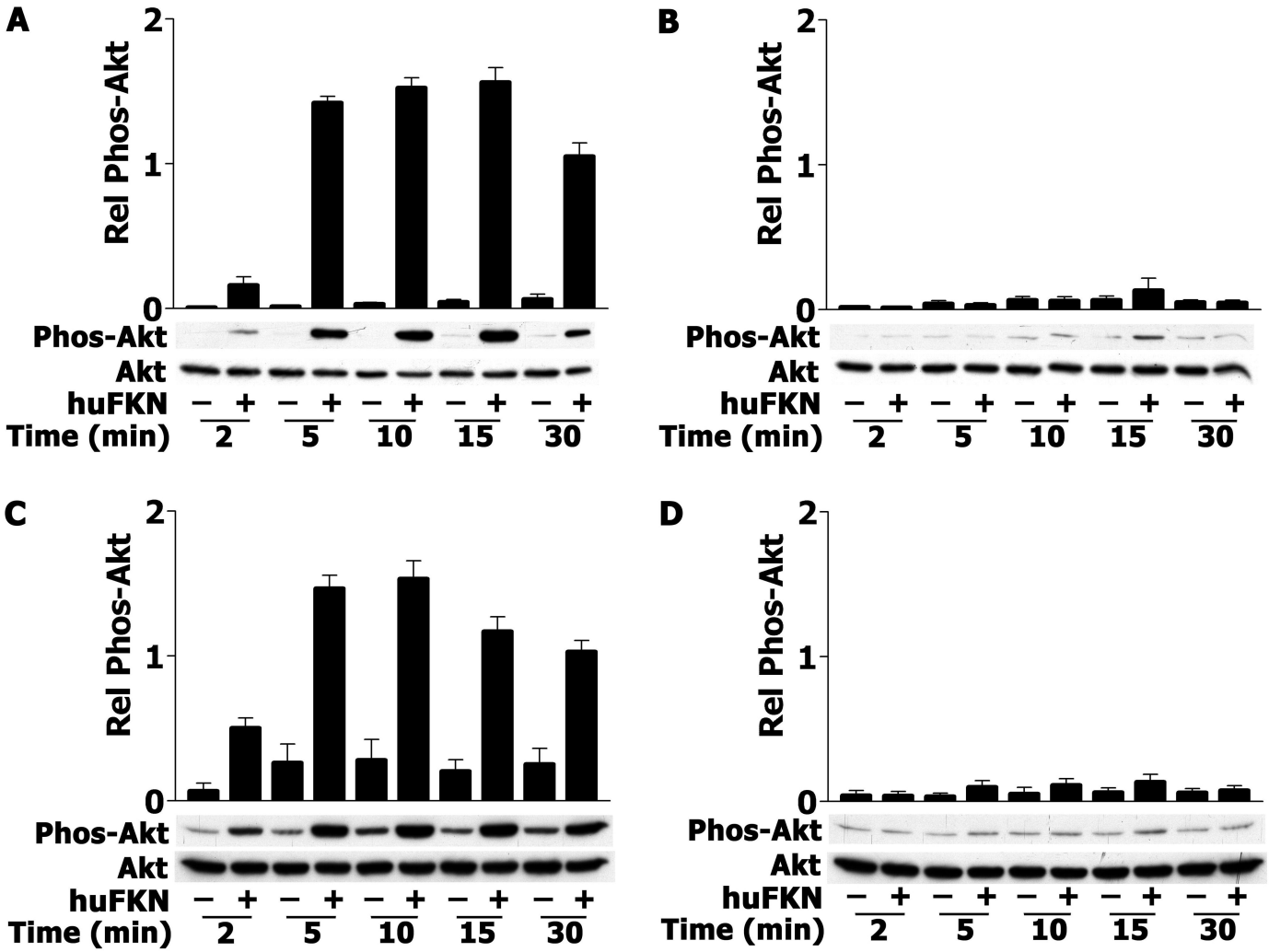

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Figure 7