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Characterizing the Dynamics and Functional Role of Site-Specific Phosphorylation of G Protein-Coupled Receptors

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CHARACTERIZING THE DYNAMICS AND FUNCTIONAL ROLE OF SITE-SPECIFIC PHOSPHORYLATION OF G PROTEIN-COUPLED RECEPTORS

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ABSTRACT

Phosphorylation of G protein-coupled receptors (GPCRs) by GPCR kinases (GRKs) and the subsequent recruitment of arrestin is a well-established paradigm that initiates the process known as desensitization. However, an emerging theme in GPCR regulation is the possibility of differential regulation dictated by the phosphorylation pattern elicited by the different members of the GRK family. Therefore, we have used small interfering RNA-mediated knock down of the GRKs and arrestins in an attempt to better understand how phosphorylation regulates the activity and signaling of the M_3 muscarinic acetylcholine receptor (M_3 mAChR) and CXCR4, two receptors endogenously expressed in HEK293 cells.

Using a two-pronged approach of assaying calcium mobilization and ERK activation, we were able to define and monitor changes in both the G protein-dependent and –independent signaling pathways. We found that GRK2, 3, and 6, and arrestin2 and 3 each has a distinct and separable role in regulating the activity of each receptor. Interestingly, knock down of GRK5 did not effect signaling via either receptor. Our studies with the M_3 mAChR suggest that signaling is strictly through a G protein-dependent manner and relief of inhibitory constraints (GRKs and arrestins) subsequently enhances receptor function. In contrast, CXCR4 uses both a G protein-dependent and –independent (arrestin-dependent) means of signaling. Notably, arrestin-dependent signaling requires both GRK3 and 6.

Based on our studies examining the role of the GRKs in receptor-mediated signaling, we further characterized agonist-promoted phosphorylation of CXCR4. Therefore, we created and characterized a cell line stably expressing Flag CXCR4 to allow for purification of CXCR4 and mass spectrometric analysis. Importantly, we show that CXCR4 regulation is conserved when stably overexpressed. Tandem mass spectrometry and phospho-specific antibodies were used to identify sites of agonist-promoted phosphorylation. These studies demonstrated that Ser-321, Ser-324, Ser-325, Ser-330, Ser-339 and two sites between Ser-346-352 were phosphorylated. Use of phospho-specific antibodies, RNA interference and specific inhibitors revealed that Ser-324/325 was rapidly phosphorylated by protein kinase C and GRK6 upon agonist treatment while Ser-339 was specifically and rapidly phosphorylated by GRK6. Ser-330 also was phosphorylated by GRK6, albeit with slower kinetics.

Taken together, these results clearly demonstrate that GPCRs are dynamically regulated by a number of proteins in a coordinated manner and clear differences exist between receptors expressed within the same cell type. For CXCR4 specifically, we can now begin to address the functional role of site- and kinase-specific phosphorylation of CXCR4 in a variety of tissues. More importantly, we can also begin to understand whether or not there is altered regulation of CXCR4 in a variety of diseases.

DEDICATION

I dedicate this work…

To the memory of my dad who showed me what true strength and courage are;

To my mom Pat for all of her sacrifices and support;

To my wife Elaine who is a constant source of inspiration and support;

and

To my sons John and Matthew who are and always will be my greatest achievements

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ABBREVIATIONS

AC……..…...Adenylyl cyclase

AIP4…….Atrophin1 interacting protein 4

AlF_4^-……...Aluminum fluoride

AP-2……..…Adaptor complex 2

$AT_{1a}R$……...Angiotensin receptor subtype 1a

β_2AR……..……..beta2-adrenergic receptor

Bis I……..Bisindolylmaleimide I

Bis V……...Bisindolylmaleimide V

cAMP…….3’5’-Cyclic adenosine monophosphate

CCP……..….Clathrin coated pit

CK1α……..Casein kinase 1 alpha

Da……..Dalton

DAG……..Diacylglycerol

DDM……...n-dodecyl-β-D-maltoside

DMEM…….Dulbecco’s Modified Eagle Medium

DTT…….Dithiothreitol

ERK……...Extracellular-signal regulated kinase

GAP……..……GTPase activating protein

GDP……Guanosine 5’-diphosphate

GEF……...Guanine nucleotide exchange factor

GIRK……..G protein-activated inwardly rectifying K^+ channel

GPCR……G protein-coupled receptor

GRK……..G protein-coupled receptor kinase

GTP……..Guanosine 5’-trisphosphate

HEK293…….Human Embryonic Kidney 293 cells

IP_3……...Inositol (1,4,5)-trisphosphate

JNK…….Jun N-terminal kinase

K_d……Dissociation constant

LC/MS/MS……..Liquid chromatography tandem mass spectrometry

mAChR……...Muscarinic acetycholine receptor

MAPK……..Mitogen activated protein kinase

MEK……MAP-ERK kinase

PBS…….Phosphate buffered saline

pFHHSiD……paraflurohexahydrosiladiphenidol

PI……Phosphatidylinositol, Phosphoinositide

PI3K……...Phosphoinositide-3’-kinase

PIP_2……..…Phosphatidylinositol (4,5)-bisphosphate

PKA……..Protein kinase A

PKC……..Protein kinase C

PLC…….Phospholipase C

PS……..Phospatidylserine

pS……..Phospho-serine

PTX…….Pertussis toxin

RGS……..Regulator of G protein signaling

RNAi……...RNA interference

SDF-1……...Stomal cell-derived factor-1/CXCL12

SFM……..Serum free DMEM

siRNA…….Small interfering RNA

TBS-T……...Tris buffered saline with Tween 20

WHIM……..*W*arts, *H*ypogammaglobulinemia, *I*nfection, and *M*yelokathexis

PUBLICATION ARISING FROM THESIS WORK

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CHAPTER I

Introduction

G PROTEIN-COUPLED RECEPTORS

Cellular homeostasis is maintained through an organism's ability to transduce a large number of extracellular stimuli into intracellular signaling cascades through interactions with proteins spanning the cellular membrane. One of the largest families of these cell surface proteins is the G protein-coupled receptors (GPCRs) (Pierce et al., 2002), comprising ~3% of the human genome and encoding for greater than 800 receptors (Bai, 2004). GPCRs bind to a wide variety of extracellular ligands including biogenic amines, amino acids, peptides, photons, and odorants resulting in diverse cellular responses such as proliferation, differentiation, cell survival, and cell motility (Marinissen and Gutkind, 2001).

Based on their primary amino acid sequence, GPCRs were predicted to contain seven transmembrane spanning α-helices connected by alternating intracellular and extracellular loops (Hargrave and McDowell, 1992), which was confirmed by the crystal structures of both rhodopsin (Palczewski et al., 2000) and the β2-adrenergic receptor (β_2AR) (Rasmussen et al., 2007). The N-terminus, extracellular loops, and transmembrane domains are involved in ligand binding, while the intracellular loops and C-terminus are involved in signaling and receptor regulation. Despite sharing the same topology, GPCRs are further subdivided based on conserved structural features and amino acid motifs into five families: glutamate, rhodopsin, adhesion, fizzled/taste, and secretin (Fredriksson et al., 2003). The rhodopsin family constitute ~90% of all GPCRs and is the most well studied class of GPCRs (Fredriksson et al., 2003).

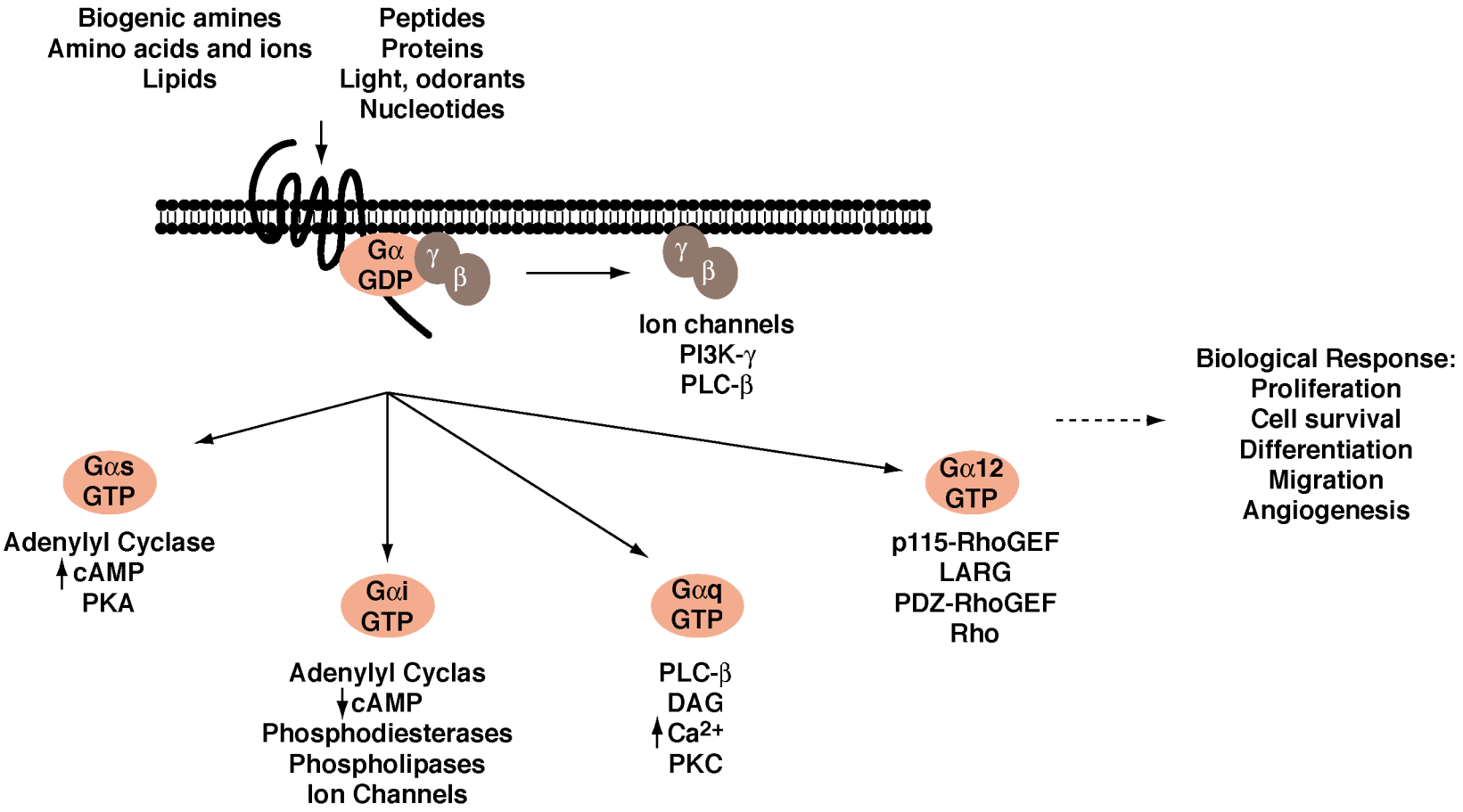
Signal Transduction by GPCRs

GPCRs respond to a myriad of extracellular stimuli, converting these cues into distinct intracellular signaling events (Figure 1). As the name implies, GPCRs couple to and activate the heterotrimeric family of guanine nucleotide binding proteins (G proteins), which consist of Gα, which contains the nucleotide binding pocket, and the functional heterodimer of Gβγ (Oldham and Hamm, 2008). In the inactive state, Gα is bound to guanosine diphosphate (GDP) and complexed with Gβγ. Upon ligand binding, GPCRs undergo a conformational change that allows them to act as a guanine nucleotide exchange factor (GEF) and catalyze the exchange of GDP for guanosine triphosphate (GTP) on the Gα subunit. This causes a conformational change of the Gα subunit, resulting in the dissociation of the heterotrimer into Gα and Gβγ and the subsequent activation of a number of second messenger effectors such as adenylyl cyclase (AC), phospholipase C isoforms (PLC), and ion channels (Flower, 1999; Marinissen and Gutkind, 2001). The G proteins are molecular switches, and through the hydrolysis of GTP to GDP, return to the inactive state. The Gα subunit has intrinsic, albeit slow, GTPase activity. Therefore, proteins known as regulators of G protein signaling (RGS) (Willars, 2006) act as GTPase activating proteins (GAPs) and catalyze the hydrolysis of GTP to GDP, thereby shutting off signaling and allowing the heterotrimer to re-associate.

Figure 1. Signal transduction by GPCRs

GPCRs bind to a number of extracellular stimuli including biogenic amines, amino acids, lipids, proteins, photons and odorants leading to activation of the heterotrimeric G proteins. Exchange of GDP for GTP in the nucleotide-binding pocket of $G\alpha$ subunits results in activation and dissociation of the heterotrimer into $G\alpha$ and $G\beta\gamma$ subunits. The dissociated heterotrimer is then able to interact with and activate a number of effectors and signaling pathways that ultimately result in cellular responses such as proliferation, differentiation, cell survival and cell motility.

GPCR Signaling



The Heterotrimeric G Proteins

As described above, there are three subunits that comprise the heterotrimeric G proteins: Gα, Gβ, and Gγ. There are currently 16 different Gα subunits, subdivided into four different classes based on sequence homology: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (McCudden et al., 2005; Simon et al., 1991). All Gα proteins, with the exception of the photoreceptor-specific $G\alpha_t$, are N-terminally modified with palmitate, a 16-carbon fatty acid. The $G\alpha_i$ class is additionally modified with myristate, a 14-carbon saturated fatty acid. These fatty acid modifications are important for membrane localization of the Gα subunits (McCudden et al., 2005). Each class of Gα subunits has well known and characterized cellular targets, known as effectors. The $G\alpha_s$ class stimulates the activity of AC, increasing the intracellular concentration of cyclic adenosine monophosphate (cAMP) (Ross and Gilman, 1977). The $G\alpha_i$ class, initially identified for its ability to inhibit AC activity (Hildebrandt and Birnbaumer, 1983), has a growing list of effectors that includes phosphodiesterases and phospholipases and are critical for sensory transduction (McCudden et al., 2005). The $G\alpha_q$ class activates PLC resulting in the subsequent generation of the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Rhee, 2001). Finally, the $G\alpha_{12/13}$ class stimulates the activity of the RhoGEFs (McCudden et al., 2005).

There is nearly as much diversity amongst the Gβ and Gγ subunits. Currently, there are 5 Gβ and 12 Gγ subunits, which creates the possibility for forming a large number of Gβγ heterodimers (McCudden et al., 2005). All Gγ

subunits are prenylated post-translationally, with either a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group added to a C-terminal CAAX motif. Similar to the Gα subunits, this modification is critical for the membrane localization of Gβγ. Gβγ is a functional heterodimer and is able to activate a number of effectors following dissociation from Gα. The first effectors identified were the G-protein-regulated inward-rectifier K^+ channels (GIRK or $K_{ir}3$ channels) (Logothetis et al., 1987). Since then, Gβγ has been shown to activate a number of other effectors including PLC-β and -ε (Boyer et al., 1992; Wing et al., 2001), phosphoinositide-3' kinase-γ (PI3Kγ) (Stephens et al., 1994), and various AC isoforms (Tang and Gilman, 1991).

GPCRs constitute one of the largest families of cell surface receptors and are critically involved in many aspects of biology. Currently, GPCRs are targeted by ~30% of marketed drugs (Jacoby et al., 2006) and as more and more GPCRs are found to be involved in various pathologies such as cancer, HIV, and cardiovascular disease, a better understanding of the molecular mechanisms regulating receptor activity and signaling is needed.

REGULATION OF GPCRs BY G PROTEIN-COUPLED RECEPTOR KINASES AND ARRESTINS

The activity of GPCRs is tightly regulated to ensure the proper magnitude and duration of signaling cascades within the cell. There are three principal modes of GPCR regulation: desensitization, where receptors become refractory to continued stimulation; internalization, a process that removes receptors from

the cell surface; and down regulation, where receptors are trafficked to lysosomes for degradation (Figure 2).

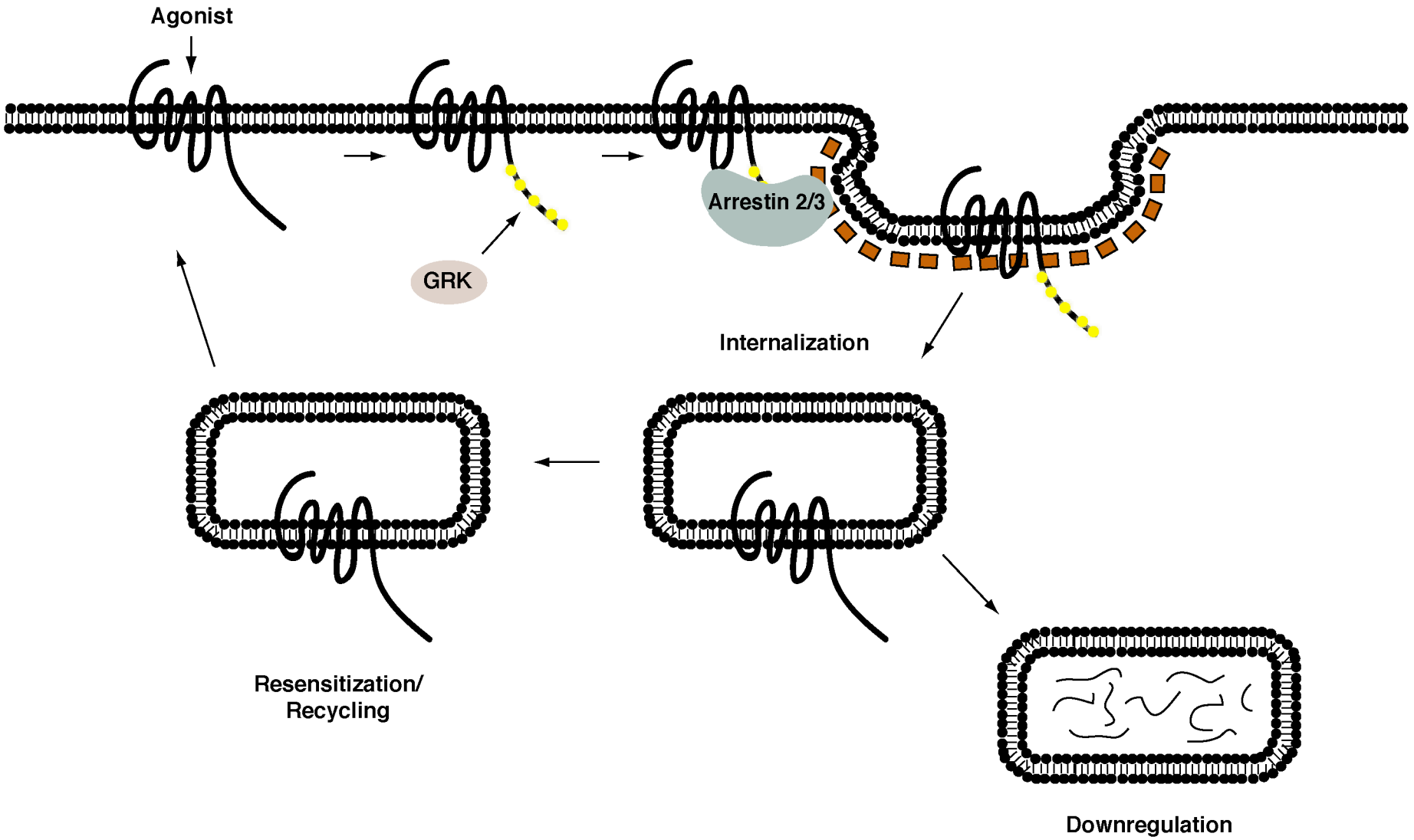
Homologous and Heterologous Desensitzation

Desensitization is a critical step in GPCR regulation, effectively shutting off receptor signaling. The process is initiated by phosphorylation of intracellular serine/threonine residues and is classified as either heterologous (agonist-independent) or homologous (agonist-dependent). Heterologous desensitization is typically mediated by the second messenger kinases, protein kinase A (PKA) and protein kinase C (PKC), which are activated by a number of cellular stimuli including receptor tyrosine kinases (RTKs) and GPCRs (Ferguson, 2001). Phosphorylation is therefore agonist-independent and results in a decreased affinity of the GPCR-G protein interaction. Homologous desensitization, on the other hand, is classically mediated by G protein-coupled receptor kinases (GRKs). Unlike second messenger kinases, GRKs specifically phosphorylate the activated, agonist-occupied form of the receptor and this usually results in the recruitment of the arrestin family of proteins (Krupnick and Benovic, 1998).

Figure 2. Regulation of GPCRs by GRKs and Arrestins

Upon receptor activation, GRKs phosphorylate serine/threonine residues of the third intracellular loop and C terminal tail, initiating the process of desensitization. Arrestins are then recruited to and bind the activated, phosphorylated receptor, uncoupling it from G protein and terminating signaling. Additionally, the arrestins act as adaptor proteins and complex with clathrin and AP-2 to target the receptors to clathrin-coated pits for internalization. In the endosomal compartment, ligand dissociates and the receptors are dephopshorylated. The receptors are then either sorted to the lysosome for degradation (down regulation) or recycled back to the plasma membrane (resensitization).

Regulation of GPCR Signaling and Trafficking



G Protein-Coupled Receptor Kinases

There are currently 7 members of the GRK family, which are divided into three subfamilies based on sequence homology: (i) GRK1 (rhodopsin kinase) (Shichi and Somers, 1978; Lorenz et al., 1991) and GRK7 (Weiss et al., 1998); (ii) GRK2 (β-adrenergic receptor kinase 1, βARK1) (Benovic et al., 1986; Benovic et al., 1989) and GRK3 (β-adrenergic receptor kinase 2, βARK2) (Benovic et al., 1991); and (iii) GRK4 (Ambrose et al., 1992), GRK5 (Kunapuli and Benovic, 1993), and GRK6 (Benovic and Gomez, 1993). GRK2, 3, 5 and 6 are ubiquitously expressed and have the ability to interact with and phosphorylate various GPCRs. Conversely, GRK1, 4, and 7 have a more restricted pattern of expression, being limited to the retina (GRK1 and 7) or the brain, testis, and kidneys (GRK4) (Krupnick and Benovic, 1998).

The overall topology of the GRKs is conserved among the subfamilies, with a moderately conserved N-terminal domain, a highly conserved central catalytic domain, and variable C-terminal domain (Krupnick and Benovic, 1998). The central catalytic domain promotes phosphorylation of a variety of substrates and is most closely related to the PKA/PKC family of kinases (Hanks and Hunter, 1995). The N-terminus of GRK is thought to not only mediate the interaction with substrates (Palczewski et al., 1991; Pronin et al., 1997), but also result in full activation of the kinase (Pao and Benovic, 2005). The GRKs also contain a RGS-like domain within the N terminus (Siderovski et al., 1996). However, to date, only GRK2 and 3 have been shown to bind to Gα subunits in an aluminum fluoride (AlF_4^-) dependent manner (Carman et al., 1999b). This interaction

occurs via a region that is unique to GRK2 and 3 (Sterne-Marr et al., 2003), and regulates $G\alpha_q$ without enhancing its GTPase activity (Carman et al., 1999b; Sallese et al., 2000), demonstrating a novel, phosphorylation-independent means of GPCR regulation. The N-terminus of the GRK4 family has a unique, highly conserved, phosphatidylinositol (4,5)-bisphosphate (PIP_2) binding site, which may function to enhance the catalytic activity of the kinases (Pitcher et al., 1996).

The most divergent region of the GRKs is the C-terminal domain, which is responsible for membrane localization (Penn et al., 2000). GRK1, 2, and 3 are predominantly cytoplasmic, needing to translocate to the membrane upon receptor activation. GRK1 is post-translationally modified by farnesylation of a C-terminal CAAX motif and recruited to the plasma membrane upon rhodopsin activation (Inglese et al., 1992). GRK2 and 3 contain both a pleckstrin homology (PH) domain and a $G\beta\gamma$ binding site (Pitcher et al., 1992; Pitcher et al., 1995). The concomitant interaction with PIP_2 and free $G\beta\gamma$ targets the kinases to the plasma membrane upon receptor activation. In contrast, GRK4 through GRK7 are constitutively associated with the plasma membrane. GRK4 and 6 maintain their plasma membrane localization through palmitoylation of one or more C-terminal cysteine residues (Premont et al., 1996; Stoffel et al., 1994). GRK5 is predominantly associated with the plasma membrane through electrostatic interactions between a polybasic region and membrane phospholipids (Pitcher et al., 1996). Finally, similar to GRK1, GRK7 contains a C-terminal CAAX motif. However, in contrast to GRK1, GRK7 is post-translationally modified with a

geranylgeranyl group and thereby constitutively associated with the plasma membrane (Weiss et al., 2001).

Regulation of GRK Activity

In addition to membrane recruitment and activation by GPCRs, the activity of the GRKs can be modulated by a number of intracellular molecules including phospholipids, site-specific phosphorylation, and protein/protein interactions (Penela et al., 2003). The GRKs are lipid-dependent enzymes. Interactions with PIP_2 and other acidic phospholipids (e.g., phosphatidylserine (PS)) enhances the ability of GRK2 to phosphorylate membrane-bound receptors as well as soluble substrates (DebBurman et al., 1996; DebBurman et al., 1995; Pitcher et al., 1995). Interestingly, PIP_2 also inhibited GRK2-mediated phosphorylation of agonist-occupied M2 muscarinic acetylcholine receptor if used at high micromolar concentrations or in the absence of $G\beta\gamma$ (DebBurman et al., 1996; DebBurman et al., 1995). GRK5 has two phospholipid binding domains, in the N- and C-terminus, which are highly conserved among the GRK4 subfamily of kinases. The N-terminal binding site shows a great deal of specificity for PIP_2, which enhances GRK5-mediated receptor phosphorylation without affecting autophosphorylation or phosphorylation of soluble substrates (Pitcher et al., 1996). The C-terminal binding site, which shows little lipid specificity, enhances GRK5 autophosphorylation and activity towards a number of different substrates (Kunapuli et al., 1994; Pronin et al., 1998).

A number of protein kinases are able to phosphorylate the GRKs, thereby modulating their cellular localization and catalytic activity. PKA-mediated

phosphorylation of GRK2 on Ser685 increases its affinity for Gβγ and enhances plasma membrane translocation and receptor phosphorylation (Cong et al., 2001). Similarly, PKC-mediated phosphorylation of GRK2 on Ser29 enhances membrane recruitment and receptor phosphorylation (Winstel et al., 1996). In contrast, the catalytic activity of GRK5 is drastically inhibited by PKC-mediated phosphorylation of two C-terminal serine residues (Pronin and Benovic, 1997a). Interestingly, this region of GRK5 also contains inhibitory autophosphorylation sites (Pronin et al., 1998). In addition to the second messenger kinases, GRK2 is phosphorylated by both c-Src and extracellular signal-regulated kinase (ERK). c-Src, a tyrosine kinase, phosphorylates and enhances the activity of GRK2 towards both receptor and soluble substrates (Fan et al., 2001; Sarnago et al., 1999). In addition, c-Src phosphorylation also plays a critical role in the degradation of GRK2 (Penela et al., 2001). In contrast, ERK phosphorylates GRK2 on Ser670 and impairs both Gβγ binding and catalytic activity (Pitcher et al., 1999).

The calcium binding proteins recoverin and calmodulin are also important regulators of the GRK activity. Recoverin, which is present in photoreceptor cells, is able to bind to and inhibit the activity of GRK1 (Klenchin et al., 1995). Calmodulin is able to inhibit the activity of GRK2, 3, 5, and 6, however, it is highly specific for GRK5 and 6 (Pronin et al., 1997b). The calmodulin binding sites have been mapped to both the N and C terminal domains of GRK5, which disrupts membrane association, substrate phosphorylation and enhances inhibitory autophosphorylation (Pronin et al., 1998; Pronin et al., 1997b). Finally,

the activity of the GRKs is inhibited through interactions with caveolin and α-actinin, which may be a way to suppress GRK activity in particular microdomains (Carman et al., 1999a; Freeman et al., 2000).

Arrestins

The arrestins are a family of proteins classically involved in shutting off signaling by binding to activated, phosphorylated GPCRs and uncoupling them from their cognate G protein (Krupnick and Benovic, 1998). However, their biological functions are ever expanding and the arrestins are now known to regulate receptor internalization, G protein independent signaling, and gene expression (DeWire et al., 2007; Moore et al., 2007).

In mammals, there are four members of the arrestin family that have been cloned and characterized. Based on function, localization and sequence homology, they are subdivided into two groups: the visual arrestins (arrestin1 and 4), and the non-visual arrestins (arrestin2 and 3, also known as β-arrestin1 and 2). Arrestin1, originally termed S-antigen, was found to translocate from the cytosol to rod outer segments (ROS) upon rhodopsin activation and regulate light dependent signal transduction (Kuhn et al., 1984; Pfister et al., 1985). Purification of arrestin1 allowed for the subsequent cloning of a bovine arrestin1 and of arrestin4 (Murakami et al., 1993; Shinohara et al., 1987; Yamaki et al., 1987). As arrestin1 and arrestin4 are restricted to the visual system, they are believed to exclusively regulate photoreceptor GPCRs (Krupnick and Benovic, 1998). The existence of a non-visual arrestin was initially postulated during studies examining the regulation of the β_2AR by GRK2 (Benovic et al., 1987).

The ability of GRK2 to desensitize purified β_2AR became attenuated as the GRK2 preparations became more homogenous, suggesting the loss of an important cofactor for β_2AR desensitization. Desensitization was restored by the addition of arrestin1, albeit at very high concentrations (Benovic et al., 1987). Subsequently, a protein termed β-arrestin (arrestin2) was cloned, which shared high homology and function to arrestin1 (Lohse et al., 1990). Unlike arrestin1, arrestin2 is not restricted to one particular tissue, but is ubiquitously expressed. Soon thereafter, a second ubiquitously expressed non-visual arrestin was cloned and termed β-arrestin2 (arrestin3) (Attramadal et al., 1992). The ubiquitous nature of arrestin2 and 3 suggest that they are involved in the regulation of a number of GPCRs.

Importantly, arrestins are able to recognize both the activation and phosphorylation state of GPCRs, suggesting that they recognize domains specifically exposed upon receptor activation. Studies have determined that the N terminal domain of arrestin is involved in receptor recognition of a number of GPCRs (Gurevich and Benovic, 1993a; Gurevich et al., 1995; Gurevich et al., 1993b). Extensive mutagenesis of arrestin1 has mapped the phosphorylation recognition domain to residues 158-185 (Gurevich and Benovic, 1993a). Subsequently, three residues (Arg171, Arg175, and Lys176) were found to be critical for the interaction with phosphorylated rhodopsin (Kieselbach et al., 1994). Interestingly, neutralization or charge reversal of Arg175 resulted in phosphorylation-independent binding to light activated rhodopsin, suggesting that this region acts as phospho-sensitive switch (Gurevich and Benovic, 1993a).

Mutation of the corresponding residue in arrestin2 (Arg169) to glutamate, resulted in a similar phosphorylation-independent binding to the β_2AR (Kovoor et al., 1999). Based on these and other studies, it is thought that arrestin undergoes a conformational change upon binding to activated, phosphorylated receptors exposing regions that allows them to interact with a number of other proteins (Gurevich and Gurevich, 2006).

The Role of Arrestins in Receptor Desensitization and Trafficking

The arrestins are classically known to shut off, or "arrest", GPCR-mediated signaling events through their ability to bind to activated, phosphorylated receptors and uncouple them from their cognate G protein (Krupnick and Benovic, 1998). Moreover, arrestins interact with cAMP phosphodiesterases (Perry et al., 2002) and diacylglycerol kinases (Nelson et al., 2007), allowing them to dually desensitize GPCRs via Gs and Gq uncoupling as well as enhancing the rate of cAMP degradation and PIP_2 regeneration, respectively.

Following desensitization, activated GPCRs are removed from the cell surface by a process known as internalization. The most common route for GPCR internalization is through specialized microdomains known as clathrin coated pits (CCPs), although other mechanisms do exist (Marchese et al., 2003a). A role for arrestin in receptor internalization was first demonstrated in studies using a mutated β_2AR that was impaired in agonist induced phosphorylation and internalization (Ferguson et al., 1996). Overexpression of either arrestin2 or 3 was able to promote internalization of this receptor whereas

various arrestin mutants inhibited internalization of the β_2AR. Mechanistic insight into this arrestin-mediated internalization was gained from the observations that arrestin2 and 3 directly interact with clathrin (Goodman et al., 1996), clathrin associated protein (AP)-2 (Laporte et al., 2000; Kim and Benovic, 2002), and phosphoinositides, which is critical for targeting GPCRs to CCPs (Gaidarov et al., 1999).

Once internalized, receptors can either be recycled to the plasma membrane in a process known as resensitization or trafficked to the lysosomes in a process known as down regulation (Marchese et al., 2008). While the molecular mechanisms mediating differential sorting of GPCRs are not well established, the stability of the GPCR/arrestin complex may be a contributing factor. For example, GPCRs have been separated into “Class A” and “Class B” receptors based on how they interact with the non-visual arrestins (Oakley et al., 2001). Class A receptors (e.g., β_2AR) transiently associate with the non-visual arrestins, which may allow for rapid dephopshorylation and recycling back to the plasma membrane, essentially resetting the system. Class B receptors (e.g., angiotensin 1a receptor (AT1aR)) form stable complexes with the non-visual arrestins and co-traffic into endosomes. This stable association has been proposed to sterically hinder dephosphorylation and prevent receptor resensitization (Oakley et al., 2001). Additionally, arrestin2 has been shown to be critical for the lysosomal sorting of CXCR4 (Bhandari et al., 2007), a GPCR known to undergo agonist-induced down regulation (Marchese and Benovic, 2001).

The Role of Arrestins in GPCR Signaling

Studies over the past several years have elucidated a novel role for the arrestins: acting as scaffolds for activation of a number of signaling pathways, also known as G protein-independent signaling. Initial evidence for arrestin-mediated signaling came from results showing that a dominant negative form of arrestin, which blocks internalization, inhibited full activation of ERK1/2 (Daaka et al., 1998). Soon thereafter, arrestin was shown to interact with c-Src, linking this non-receptor tyrosine kinase to GPCR-mediated signaling (DeFea et al., 2000a; Luttrell et al., 1999). Subsequent studies revealed that arrestin was able to scaffold specific components of the mitogen activated protein kinase (MAPK) cascade including Raf-1, MEK1, ERK1/2 (DeFea et al., 2000b; Luttrell et al., 2001) and JNK3 (McDonald et al., 2000). Though no direct interaction has ever been established, p38 activation has been shown to be arrestin dependent following activation of a number of different GPCRs (Bruchas et al., 2006; Miller et al., 2003; Sun et al., 2002).

Certain ligands appear to have the ability to preferentially activate a particular downstream signaling pathway, a phenomenon known as biased agonism. For GPCRs, this would mean preferential activation of either a G protein-dependent or –independent pathway. This was first identified for the AT1aR using a specific mutant of the ligand angiotensin, known as AngII(SII). This mutant ligand was unable to activate G protein-dependent signaling (i.e., phosphoinositide (PI) hydrolysis) but was able to activate ERK1/2 in an arrestin-dependent manner (Wei et al., 2003). Similar mechanisms have been uncovered

for both the vasopressin and β2 adrenergic receptors (Azzi et al., 2003; Drake et al., 2008; Wisler et al., 2007).

THE CHEMOKINE RECEPTOR CXCR4

Chemokines are 8-10 kDa cytokines that are classified into four groups (CXC, CC, C, and CX3C) based on the position of the first two cysteines (Zlotnik and Yoshie, 2000). Chemokine receptors belong to the GPCR superfamily and couple to the pertussis toxin sensitive G_i proteins (Murphy et al., 2000). In general, chemokines/chemokine receptors exhibit promiscuity, being able to bind multiple receptors/ligands, though 6 of the 18 chemokine receptors bind a single ligand (Balkwill, 2004a). One of the best studied chemokine receptors is CXCR4, primarily due to its role as a co-receptor for HIV entry (Feng et al., 1996) as well as its ability to mediate the metastasis of a variety of cancers (Zlotnik, 2006b).

CXCR4 is a 352 amino acid rhodopsin-like GPCR and selectively binds the CXC chemokine Stromal Cell-Derived Factor 1 (SDF-1) also known as CXCL12 (Fredriksson et al., 2003; Murphy et al., 2000). Classically, two alternatively spliced isoforms of SDF have been identified. SDF-1α is an 89 amino acid protein that is the predominantly expressed form of SDF-1 while SDF-1β contains a four amino acid extension at the carboxyl terminus (Shirozu et al., 1995). SDF-1α and β bind to CXCR4 with a comparable affinity (K_d of 7.5 and 13.7 nM, respectively) (Hesselgesser et al., 1998). Recently, an additional four splice variants that contain 30 (SDF-1γ), 31 (SDF-1δ), 1 (SDF-1ε), and 51 (SDF-1ϕ) amino acid extensions at the carboxyl terminus compared to SDF-1α have

been identified (Yu et al., 2006). Each of these isoforms stimulates cell migration in a CXCR4-dependent manner. However, as they have a differential tissue distribution, their functional significance is currently unknown. Mice that lack either SDF-1 or CXCR4 exhibit an almost identical phenotype of late gestational lethality and defects in B cell lymphopoiesis, bone marrow colonization, and cardiac septum formation (Nagasawa et al., 1996; Zou et al., 1998). These and other studies reveal that CXCR4 is essential for development, hematopoeisis, organogenesis, and vascularization (Ma et al., 1998; McGrath et al., 1999; Nagasawa et al., 1996; Nagasawa et al., 1998; Tachibana et al., 1998; Zou et al., 1998), in addition to functioning as a classical chemokine receptor (i.e., directed chemotaxis) in the adult (Moser and Loetscher, 2001; Murphy, 1994).

Given that CXCR4 plays a prominent role in HIV (Agrawal et al., 2006; Lusso, 2006; Reeves and Piefer, 2005) and cancer metastasis (Burger and Kipps, 2006; Kucia et al., 2005; Zlotnik, 2006a; Zlotnik, 2006b), the knowledge of the factors that shape signaling, receptor regulation, and receptor expression, and how dysregulation of these pathways may contribute to disease progression is crucial.

REGULATION OF CXCR4 EXPRESSION AND FUNCTION

Transcriptional Control of CXCR4

In order to understand the role of CXCR4 in disease, a fundamental understanding of the factors regulating expression is critical. While CXCR4 was initially cloned from leukocytes (Loetscher et al., 1994; Nomura et al., 1993), it

has since been shown to be expressed in a number of tissues in addition to cells of hematopoetic lineages (Rossi and Zlotnik, 2000). The promoter region of CXCR4 contains a number of predicted regulatory consensus sequences (Caruz et al., 1998; Moriuchi et al., 1997; Wegner et al., 1998), however, the basal transcription is mainly controlled by the opposing actions of two transcriptional regulators. Functional characterization of the CXCR4 promoter has revealed that Nuclear Respiratory Factor-1 (NRF-1) is the major transcription factor positively regulating the transcription of CXCR4 (Moriuchi et al., 1997; Wegner et al., 1998), although a potential role for an additional transcription factor, SP-1, has also been suggested (Wegner et al., 1998). This work also defined a negative regulatory element upstream (near position –300 of the transcriptional start site) that may be mediated by Ying Yang 1 (YY1) (Moriuchi et al., 1999b).

In addition to the basal regulation of CXCR4 transcription, a number of signaling molecules also have been shown to affect CXCR4 transcription. For example, the expression of CXCR4 can be increased as a result of intracellular second messengers such as calcium (Moriuchi et al., 1997) and cyclic AMP (Cristillo et al., 2002), by the cytokines interleukin-2 (IL-2) (Moriuchi et al., 1997), IL-4 (Jourdan et al., 2000), IL-7 (Jourdan et al., 2000), IL-10 (Wang et al., 2001), IL-15 (Jourdan et al., 2000), TGF-1β (Wang et al., 2001), and simultaneous CD3 and CD28 engagement (Moriuchi et al., 1997), and by growth factors such as basic fibroblast growth factor (bFGF) (Feil and Augustin, 1998; Salcedo et al., 1999), vascular endothelial growth factor (VEGF) (Salcedo et al., 1999), and epidermal growth factor (EGF) (Phillips et al., 2005). On the other hand,

inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (Feil and Augustin, 1998; Gupta et al., 1998; Han et al., 2001), interferon-γ (INF-γ) (Gupta et al., 1998), and IL-1β (Gupta et al., 1998) have all been shown to attenuate CXCR4 expression.

These data clearly show that there is dynamic regulation of CXCR4 transcription as the result of physiological stimuli. Of additional interest are those factors that regulate CXCR4 expression and affect disease progression, such as modulating HIV infection. Alterations in NRF-1 or YY1 activity can lead to an increase or decrease in transcription of CXCR4, respectively, which certain viruses appear to have taken advantage of. The human T lymphotropic virus type I transactivator Tax protein interacts with and enhances NRF-1 activity, which in infected individuals may enhance susceptibility to HIV infection or disease progression (Moriuchi et al., 1999a). In contrast, individuals infected with human herpes virus 6 have a decrease in cell surface expression of CXCR4 (Yasukawa et al., 1999). Investigation into the underlying mechanism has revealed that there is an increase in YY1 binding through a decreased association with c-Myc, a natural suppressor of YY1 activity (Hasegawa et al., 2001).

Regulation of CXCR4 Protein Expression

A number of co-translational modifications contribute to the expression and function of CXCR4. Within the extracellular domain of CXCR4, there are two potential N-linked glycosylation sites, Asn11 and Asn176 (Berson et al., 1996). Both sites undergo glycosylation when CXCR4 is expressed in Sf9 insect cells (Zhou and Tai, 1999), however, only Asn11 appears to be glycosylated in

mammalian cells (Chabot et al., 2000). SDF and the HIV-1 glycoprotein gp120 bind to a non-overlapping region of the N-terminus of CXCR4 (Brelot et al., 2000; Brelot et al., 1997; Chabot et al., 1999; Doranz et al., 1999; Kajumo et al., 2000; Zhou et al., 2001) and glycosylation has opposing effects on each process. Mutation of Asn11 to glutamine leads to enhanced CD4-dependent binding of both CXCR4-specific and duel tropic (CCR5 and CXCR4) HIV-1 isolates (Brelot et al., 1997; Thordsen et al., 2002; Wang et al., 2004). Conversely, mutation of Asn11 to glutamine (Wang et al., 2004) or leucine (Zhou and Tai, 1999) disrupts SDF binding and diminishes signal transduction (Wang et al., 2004). Thus, glycosylation of CXCR4 is important for SDF binding and helps to inhibit the use of CXCR4 as an HIV-1 co-receptor.

CXCR4 has also been shown to undergo tyrosine sulfation, a modification catalyzed by tyrosyl protein sulfotransferase within the trans-golgi network. There are three extracellular tyrosines in CXCR4 that are modified by sulfation, Tyr7, Tyr12, and Tyr21, with Tyr21 accounting for the majority of sulfate incorporation (Farzan et al., 2002a). Functionally, tyrosine sulfation of CXCR4 doesn't regulate co-receptor usage by HIV-1 (Farzan et al., 2002a) as is observed with CCR5 (Farzan et al., 1999), however, similar to CCR2b (Preobrazhensky et al., 2000), CCR5 (Farzan et al., 2002b), and CX_3CR1 (Fong et al., 2002a), this is an important modification for ligand binding (Farzan et al., 2002a). Indeed, the structural basis for sulfotyrosine-SDF interaction reveals that sulfotyrosine 21 binds to a specific site on SDF-1 that includes Arg47, while sulfotyrosine 7 and 12 occupy positively charged clefts of a SDF homodimer

(Veldkamp et al., 2008; Veldkamp et al., 2006). An additional N-terminal modification that has been identified in CXCR4 is addition of a chondroitin sulfate chain at serine 18, although no functional consequence of this modification has been identified (Farzan et al., 2002a).

Oligomerization

An emerging theme in GPCR signaling is the formation of homo- and heterodimers (Angers et al., 2002). CXCR4 exhibits significant heterogeneity in cells, which may be a result of ubiquitination, differential glycosylation, or the formation of oligomers (Lapham et al., 2002; Sloane et al., 2005). It's been suggested that CXCR4 has the ability to homodimerize in the absence of ligand (Babcock et al., 2003; Issafras et al., 2002; Percherancier et al., 2005; Toth et al., 2004), an event that most likely occurs soon after protein translation (Babcock et al., 2003). However, two reports suggest that SDF can also enhance dimerization (Toth et al., 2004; Vila-Coro et al., 1999). Interestingly, CXCR4 homodimers have been suggested to form between wild type and C terminally truncated mutations of CXCR4 in patients with WHIM syndrome, effectively enhancing the activity of the wild type receptor (Lagane et al., 2008). There have also been reports of CXCR4 forming heterodimers with CCR2, T cell receptors, and CD4, which may affect the functionality of CXCR4 as a co-receptor for HIV (Basmaciogullari et al., 2006; Kumar et al., 2006; Mellado et al., 1999; Percherancier et al., 2005; Rodriguez-Frade et al., 2004; Toth et al., 2004). Recently, heterodimerization between CXCR4 and the delta opioid receptor in immune cells has been shown to functionally inactivate each receptor, providing

another layer of potential regulation of GPCR activity (Pello et al., 2008). Finally, while some studies suggest that CXCR4 does not heterodimerize with CCR5 (Babcock et al., 2003; Issafras et al., 2002), CD4+ cells isolated from patients with a CCR5Δ32 mutant, a loss-of function mutation that prevents cell surface expression of CCR5, have reduced expression of CXCR4 (Agrawal et al., 2004). Moreover, these studies show that CCR5Δ32 and CXCR4 can interact resulting in reduced cell surface expression of CXCR4 and enhanced resistance to HIV infection (Agrawal et al., 2004). More recently, it has been shown that CXCR4 and CCR5 form heterodimers at the immunological synapse, having a specific function and affecting T cell responses to antigen (Contento et al., 2008).

The functional consequences of homo- or heterodimerization of GPCRs are currently not well understood. However, it has been suggested that homodimerization of CXCR4 is necessary to elicit G protein independent activation of JAK/STAT as well as enhance the response of CXCR4 to SDF (see below). Heterodimerization may be a means of achieving an additional level of regulation. For example, it has recently been proposed that non-agonist occupied CCR5 may be phosphorylated by GRK2 activated as a result of heterodimer formation and activation of C5a (Huttenrauch et al., 2005). Taken together, homo- and hetero-oligomerization of CXCR4 may be a way of regulating signaling while also allowing for alternative, non-classical, signaling pathways upon activation.

REGULATION OF CXCR4 SIGNALING

SDF Binding

The interaction between SDF and CXCR4 has been proposed to occur through a two-step process (Crump et al., 1997). The initial interaction between residues 12-17 of SDF and 2-36 of CXCR4 are believed to result in a conformational change in the receptor (Huang et al., 2003). This conformational change facilitates interaction between the first eight amino acids of SDF and an exposed binding pocket in CXCR4 that involves residues in both the second (Asp187) and third (Glu268) extracellular loops (Brelot et al., 2000; Zhou et al., 2001). As this interaction requires the integrity of both SDF and CXCR4, it is not surprising that proteases are able to inhibit this interaction. During an inflammatory response, neutrophil released cathepsin G and neutrophil elastase have the ability to inactivate SDF by cleaving the N-terminal residues necessary for interacting with CXCR4 (Delgado et al., 2001; Valenzuela-Fernandez et al., 2002). Additionally, the widely expressed cell surface protease dipeptidase 26 (CD26) is also able to cleave and inactivate SDF (Christopherson et al., 2002; Huhn et al., 2000; Lambeir et al., 2001). To date, only neutrophil elastase has been shown to cleave the N terminal domain of CXCR4, effectively disrupting interaction with SDF (Valenzuela-Fernandez et al., 2002). Therefore, inflammatory responses promote the release of factors that positively and negatively regulate the receptor. When taken together, these data highlight the exquisite interplay between a variety of factors that are able to shape and

influence the SDF-CXCR4 signaling axis, ensuring that the proper physiological response is elicited.

SDF-1 is also able to interact with glycosaminoglycans, such as heparin sulfate, and is most likely immobilized in vivo allowing for gradient formation (Hoogewerf et al., 1997; Tanaka et al., 1993). Furthermore, this association may induce the oligomerization of SDF-1 (Sadir et al., 2001), a phenomenon observed at high SDF-1 concentrations (Crump et al., 1997; Dealwis et al., 1998; Fernandez and Lolis, 2002; Holmes et al., 2001), that may promote CXCR4 oligomerization and enhanced function. In fact, it has been shown that the combination of glycosaminoglycans and SDF-1 enhanced migration when compared to SDF alone (Netelenbos et al., 2002). Moreover, SDF-1 mediated inhibition of HIV X4 isolates was enhanced in the presence of heparin sulfate (Valenzuela-Fernandez et al., 2001). Interestingly, it has been recently shown that a constitutive homodimer of SDF-1 completely inhibits CXCR4-mediated chemotaxis without affecting calcium mobilization, suggesting that SDF dimerization may preferentially activate certain signaling pathways (Veldkamp et al., 2008).

It may also be possible to sensitize CXCR4 to have a greater response to lower SDF-1 concentrations. Recent evidence suggests that products released during inflammatory responses (Majka et al., 2000) or platelet activation (Janowska-Wieczorek et al., 2001; Wysoczynski et al., 2005) “prime” the SDF response enhancing hematopoetic stem/progenitor cell migration at lower SDF concentrations (Janowska-Wieczorek et al., 2001; Majka et al., 2000;

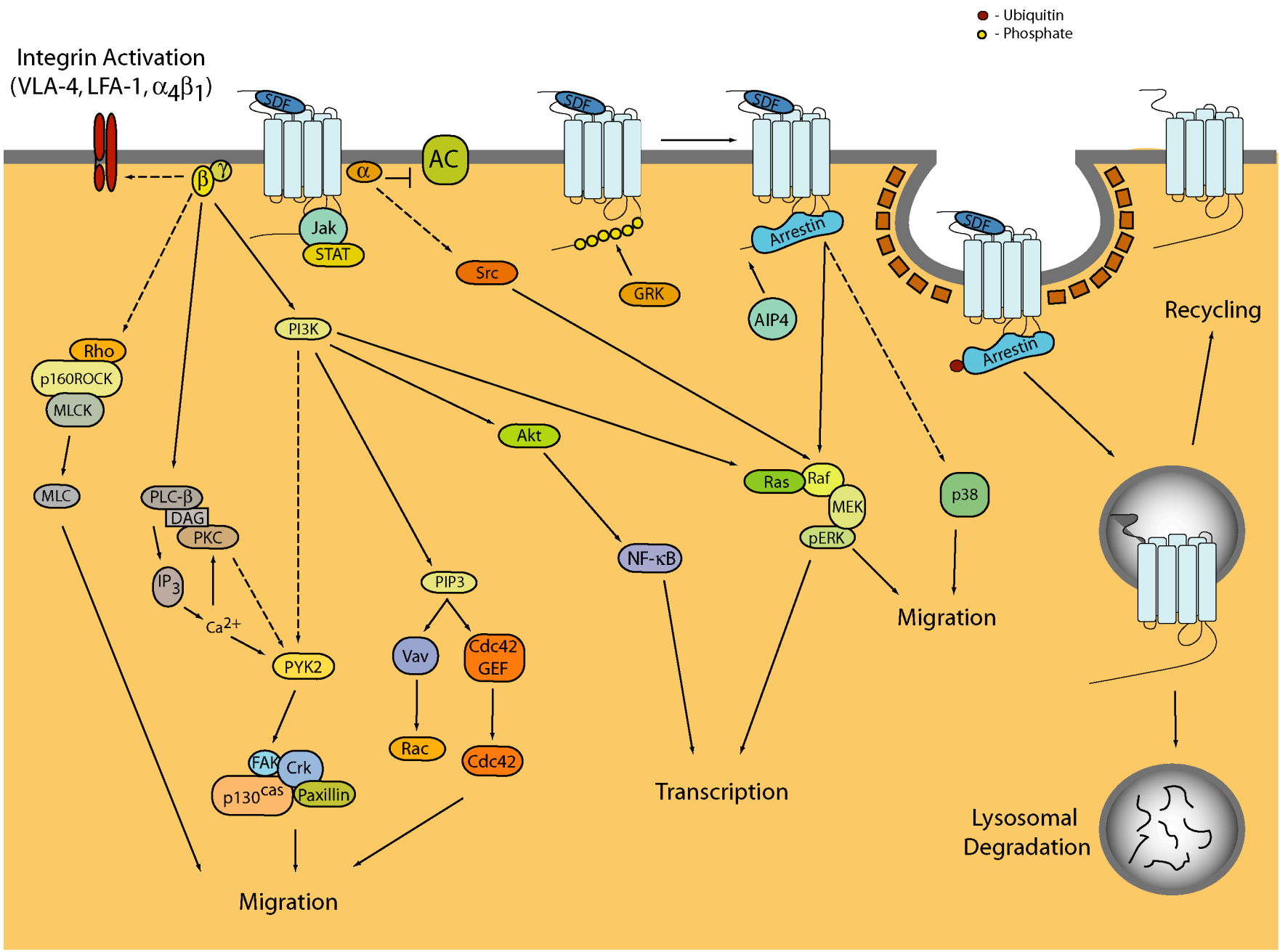
Wysoczynski et al., 2005). This phenomenon may be the result of changing the membrane localization of CXCR4 through incorporation into lipid rafts (Wysoczynski et al., 2005). A number of studies have suggested that lipid raft localization is required for proper function of CXCR4 (Le et al., 2005; Nguyen et al., 2005; Nguyen and Taub, 2002) and recently it has been shown that SDF stimulation promotes the incorporation of Src tyrosine kinases, focal adhesion kinase, PI3 kinase and the small G protein Rac into lipid rafts (Wysoczynski et al., 2005). This agonist promoted clustering of receptor and effectors into lipid rafts might be a way of ensuring that the proper signaling pathways are activated.

G Protein Signaling

Upon activation of CXCR4, a number of signaling pathways are activated leading to a variety of biological responses (Figure 3) (Kucia et al., 2004). As CXCR4 couples to the G_i family of proteins, the use of pertussis toxin (PTX), which ADP-ribosylates $G\alpha_i$ and inhibits GPCR/G_i coupling, is a useful tool to delineate pathways that are G protein-dependent and -independent. To date, the majority of signaling pathways and biological outcomes of CXCR4 activation are PTX-sensitive and therefore dependent on activation of G_i proteins. Activated G_i is able to inhibit adenylyl cyclase as well as activate the Src family of tyrosine kinases while liberated $G\beta\gamma$ recruites GRK2/3 to the plasma membrane and activates PLC-β and phosphoinositide-3 kinase (PI3K) ultimately leading to the regulation of processes such as gene transcription, cell migration, and cell adhesion (Figure 3).

Figure 3. Signal transduction pathways and regulation of CXCR4

SDF binding to CXCR4 leads to the activation of multiple G protein-dependent signaling pathways, resulting in diverse biological outcomes such as migration, adhesion, and transcriptional activation. Pathways activated and outcomes elicited may differ between CXCR4 + cell types. Two potential G protein-independent pathways have been described. Tyrosine phosphorylation of CXCR4 results in the recruitment and activation of the JAK/STAT pathway, while p38 and ERK activation has been shown to be partially dependent on arrestin3. Following activation, GRK phosphorylation results in the recruitment of arrestin2/3 and subsequent internalization. CXCR4 is also ubiquitinated by AIP4 at the plasma membrane, which results in its sorting to and degradation in lysosomes. However, a portion of the internalized receptor may also recycle back to the plasma membrane. Adapted from Busillo, J.M. and Benovic, J.L. (2007) Regulation of CXCR4 signaling. *Biochim Biophys Acta,* **1768**, 952-963.


- Ubiquitin
- Phosphate
Integrin Activation
(VLA-4, LFA-1, $\alpha_4\beta_1$)
SDF
Jak
STAT
AC
α
β
γ
Src
GRK
Arrestin
AIP4
Recycling
Rho
p160ROCK
MLCK
MLC
PI3K
Akt
PLC-β
DAG
PKC
IP_3
Ca^{2+}
PYK2
PIP3
Vav
Cdc42 GEF
Rac
Cdc42
FAK
Crk
$p130^{cas}$
Paxillin
Ras
Raf
MEK
pERK
p38
NF-κB
Migration
Transcription
Lysosomal Degradation
Migration

G Protein Independent Signaling

Activation of the JAK/STAT pathway by CXCR4 has been proposed to be G protein independent (Vila-Coro et al., 1999). SDF induced the transient association of JAK2 and JAK3 with CXCR4, leading to the activation and nuclear translocation of a number of STAT proteins. While JAK/STAT activation was G protein-independent, pretreatment with PTX led to a prolonged association of JAK with CXCR4 suggesting that G protein coupling is involved in JAK/STAT-receptor complex recycling (Vila-Coro et al., 1999).

The non-visual arrestins have classically been considered to shut off signal transduction following receptor activation, a process termed desensitization (Krupnick and Benovic, 1998). Indeed, lymphocytes isolated from arrestin3 knock out mice display attenuated desensitization and enhanced G protein coupling of CXCR4 (Fong et al., 2002b). However, these mice also display a decreased chemotactic response to SDF, possibly due to the ability of arrestin3 to promote signaling (Fong et al., 2002b). In addition to signal termination, arrestins are able to act as scaffolds for a number of signaling molecules (DeFea et al., 2000b; Luttrell et al., 1999). These interactions may serve to propagate signaling or even create a platform to allow for activation of the proper signaling cascade (Lefkowitz and Shenoy, 2005). Consistent with these observations, it has been reported that arrestin2 and 3 enhance CXCR4-mediated ERK activation (Cheng et al., 2000) and arrestin3 is involved in p38 activation and migration following SDF stimulation (Sun et al., 2002). Taken

together, non-visual arrestins may play a role in regulating CXCR4/G_i interaction as well as SDF-promoted signaling and cell migration.

Regulation of Signaling

As described earlier, three processes primarily regulate GPCRs: desensitization (homologous and heterologous), internalization, and degradation. The process of homologous desensitization, or becoming refractory to continued stimulation, is initiated by GRK phosphorylation of serine/threonine residues of the third intracellular loop or cytoplasmic tail (C-tail) following receptor activation (Krupnick and Benovic, 1998). This phosphorylation allows for the subsequent binding of arrestin2 and/or arrestin3, effectively uncoupling the receptor from further G protein activation and often targeting the receptor for internalization (Krupnick and Benovic, 1998).

Upon SDF activation, CXCR4 is rapidly phosphorylated and internalized (Haribabu et al., 1997; Orsini et al., 1999; Signoret et al., 1997; Signoret et al., 1998). Removing the 45 amino acid C-tail of CXCR4, which contains 15 serine and 3 threonine residues, eliminates agonist-promoted phosphorylation (Haribabu et al., 1997), enhances receptor activity, and attenuates receptor internalization (Signoret et al., 1997). Truncation and alanine scanning mutagenesis has suggested multiple regions in the CXCR4 C-tail as potential phospho-acceptor sites (Orsini et al., 1999; Signoret et al., 1998). Mutation of Ser338 and Ser339 resulted in reduced SDF-promoted phosphorylation of CXCR4 as did truncation of the C-terminal 7 amino acids, which removes serines 346, 347, 348, 351, and 352 (Orsini et al., 1999). Recently, a phospho-specific

antibody directed against phospho-Ser339 also revealed increased phosphorylation of Ser339 following SDF stimulation (Woerner et al., 2005). Interestingly, increased phosphorylation of Ser339 was also observed following EGF or phorbol ester treatment (Woerner et al., 2005), suggesting that this may be a potential PKC phosphorylation site. To date, the GRKs responsible for phosphorylation of CXCR4 have not been identified, although GRK2 (Cheng et al., 2000; Orsini et al., 1999), GRK3 (Balabanian et al., 2008), and GRK6 (Fong et al., 2002b; Vroon et al., 2004) have been implicated. Overexpression of GRK2 was able to enhance SDF-mediated internalization of CXCR4, which was further increased by the co-expression of arrestin3 (Cheng et al., 2000; Orsini et al., 1999). Interestingly, GRK2 has also been suggested to negatively regulate CXCR4 signal transduction at a level downstream of the receptor, possibly via interaction with MEK (Jimenez-Sainz et al., 2006). Overexpression of GRK3 was shown to restore internalization of wild type, but not of a CXCR4 mutant lacking the last 15 amino acids in fibroblast cells (Balabanian et al., 2008). Accordingly, siRNA-mediated knock down of GRK3, but not GRK2, led to a reduction in CXCR4 internalization. Furthermore, overexpression of GRK3 was able to restore normal CXCR4-mediated G protein activation and migration in cells isolated from patients diagnosed with WHIM syndrome despite expressing wild type CXCR4 (Balabanian et al., 2008). Lymphocytes and neutrophils isolated from mice with a targeted disruption of GRK6 showed enhanced CXCR4 function and a lack of desensitization (Fong et al., 2002b; Vroon et al., 2004), which was not seen in cells isolated from mice lacking GRK5 (Fong et al., 2002b). These

data suggest that there may be multiple kinases regulating CXCR4 in response to SDF stimulation. As has recently been suggested for the angiotensin receptor (Ahn et al., 2004; Kim et al., 2005), vasopressin receptor (Ren et al., 2005), and β_2AR (Shenoy et al., 2006; Violin et al., 2006), the coordinated action of these kinases may be necessary for proper receptor regulation by dictating specific interactions through alternative phosphorylation patterns.

Many GPCRs also undergo a process termed heterologous desensitization, which is mediated by the activation of second messenger dependent protein kinases such as PKA and PKC. Sequence analysis of CXCR4 shows that multiple serines in the C-tail are potential PKC phosphorylation sites. Consistent with this, direct activation of PKC using phorbol esters results in phosphorylation (Haribabu et al., 1997) and internalization (Orsini et al., 1999; Signoret et al., 1997; Signoret et al., 1998) of CXCR4. Although the sites of phorbol ester induced phosphorylation of CXCR4 have not been completely determined, a significant decrease in phorbol ester induced internalization was observed when either Ser324 and Ser325 or Ser338 and Ser339 were mutated (Signoret et al., 1998) while phorbol ester treatment induced phosphorylation of Ser339 (Woerner et al., 2005). More physiologically relevant stimuli that lead to PKC activation such as T or B cell receptor engagement (Guinamard et al., 1999; Peacock and Jirik, 1999), formyl peptide receptor activation (Li et al., 2001; Selleri et al., 2005), CXCR1 activation (Richardson et al., 2003), CXCR2 activation (Suratt et al., 2004), or CCR5 activation (Hecht et al., 2003) are also able to induce CXCR4 internalization. Interestingly, T cells overexpressing the

HIV-1 protein Tat, had enhanced SDF-mediated internalization of CXCR4 that was attributed to activation of the atypical PKC, PKCζ (Hidalgo-Estevez et al., 2008). The functional significance of this is currently unknown, though it may affect homing of memory T cells, which have been shown to express CXCR4 (Nanki et al., 2000).

Phosphorylation of tyrosine residues in CXCR4 has also been observed following both SDF (Vila-Coro et al., 1999) and cytokine activation (Wang et al., 2001), although the residues that are phosphorylated are currently unknown. SDF-promoted tyrosine phosphorylation may promote the activation of the JAK/STAT pathway (Vila-Coro et al., 1999; Zhang et al., 2001), while cytokine-induced tyrosine phosphorylation may be a way of promoting ligand-independent internalization of CXCR4 (Wang et al., 2001).

Internalization and Degradation

As outlined above, the non-visual arrestins also act as adaptor proteins and, through their interaction with clathrin and AP-2, target GPCRs to CCPs for internalization (Moore et al., 2007). CXCR4 is thought to internalize in an arrestin- and clathrin-dependent manner (Cheng et al., 2000; Marchese et al., 2003b; Orsini et al., 1999). Moreover, mutation of potential phospho-acceptor sites has identified regions important for constitutive (Futahashi et al., 2007) and heterologous or homologous internalization of CXCR4 (Orsini et al., 1999; Signoret et al., 1998) (Figure 4).

Figure 4. Amino acid sequence of the C terminal tail of CXCR4

The C terminal tail of CXCR4 contains 15 serine and 3 threonine residues. Truncation and alanine scanning mutagenesis has identified multiple residues as potential phospho-acceptor sites (highlighted in yellow) as well as those residues important for degradation (highlighted in red). Evidence to date suggests that multiple GRKs are responsible for homologous desensitization of CXCR4. Additionally, multiple residues are potential PKC phosphorylation sites.

S324/5A - Reduces PKC-mediated and SDF-promoted internalization

S324/5A - Eliminates SDF-promoted degradation

K327/331/334R - Eliminates SDF-promoted degradation

S330A - 50% reduction in degradation with no effect on internalization

S338/9A - Reduces SDF-promoted internalization and phosphorylation

S341/Stop - Reduces SDF-promoted internalization and phosphorylation

Upon internalization, GPCRs can be recycled back to the plasma membrane or sorted to the lysosome for degradation (Marchese et al., 2003a). CXCR4 can recycle back to the plasma membrane following PKC-mediated internalization (Signoret et al., 1997), however, the receptor recycles poorly following SDF stimulation (Tarasova et al., 1998). In fact, CXCR4 has been shown to be ubiquitinated, sorted to the lysosome, and degraded (Marchese and Benovic, 2001), a process mediated by the E3 ubiquitin ligase AIP4 (Marchese et al., 2003b). Based on electrophoretic mobility shift, the receptor is most likely mono-ubiquitinated on one of three lysines residues (Lys327, Lys331, or Lys333) in the C-tail. Mutation of these three residues to arginine eliminates ubiquitination and degradation of the receptor (Marchese and Benovic, 2001). Interestingly, mutation of Ser330 to alanine partially inhibited degradation of CXCR4 without affecting receptor internalization while mutation of Ser324 and Ser325 partially inhibited SDF-promoted internalization but completely disrupted degradation (Marchese and Benovic, 2001). Ubiquitination of CXCR4 occurs at the cell surface and the rate limiting step for degradation appears to be sorting to the lysosome for degradation (Marchese et al., 2003b). Interestingly, recent evidence has shown that arrestin2 mediates sorting of CXCR4 into the degradative pathway (Bhandari et al., 2007). Taken together, these data suggest that phosphorylation of specific residues may dictate the fate of the receptor following internalization.

CXCR4 DYSREGULATION IN DISEASE

WHIM Syndrome

Heterozygous mutations in the gene encoding CXCR4 leads to a rare combined immunodeficiency characterized by warts, hypogammaglobulinemia, recurrent bacterial infection, and myelokathexis, known as WHIM syndrome (Diaz and Gulino, 2005; Gulino, 2003). WHIM syndrome is currently the only immunological disease associated with mutations to a chemokine receptor (Diaz and Gulino, 2005). The mutations identified to date (one frameshift and three nonsense mutations) all truncate the C-terminal tail of CXCR4 (Figure 5) eliminating 10 to 19 of the distal tail amino acids, including a number of potential phosphorylation sites (Gulino et al., 2004; Hernandez et al., 2003). This leads to the expression of a receptor with altered regulation. Following activation, there is a lack of desensitization (Balabanian et al., 2005; Gulino et al., 2004), enhanced chemotaxis (Gulino et al., 2004; Kawai et al., 2005), an increase in F- actin polymerization (Balabanian et al., 2005), enhanced calcium mobilization (Kawai et al., 2005), and a decrease in SDF promoted internalization (Balabanian et al., 2005; Kawai et al., 2005), although one report found no difference in calcium mobilization or internalization (Gulino et al., 2004).

Figure 5. Amino acid sequence of the C terminal tail of CXCR4 and known WHIM syndrome mutations

Amino acid sequence of CXCR4 as a result of the various germline mutations identified to date resulting in WHIM syndrome. These C terminal truncations result in expression of a receptor with altered regulation. In addition to these identified mutations, two patients have been identified with WHIM syndrome despite expressing wild type CXCR4, suggesting the loss of a downstream regulatory protein.


308
320
330
340
350
WT CXCR4 KFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS
R334X KFKTSAQHALTSVSRGSSLKILSKGK (Δ19)
G336X KFKTSAQHALTSVSRGSSLKILSKGKRG (Δ17)
S338X KFKTSAQHALTSVSRGSSLKILSKGKRGGHS (Δ14)
S339fs342X KFKTSAQHALTSVSRGSSLKILSKGKRGGHSCFH (Δ11)
E343X KFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVST (Δ10)

Interestingly, WHIM syndrome has been reported in two patients expressing a wild type CXCR4 (Balabanian et al., 2005). Functional assays using cells isolated from these patients revealed that, consistent with classical WHIM cases, there was a lack of desensitization and internalization of CXCR4 following SDF stimulation. The lack of germline mutations in these receptors suggests that there is a change in some downstream regulator such as a GRK or arrestin. Indeed, it has recently been shown that GRK3 levels are specifically decreased in one of the two patients (Balabanian et al., 2008) and re-expression of GRK3 was able to rescue receptor internalization and normalize actin polymerization and cell migration. It is interesting to note that mice lacking either GRK6 (Fong et al., 2002b; Vroon et al., 2004) or arrestin3 (Fong et al., 2002b) also have enhanced receptor function in response to SDF stimulation, similar to those seen in WHIM syndrome, suggesting that tissue specific differences in protein expression or activity possibly differentially regulates CXCR4 activity.

Cancer

The expression of CXCR4 has been detected in 23 different cancers of various origins (Balkwill, 2004b) and is the most common chemokine receptor expressed on cancer cells (Zlotnik, 2006a). The expression of CXCR4 on hematopoetic malignancies is not surprising given the critical role of the receptor in development of these cells (Ara et al., 2003; Egawa et al., 2001; Lapidot and Kollet, 2002; Tachibana et al., 1998; Zou et al., 1998). However, in a variety of other cancers, CXCR4 expression is enhanced compared to the adjacent normal tissue, which may have little or no CXCR4 (Muller et al., 2001; Scotton et al.,

2001; Sun et al., 2003). A potential underlying mechanism for this may result from changes that occur within the vasculature or O_2 carrying capacity of cells leading to hypoxic conditions during tumor progression (Hirota and Semenza, 2006). Hypoxia induces the activation of hypoxia inducible factor 1 (HIF-1) which in turn promotes expression of a number of target genes (Hirota and Semenza, 2006) including CXCR4 (Schioppa et al., 2003; Staller et al., 2003; Zagzag et al., 2005). Further evidence regarding the role of HIF-1 came from studies of the tumor suppressor von Hipple Lindau (VHL). Inactivating mutations of VHL, which normally targets HIF-1 for degradation, account for the increased CXCR4 expression in renal cell carcinomas (Schioppa et al., 2003; Staller et al., 2003; Zagzag et al., 2005).

A number of other factors also have the ability to enhance CXCR4 expression specifically during cancer progression. For example, vascular endothelial growth factor (VEGF) (Bachelder et al., 2002) or activation of nuclear factor kappa B (NF-κB) (Helbig et al., 2003) enhances CXCR4 expression in breast cancer promoting invasion and metastasis, respectively. Additionally, it has been shown that CXCR4 expression can be induced by the oncoproteins PAX3-FKHR (Libura et al., 2002; Tomescu et al., 2004) and RET/PTC (Castellone et al., 2004). CXCR4 expression as a result of the PAX3-FKHR fusion leads to enhanced migration and adhesion of rhabdomyosarcoma cells (Libura et al., 2002), while RET/PTC induced expression enhanced the transforming ability of breast cancer cells (Castellone et al., 2004). Furthermore,

altering the activity of (Lee et al., 2005) or deletion of (de Nigris et al., 2008) YY1 results in decreased or enhanced CXCR4-mediated metastases, respectively.

Increased cell surface expression of CXCR4 may also be the result of altered regulation, independent of effects on transcription/translation. Ubiquitination of CXCR4 is a modification regulating the expression of CXCR4 post-translationally (Marchese and Benovic, 2001; Marchese et al., 2003b). It has been found that HER2/neu positive cancer cells have increased expression of CXCR4 as a result of inhibition of receptor ubiquitination (Li et al., 2004). Expression of AIP4, the E3 ubiquitin ligase responsible for ubiquitination of CXCR4 (Marchese et al., 2003b), was able to reverse this effect (Li et al., 2004). Moreover, the recent finding that cytokine-independent survival kinase (CISK) associates with and inhibits AIP4 function (Slagsvold et al., 2006) provides a potential link between HER2 positive cancers and the attenuated degradation of CXCR4 (Li et al., 2004). It will be interesting to examine if altered CXCR4 ubiquitination is a global phenomenon in CXCR4-overexpressing cancers or if this effect is specific to HER2/neu expressing cancers.

It is expected that the functional consequence of CXCR4 expression on cancer cells would be varied based on the numerous roles of the CXCR4-SDF signaling axis. For example, the combination of CXCR4 expression and interaction with stromal or nurse-like cells in chronic lymphocytic leukemia (Burger et al., 2000) and multiple myeloma (Damiano et al., 1999) may account for resistance to spontaneous/drug induced apoptosis and cell adhesion-mediated drug resistance, essentially providing a protective niche. Tumor

progression is also affected by CXCR4-SDF-1 signaling through the induction of tumor-associated integrin activation and signaling (Hartmann et al., 2005). Finally, in addition to mediating metastases (see below), CXCR4-SDF-1 signaling may affect the survival of the tumor at the metastatic site. Enhanced CXCR4 signaling has been shown to down-regulate phosphoglycerate kinase (PGK), a key regulator of angiogenesis, activating angiogenic pathways and promoting tumor survival (Wang et al., 2007).

Since SDF is a chemokine, an attractive hypothesis is that CXCR4 expression correlates with metastasis. Consistent with this, activation of CXCR4 stimulates the production of matrix metalloproteases (Fernandis et al., 2004; Janowska-Wieczorek et al., 2000; Samara et al., 2004; Spiegel et al., 2004) potentially facilitating the ability of cancers to egress from the primary tumor site. Furthermore, SDF signaling is also able to enhance integrin activity (Campbell et al., 1998; Glodek et al., 2003; Wright et al., 2002) enhancing cell adhesion under flow conditions. Upon entering the blood or lymphatic systems, if CXCR4 truly mediates metastasis, tumors would preferentially migrate and adhere to areas that highly express SDF-1. Breast cancer follows this distinct pattern of metastasis, namely to lymph nodes, lung, liver, and bone marrow all of which highly express SDF-1 (Allinen et al., 2004; Muller et al., 2001). Accordingly, neutralizing antibodies to CXCR4 (Muller et al., 2001) or siRNA knock down (Lapteva et al., 2005; Liang et al., 2005) inhibit metastasis and growth of breast cancer cells. Other cancers, such as small cell lung cancer, thyroid, neuroblastoma, hematological and hepatic malignancies also metastasize to

areas with high SDF-1 expression (Burger et al., 2003; Geminder et al., 2001; Hwang et al., 2003; Kijima et al., 2002; Schimanski et al., 2006). In spite of this evidence, studies attempting to correlate expression with metastatic potential have yielded mixed results. Whereas CXCR4 expression increased with aggressiveness of prostate tumors (Sun et al., 2003) there was not a significant correlation of CXCR4 expression and distant breast cancer cell metastasis (Kato et al., 2003), although the extent of nodal metastasis was greater in cells expressing high levels of CXCR4 compared to those with lower levels (Kato et al., 2003). Recently, CXCR4 expression on hepatocellular carcinoma was suggested to correlate with local tumor progression, lymphatic and distant metastasis, as well as negatively impact the 3-year survival rate of these patients (Schimanski et al., 2006).

On the other hand, cancers such as lymphomas, glioma, ovarian, and pancreatic have a high expression of SDF-1 at the primary site (Corcione et al., 2000; Koshiba et al., 2000; Scotton et al., 2002; Zhou et al., 2002). Additionally, colonic epithelia normally express CXCR4 (Jordan et al., 1999). Thus, the CXCR4-SDF-1 interaction could be retaining tumor cells that originate at these sites, analogous to the retention of B-cells and neutrophils in the bone marrow during development. Epigenetic mechanisms that negatively regulate the expression of SDF or CXCR4 may be necessary in order for metastasis to occur. One example is DNA methylation, a modification typically associated with inactivation of tumor suppressors (Jones and Baylin, 2002). It has recently been shown that methylation of the SDF promoter in colonic epithelium promotes

metastasis of these tumors (Wendt et al., 2006). The CXCR4 promoter is also methylated in a number of pancreatic cancers, decreasing mRNA and protein levels (Sato et al., 2005). Though not addressed in the study, this may be a mechanism that allows pancreatic cancers to metastasize from these sites.

As detailed above, the C-tail is absolutely critical for proper regulation of CXCR4. Interestingly, expression of a C-tail truncated mutant of CXCR4 in MCF-7 mammary carcinoma cells led to an epithelial-to-mesenchymal transition (Ueda et al., 2006). Oligomicroarray analysis showed that there was a down regulation of E-cadherin and Zonula occludens, thereby disrupting cell-to-cell contacts, with a concomitant increase in ERK activation. There was also an increased expression of a number of growth factor receptors. While there have been no cancers described as a result of truncation of CXCR4, this may give insight into the signaling pathways critical for cancer progression and metastasis.

Recent evidence also suggests that, in some breast cancers, receptor expression and functional activity are not linked (Holland et al., 2006). Examining a variety of breast cancer cell lines, ranging from untransformed but immortalized to highly invasive, it was concluded that receptor expression alone does not lead to the acquisition of an invasive phenotype. Specifically, it was speculated that there were alterations in G protein coupling to the receptor. Untransformed or transformed non-invasive cells were not able to properly couple to G_i, and therefore, were not able to elicit Ca^{2+} mobilization, ERK activation or migration; signaling pathways conserved in the invasive lines. Interestingly, as B cells develop into mature cells, they progressively lose the ability to respond to SDF-1

even though surface expression of CXCR4 remains relatively high (Fedyk et al., 1999; Honczarenko et al., 1999). However, as they further differentiate into plasma cells, they regain responsiveness to SDF (Hargreaves et al., 2001). The underlying mechanisms regulating this phenomenon in B cells are currently not known, though similar mechanisms may be occurring as a result of the transition to a more malignant phenotype in these breast cancer cells.

OBJECTIVES

An emerging theme in GPCR regulation is the possibility of differential regulation dictated by the phosphorylation pattern, or “barcode”, elicited by the different members of the GRK family. Given the multifaceted role CXCR4 plays in diverse processes from development to cancer metastasis, CXCR4 is a very intriguing therapeutic target. An ample body of work has been generated in delineating potential pathways that mediate specific effects (e.g., leading to metastasis), however, a detailed basic understanding of receptor regulation is lacking. Understanding the precise mechanisms regulating CXCR4 function at the receptor level should provide insight into attractive therapeutic targets in this pathway. Furthermore, this will allow for translational research opportunities to dissect the specifics of how receptor regulation is altered in disease.

Our objectives were to 1) establish a model system in which to characterize the regulation of GPCRs, 2) use this model system to identify agonist-promoted sites of phosphorylation of CXCR4, 3) identify the kinases that

mediate site-specific phosphorylation, and 4) characterize the functional role of site-specific phosphorylation.

Classically, the β_2AR and rhodopsin have served as elegant models for establishing the current paradigms of GPCR signaling and regulation. However, accumulating evidence suggests that the molecular mechanisms that underlie receptor regulation are extremely diverse and receptors need to be studied individually. Therefore, we chose to establish a cellular system that would allow us to systematically analyze the proteins involved in receptor regulation as well as biochemically characterize receptor phosphorylation. Human Embryonic Kidney 293 (HEK293) cells are an excellent model system as they express a number of GPCRs (including CXCR4), GRK2, 3, 5, 6, the non-visual arrestins, and multiple PKC isoforms endogenously. In order to validate HEK293 cells as a model system, our aims included 1) screening HEK293 cells for a candidate receptor endogenously expressed, 2) identify downstream signaling pathways that can be used to characterize receptor regulation, and 3) systematically analyze proteins known to be involved in regulating the candidate receptor using small molecule inhibitors and small interfering RNA.

CXCR4 is primarily phosphorylated on multiple residues of the C terminal tail, which contains 15 serine and 3 threonine residues (Haribabu et al., 1997; Orsini et al., 1999). To date, GRK2 (Orsini et al., 1999; Cheng et al., 2000), GRK3 (Balabanian et al., 2008), GRK6 (Fong et al., 2004; Vroon et al., 2004), and PKC (Signoret et al., 1997; Orsini et al., 1999) have been implicated in regulating CXCR4, though the specific sites of phosphorylation, kinase

specificity, and functional role of site-specific phosphorylation are unknown. Therefore, to identify agonist promoted sites of phosphorylation and what kinase(s) are involved, our aims included 1) establish a HEK293 cell line that stably expressed Flag tagged CXCR4 at moderate levels, 2) affinity purify CXCR4 and use tandem mass spectrometry to identify SDF-1-promoted sites of phosphorylation, 3) develop phospho-specific antibodies against the identified sites, and 4) use a combination of small molecule inhibitors and siRNA to define kinase-specific sites of phosphorylation.

Accumulating evidence suggests that site- and tissue-specific phosphorylation of GPCRs has distinct effects on both receptor regulation and signaling (Tobin et al., 2008). Targeted deletion of GRK6 in mice results in enhanced receptor function following SDF-1 stimulation. However, neutrophils have enhanced while T cells are deficient in SDF-1-mediated chemotaxis. Interestingly, T cells isolated from mice specifically lacking arrestin3 display a similar phenotype as those from GRK6 mice. Furthermore, arrestin3 has been shown to enhance SDF-1-mediated chemotaxis and activation of MAPK signaling pathways (Cheng et al., 2000; Sun et al., 2002). Therefore, our final aims were to 1) characterize the roles of the GRKs and non-visual arrestins in regulating signal transduction of endogenous CXCR4 in HEK293 cells, and 2) ensure that stable over-expression of CXCR4 does not alter this regulation.

We were able to establish a model system by studying the regulation of the M_3 muscarinic acetylcholine receptor (M_3 mAChR), a Gq-coupled receptor. Consistent with what is currently known, we have shown that GRK2, GRK3,

casein kinase 1α, and the non-visual arrestins negatively regulate receptor activity (Budd et al., 2000; Wu et al., 2000; Willets et al. 2001; Willets et al., 2002; Willets et al., 2003), as demonstrated by changes in calcium mobilization and ERK activation. Furthermore, we were also able to show that GRK2 regulated calcium mobilization and ERK activation through interactions with Gq. Finally, we were also able to validate the combination of small molecule inhibitors and siRNA-mediated silencing as a method to unravel and better understand the complex regulatory mechanisms in place for any given GPCR.

Using this model system, we were then able to identify sites of phosphorylation by optimizing an affinity based purification strategy for CXCR4 from HEK293 cells. Following stimulation and purification, CXCR4 was subjected to proteolytic digestion and tandem mass spectrometry to identify agonist-promoted sites of phosphorylation. Using the mass spectrometry data and previous studies (Marchese et al., 2001), phospho-specific antibodies were successfully generated against phospho-Ser324/325 (pS324/5) and pS330. Furthermore, we also used a previously characterized antibody that is specific for pSer339 (Woerner et al., 2005). Using these antibodies, we were then able to characterize both the kinetics and kinase specificity at these residues. We provide novel evidence for a role of PKC in phosphorylating Ser324/5 following SDF stimulation. Additionally, we show that GRK6 phosphorylates multiple residues with distinct kinetics.

Analyzing calcium mobilization and ERK1/2 activation following systematic knocking down of GRK2, 3, 5, and 6, arrestin2, and arrestin3 has given

substantial insight into the functional role of site-specific phosphorylation. Importantly, we show that the endogenous and overexpressed receptors are regulated in a similar manner. Furthermore, we demonstrate that the GRKs and arrestins differentially regulate signaling. Together, this data suggests that CXCR4 function and signaling are dynamically regulated by phosphorylation and subsequent protein/protein interactions. Moreover, we have developed tools to allow for a complete analysis of CXCR4 function in a variety of tissues and disease states.

Chapter II

M_3 Muscarinic Acetylcholine Receptor-Mediated Signaling is Regulated by Distinct Mechanisms

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John M. Busillo's contributions:

- Designed and performed experiments for Figures:
  - Figure 6 – A, B, and C
  - Figure 7 – A and B
  - Figure 10 – A, B, C and D
  - Figure 11 – C and D
- Designed and illustrated Figure 12
- Authored initial draft and subsequent revisions

INTRODUCTION

Activation of G protein-coupled receptors (GPCRs) by agonist occupancy leads to a conformational change in the receptor that promotes the activation of heterotrimeric G proteins, which in turn activate a variety of effectors leading to downstream signaling events (Pierce et al., 2002). Activated GPCRs are regulated by three principal mechanisms: desensitization, internalization, and down-regulation. Receptor desensitization is initiated by the phosphorylation of serine/threonine residues by GPCR kinases (GRKs) which promotes the high affinity binding of arrestins, uncoupling the receptor from G protein and terminating signaling (Krupnick and Benovic, 1998).

There are seven members of the GRK family that are grouped into three subfamilies based on sequence and functional similarity: GRK1 and GRK7; GRK2 and GRK3; and GRK4, GRK5, and GRK6. GRK2, GRK3, GRK5, and GRK6 are ubiquitously expressed, while GRK1, GRK4, and GRK7 have a restricted expression pattern. Much of the research determining specific GPCR-GRK interaction has relied on techniques such as heterologous overexpression, dominant-negative constructs, and more recently RNA interference (Krupnick and Benovic, 1998; Iwata et al., 2005; Kim et al., 2005).

The non-visual arrestins, arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2) bind to activated, phosphorylated GPCRs subsequently terminating G protein activation and targeting the receptors to clathrin coated pits for internalization (Moore et al., 2007). Arrestins have also been shown to act as scaffolding

proteins to promote downstream signaling events, such as activation of mitogen-activated protein kinases (Lefkowitz and Shenoy, 2005).

The muscarinic acetylcholine receptors (mAchRs) represent a subfamily of GPCRs with five subtypes, M_1 – M_5. The M_3 mAchR couples to Gq resulting in phospholipase C-β (PLC-β) activation, and production of inositol trisphosphate (IP_3) and diacylglycerol (DAG), which leads to calcium release from intracellular stores and protein kinase C (PKC) activation. Additionally, the M_3 mAchR can activate extracellular signal-regulated kinase (ERK), although the mechanism by which this occurs is unclear. Upon activation, the M_3 mAchR is rapidly phosphorylated on serine/threonine residues within the third intracellular loop (Tobin et al., 1997) and C terminal tail (Budd et al., 2000), although it is unclear which kinases mediate receptor phosphorylation and regulation. Wu et al. showed that GRK2 phosphorylates the M_3 mAchR in a Gβγ dependent manner and mapped the phosphorylation sites to ^{331}SSS^{333} and ^{348}SASS^{351} in the third intracellular loop (Wu et al., 2000). GRK3 also has the ability to phosphorylate the receptor but receptor regulation by GRK3 appears to occur primarily through modulation of PLC-β activity (Willets et al., 2001; Willets et al., 2002; Willets et al., 2003). Willets and coworkers also showed that GRK6 regulates the M_3 mAchR by phosphorylation while GRK2 and GRK5 were found to have no effect (Willets et al., 2001; Willets et al., 2002; Willets et al., 2003). In addition to GRK-mediated phosphorylation, casein kinase 1a (CK1α) has also been shown to phosphorylate the M_3 mAchR in an agonist dependent manner although this alone was insufficient to mediate receptor desensitization (Budd et al., 2000).

Finally, arrestins do not appear to be required for M_3 mAchR internalization (Lee et al., 1998; Mundell and Benovic, 2000), but are involved in receptor desensitization with no discernable specificity between arrestin2 and arrestin3 (Mundell and Benovic, 2000).

One major unanswered question regarding the physiological regulation of GPCRs is to understand which GRKs and arrestins regulate a given receptor subtype. To date, a limited number of GRKs and arrestins have been identified, whereas more than 700 mammalian GPCRs have been cloned (Gainetdinov et al., 2004). Studies over the past decade have defined the ability of individual GRKs, second messenger dependent kinases (e.g., PKA or PKC), and arrestins to regulate GPCRs in model systems. However, the mechanisms by which GRKs target endogenous GPCRs are still unknown. Using either wild type GRK2, kinase dead GRK2, or mutants deficient in Gaq binding, we previously showed that the human H1 histamine receptor was specifically regulated by GRK2 mainly through regulation of activated Gq (Iwata et al., 2005). In this report, we used RNA interference to target proteins specifically involved in the agonist dependent regulation of the endogenous M_3 mAchR in HEK293 cells. We found that there was differential GRK-mediated regulation of this receptor as assessed by calcium signaling and ERK activation. In addition, knockdown of either arrestin2 or arrestin3 resulted in enhanced signaling from the receptor, with different temporal effects. Furthermore, we show that, in addition to GRKs, CK1α has a negative role in M_3 mAchR mediated calcium mobilization. Taken

together, our results show that multiple proteins mediate agonist-dependent regulation of M_3 mAchR signaling.

EXPERIMENTAL PROCEDURES

Materials

HEK293 cells were from Microbix Biosystems, Inc (Toronto, Canada) while carbachol was from EMD Biosciences (San Diego, CA). Pirenzepine and p-fluorohexahydro-sila-difenol (pFHHsiD) were from Sigma-Aldrich (St. Louis, MO) and Lipofectamine™ 2000 and Opti-MEM® were from Invitrogen (Carlsbad, CA). Phospho-specific p44/p42 polyclonal antibody was from Cell Signaling Technologies (Beverly, MA). Polyclonal ERK2, CK1a and GRK3 antibodies were from Santa Cruz (Santa Cruz, CA). Anti-β-arrestin monoclonal antibody was from BD Biosciences Pharmagen (San Diego, CA). Anti-GRK4-6 monoclonal antibody was from Upstate Cell Signaling Solutions (Waltham, MA) while the GRK2 monoclonal antibody was produced in our laboratory and anti-a-tubulin monoclonal antibody was from Sigma (St. Louis, MO).

Synthesis of small interfering RNAs (siRNAs)

All siRNAs were chemically synthesized by Dharmacon, Inc (Chicago, IL). The GRK2, GRK5 and CK-1a siRNAs were reported previously (Iwata et al., 2005; Kim et al., 2005; Liu et al., 2002). The GRK3 siRNA sequence was 5′-GCAGAAGUCGACAAAUUUA-3′ while 5′-GCGCUUGGCCUACGCCUAU-3′ was used for GRK6. Arrestin2 and 3 siRNAs were purchased as a SMARTpool®.

Non-specific control siRNA VIII (5´-AAACUCUAUCUGCACGCUGAC-3´) was used as the control for all siRNA experiments.

Cell Culture and siRNA transfection

HEK293 cells were maintained in Dulbecco's modified Eagles Media supplemented with 10% FBS, 25 mM HEPES, pH 7.2, and 0.1 mM non-essential amino acids in a 5% CO_2 incubator at 37˚C. For transfection of GRK and casein kinase siRNAs, HEK293 cells grown to 85 to 90% confluence in 100-mm dishes were transfected with 600 pmol of siRNA using Lipofectamine 2000 in Opti-MEM. After 6 hr, cells were split 1:2 and a second transfection of 600 pmol was performed 24 hr after the initial transfection. Forty-eight hr after the second transfection, cells were split for assay the following day. For arrestin SMARTpool® siRNAs, cells ~70% confluent were transfected with 600 pmol of siRNA corresponding to either arrestin2 or arrestin3. Forty-eight hr later, cells were split for assay the following day. Control siRNA was transfected in a similar fashion as described above for each transfection condition.

Immunoblotting

To analyze siRNA target proteins, siRNA transfected HEK293 cells in a 6-well plate were washed twice with ice cold PBS and lysed with buffer (20 mM HEPES, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100 and one tablet of Complete Inhibitor (Roche) per 50 ml) at 4°C on a rocker for 30 min. The lysates were centrifuged at 4°C at 30,000 rpm in a TLA45 rotor for 30 min. The supernatants were electrophoresed on a 10% SDS polyacrylamide gel,

transferred to nitrocellulose, and immunoblotted using monoclonal anti-GRK2 (1:1000), polyclonal anti-GRK3 (1:200), monoclonal anti-GRK4-6 (1:3000), monoclonal anti-β-arrestin-1 (1:1000) or polyclonal anti-CK1a (1:200), HRP-labeled secondary antibodies, and chemiluminescence. The blots were stripped and reprobed using an anti-tubulin (1:7500) monoclonal antibody.

Measurement of intracellular calcium mobilization

Calcium mobilization was performed as previously described with slight modifications (Iwata et al., 2005). In brief, HEK293 cells transfected with siRNAs were harvested with Cellstripper (Mediatech, Herndon, VA), washed twice with phosphate-buffered saline, and resuspended at 5 x 10^6 cells/ml in Hanks' balanced salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml glucose) (Invitrogen) containing 0.025% bovine serum albumin. The cells were then loaded with 3 μM Fura-2 acetoxymethyl ester derivative (Fura-2/AM) (Molecular Probes, Eugene, OR) for 30 min at 37°C. The cells were washed once in Hanks' solution, resuspended in Hanks' solution containing 0.025% bovine serum albumin, incubated at room temperature for 15 min, washed twice in Hanks' solution, and then resuspended in Hanks' at a concentration of 3 x 10^7 cells/ml. A typical experiment contained 1.5 x 10^6 cells/1.6 ml in a quartz cuvette and stimulation with different concentrations of carbachol. Calcium mobilization was measured using excitation at 340 and 380 nm and emission at 510 nm in a fluorescence spectrometer (LS55, Perkin-Elmer Life Sciences). Calibration was performed using 0.1% Triton X-100 for total

fluorophore release and 15 mM EGTA to chelate free calcium. When antagonists were used, cells were preincubated with the indicated antagonist for 30 seconds prior to starting the fluorescent spectrometer and an additional 30 seconds prior to stimulation with carbachol. Intracellular calcium concentrations were calculated using a fluorescence spectrometer measurement program.

ERK activation assays

HEK293 cells, ~90% confluent in 6-well plates, were serum starved for at least 6 hr. Following serum starvation, cells were stimulated with 100 μM carbachol as indicated and washed once with ice cold PBS. Lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.2, 150 mM NaCl, 10 mM EDTA, 1 μM sodium orthovanadate, 3 mM sodium pyrophosphate, 10 mM sodium fluoride, and 1 Complete Inhibitor tablet per 50 ml) was added and plates were stored at –80˚C until harvesting. Cells were thawed and scraped into lysis buffer on ice, vortexed briefly, and debris was cleared by centrifugation at 14,000 rpm for 15 min. Equal amounts of whole cell lysate were separated by electrophoresis on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and proteins detected by immunoblotting. Nitrocellulose membranes were blocked for 1 hr at room temperature in a 1:3 dilution of ODYSSEY® blocking buffer (LI-Cor® Biosciences). A mixture of primary antibodies directed at ERK2 (monoclonal, Santa Cruz) and phospho-ERK1/2 (polyclonal, Cell Signaling Technologies) in 100% ODYSSEY® blocking buffer were incubated overnight at 4˚C. Nitrocellulose membranes were washed with Tris Buffered Saline containing 0.1% Tween-20 (TBS-T) over 40

min. The membranes were then incubated for 1 hr at room temperature with a mixture of goat anti-rabbit Alexa® Fluorophore 680 conjugated (Molecular Probes) and goat anti-mouse IRDye 800 conjugated (Rockland Immunochemicals) antibodies. Following a 1 hr incubation, the membranes were washed with TBS-T for 60 min. Fluorescence was detected simultaneously using the ODYSSEY® infrared imaging system (LI-Cor® Biosciences). When antagonists were used, cells were incubated at 37°C with the indicated antagonist for 5 minutes prior to stimulation with carbachol. Fluorescence intensity of phosphorylated ERK2 was normalized to total ERK2 fluorescence, and data are represented as fold-increase over basal (+/- SEM).

Statistical Analysis

Results were analyzed using a paired, two-tailed, students T-Test with significance at $p \leq 0.05$.

RESULTS

Pharmacological characterization of the muscarinic acetylcholine receptor subtype endogenously expressed in HEK293 cells

Using RNAi, we have previously shown that GRK2 regulates the endogenously expressed H1 histamine receptor in HEK293 cells (Iwata et al., 2005). We wanted to expand this approach to determine the regulation of other endogenous GPCRs. Previous work has shown that HEK293 cells respond to stimulation with carbachol, a non-specific mAchR agonist, with robust IP_3 production and calcium mobilization that had been attributed to the M_1 mAchR

subtype (Mundell and Benovic, 2000). However, a recent microarray analysis of commonly used cell lines suggested that the mAchR endogenously expressed in these cells is the M_3 receptor subtype (Hakak et al., 2003). In light of this, we sought to pharmacologically determine which mAchR subtype is actually expressed in HEK293 cells. Cells loaded with the ratiometric calcium indicator Fura-2/AM display a robust increase in calcium mobilization in response to carbachol stimulation (Figure 6A) with an EC_{50} of 20 μM (data not shown). Incubation with the antagonist p-FHHsiD, which has some selectivity for the M_3 (pKi = 7.1) (de la Vega et al., 1997) completely inhibited calcium mobilization in response to carbachol while the selective M_1 mAchR antagonist pirenzepine, only slightly inhibited calcium mobilization (Figure 6A). This result is in line with previous reports demonstrating that pirenzepine selectively inhibits the M_1 mAchR (pKi 8.0), but at higher concentrations is able to inhibit the M_3 subtype (pKi 6.7) (de la Vega et al., 1997). In addition, there was no calcium response when the cells were stimulated with the M_1/M_4 mAChR-selective agonist McN-A-343 (data not shown).

To further investigate the subtype of mAchR expressed, we also analyzed the effects of the M_1 and M_3 selective antagonists on carbachol-stimulated ERK activation. GPCRs activate ERK1/2 via a number of pathways (Werry et al., 2005) and both the M_1 and M_3 mAchRs have been shown to activate ERK1/2 in a number of cell types (Budd et al., 1999; Guo et al., 2001). Carbachol-mediated ERK activation in HEK293 cells is dose dependent (EC_{50} ~8 μM), peaking at 5 min and returned to basal levels by 60 min (Figure 6B, top panel). The addition of

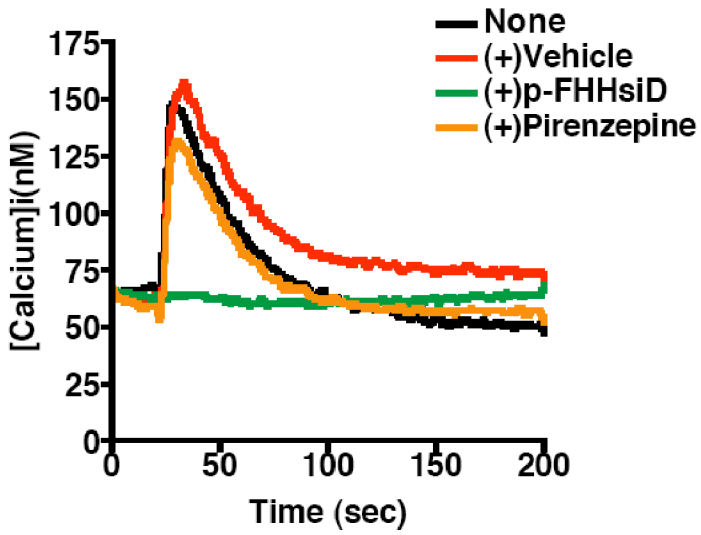
p-FHHsiD completely blocked ERK1/2 activation in response to carbachol, whereas pirenzepine had no effect (Figure 6B). These results confirm that the primary mAchR subtype in HEK293 cells is the M_3.

We also wanted to determine whether PKC was responsible for ERK activation following M_3 mAchR stimulation. Previous evidence suggests that the novel PKC isoforms are responsible for M_3 mAchR-mediated ERK activation, including PKCε in SK-N-BE2(C) cells (Kim et al., 1999) and a calcium independent PKC in Chinese hamster ovary cells (Wylie et al., 1999). Furthermore, it has been shown recently that the M_3 mAchR was shown to regulate the Kir 3.1/3.2 potassium channel through activation of PKC-δ in HEK293 cells (Brown et al., 2005). To establish whether PKC-δ is involved in M_3 mAchR-mediated ERK activation, we used bisindolylmaleimide I (Bis I), a general PKC inhibitor, and rottlerin, which selectively inhibits PKC-δ (Gschwendt et al., 1994). Rottlerin significantly inhibited carbachol-mediated ERK activation while Bis I only partially inhibited ERK activation (Figure 6C). The specificity of these inhibitors was confirmed by the demonstration that rottlerin had minimal effects on PMA-induced ERK activation while Bis I completely inhibited PMA-promoted ERK activation (Figure 6C). Taken together, we conclude that HEK293 cells endogenously express the M_3 mAchR and that carbachol-mediated activation of the ERK1/2 cascade is dependent on PKC-δ.

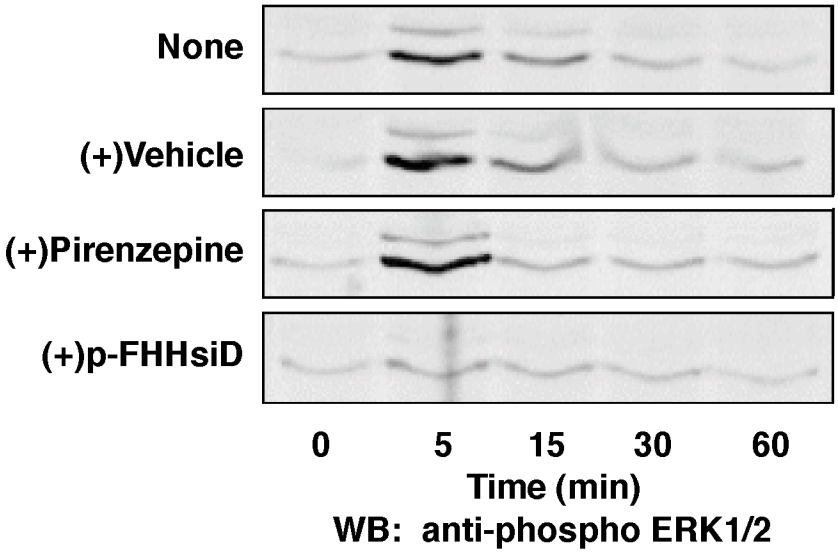
Figure 6. Charactrization of the Muscarinic Acetylcholine Receptor Subtype Endogenously Expressed in HEK293 Cells

A) HEK293 cells loaded with the ratiometric calcium indicator Fura2/AM were incubated with 100 nM pirenzepine (green), 1 μM p-FHHsiD (orange), vehicle (red), or carbachol alone (black) and stimulated with 100 μM carbachol. Changes in calcium mobilization were assayed by monitoring the change in Fura-2AM fluorescence. Shown is a representative tracing from three independent experiments. *B)* Following a 6 hr serum starve, HEK293 cells were incubated with 100 nM pirenzepine, 1 μM p-FHHsiD, vehicle, or carbachol alone and stimulated with 100 μM carbachol for the indicated times. Cells from a 6-well plate were harvested and equal amounts of total cellular lysate were separated by SDS-PAGE and probed for phospho-ERK1/2 as described in Materials and Methods. Shown is a representative immunoblot of three independent experiments. *C)* Cells were treated with Bis I (2.5 μM), Bis V (2.5 μM) or rottlerin (5 μM) for 30 min prior to stimulation with carbachol (100 μM) for 5 min or PMA (100 nM) for 15 min.

A.

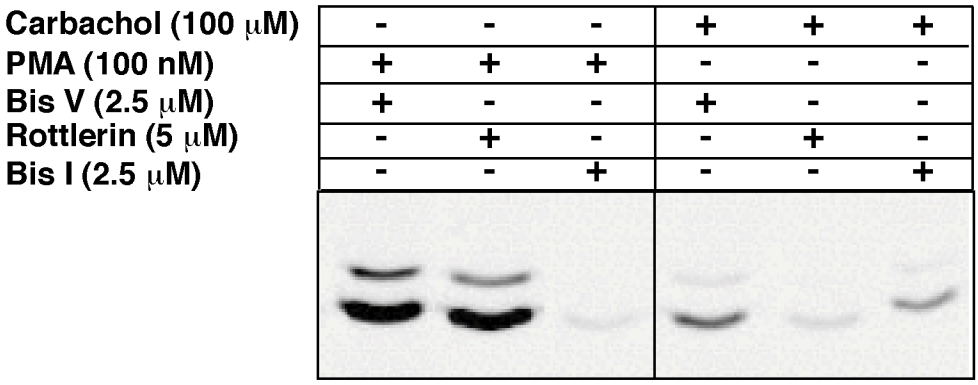


B.



WB: anti-phospho ERK1/2

C.



WB: anti-phospho ERK1/2

Regulation of M_3 mAchR-mediated calcium mobilization in HEK293 cells

We next evaluated the effect of knocking down various regulatory proteins on M_3 mAchR signaling. Since the phosphorylation of activated GPCRs by GRKs is often an early step in signal termination, we initially determined the effect that GRK knockdown would have on calcium mobilization following carbachol treatment. As shown in Figure 7A and 7B, we were able to selectively and specifically knockdown each of the four individual GRKs expressed in HEK293 cells. A modest increase in GRK3 expression was observed when other GRKs, in particular GRK2, were knocked down (Figure 7B).

Knockdown of GRK2, GRK3, and GRK6 led to increases of 210% ($p<0.001$), 190% ($p<0.05$) and 230% ($p<0.001$), respectively, in the peak calcium transients, whereas knockdown of GRK5 had no effect on calcium mobilization (Figure 8A and 8B). This effect was also observed when methacholine was used to activate the M_3 mAchR (data not shown). These data suggest that multiple GRKs are involved in the desensitization of the M_3 mAChR.

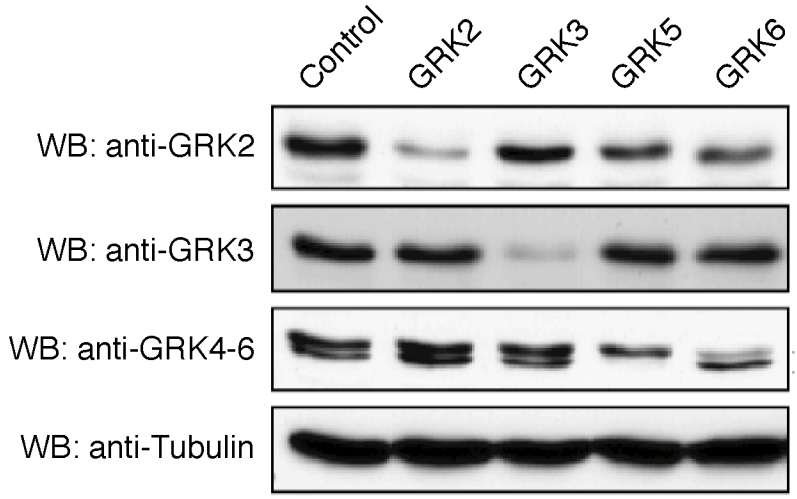
GRK2 interaction with G_q is primarily responsible for increased calcium mobilization

The enhanced mobilization of calcium seen following silencing of GRK2 may arise from phosphorylation-dependent and/or phosphorylation-independent mechanisms (Ribas et al., 2007). Therefore, we next sought to further delineate the underlying mechanism observed for calcium mobilization when GRK2 was knocked down. Because we showed previously that GRK2 interacts with $G\alpha_q$

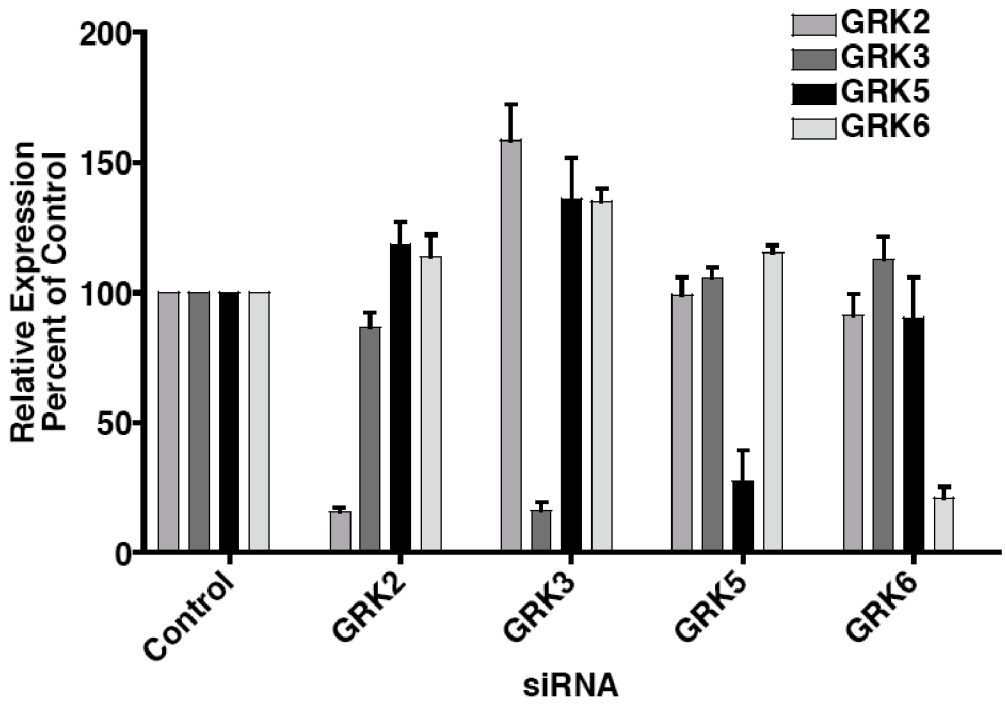
Figure 7. Knock Down of Endogenous GRK Isoforms in HEK293 Cells

A) HEK293 cells were transfected twice within a 24 hr interval with GRK-specific or non-specific control siRNA. 72 hr after the second transfection, cells were harvested and equal amounts of total cellular lysate was separated by 10% SDS-PAGE, transferred to nitrocellulose and incubated with the indicated antibodies. Blots were stripped and re-probed for α-tubulin to control for loading. Shown is a representative immunoblot. *B)* Mean relative level of GRK expression following siRNA quantified by densitometry from five separate experiments.

A.


Control
GRK2
GRK3
GRK5
GRK6
WB: anti-GRK2
WB: anti-GRK3
WB: anti-GRK4-6
WB: anti-Tubulin

B.

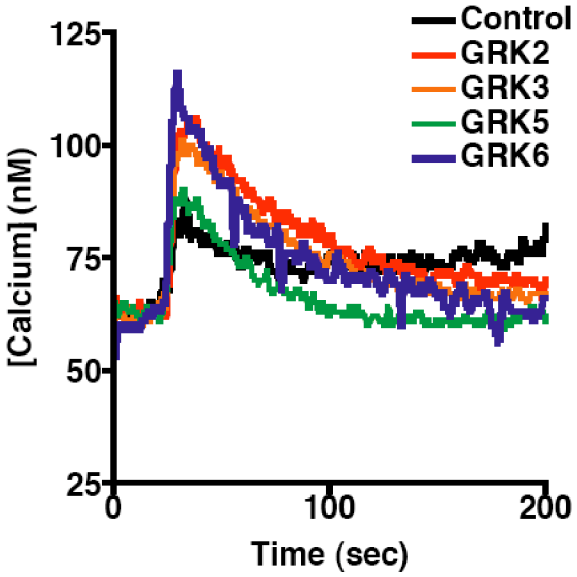

GRK2
GRK3
GRK5
GRK6
200
150
100
50
0
Relative Expression
Percent of Control
Control
GRK2
GRK3
GRK5
GRK6
siRNA

through the RGS-homology domain of GRK2 (Carman et al., 1999), the increase in peak calcium mobilization could be a result of a loss of receptor phosphorylation, a loss of the ability of GRK2 to inhibit activated $G\alpha_q$, or both. To address this, we generated cell lines that stably express either wild-type bovine GRK2, kinase dead GRK2 (K220R), GRK2 point mutants defective in binding $G\alpha_q$ (R106A, D110A), or a GRK2 mutant that was both kinase-dead and Ga-deficient (R106A/K220R). Cloned cell lines expressing wild type or mutant bovine GRK2 at levels close to endogenous GRK2 levels (1- to 5-fold overexpression) were selected for study (Figure 8C). SDS-PAGE revealed that bovine GRK2 ran slightly slower than endogenous human GRK2 when expressed in HEK293 cells (Figure 8C). Stable expression of either wild type or the kinase dead mutant reduced carbachol-stimulated calcium mobilization by ~50% (Figure 8D). In striking contrast, stable expression of the $G\alpha_q$-binding deficient mutants (R106A and D110A) or the double mutant (R106A/K220R) had no effect on calcium mobilization (Figure 8D). This suggests that GRK2 primarily regulates the activity of the M_3 mAchR through its ability to interact with the activated pool of $G\alpha_q$.

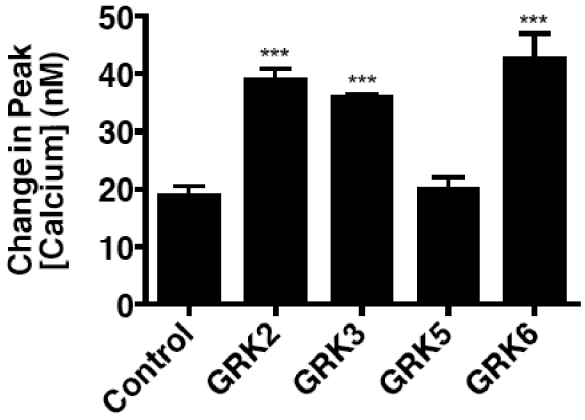
Figure 8. GRK-Mediated Regulation of Calcium Mobilization Following M_3 Muscarinic Acetylcholine Receptor Activation

A) Effect on calcium mobilization. 72 hr after the second siRNA transfection, HEK293 cells were loaded with Fura2/AM and stimulated with 10 μM carbachol. *B)* Mean (+/- SEM) increase in the peak calcium transient following stimulation with 10 μM carbachol from five individual experiments (*p<0.05, ***p<0.001 using two-tailed T test). *C)* Representative immunoblot showing relative levels of GRK2 stably expressed in HEK293 cells. *D)* Calcium mobilization in HEK293 cells stably expressing bovine GRK2. Mean (+/- SEM) increase in peak calcium mobilization in cells expressing vector (pcDNA3), wild type, Gq-binding deficient (R106A; D110A), kinase-dead (K220R), or the Gq-binding deficient/kinase dead (R106A/K220R) bovine GRK2 (*p<0.05 for GRK2-K220R, ***p<0.001 for wild type GRK2).

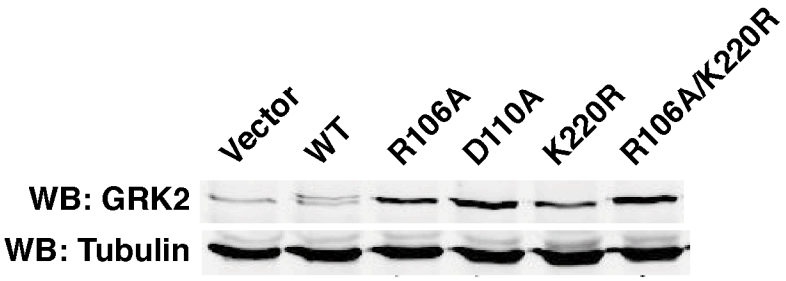
A.


Control
GRK2
GRK3
GRK5
GRK6
125
100
75
50
25
[Calcium] (nM)
0
100
200
Time (sec)

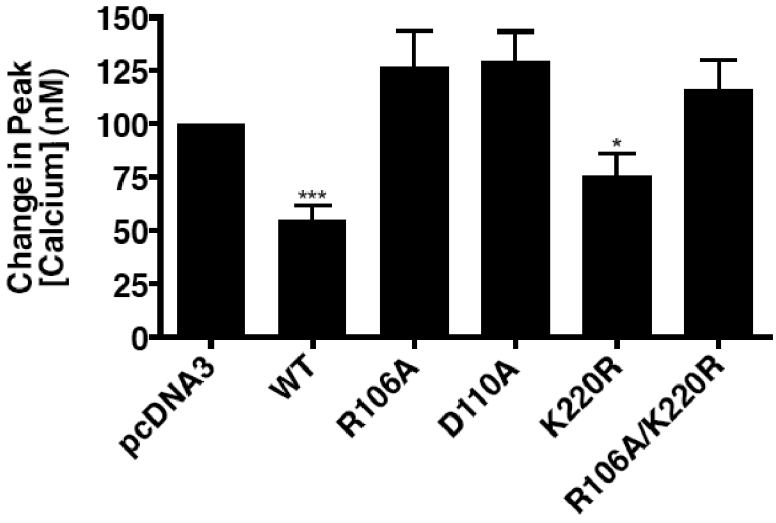
B.


50
40
30
20
10
0
Change in Peak
[Calcium] (nM)
***
***
***
Control
GRK2
GRK3
GRK5
GRK6

C.


Vector
WT
R106A
D110A
K220R
R106A/K220R
WB: GRK2
WB: Tubulin

D.


150
125
100
75
50
25
0
Change in Peak
[Calcium] (nM)
***
*
pcDNA3
WT
R106A
D110A
K220R
R106A/K220R

The non-visual arrestins negatively regulate M_3 mAchR-promoted calcium mobilization

Our data suggest that GRK-mediated phosphorylation of the M_3 mAchR may contribute to subsequent desensitization. Because GRK phosphorylation often promotes arrestin binding, we next determined the effect siRNA knockdown of arrestin2 and arrestin3 had on calcium mobilization. Pooled siRNAs targeting either arrestin2 or arrestin3 specifically reduced protein expression by ~90% (Figure 9A and 9B). As shown in Figure 9, C and D, knockdown of either arrestin2 or arrestin3 resulted in a significant increase in the peak calcium transient upon stimulation with carbachol. The increase seen with arrestin3 was slightly higher (74% increase) than that seen with arrestin2 (65% increase), although silencing of arrestin3 also led to an increase in the prolonged phase of the calcium transient (Figure 9C), suggesting prolonged IP_3 production.

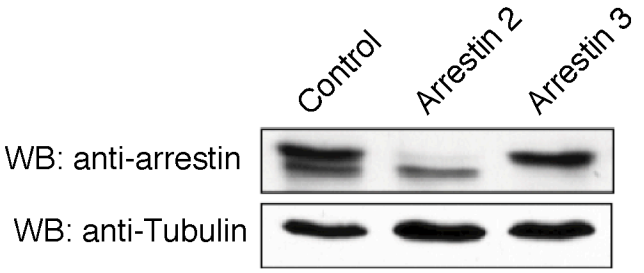
Regulation of M_3 muscarinic acetylcholine receptor-mediated activation of the ERK cascade

We next focused on understanding the roles of GRKs and arrestins in regulating activation of ERK1/2 following M_3 mAchR stimulation. The kinetics of ERK1/2 activation showed a consistent peak at 5 min that returned to basal levels by 60 min (Figure 6C). As shown in Figure 10, A and B, knocking down

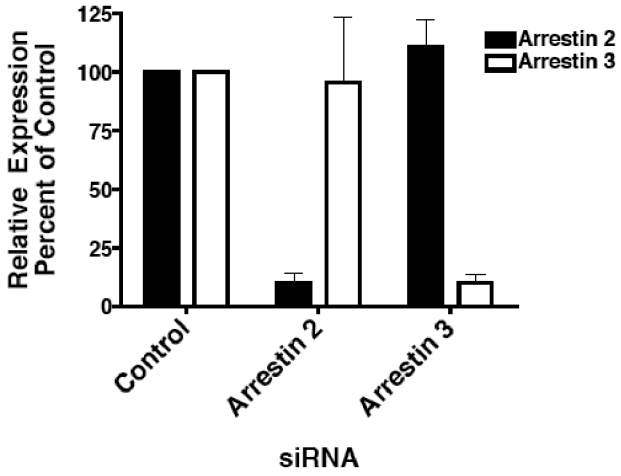
Figure 9. Effect of Arrestin Knockdown on Calcium Mobilization Following M_3 Muscarinic Acetylcholine Receptor Activation

A) Cells were transfected with SMARTpool siRNA and harvested 72 hr later. Blots were incubated with a monoclonal antibody for arrestin2 that cross-reacts with arrestin3. Blots were stripped and re-probed for α-tubulin to control for loading. Shown is a representative immunoblot. *B)* Mean relative level of arrestin expression following siRNA quantified by densitometry from five separate experiments. *C)* Effect on calcium mobilization. Cells were harvested 72 hr post-transfection and processed as described previously. Shown is a representative calcium trace from five independent experiments. *D)* Mean (+/- SEM) increase in the peak calcium transient following stimulation with 100 μM carbachol from five individual experiments (* $p<0.05$, *** $p<0.001$ using two-tailed T test).

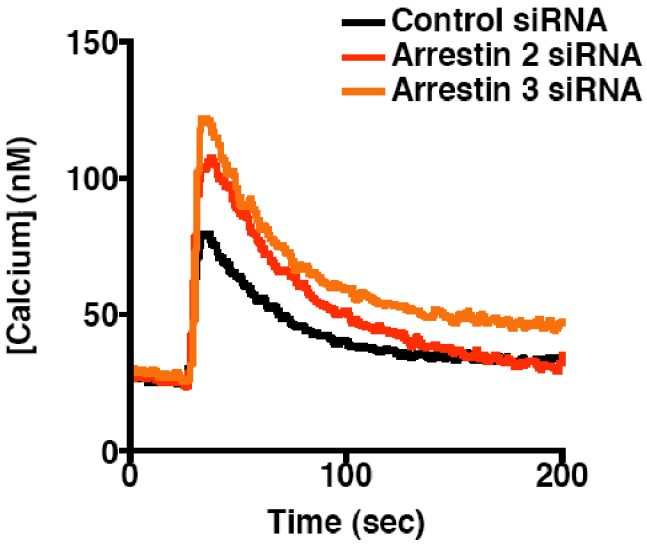
A.


Control
Arrestin 2
Arrestin 3
WB: anti-arrestin
WB: anti-Tubulin

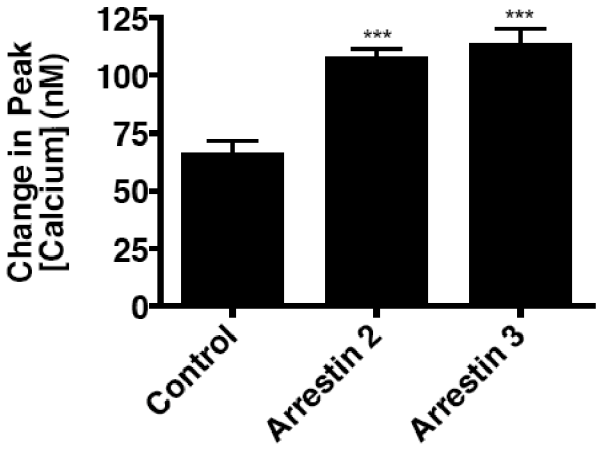
B.


Relative Expression
Percent of Control
125
100
75
50
25
0
Arrestin 2
Arrestin 3
Control
Arrestin 2
Arrestin 3
siRNA

C.


Control siRNA
Arrestin 2 siRNA
Arrestin 3 siRNA
[Calcium] (nM)
150
100
50
0
0
100
200
Time (sec)

D.


Change in Peak
[Calcium] (nM)
125
100
75
50
25
0
***
***
Control
Arrestin 2
Arrestin 3

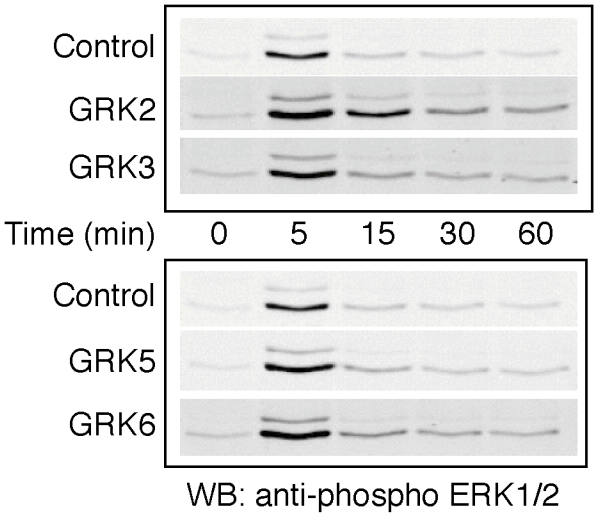
GRK2 resulted in a 2.5-fold increase in the peak of ERK1/2 activation and prolonged ERK1/2 activation (Figure 10B). Silencing of GRK5 or GRK6 also enhanced ERK1/2 activation following a 5-min stimulation, although the effects were modest and not statistically significant (1.3- and 1.5-fold increase, respectively) (Figure 10A and 10B). GRK knockdown did not change basal phospho-ERK1/2 levels (data not shown). It is interesting that in contrast to calcium mobilization, knocking down GRK3 had no effect on ERK1/2 activation (Figure 10, A and B). Collectively, these data demonstrate that signaling pathways downstream of M_3 mAchR activation are regulated by multiple GRKs in HEK293 cells, in a separate but coordinated fashion.

In contrast to some GPCRs (Ahn et al., 2004; Lefkowitz and Shenoy, 2005), internalization is not required for M_3 mAchR-mediated ERK activation (Budd et al., 1999). Thus, it was not surprising that knockdown of either arrestin2 or arrestin3 resulted in an ~2-fold increase in ERK activation, with differential temporal effects (Figure 10, C and D). Silencing of arrestin2 led to enhanced ERK1/2 activation at 5 min, whereas silencing of arrestin3 led to both enhanced and prolonged activation (Figure 10D). These data suggest that under normal physiological conditions, either arrestin2 or arrestin3 is sufficient to negatively regulate acute signaling events upon M_3 mAchR activation, although arrestin3 appears to play a larger role in terminating signaling in response to prolonged agonist exposure.

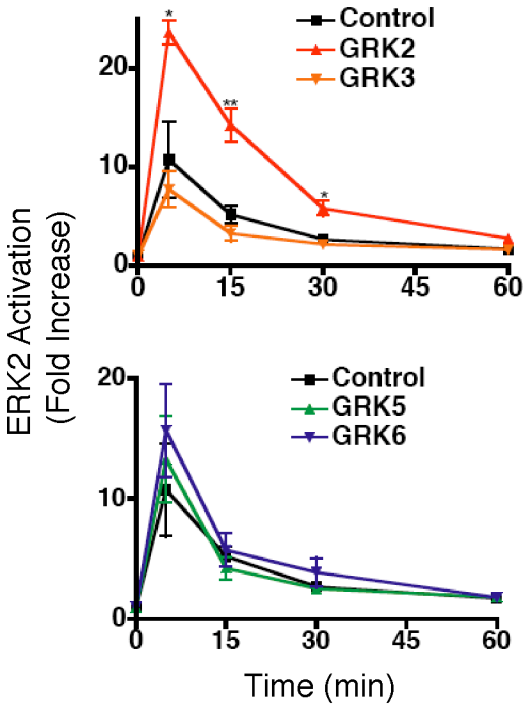
Figure 10. Effect of GRK and Arrestin Knockdown on M_3 Muscarinic Acetylcholine Receptor ERK Activation

A) Effect of GRK knockdown on ERK1/2 activation. Following a 6 hour serum starve, cells were treated with 100 μM carbachol for indicated times. Shown is a representative immunoblot from six independent experiments. *B)* Mean fold increase in ERK2 activation. Blots were incubated simultaneously with fluorophore conjugated primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence and data are presented as fold-increase in ERK2 activation over basal (n=6, +/- SEM; *p<0.05, **p<0.01). *C)* Effect of arrestin knockdown on ERK1/2 activation. Following a 6 hour serum starve, cells were treated with 100 μM carbachol for indicated times. Shown is a representative immunoblot from eight independent experiments. *D)* Mean fold increase in ERK2 activation. Blots were incubated simultaneously with fluorophore conjugated primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence and data are presented as fold-increase in ERK2 activation over basal (n=8, +/- SEM; **p<0.01).

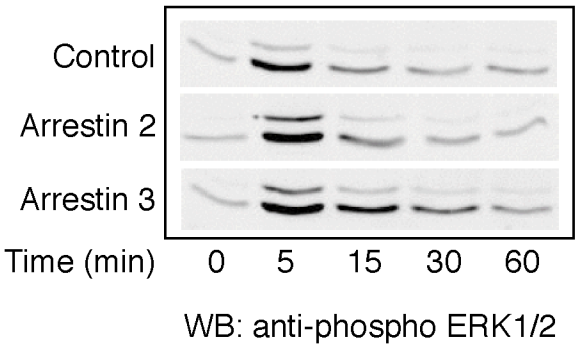
A.


Control
GRK2
GRK3
Time (min)
0
5
15
30
60
Control
GRK5
GRK6
WB: anti-phospho ERK1/2

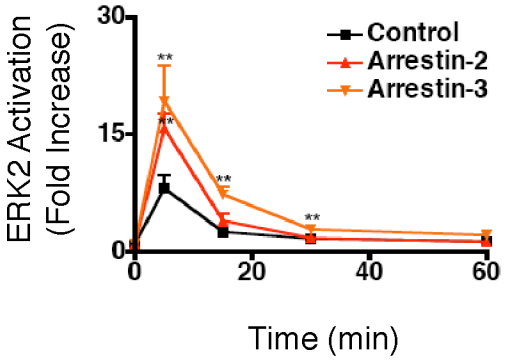
B.


ERK2 Activation
(Fold Increase)
Control
GRK2
GRK3
Control
GRK5
GRK6
Time (min)

C.


Control
Arrestin 2
Arrestin 3
Time (min)
0
5
15
30
60
WB: anti-phospho ERK1/2

D.


ERK2 Activation
(Fold Increase)
Control
Arrestin-2
Arrestin-3
Time (min)

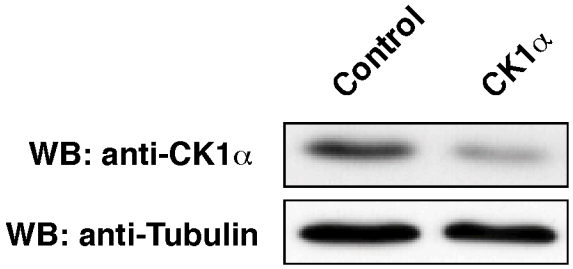
Regulation of the M_3 muscarinic acetylcholine receptor by casein kinase 1α

CK1α also phosphorylates the M_3 receptor in an agonist dependent manner although it does not appear to be required for desensitization of the receptor (Budd et al., 2000; Budd et al., 2001; Tobin et al., 1997). CK1α has also been shown to phosphorylate the M_1 mAchR and rhodopsin in vitro (Tobin et al., 1997; Waugh et al., 1999). To determine whether CK1α has a role in regulating the endogenous M_3 mAchR, HEK293 cells were transfected with CK1α siRNA that specifically reduced CK1α protein levels to ~40% of that seen in control cells (Figure 11A). Knockdown of CK1α resulted in a significant increase (62%, $p<0.01$, $n=4$) in the peak calcium transient compared to cells treated with control siRNA (Figure 11B). To determine if this effect was specific to CK1α mediated regulation of the M_3 mAchR and not to some other aspect of the G_q signaling pathway, we also tested the ability of CK1α to regulate the histamine H1 receptor which is regulated by GRK2 in HEK293 cells (Iwata et al., 2005). Knockdown of CK1α had no effect on calcium mobilization upon stimulation with 100 μM histamine (data not shown), suggesting that the effect of CK1α knockdown was specific for M_3 mAchR signaling. Interestingly, knockdown of CK1α had no effect on carbachol-mediated activation of ERK1/2 (Figures 11, C and D). These data demonstrate that, in addition to the GRK family, the agonist activated M_3 mAchR is also regulated by CK1α.

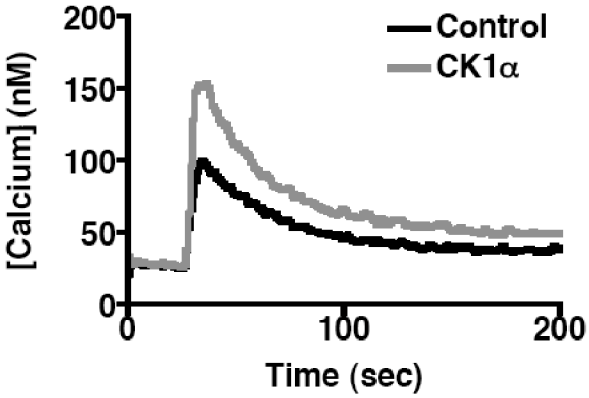
Figure 11. Effect of CK1α Knockdown on M_3 Muscarinic Acetylcholine Receptor Signaling

A) 72 hr after the second siRNA transfection, cells were harvested and equal amounts of total cellular lysate were separated by SDS-PAGE and immunoblotted for CK1α using a specific antibody. Blots were stripped and re-probed for α-tubulin to control for loading. Shown is a representative immunoblot. *B)* Effect on calcium mobilization. 72 hr after the second siRNA transfection, cells were loaded with Fura-2/AM and stimulated with 100 μM carbachol. Shown is a representative tracing from five independent experiments. *C)* Effect on ERK1/2 activation. Following a 6 hr serum starve, cells were stimulated with 100 μM carbachol for indicated times. Shown is a representative immunoblot from eight independent experiments. *D)* Mean activation of ERK2. Blots were incubated simultaneously with fluorophore conjugated primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence and data are presented as fold-increase over basal (n=8, +/- SEM).

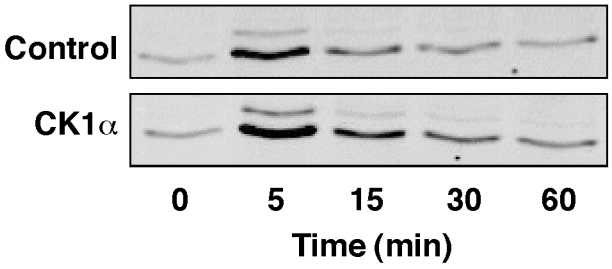
A.


Control
CK1α
WB: anti-CK1α
WB: anti-Tubulin

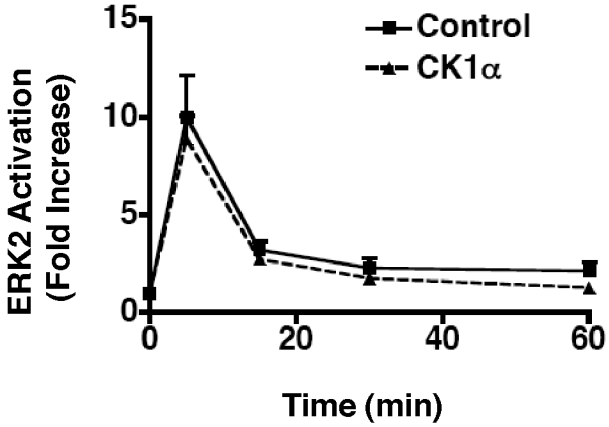
B.


Control
CK1α
[Calcium] (nM)
200
150
100
50
0
0
100
200
Time (sec)

C.


Control
CK1α
0
5
15
30
60
Time (min)

D.


Control
CK1α
ERK2 Activation
(Fold Increase)
15
10
5
0
0
20
40
60
Time (min)

DISCUSSION

GPCRs transduce extracellular stimuli into specific intracellular signals that regulate a variety of cellular functions. GPCR desensitization is classically mediated by members of the GRK family, which specifically phosphorylate the agonist-occupied receptor, promoting the subsequent high-affinity binding of arrestins. For most GPCRs, the specificity of GRKs and arrestins in cells remains poorly defined. In this report, we used a siRNA-based approach in HEK293 cells to characterize the role of these proteins in M_3 mAchR signaling. We found that the M_3 mAchR displays a complex pattern of regulation, such that GRK2, GRK3, GRK6, arrestin2, arrestin3, and CK1α all participate to negatively regulate calcium signaling upon receptor activation.

Previously, it was shown that GRK2 can be recruited to and phosphorylate the M_3 mAchR at two separate serine clusters within the third intracellular loop (Wu et al., 2000). In addition to receptor phosphorylation, GRK2 is able to bind both GTP-bound Gα_q (Carman et al., 1999) and free G$\beta\gamma$ (Pitcher et al., 1992). The crystal structure of GRK2 (Tesmer et al., 2005) suggests that it may simultaneously sequester both active Gα_q and free G$\beta\gamma$, which in addition to receptor phosphorylation may increase the strength and effectiveness of GRK2-mediated receptor regulation. Previously, we and others demonstrated that GRK2 regulated GPCRs, such as the H1 histamine (Iwata et al., 2005), M_1 mAchR (Willets et al., 2005), metabotropic glutamate (Dhami et al., 2005) and mouse cytomegalovirus GPCR M33 (Sherrill and Miller, 2006), involved the regulation of G_q. Studies analyzing GRK-mediated regulation of the M_3 mAchR in

SH-SY5Y cells have shown that GRK3 and GRK6 differentially regulate the receptor whereas GRK2 and GRK5 did not appear to be involved (Willets et al., 2001; Willets et al., 2002; Willets et al., 2003). Overexpressed GRK3 could phosphorylate the M_3 mAchR, however, GRK3-mediated regulation appeared to be the result of altering the activity of PLC-β and not via receptor phosphorylation (Willets et al., 2001; Willets et al., 2002; Willets et al., 2003). In contrast, overexpressed GRK6 could phosphorylate the M_3 mAchR leading to a decrease in signaling. This effect was reversed upon expression of a kinase dead GRK6 (Willets et al., 2003).

Using siRNA coupled with stable expression of low levels of various GRK2 mutants, we found that the enhanced calcium mobilization observed upon GRK2 knockdown is primarily due to a loss in regulation of activated G_q following M_3 mAchR stimulation (Figure 8). Furthermore, we showed that loss of GRK2 leads to enhanced and prolonged activation of the ERK1/2 cascade (Figure 10). The observed effects of GRK2 knock down are 2-fold: the enhanced calcium mobilization seems to be primarily due to the loss of inhibition of activated G_q, whereas the enhanced and prolonged activation of ERK1/2 probably reflects enhanced DAG production/PKC-δ activation and a relief of inhibition of mitogen activated protein kinase kinase 1 (MEK1) (Jiménez-Sainz et al., 2006). However, we cannot completely rule out the possibility that GRK2 also mediates receptor phosphorylation since endogenous M_3 mAchR levels are too low to evaluate phosphorylation (Tovey and Willars, 2004).

We have also found that GRK3 and GRK6 negatively regulate calcium mobilization following M_3 mAchR stimulation. While knockdown of either kinase led to significant increases in calcium mobilization (Figure 8A and 8B), silencing of GRK3 had no effect on activation of ERK1/2 while loss of GRK6 had only a minor effect (Figure 10A and 10B). The possibility exists that there is overlap between these kinases and that regulation might involve a competition for receptor binding as has been suggested for the angiotensin receptor (Kim et al., 2005). These previous studies suggested that GRK2 and GRK3 negatively regulate while GRK5 and GRK6 positively regulate ERK1/2 activation and that differences in the phosphorylation pattern mediated by GRK2/3 or GRK5/6 could alternatively promote the binding of arrestin2 or arrestin3, respectively (Kim et al., 2005). However, our results suggest that the M_3 mAchR is not subject to this type of overlapping regulation. Furthermore, the GRKs do not play a positive role in M_3 mAchR signaling. There is a growing number of non-receptor substrates that have been identified for the GRKs (Ribas et al., 2007), and in line with previous findings, GRK3 could be primarily regulating PLC-β activity via binding to $G\beta\gamma$ or $G\alpha_q$ (Willets et al., 2001). This might allow for a very rapid and robust production of IP_3 and subsequent calcium release that is not evident at later time points because other kinases (e.g., GRK6) may phosphorylate the receptor resulting in desensitization. In additional, mechanisms regulating downstream signaling events (e.g., IP_3 hydrolysis, calcium reuptake, etc) also shape both calcium mobilization and ERK1/2 activation responses following carbachol stimulation. As we have identified three GRKs that are involved in M_3 mAchR

regulation, multiple proteins may need to be knocked down simultaneously to produce more prolonged signaling.

We reported previously that an ~50% reduction in arrestin levels using antisense strategies had no effect on calcium mobilization in HEK293 cells (Mundell and Benovic, 2000). In the present study, we were able to reduce protein levels by ~90% and show that the loss of either arrestin2 or arrestin3 enhanced the peak calcium transient seen upon activation of the M_3 mAchR (Figure 9, C and D). Taking into consideration previous reports demonstrating that the M_3 mAchR internalizes in an arrestin-independent manner (Lee et al., 1998), our results suggest that arrestins primarily mediate desensitization of the M_3 mAchR following agonist activation. Consistent with this and with previous reports (Budd et al., 1999), knockdown of either arrestin2 or arrestin3 also enhanced ERK1/2 activation (Figure 10, C and D). This is in contrast to the emerging paradigm that has been proposed for a number of other GPCRs where arrestins promote G protein-independent signaling pathways (reviewed in Lefkowitz and Shenoy, 2005) or even have opposing effects to one another as has been shown for the angiotensin II receptor (Ahn et al., 2004). In light of the fact that HEK293 cells express similar levels of endogenous arrestin2 and arrestin 3 (J.L.B, unpublished results), our data suggest an inherent specificity for the M_3 mAchR by arrestin3 as both calcium mobilization and ERK activation were enhanced and prolonged with arrestin3 knockdown. This also suggests that the PLC-β/PKC arm of signaling is responsible for ERK activation, consistent with previous reports (Budd et al., 1999; Kim et al., 1999; Wylie et al., 1999). It is

interesting that arrestins can also terminate muscarinic receptor signaling by recruiting diacylglycerol kinases and enhancing the degradation of the second messenger DAG, thereby coordinately terminating GPCR/G protein interaction and second messenger generation (Nelson et al., 2007). Taken together, the prolonged ERK activation observed following GRK2 and arrestin3 knockdown can be attributed to enhanced G_q activity, sustained DAG production and subsequent PKC-δ activation (Figure 12).

CK1α has a variety of functions within the cell (Knippschild et al., 2005) and recently has been shown to regulate heterologously expressed M_3 mAchR in HEK293 and COS7 cells (Budd et al., 2000; Tobin et al., 1997). These studies showed that CK1α phosphorylated the receptor in an agonist dependent manner, and that deletion of a portion of the third intracellular loop or transient expression of a dominant-negative CK1α construct caused an increase in IP_3 production upon receptor stimulation. Similarly, in the present study, we demonstrate that CK1α knockdown results in enhanced calcium mobilization upon M_3 receptor activation, suggesting that CK1α is also involved in desensitization of endogenous M3 mAchR in HEK293 cells. Knockdown of CK1α had no effect on calcium mobilization upon H1 histamine receptor activation, demonstrating that this effect was specific to the M_3 mAchR. Previous studies have also shown that expression of a peptide corresponding to the CK1α binding region or overexpression of a mutated receptor lacking a portion of the third intracellular loop led to a decrease in ERK1/2 activation upon receptor stimulation, suggesting that CK1α-mediated phosphorylation was necessary for ERK

activation (Budd et al., 2001). While we show that knockdown of CK1α has no effect on ERK1/2 activation (Figure 11, C and D), indicating CK1α only plays a partial role in regulation of M_3 mAchR similar to GRK3 and GRK6, this may be due to the fact that we only achieved ~60% knockdown of CK1α. It is interesting to note that the peptide expressed in previous studies to sequester CK1α also contained a portion of the Gβγ binding site of the third intracellular loop (Budd et al., 2001; Wu et al., 2000). While free Gβγ was preferred, the heterotrimeric G protein complex could also bind to this region (Wu et al., 2000). Therefore, overexpression of this peptide could result in sequestration of the G protein, decreasing activation of downstream signaling. The third intracellular loop of the M_3 mAchR contains 12 putative CK1α phosphorylation motifs (Tobin, 2002), two of which overlap with the proposed GRK2 phosphorylation sites (Wu et al., 2000). Thus, under physiological conditions, there could be competition between these kinases for receptor binding and phosphorylation.

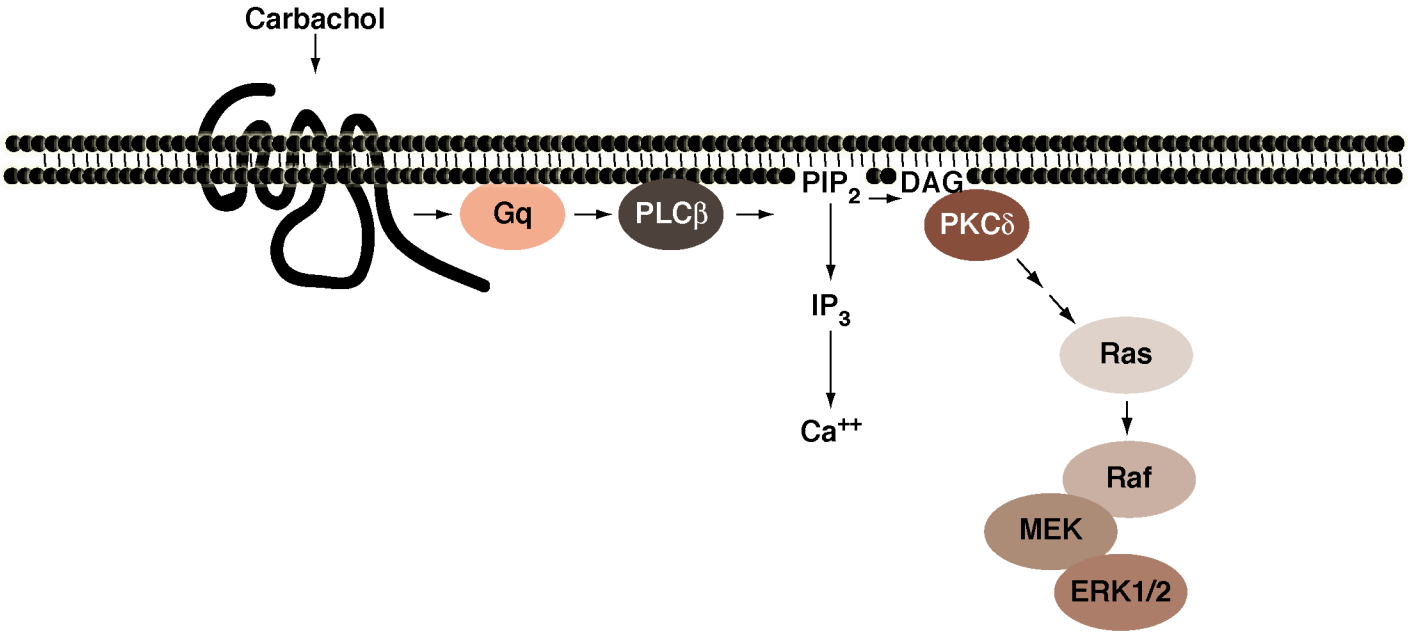
In this study, we demonstrate that multiple proteins coordinately regulate the activity of the endogenous M_3 mAchR in HEK293 cells (Figure 12). Knockdown of GRK2, GRK3, GRK6, and CK1α, but not GRK5, enhanced receptor calcium signaling, suggesting that multiple kinases regulate downstream signaling following M_3 mAchR activation. The effect of GRK2 on calcium flux could be enhanced by both wild type and a kinase-dead mutant but not by Gaq-binding defective mutants demonstrating that GRK2 primarily regulates activated Gq. Interestingly, only silencing of GRK2 led to both an enhanced and prolonged ERK activation. Consistent with our findings that GRK2 primarily regulated Gq

activity, this is likely a result of enhanced activation of the Gq/PLC-β/PKC-δ signaling pathway. Finally, both arrestin2 and arrestin3 are involved in negatively regulating the M_3 mAchR as knockdown of either protein enhanced calcium mobilization and ERK activation. Overall, our data suggest that multiple proteins dynamically regulate M_3 mAchR-mediated signal transduction.

Figure 12. Regulation of the endogenous M_3 mAChR in HEK293 cells

A) carbachol binding to the M_3 mAChR results in activation of the G_q family of heterotrimeric G proteins, leading to the dissociation of G_q and $G\beta\gamma$. Activated G_q activates PLC-β, resulting in the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to form the second messengers IP_3 and DAG. IP_3 interacts with the IP_3 receptor located at the endoplasmic reticulum, resulting in a robust but transient increase in cytosolic calcium. The formation of DAG recruits and activates the novel PKC isoform PKC-δ. Once activated, PKC-δ leads to the activation of a Ras-Raf-MEK-ERK1/2 cascade. *B*) phosphorylation of the M_3 mAChR by GRK6 and possibly CK1α recruits arrestin2 and arrestin3 to the receptor, preventing further G protein activation and terminating signaling. In addition, arrestins are able to recruit diacylglycerol kinases (DGK) to the membrane and terminate the PKC-dependent arm of the signaling cascade. GRK2 and GRK3, through a conserved RGS domain, are able to interact with and sequester free G_q and prevent activation of PLC-β. This results in the inhibition of both calcium mobilization and activation of the ERK1/2 cascade. GRK2 is also able to regulate the activation of the ERK1/2 cascade by interacting with and negatively regulating the activity of MEK1.

A.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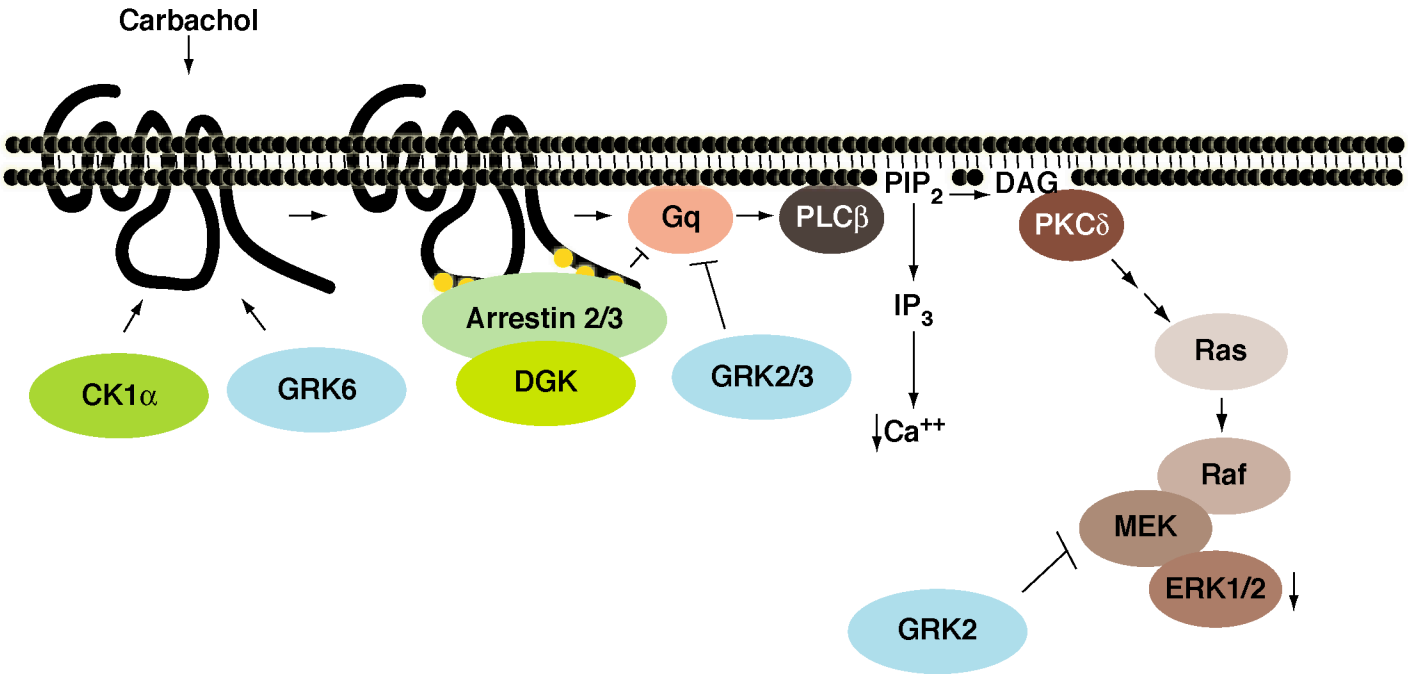
Gq
PLCβ
PIP2
DAG
PKCδ
IP3
Ca++
Ras
Raf
MEK
ERK1/2

B.


Carbachol
CK1α
GRK6
Arrestin 2/3
DGK
Gq
PLCβ
GRK2/3
PIP2
DAG
PKCδ
IP3
Ca++
Ras
Raf
MEK
ERK1/2
GRK2

FOOTNOTES

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JL and JMB contributed equally to this work.

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Chapter III

Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling

John M. Busillo and Jeffrey L. Benovic

INTRODUCTION

CXCR4 is a widely expressed chemokine receptor that is essential for development, hematopoiesis, organogenesis, and vascularization (Busillo and Benovic, 2007). CXCR4 also plays a prominent role in a number of diseases including WHIM syndrome (Hernandez et al., 2003), HIV-1 entry (Feng et al., 1996) and cancer progression and metastasis (Zlotnik, 2006). Interestingly, WHIM syndrome is the direct result of C-terminal truncations of CXCR4 which result in enhanced receptor function (Diaz and Gulino, 2005). Moreover, CXCR4 is expressed in at least 23 different types of cancer (Balkwill, 2004) and accumulating evidence suggests that this results in dysregulation of CXCR4 transcription, signaling and trafficking (Busillo and Benovic, 2007).

Protein phosphorylation is the most prevalent post-translational modification and plays a major role in regulating protein function (Cohen, 1992; Manning et al., 2002). Importantly, phosphorylation is one of the earliest events in regulating G protein-coupled receptor [GPCR] signaling, initiating a process known as desensitization (Krupnick and Benovic, 1998; Pitcher et al., 1998). Agonist-promoted desensitization is primarily mediated by members of the GPCR kinase [GRK] family, which specifically phosphorylate agonist-occupied GPCRs (Krupnick and Benovic, 1998; Pitcher et al., 1998; Gainetdinov et al., 2004). This results in the recruitment and high affinity binding of arrestins, which function to uncouple the receptor from G protein, target receptors for internalization, and promote G protein-independent signaling (DeWire et al., 2007; Moore et al., 2007). While the specific protein kinases that mediate phosphorylation of

individual GPCRs have not been well defined, site-specific and tissue-specific phosphorylation of GPCRs likely have distinct effects on signaling (Tobin et al., 2008).

Upon activation, CXCR4 is rapidly phosphorylated within its 45 amino acid serine/threonine-rich C-terminal tail. Previous studies have suggested a number of potential phosphorylation sites critical for agonist (CXCL12)- and PKC-mediated receptor internalization (Signoret et al., 1998; Orsini et al., 1999) and degradation (Marchese and Benovic, 2001). In addition, GRK2 (Orsini et al., 1999; Cheng et al., 2000; Jiminez-Sainz et al., 2006), GRK3 (Balabanian et al., 2008), GRK6 (Fong et al., 2002; Vroon et al., 2004), and PKC (Signoret et al., 1997; Orsini et al., 1999) have been implicated in CXCR4 regulation, although the sites of phosphorylation, the kinases involved in the phosphorylation of specific sites, and the functional role of site-specific phosphorylation remain largely unknown.

In order to better understand the role of phosphorylation in regulating CXCR4 signaling, we sought to identify agonist-promoted sites of phosphorylation and the kinases that mediate site-specific phosphorylation. Using liquid chromatography tandem mass spectrometry [LC/MS/MS] and phospho-specific antibodies, we identified seven serine residues that are phosphorylated in response to CXCL12 stimulation. We show that phosphorylation of these sites occurs with distinct kinetics and kinase specificity: namely Ser-324/325 phosphorylation is rapid, transient and is primarily mediated by PKC and GRK6; Ser-330 phosphorylation is delayed and is mediated by

GRK6; and Ser-339 is phosphorylated rapidly by GRK6. Finally, we show that GRK-mediated phosphorylation of CXCR4 and arrestin binding have differential effects on calcium mobilization and ERK1/2 activation following CXCR4 activation.

RESULTS

Phospho-site mapping of CXCR4 by mass spectrometry

CXCR4 is rapidly phosphorylated and internalized following agonist-activation (Haribabu et al., 1997; Signoret et al., 1997; Orsini et al., 1999). Truncation of the C-terminal tail of CXCR4, which contains 15 serine and 3 threonine residues, eliminates agonist-promoted phosphorylation, attenuates internalization, and enhances receptor activity (Haribabu et al., 1997; Signoret et al., 1997). Since alanine scanning mutagenesis suggested that multiple regions of the C-tail may be phosphorylated following CXCL12 stimulation (Orsini et al., 1999), we decided to use mass spectrometry to better define the specific sites phosphorylated in CXCR4. We initially made a cell line stably expressing Flag-tagged CXCR4 to enable rapid purification of the receptor, as previously demonstrated for the β_2-adrenergic receptor (β_2AR) (Trester-Zedlitz et al., 2005). HEK293 cells were chosen as a model cell because they express CXCR4 endogenously, though at very low levels (~20 fmol/mg membrane protein). A clonally selected HEK293 cell line expressing Flag-tagged CXCR4 at ~0.5 pmol/mg (termed Flag CXCR4 cells) was chosen for further study. CXCL12 stimulation of endogenous CXCR4 in HEK293 cells resulted in robust calcium

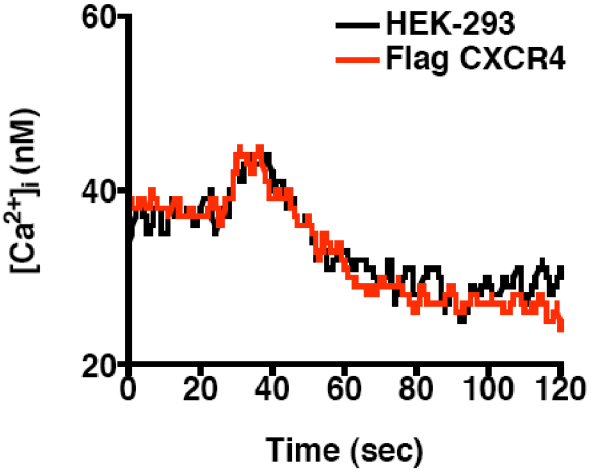
mobilization and ERK1/2 activation (Figures 13A and B), although we were unable to detect CXCL12-mediated inhibition of cAMP production or activation of p38 or AKT (data not shown). Stable-expression of CXCR4 did not enhance CXCL12-mediated calcium mobilization (Figure 13A) but did lead to an ~2.5 fold increase in activation of ERK1/2 (Figure 13B). In addition, as an indirect measure of receptor phosphorylation, we looked at the ability of CXCL12 to induce an electrophoretic mobility shift of CXCR4 on SDS-PAGE. Stimulation of either HEK293 cells (Figure 13C) or Flag CXCR4 cells (Figure 13D) resulted in a rapid retardation of electrophoretic mobility, consistent with receptor phosphorylation. Thus, the Flag CXCR4 cells appear to be a good model system in which to further characterize CXCR4 phosphorylation.

Mass spectrometry has become a valuable tool for identifying amino acids that are post-translationally modified (Carr et al., 2005), a strategy recently employed for the β_2AR (Trester-Zedlitz et al., 2005). Since phosphorylation adds ~80 daltons [Da] to the molecular mass of a peptide, peptides with changes of 80 Da (or multiples thereof) from the theoretical mass can be identified, trapped and subsequently fragmented by MS/MS to provide site-specific information on phosphorylation (Carr et al., 2005). To identify sites of phosphorylation on CXCR4, Flag CXCR4 cells were treated with CXCL12 for 10 min and the receptor was then affinity purified on an anti-Flag column (Figure 14A). This procedure resulted in ~80% recovery of the receptor and yielded ~0.5 mg of purified CXCR4 per preparation (Figure 14B). Duplicate samples of purified

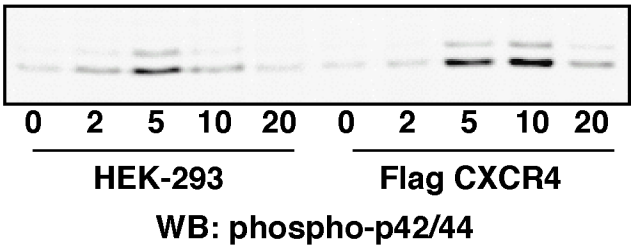
Figure 13. Establishing and characterizing HEK293 cells that stably express CXCR4

A) HEK293 cells or cells stably expressing Flag CXCR4 (Flag CXCR4 cells) were loaded with the ratiometric calcium indicator Fura-2/AM prior to stimulation with CXCL12 (100 nM). The change in intracellular calcium was calculated by monitoring the change in fluorescence of Fura-2/AM. Shown is a representative trace of calcium mobilization from three independent experiments. *B)* Following a 6 hr serum starvation, HEK293 cells or Flag CXCR4 cells were stimulated with CXCL12 for the times indicated. Shown in a representative Western blot from three independent experiments. CXCL12-promoted retardation of electrophoretic mobility of endogenous CXCR4 *(C)* or Flag tagged CXCR4 *(D)*. Following a 6 hr serum starvation, cells were stimulated with 100 nM CXCL12 for the indicated time. Crude membranes were prepared and 50 μg of solubilized protein (endogenous) or equal volume of whole cell lysate (Flag CXCR4) was separated by 10% SDS-PAGE. Shown is a representative Western blot from four independent experiments.

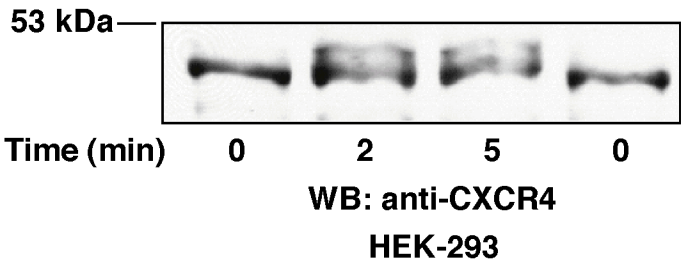
A.


HEK-293
Flag CXCR4
60
40
20
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (sec)

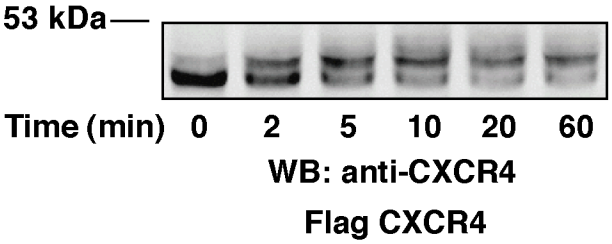
B.


0 2 5 10 20 0 2 5 10 20
HEK-293
Flag CXCR4
WB: phospho-p42/44

C.


53 kDa
Time (min) 0 2 5 0
WB: anti-CXCR4
HEK-293

D.


53 kDa
Time (min) 0 2 5 10 20 60
WB: anti-CXCR4
Flag CXCR4

CXCR4 were then digested with either Lys-C or chymotrypsin and the resulting peptides were subjected to LC/MS/MS. This identified peptides containing 38 of the 45 C-terminal residues of CXCR4 including residues 310-328 and 334-352, and several of the peptides were phosphorylated (Table 1). Figure 14C shows a representative mass spectrum of a peptide (Thr-318 to Leu-328) obtained from a chymotryptic digest, which contains five potential phosphorylation sites (Thr-318, Ser-319, Ser-321, Ser-324, and Ser-325). The peak occurring with a mass ratio of ~600 demonstrates that this peptide has two phosphates attached (M-$(H_3PO_4)_2$). The loss of phosphates upon fragmentation was apparent when Ser-324 and Ser-325 were present in the fragment (see peaks b9-$(H_3PO_4)_2$ and [b10-$(H_3PO_4)_2]^{2+}$) but not in a peptide containing Thr-318, Ser-319 and Ser-321 (peak b4), suggesting that both Ser-324 and Ser-325 are phosphorylated. These results were confirmed from two separate experiments as well as with peptides derived from Lys-C digestion (Table 1). Since peptides containing Ser-325 phosphorylation alone were also observed, we speculate that Ser-325 is phosphorylated before Ser-324 although we were unable to generate a pS325-specific antibody to directly test this. Overall, these studies identified six sites of phosphorylation following a 10 min stimulation with CXCL12: Ser-321, Ser-324, Ser-325, one residue from Ser-338 to Ser-341, one residue from Ser-346 to Ser-348, and either Ser-351 or Ser-352 (Figure 14D). The specific sites of phosphorylation that occur from Ser-338 to Ser-352 were not able to be specifically determined by LC/MS/MS due to the serine-rich nature of this region.

Figure 14. Purification and mass spectrometry analysis of CXCR4

A) Flag CXCR4 was purified from five 15-cm plates following a 10 min stimulation with 50 nM CXCL12. The bulk of the receptor (~80%) elutes in fractions 2 and 3 and is highly purified as shown by Coomassie blue staining *(B)*. *C)* Shown is a representative mass spectrum of the peptide Thr-318 to Leu-328 following a chymotrypsin digest demonstrating that CXCR4 is phosphorylated on Ser-324 and Ser-325. *D)* Amino acid sequence of the C terminal tail of CXCR4. Residues highlighted in red are those that are predicted to be phosphorylated by mass spectrometry. Brackets under Ser-338-Ser-341, Ser-346-Ser-348, and Ser-351/Ser-352 indicate that one residue in each cluster is phosphorylated, though the exact residue was not identified by mass spectrometry.

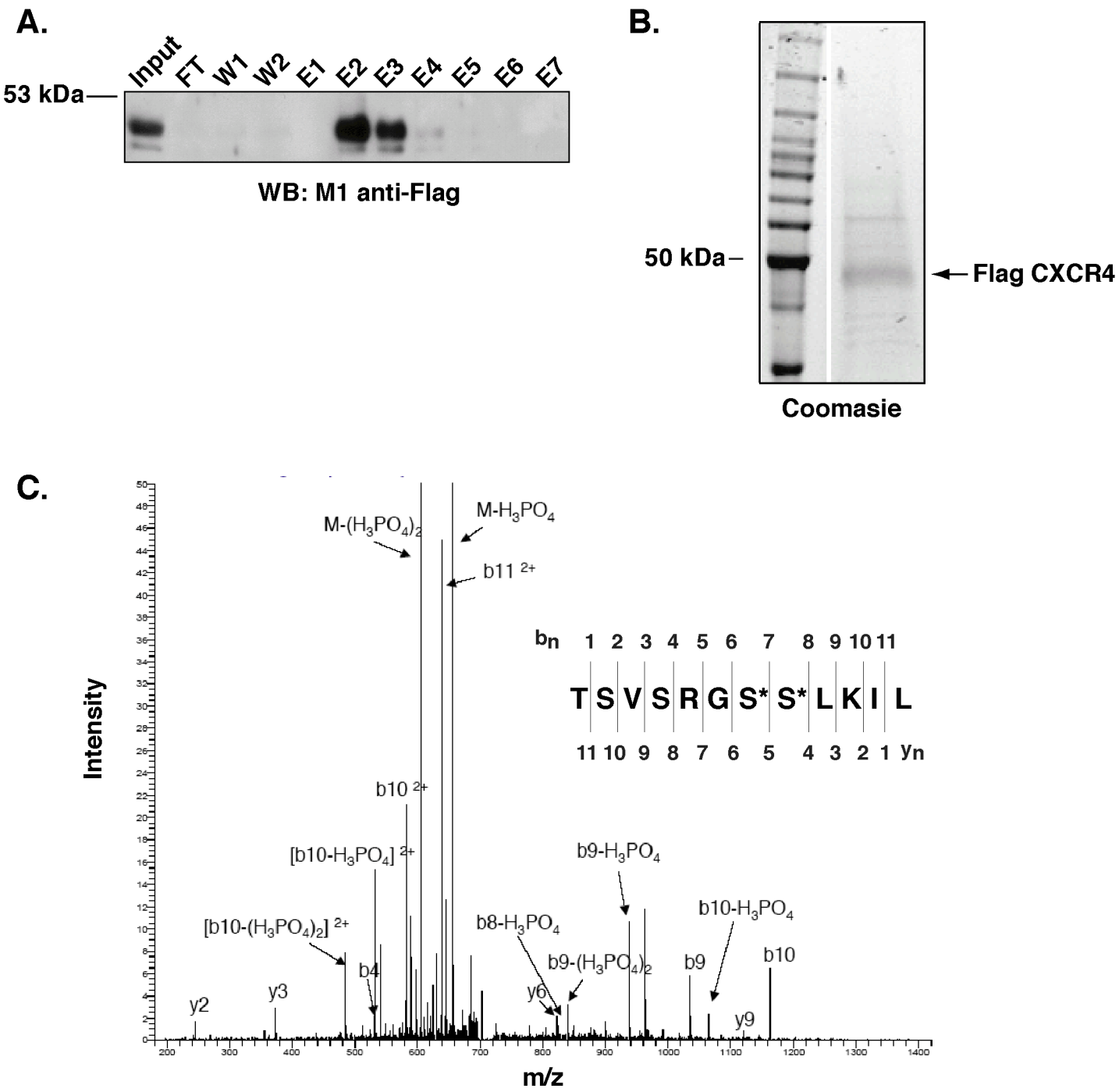

A.
Input
FT
W1
W2
E1
E2
E3
E4
E5
E6
E7
53 kDa
WB: M1 anti-Flag
B.
50 kDa
Flag CXCR4
Coomasie
C.
Intensity
M-(H3PO4)2
M-H3PO4
b11 2+
bn 1 2 3 4 5 6 7 8 9 10 11
T S V S R G S* S* L K I L
11 10 9 8 7 6 5 4 3 2 1 yn
b10 2+
[b10-H3PO4] 2+
b9-H3PO4
[b10-(H3PO4)2] 2+
b8-H3PO4
b10-H3PO4
b9-(H3PO4)2
b9
b10
b4
y3
y6
y2
y9
m/z
D.
308
320
330
340
350
KFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS

Table 1		
Lys-C Digestion		
Peptide	**Predicted m/z Ratio**	**Observed m/z Ratio**
^{311}TSAQHALTSVSRGSSLK^{326}	1730	1730
^{311}TSAQHALTSVSRGSS*LK^{326}	1730	1810
^{311}TSAQHALTSVSRGS*S*LK^{326}	1730	1890
^{311}TSAQHALTSVS*RGSSLK^{326}	1730	1810
^{311}TSAQHALTSVS*RGSS*LK^{326}	1730	1890
^{334}RGGHSSVSTESESSSFHSS^{352}	1952	1951
^{334}RGGHSSVSTESESSSFH[SS]^{352}	1952	2031
^{334}RGGHSSVSTESE[SSS]FHSS^{352}	1952	2031
^{334}RGGH[SSVS]TESESSSFHSS^{352}	1952	2031
Chymotrypsin Digestion		
Peptide	**Predicted m/z Ratio**	**Observed m/z Ratio**
^{316}ALTSVSRGSSLKIL^{328}	1432	1432
^{316}ALTSVSRGSS*LKIL^{328}	1432	1511
^{316}ALTSVSRGS*S*LKIL^{328}	1432	1591
^{316}ALTSVSRGSSL^{326}	1077	1077
^{316}ALTSVSRGSS*L^{326}	1077	1157
^{318}TSVSRGSSLKIL^{328}	1248	1248
^{318}TSVSRGS*S*LKIL^{328}	1248	1408
^{310}KTSAQHALTSVSRGSSLKIL^{328}	2084	Not observed
^{310}KTSAQHALTSVSRGS*S*LKIL^{328}	2084	2244

*** Denotes phosphorylated residues as predicted by LC/MS/MS**

[ ] Denotes one of the highlighted residues is phosphorylated as predicted by LC/MS/MS

Protein kinase C is primarily responsible for phosphorylation of Ser-324/5

Previous mutagenesis studies have identified Ser-324/5 as critical for CXCL12-induced internalization (Orsini et al., 1999) and degradation (Marchese and Benovic, 2001) as well as PMA-promoted internalization (Signoret et al., 1997; Orsini et al., 1999). To further characterize Ser-324/5 phosphorylation, we generated a phospho-specific antibody to evaluate the kinetics of phosphorylation and kinase-specificity for these residues. A 10 min stimulation with CXCL12 results in robust phosphorylation of Ser-324/5 that was blocked by pre-incubation with the immunizing phospho-peptide but not with vehicle or unphosphorylated peptide (Figure 15A). Flag CXCR4 cells were then stimulated for various times with CXCL12 and cell lysates were electrophoresed and blotted with anti-pS324/5 to assess the kinetics of phosphorylation. Phosphorylation of Ser-324/5 rapidly increased peaking at ~4-fold over basal within 5-10 min and returned to near basal levels within 60 min (Figure 15B). Furthermore, the increase in pS324/5 immuno-reactivity parallels the observed reduction in electrophoretic mobility of CXCR4 following stimulation with CXCL12 (Figures 13D and 15B).

CXCR4, like other chemokine receptors, primarily couples to the Gi family of heterotrimeric G proteins (Busillo and Benovic, 2007). In order to determine if G protein activation was involved in receptor phosphorylation, cells were pretreated with pertussis toxin prior to CXCL12 stimulation. Pertussis toxin pretreatment significantly attenuated both the rate and extent of Ser-324/5 phosphorylation (Figure 15C). This suggests the possibility that activation of

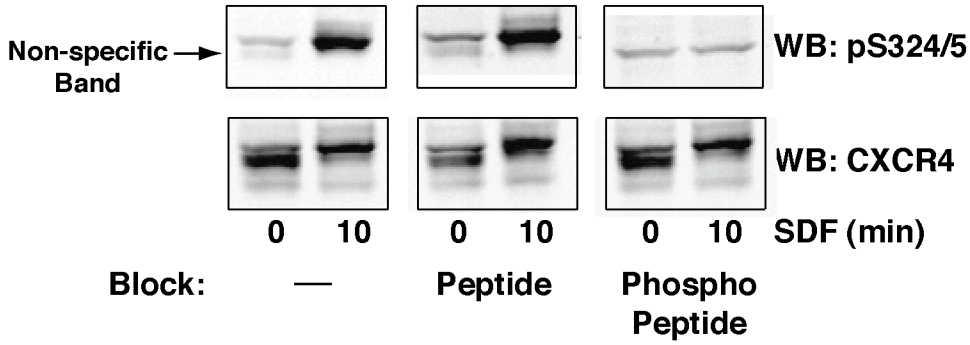
second messenger dependent kinases such as PKC are largely responsible for phosphorylation of these residues. Indeed, the primary amino acid sequence of CXCR4 reveals that both Ser-324 and Ser-325 fall within a PKC consensus motif (RGSSLK).

To initially address the role of PKC in agonist-promoted phosphorylation of Ser-324/5, cells were pretreated with the broad-spectrum PKC inhibitor Bisindolylmaleimide I [Bis I] or the negative control Bisindolylmaleimide V [Bis V] (Toullec et al., 1991) prior to CXCL12 stimulation. Bis I treatment led to a significant reduction in basal phosphorylation as well as CXCL12-promoted phosphorylation of Ser-324/5 (Figure 16A). Since Bis I inhibits conventional and novel PKC isoforms, we attempted to better define the PKC subtype(s) involved in Ser-324/5 phosphorylation by pretreating cells with either Gö 6976, which inhibits the conventional PKC isoforms (α, β_I, and β_{II}) (Martiny-Baron et al., 1993) or rottlerin, which is reported to inhibit PKCδ (Gschwendt et al., 1994). Treatment with rottlerin, but not with Gö 6976, led to a significant reduction of Ser-324/5 phosphorylation (Figure 16B). However, since rottlerin can have off-target effects (Soltoff, 2007), we attempted to confirm the role of PKCδ in CXCR4 phosphorylation using siRNA treatment. PKCδ levels could be effectively reduced in Flag CXCR4 cells by siRNA treatment and this resulted in a partial but significant decrease in Ser-324/5 phosphorylation (Figure 16C). In contrast, knock down of PKCα had no effect on Ser-324/5 phosphorylation (Figure 16C). Taken together, these data demonstrate that PKC plays a major role in phosphorylating Ser-324/5 following CXCL12 stimulation. The significant effect

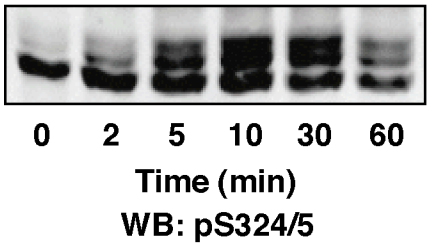
Figure 15. Characterization and pertussis toxin sensitivity of anti-pSer-324/5 (pS324/5)

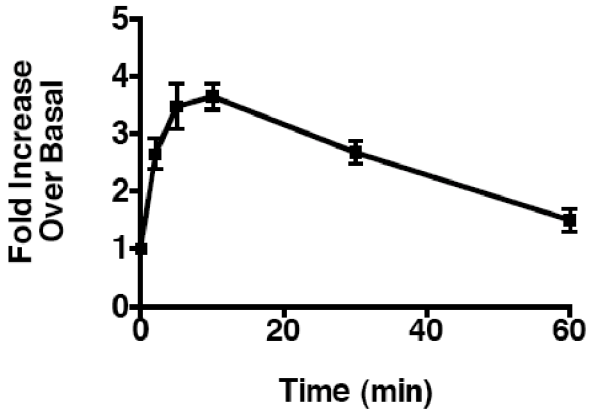
A) Shown is a representative Western blot demonstrating the specificity of the pS324/5 antibody. 10 μg of purified antibody was incubated for 10 min with vehicle (PBS), 10 μg of peptide (C-Ahx-RGSSLKIL) or 10 μg of phospho-peptide (C-Ahx-RG(pS)(pS)LKIL) prior to overnight incubation with the nitrocellulose blots. *B)* Cells stably expressing Flag CXCR4 were stimulated at the time points indicated with 100 nM CXCL12. Lysates were processed and separated to visualize the agonist promoted gel shift of CXCR4. Blots were incubated overnight with 1:1000 dilution of crude pS324/5 antibody. pS3245 was normalized to total CXCR4 and data is presented as fold increase over basal (± S.E.M., n=4). *C)* Cells stably expressing Flag CXCR4 were treated overnight with vehicle (PBS) or pertussis toxin (100 ng/ml) prior to stimulation with 100 nM CXCL12. Left panel: representative Western blot using purified anti-pS324/5. Right panel: pS324/5 was normalized to total CXCR4 and data is presented as percent maximum of vehicle treated cells (± S.E.M., n=3; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

A.

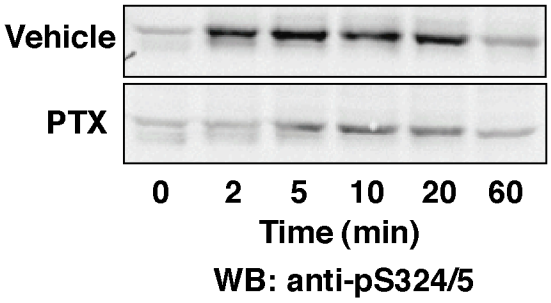

Non-specific Band
WB: pS324/5
WB: CXCR4
0 10 0 10 0 10 SDF (min)
Block: — Peptide Phospho Peptide

B.


0 2 5 10 30 60
Time (min)
WB: pS324/5


Fold Increase Over Basal
0 1 2 3 4 5
0 20 40 60
Time (min)

C.


Vehicle
PTX
0 2 5 10 20 60
Time (min)
WB: anti-pS324/5

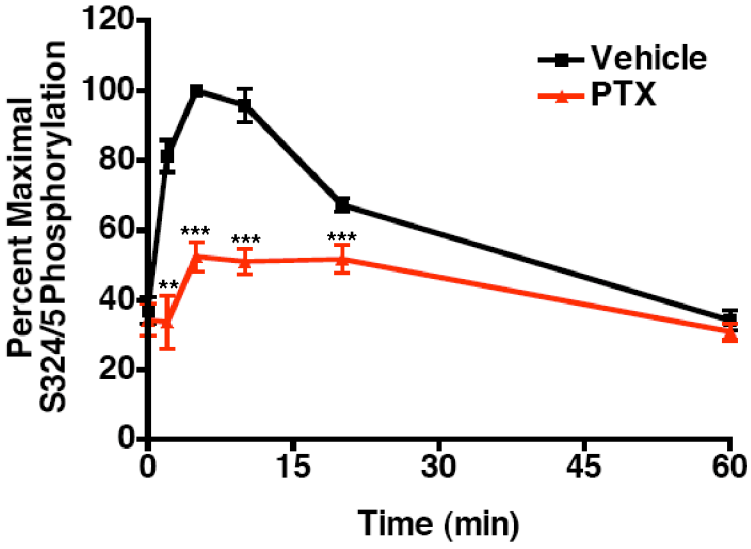
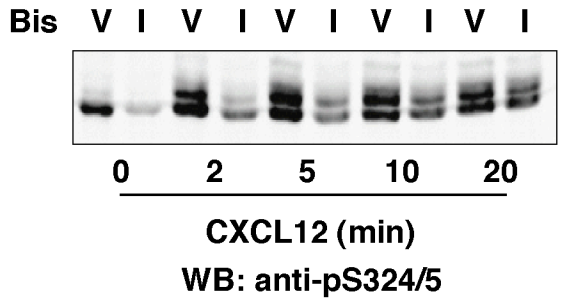
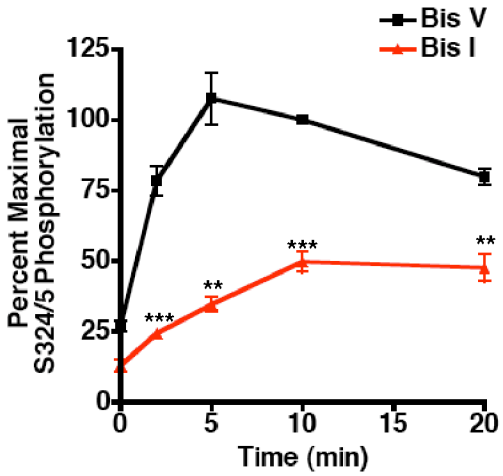

Percent Maximal S324/5 Phosphorylation
0 20 40 60 80 100 120
0 15 30 45 60
Time (min)
Vehicle
PTX
**
***
***
***

Figure 16. PKC is primarily responsible for Ser-324/5 phosphorylation following CXCL12 stimulation

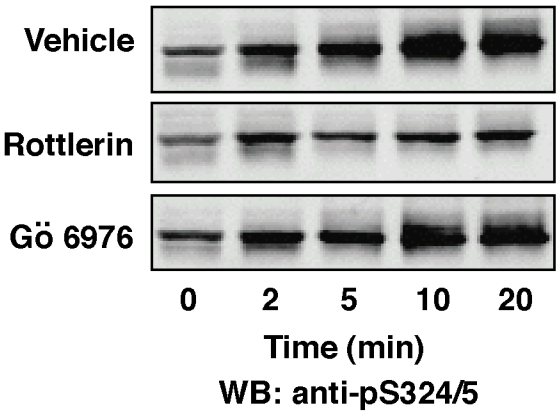
A) Cells stably expressing Flag CXCR4 were serum starved for 6 hr. 30 min prior to CXCL12 stimulation, cells were pretreated with 2.5 μM Bis I or Bis V. Left panel: representative Western blot using crude anti-pS324/5 antibody. Blots were processed as to visualize the gel shift. Right panel: pS324/5 was normalized to total CXCR4 and data is presented as percent maximal Ser-324/5 phosphorylation compared to control (Bis V) (± S.E.M., n=4). *B)* To better define the PKC isoforms responsible for CXCR4 phosphorylation, cells were pretreated with vehicle (DMSO), 1 μM Gö 6976, or 5 μM Rottlerin for 30 min prior to stimulation with CXCL12. Left panel: Representative Western blot using purified anti-pS324/5 antibody. Right panel: pS324/5 was normalized to total CXCR4 and data is presented as percent maximal Ser-324/5 phosphorylation compared to control (± S.E.M., n=4). *C)* Cells were treated with PKC-specific siRNAs. Left panel: lysates were prepared 72 hr post-transfection and blotted for PKCα and PKCδ as indicated. Shown is representative Western blot demonstrating efficiency and specificity of PKCα and PKCδ knock down. Right panel: 72 hr post-transfection, cells were serum starved for 6 hr prior to stimulation with CXCL12 (± S.E.M., n=4; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

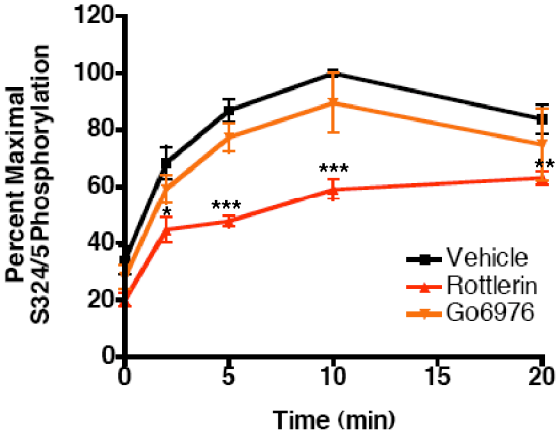
A.


Bis V I V I V I V I V I
0 2 5 10 20
CXCL12 (min)
WB: anti-pS324/5

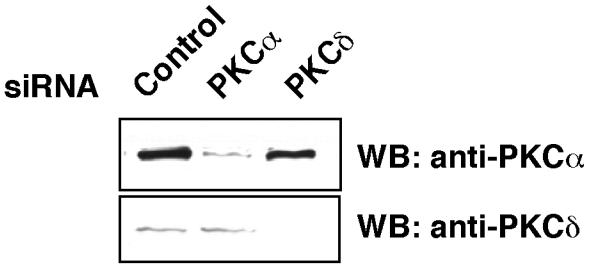

Bis V
Bis I
Percent Maximal S324/5 Phosphorylation
125
100
75
50
25
0
0 5 10 15 20
Time (min)
***
**
***
**

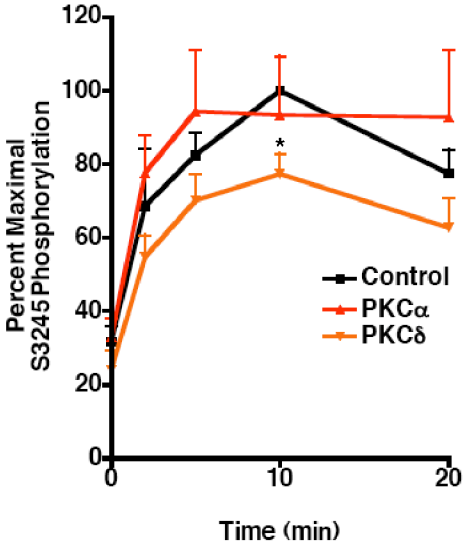
B.


Vehicle
Rottlerin
Gö 6976
0 2 5 10 20
Time (min)
WB: anti-pS324/5


Percent Maximal S324/5 Phosphorylation
120
100
80
60
40
20
0
0 5 10 15 20
Time (min)
Vehicle
Rottlerin
Go6976
*
***
***
**

C.


siRNA
Control
PKCα
PKCδ
WB: anti-PKCα
WB: anti-PKCδ


Percent Maximal S3245 Phosphorylation
120
100
80
60
40
20
0
0 10 20
Time (min)
Control
PKCα
PKCδ
*

of Bis I pretreatment coupled with the fact that knock down of PKCδ only partially decreases phosphorylation, suggests that PKCδ as well as additional PKC isoforms likely mediate CXCR4 phosphorylation.

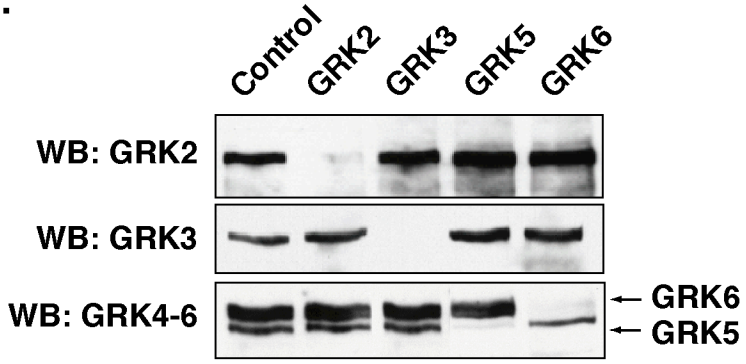
GRK6 contributes to Ser-324/5 phosphorylation

As some phosphorylation of Ser-324/5 is still evident following pertussis toxin treatment or PKC inhibition, we hypothesized that GRKs also contribute to phosphorylation of these residues. The GRKs consist of seven members, four of which (GRK2, 3, 5, and 6) are expressed in HEK293 cells (Ren et al., 2005; Luo et al., 2008). Since there are no specific GRK inhibitors available, we assessed the effect of individual GRK knock down on Ser-324/5 phosphorylation. While efficient and specific knock down of each of the individual GRKs expressed in HEK293 cells was achieved (Figure 17A), only GRK6 knock down had a significant effect on Ser-324/5 phosphorylation (Figure 17B). Since our results suggest that both GRK6 and PKC contribute to Ser-324/5 phosphorylation, we also evaluated the effect of PKC inhibition and GRK6 knock down. While PKC inhibition or GRK6 knock down alone resulted in an ~50% or ~40% reduction in phosphorylation, respectively, the combination resulted in an almost complete loss of Ser-324/5 phosphorylation (Figure 17C). These data demonstrate that both PKC and GRK6 are needed for maximal agonist-promoted phosphorylation of Ser-324/5 following CXCL12 stimulation.

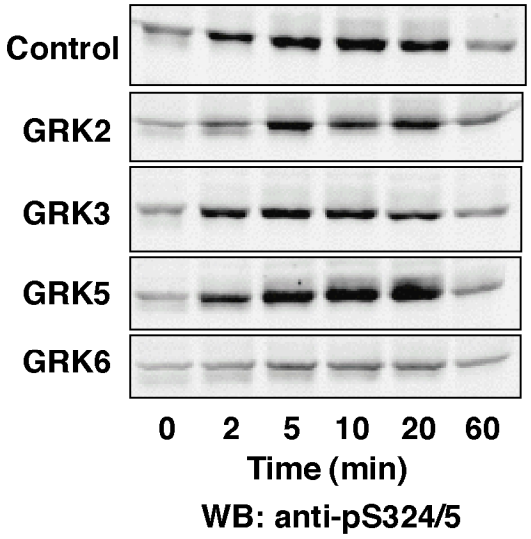
Figure 17. GRK6 contributes to Ser-324/5 phosphorylation following CXCL12 stimulation

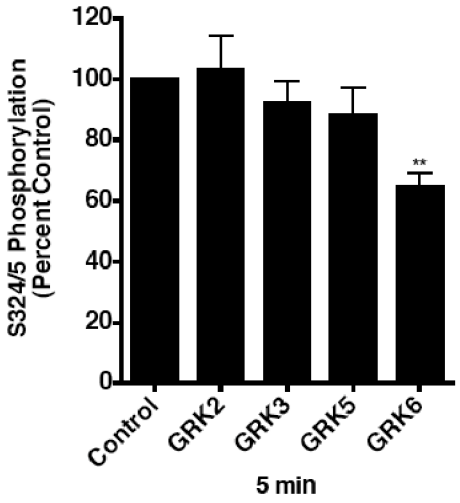
A) Representative Western blot demonstrating specific and efficient knock down of GRKs endogenously expressed in HEK293 cells 72 hr post-transfection. *B)* Knock down of GRK6, but not GRK2, GRK3 or GRK5 led to a significant reduction in phosphorylation of Ser-324/5. Left panel: representative Western blot using purified anti-pS324/5. Right panel: comparison of pS324/5 phosphorylation following a 5 min stimulation with CXCL12. pS324/5 was normalized to total CXCR4 and data is presented as percent of control at 5 min (± S.E.M., n=4). *C)* GRK6 knock down and PKC inhibition almost completely abolishes phosphorylation of Ser-324/5. Cells transfected with GRK6 siRNA were pretreated with 2.5 μM Bis I or Bis V 30 min prior to stimulation with CXCL12. Left panel: representative Western blot using purified anti-pS324/5. Right panel: pS324/5 was normalized to total CXCR4 and data is presented as percent maximal phosphorylation of Ser-324/5 as compared to control/Bis V treated cells (± S.E.M., n=3; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

A.

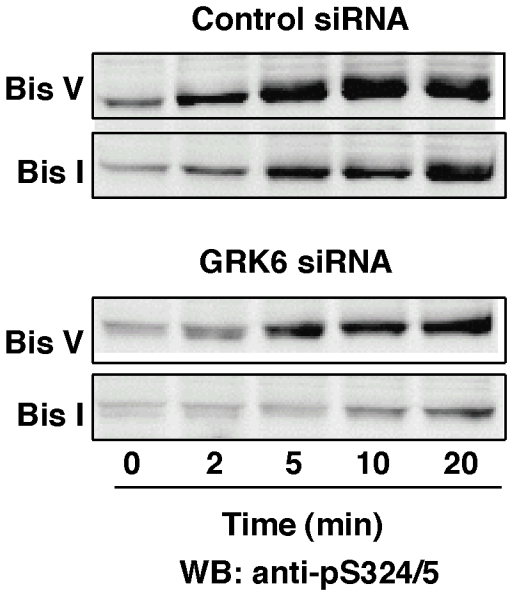

Control
GRK2
GRK3
GRK5
GRK6
WB: GRK2
WB: GRK3
WB: GRK4-6
GRK6
GRK5

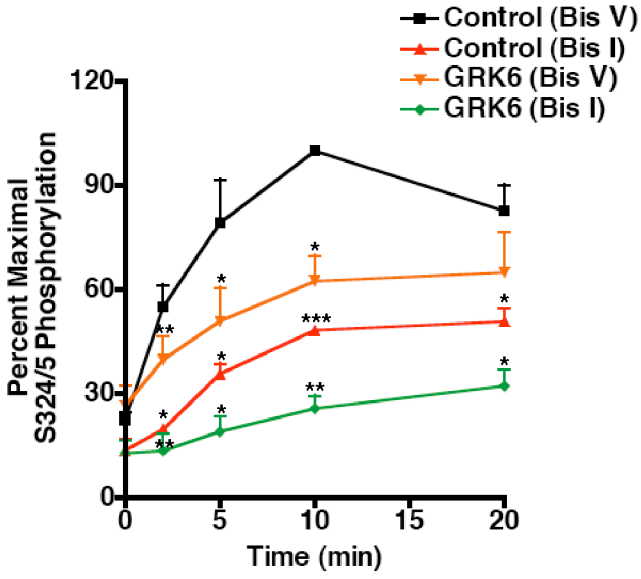
B.


Control
GRK2
GRK3
GRK5
GRK6
0 2 5 10 20 60
Time (min)
WB: anti-pS324/5


S324/5 Phosphorylation (Percent Control)
120
100
80
60
40
20
0
**
Control
GRK2
GRK3
GRK5
GRK6
5 min

C.


Control siRNA
Bis V
Bis I
GRK6 siRNA
Bis V
Bis I
0 2 5 10 20
Time (min)
WB: anti-pS324/5


Control (Bis V)
Control (Bis I)
GRK6 (Bis V)
GRK6 (Bis I)
Percent Maximal S324/5 Phosphorylation
120
90
60
30
0
0 5 10 15 20
Time (min)

Ser-330 and Ser-339 are phosphorylated by GRK6

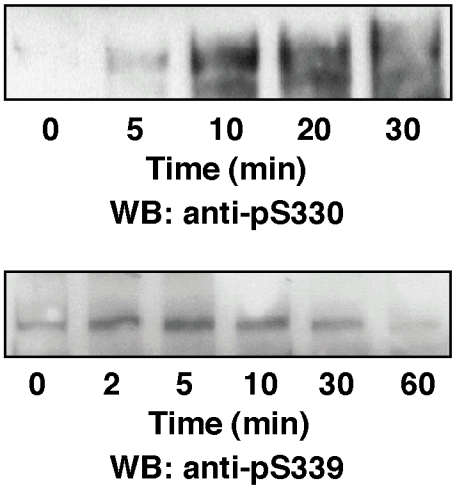
Previous studies have demonstrated a prominent role of Ser-330 in regulating CXCL12-promoted degradation (Marchese and Benovic, 2001) while Ser-339 contributes to receptor internalization (Signoret et al., 1998; Orsini et al., 1999). Although a peptide containing Ser-330 was not observed in our mass spectrometry analysis, we generated and characterized a phospho-specific antibody to this site. In addition, a phospho-specific antibody has been generated against Ser-339 (Woerner et al., 2005) and phosphorylation within this region was also detected by mass spectrometry (Figure 14D). As shown in Figure 18A, both Ser-330 and Ser-339 undergo agonist-promoted phosphorylation, albeit with different kinetics. Phosphorylation of Ser-330 is relatively slow peaking at ~20 min, whereas phosphorylation of Ser-339 is very rapid peaking at ~2 min (Figure 18A). Interestingly, despite the kinetic differences, we found that both Ser-330 and Ser-339 are primarily phosphorylated by GRK6 (Figures 18B and C).

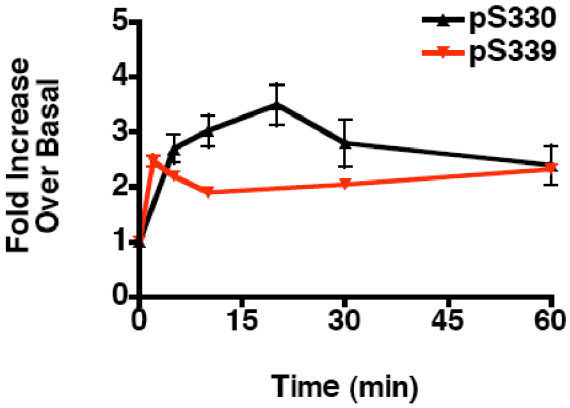
Overall, we found that GRK6 phosphorylates multiple sites within the C terminal tail of CXCR4 including Ser-324/5, Ser-330 and Ser-339 while GRK2, 3, and 5 do not contribute to CXCR4 phosphorylation at these sites. Additional studies demonstrate that PKC inhibition had no effect on CXCL12-promoted phosphorylation of Ser-330 and Ser-339 (data not shown), demonstrating that phosphorylation of these residues is completely GRK6-dependent in response to CXCL12.

Figure 18. Ser-330 and Ser-339 are GRK6 substrates

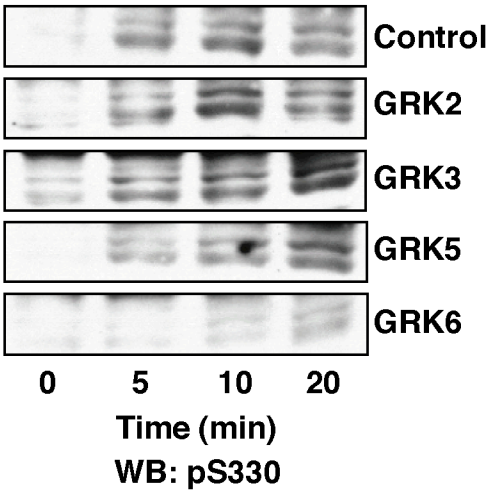
A) Cells expressing Flag CXCR4 were serum starved for 6 hr prior to stimulation with 100 nM CXCL12 for times indicated. An equal volume of lysate was separated by SDS-PAGE and blotted with purified anti-pS330 (top left panel) or anti-pS339 (bottom left panel). pS330 blots were processed in order to visualize gel shift of CXCR4. Right panel: pS330 or pS339 blotting was normalized to total CXCR4 and data presented as fold increase over basal (± S.E.M., n=4). *B)* 72 hr post-transfection, an equal volume of cell lysate was separated to visualize the gel shift of CXCR4 and blotted using purified anti-pS330. Shown are representative Western blots of four separate experiments. *C)* 72 hr post-transfection, an equal volume of cell lysate was separated by SDS-PAGE and blotted using anti-pS339. Shown are representative Western blots from four separate experiments.

A.

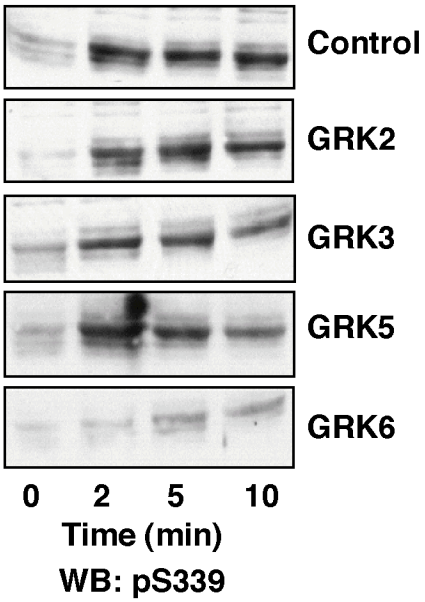

0 5 10 20 30
Time (min)
WB: anti-pS330
0 2 5 10 30 60
Time (min)
WB: anti-pS339


pS330
pS339
Fold Increase Over Basal
0
1
2
3
4
5
0 15 30 45 60
Time (min)

B.


Control
GRK2
GRK3
GRK5
GRK6
0 5 10 20
Time (min)
WB: pS330

C.


Control
GRK2
GRK3
GRK5
GRK6
0 2 5 10
Time (min)
WB: pS339

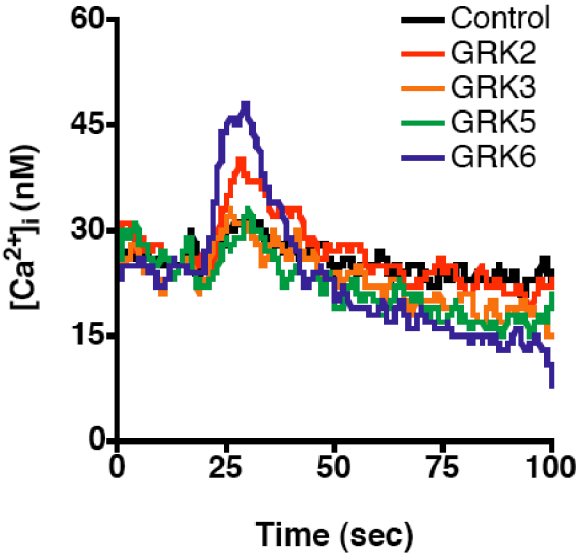
GRKs differentially regulate CXCR4 signaling in HEK293 cells

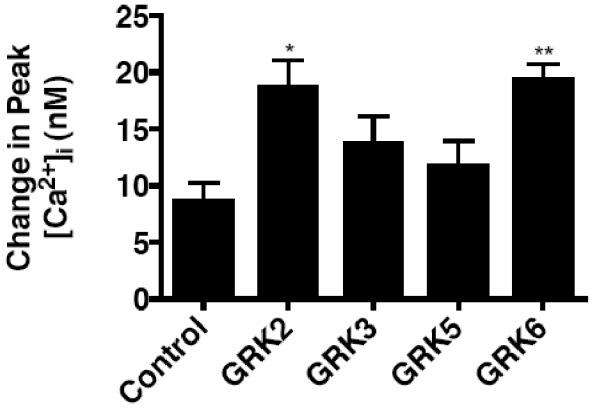
Phosphorylation of GPCRs is one of the earliest mechanisms of regulation, initiating the process of desensitization (Krupnick and Benovic, 1998). Recent evidence suggests that differential phosphorylation of GPCRs can have specific but disparate effects on receptor regulation (Kim et al., 2005; Ren et al., 2005; Torrecilla et al., 2007; Luo et al., 2008). Since CXCR4 activation in HEK293 cells leads to calcium mobilization (Figure 13A) and activation of ERK1/2 (Figure 13B), we next evaluated the functional role of GRKs in regulating CXCR4-mediated signaling. Knock down of GRK2 or GRK6, but not GRK3 or GRK5, led to a statistically significant increase in the peak calcium transient observed following CXCL12 stimulation of endogenous CXCR4 in HEK293 cells (Figure 19A). Interestingly, knock down of GRK2 led to an ~30% increase in ERK1/2 activation while knock down of GRK3 or GRK6 led to an ~40% reduction in ERK1/2 activation (Figures 19B and C). In contrast, inhibition of PKC by Bis I had no effect on activation of ERK1/2 following CXCL12 activation (data not shown).

Figure 19. GRKs differentially regulate signaling following activation of endogenous CXCR4 in HEK293 cells

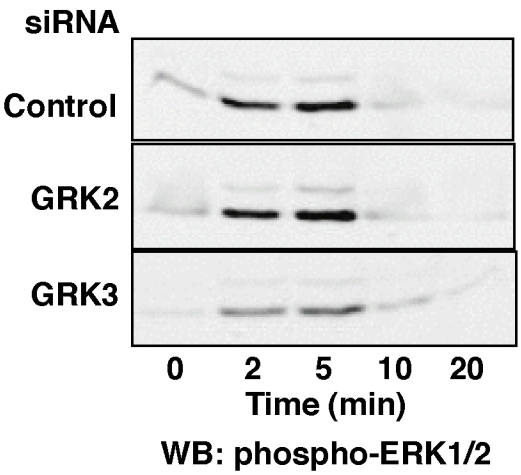
A) HEK293 cells were loaded with the ratiometric calcium indicator Fura-2A/M 72 hr after siRNA transfection. Cells were stimulated with 100 nM CXCL12 and changes in intracellular calcium were calculated from changes in fluorescence. Left panel: shown is a representative trace from six separate experiments. Right panel: mean (± S.E.M.) increase in peak calcium transient calculated from six separate experiments. *B)* Effect of GRK knock down on CXCL12-mediated activation of ERK1/2. 72 hr post-transfection, cells were serum starved for 6 hr prior to stimulation with CXCL12 (100 nM). Shown is a representative Western blot from five independent experiments. *C)* Left panel: pERK2 was normalized to total ERK2 and data are presented as percent maximal ERK2 activation as compared to control (± S.E.M., n=4). Right panel: comparison of maximal ERK2 activation (5 min) following stimulation with CXCL12 (100 nM) (± S.E.M., n=4; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

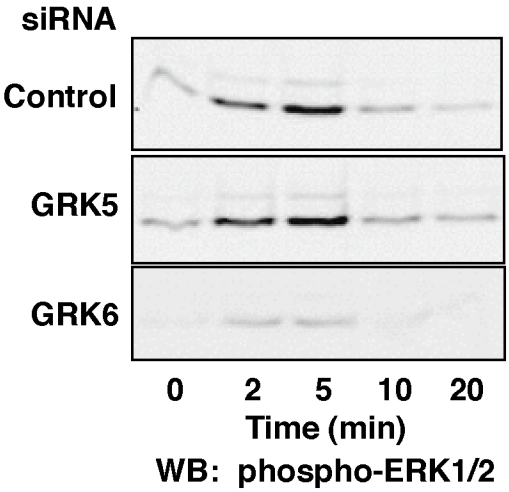
A.


Control
GRK2
GRK3
GRK5
GRK6
[Ca2+]i (nM)
Time (sec)

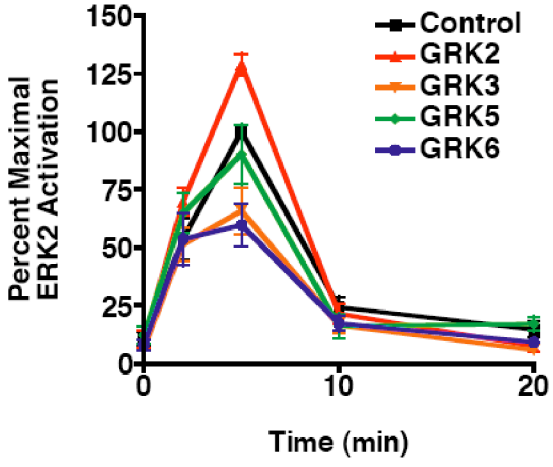

Change in Peak [Ca2+]i (nM)
Control
GRK2
GRK3
GRK5
GRK6

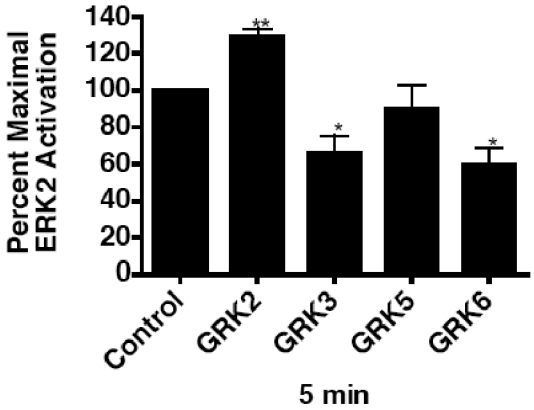
B.


siRNA
Control
GRK2
GRK3
Time (min)
WB: phospho-ERK1/2


siRNA
Control
GRK5
GRK6
Time (min)
WB: phospho-ERK1/2

C.


Percent Maximal ERK2 Activation
Control
GRK2
GRK3
GRK5
GRK6
Time (min)

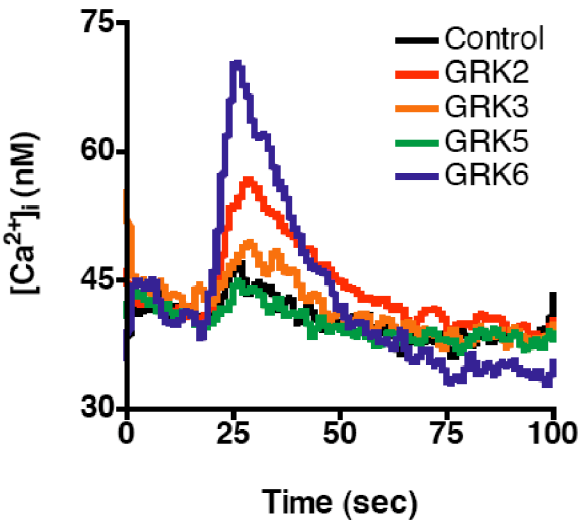

Percent Maximal ERK2 Activation
Control
GRK2
GRK3
GRK5
GRK6
5 min

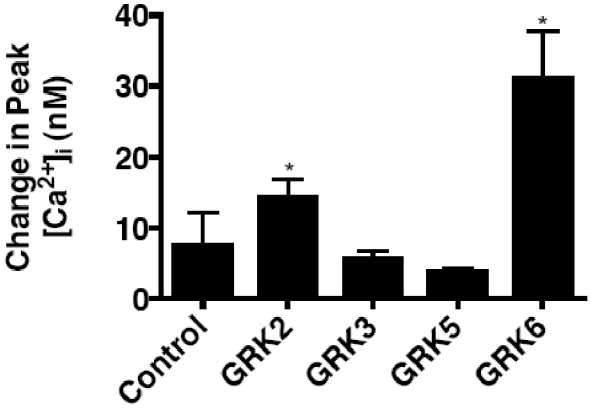
We also evaluated whether the stable over-expression of CXCR4 altered GRK-mediated regulation. Similar to endogenous CXCR4, knock down of either GRK2 or GRK6 in the Flag CXCR4 cells enhanced calcium mobilization, although GRK6 had the larger effect (Figure 20A). Similarly, knock down of GRK2 enhanced while knock down of GRK3 or GRK6 decreased activation of ERK1/2 (Figures 20B and C). Thus, stable over-expression of CXCR4 in HEK293 cells did not alter the signaling or regulation of CXCR4. Our data suggest that phosphorylation of CXCR4 by GRK6 and possibly GRK2 uncouples the receptor from activation of Gi, decreasing calcium mobilization. In contrast, GRK3 and GRK6-mediated phosphorylation of CXCR4 positively regulates activation of ERK1/2.

Figure 20. Differential GRK-mediated regulation is conserved in Flag CXCR4 cells

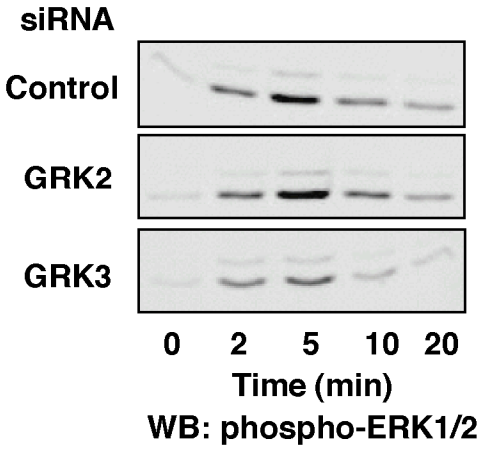
Cells expressing Flag CXCR4 were analyzed for calcium flux *(A)* and ERK1/2 activation *(B and C)* exactly as described in the legend to Figure 19.

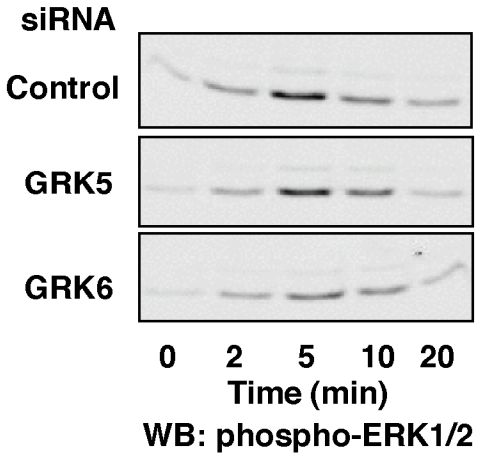
A.


75
60
45
30
0
25
50
75
100
Time (sec)
[Ca2+]i (nM)
Control
GRK2
GRK3
GRK5
GRK6

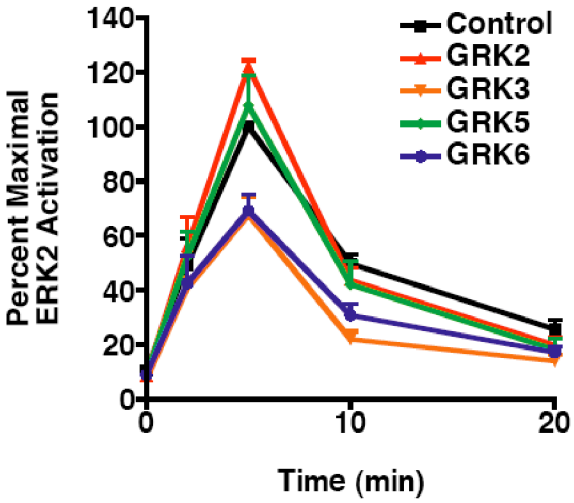

40
30
20
10
0
Change in Peak
[Ca2+]i (nM)
*
*
Control
GRK2
GRK3
GRK5
GRK6

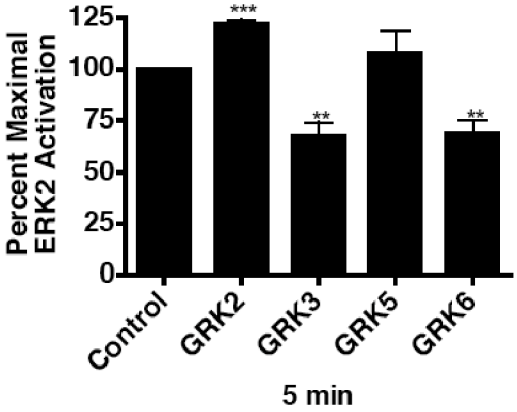
B.


siRNA
Control
GRK2
GRK3
0
2
5
10
20
Time (min)
WB: phospho-ERK1/2


siRNA
Control
GRK5
GRK6
0
2
5
10
20
Time (min)
WB: phospho-ERK1/2

C.


140
120
100
80
60
40
20
0
0
10
20
Time (min)
Percent Maximal
ERK2 Activation
Control
GRK2
GRK3
GRK5
GRK6

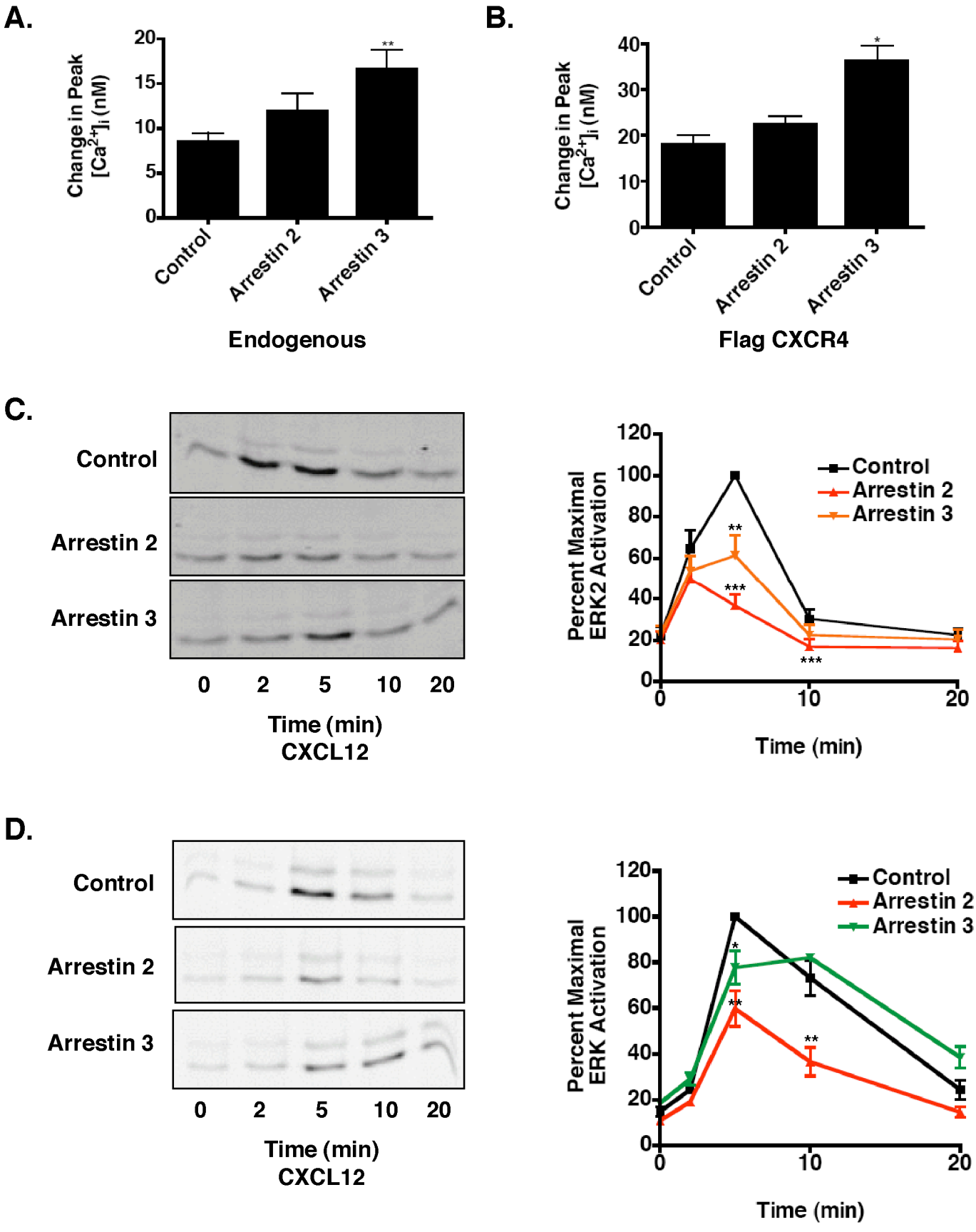

125
100
75
50
25
0
***
**
**
Control
GRK2
GRK3
GRK5
GRK6
5 min
Percent Maximal
ERK2 Activation

Arrestins differentially regulate signaling following CXCR4 activation

Agonist-dependent phosphorylation of many GPCRs leads to the recruitment of the non-visual arrestins, arrestin2 and arrestin3 (also termed β-arrestin1 and β-arrestin2, respectively), which effectively uncouples the receptor from heterotrimeric G proteins as well as targeting the receptor for internalization (Moore et al., 2007). Recent evidence has highlighted the ability of arrestins to nucleate signaling events in addition to their classical role in desensitization (DeWire et al., 2007). Therefore, we next examined the effect of siRNA-mediated knock down of arrestin2 and 3 on CXCR4 signaling in HEK293 cells. Knock down of arrestin3 led to a significant increase, whereas arrestin 2 had only a modest effect, in the peak calcium transient observed following CXCL12 stimulation of endogenous (Figure 21A) or overexpressed CXCR4 (Figure 21B). Conversely, knock down of arrestin2 led to a significant reduction in ERK1/2 activation following CXCL12 stimulation of endogenous (Figure 21C) or overexpressed (Figure 21D) CXCR4, while knock down of arrestin3 had lesser effects. These results reveal that both arrestins contribute to regulating CXCR4 signaling although arrestin3 appears to play the primary role in desensitization of calcium mobilization while arrestin2 plays the primary role in activation of ERK1/2 signaling.

Figure 21. Non-visual arrestins differentially regulate CXCR4-mediated signaling

A) Mean (± S.E.M.) increase in peak calcium transient following stimulation of endogenous CXCR4 calculated from seven separate experiments. *B)* Mean (± S.E.M.) increase in peak calcium transient following stimulation of Flag CXCR4 calculated from three separate experiments. *C and D)* Effect of arrestin knock down on ERK1/2 activation following activation of HEK293 *(C)* or Flag CXCR4 *(D)* cells. 72 hr post-transfection, cells were serum starved for 6 hr prior to stimulation with CXCL12 (100 nM). Left panels: shown are representative Western blots from seven *(C)* and four *(D)* separate experiments. Right panels: pERK2 was normalized to total ERK2 and data is presented as percent maximal ERK2 activation as compared to control (± S.E.M.; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).


A.
Change in Peak [Ca2+]i (nM)
20
15
10
5
0
**
Control
Arrestin 2
Arrestin 3
Endogenous
B.
Change in Peak [Ca2+]i (nM)
40
30
20
10
0
*
Control
Arrestin 2
Arrestin 3
Flag CXCR4
C.
Control
Arrestin 2
Arrestin 3
0
2
5
10
20
Time (min)
CXCL12
Percent Maximal ERK2 Activation
120
100
80
60
40
20
0
**
***
***
Control
Arrestin 2
Arrestin 3
0
10
20
Time (min)
D.
Control
Arrestin 2
Arrestin 3
0
2
5
10
20
Time (min)
CXCL12
Percent Maximal ERK Activation
120
100
80
60
40
20
0
*
**
**
Control
Arrestin 2
Arrestin 3
0
10
20
Time (min)

DISCUSSION

Phosphorylation has long been recognized as an initiating step in the process of GPCR desensitization (Krupnick and Benovic, 1998; Pitcher et al., 1998). Characterizing the mechanisms involved in receptor regulation under "normal" physiological conditions will add significantly to our understanding of receptor signaling as well as receptor dysregulation in disease. CXCR4 has emerged as a prominent GPCR due to its reported role in cancer progression and metastasis (Zlotnik, 2006) and recent studies have provided evidence for the dysregulation of CXCR4 in cancer cells (Li et al., 2004; Slagsvold et al., 2006). Given the wealth of knowledge on the role of CXCR4-mediated signaling in cancer (Kucia et al., 2005), it is surprising that the regulation of CXCR4 function is not better understood. In this report, we characterize site-specific phosphorylation of CXCR4 and provide evidence that kinase-specific phosphorylation has distinct effects on CXCR4 signaling.

Since mutagenesis and metabolic labeling studies have suggested that multiple regions of the C terminal tail of CXCR4 may be phosphorylated (Orsini et al., 1999), we used mass spectrometry and phospho-specific antibodies to define specific sites of phosphorylation. Using these approaches we found that CXCL12 promotes the phosphorylation of 7 serines in the C terminal tail (Figure 14D), although we cannot rule out the presence of additional sites. We confirmed previous studies suggesting that Ser-324 and Ser-325 are phosphorylated (Signoret et al., 1998; Orsini et al., 1999) as well as one study showing that Ser-339 is phosphorylated (Woerner et al., 2005) (Figure 15C and D). In addition, we found that Ser-321 is phosphorylated, a site that has not been previously

implicated in CXCR4 regulation, and we demonstrated that Ser-330 is also phosphorylated in response to CXCL12 (Figure 18A).

Phospho-specific antibodies have provided a powerful tool to enable characterization of phosphorylation kinetics for a number of GPCRs (Pollok-Kopp et al., 2003; Schulz et al., 2004; Tran et al., 2004). Here, we successfully used phospho-specific antibodies against Ser-324/5, Ser-330 and Ser-339 and found that phosphorylation at these sites occurs with disparate kinetics, peaking at 5-10 min, ~20 min, and ~2 min following CXCL12 stimulation, respectively. Based on these results, we hypothesize that phosphorylation at these sites would have distinct effects on desensitization, signaling, and/or trafficking following CXCR4 activation. In this regard, previous studies have suggested that Ser-324/5 and Ser-330 play an important role in CXCR4 degradation (Marchese and Benovic, 2001) while CXCR4 recycles to the plasma membrane with varying efficiencies in different cell lines (Amara et al., 1997; Tarasova et al., 1998; Marchese et al., 2003; Venkatesan et al., 2003). These findings suggest cell type differences in Ser-324/5 or Ser-330 phosphorylation may regulate differential CXCR4 sorting. These phospho-specific antibodies should enable a more in-depth analysis of CXCR4 phosphorylation in various tissues and cells.

To address if site-specific phosphorylation can differentially dictate the regulation of CXCR4 signaling, we evaluated the role of site- and kinase-specific phosphorylation in CXCR4-mediated signaling. We provide evidence for PKCδ in selectively phosphorylating Ser-324/5 following CXCL12 stimulation with no effect on Ser-330 or Ser-339 (Figure 16 and data not shown). Previously, PKC

has been thought to be primarily involved in heterologous desensitization of CXCR4, downstream of a number of receptors (Busillo and Benovic, 2007). Interestingly, Ser-324 and Ser-325 have been shown to play a prominent role in PKC-mediated internalization of CXCR4 (Signoret et al., 1998; Orsini et al., 1999), although there have been conflicting reports for the role of these residues in CXCL12-mediated internalization. For example, mutation of Ser-324/5 to alanine had no effect on internalization in Mv-1-Lu cells (Signoret et al., 1998) but effectively attenuated internalization in HEK293 cells (Orsini et al., 1999). Since Ser-324/5 is robustly phosphorylated following CXCL12 stimulation in HEK293 cells, cell type dependent differences in trafficking may be attributed to differences in phosphorylation or in subsequent protein/protein interactions. In addition, since PKC inhibition had no effect on CXCR4 activation of ERK1/2 (data not shown), Ser-324/5 phosphorylation does not appear to participate in CXCR4 signaling or in arrestin recruitment (discussed below).

GRK6 was found to contribute to Ser-324/5 phosphorylation and be principally responsible for Ser-330 and Ser-339 phosphorylation (Figures 17 and 18). While no GRK-specific phosphorylation motifs have been identified, GRK2 and 3 prefer acidic residues N terminal to the phosphorylation site while GRK5 and 6 prefer basic residues (Pitcher et al., 1998). Consistent with our GRK6 results, there are basic residues located N terminally to Ser-324/5, Ser-330 and Ser-339 (Figure 14D). Previous studies have shown that the deletion of GRK6 in a mouse had marked effects on the activity of CXCR4, leading to enhanced function and a lack of desensitization (Fong et al., 2002; Vroon et al., 2004).

Surprisingly, loss of GRK6 had different effects on CXCL12-mediated chemotaxis in neutrophils and T cells, suggesting that the regulation of CXCR4 function by phosphorylation may be cell type dependent (Fong et al., 2002; Vroon et al., 2004). While these previous studies did not evaluate signaling, our work has shown that loss of GRK6 significantly increased calcium mobilization while reducing ERK1/2 activation. We propose that GRK6-mediated phosphorylation of CXCR4 plays an essential role in the recruitment of arrestins to the receptor as discussed below.

Two residues at the extreme C terminus of CXCR4 (one between Ser-346-348 and either Ser-351 or Ser-352) are also phosphorylated in response to CXCL12 stimulation (Figure 14D). Based on the acidotropic nature of these serines, we postulate that these residues are phosphorylated by GRK2 and/or GRK3 (Figure 14D). Interestingly, recent studies suggest that GRK3 phosphorylates the far C-terminal region of CXCR4 (Balabanian et al., 2008). Over-expression of GRK3 enhanced internalization of wild type CXCR4 but not a C-terminally truncated CXCR4 lacking the last 15 amino acids (which contains 10 serines and threonines). Since previous work has demonstrated that Ser-338 is not phosphorylated in response to CXCL12 (Woerner et al., 2005) and we have shown that Ser-339 is phosphorylated by GRK6 (Figure 18C), we speculate that GRK3 is responsible for phosphorylating a serine within the Ser-346-348 cluster as well as either Ser-351 or Ser-352 in response to CXCL12 stimulation. Furthermore, as knock down of GRK3 had no effect on calcium mobilization but significantly decreased ERK1/2 activation (Figures 19 and 20), we propose that

GRK3-mediated phosphorylation of C-terminal residues acts in concert with GRK6-mediated phosphorylation to participate in ERK1/2 activation by stabilizing CXCR4 interaction with arrestin (Figure 22).

Interestingly, we found that GRK2 negatively regulates CXCR4-mediated activation of both calcium flux and ERK1/2 (Figures 19 and 20). Previous over-expression studies showed that GRK2 enhances CXCR4 internalization (Orsini et al., 1999; Cheng et al., 2000) and negatively regulates CXCL12-mediated ERK activation downstream of the receptor in HEK293 cells, possibly through interaction with MEK (Jimenez-Sainz et al., 2006). While we cannot rule out the possibility that GRK2 directly phosphorylates CXCR4, we hypothesize that the observed effects of GRK2 knock down occur downstream of CXCR4. This could include interaction with free $G\beta\gamma$ (Pitcher et al., 1992; Carman et al., 1998), resulting in inhibition of $G\beta\gamma$-mediated activation of PLC-β (Camps et al., 1992) and the observed increase in calcium mobilization when GRK2 levels are reduced. In addition, GRK2 inhibition of MEK would result in enhanced ERK1/2 activation when GRK2 levels are reduced (Jimenez-Sainz et al., 2006). It is also possible that there may be tissue specific differences in site-specific phosphorylation of CXCR4 by GRK2 and GRK3.

The positive role of GRK3 and GRK6 on ERK1/2 activation prompted us to evaluate the role of arrestins in CXCR4 signaling. Previous studies have suggested that arrestin3 is involved in desensitization, internalization, and activation of p38 and ERK1/2 (Orsini et al., 1999; Cheng et al., 2000; Fong et al., 2002; Sun et al., 2002), whereas arrestin2 is involved in sorting CXCR4 to the

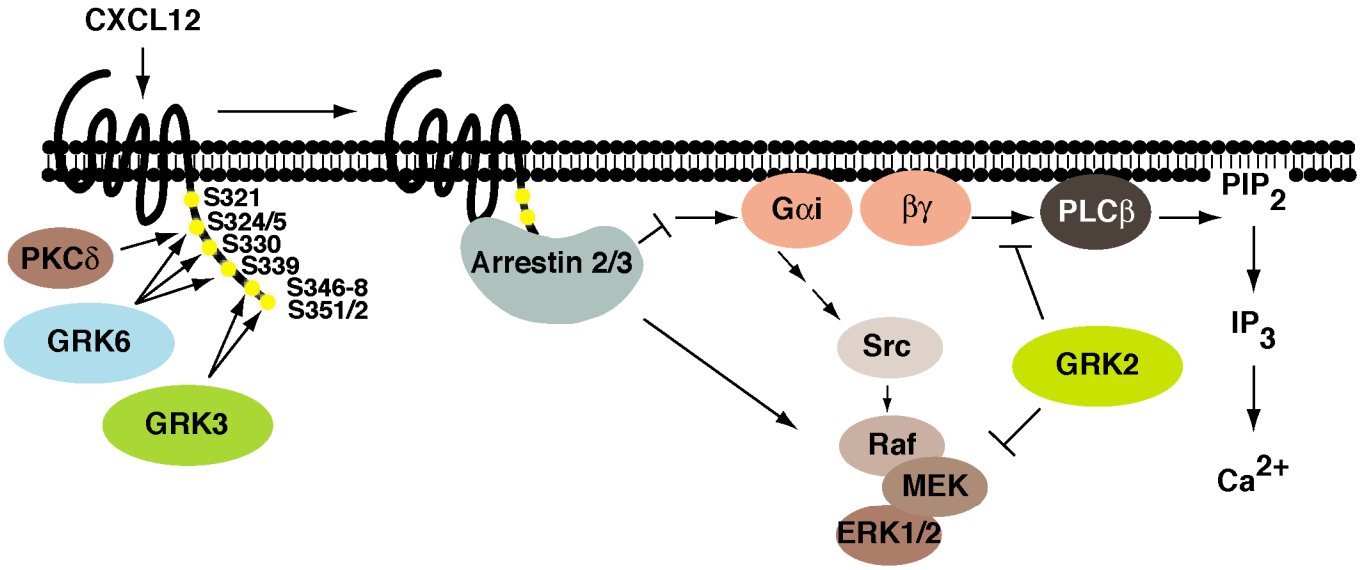
lysosomes for degradation (Bhandari et al., 2007). Similar to studies on the angiotensin and vasopressin receptors (Ahn et al., 2004; Ren et al., 2005), we have found that arrestin2 and arrestin3 have different effects on CXCR4 signaling. Our results suggest that arrestin3 plays a primary role in desensitizing G protein activation (i.e., inhibiting calcium mobilization), a pathway that appears dependent on GRK6 phosphorylation of CXCR4. Conversely, arrestin2 plays a positive role in ERK1/2 activation and appears to require phosphorylation of the receptor by both GRK3 and GRK6. This pattern of arrestin-mediated activation of ERK1/2 is somewhat unique since most GPCRs either require both arrestins (DeWire et al., 2007) or arrestin3 (Ahn et al., 2004; Ren et al., 2005) for ERK activation although there is one report that PAR1 selectively utilizes arrestin2 (Kuo et al., 2006). In addition, the involvement of multiple GRKs in receptor regulation is analogous to studies on the β_2AR where GRK2- and GRK6-mediated phosphorylation mediates arrestin3 binding, although GRK6 plays the prodominant role (Violin et al., 2006). We hypothesize that the observed decrease in ERK activation is likely due to a decrease in the extent of arrestin2 recruitment with GRK3 or GRK6 knock down and the inability to form a stable CXCR4-arrestin2 complex (Tohgo et al., 2003; Jafri et al., 2006). Another possibility is that CXCR4 phosphorylation by GRK3 and GRK6 allows arrestin2 to adopt a conformation (Kim et al., 2005; Ren et al., 2005) that allows for full activation of ERK1/2.

In summary, our results support a model where GRK6-mediated phosphorylation leads to recruitment of arrestin3 (Figure 22). This serves to

uncouple CXCR4 from activation of Gi thereby regulating calcium release. In contrast, GRK3- and GRK6-mediated phosphorylation of CXCR4 promotes interaction with arrestin2 and results in full activation of ERK1/2. GRK2, on the other hand, likely attenuates calcium release and activation of ERK1/2 through its interaction with $G\beta\gamma$ and MEK, respectively. While we cannot specifically pinpoint the phosphorylation sites that mediate differential arrestin association, the finding that PKC inhibition does not affect ERK1/2 activation suggests that Ser-324/5 phosphorylation does not contribute to arrestin binding. These results provide a foundation to better understand how CXCR4 is regulated and identify distinct regulatory molecules that can be targeted to modulate CXCR4 signaling in disease.

Figure 22. Regulation of CXCR4 activity and signaling

Upon activation, CXCR4 is phosphorylated on seven residues: Ser-321, Ser-324, Ser-325, Ser-330, Ser-339, a residue from Ser-346-S348, and either Ser-351 or Ser-352. We propose that GRK6-mediated phosphorylation results in the recruitment of arrestin3 to CXCR4, thereby attenuating G protein activation and calcium release. GRK2 also attenuates G protein activation and calcium release most likely by regulating $G\beta\gamma$ activation of PLCβ. We also propose that phosphorylation by GRK3 and GRK6 results in more stable interaction with arrestin2 allowing for activation of ERK1/2. In contrast, GRK2 inhibits ERK1/2 by regulating the activity of MEK. Although robustly phosphorylated by PKC and, to a lesser extent by GRK6, phosphorylation of Ser-324/5 does not contribute to signaling.


CXCL12
S321
S324/5
S330
S339
S346-8
S351/2
PKCδ
GRK6
GRK3
Arrestin 2/3
Gαi
βγ
PLCβ
PIP2
IP3
Ca2+
Src
GRK2
Raf
MEK
ERK1/2

EXPERIMENTAL PROCEDURES

Cell culture and RNAi transfections

HEK293 cells (Microbix, Toronto, Canada) were maintained in complete Dulbecco's modified Eagle's media [DMEM] with 10% fetal bovine serum and 25 mM HEPES, pH 7.4. Cells stably expressing CXCR4 were selected and maintained in complete DMEM supplemented with 0.8 mg/ml G418 and penicillin/streptomycin. HEK293 cells were plated in fresh complete DMEM 24 hr prior to RNAi transfection. All siRNAs were synthesized by Dharmacon (Lafayette, CO) with the ON-TARGET plus modification. Four separate siRNAs were reconstituted and pooled at a final concentration of 15 pmol/μl. GRKs were targeted against the following sense strands: GRK2 – *5' GGGACGUGUUCCAGAAAUU 3'; 5' GCUCGCAUCCCUUCUCGAA 3'; 5' GGAAUCAAGUUACUGGACA 3'; 5' GCAAUAAGUUCACACGGUU 3'*; GRK3 – *5' GGAGUGUGAUGCAGAAGUA 3'; 5' GAGGAUACCAAAGGGAUUA 3'; 5' GGGAAGGACUGUAUUAUGC 3'; 5' GAACACGUACAAAGUCAUU 3';* GRK5 – *5'CCAACACGGUCUUGCUGAA 3'; 5' GGGAGAACCAUUCCACGAA 3'; CAAACCAUGUCAGCUCGAA 3'; 5' GAUUAUGGCCACAUUAGGAUU 3';* GRK6 – *5'GGUGAAGAAUGAACGGUAC 3'; 5' GAGCUUGGCCUACGCCUAU 3'; GCACGUAACGCAGAAUUUU 3'; 5' CGCCAAGAUUGCUGUGGAA 3'*. PKCδ was targeted against the following sense strands: *5' CCAUGAGUUUAUCGCCACC 3'; 5' CAGCACAGAGCGUGGGAAA 3'*. The arrestin and PKCα siRNAs have been previously described (Luo et al., 2008; Oliva et al., 2008). The PKC siRNAs were reconstituted individually at 60

pmol/μl. Prior to transfection, 300 pmol of each were combined for a total of 600 pmol/transfection. Non-targeting siRNA pooled control modified with the ON-TARGET plus modification was used for all experiments. HEK293 cells, ~65 to 70% confluent, were transfected with 600 pmol of siRNA using Lipofectamine 2000 (Invitrogen) in OPTI-MEM (Invitrogen) per the manufacturer's instructions. Cells were maintained in low-serum media for 4 hr, at which point an equal volume of 2X complete media (20% FBS and 25 mM HEPES) was added. Cells were split 48 hours post-transfection for assay the following day.

Purification of Flag CXCR4

Purification of Flag-tagged CXCR4 was performed as previously described for the β_2AR (Trester-Zedlitz et al., 2005) with minor modifications. Cells grown to ~90% confluency in five 15-cm plates were washed twice with phosphate buffered saline [PBS]. Cells were then incubated in serum free DMEM media [SFM] for 6 hr, the media was aspirated, and replaced with fresh SFM containing 50 nM CXCL12 for 10 min. Cells were scrapped into 10 ml of hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 2.5 mM $CaCl_2$, 10 mM NaF, and one Complete Protease inhibitor tablet (EDTA-free)) and lysed by 10 strokes in a Dounce homogenizer (tight pestle). Membranes were pelleted by centrifugation at 40,000 x *g* for 20 min at 4°C. Pellets were resuspended in 10 ml of Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM NaF, 1% dodecyl maltoside [DDM], 2.5 mM $CaCl_2$, and one Complete protease inhibitor tablet (EDTA-free)) and homogenized by 20 strokes in a Dounce homogenizer. Cellular debris was

cleared by centrifugation at 40,000 x *g* for 20 min at 4°C. The resulting supernatant was passed over an M1 anti-flag affinity (Sigma-Aldrich, St Louis MO) column (0.4 ml resin/10 ml of lysate, flow rate of 5 ml/hr) equilibrated in Buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% DDM, 2.5 mM $CaCl_2$) at room temperature. The column was washed once with 5 ml of Buffer C (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% DDM, 2.5 mM $CaCl_2$) at a rate of 10 ml/hr followed by an additional wash with 5 ml of Buffer B. Bound receptor was then eluted from the column in Buffer D (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% DDM, 200 μM Flag peptide, 1 μM AMD 3100, 10 mM EDTA) in 1 ml fractions and immediately snap frozen in liquid nitrogen and stored at –80°C. Fractions containing CXCR4 (by Western blot) were concentrated using Microcon ultracell YM-10 concentrators (Millipore Cooperation, Billerica, MA). Purified CXCR4 was electrophoresed on a 4-20% SDS-Glycine gradient gel (Invitrogen, Carlsbad CA), stained with Coomassie blue, de-stained, excised and shipped on ice for LC/MS/MS analysis.

LC/MS/MS procedure

Gel pieces were transferred to siliconized tubes, washed and destained overnight in 200 µl of 50% methanol. The gel pieces were dehydrated in acetonitrile and then rehydrated in 30 µl of 10 mM dithiothreitol [DTT] in 0.1 M ammonium bicarbonate and incubated at room temperature for 30 min. The DTT solution was removed and the sample was alkylated in 30 µl of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min.

The gel pieces were dehydrated in 100 µl of acetonitrile, rehydrated in 100 µl of 0.1 M ammonium bicarbonate, dehydrated in 100 µl of acetonitrile, completely dried by vacuum centrifugation and then rehydrated in 20 µl of 50 mM ammonium bicarbonate containing 20 ng/µl Lys-C or chymotrypsin and incubated overnight at 37^{o}C. Peptides were extracted from the polyacrylamide in two 30 µl aliquots of 50% acetonitrile/5% formic acid and the extracts were combined and evaporated to 15 µl for MS analysis.

The LC/MS system consisted of a Thermo Electron LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm x 75 µm id Phenomenex Jupiter 10 µm C18 reversed-phase capillary column. Extract (1-5 µl) was injected and peptides eluted from the column using an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 µl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra (ICR; 100K resolution) to determine peptide molecular weights and five product ion spectra (ion trap) to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 1500 CAD spectra of ions ranging in abundance over several orders of magnitude. The data were analyzed by database searching using the Sequest search algorithm against CXCR4. Putative phosphorylated peptides were confirmed by manual analysis.

Generation of polyclonal antibodies specific for pS324/5 and pS330

Polyclonal antibodies specific for pS324/5 and pS330 were generated by Open Biosystems (Huntsville, AL). Briefly, peptides corresponding to phosphorylated CXCR4 at Ser-324/5 (C-Ahx-RG(pS)(pS)LKIL where Ahx is amino hexonic acid) and Ser-330 (CLKIL(pS)KGKRGGH) were synthesized, coupled to hemocyanin and used to immunize rabbits following a standard immunization protocol. Crude sera from each rabbit was collected at days 28, 56 and 72 following primary immunization and tested for immuno-reactivity. Antibody was purified from pooled bulk sera (days 56 and 72) from animal E5199 (pS324/5) and E5198 (pS330) using the immunizing peptide, concentrated to 1 mg/ml, and aliquots were stored at –80°C. Antibody specificity was evaluated by preincubating 10 μg of purified antibody with vehicle (PBS), 10 μg of the immunizing peptide or 10 μg of the non-phosphorylated peptide.

Calcium mobilization and ERK1/2 activation

Calcium mobilization was performed as previously described (Luo et al., 2008). For analyzing ERK1/2 activation, cells were plated into 6-well plates 24 hr prior to stimulation. Confluent cells were washed twice with PBS and maintained in 1 ml of SFM for 6 hr at 37°C prior to stimulation. Following stimulation, media was aspirated on ice and cells were lysed by the addition of 300 μl of 2x SDS sample buffer and stored at –80°C until processed. Lysates were thawed on ice, sonicated for 10 sec (10% amplitude) and allowed to sit at room temperature for 30 min prior to electrophoresis. Equal volumes were separated by 10% SDS-

PAGE, transferred to nitrocellulose, blocked with ODYSSEY® blocking buffer (Li-Cor® Biosciences) and blotted overnight with a mixture of anti-phospho-p42/44 (Cell Signaling Technologies, Boston MA) and anti-ERK2 (Santa Cruz, Santa Cruz, CA). The following day, blots were washed extensively with Tris Buffered Saline containing 0.1% Tween-20 [TBS-T], incubated with a mixture of goat anti-rabbit Alexa® Fluorophore 680 conjugated (Molecular Probes) and goat anti-mouse IRDye 800 conjugated secondary (Rockland Immunochemicals) antibodies (1:5000) for 1 hr at room temperature. Blots were washed extensively with TBS-T and visualized using the ODYSSEY® infrared imaging system (Li-Cor® Biosciences).

Detection of pS324/5, pS330 and pS339

HEK293 cells stably expressing Flag CXCR4 were stimulated and processed as described for ERK1/2 activation. An equal volume of cell lysate was separated by 10% SDS-PAGE, transferred to nitrocellulose and blocked for 1 hr in 0.25% gelatin. Blots were incubated overnight at 4°C with a mixture of anti-CXCR4 (BD Bioscience) and anti-pS324/5, anti-pS330, or anti-pS339 primary antibodies. Blots were extensively washed with TBS-T and incubated with a mixture of goat anti-rabbit Alexa® Fluorophore 680 conjugated (Molecular Probes) and goat anti-rat IRDye 800 conjugated secondary (Rockland Immunochemicals) antibodies for 1 hr at room temperature. Blots were developed as described above for phospho-ERK. Phospho-CXCR4 was then normalized to total CXCR4 and is represented as percent maximum. For assays

with PKC inhibition, cells were pretreated with vehicle (DMSO) or appropriate inhibitor for 30 min prior to stimulation with CXCL12.

Electrophoretic mobility shift assay

An equal volume of cell lysate was separated by 10% SDS-PAGE for 1 hr 40 min at ~135V, transferred to nitrocellulose and blocked for 1 hr in 5% milk in TBS-T. Blots were subsequently probed for CXCR4 as described above for phospho-CXCR4.

Statistical Analysis

All data are represented as the mean ± standard error of the mean [S.E.M.]. Data were analyzed using a two-tailed student's t-test with significance set at $p \leq 0.05$.

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Chapter IV

Discussion

Being one of the largest families of cell surface receptors, GPCRs are critically involved in nearly every physiological process. Accordingly, strict regulatory mechanisms need to be in place to ensure proper spatial and temporal control of receptor activity and signaling. Research over the past 20 years has significantly enhanced our understanding of these regulatory mechanisms and established an elegant paradigm of GPCR regulation (Moore et al., 2007; DeWire et al., 2007). However, it is becoming increasingly clear that not all GPCRs fit this paradigm and a comprehensive analysis of receptor regulation is needed. Therefore, we investigated the molecular mechanisms that underlie regulation of GPCR signaling. Specifically, we looked at the roles of the GRKs and arrestins in regulating signal transduction following activation of two endogenous GPCRs expressed in HEK293 cells: the M_3 mAChR and CXCR4. In addition, using CXCR4 as a model receptor, we identified agonist-promoted sites of phosphorylation by mass spectrometry. Using a combination of phospho-specific antibodies, RNA interference, and specific inhibitors, we then characterized the kinetics and kinases involved in agonist-promoted phosphorylation. Together, these studies have significantly enhanced our understanding of CXCR4 regulation and provide the groundwork for understanding dysregulation of CXCR4 in disease.

Differential Regulation of GPCR-Mediated Signaling Events By GRKs and Arrestins

The role of GRKs in initiating the process of desensitization is a well-established paradigm in GPCR regulation (Krupnick and Benovic, 1998).

However, emerging evidence has uncovered a previously unappreciated role for the GRKs and arrestins in initiating G protein-independent signaling cascades following receptor activation (Reiter and Lefkowitz, 2006). Seminal studies with the angiotensin and vasopressin receptors have provided evidence for the following model: GRK2/3, although primarily responsible for receptor phosphorylation, negatively regulate arrestin-dependent ERK activation. Conversely, GRK5/6 play a lesser role in overall receptor phosphorylation, but positively regulate arrestin-dependent ERK activation (Kim et al., 2005; Ren et al., 2005). There are two important features implicit in this model: 1) GPCRs are regulated by numerous GRKs in a coordinated fashion, and 2) GRK-specific phosphorylation of GPCRs has distinct consequences on receptor activity and signaling. As similar phenomena have been described for the β_2AR (Shenoy SK et al., 2006) and follicle-stimulating hormone receptor (Kara E et al., 2006), it has been suggested that this model is applicable to all GPCRs.

As the above-mentioned studies primarily used stable overexpression of individual receptors, we wanted to better understand how phosphorylation regulates signaling of endogenous GPCRs. Therefore, we initially analyzed how siRNA-mediated knock down of the GRKs affected signaling pathways activated by the M_3 mAChR (Gq-coupled) and CXCR4 (Gi-coupled), two receptors endogenously expressed in HEK293 cells. Using a two-pronged approach of assaying calcium mobilization and ERK activation, we were able to define and monitor changes in both the G protein-dependent and –independent signaling pathways. We found that GRK2, 3, and 6, and arrestin2 and 3 each has a

distinct and separable role in regulating the activity of each receptor. Interestingly, knock down of GRK5 did not effect signaling via either receptor.

Our studies with the M_3 mAChR suggest that signaling is strictly through a G protein-dependent manner and relief of inhibitory constraints (GRKs and arrestins) subsequently enhances receptor function. In contrast, CXCR4 uses both a G protein-dependent and –independent (arrestin-dependent) means of signaling. Intriguingly, and in contrast to previous studies (Kim et al., 2005; Ren et al., 2005; Kara et al., 2006; Shenoy et al., 2006), G protein-independent signaling requires both GRK3 and 6, while GRK2 was inhibitory. It is interesting to note that, to date, GRK2 is largely a negative regulator and GRK6 is largely a positive regulator of ERK activation. While this is the first demonstration that GRK2/3 and GRK5/6 cooperate in terms of signaling, it has been recently shown that GRK2 and 6 are required for the recruitment and high affinity interaction of arrestin3 with the β_2AR (Violin et al., 2006). As more is learned regarding differences in receptor phosphorylation, the underlying mechanisms should become apparent.

We have also shown that arrestin2 and 3 differentially regulate the activity and signaling of CXCR4. Specifically, only knock down of arrestin3 led to enhanced calcium mobilization following CXCR4 activation, suggesting a loss of desensitization. In contrast, arrestin2 is primarily responsible for the arrestin-dependent phase of ERK activation. This apparent reciprocal regulation has been described for both the angiotensin (Ahn et al., 2004) and PAR1 receptor (Kuo et al., 2006), where arrestin3 and 2 positively regulated signaling,

respectively. However, in contrast to the PAR1 receptor (Kuo et al., 2006), arrestin3 is not completely inhibitory and seems to be required for early activation of ERK1/2, suggesting a certain degree of codependence (reviewed in DeWire et al., 2007). In fact, overexpression of arrestin2 and 3 enhances ERK activation of transiently expressed CXCR4 in HEK293 cells (Cheng et al., 2000). Interestingly, G protein-dependent and arrestin-dependent signaling are not temporally distinct (Ahn et al., 2004). How arrestin modulates the subcellular localization (Luttrell et al., 2001; Tohgo et al., 2002; Ahn et al., 2004) of these signaling molecules, remains to be seen.

Taken together, these results clearly demonstrate that 1) GPCRs are dynamically regulated by a number of proteins in a coordinated manner; 2) clear differences exist between receptors expressed within the same cell type; and 3) a comprehensive evaluation of individual receptors is needed to truly appreciate and understand the intricacies of receptor regulation and signaling. One major limitation of assaying endogenous GPCRs is the inability to directly assess whether or not the observed effect following GRK knock down is due to a loss of receptor phosphorylation or through interaction with some downstream signaling component. This is a critical distinction as the functional consequences of GRK-mediated signaling are just beginning to be uncovered. As we have demonstrated for the M_3 mAChR, using specific point mutants helped delineate that GRK2 primarily regulated Gαq following receptor activation. As the GRKs represent important pharmacological targets (Premont RT and Gainetdinov RR, 2007) that interact with a number of proteins (Ribas et al., 2007), the ability to

specifically target and disrupt these interactions would be a benefit therapeutically.

Determining Site- and Kinase-Specific Phosphorylation of GPCRs

In order to elucidate a functional role of site- or GRK-specific phosphorylation, the sites phosphorylated and the kinases involved need to be determined. Based on our initial studies examining the role of the GRKs in receptor-mediated signaling, we wanted to further characterize agonist-promoted phosphorylation of CXCR4. With very few exceptions, the sites of agonist-promoted phosphorylation of GPCRs are largely unknown. CXCR4 is extensively phosphorylated on as many as 18 potential phospho-acceptor sites in the C terminal tail (Haribabu et al, 1997; Orsini et al., 1999). Therefore, in order to determine agonist-promoted sites of phosphorylation of CXCR4, we needed to develop a number of biochemical approaches. With the identification of mass spectrometry-friendly detergents (Cadene et al., 2000) and epitope tag-based purification (Kobilka 1995), it is now feasible to readily analyze non-visual GPCRs by mass spectrometry for a myriad of post-translational modifications (Trester-Zedlitz et al., 2005). Importantly, as has been demonstrated with the β_2AR, differences in receptor phosphorylation clearly exist between in vitro and "in vivo" studies (Fredericks et al., 1996; Trester-Zedlitz et al., 2005). Therefore, in an attempt to more closely mimic biologically relevant regulation, we chose to purify CXCR4 from cell culture.

Mass spectrometry identified 6 sites of phosphorylation following SDF-stimulation: Ser321, Ser324, Ser325, one between Ser338-341, and two

between Ser346-352. Unfortunately, the ability of mass spectrometry to identify sites of phosphorylation is limited, to a certain degree, by the nature of the peptides. Using the endopeptidases Lys-C and chymotrypsin, we were able to generate and identify peptides derived from the C terminal tail and portions of intracellular loops 2 and 3 of CXCR4. However, a complete complement of peptides was not observed during the mass spectrometric analysis. Namely, peptides containing Ser330, the seventh phospho-acceptor site, were not observed. This demonstrates that a variety of techniques are needed in order to comprehensively characterize receptor phosphorylation.

Having identified sites of phosphorylation, we next wanted to determine the kinetics and kinase-specificity of phosphorylation. Therefore, using both the mass spectrometry results and previous studies (Marchese et al., 2001), we successfully generated and characterized antibodies directed against pS324/5 and pS330. Additionally, we were able to use a previously characterized antibody directed at pS339 (Woerner et al., 2005). These antibodies proved to be invaluable for identifying and characterizing the kinetics of and kinase-mediated phosphorylation of CXCR4. Unfortunately, attempts to generate an antibody directed at pS321, a novel site of phosphorylation, were unsuccessful. Furthermore, as we were not able to pinpoint the exact residues phosphorylated between Ser346-352, we were unable to generate antibodies against these residues.

We have found that GRK6 phosphorylates four serine residues (Ser324/5, Ser330, and Ser339) and appears to be the kinase primarily responsible for

CXCR4 phosphorylation. Our data also suggest that GRK3 is responsible for phosphorylation of two residues between Ser346-352. Finally, we have provided evidence for a direct and novel role of PKC in agonist-promoted phosphorylation of CXCR4. Overall, our results are consistent with the C terminal tail acting as the primary site of phosphorylation (Haribabu et al., 1997). However, we cannot exclude that residues of the intracellular loops may also be phosphorylated. In fact, tyrosyl phosphorylation of the first or second intracellular loop has also been suggested to occur and activate the JAK/STAT pathway independent of G protein activation (Villa-Coro et al., 1999).

Functional Significance of Site-Specific Phosphorylation of GPCRs

Can a particular functional outcome be directly linked to site-specific phosphorylation of a GPCR? If so, delineating whether or not certain phospho-sites are critical for receptor desensitization, trafficking, and signaling would be of particular interest. Furthermore, linking individual kinases with site-specific phosphorylation (and function) would provide significant insight into receptor regulation and, possibly, a novel area of therapeutic research. To date, the only comprehensive study linking kinase-specific phosphorylation with a functional outcome has been with smoothened, a 7 transmembrane receptor closely related to GPCRs (Zhang et al., 2004; Fredriksson et al., 2003). CXCR4 is of particular interest as it plays a critical role in diverse physiological processes and is one of the most commonly expressed receptors found on tumor cells, detected in more than 20 distinct tumor types (reviewed in Busillo and Benovic, 2007). Here, we have demonstrated that the GRKs and arrestins differentially regulate activity and

signaling of CXCR4, providing insight into the functional significance of site-specific phosphorylation of CXCR4. While these results have direct implications for CXCR4, the methods developed here are applicable to all GPCRs.

Much of the research on CXCR4 over the past 10 years has focused on understanding the signaling pathways critical for tumor progression and metastases (Vandercappellen et al., 2008). Of these pathways, activation of MAP kinases (i.e., ERK1/2, p38, and JNK) has been linked to cellular migration, proliferation and survival (reviewed in Kucia et al., 2005). Interestingly, G protein-dependent and -independent MAP kinase activation are spatially distinct (Luttrell et al., 2001; Tohgo et al., 2002; Ahn et al., 2004), providing a target of potential therapeutic intervention. Notably, we have found that GRK3 and 6 positively regulate arrestin-dependent ERK activation. As knock down of GRK6 significantly enhances receptor activity we would predict that targeted disruption of GRK6 would lead to global enhancement of CXCR4 activity. Conversely, disrupting GRK3 would be predicted to have no effect on receptor activity while specifically altering arrestin-dependent ERK activation. However, the differences between G protein-dependent and –independent signaling downstream of CXCR4 activation are currently unknown and warrant further investigation.

In addition to the potential importance in signaling, receptor phosphorylation also drives specific protein/protein interactions, namely with arrestin2 and 3. Our results suggest that recruitment of the arrestins is specifically driven by the differences in receptor phosphorylation, specifically of residues within the last 15 amino acids of CXCR4. All know WHIM syndrome

truncations, which significantly enhance receptor function, occur within this region (Diaz and Gulino, 2005). Furthermore, deletion of either GRK6 or arrestin3 in a mouse enhances receptor function (Fong et al., 2002). Consistent with these observations, knock down of GRK6 and arrestin3 significantly enhances calcium mobilization. Of the 4 sites phosphorylated by GRK6, Ser339 occurs the quickest, peaking within 2 minutes of stimulation. Moreover, overexpression of arrestin3 is unable to rescue desensitization and internalization of CXCR4 lacking the last 15 amino acids (Balabanian et al., 2008). Collectively, based on these results, we could hypothesize that phosphorylation of Ser339 is responsible for the initial recruitment of arrestin3 to CXCR4. Notably, GRK6 phosphorylation is also primarily responsible for the recruitment of arrestin3 to the β_2AR in HEK293 cells (Violin et al., 2006). On the other hand, the interaction of arrestin2 with CXCR4 appears to be driven by GRK6- and GRK3-mediated phosphorylation of Ser 330 and Ser339 and Ser346-352, respectively. However, it is important to note that we were unable to determine the kinetics of GRK3-mediated phosphorylation of Ser346-352.

Intriguingly, PKC-mediated phosphorylation of Ser324/5, though robust, does not appear to contribute to either the recruitment of arrestin or G protein-independent signaling. Given the critical role of these residues in receptor degradation (Marchese and Benovic, 2001), it is reasonable to hypothesize that PKC-mediated phosphorylation drives receptor degradation. In fact, phosphorylation of Ser324/5 appears to be required for the interaction of AIP4 with CXCR4 and subsequent receptor ubiquitination (A. Marchese, personal

communication). Accordingly, preliminary studies have suggested that PKC inhibition is sufficient to completely block receptor degradation (data not shown). Additionally, arrestin2 interacts with both CXCR4 and AIP4 at the endosome, directing CXCR4 into the degradative pathway (Bhandari et al., 2007). This interaction could be largely driven/stabilized by phosphorylation of Ser330, which we found to peak between 10 and 20 min of SDF-stimulation.

A conservative estimate puts GPCRs as targets for ~30% of currently marketed pharmaceutical drugs (Jacoby et al., 2006). The overall significance of the research presented here, and linking site-specific phosphorylation to a functional outcome in general, will have a broad impact on both basic and clinical research. For CXCR4 specifically, we can now begin to address the functional role of site- and kinase-specific phosphorylation of CXCR4 in a variety of tissues. More importantly, we can also begin to have a better understanding of whether or not there is altered regulation of CXCR4 in a variety of diseases. It is clear that from the work presented here that CXCR4 phosphorylation is not just an off switch for receptor activity, but in fact drives signaling pathways and likely a variety of protein-protein interactions. How these pathways are integrated *in vivo* presents a significant, but interesting challenge for future research.

Future Directions

The work presented here has significantly enhanced our current understanding of the mechanisms regulating CXCR4 activity and signaling. However, despite these advances, additional biochemical and cellular studies are warranted to further expand on the novel insights gained here.

One of the novel findings of the research presented in this thesis was the demonstration that individual kinases phosphorylate distinct sites on CXCR4. It is expected that receptor regulation and site-specific phosphorylation would largely depend on tissue-specific differences in protein expression. In fact, cell type-dependent phosphorylation has been noted for the M_3 mAChR (Torrecilla et al., 2007). Importantly, these studies revealed both common and cell type-specific sites of phosphorylation (Torrecilla et al., 2007). Using the phospho-specific antibodies described here will allow us to quickly assess site-specific phosphorylation in a variety of tissues. Furthermore, and of substantial interest, these could be used to determine if kinase-specificity is conserved in tissues and tumors or is it a result of the relative expression of the individual GRKs (Violin et al., 2006).

Our data show a direct, and novel role for PKC as the primary kinase responsible for agonist-promoted phosphorylation of Ser324/5. Determining to what extent PKC is involved in Ser324/5 phosphorylation in a variety of tissues would help substantiate our findings. Furthermore, preliminary results suggest that PKC is critically involved in regulating CXCR4 degradation. Inhibition of PKC significantly blocks agonist-promoted degradation of CXCR4, however, we are currently unable to distinguish if this is due to alterations in receptor trafficking, ubiquitination, or combination of the two. Extensive studies examining cellular localization and receptor ubiquitination are warranted to fully delineate how PKC, and to a larger extent Ser324/5 phosphorylation, affects receptor degradation.

We have observed that phosphorylation of CXCR4 occurs with clear kinetic differences. However, in the experiments presented here, we have not fully addressed the biological relevance of this. Our current hypothesis is that phosphorylation of Ser339 is critical for arrestin3 recruitment, while phosphorylation of Ser330, Ser339, and Ser346-352 are critical for forming a stable arrestin2/CXCR4 complex. In order to address arrestin recruitment and interaction, we are currently investigating whether CXCR4 results in a conformational change of arrestin2 and 3 by intramolecular bioluminescent energy transfer (BRET) (Charest et al., 2005). These studies should additionally provide both the kinetics and the relative stability of arrestin recruitment and interaction with CXCR4, respectively. If positive, these studies would then be expanded to look at how the knock down of GRK3 and GRK6 and specific receptor point mutants affect these parameters. Furthermore, detailed cellular localization studies using individual point mutants would provide further functional insight into site-specific phosphorylation.

How the GRKs and arrestins contribute to cancer progression and metastases in CXCR4-positive tumors is also of significant interest. Specifically, what arrestin-dependent signaling pathways are activated that contribute to cancer progression and metastases of these tumors? Interestingly, to date there have been no studies describing cancer progression/metastases in patients with WHIM syndrome. All of the WHIM mutations described to date either truncate the last 10-15 amino acids of CXCR4 or have selective loss of GRK3 (Diaz and Gulino, 2005; Balabanian et al., 2008), which we would predict are critical for

activation of arrestin2-dependent signaling. A simple way to address this question would be to compare the signaling pathways activated downstream of wild type or C terminally truncated (to Ser338) CXCR4 by western blot or microarray analysis. Given the clear role of arrestins in G protein-independent signaling and the recent demonstration that arrestin directly regulates gene transcription (Kang et al., 2005), it would be interesting to study how CXCR4 affecs the subcellular localization of arrestin following activation. Immunohistochemical studies analyzing the expression pattern of arrestin in breast cancer tissue have been initiated in the laboratory. Using a similar approach for the individual GRKs could provide correlations between CXCR4 expression and any alterations in the levels of the GRKs.

Finally, we have described an efficient and rapid protocol for purifying activated, phosphorylated CXCR4. Using this protocol, it may be possible to determine proteins that specifically interact with CXCR4 in an agonsit-dependent manner. However, it should be noted that the relative affinities of these interactions may be weak and would need experimental manipulation (i.e., chemical crosslinking) to maintain. Additionally, with further optimization, activated phosphorylated CXCR4 may be purified to sufficient homogeneity to allow for crystallization and structural determination. To date, the crystal structures of a handful of GPCRs have been solved (Palczewski et al., 2001; Cherezov et al., 2007; Rasmussen et al., 2007; Jaakola et al., 2008; Park et al., 2008). Each of these structures has had a substantial impact, however with the exception of metarhodopsin (Park et al., 2008), they are inactive and, for some,

are chimeras that allowed for receptor stabilization and crystallization (Cherezov et al., 2007; Jaakola et al., 2008). While undoubtedly a daunting task, the possibility of obtaining a crystal structure of a GPCR in its active form, and possibly complexed with other proteins (e.g., G proteins or arrestin), would be a significant revelation for the field of GPCR biology.

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