Nuclear localisation and function of the G protein-coupled Receptor Kinase 4 subfamily

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I, Laura Ruth Johnson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Publications

Papers

G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localisation sequence. Johnson, L.R., Scott, M.G.H. & Pitcher, J.A. Mol Cell Biol. 2004 Dec; 24(23):10169-79.

GRK5 DNA-binding ability and kinase activity are required for inhibition of hypertrophy in myocytes. Johnson, L.R. & Pitcher, J.A. PNAS paper in revision.

The G protein-coupled receptor kinase 4 subfamily members contain nuclear localization sequences. Johnson, L.R. & Pitcher, J.A. JBC paper in preparation.

Posters/Presentations

Nuclear Localisation of the GRK4 Subfamily of GRKs. Johnson, L.R. & Pitcher, J.A. Keystone Symposium, G Protein-Coupled Receptors: Evolving Concepts and New Techniques. *February 12 - 17, 2006.* Keystone, Colorado. Selected for oral presentation.

Differential Regulation of the Nuclear Localisation of GRK4 Subfamily Members. Johnson, L.R. & Pitcher, J.A. BioScience 2005: From genes to systems. *July 17 - 21, 2005.* Glasgow, UK.

Differential Regulation of the Nuclear Localisation of G Protein-Coupled Receptor Kinase 4 Subfamily Members. Johnson, L.R. & Pitcher, J.A. 4th BPS Focused Meeting on Cell Signalling. *April 11 - 12, 2005.* Leicester, UK. Selected for oral presentation.

Abstract

G protein-coupled receptor kinases (GRKs) are a family of serine/threonine kinases that phosphorylate and mediate densensitisation of agonist-occupied G protein-coupled receptors (GPCRs). Recent evidence suggests, however, additional cellular functions for these enzymes.

The seven mammalian GRKs are divided into the GRK1-, GRK2- and GRK4-like subfamilies. I have discovered that GRK4 subfamily members contain functional nuclear localisation sequences (NLSs). The location of the NLSs of GRK5 and 6 is similar whereas the NLS of GRK4 is more N-terminal. Although all members of the GRK4 subfamily contain NLSs the nuclear localisation of these kinases is differentially regulated by Ca^{2+}/CaM. Furthermore, the NLS of GRKs 5 and 6, but not GRK4, mediate DNA-binding in vitro, suggesting differential nuclear signalling roles for these enzymes.

I investigated structural determinants of GRK5 required for DNA-binding and nuclear localisation. The ability of GRK5 to bind DNA requires an intact NLS and an N-terminally located calmodulin (CaM) binding domain. Additionally, CaM-dependent autophosphorylation of GRK5 reduces the affinity of GRK5 for DNA. Overexpression of GRK5 in cultured rat neonatal cardiac myocytes rescues hypertrophy induced by phenylephrine, an α-adrenergic receptor agonist, or by activation of PLC. Hypertrophy is not rescued by over expression of GRK5 with a mutant NLS (GRK5ΔNLS), mutant N-terminal CaM binding site (GRK5NTPB) or a catalytically in active version of the kinase (GRK5K215R). That GRK5 and

GRK5ΔNLS show similar kinase activity *in vitro* suggests that both the kinase activity and the DNA-binding ability of GRK5 contributes to its role in rescuing hypertrophy. Since GRK5 rescues hypertrophy induced downstream of adrenergic receptors, by PLC activation, GRK5 DNA-binding ability and kinase activity in the nucleus would appear to be required for this effect. Clearly, the GRK4 subfamily of GRKs is more diverse than previously appreciated and play a role in cellular signalling in the nucleus distinct from GPCR desensitisation.

To

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Abbreviations used in this thesis

αAR: α-adrenergic receptor

α_{1B}AR: α_{1B}-adrenergic receptor

βAR: β-adrenergic receptor

βarr: βarrestin

β-MHC: β-myosin heavy chain

α-MHC: α-myosin heavy chain

7TMRs: seven transmembrane receptors

AC: adenylyl cyclase

ACE: angiotensin converting enzyme

ACh: acetylcholine

ANF: atrial natriuretic factor

AngII: angiotensin II

$AngII_{1A}R$: angiotensin II type IA receptor

Ao: angiotensinogen

AP: autophosphorylation

aPKC: atypical protein kinase C

AR: adrenergic receptor

ATP: adenosine triphosphate

ATR: angiotensin receptor

bHLH: basic helix-loop-helix

BSA: bovine serum albumin

Ca^{2+}/CaM: calcium-calmodulin

Ca^{2+}: calcium

CALM: clathrin assembly lymphoid myeloid leukemia

CaM: calmodulin

CaMBP/m: cytosolic CaM binding sequestrant

CaMBP/n: nuclear CaM binding sequestrant

cAMP: cyclic adenosine monophosphate

CAMTA2: calmodulin binding transcription activator 2

CASTing: cyclic amplification of targets

CBP: cAMP response element-binding-protein

CCPs: clathrin coated pits

CCR: chemokine receptor

CDKs: cyclin dependent kinases

cGMP: cyclic guanosine monophosphate

CHF: chronic heart failure

ChIP: chromatin immunoprecipitation

CHO: Chinese hamster ovary

CHO-M3: Chinese hamster ovary cells expressing muscarinic M3 receptor

cPKC: classical PKC

CRM: chromosomal region maintenance

CsA: cyclosporine A

CSP: calcium sensor protein

CTPB: C-terminal polybasic

CXCR: chemokine receptor

DAG: diacylglycerol

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

E. coli: Escherichia coli

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ENaCs: epithelial sodium channels

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

ERM: ezrin-radixin-moesin

ET_1: endothelin-1

ET_1R: endothelin-1 receptor

EYFP: enhanced yellow fluorescent protein

FCS: foetal calf serum

G protein: GTP-binding protein

GAP: GTPase activating protein

GEF: guanine nucleotide exchange factor

GIRK: G protein-coupled inwardly rectifying potassium channel

GIT: G protein-coupled receptor kinase-interacting protein

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

HATs: histone acetyltransferases

HCC: hepatocellular carcinoma

HDAC: histone deacetylase

HEK-293: human embryonic kidney 293

HEp2: human epithelial 2

HF: heart failure

IP_3: 1,4,5-trisphosphate

IκB: inhibitor of kappa B

JAK: janus-family tyrosine kinase

JNK: c-Jun amino-terminal kinase

LB: luria broth

M2MR: M2 muscarinic receptor

M3MR: M3 muscarinic receptor

MAPK: mitogen-activated protein kinase

MEF2: myocyte enhancer factor 2

MEK1: MAPK/ERK kinase 1

MK2: MAPK-activated protein kinase 2

MKK: mitogen activated protein kinase kinase

MR: muscarinic receptor

mRNA: messenger ribonucleic acid

NES: nuclear export sequence

NFAT: nuclear factor of activated T cells

NFκB: nuclear factor kappa B

NHERF: Na^+/H^+ exchanger regulatory factor

NLS: nuclear localisation sequence

NPC: nuclear pore complex

nPKC: novel PKC

NTPB: N-terminal polybasic

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDZ: PSD-95, Dlg, ZO-1 homology

PE: phenylephrine

PH: pleckstrin homology

PI3K: phosphoinositide 3–kinases

PIP_2: phosphatidylinositol 4,5-bisphosphate

PIP_3: phosphatidylinositol 3,4,5-trisphosphate

PKA: protein kinase A, cAMP-dependent protein kinase

PKC: protein kinase C or Ca^{2+}-dependent protein kinase

PKI: protein kinase C inhibitor

PLC: phospholipase C

PMA: phorbol ester 12-tetradecanoylphorbol-13 acetate

PMSF: phenylmethanesulfonyl fluoride

RGS: regulator of G protein signalling

RH: regulator of G protein signalling homology domain

RNA: ribonucleic acid

RNAi: ribonucleic acid interference

ROS: rod outer segments

RPMI: Roswell Park Memorial Institute

R-Smads: receptor activated smads

RT: room temperature

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS polyacrylamide gel electrophoresis

SHHF: spontaneously hypertensive heart failure

SR: sarcoplasmic reticulum

SRF: serum response factor

ssDNA: single stranded deoxyribonucleic acid

STAT: signal transducers and activators of transcription

TEMED: N, N, N′, N′-tetramethyl-ethylenediamine

TF: transcription factor

TPA: 12-o-tetradecanoylphorbol-13-acetate

TSA: trichostatin A

WT: wildtype

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1. Introduction

1.1. GRKs

G protein-coupled receptor kinases (GRKs) are a seven-member family of serine/threonine kinases, depicted in Figure 1.1. GRKs were originally identified as kinases that phosphorylate agonist occupied, or activated, G protein-coupled receptors (GPCRs) initiating their desensitisation. However, it is now evident that these kinases phosphorylate a range of receptor and soluble substrates. The implications of this are discussed in Section 1.3.

1.1.1. GRK structure

Based on sequence similarity and functionality, the seven mammalian GRKs have been divided into three subfamilies shown in Figure 1.1 (Pitcher et al., 1998; Vatter et al., 2005):

- GRK1 subfamily (47% sequence homology)
    - GRK1 (rhodopsin kinase)
    - GRK7 (cone opsin kinase)
- GRK2 subfamily (84% sequence homology)
    - GRK2 (β-Adrenergic receptor kinase 1)
    - GRK3 (β-Adrenergic receptor kinase 2)
- GRK4 subfamily (70% sequence homology)
    - GRK4α, β, γ, δ
    - GRK5
    - GRK6A, B, C (and D in mouse)

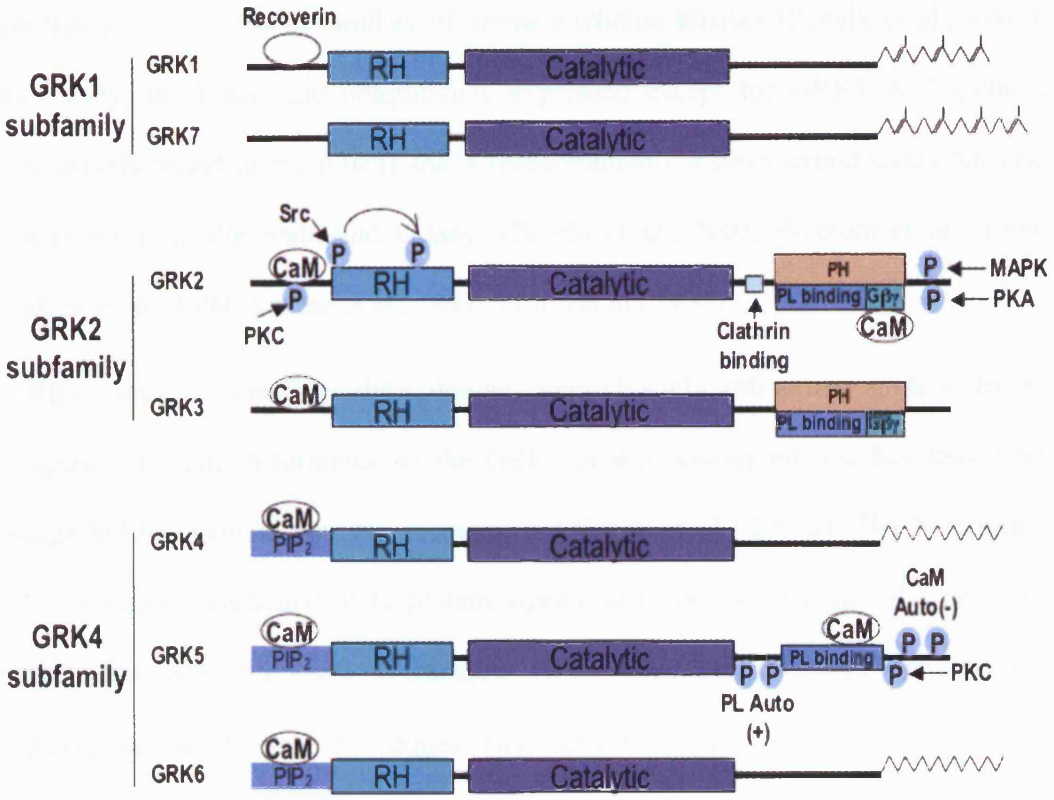


Figure 1.1 GRK Structure.

The schematic shows the multi-domain structure of the GRKs along with key regulatory elements and sites of protein interactions that have been mapped. The lipid modifications depicted are farnesylation (GRK1), geranylgeranylation (GRK7) and palmitoylation (GRKs 4 and 6). Auto (±), stimulatory or inhibitory autophosphorylation sites; CaM, calcium-calmodulin; MAPK, mitogen-activated protein kinase; PL, phospholipids; PIP_2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology; PKC, protein kinase C; RH, regulator of G protein signalling homology domain (Reproduced from Penela et al., 2003).

As indicated above, GRKs 4 and 6 undergo alternative splicing to give, respectively, four and three distinct splice variants (Premont et al., 1996). GRKs are well conserved from nematodes (*Caenorhabditis elegans*) and flies *(Drosophila melanogaster)* to mammals indicating their importance in biological functions (Pitcher et al., 1998). GRKs are most related to the protein kinase C (PKC) and

protein kinase A (PKA) families of serine/threonine kinases (Penela et al., 2003). Generally, the GRKs are ubiquitously expressed except for GRK1 & 7 (almost exclusively found in the retina) and 4 (predominantly testes/spermatocytes but also lower levels in the brain and kidney) (Penela et al., 2003; Premont et al., 1996; Sallese et al., 1994; Sallese et al., 1997; Virlon et al., 1998).

GRKs contain a central catalytic domain approximately 260 amino acids in length (Figure 1.1). The N-terminus of the GRKs is well conserved and has thus been suggested to meditate receptor recognition (Willets et al., 2003a). The N-terminus also contains a regulator of G protein signalling homology domain (RH domain), which has been proposed to regulate GPCR signalling in a phosphorylation-independent mechanism by sequestering activated Gα subunits. This has been demonstrated for GRK2 which sequesters $G\alpha q_{GTP}$, suppressing Gαq-mediated phospholipase C (PLC)-β activation (Carman et al., 1999). RH domain ligands for the other GRKs have yet to be identified. However, GRK4 has been reported to promote phosphorylation-independent desensitisation of the $GABA_B$ receptor (Perroy et al., 2003). GRK4 catalytic activity is not required for $GABA_B$ desensitisation perhaps suggesting that GRK4 promotes desensitisation by binding to $G\alpha i/o_{GTP}$, preventing functional coupling of the receptor to its cognate effector (Perroy et al., 2003). The C-terminus of GRKs is more variable and constitutes the major site of posttranslational modifications and protein-protein interactions which act to regulate GRK catalytic activity and cellular localisation, discussed further in Section 1.4 (Pitcher et al., 1998). The crystal structure of two GRK2 and GRK6, has been determined and reveals some interesting insights into their function (Lodowski et al., 2003a;

Lodowski et al., 2006). The kinase domain of these GRKs appears to adopt an open conformation similar to other kinases such as PKA. The open and presumably inactive conformation of GRKs suggests that GPCR binding may activate these kinases by inducing kinase domain closure (Lodowski et al., 2006). However, differences between GRK2 and GRK6 3D conformation were noted, highlighting potential differences between the GRKs. In particular, the crystal structure of GRK6 reveals that it exists as a dimer. Dimerisation does not appear to be required for receptor phosphorylation (Lodowski et al., 2006) and a function for GRK6 dimerisation has yet to be reported.

1.2. GRK function – GPCR desensitisation

1.2.1. GPCR structure

GPCRS or seven-transmembrane spanning receptors (7TMRs), make up the largest family of plasma membrane receptors and the most frequent therapeutic drug target (Pierce et al., 2002). There are over 800 genes encoding GPCRs in the human genome (Jacoby et al., 2006), many of which are yet to be deorphanised (Lefkowitz, 2004). GPCRs are regulated by a diverse range of agonists from hormones, neurotransmitters, chemokines and calcium ions to odorants for bitter and sweet taste and light (Lefkowitz, 2004; Pierce et al., 2002).

Based on sequence homology alone, GPCRs have been divided into three subfamilies, A, B and C (Pierce et al., 2002). The families share over 25% sequence homology in the transmembrane spanning region along with shared motifs and conserved amino acids (Pierce et al., 2002). Family A, the largest family, includes

amongst others retinal light receptors (rhodopsin), adrenergic receptors (ARs, types α and β) and olfactory receptors. Family B is made up of only 25 members including receptors for gastrointestinal peptide hormones. The members of this family appear to couple predominantly to the G protein G_s, to activate adenylyl cyclase (AC). Family C is comparatively small and contains the metabotropic glutamate receptor family, $GABA_B$, and the calcium (Ca^{2+}) sensing receptors. Family C receptors are typified by a large extracellular amino terminus, which seems to be critical for ligand binding and activation.

Determination of the crystal structure of rhodopsin (Figure 1.2A) confirmed what biochemical experiments had previously indicated, that GPCRs share structural features such as the seven α transmembrane helices which are connected by six loops of differing lengths (Palczewski et al., 2000). The N-terminus of rhodopsin is extracellular and includes a conserved disulfide bridge that arranges the seven transmembrane helix motifs (Figure 1.2A). The C-terminus of the receptor is intracellular and contains key residues that allow interactions between the transmembrane helices and the cytoplasmic surface (Figure 1.2A).

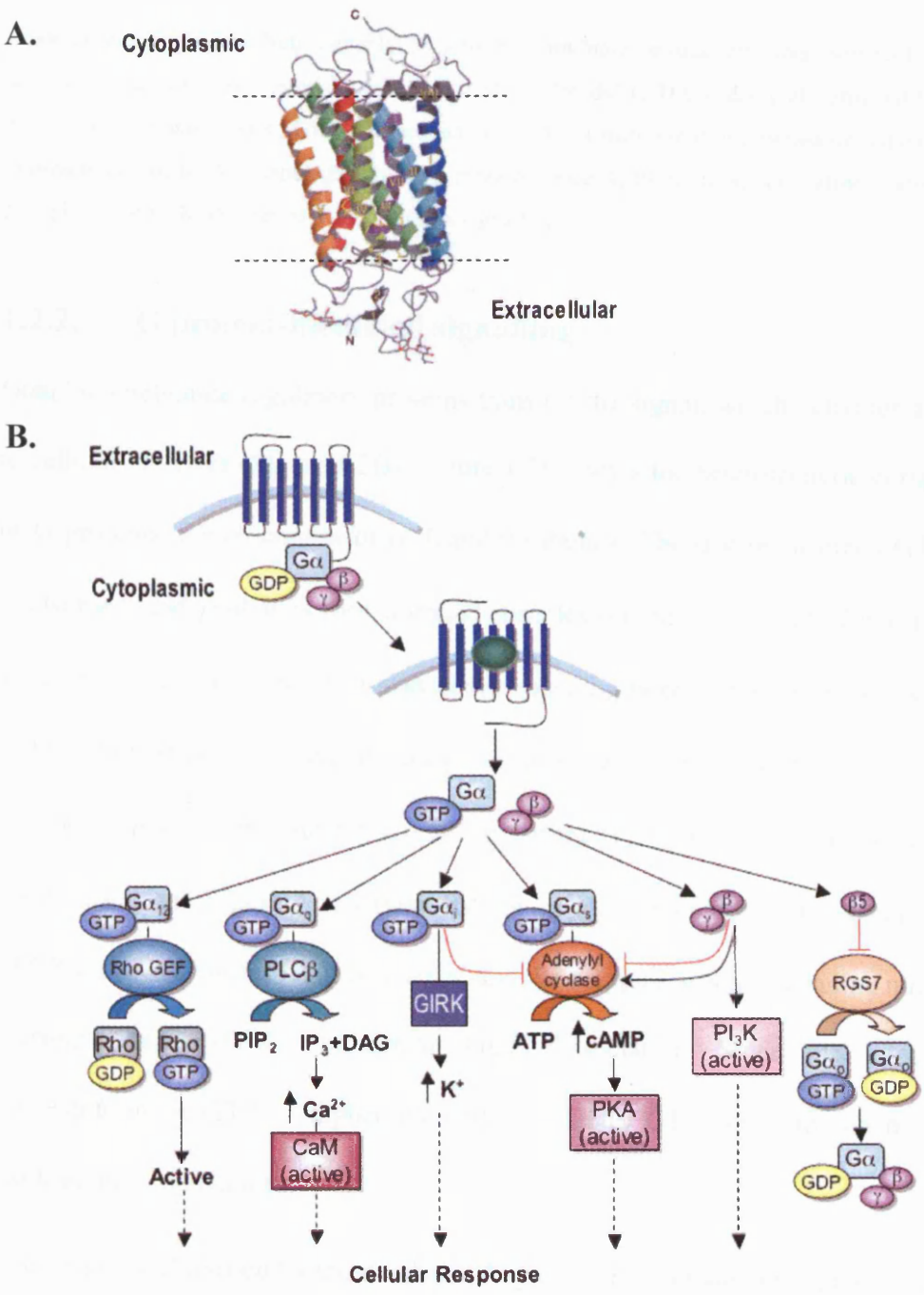


Figure 1.2 Classical GPCR signalling.

A. 2.8Å Crystal structure of rhodopsin, the light activated GPCR found in the retina. Rhodopsin is represented as a ribbon drawing parallel to the plane of the membrane (stereoview) (Reproduced from Palczewski et al., 2000). B. Classic examples of GPCR signalling. Following agonist binding, a conformational change within the receptor promotes GDP release from the α subunit of the heterotrimeric G protein and GTP loading. This results in G protein activation and dissociation into α

subunits and βγ dimers, both capable of activating multiple cellular effectors, some of which are depicted (Adapted from Pierce et al., 2002). CaM, calmodulin; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; GIRK, G protein-coupled inwardly rectifying potassium channels; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PI_3K, phosphoinositide 3–kinase; PLC, phospholipase C; RGS, regulator of G protein signalling.

1.2.2. G protein-mediated signalling

Guanine-nucleotide regulatory proteins transmit the signal, which activates a GPCR, to cellular effectors (Figure 1.2B). Figure 1.2B shows the heterotrimeric composition of G proteins, which consist of α, β and γ subunits. The α subunit binds GTP/GDP whilst the β and γ subunits form a linked complex (Gainetdinov et al., 2004; Pierce et al., 2002). There are sixteen human genes encoding twenty-three known α subunits, and five human β and twelve human γ subunit genes identified to date (McCudden et al., 2005). In its inactive state, the α subunit of the G protein is GDP bound and binds the βγ complex (Figure 1.2B) (Gainetdinov et al., 2004). Agonist occupancy of a GPCR induces a conformational change and the receptor acts as a guanine nucleotide exchange factor (GEF), promoting GDP dissociation. Since the intracellular concentration of GTP is higher than that of GDP, GTP binds to the α subunit resulting in its activation.

G proteins are classified by the α subunit they contain. Four subfamilies of α subunits are known; G_s proteins which stimulate AC, G_i proteins which inhibit ACs and activate G protein-coupled inwardly rectifying potassium channels (GIRKs), Gq proteins which activate PLCβ and G_{12} proteins which activate Rho GEFs. The Gβγ complex also activates or inhibits a large number of effectors including but not limited to GIRKs (Huang et al., 1997; Kunkel and Peralta, 1995), Ca^{2+} channels

(Delmas et al., 2000; Kammermeier et al., 2000), ACs (Tang and Gilman, 1991), mitogen-activated protein kinases (MAPKs) (Faure et al., 1994), phosphoinositide 3-kinases (PI3Ks) (Stephens et al., 1994) and GRK2 (Lodowski et al., 2003b). Gβ5 has been shown to bind directly to regulator of G protein signalling (RGS) proteins which act to turn off G protein mediated signalling by promoting Gα-mediated GTP hydrolysis thereby acting as a GTPase activating protein (GAP) (Levay et al., 1999; Snow et al., 1998). Gβ5 binding to RGS7, inhibits the ability of RGS7 to bind to Gαo and has been suggested to be a RGS7 inhibitor (Levay et al., 1999). It was traditionally the view that Gα activation results in its dissociation from the βγ complex (Gilman, 1987). However, it has emerged that this is not necessarily the case for all Gα subunits, certain Gαi subunits do not dissociate from the heterotrimeric complex but appear to undergo a rearrangement which permits an interaction with effector subunits (Frank et al., 2005). Typical signalling pathways regulated by the heterotrimeric G proteins are depicted in Figure 1.2B. The combinatorial possibilities of α, β and γ subunits and the fact that different heterotrimeric complexes can couple to multiple GPCRs, provides huge potential variety in terms of signal propagation from GPCRs.

1.2.3. GRK and βarrestin-mediated GPCR desensitisation

An important characteristic of GPCRs is that continuous stimulation results in receptor desensitisation. Rapid GPCR desensitisation is mediated, in large part, by receptor phosphorylation by two distinct classes of protein kinases. Firstly, second-messenger regulated kinases such as PKA participate in a feedback mechanism by which they are activated by GPCRs and then, in turn, phosphorylate the GPCRs,

inhibiting their coupling to G proteins and terminating signalling (Reviewed inFerguson, 2001). Here however I will focus on the second phosphorylation-dependent mechanism of GPCR desensitisation, that mediated by GRKs.

Agonist dependent recruitment of GRKs to GPCRs leads to specific phosphorylation of serine and threonine residues in the C-terminal tail or third intracellular loop of agonist occupied receptors (Ferguson, 2001; Gainetdinov et al., 2004; Willets et al., 2003a). Arrestins, a family of adaptor proteins bind GRK phosphorylated GPCRs preventing G protein coupling and thus GTP loading of the α subunit (Luttrell and Lefkowitz, 2002). This terminates further G protein-mediated intracellular signalling from this receptor. There are four arrestin family members, rod arrestin (arrestin-1), cone arrestin (arrestin 4), βarrestin 1 (arrestin 2) and βarrestin 2 (arrestin 3), all of which share high sequence homology but as the names suggest, rod arrestin is found solely in rod outer segments of the retina in the eye and cone arrestin in the cone photoreceptors of the eye and pineal gland (Pierce and Lefkowitz, 2001). β-Arrestin 1 (βarr-1) and β-arrestin 2 (βarr-2) (shown schematically in Figure 1.3) are ubiquitously expressed, with particularly high levels in the brain. There are however, some differences in distribution between the two subtypes, βarr-2 being more widely distributed in rat brain than βarr-1. Conversely, βarr-2 is expressed at higher levels than βarr-1 in the striatum, hypothalamus and hippocampus whereas βarr-1 is more highly expressed in the olfactory bulb (Pierce and Lefkowitz, 2001). Agonist mediated translocation of βarrs to GPCRs defines two major classes of receptors (Oakley et al., 2000). Class A receptors, which bind both biogenic amines and peptide ligands, such as the β_2-adrenergic receptor (β_2AR), Dopamine D1A receptor

and the α_{1B}-adrenergic receptor (α_{1B}AR), bind to βarr-2 with higher affinity than βarr-1. Conversely, class B receptors, which bind peptide ligands, such as the Angiotensin II type IA receptor ($AngII_{1A}R$) and vasopressin V2 receptor, bind to both βarr-1 and βarr-2 with similarly high affinity. It appears that residues in the C-terminal tail of GPCRs determine their classification as either class A or B receptors rather than their ligand (Oakley et al., 2000). Thus it might be possible to predict which βarr isoform will desensitise which GPCRs in a particular cellular environment.

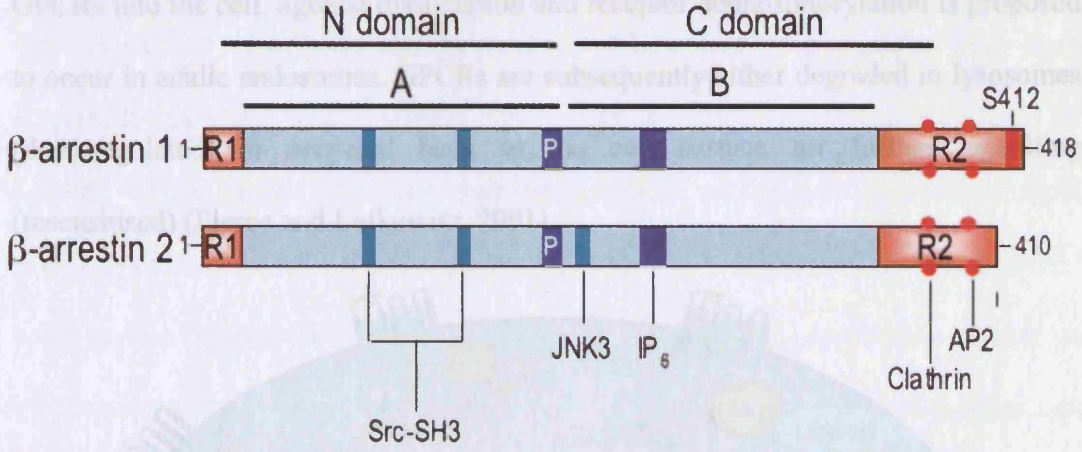


Figure 1.3. βarrestin Family.

Domain architecture of the βarrestins (βarrs). The βarrs contain two major structural domains, the N-terminal domain (A) and the C-terminal domain (B) separated by a phosphate sensor domain (P). It has been proposed that the N-terminal domain specifically recognises activated GPCRs whilst the B domain provides further receptor-binding capability. Regulatory domains are present at the N- and C-termini of βarrs. The R2 region contains binding motifs for clathrin and AP2, components of the endocytosis machinery and the primary site of βarr-1 phosphorylation, S412. Other motifs in βarrs recognise and bind IP_6, Src-SH3 and Jnk3. βarrs also bind to a range of other signalling molecules not shown here including Src-SH1, Ask1, NSF, Mdm2 and filamin (Reproduced from Luttrell and Lefkowitz, 2002).

As well as acting to prevent coupling to G proteins arrestins mediate the

internalisation of GPCRs (Figure 1.4). βarrs act as adaptors between the GPCRs and clathrin-coated pits (CCPs). βarrs bind to clathrin (Goodman et al., 1996) and the β2-adaptin subunit of the AP2 complex (Laporte et al., 1999). By virtue of this ability to bind to components of CCPs, they recruit GPCRs into such membrane invaginations and therefore direct their internalisation (Figure 1.4). GRK2, whilst promoting βarr binding to the receptor also recruits PI3K which is required to generate D3-phosphoinositides, required for receptor internalisation (Naga Prasad et al., 2001; Naga Prasad et al., 2002). As depicted in Figure 1.4, following internalisation of GPCRs into the cell, agonist dissociation and receptor dephosphorylation is proposed to occur in acidic endosomes. GPCRs are subsequently either degraded in lysosomes (downregulated) or recycled back to the cell surface for further signalling (resensitised) (Pierce and Lefkowitz, 2001).

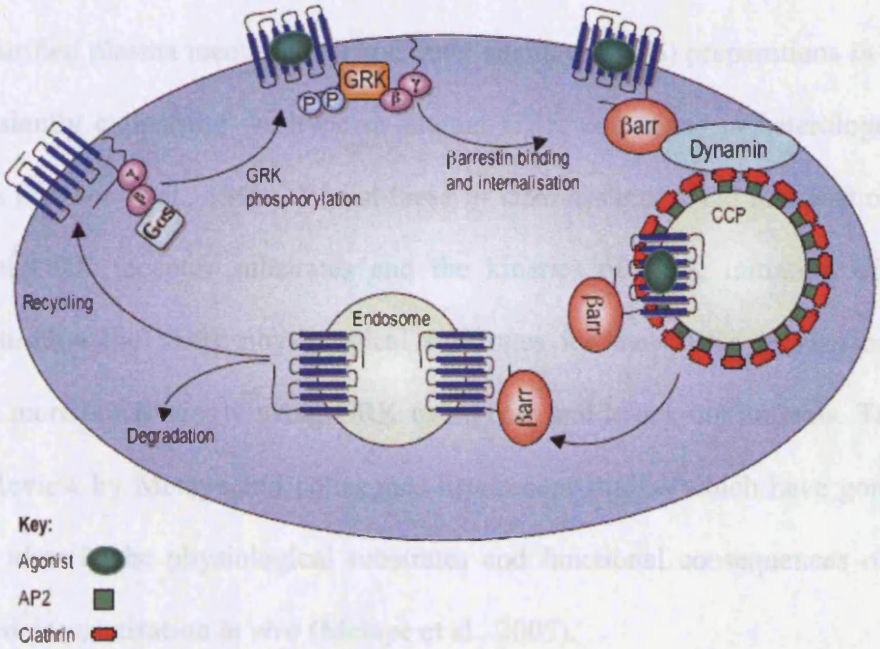


Figure 1.4. GPCR homologous desensitisation and trafficking.

Agonist binding to the GPCR induces a conformational change within the receptor which acts as a

GEF to activate the G protein. Activation of the receptor causes the dissociation of the activated G protein α and βγ subunits, which activate effector substrates in the cell. GRK recruitment to the receptor varies. Free βγ dimers recruit GRKs 2 or 3, GRKs 4 and 6 are permanently tethered to the membrane via lipid modifications at their C-termini and GRK5 binds to phospholipids in the plasma membrane via a PIP_2 binding domain. Recruited GRKs specifically phosphorylate agonist occupied GPCRs. This phosphorylation event recruits βarr, which binds to the phosphorylated tail of the GPCR and targets the receptor to a clathrin-coated pit (CCP). The receptor is internalised into acidic endosomes, dephosphorylated and then either follows a pathway for degradation or recycling back to the cell surface for a further round of signalling (Reproduced from Pierce et al., 2002).

1.2.4. GPCR desensitisation *in vivo*

Since the discovery of the GRK subfamily much work has focused on the ability these kinases to desensitise specific GPCRs. A multitude of approaches have been employed to answer such questions (Pitcher et al., 1998), including the ability of purified GRKs to phosphorylate purified receptors reconstituted in phospholipid vesicles (Pitcher et al., 1998). The subtype specificity of GRKs has also been studied using purified plasma membrane or rod outer segment (ROS) preparations *in vitro* or by transiently expressing wildtype or mutant GRK constructs in heterologous cell systems (Pitcher et al., 1998). Whilst these *in vitro* systems yield information about potential GRK receptor substrates and the kinetics of GRK initiation of GPCR desensitisation the likely physiological substrates for the GRKs *in vivo* has been defined more convincingly using GRK transgenic and knock-out animals. Table 1.1 and a Review by Metaye and colleagues lists recent studies which have gone some way to identify the physiological substrates and functional consequences of GRK-mediated desensitisation *in vivo* (Metaye et al., 2005).

Table 1.1: GRK-mediated regulation of GPCRs *in vivo*

(Adapted from Metaye et al., 2005)

GRK Subfamily	GRK subfamily member	GRK Genetic Alteration	Targeted GPCR	Functional Implication	Reference
GRK1	**GRK1**	Knockout	Rhodopsin	• Prolonged rhodopsin responsiveness • Retinal apoptotic degeneration	(Chen et al., 1999; Lyubarsky et al., 2000)
GRK2	**GRK2**	Homozygous knockout	N/A	• Lethal phenotype identical to 'thin myocardium syndrome' observed in mice with inactive transcription factors RXRα, N-*myc*, TEF-1 and WT-1	(Jaber et al., 1996)
		Heterozygous knockout	Chemokine receptor (CCR5)	• Enhanced T cell chemotaxis	(Vroon et al., 2004a)
		Heterozygous knockout	β-Adrenergic receptors (β-AR)	• Increase in β-AR stimulated cardiac contractility	(Rockman et al., 1998b)
		Vascular smooth muscle overexpression	β-ARs	• Attenuated β-AR stimulated vasodilation, elevated blood pressure and hypertrophy	(Eckhart et al., 2002)
		Cardiac overexpression	β-ARs	• Diminished β-AR stimulated ventricular contraction	(Koch et al., 1995)

GRK Subfamily	GRK subfamily member	GRK Genetic Alteration	Targeted GPCR	Functional Implication	Reference
GRK2 (Cont.)	**GRK2**	Cardiac overexpression	Angiotensin-II type IA receptor ($AngII_{IA}R$)	• Diminished Angiotensin-II stimulated cardiac contraction and heart rate	(Rockman et al., 1996)
		Cardiac overexpression	α_{1B}-Adrenergic receptor (α_{1B}-AR)	• No effect compared to controls	(Eckhart et al., 2000)
	GRK3	Knockout	M2/M3 Muscarinic receptors	• Enhanced cholinergic stimulation of airway responses	(Walker et al., 1999)
		Knockout	κ-opioid receptor	• Loss of Kappa opioid tolerance	(Xu et al., 2004)
		Cardiac overexpression	β-AR	• No cardiac effect compared to controls	(Iaccarino et al., 1998a)
		Cardiac overexpression	$AngII_{IA}R$	• No cardiac effect compared to controls	(Iaccarino et al., 1998a)
		Cardiac overexpression	α_{1B}-AR	• Diminished α_{1B}-AR cardiac signalling	(Eckhart et al., 2000)
		Cardiac overexpression	Thrombin receptor	• Attenuated MAPK p42/p44 signalling via the Thrombin receptor	(Iaccarino et al., 1998a)
GRK4	**GRK4γ (A142V)**	Overexpression	Dopamine-D1 receptor	• Hypertension as a result of attenuation of renal D1R regulation of fluid and electrolyte balance	(Felder et al., 2002)

GRK Subfamily	GRK subfamily member	GRK Genetic Alteration	Targeted GPCR	Functional Implication	Reference
GRK4 (Cont.)	**GRK5**	Knockout	Dopamine D1/D2 receptors	• No effect on locomotion compared to controls	(Gainetdinov et al., 1999)
		Knockout	M2/M3 Muscarinic receptors	• Excessive muscarinic receptor-mediated opposition of airway smooth muscle relaxation • Enhanced hypothermia, hypoactivity, tremor, salivation following muscarinic stimulation (oxotremorine)	(Gainetdinov et al., 1999; Walker et al., 2004)
		Knockout	Serotonin type IA receptor	• No effect compared to controls	(Gainetdinov et al., 1999)
		Knockout	Chemokine receptor (CXCR4)	• No effect on chemotactic responses	(Fong et al., 2002)
		Knockout	N/A	• Age-dependent increase in hippocampal defects, extracellular β-amyloid fibrillar deposits, degenerating axons, loss of synaptic proteins and consistent selective working memory impairment	(Suo et al., 2006)

GRK Subfamily	GRK subfamily member	GRK Genetic Alteration	Targeted GPCR	Functional Implication	Reference
GRK4 (Cont.)	**GRK5**	Knockout	N/A	• Decreased PDGF-stimulated PDGFRβ serine phosphorylation in smooth muscle cells compared to wildtype with a concomitant increase in PI hydrolysis and thymidine incorporation. • Decreased PDGFRβ association with the phosphatase Shp2 and Src activation	(Wu et al., 2006)
		Cardiac overexpression	β-ARs	• Diminished β-AR stimulated cardiac contraction	(Rockman et al., 1996)
		Cardiac overexpression	$AngII_{1A}R$	• No cardiac effect compared to controls	(Rockman et al., 1996)
		Cardiac overexpression	α_{1B}-AR	• Partially diminished cardiac α_{1B}-AR signalling	(Eckhart et al., 2000)
	GRK6	Knockout	Dopamine D_2-like receptors	• Supersensitivity to cocaine and amphetamine stimulated locomotion	(Gainetdinov et al., 2003)
		Knockout	Chemokine receptor (CXCR4)	• Impaired T cell chemotaxis • Enhanced chemotaxis of bone marrow derived neutrophils and impaired neutrophil mobilisation	(Fong et al., 2002; Vroon et al., 2004b)

1.3. GRK function – signalling roles

GRKs are best known for their role in promoting desensitisation of GPCRs however it is evident from the number of non-receptor substrates and binding partners that have been identified for these kinases that they perform additional signalling functions (Penela et al., 2006; Penela et al., 2003). Non-receptor substrates and binding partners of GRK2, the most intensively studied member of the GRK family, are shown in Figure 1.5. The functional relevance of many of the GRK2-mediated non-GPCR phosphorylation events shown in Figure 1.5 remains somewhat unclear. GRK2-mediated phosphorylation of the inhibitory gamma subunit of cyclic guanosine monophosphate (cGMP) phosphodiesterase stimulates epidermal growth factor receptor (EGFR)-mediated activation of p42/p44 MAPK in human embryonic kidney cells (HEK-293 cells) (Wan et al., 2001; Wan et al., 2003). GRK2-mediated epithelial Na^{2+} channel (ENaC) phosphorylation inhibits Nedd4-2-mediated Na^{+} channel ubiquitination and internalisation (Dinudom et al., 2004). Phosphorylation of phosducins by GRK2 inhibits an interaction between phosducins and Gβγ thereby promoting Gβγ signalling (Ruiz-Gomez et al., 2000).

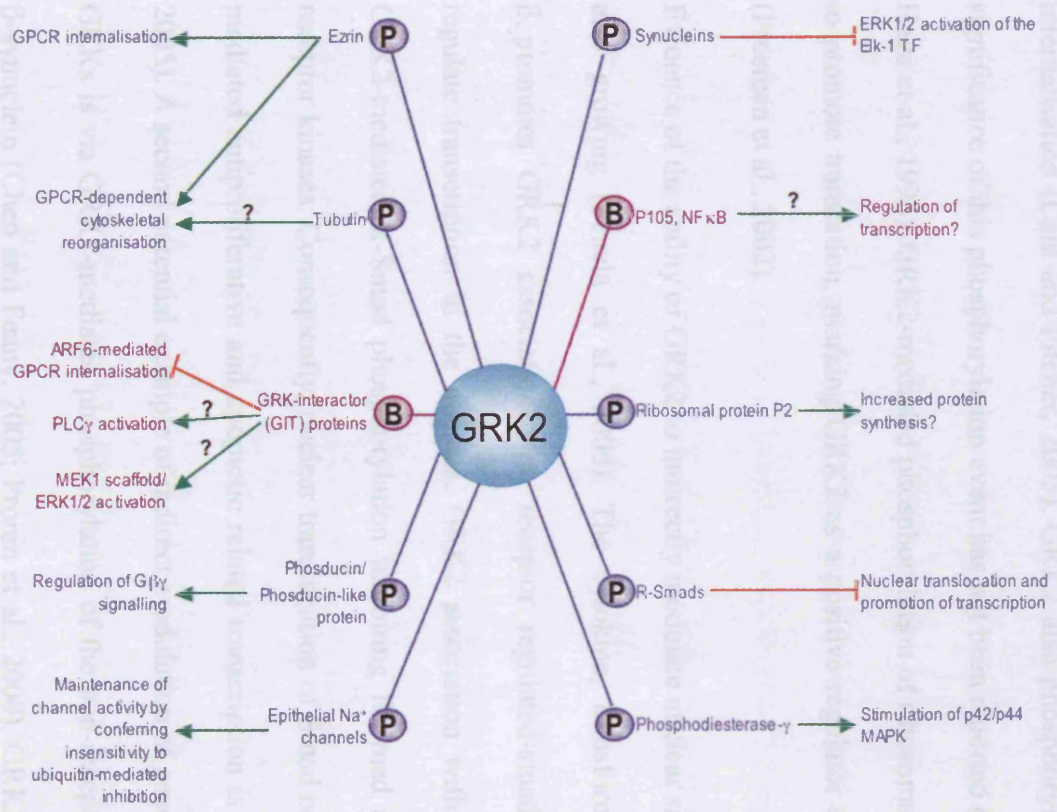
(Cant and Pitcher, 2005)

(Cant and Pitcher, 2005; Carman et al., 1998; Haga et al., 1998)

(Haendeler et al., 2003; Premont et al., 1998; Yin et al., 2004; Yin et al., 2005)

(Ruiz-Gomez et al., 2000)

(Dinudom et al., 2004)



(Chen and Feany, 2005; Iwata et al., 2001a; Iwata et al., 2001b; Pronin et al., 2000)

(Parameswaran et al., 2006)

(Freeman et al., 2002)

(Ho et al., 2005)

(Wan et al., 2001; Wan et al., 2003)

Figure 1.5. GRK2 non-receptor substrates and binding partners.

GRK2 is involved in a wide range of intracellular signalling pathways distinct from its role in desensitizing GPCRs. GRK2 phosphorylates (P) and binds to (B) a wide range of molecules. GRK2 substrates are shown in blue, whilst binding partners are shown in purple. The results of either of these actions can promote (green arrow) or inhibit (red inhibitory arrow) various signalling pathways including cytoskeletal organisation, cytosolic signalling and nuclear signalling. Unconfirmed steps are indicated with a '?'. B, binding; P, phosphorylation; TF, transcription factor.

A role for GRK2 in mediating cytoskeletal organisation has also emerged. Phosphorylation of ezrin, a member of the ezrin-radixin-moesin (ERM) protein family, by GRK2 results in activation of this protein and promotes GPCR-dependent cytoskeletal reorganization. GRK2-mediated activation of Ezrin is required for β_2AR internalisation (Cant and Pitcher, 2005). GRK2 also phosphorylates tubulin, but the significance of this phosphorylation event has not been reported (Carman et al., 1998; Haga et al., 1998). GRK2-mediated phosphorylation of ribosomal subunit P2 appears to promote translation, marking GRK2 as a positive regulator of gene transcription (Freeman et al., 2002).

Evidence of the ability of GRK2 to indirectly modulate nuclear signalling pathways is also growing (Penela et al., 2006). The cytokine, transforming growth factor-β, promotes GRK2 association with receptor regulated-smads (R-smads) which regulate transcription in the nucleus. GRK2 association with R-smads results in GRK2-mediated R-Smad phosphorylation inhibiting R-Smad activation by type-1 receptor kinases. Consequently, nuclear translocation of Smad complexes and Smad-mediated antiproliferative and apoptotic related transcription is inhibited (Ho et al., 2005). A second potential example of indirect modulation of gene transcription by the GRKs is via GRK2-mediated phosphorylation of the non-receptor substrates α- and β-synuclein (Chen and Feany, 2005; Pronin et al., 2000). GRK2 phosphorylation of α-synuclein enhances α-synuclein aggregate formation in a Drosophila model of Parkinson disease (Chen and Feany, 2005). Although the function of α-synuclein is not completely understood it has been shown to inhibit ERK2-mediated phosphorylation and activation of the transcription factor Elk-1 (Iwata et al., 2001a;

Iwata et al., 2001b). Furthermore, GRK5 was also found to phosphorylate α-synuclein, suggesting that this signalling pathway might be modulated by more than one GRK (Pronin et al., 2000).

Binding partners of the GRKs are also increasing in number. GRK2 has been shown to bind to and recruit G protein coupled receptor kinase-interacting protein 1 (GIT1) to the plasma membrane (Premont et al., 1998). GIT's have multiple cellular functions (Hoefen and Berk, 2006), but of particular importance to GRK signalling is the ability of GIT-1 (G protein coupled receptor kinase-interacting protein-1) to act as an ARF-GAP inhibiting receptor internalisation mediated by the small GTPase Arf6 (Premont et al., 1998). GIT-1 also interacts with and activates PLC, promoting phosphatidylinositol 4,5-bisphophate (PIP_2) hydrolysis and promotes MAPK/ERK kinase 1 (MEK1) activation of extracellular signal-regulated kinase (ERK)-1/2 (Hoefen and Berk, 2006). It is unclear if GRK2-dependent translocation of GIT-1 to the plasma membrane actively promotes all of these GIT-1 mediated processes but as GRK2 is localised to GPCRs where activation of these pathways is initiated, it might not be unreasonable to think that GRK2 promotes more than one of GIT-1's signalling functions. *In vitro* GRK5 binds to and phosphorylates the NFκB1 (nuclear factor κB1) precursor, p105, and appears to negatively regulate lipopolysaccharide-stimulated ERK activation (Parameswaran et al., 2006). The relevance of these findings, in relation to toll-like receptor signalling which regulates innate and adaptive immunity, are yet to be fully explored.

Evidence for cellular signalling roles of the GRKs has focused particularly on GRK2 but other members of the GRK family might also have roles similar to GRK2 in

regulating cellular signalling.

1.4. Regulation of GRK activity

GRKs are subject to a wide range of regulatory mechanisms including alteration of their expression levels, subcellular localisation and interactions with other cellular proteins, all of which affect their cellular activity (Reviewed in Penela et al., 2003).

1.4.1. Regulation of GRKs by calcium binding proteins

Ca^{2+} plays a major role in intracellular signalling, activating calcium-sensor proteins (CSPs) which, either directly or indirectly, activate other target molecules that regulate biochemical changes within the cell. (Ikura et al., 2002; Santella et al., 2005). Calmodulin (CaM) is a universal and well-studied CSP (Chin and Means, 2000). CaM is expressed in all eukaryotic cells and regulates a range of effectors including the CaM-dependent ACs (Ferguson and Storm, 2004), PLC (McCullar et al., 2003) and protein kinases including but not limited to the CaM kinases (Sheng et al., 1991), sphingosine kinase (Young et al., 2003), the cyclin dependent protein kinases (Agell et al., 1998) and the GRKs (Sallese et al., 2000a). CaM also regulates receptors at the plasma membrane including ion channels (Chin and Means, 2000). In addition, CaM has a number of nuclear roles including regulation of transcription factors, particularly the basic helix-loop-helix (bHLH) group of transcription factors (Agell et al., 1998; Hermann et al., 1998). Thus, activation of CaM by increasing Ca^{2+} intracellular concentration has wide ranging effects.

1.4.1.1. Regulation of the GRK1 subfamily by Recoverin

CSP regulation of GRKs was first identified in studies of retinal phototransduction.

Ca^{2+} activated Recoverin, a CSP expressed primarily in vertebrate photoreceptor cells binds to and inhibits GRK1 (rhodopsin kinase) catalytic activity (Figure 1.1) (Chen et al., 1995; Klenchin et al., 1995). In the dark, when rhodopsin is inactive, basal Ca^{2+} levels are high and thus Recoverin is activated. Recoverin binds to GRK1 and inhibits its catalytic activity and thus rhodopsin phosphorylation. Recoverin mediated inhibition of GRK1 is relieved upon light exposure when Ca^{2+} levels decrease, permitting GRK1 to phosphorylate rhodopsin and initiate its desensitisation (Kohout and Lefkowitz, 2003). The effect of Recoverin on GRK7 activity has not been reported.

1.4.1.2. Regulation of the GRK2 subfamily by CaM

The ubiquitous CSP, CaM, inhibits the kinase activity of the non-retinal GRKs (Figure 1.1) (Sallese et al., 2000a). Ca^{2+}/CaM inhibits GRK2 and 3 mediated phosphorylation of rhodopsin *in vitro* with an IC_{50} of 2μM (Chuang et al., 1996; Haga et al., 1997; Pronin et al., 1997). GRK2 and 3 contain an N-terminal CaM binding site between residues 18-37 (shown in Figure 1.1) and bind Ca^{2+}/CaM directly (Pronin et al., 1997).

1.4.1.3. Regulation of the GRK4 subfamily by CaM

Analysis of the regulation of the GRK4 subfamily by Ca^{2+}/CaM reveals that GRK4, 5 and 6 exhibit different affinities for Ca^{2+}/CaM. Purified GRK5 kinase activity, as assessed by rhodopsin phosphorylation, is inhibited by Ca^{2+}/CaM with an IC_{50} of approximately 40-50nM (Haga et al., 1997; Pronin et al., 1997). Ca^{2+}/CaM is therefore approximately fourty- to fifty-fold more potent in inhibiting GRK5-

mediated rhodopsin phosphorylation than that of GRKs 2 and 3 (Iacovelli et al., 1999).

Ca^{2+}/CaM has been shown to bind to GRK5 directly with a Kd of approximately 10nM (Iacovelli et al., 1999; Pronin et al., 1997). Two Ca^{2+}/CaM binding sites have been identified in GRK5 (shown in Figure 1.1), one in the N-terminus between residues 20-39 and one in the C-terminus between residues 547-562 (Pronin et al., 1998; Pronin et al., 1997). Ca^{2+}/CaM binding at the N-terminus of GRK5 prevents GRK5 from binding to and phosphorylating receptor substrates rather than inhibiting its kinase activity directly since Ca^{2+}/CaM bound GRK5 can phosphorylate soluble substrates such as casein (Chuang et al., 1996). The N-terminal Ca^{2+}/CaM binding site of GRK5 is coincident with the PIP_2-binding site of this kinase (shown in Figure 1.1) (Kunapuli et al., 1994; Pronin et al., 1997). PIP_2 binding to the N-terminal polybasic domain of GRK5 promotes receptor but inhibits soluble substrate phosphorylation (Freeman et al., 2000; Pitcher et al., 1996). In contrast, CaM or α-actinin binding to this same region inhibits specifically GRK5 plasma membrane localisation and GRK5-mediated GPCR phosphorylation but does not affect catalytic activity. Thus, CaM or α-actinin bound GRK5 phosphorylates exclusively soluble substrates (Freeman et al., 2000). Finally, actin binding to the N-terminal polybasic domain of GRK5 inhibits its catalytic activity (Freeman et al., 1998). So GRK5 activity can be completely inhibited by binding of actin, but can be targeted towards receptor or soluble substrates by binding of PIP_2 or CaM/α-actinin respectively. The interplay between these cytosolic molecules in determining GRK5 kinase activity and subcellular localisation are not yet fully understood and might be expected to be

complex.

Ca^{2+}/CaM binding to the C-terminus of GRK5 induces GRK5 inhibitory autophosphorylation at distinct sites within the kinase (serine 579, serine 583 and serine 584), shown in Figure 1.1, which serves to inactivate catalytic activity of GRK5 (Levay et al., 1998; Pronin et al., 1998; Pronin et al., 1997). So whilst Ca^{2+}/CaM binding at the N-terminus primarily inhibits GRK5-mediated receptor phosphorylation by directly competing with the receptor to bind to GRK5, Ca^{2+}/CaM binding at the C-terminus also directly inactivates GRK5s kinase activity. Ca^{2+}/CaM-mediated autophosphorylation of GRK5 will prolong GRK5 catalytic inactivity long after Ca^{2+}/CaM dissociation and presumably GRK5 kinase activity is reactivated following de-phosphorylation of these sites.

The catalytic activity of GRKs 4 and 6 is also inhibited by Ca^{2+}/CaM binding. Partially purified GRK4α-mediated phosphorylation of rhodopsin, the only splice variant demonstrated to phosphorylate rhodopsin *in vitro,* is inhibited by Ca^{2+}/CaM with an IC_{50} of approximately 80nM (Sallese et al., 1997). The IC_{50} of Ca^{2+}/CaM inhibition of GRK6 catalytic activity, determined using lysates from COS cells overexpressing GRK6, is 0.7μM (Pronin et al., 1997). These measurements reveal that GRKs 4 and 6 are less sensitive than GRK5 to Ca^{2+}/CaM-mediated kinase inhibition but are still thirty-fold more sensitive to Ca^{2+}/CaM than GRKs 2 and 3 (Iacovelli et al., 1999). The N-terminal CaM binding site of GRK5 is well conserved within the GRK4 subfamily members but is absent in GRK4β and δ since the CaM binding site lies within an alternatively spliced exon of these splice variants (shown in Figure 1.1) (Iacovelli et al., 1999; Sallese et al., 1997). Like GRK5, GRK4α binds to

CaM directly as determined using CaM-conjugated Sepharose 4B (Sallese et al., 1997). However, GRK4β, γ and δ did not bind to CaM in the same experiment, perhaps suggesting that these splice variants are not regulated by Ca^{2+}/CaM (Sallese et al., 1997). The N-terminal high affinity Ca^{2+}/CaM binding site of GRK5 is not as well conserved in GRKs 2 and 3 suggesting a potential explanation for the reduced affinity of GRK2 and 3 for Ca^{2+}/CaM compared to GRK5 (Iacovelli et al., 1999). The C-terminal Ca^{2+}/CaM binding site of GRK5 appears to be at least partially conserved in GRKs 4α, 4β and 6 but the significance of this binding site has not been thoroughly investigated. The fact that GRK4β does not bind CaM *in vitro* suggests that at least in this kinase the binding site is not functional although this has not been definitively investigated (Sallese et al., 1997).

The functional implications of Ca^{2+}/CaM inhibition of GRK-mediated receptor phosphorylation are potentially far reaching. Ca^{2+}/CaM binding to GRK5 has already been demonstrated to inhibit receptor phosphorylation and perhaps therefore promote kinase activity towards soluble substrates (Chuang et al., 1996; Freeman et al., 2000). Ca^{2+}/CaM-mediated inhibition of GRKs can be envisaged in other cellular settings, for example, a rise in intracellular Ca^{2+} mediated by activation of a Gq-coupled receptor would ultimately inhibit GRK activity. This might provide a feedback mechanism to inhibit GRK-mediated GPCR desensitisation. Indeed, GRK2-mediated agonist-dependent internalisation of the M2 Muscarinic receptor (M2MR) in Chinese hamster ovary (CHO) cells was inhibited after treatment with a Ca^{2+} ionophore (Haga et al., 1997). It seems likely, due to the ubiquitous nature of Ca^{2+} signalling, that the roles of Ca^{2+}/CaM-dependent inhibition of GRKs has not yet been fully elucidated.

1.4.2. Regulation of GRK cellular localisation

The ability of GRKs to phosphorylate and desensitise receptors depends on their localisation at the plasma membrane in close proximity to the GPCR. Various C-terminal modifications play an important role in regulating how GRKs interact with GPCRs at the plasma membrane during receptor signalling. GRKs have variable C-termini and utilise distinct mechanisms for binding to the plasma membrane (shown in Figure 1.1). Some of the GRK family members are post-translationally modified at their C-termini. GRKs 1 and 7 contain a C-terminal CAAX motif which directs isoprenylation of these kinases which is essential for light-dependent membrane association (Figure 1.1) (Chen et al., 1999). GRK4α, β, γ, δ and GRK6A are all C-terminally modified by palmitoylation, a reversible post-translational modification (Figure 1.1) (Premont et al., 1996; Stoffel et al., 1994). GRK6A contains three C-terminal cysteine residues (561, 562, 565), mutation of which prevents GRK6A palmitoylation (Figure 1.1) (Stoffel et al., 1994). Thus, palmitoylation of GRK6A occurs at one or more of these cysteine residues. The exact location of the site of palmitoylation of GRK4 is not definitively established but all GRK4 splice variants contain a C-terminal cysteine residue (α: C563, β: C531, γ: C517 and δ: C485) homologous to C561 in GRK6A, as well as a second cysteine residue at the very C-terminus of all splice variants. These represent potential sites of palmitoylation in GRK4. GRK5 contains two lipid-binding sites, one at its N-terminus (residues 22-29) and one at its C-terminus (547-560) (Figure 1.1) (Pitcher et al., 1996). GRKs 4α, 4γ and 6A, B and C all appear to contain the N-terminal lipid binding site present in GRK5, suggesting that these kinases might also be regulated by lipid binding (Figure

1.1) (Pitcher et al., 1996). The C-terminal lipid-binding site of GRK5 does not show strict lipid specificity and this interaction induces intramolecular stimulatory autophosphorylation, primarily at serine 484 and threonine 485 (Kunapuli et al., 1994). This autophosphorylation increases GRK5 catalytic activity towards receptor but not soluble substrates. GRKs 2 and 3 are not posttranslationally modified at their C-terminus, but instead possess a phospholipid (PIP_2) binding pleckstrin homology domain (PH domain) that extends into the Gβγ binding domain of these kinases (Figure 1.1). Following GPCR activation, Gβγ subunits are released from the heterotrimeric G protein complex and bind to GRKs 2 and 3, recruiting these kinases to the plasma membrane (Pitcher et al., 1992; Pitcher et al., 1995). Thus, GRK2 and 3 are cytosolic kinases that are only present at the membrane following GPCR stimulation.

1.4.3. Regulation of GRKs by other kinases

Several kinases have been shown to regulate GRKs by phosphorylation, although work has generally focused on regulatory phosphorylations affecting the activity of GRKs 2 and 5. Here I will discuss regulation of GRKs by PKC which is most relevant to this thesis.

PKC is activated by intracellular Ca^{2+} release, such as that stimulated by Gq-coupled GPCRs. PKC phosphorylates GRK2 and GRK5 *in vitro* (Figure 1.1) (Chuang et al., 1995; Krasel et al., 2001; Pronin and Benovic, 1997; Winstel et al., 1996). PKC phosphorylation of GRK2 at serine 29 enhances its phosphorylation of GPCRs without affecting GRK2-mediated phosphorylation of soluble substrates implying that PKC phosphorylation promotes membrane association of GRK2 without

impairing its catalytic activity (Krasel et al., 2001). Phosphorylation at serine 29 within the N-terminal CaM binding region of GRK2 relieves the inhibitory interaction between GRK2 and CaM (Krasel et al., 2001). Conversely, phosphorylation of GRK5 by PKC inhibits its catalytic activity towards both receptor and soluble substrates (Pronin and Benovic, 1997; Pronin et al., 1998). Inhibitory PKC phosphorylation of GRK5 occurs at its C-terminus (ser-566, ser-568, ser-572) in the same region that CaM induces inhibitory autophosphorylation of GRK5 (Figure 1.1) (Pronin et al., 1998). PKC thus seems to have opposing effects, activating GRK2 while inhibiting GRK5.

1.4.4. Regulation of GRK expression

GRKs appear to be expressed at relatively low levels, such that their expression is limiting for agonist-dependent desensitisation of GPCRs (Penn and Benovic, 1994). Regulation of GRK expression during times of persistent GPCR signalling is required to prevent chronic activation of GPCRs. Relatively little is understood about the regulation of GRK gene transcription. However, it appears that varying signals can alter expression levels of GRKs depending on the signal and cell type. Increased circulation of catecholamines and stimulation of βARs correlates with an increase in GRK2 expression in the heart (Iaccarino et al., 1998b). In aortic smooth muscle cells, induction of physiological vasoconstriction and hypertrophy by agents such as phorphol esters or Gq activation results in increased transcriptional activity at the GRK2 promoter, whilst proinflammatory cytokines decrease transcriptional activity at the GRK2 promoter (Ramos-Ruiz et al., 2000). This suggests that GRK2 expression might be modulated by cell type specific signalling pathways. Recently, it

has been reported that GRK2 translation is downregulated in C6 rat glioma cells following exposure to hydrogen peroxide, the full mechanism of which is yet to be determined but appears to involve activation of cyclin dependent kinases (CDKs) (Cobelens et al., 2006). Further work is required to elucidate the regulatory mechanisms controlling GRK expression under both normal and disease states.

Regulating protein stability is another mechanism by which the function of GRKs may be modulated. GRK2, for example, has a short half-life of approximately 1 hour (Penela et al., 1998). Persistent isoproterenol stimulation of the β_2AR in HEK-293 cells, stimulates polyubiquitination of GRK2, continuously promoting its degradation by the proteasome (Penela et al., 1998). Kinase activity appears to be required for GRK degradation since the catalytically inactive mutant of GRK shows greater stability than wildtype (Penela et al., 1998). In this heterologous system, βarr-1 or -2 recruits c-Src to GRK2 which phosphorylates tyrosine residues within the kinase marking it for degradation via the proteasome (Penela et al., 2001). This mechanism causes downregulation of steady-state kinase levels. Regulation of GRK stability is yet to be fully understood.

1.5. GRKs and disease

GPCRs are the largest class of cell surface receptors and their dysfunction has been implicated in a number of human disease states (Johnson and Lima, 2003). GRK-mediated GPCR desensitisation regulates the number and function of GPCRs at the plasma membrane and thus GRKs have the potential to influence cellular signalling-mediated by these receptors in disease. Additionally, as discussed in Section 1.2.5, studies using transgenic animals not only reveal physiological receptor substrates for

the GRKs but also their potentially direct involvement in disease progression. Naturally occurring alterations in the expression of GRKs has also revealed the importance of GRKs in maintaining normal cellular function.

In patients suffering from Oguchi disease, point mutations in the catalytic region of GRK1, deletions at the C-terminus and deletion of the entire of exon 5 have been reported (Cideciyan et al., 1998; Khani et al., 1998). The absence of GRK-mediated rhodopsin desensitisation results in enhanced rhodopsin signalling causing Oguchi disease, an autosomal recessive form of retinitis pigmentosa apparent as night blindness (Cideciyan et al., 1998; Khani et al., 1998). Sufferers experience prolonged dark adaptation, abnormal sensitivity to light and, with excessive light exposure, retinal degeneration (Metaye et al., 2005).

Alterations in the expression of the ubiquitous GRKs have been observed in a number of human pathologies, of which I will only discuss a few (Metaye et al., 2005). In patients with rheumatoid arthritis a significant decrease in GRK2 (~55%) and GRK6 (~60%) expression was observed in peripheral blood mononuclear cells (Lombardi et al., 1999). Reduction in the expression of these GRKs likely results in increased sensitivity of β_2ARs in these cells, as administration of β2AR antagonists in murine models of rheumatoid arthritis delays the onset of the disease (Levine et al., 1988; Lombardi et al., 1999).

Changes in the expression levels of GRKs in a number of human cancers has also been reported (Metaye et al., 2005). In human differentiated thyroid carcinoma a decrease in GRK5 expression is observed which correlates with increased signalling from the thyrotropin receptor (Metaye et al., 2002), suggesting an inhibitory role for

GRK5 in hormonal-mediated cell proliferation.

Chronic treatment of rats with mood stabilisers such as lithium or carbamazepine results in a significant increase in membrane association of GRK3 in the frontal cortex. Membrane association of GRK3 is correlated with increased activity. Mood stabiliser treatments may thus promote GRK3 mediated GPCR desensitisation correcting supersensitivity of D2 dopaminergic receptors and other GPCRs seen in bipolar disorder (Ertley et al., 2006). A single nucleotide polymorphism in the promoter of GRK3 is linked with a susceptibility locus for bipolar disorder (Barrett et al., 2003). The P-5 SNP had an estimated allele frequency of 3% in bipolar subjects suggesting that dysregulation in GRK3 expression and thus GPCR desensitisation predisposes to onset of bipolar disorder.

Abnormalities in GRK2 function and expression are observed in a mouse model of Alzheimer's disease (Suo et al., 2004). Treatment of microglial cells with β-amyloid, the component that is deposited in the brains of sufferers, reduces the amount of GRKs 2 and 5 associated with the membrane in microglial cells, inhibiting desensitisation of thrombin receptors in this model, perhaps marking these GRKs as potential therapeutic targets in Alzheimer's disease (Suo et al., 2004).

Although GRK4 shows limited tissue distribution, aberrant expression of this kinase in kidney has been found to contribute to essential hypertension. Single nucleotide polymorphisms in GRK4γ have been shown to enhance GRK4γ kinase activity, against receptor substrates such as the Dopamine-D1 receptor in the renal proximal tubule, impairing the ability of D1R to regulate fluid and electrolyte balance which results in genetic hypertension (Felder et al., 2002; Speirs et al., 2004; Williams et al.,

2004).

The strongest evidence supporting a causative role for the GRKs in disease progression come from studies investigating their role in heart failure (HF) and cardiac hypertrophy discussed in the next Section, 1.6.

1.6. Heart failure and cardiac hypertrophy

HF has reached epidemic proportions in the western world and the lifetime risk for developing HF is one in five for both men and women (Lloyd-Jones et al., 2002). In HF the heart is unable to meet the metabolic demands of the peripheral tissues (Ritter and Neyses, 2003). The disease is typified by a progression of events, which begins when part of the heart is damaged, for example by hypoxia, myocardial infarction (necrosis of heart tissue due to lack of blood supply) or hypertension (increased blood pressure due to narrowed arteries containing fatty deposits). Initially, the heart can generally preserve function but slowly over time the rest of the heart begins to fail. Death ensues when the heart develops fatal rhythm disorders and stops pumping oxygenated blood to the body (Ritter and Neyses, 2003).

Generally, pathological cardiac hypertrophy is a prerequisite for the development of HF. Hypertrophy is an adaptive mechanism in which the heart increases its mass in response to signals initiated by biomechanical stress. Although hypertrophy initially compensates for the lack of wall tension in the heart, it is ultimately a maladaptive process which is generally associated with progression into decompensated HF (Mathew et al., 2001; Vakili et al., 2001).

In order to cope with increased biomechanical stress the cardiac muscle must increase

its capacity. Since myocytes cannot divide, hypertrophic signalling results in an increase in cardiac myocyte size by altering transcription of contractile and extracellular matrix proteins and increasing their rate of protein synthesis (Frey et al., 2004). Heightened organisation of the sarcomere occurs, chiefly by altering the composition of the myosin subunit. Transcription of α-MHC (myosin heavy chain) is downregulated whilst transcription of β-MHC is upregulated (Schwartz et al., 1993). This switch ultimately results in increased heart muscle contractility (Morano, 1999). These transcriptional changes represent induction of the fetal gene program and initiate cellular changes that lead to development of cardiac hypertrophy.

1.6.1. Chromatin modification

Nuclear deoxyribonucleic acid (DNA) is packaged into chromatin by proteins called histones (Figure 1.6). Chromatin is made up of nucleosomes which comprises 146 base pairs of DNA wrapped around a histone octamer (two copies each of histones H2A, H2B, H3 and H4) (Backs and Olson, 2006). Nucleosomes condense DNA, limiting access of transcription factors, and therefore acting to regulate gene transcription. Residues within the tails of histones are postranslationally modified by acetylation, methylation and phosphorylation creating a 'histone code' which determines if a region of chromatin is transcriptionally active or silent (Jenuwein and Allis, 2001). Acetylation of lysine residues in histone tails by histone acetyltransferases (HATs) is commonly associated with activation of gene transcription (Figure 1.6). Histone acetylation destabilises the chromatin structure allowing access to gene promoters. In contrast histone deacetylases (HDACs) remove acetyl modifications repressing transcription (Figure 1.6).

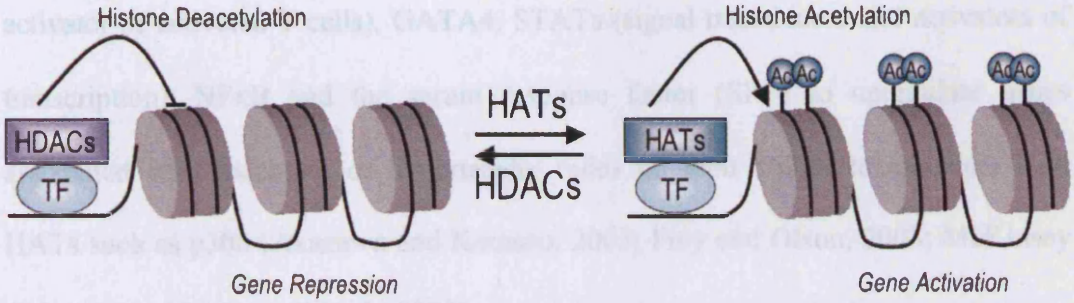


Figure 1.6. Generalised model of histone acetylation and deacetylation.

DNA and chromatin are assembled into nucleosomes. HATs and HDACs are recruited to DNA by association with transcription factors (TFs). Acetylation of histone tails results in chromatin relaxation and gene activation. Deacetylation opposes transcriptional activation by promoting chromatin condensation (Adapted from Backs and Olson, 2006).

1.6.2. HATs

There are at least five families of HATs (Roth et al., 2001). The most extensively studied HATs are p300 and its associated cofactor cAMP response element-binding protein (CBP) (Backs and Olson, 2006). p300 has inherent HAT activity and is capable of modifying both chromatin and transcription factors to promote gene transcription. p300 is essential for cardiac development since mice in which p300 is ablated die early during gestation due to inadequate expression of muscle structural proteins including β–MHC and α-actinin (Yao et al., 1998). p300-mediated promotion of transcription is enhanced following stimulation of α-ARs with phenylephrine (PE), which induces hypertrophy in cultured myocytes (Gusterson et al., 2002). Furthermore, PE-induced hypertrophy is stimulated by overexpression of CBP/p300 and blocked by overexpression of p300/CBP dominant negative mutants or antisense (Gusterson et al., 2002; Gusterson et al., 2003). The ability of cardiac transcription factors such as MEF2 (myocyte enhancer factor 2), NFAT (nuclear

activator of activated T cells), GATA4, STATs (signal transducers and activators of transcription), NFκB and the serum response factor (SRF) to upregulate genes associated with induction of hypertrophy relies on their ability to associate with HATs such as p300 (Akazawa and Komuro, 2003; Frey and Olson, 2003; McKinsey and Olson, 2004; Purcell et al., 2001).

1.6.3. HDACs

HATs are opposed by HDACs, of which there are three classes, I, II and III (Thiagalingam et al., 2003). Intriguingly, the nuclear class I HDACs have been reported to promote hypertrophic growth (Antos et al., 2003) whilst the class II family, which is localised throughout the nucleus and cytoplasm, are strongly linked to repression of hypertrophic growth (Zhang et al., 2002). The class II HDACs have a large N-terminal extension not present in class I HDACs which serves to mediate interactions with transcriptional repressors or activators as well as being a site for regulatory phosphorylation events which promote their nuclear export (Verdin et al., 2003). The role of class III HDACs in regulating cardiac transcription is yet to be fully explored (Backs and Olson, 2006).

Mice in which the prototypical class II HDACs such as HDAC5 or HDAC9 are ablated develop spontaneous cardiac hypertrophy aged six months induced via a calcineurin-dependent pathway (discussed in Section 1.6.4.4). This suggests these HDACs participate in specific Ca^{2+}-dependent signalling pathways that act to repress cardiac growth (Chang et al., 2004; Zhang et al., 2002). Class II HDACs are shuttled from the nucleus to the cytoplasm in response to stress signals promoting cardiac growth (Harrison et al., 2004; Vega et al., 2004). The nuclear export of HDAC 5 and

9 frees cardiac transcription factors, which associate with HATs, increasing local acetylation and induction of hypertrophic gene transcription (Zhang et al., 2002). Indeed, MEF2 binds class II HDACs and HATs competitively and is thus able to respond to both positive and negative hypertrophic transcriptional signals (McKinsey et al., 2001; McKinsey et al., 2002). Class II HDACs also regulate the transcriptional cofactor calmodulin binding transcription activator 2 (CAMTA2) (Song et al., 2006b). Recruitment of CAMTA2 to the atrial natriuertic factor (ANF) promoter occurs via an interaction with the homeodomain-containing cardiac transcription factor Nkx2-5 where it acts as a coactivator of Nkx2-5-mediated transcription (Song et al., 2006b). Nkx2-5 is essential in mice for normal cardiac development (Prall et al., 2002). The Class II HDAC, HDAC5 binds to CAMTA2, preventing its interaction with Nkx2-5 (Song et al., 2006b). Phosphorylation of HDAC5 by PKC or PKD results in its nuclear export which relieves HDAC5-mediated repression of CAMTA2 (Song et al., 2006b). Overexpression of CAMTA2 in myocytes in culture or in the hearts of mice results in hypertrophy and HF. Notably, hypertrophy is not observed following overexpression of a mutant CAMTA2 which cannot associate with Nkx2-5 (Song et al., 2006b). Since CAMTA2 is activated by CaM, activation of this coactivator is likely to be linked to Ca^{2+} excitation coupling which is required for the induction of hypertrophy. These findings support the proposed role for class II HDACs as inhibitors of hypertrophy. Paradoxically however, general inhibitors of HDACs such as trichostatin A (TSA) do not promote hypertrophy, but promote a dose dependent blockade of hypertrophy, improving cardiac function (Antos et al., 2003). These reports suggest that the function of Class I HDACs in promoting

hypertrophy may suppress the inhibitory effects of the Class II HDACs. Clearly, the role of histone modification in regulating the fetal gene program and cardiac hypertrophy is a complex but important avenue for research in understanding the regulation of cardiac growth.

1.6.4. Signalling pathways which regulate hypertrophy

GPCRs play an important role in the regulation of cardiac function, particularly the α- and β-ARs, angiotensin receptors (ATRs) and muscarinic receptors (MRs). Agonist stimulation of these receptors regulates cardiac contractility and promotes gene transcription, particularly those of the fetal gene program, illustrated in Figure 1.7. In this section I briefly describe the signalling pathways downstream of cardiac GPCRs that have been implicated in regulating cardiac hypertrophy and progression to HF. The role of the GRKs in regulating the signalling cascades is also discussed.

1.6.4.1. Gq/G_{11} signalling

In the heart, ATRs, Endothelin-I receptors (ET_1Rs) and αARs (α_{1A} and α_{1B} in myocytes) all couple to Gq/G_{11}, activating PLC, thereby increasing intracellular Ca^{2+} in myocytes (Figure 1.7) (Ritter and Neyses, 2003). Moreover, stimulation of these receptors results in activation of the transcription factor, NFκB, which promotes hypertrophic growth in cardiac myocytes (Figure 1.7) (Hirotani et al., 2002; Purcell et al., 2001).

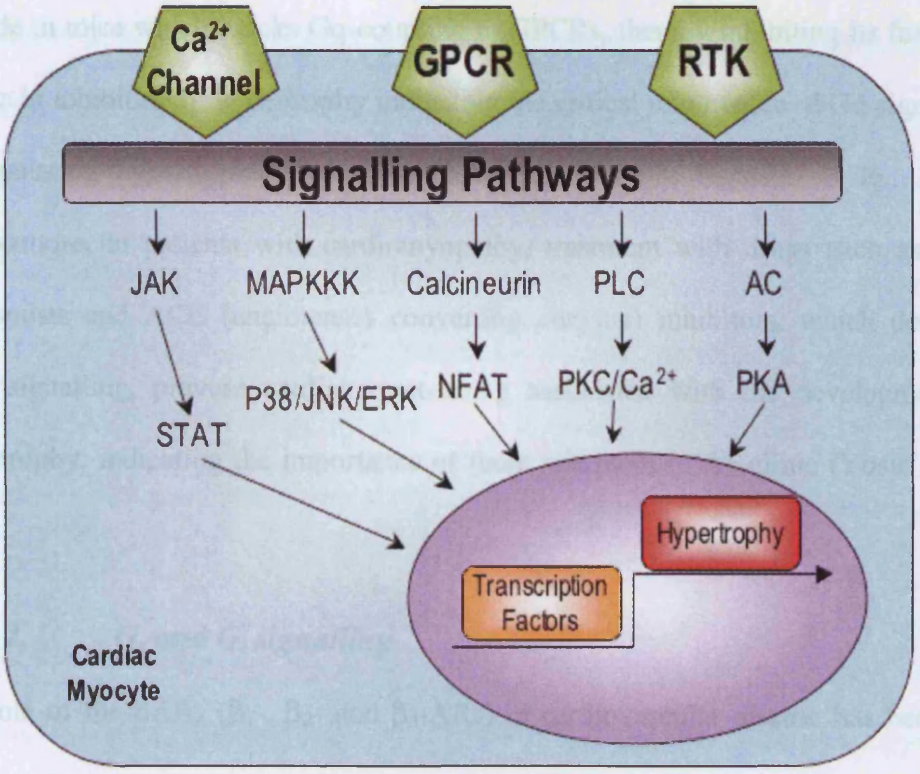


Figure 1.7. Signalling pathways in myocardial hypertrophy.

Biomechanical pressure activates calcium channels, receptor tyrosine kinases (RTK) and G-protein-coupled receptors (GPCRs) inducing activation of intracellular signalling cascades including the JAK-STAT, MAPK, Calcineurin-NFAT, PLC and adenylyl cyclase (AC) pathways. Activation of these intracellular signalling pathways results in hypertrophic gene transcription, which mediates an increase in cellular growth. Abbreviations: AC, adenylyl cyclase; ERK, extracellular-signal-regulated kinase; JAK, Janus kinase; JNK, Jun N-terminal kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; NF-AT, nuclear factor of activated T cells; p38, p38 kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; STAT, signal transducer and activator of transcription (Reproduced from Ritter and Neyses, 2003).

In rodents, activation of ATRs, ET_1Rs and α_1ARs has been shown to be sufficient to induce hypertrophy (Ichikawa et al., 1996; Keys and Koch, 2004; Rockman et al., 2002; Shah and Catt, 2003). Overexpression of ATRs, ET_1Rs, α_1ARs or Gq itself also results in hypertrophy in rodents (D'Angelo et al., 1997; Koch et al., 2000; Paradis et al., 2000; Sakata et al., 1998). Consistent with these observations, overexpression of a

peptide in mice which blocks Gq-coupling to GPCRs, thereby inhibiting its function, results in inhibition of hypertrophy indicating the critical importance of Gq signalling in regulating hypertrophic growth (Akhter et al., 1998; Esposito et al., 2001). Furthermore, in patients with cardiomyopathy, treatment with drugs such as ATR antagonists and ACE (angiotensin converting enzyme) inhibitors, which decrease ATR signalling, prevent cardiac remodeling associated with the development of hypertrophy, indicating the importance of these receptors in the clinic (Yusuf et al., 2000).

1.6.4.2. G_s and G_i signalling

The role of the βARs (β_1-, β_2- and β_3-ARs) in cardiovascular disease has been the subject of much investigation (Rockman et al., 2002). Acute βAR stimulation is a powerful mechanism by which cardiac output is increased but chronic stimulation leads to hypertrophic cardiac remodeling and myocyte apoptosis. The β_1AR is the predominant subtype expressed in heart representing 75-80% of the total βAR present (Rockman et al., 2002). Both β_1–and β_2–ARs couple to G_s and activate AC and PKA (Figure 1.7), regulating cardiac contractility and heart rate (Keys and Koch, 2004), but the β_2 receptor can also couple to G_i (Daaka et al., 1997), inhibiting AC whilst activating MAPK cascades, adding an extra layer of regulation to the cardiac system.

Most β_1AR knockout mice die prenatally, those that do survive to adulthood have a dysfunctional cardiac response to exercise (Rohrer et al., 1996). There is no β-agonist-dependent change in cardiac function in these mice suggesting that the β_1AR controls heart rate. Consistent with this observation, overexpression of β_1AR in hearts

of transgenic mice increases cardiac contractility and responsiveness to the βAR agonist isoproterenol (Bisognano et al., 2000; Engelhardt et al., 2002). Eventually however, these mice exhibit a progressive loss in cardiac function and hypertrophy.

β_2AR knockout mice have no defect in cardiac physiology indicating that the β_2AR does not contribute to cardiac function under normal conditions in mice (Rohrer, 1998). β_2AR overexpression in transgenic mice, however, results in increased contractility and heart rate although there is little pathology observed in these mice upon ageing (Koch et al., 2000).

Cardiac overexpression of $G\alpha_s$ in mice results in increased cardiac contractility and development of HF with age (Geng et al., 1999). This suggests that acute stimulation of the β-adrenergic system could contribute to cardiac failure. Mice overexpressing a novel $G\alpha_i$ coupled receptor (Ro1) exhibit, as might be predicted, impaired cardiac contractility. Since G_i is up-regulated in human HF, this indicates a role for $G\alpha_i$ signalling in development of HF (Baker et al., 2001; Eschenhagen et al., 1992).

1.6.4.3. Calcineurin-NFAT signalling

Ca^{2+} signalling plays a vital role in hypertrophic growth in response to biomechanical pressure (Bers and Guo, 2005). Increases in intracellular Ca^{2+} results in the activation of an array of Ca^{2+} sensor proteins. One major hypertrophic signalling pathway activated by the increase in intracellular Ca^{2+} is the calcineurin-NFAT pathway (Figure 1.7). Calcineurin is a serine-threonine phosphatase activated by Ca^{2+}/CaM (Frey and Olson, 2003; Schulz and Yutzey, 2004). Calcineurin dephosphorylates transcription factors from the NFAT family, causing exposure of their nuclear

localisation sequence (NLS) and activating their ability to translocate to the nucleus and turn on gene transcription. Constitutive activation of calcineurin in the hearts of mice results in HF (Molkentin et al., 1998). Inhibition of calcineurin with Cyclosporine A (CsA) results in inhibition of Angiotensin II (AngII)- or PE-dependent hypertrophy in rodents (Olson and Williams, 2000). Transgenic mice expressing a constitutively active nuclear mutant of NFAT3 also develop HF, indicating this transcription factor regulates induction of cardiac hypertrophy (Figure 1.7) (Molkentin et al., 1998). NFAT signalling is regulated by a range of other molecules, including MAPKs, suggesting that this significant pathway may represent a point of intersection for many hypertrophic signals (Figure 1.7) (Crabtree and Olson, 2002).

1.6.4.4. PI3K/Akt/GSK-3-dependent signalling

PI3Ks phosphorylate both proteins and lipids and are involved in a wide array of signalling functions including cell metabolism and survival along with gene expression (Cantley, 2002). PI3Ks are activated by many receptors including the ARs (Schluter et al., 1998; Zhu et al., 2001). PI3Kγ is specifically activated during pressure overload hypertrophy in mouse heart by Gβγ released from activated Gαq (Naga Prasad et al., 2000). Consistent with this observation, overexpression of a constitutively active mutant of PI3K in mice results in cardiac hypertrophy (Figure 1.7) (Shioi et al., 2000). As discussed in Section 1.6.4.2, βAR dysfunction observed in HF is proposed, at least in part, to arise as a consequence of enhanced GRK expression and GRK-mediated βAR desensitisation. GRK2/PI3K complex formation is required for βarr-mediated βAR internalisation (Naga Prasad et al., 2005). The

agonist–dependent recruitment of GRK2 to activated βARs targets PI3K to the receptor where it produces D3 phosphatidylinositols. Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) recruits endocytic proteins including βarrs, to the receptor to facilitate receptor internalisation (Naga Prasad et al., 2002). Overexpressing a catalytically inactive form of PI3Kγ inhibits this interaction in a murine model of HF induced by overexpression of calsequestrin, and results in normalised βAR levels and preserved βARs responsiveness to the adrenergic agonist isoproterenol (Perrino et al., 2005). This defines PI3K as a potential therapeutic target for inhibition of GRK-mediated downregulation of βARs.

Akt, a PI3K effector, is a serine/threonine kinase which promotes protein translation by phosphorylating and inhibiting GSK-3β and stimulates gene transcription by phosphorylation and activating the NFAT transcription factors (Frey and Olson, 2003). GSK-3β inhibits cardiac hypertrophy when overexpressed in mice (Antos et al., 2002). In cardiac myocytes in culture, βAR stimulation of ANF transcription mediated by the GATA4 transcription factor was inhibited by GSK-3β (Akazawa and Komuro, 2003). Confirmation that GSK-3β suppresses cardiac hypertrophy by preventing upregulation of the fetal gene program *in vivo* is yet to be explored.

1.6.4.5. MAPK signalling

MAPKs provide a major pathway for relaying signals from the plasma membrane to the nucleus. Three main MAPK pathways exist, the ERKs, c-Jun amino-terminal kinases (JNKs) and the p38 MAPKs (Johnson and Lapadat, 2002). The latter two cascades are responsive not only to signals from receptors such as GPCRs but by

stress stimulants such as ischemia (Sugden and Clerk, 1998). A major role for these pathways in promoting cardiac hypertrophy was demonstrated by inhibiting all three cascades by adenoviral transfer of MAPK phosphatase-1, a phosphatase which inhibits all MAPK cascades (Bueno et al., 2001). Isoproterenol stimulation or pressure overload aortic banding of these mice does not induce cardiac growth suggesting that MAPK cascades positively regulate cardiac hypertrophy (Bueno et al., 2001).

Each of the MAPK pathways appears to have distinct signalling functions during induction of hypertrophy. Overexpression of MEK1, an activator of ERK1/2, but not JNKs or p38 MAPKs in mice promotes cardiac hypertrophy (Bueno et al., 2000). Moreover, ERK can activate GATA4 cardiac transcription factors which regulate expression of a range of fetal program genes including α-MHC and ANF (Akazawa and Komuro, 2003).

The role of p38 in the regulation of hypertrophy is somewhat unclear as different results have been obtained in cultured myocytes versus studies in transgenic animals. Overexpression of activated MAP kinase kinase-(MKK)-3 or -6, upstream activators of p38, induces hypertrophy and ANF expression in cultured cardiac myocytes (Wang et al., 1998a). Different isoforms of p38 have been attributed different signalling roles, that of p38α in promoting myocyte apoptosis whilst p38β promotes hypertrophy (Wang et al., 1998a). However, studies of p38 in transgenic animals, have suggested that p38 does not promote hypertrophy (Liang and Molkentin, 2003) since overexpression of dominant negative mutants of MKK3, MKK6 and p38α develop cardiac hypertrophy following pressure-overload or with increasing

age (Braz et al., 2003). The discordance between *in vitro* and *in vivo* results requires further investigation.

JNK is activated in cardiac myocytes by mechanical stretching or agonist stimulation by ET_1, PE or AngII (Choukroun et al., 1998; Ramirez et al., 1997; Yano et al., 1998). In a similar fashion to p38, there is some controversy as to the function of JNK in the development of hypertrophy. Overexpression of MKK7, an upstream activator of JNK in cultured myocytes results in JNK activation and development of hypertrophy (Wang et al., 1998b). However, mice expressing activated MKK7 in the heart did not develop hypertrophy but lethal juvenile cardiomyopathy (Petrich et al., 2003).

Whilst a role for ERK in promoting cardiac hypertrophy has been demonstrated, the role of the p38 and JNK MAPKs in juvenile (e.g. neonatal myocytes) versus adult cardiac tissue (transgenic or knock-out mice) remains controversial.

1.6.4.6. PKC signalling

The PKC family consists of at least twelve serine/threonine kinases which are grouped into three subfamilies, classical (cPKC:α, β and γ), novel (nPKC: δ, ε, η and θ) and atypical (aPKC: μ, ξ and ι) (Salamanca and Khalil, 2005). cPKC enzymes are activated by Ca^{2+} and diacylglycerol (DAG), aPKC by DAG, and nPKC independently of Ca^{2+} or DAG possibly by other PKCs (Salamanca and Khalil, 2005).

PKC regulation of hypertrophy is complex since the PKC isoforms vary in expression level in different cardiac cell types and at different developmental stages (Bowling et

al., 1999; Sabri and Steinberg, 2003). In cultured myocytes, constitutively active PKC-β stimulates promoters of the β-MHC gene, which is upregulated during cardiac hypertrophy (Kariya et al., 1994).

1.6.4.7. JAK-STAT signalling

JAK (janus-family tyrosine kinase) activation results in their tyrosine-phosphorylation and activation of a family of latent cytoplasmic transcription factors called STATs. Activated STATs dimerise via a intermolecular SH2-phosphotyrosine interaction and then translocate to the nucleus to regulate gene expression (Kunisada et al., 2000). Cardiac-specific overexpression of STAT3 in mice results in hypertrophy (Hoshijima and Chien, 2002). Gp130, a promiscuous cytokine receptor which activates both MAPK and STAT3, has also been linked to development of hypertrophy in mice overexpressing IL-6 and IL-6 receptors (Mascareno et al., 1998). Increased STAT activity has been reported in the myocardium of the spontaneously hypertensive rat, in particular STAT3, STAT5a and STAT6 binding to the Angiotensinogen (Ao) promoter (Booz et al., 2002). Ao is the precursor of AngII and therefore STAT signalling produces a hypertrophic phenotype by generating AngII. Production of Ao by hypertrophic AngII signalling thus represents an autocrine loop further enhancing the hypertrophic phenotype (Liu et al., 1997). Interestingly though, STAT3 and STAT1 appear to have opposing actions in determining cardiac myocyte survival (Booz et al., 2002). Ischemia induces STAT1 and apoptosis whereas STAT3 is cardioprotective in this setting (Hattori et al., 2001; Stephanou et al., 2000; Stephanou et al., 2001). JAK-STAT signalling in cardiac muscle is complex since it participates in hypertrophic growth, cell survival and apoptotic pathways.

1.6.4.8. The role of GRKs in cardiac hypertrophy

GRKs, like GPCRs play a role in cardiac function. GRKs 2, 3 and 5 are the predominant subtypes expressed in human heart and are all significantly upregulated in a range of cardiomyopathies (Dzimiri et al., 2004; Ungerer et al., 1993; Vinge et al., 2001; Yi et al., 2002). GRK2 protein is upregulated 3-fold and GRK5 protein 2.6-fold in the myocardium of rats with congestive heart failure (Vinge et al., 2001). Interestingly, upregulation of GRKs often occurs before detectable onset of clinical HF and in this capacity represents a novel marker of cardiac function with the potential to indicate for therapeutic intervention before complete HF ensues (Hata et al., 2006; Iaccarino et al., 2005).

As described earlier, GRK2 knockout mice are embryonic lethal and exhibit major cardiac abnormalities indicating an important role for this GRK in regulating cardiac development (Jaber et al., 1996). The cardiac failure observed during development of GRK2 knockout mice is also seen in mice that have targeted knockdown of cardiac transcription factors (Jaber et al., 1996). GRK2 is upregulated in human chronic heart failure (CHF) (Ungerer et al., 1993; Ungerer et al., 1994). Overexpression of GRK2 in the heart of mice during adulthood results in diminished left ventricular contractility in response to isoproterenol stimulation (Koch et al., 1995). Consistent with these results, inhibition of GRK2 activity by overexpression of an inhibitory C-terminal construct, corresponding to 194 amino acids of GRK2 which contains the $G\beta\gamma$ binding domain (GRK2ct), in mice results in prevention of cardiac failure (Koch et al., 1995). These results suggest that upregulation of myocardial GRK2 is detrimental to cardiac function whereas inhibition of GRK2 enhances cardiac

contractility (Petrofski and Koch, 2003). Indeed, transfection of GRK2ct into a genetic model of murine HF (termed $MLP^{-/-}$) significantly improves heart contractility in these mice and prevents development of cardiomyopathy (Rockman et al., 1998a). Similarly, mating transgenic mice overexpressing GRK2ct with transgenic mice with severe cardiomyopathy due to overexpression of calsequestrin, a sarcoplasmic reticulum (SR)-Ca^{2+}-binding protein, results in an increased survival age with significantly improved cardiac function (Harding et al., 2001). Hence, GRK2 is a potential therapeutic target for correcting cardiac function. However, whilst GRK2ct has been delivered successfully to the hearts of rats and rabbits, this method of delivery in human patients is not practical.

GRK5 messenger RNA (mRNA) is significantly increased in the left-ventricle of patients with volume overload and dilated cardiomyopathy (Dzimiri et al., 2004). Consistent with this observation, cardiac overexpression of GRK5 in mice results in impaired cardiac function (cardiac output and contractility) (Chen et al., 2001) and GRK5 expression (mRNA and protein levels) is upregulated in rat models of CHF (Oyama et al., 2005; Vinge et al., 2001). This suggests GRK5, as well as GRK2, regulates cardiac function and the progression of hypertrophy to HF. Interestingly, in a rat model of CHF, GRK2 and GRK5 display a distinct and differential distribution following myocardial infarction (Vinge et al., 2001). GRK2 is detected in the ischemic region, primarily in endothelial cells, whereas GRK5 is found in several cell types, but principally in myocytes. This points towards distinct functions for GRKs 2 and 5 in cardiac tissue (Vinge et al., 2001).

Based on a wealth of data it is therefore hypothesised that the loss of adrenergic

signalling in cardiac tissue associated with HF is due, at least in part to increased GRK-mediated desensitisation and later downregulation of receptors. Currently, blockers of βAR function (β-blockers) are used widely to inhibit myocardial disease with significant improvement in patient survival (Rockman et al., 2002). Whilst upregulation of βAR signalling initially compensates for an increase in biomechanical pressure, chronic stimulation ultimately leads to cardiac failure and so blockers of βAR function are thought to maintain normal heart function by preventing GRK-mediated desensitisation and downregulation of adrenergic receptors (Koch and Rockman, 1999). Clearly the role of GRKs in disease, certainly heart disease, is complicated and requires further investigation.

1.6.5. Cardiac hypertrophy – therapeutic opportunities

It is clear that initiation and inhibition of cardiac hypertrophy involves a multitude of signalling pathways that form an intricate and integrated web. Certainly new therapeutic approaches developed to tackle cardiac hypertrophy will have to consider a plethora of signalling molecules and targets.

Work in animal models has revealed a wealth of information about the action of pharmacological agents. ACE inhibitors and AR antagonists prevent ventricular remodeling, improve cardiac function and increase survival rates in models of myocardial infarction (Liu et al., 1997). βAR blockers such as propranolol and bisprolol and Ca^{2+} antagonists have also been shown to improve cardiac function (Leitch et al., 1998; Sharpe, 1996). These drugs are currently approved for use in treatment of HF in the clinic. It is anticipated that a more detailed understanding of

the molecular events leading to hypertrophy and decompensated HF will reveal new targets for pharmaceutical intervention. It seems reasonable that the GRKs and GRK-binding proteins might prove useful therapeutic targets for the prevention of hypertrophy.

1.7. Nuclear trafficking

One of the major characteristics of eukaryotic cells is the presence of intracellular compartments or organelles such as the nucleus. This allowed eukaryotic cells to become more complex, signalling molecules and their functions could be localised to specific intracellular compartments, increasing cellular efficiency. A pertinent example of this is the compartmentalisation of the genetic contents of the cell into the nucleus. The nucleus is a defined organelle with its own double membrane, the nuclear envelope. Central to cellular function is macromolecular transport into and out of the nucleus via protein channels called the nuclear pore complex (NPC) (Figure 1.8A). This allows the cell to respond to cues generated at the plasma membrane and transmitted through the cytosol, by initiating gene expression from which instructions are sent back out to the cytosol. Nuclear trafficking is thus a regulated and complicated process. It is possible for small molecules up to 40 kDa to diffuse through the nuclear pores along a concentration gradient but molecules larger than this require active transport into the nucleus (Yoneda, 2000).

1.7.1. Nuclear targeting of cellular molecules

Active nuclear import and export of proteins larger than 40-60 kDa is regulated by the presence of defined amino acid motifs within the molecule called NLSs or nuclear export sequences (NESs). In order for a protein to be directly imported or exported

from the nucleus, it must possess one of these signals. Of course, it is possible for proteins, which do not contain such motifs, to enter the nucleus but they must first associate with a molecule which contains this structural code.

1.7.1.1. Nuclear localisation sequences

The first 'classical' NLS was characterised as a cluster of basic amino acids in the SV40 large T-antigen (Yoneda, 2000). The importance of NLSs was realised when ablation of certain residues in molecules caused their nuclear exclusion while addition of NLSs to proteins resulted in their nuclear localisation (Cokol et al., 2000). NLSs are classified as monopartite or bipartite (Boulikas, 1993). Characteristically a monopartite motif is a cluster of basic residues following a helix-breaking residue. A bipartite motif is similar but contains two clusters of basic residues separated by nine to twelve amino acids (Cokol et al., 2000). However, many NLSs have been identified that do not conform to these general rules. Interestingly, a quarter of all known NLS sequences bind DNA (Cokol et al., 2000). NLSs must show appropriate 3D conformation and be accessible for nuclear import to occur. Thus NLS exposure is a common mechanism whereby the nuclear localisation of proteins is modulated. For example, phosphorylation near to a NLS can prevent nuclear import. Mutation of a putative PKA site adjacent to the NLS in adenomatous polyposis coli protein inhibits nuclear import *in vitro* (Zhang et al., 2000). Cell cycle-regulated phosphorylation of the *Saccharomyces cerevisiae* transcription factor, Swi6, regulates its nuclear import (Sidorova et al., 1995). Hypophosphorylation of Swi6 during late mitosis phase through to G1 permits Swi6 nuclear localisation whereas hyperphosphorylation imparts cytoplasmic localisation during the remainder of the

cycle (Sidorova et al., 1995). *In vitro* it has been shown that phosphorylation in the vicinity of a NLS prevents the NLS interacting with α-importins, thus preventing nuclear import (Harreman et al., 2004).

1.7.1.2. Nuclear export sequences

NESs are a more recent discovery than NLSs (la Cour et al., 2004). NESs are typically composed of four or five hydrophobic residues within a region of about ten residues. In contrast to NLSs, NESs are hydrophobic rich amino acid motifs with a high proportion of leucine or isoleucine amino acids, although, NESs also commonly contain isoleucine, valine, methionine and phenylalanine residues (Yoneda, 2000). The leucine residues at the C-terminus (rather than the N-terminus) of the motif are most highly conserved and deemed most important for NES function (Schneider and Stephens, 1990; Wen et al., 1995). The flanking residues of a NES may also contribute to the ability of a NES to mediate nuclear import (Kutay and Guttinger, 2005). In the same way that NLSs do not all conform to a single consensus sequence, only 36% of NES conform to the classical L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI] consensus sequence (where x = any amino acid and numbers indicate the number of amino acids) (Kutay and Guttinger, 2005; la Cour et al., 2004). Leucine rich NESs were originally identified in the HIV rev protein and PKA inhibitor (PKI) (Fischer et al., 1995; Wen et al., 1995).

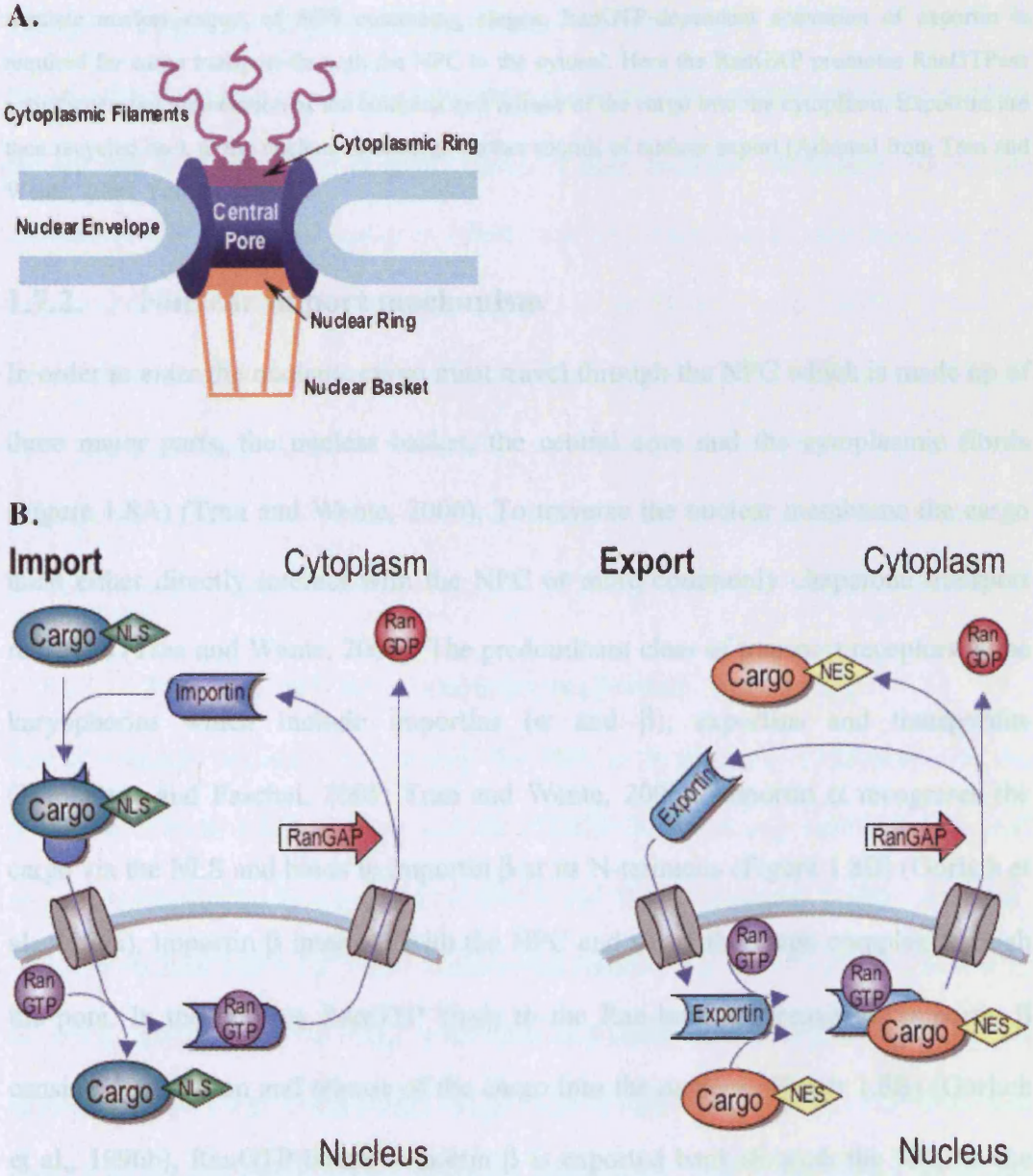


Figure 1.8. Nuclear trafficking via the NPC.

A. Schematic representation of the structure of the NPC. The NPC sits in the nuclear envelope membrane and consists of a central pore (dark blue) with cytoplasmic rings either side in the cytoplasm and nucleus. The cytoplasmic side contains cytoplasmic filaments and the nuclear side a nuclear basket. B. Mechanism of nuclear import and export via the NPC. During import, a cargo containing a NLS is recognised by the importin complex which directs travel through the pore into the nucleus. In the nucleus, RanGTP binds to the complex, releasing the cargo and initiating recycling of the importin back to the cytosol via the NPC. In the cytosol, a Ran GAP promotes Ran GTPase activity readying the importin for a further round of nuclear import. In a similar fashion to importins, exportins

regulate nuclear export of NES containing cargos. RanGTP-dependent activation of exportin is required for cargo transport through the NPC to the cytosol. Here the RanGAP promotes RanGTPase activity causing dissociation of the complex and release of the cargo into the cytoplasm. Exportins are then recycled back to the nucleus to undergo further rounds of nuclear export (Adapted from Tran and Wente, 2006; Yoneda, 2000).

1.7.2. Nuclear import mechanism

In order to enter the nucleus, cargo must travel through the NPC which is made up of three major parts, the nuclear basket, the central core and the cytoplasmic fibrils (Figure 1.8A) (Tran and Wente, 2006). To traverse the nuclear membrane the cargo must either directly interact with the NPC or more commonly chaperone transport receptors (Tran and Wente, 2006). The predominant class of transport receptors is the karyopherins which include importins (α and β), exportins and transportins (Pemberton and Paschal, 2005; Tran and Wente, 2006). Importin α recognises the cargo via the NLS and binds to importin β at its N-terminus (Figure 1.8B) (Gorlich et al., 1996a). Importin β interacts with the NPC and steers the cargo complex through the pore. In the nucleus RanGTP binds to the Ran-binding domain of importin β causing dissociation and release of the cargo into the nucleus (Figure 1.8B) (Gorlich et al., 1996b). RanGTP-bound importin β is exported back through the NPC to the cytoplasm (Figure 1.8B). Importin α must form a trimeric complex with CAS, an importin β related molecule, and RanGTP to traverse the NPC to the cytoplasm (Kutay et al., 1997). In the cytoplasm, RanGTP-bound importin is hydrolysed preparing the carrier for further rounds of nuclear import (Figure 1.8B).

1.7.3. Nuclear export mechanism

In a similar manner to nuclear import, cargo molecules are exported from the nucleus by an active mechanism (Figure 1.8B). Cargo proteins containing NESs are complexed with an export receptor, CRM1 (chromosomal region maintenance), also called exportin 1, and RanGTP in the nuclear compartment (Yoneda, 2000). Exportin 1 behaves essentially as importin β does during the import process. Exportin 1 binds the NES containing cargo and RanGTP in the nucleus and directs the complex through the NPC to the cytoplasm where again, RanGTP hydrolysis dissociates the complex (Figure 1.8B) (Fornerod et al., 1997; Fukuda et al., 1997; Stade et al., 1997).

1.7.4. The role of Ran in nucleocytoplasmic trafficking

Ran is critically required for crossing the NPC as it provides directionality to the pore. Ran exhibits a low intrinsic activity of GDP/GTP exchange and hydrolysis and so its activation state is regulated by GEFs and GAPs (Yoneda, 2000). RanGEF, which loads Ran with GTP is restricted to the nucleus, and thus it is here that it is in its active form, ready for cargo unloading and initiation of nuclear export in the nucleus (Figure 1.8B). Furthermore, since there is substantially more GTP in the cell than GDP, this exchange occurs readily in the nucleus. RanGAPs contain NESs and are thus permanently excluded from the nucleus, only allowing RanGTP hydrolysis in the cytosol (Figure 1.8B). RanGAPs are localised to the cytoplasmic fibrils of the NPC (Figure 1.8A) which provides an efficient mechanism for rapid hydrolysis and therefore, cargo release once the nuclear export complex reaches the cytoplasm (Figure 1.8B) (Yoneda, 2000). Consequently, a RanGDP/RanGTP gradient exists across the nuclear membrane and provides directionality for nuclear transport.

1.7.5. Regulation of nuclear protein localisation

Whilst NLSs and NESs directly mediate nuclear import and export via the NPC, mechanisms exist to regulate the nuclear localisation of a protein under certain conditions.

1.7.5.1. Phosphorylation

Phosphorylation of proteins has been shown to regulate their nuclear localisation. STAT1 transcription factor activation requires dimerisation mediated by tyrosine phosphorylation. This structural change allows nuclear import and DNA binding. The NES of these transcription factors, is found in the DNA binding region and is hidden when bound to DNA. Crystal structure data indicates that dephosphorylation of the tyrosine residues might unmask the NES and promote nuclear export of the STATs (Reich and Liu, 2006). p42/p44 MAPK phosphorylation of serine 641 or serine 643 in HIF-1a promotes its nuclear accumulation and transcriptional activity (Mylonis et al., 2006). MAPK phosphorylation promotes nuclear localisation of HIF1a by inhibiting its CRM1-dependent nuclear export. Similarly, growth factor mediated translocation of the nuclear kinase Akt is mediated by PI3K activation. AKT phosphorylation via a PI3K-dependent mechanism promotes AKT nuclear translocation (Xuan Nguyen et al., 2006). In contrast, the nuclear export of the ribosomal S6 kinases is promoted by phosphorylation. Phosphorylation of S6K-1 at serine 17 by CK2 and S6Kβ-II phosphorylation at serine 486 by PKC results in nuclear export of these kinases (Panasyuk et al., 2006; Valovka et al., 2003).

1.7.5.2. Calmodulin

CaM has been shown to promote the nuclear import of the cell cycle inhibitor p21 (Rodriguez-Vilarrupla et al., 2005). PKC phosphorylation of p21 retains this inhibitor in the cytosol whilst CaM binding prevents this phosphorylation and allows p21 nuclear import (Rodriguez-Vilarrupla et al., 2005). Conversely, Ca^{2+}/CaM can bind to the NFκB/Rel transcription factor c-Rel and inhibits its nuclear localisation (Antonsson et al., 2003). CaM therefore has the capability to both promote and inhibit nuclear localisation.

1.8. Aims of this thesis

An emerging trend over recent years has been the multiplicity of functions that components of the GPCR desensitisation machinery perform. Those of the βarrs have been studied in most depth. βarrs act as scaffolds facilitating the agonist-dependent recruitment of a multiplicity of binding partners to activated GPCRs (Lefkowitz and Whalen, 2004). Perhaps the best-characterised scaffold function of the βarrs is their ability to bind multiple components of MAPK cascades promoting efficient GPCR-mediated activation of ERK1/2 (extracellular signal-regulated kinase 1/2), JNK3 (c-Jun amino-terminal kinase 3) and p38 (Lefkowitz and Whalen, 2004). βarr-dependent GPCR-mediated MAPK activation results in the cytoplasmic retention of the activated kinase, indirectly regulating MAPK-dependent gene transcription and presumably facilitating phosphorylation of cytoplasmic substrates. Furthermore, βarrs can act as adaptors for non-receptor tyrosine kinase, c-Src, initiating MAPK signalling (Lefkowitz and Shenoy, 2005). βarr-2, but not βarr-1, contains a NES at its C-terminus (Scott et al., 2002; Wang et al., 2003) and is actively imported into the

nucleus, indicating that βarr-2 is capable of nucleocytoplasmic shuttling (Scott et al., 2002; Wang et al., 2003). Trafficking through the nucleus results in the cytoplasmic retention of βarr-2 binding partners such as Mdm2, activated ERK and JNK3 (Luttrell et al., 2001; McDonald et al., 2000; Scott et al., 2002; Wang et al., 2003), thereby indirectly regulating nuclear signalling pathways. Both βarr-1 and 2 inhibit activation of the transcription factor NF-κB. βarr binding to IκB, the inhibitory protein that regulates NF-κB activity, preventing its degradation and thereby inhibiting NF-κB-mediated transcription (Gao et al., 2004; Witherow et al., 2004). Recently a more direct nuclear function for βarr-1 has been described. Following activation of the δ-opioid receptor βarr-1 accumulates in the nucleus of HEK293 cells where it is found in a complex with histone acetylase, p300 at specific promoters (Kang et al., 2005). βarr-1-dependent recruitment of p300 promotes local histone H4 acetylation and gene transcription (Kang et al., 2005).

Cell signalling functions for the GRKs have been less well documented although the literature hints towards roles for the GRKs similar to those of the βarrs (discussed in Section 1.3). The list of soluble substrates for the GRKs is growing and includes synucleins and AKT (Penela et al., 2003) suggesting roles for the GRKs other than desensitisation of GPCRs. Indirect links between GRKs and nuclear functions, such as regulation of gene transcription have also been reported, including for example, GRK2 phosphorylation of R-Smads which inhibits their nuclear localisation and therefore their ability to regulate gene transcription (Ho et al., 2005). However, to date, no direct nuclear functions for the GRKs have been described.

My aim in this thesis was to investigate the observation that the GRK4 subfamily of GRKs is present in the nucleus when transfected in tissue culture cell lines, whereas GRK2 subfamily members are cytosolic. To do this I determined if nuclear localisation was regulated and of any relevant functional significance. Chapter 2 outlines the methods utilised in this thesis.

Chapter 3 describes the identification of a functional NLS and NES in the most studied member of the GRK4 subfamily, GRK5. The nuclear localisation of GRK5 is regulated by Ca^{2+} signalling activated by the Gq-coupled M3 muscarinic receptor (M3MR), or by activation of the Ca^{2+} sensor, CaM. CaM binding to the N-terminus of GRK5 results in nuclear export of this kinase. Furthermore, in Chapter 3, I discover that GRK5 binds directly and specifically to DNA *in vitro*, hinting for the first time, of a potential nuclear function for GRK5.

Chapter 4 focuses on a comparison of the GRK4 subfamily in terms of the regulation of their nuclear localisation and DNA-binding ability. Data in this Chapter reveals diversity among the GRK4 subfamily members. Whilst all subfamily members are localised to the nucleus of human epithelial cells (HEp2 cells), GRKs 5 and 6 but not GRK4 bind to DNA. What is more, the nuclear localisation of GRK6 splice variants are differentially regulated by Ca^{2+}.

Chapter 5 investigates a possible nuclear function for GRK5; inhibition of cardiac hypertrophy in rat neonatal myocytes. Using mutants of GRK5 I determine the structural features of GRK5 which regulate *in vitro* DNA-binding and nuclear export of this kinase. I demonstrate *ex vivo* that GRK5 overexpression in rat neonatal cardiac myocytes rescues PE induced cardiac hypertrophy as has previously been described

by Eckhart and colleagues (Eckhart et al., 2000). Furthermore, I provide evidence to suggest that rescue of cardiac hypertrophy by overexpression of GRK5 is not due to desensitisation of GPCRs but due to its activity as a nuclear kinase and its ability to bind DNA. These findings show for the first time, that GRK5 has nuclear functions. The findings from Chapters 3 – 5 will be discussed in detail in Chapter 6.

2. Materials & Methods

Tissue culture dishes were obtained from Nunc, culture medium from Gibco BRL, plasticware from Falcon or Sterilin and other reagents from Sigma, unless otherwise specified. All kits were used according to the manufacturers' instructions and solutions made with MilliQ deionised water. Buffer ingredients, cDNAs and antibodies referred to in this Chapter are listed in Tables 2.1, 2.2 and 2.3-2.6 respectively.

2.1. Cell culture

2.1.1. Cell lines

CHO cells stably expressing M3MR (CHO-M3, a generous gift from Dr Andrew Tobin, University of Leicester, UK) were maintained in modified Eagle medium alpha (α-MEM) without nucleosides containing 10% foetal calf serum (FCS), 250μg/ml G418 sulphate and penicillin and streptomycin (100 IU penicillin and 100μg of streptomycin/ml). HEp2, HeLa, HEK-293 and Cos-7 were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum and penicillin and streptomycin (100 IU penicillin and 100μg of streptomycin/ml). Cells were maintained in a humidified incubator at 37°C, 5% CO_2. Confluent monolayers were passaged every 3-4 days by trypsinising and replating at a ratio of 1:10.

2.1.2. Cell freezing

To freeze cell stocks, cells were taken from two plates of 70% confluency and resuspended in 1ml cold freezing medium on ice. Cells were frozen at –70 °C for 1

week and subsequently transferred to liquid nitrogen.

2.1.3. Cell thawing

To recover cells stored in liquid nitrogen, aliquots were rapidly thawed at 37°C, the cells diluted in 10 ml culture medium and split equally into two 9cm dishes containing 5ml of the appropriate media. Cells were maintained in a humidified incubator at 37°C, 5% CO_2.

2.1.4. Electroporation of DNA into HEp2 cells

HEp2 cells were transiently transfected using electroporation as this method gave approximately 70% transfection efficiency compared to Fugene transfection which gave approximately 50% transfection efficiency. HEp2 cells that had been plated 24h previously (75% confluency) were trypsinised, washed from the plate with DMEM and spun down at 1500 rpm for 3 min (Sorvall TC 6 centrifuge). The supernatant was discarded and the cell pellet was then washed in 10ml HEBS buffer. Washed cells were recovered by centrifugation as before, resuspended in 250µl HEBS buffer, and transferred to a $0.4cm^3$ electroporation cuvette. Unless otherwise stated, 1µg DNA was added to the cuvette and the cells mixed gently by hand. Using the Gene Electropulser II (BioRad), cells were electroporated with 2 pulses of 420V, 125µF, ∞ ohms. Cells were subsequently incubated at room temperature (RT) for 5 minutes before being plated onto coverslips in a 5cm tissue culture dish and left to express the construct(s) for up to 48h.

2.1.5. Transfection of DNA into Cos-7, HeLa, CHO-M3 and HEK-293

Cos-7, HeLa, CHO-M3 and HEK-293 cells were transiently transfected using Fugene or GeneJuice as these methods gave approximately 60-70% transfection efficiency and was practically simpler to perform than electroporation. 3µl of GeneJuice (Novagen) or Fugene (Roche) transfection reagent for every 1µg of DNA to be transfected was added to 100µl serum-free DMEM. The media was mixed and incubated at RT for 5 minutes. This mixture was then added to another 1.5ml tube containing the appropriate DNA, mixed gently by hand and incubated at RT for 30 minutes. The media/DNA mixture was then dropped onto 70% confluent cells that had been plated 24h previously in appropriate media. The cell media was mixed by gentle agitation and the cells were left to express the transfected construct(s) for up to 48h.

2.1.6. Cell lysate preparation and determination of concentration

Untransfected cells or transfected cells were washed twice with cold phosphate buffered saline (PBS) on ice. Cells were lysed by addition of 300µl of the appropriate cold lysis buffer and scraped with a cell scraper. Cell lysates were sonicated twice for 15s (Branson Sonifier 450, setting 6) and cleared by centrifugation at 13,000 rpm (Heltich bench top centrifuge Mikro 20) for 5min. Cell lysates were transferred to a clean tube and protein concentration was determined using the BioRad protein assay. BioRad Protein Assay Dye Reagent Concentrate (BioRad) was diluted 1:5 in water. 2.5µl of cell lysate was added to 1ml of reagent and vortexed to mix. The A_{595} of samples was measured using an Ultraspec 2000 spectrophotometer and protein

concentration determined by comparison with a standard curve obtained by measuring the A_{595} of known amounts of bovine serum albumin (BSA, First Link UK Ltd.). Lysates were stored at -20°C.

2.1.7. Preparation of neonatal rat cardiac myocytes

All procedures involving animals were performed in accordance with institutional guidelines for the care and use of laboratory animals. Hearts removed from 1- to 2-day-old neonatal Wistar rats were minced and digested with oxygenated digestion buffer. Eight to twelve incubations of the heart tissue in digestion buffer were performed at 37°C for 15min. 2mls calf serum were added to each incubation to stop digestion and cells recovered by centrifugation at 1000 rpm for 4min (Sorvall TC 6 centrifuge). 4ml calf serum was added to the pellet of cells and then incubated at 37°C, 5% CO_2. The cells from all digestions were subsequently pooled and centrifuged as before. Cells were resuspended in an appropriate amount of plating media (Roswell Park Memorial Institute medium (RPMI), 15% calf serum and penicillin and streptomycin (100 IU penicillin and 100µg of streptomycin/ml) and plated in 175cm^2 flasks for 1 hour at 37°C to remove fibroblasts and enrich the myocyte population in the suspension. Cells were transfected with cDNA by nucleofection (Amaxa, described in Section 2.1.8) and plated onto coverslips that had been coated with 1% gelatin/PBS (1h at 37°C). The following day, cells were washed three times with maintenance media (RPMI, 1% calf serum and penicillin and streptomycin (100 IU penicillin and 100µg of streptomycin/ml) and left in maintenance media for 48h with or without treatment as described in Section 2.3.3.

2.1.8. Transfection of neonatal rat cardiac myocytes

Cardiac myocytes are difficult to transfect efficiently. Transfection using the rat cardiomyocyte-neonatal nucleofector kit (Amaxa) gave approximately 50% transfection efficiency compared to less than 5% transfection efficiency using Fugene and was therefore deemed the most appropriate transfection method for cardiac myocytes. Cardiac myocytes were transfected using a rat cardiomyocyte-neonatal nucleofector kit (Amaxa) according to manufacturer's instructions with the following adjustments. Cells were nucleofected using program G-0012 and subsequently plated in RPMI containing 15% calf serum, penicillin and streptomycin (100 IU penicillin and 100μg of streptomycin/ml).

2.2. DNA manipulation

2.2.1. cDNA constructs

Table 2.2 lists DNA constructs used in this thesis and their suppliers. Constructs were stored at -20°C.

2.2.2. Generation of GRKΔNLS and GRK5ΔNES constructs

Putative NLSs in GRKs 4, 5 and 6 were identified using an automated tool for the analysis and determination of NLSs (Cokol et al., 2000). Putative NESs were identified using the NetNES 1.1 server which predicts leucine-rich NES in eukaryotic proteins (la Cour et al., 2004). Predicted putative localisation sequences are listed in Table 2.7.

In order to generate mutants of GRK5 in which putative NLS and NES sequences are ablated, primers were designed which converted selected residues in the protein

sequence (see Table 2.7) to alanine (see Section 2.2.3).

2.2.3. PCR

All reagents were from Promega unless otherwise stated. Primers were custom made by Invitrogen.

2.2.3.1. Site directed mutagenesis

The Quikchange® site-directed mutagenesis kit (Stratagene) was used according to manufacturer's instructions to make point mutants in the putative NLS and NES of various GRKs as detailed in Table 2.7.

pRK5-GRK5 (bovine) was used as template to create GRK5ΔNLS. Basic residues located between amino acids 388 to 394 were mutated to alanine residues using the following primers; sense primer 5' -C CAG TCG CCC TTC CGC GGC **GCT GCT** GAG **GCT** GTG **GCT GCT** GAG GAG GTG GAC CGC CGG- 3', anti-sense primer 5' -CCG GCG GTC CAC CTC CTC **AGC AGC** CAC **AGC** CTC **AGC AGC** GCC GCG GAA GGG CGA CTG G- 3'. Nucleotides in bold encode the mutated amino acids. The introduced mutation was screened by a loss of a NotI restriction site.

pRK5-GRK5 (bovine) was used as a template to create GRK5ΔNES. Basic residues located between residues 259 and 265 were mutated to alanine residues using the following primers; sense primer 5' -GCC TAC GAG ACC AAG GAT GCC **GCG** TGT **GCA G'CT GCT** ACC **GCC** ATG AAC GGC GGG GAC C- 3', anti-sense primer 5' -G GTC CCC GCC GTT CAT **GGC** GGT **AGC AG'C TGC** ACA CGC GGC ATC CTT GGT CTC GTA GG- 3'. Nucleotides in bold encode the mutated

amino acids. The introduction of the mutation was screened by the addition of a PvuII site (as indicated by ′).

pRK5-GRK4α, β, γ and δ (human) were used as templates to create GRK4ΔNLS. Basic residues between 221-225 (GRKα and γ) or 189-193 (GRKβ and δ) were mutated to alanine residues using the following primers; sense primer 5' -GCC TGC AAA AAG CTA CAA AAA **GCT** AGA ATA **G′CT GCT** AGG AAA GGT GAA GCT ATG GC- 3', anti-sense primer 5' – GC CAT AGC TTC ACC TTT CCT **AG′C AGC** TAT TCT **AGC** TTT TTG TAG CTT TTT GCA GGC – 3'. Nucleotides in bold encode the mutated amino acids. The introduction of the mutation was screened by addition of an ApeKI restriction site (as indicated by ′).

pBKΔ-GRK6A, B and C (human) and pBKΔ-GRK6A-flag, B-flag and C-flag were used as templates to create GRK6ΔNLS. Basic residues between 389-391 were mutated to alanine residues using the following primers; sense primer 5' -CG CCC TTC CAG CAG AGG **GCA G′CT GCT** ATC AAG CGG GAG GAG GTG GAG CGG C- 3', anti-sense primer 5' –G CCG CTC CAC CTC CTC CCG CTT GAT **AGC AG′C TGC** CCT CTG CTG GAA GGG CG– 3'. Nucleotides in bold encode the mutated amino acids. The introduction of the mutation was screened by the addition of a PvuII site (as indicated by ′).

Polymerase chain reactions were carried out as follows:

2μl Template (10ng)

5μl 10X Primer Reaction Buffer

1.25μl Sense Primer (100ng/μl)

1.25µl Anti-sense Primer (100ng/µl)

1µl dNTPs (10mM)

1µl Pfu Polymerase

38.5 dH_20

50µl

PCRs were cycled in a PTC-2000 Peltier Thermal Cycler (MJ Research) as follows:

1. 95°C, 30 sec
2. 95°, 30 sec
3. 55°C, 60 sec
4. 68°C, 11 min
5. Return to step 2 for 17 more cycles then step 6.
6. 4°C.

Following PCR, 1µl DpnI was added to the reaction and incubated at 37°C for 1 hour to cleave methylated parent DNA. 5µl of each PCR reaction was then transformed into competent *Escherichia coli (E. coli)* as detailed in Section 2.2.4.

2.2.4. Bacterial transformation and plasmid DNA extraction

TOP10 (Invitrogen) or DH5α ultracompetent (Invitrogen) *E. coli* were thawed on ice and mixed by hand. 50µl were aliquoted into a pre-chilled 1.5ml Eppendorf tube and 20ng of DNA or 5µl of a PCR reaction added. The cells were swirled and incubated

on ice for 30 minutes before being heat shocked at 42°C for 30 seconds. The bacteria were then incubated on ice for 2 minutes. 0.9ml pre-warmed SOC medium (Invitrogen) was added and the cells incubated at 37°C for 1h with shaking at 250 rpm (Kuhner ISF-1-W bacterial incubator). Bacteria were then plated onto Luria Broth (LB) agar plates containing 100μg/ml ampicillin or 25μg/ml kanamycin as appropriate and incubated at 37°C overnight.

Single colonies were picked from the plate and grown in either 5ml or 500ml of LB medium, containing 100μg/ml ampicillin or 25μg/ml kanamycin as appropriate, overnight at 37°C with shaking at 250 rpm (Kuhner ISF-1-W bacterial incubator). The next day, bacteria were pelleted and the plasmid DNA was extracted using either a QIAprep Spin Miniprep or Maxiprep kit (QIAGEN). To test whether the plasmid contained an insert of the right size, a restriction digest was performed using 2μl of the relevant restriction enzyme (Promega) for 1h at 37°C. Digested products were analysed on a 1% agarose tris-borate (TBE) gel containing ethidium bromide and their size determined using the 1kb Plus DNA ladder. The concentration of the plasmid DNA was determined using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech).

2.2.5. DNA sequencing

Sequencing was performed by MWG Biotech (Germany) to confirm introduction of desired mutations and the integrity of the remaining sequence. Primers used to sequence constructs are listed in Table 2.8 and 2.9. The sequences obtained were compared to the published sequences of the protein using the Gene Jockey II

Sequence Processor program (Biosoft).

2.3. Immunofluorescence techniques

2.3.1. HEp2 cells - ionophore treatment

24-48h post-transfection cells were treated as described in the figure legends. Cells treated with Ca^{2+} ionophore were incubated with 25μM A23187 (Calbiochem) for 15min, unless otherwise indicated, in medium supplemented with 2mM $CaCl_2$ at 37°C, 5% CO_2.

2.3.2. CHO-M3MR cells - drug treatment

24-48h post-transfection cells were treated as described in the figure legends. CHO-M3 expressing cells were treated with the muscarinic agonist acetylcholine (ACh, Sigma) at a concentration of 100μM in CHO-M3 eagle medium (see Section 2.1.1) for 5 min, unless otherwise stated, at 37°C, 5% CO_2. The phospholipase-C inhibitor U73122 (1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione, Calbiochem) was used at a concentration of 1μM for 1h.

2.3.3. Induction of hypertrophy in rat neonatal cardiac myocytes

The day following preparation cardiac myocytes were treated with 200μM PE (Sigma), 167nM Epidermal Growth Factor (EGF) (Sigma), 200nM AngII (Sigma) or 100nM PLC activator *m*-3M3FBS (Calbiochem) in RPMI, 1% calf serum and penicillin and streptomycin (100 IU penicillin and 100μg of streptomycin/ml) for 48h. Other stimulants of cardiac hypertrophy were also trialed, including Endothelin-1 (ET_1) (100nM) and Phorbol ester 12-tetradecanoylphorbol-13 acetate (PMA,

200nM). These agents failed to produce reproducible myocyte hypertrophy, as assessed by an increase in cell area and thus ET_1 and PMA were not used during these studies.

2.3.4. Immunofluorescent labelling

2.3.4.1. Cell lines

Cells were fixed in 4% paraformaldehyde (TAAB)/PBS for 20min and quenched for 10 min in 0.27% NH_4Cl/0.37% glycine in PBS. 1% BSA (First Link UK Ltd.)/0.2% saponin/PBS was subsequently used to block and permeabilise fixed cells. Primary antibody incubations were performed for 1h at RT or at 4°C overnight. Cells were subsequently washed with 1%BSA/0.2% Saponin/PBS and incubated with an appropriate secondary antibody for 45min at RT. YFP constructs were not subject to immunostaining. Nuclei were visualised by staining DNA with Hoechst (1/10,000 in PBS; Sigma). After washing, coverslips were mounted on slides in 90% glycerol (Sigma)/PBS/3% N-propyl-galate (Sigma). Essentially the same protocol was used for the detection of endogenous GRK5 in HEp2 cells except the fluorescently labelled secondary antibodies described above were replaced with a tyramide signal amplification kit (Molecular Probes) that was used according to the manufacturers instructions. To block anti-GRK5 C20 antibody before use in immunofluorescent labeling, the antibody was preincubated with of 10 times more blocking peptide (Santa Cruz, sc-565) than antibody (e.g. 10μg antibody: 100μg blocking peptide) for 30 min on ice.

2.3.4.2. Neonatal rat cardiac myocytes

Immunofluorescent labelling of cardiac myocytes was carried out as for cell lines described in Section 2.3.4.1 with the following modifications. Cells were permeabilised using 0.5% Triton X-100/PBS for 20 min. Cells were washed with wash buffer (0.5% BSA/0.1% glycine/PBS); and blocked in wash buffer containing 5% calf serum for 45 min. Primary antibody incubations were performed for 1h at RT. GRK5 was detected using the polyclonal GRK5 antibody (Santa Cruz) listed in Table 2.3 and myocytes were identified by labelling α-actinin with the monoclonal antibody (Sigma) listed in Table 2.3. Secondary antibody labelling and coverslip mounting was carried out as described in Section 2.3.4.1.

2.3.5. Confocal microscopy

Confocal images were taken at RT. Nikon Plan Apo 40x and 60x oil immersion lenses and a BioRad 1024 MRC confocal were used with a Nikon microscope and BioRad Lasersharp 2000 software to acquire the images. Images were optimised for contrast in Adobe Photoshop but no further manipulations were made.

2.3.6. Analysis of GRK nuclear export

To quantify ionophore-dependent nuclear export of GRKs, at least 50 appropriately transfected cells were counted and GRK distribution scored as nuclear or cytosolic. Cells were deemed to show a cytosolic distribution if no transfected protein was detected in the nucleus. At least three separate GRK transfections were scored per experiment.

2.3.7. Analysis of cardiac hypertrophy

Rat cardiac myocytes were assessed for induction of hypertrophy by measuring the change in cell area between untreated and treated samples. Following immunofluorescent staining, cells were viewed using a Zeiss Axioplan2 microscope using a Nikon plan apo 40x oil immersion lens and the cell areas of a minimum of 50 cells from three independent experiments were measured and averaged for each condition. Myocytes were identified by staining for α-actinin. Where myocytes were transfected, transfected cells were identified by appropriate immunofluorescent staining. Untransfected cells on the same coverslips were identified by a lack of immunofluorescence and measured as an internal control. Cell areas were measured by drawing around the outline of each cell visualised by α-actinin and/or GRK5 (Santa Cruz anti-GRK5 C20; see Section 2.5) staining using Improvision openlab software. Statistical differences were determined by performing a Student's t-test.

2.4. Other techniques

2.4.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Laemmli method (Laemmli, 1970) with a discontinuous SDS-PAGE and the Hoefer Scientific Instruments (HSI) vertical slab gel unit SE 600 gel system. 10% SDS-PAGE resolving gel was cast between two glass plates by polymerising 30% (v/v) acrylamide in 0.375M Tris-HCl pH8.8 and 1% sodium dodecyl sulphate (SDS), using 0.3% (v/v) ammonium persulphate and 0.07% (v/v) N, N, N′, N′-tetramethyl-ethylenediamine (TEMED). A 4% stacking gel was cast above the resolving gel by polymerising 4% (v/v) acrylamide in 0.12M Tris-

HCl pH6.8 and 1% SDS, using 0.1% (v/v) ammonium persulphate and 0.1% (v/v) TEMED.

The polymerised gel was placed in an HSI SE 600 cell and covered with gel running buffer. An appropriate volume of SDS reducing buffer was added to known concentrations of samples prior to heating at 60°C for 10 minutes. Samples were briefly centrifuged to collect the entire sample and then loaded onto the gel. Rainbow coloured protein molecular weight markers (14.3 - 220kD, Amersham Biosciences) were loaded to indicate the molecular weights of sample proteins. The gel was subject to a constant voltage of 200-300V until the dye front ran off the gel.

2.4.2. Coomassie staining of SDS-PAGE gels

To detect proteins, the gel was covered in Coomassie stain, heated for 1 minute at 750W in a microwave and then allowed to cool at RT on a shaker. The gel was then destained using Coomassie de-stain until proteins were visible and identifiable by comparison to molecular weight markers. The gel was scanned using a densitometer (BioRad) and the intensity of the appropriate purified protein band determined as a percentage of total protein present. An empty lane was scanned to determine background staining.

2.4.3. Western blotting and immunodetection

Proteins separated on the 10% SDS-PAGE were transferred onto nitrocellulose membrane (Hybond-ECL, Amersham) using a semi-dry electrophoretic transfer method. The stacking gel was discarded and the resolving gel and nitrocellulose membrane were soaked in transfer buffer and placed between 6 sheets of filter paper

that had also been soaked in transfer buffer. Horizontal electrophoretic transfer was performed in the SCIE-PLAS semi-dry electroblotting unit V20-SDB with a constant current of 0.8mA per cm^2 of nitrocellulose membrane applied for 1h 45min. Transfer was considered complete if visual transfer of the molecular weight standards to the nitrocellulose membrane had occurred.

The nitrocellulose membrane to which the proteins had been transferred was incubated in blocking buffer for 1h at RT. The immobilised proteins were subject to immunoblotting with a primary antibody diluted in blocking buffer for 1h at RT or overnight at 4°C. Following five washes over a 30 min period in TTBS a horseradish peroxidase-conjugated secondary antibody (Amersham) diluted in TTBS was added to the immunoblots for 1h at RT. The blots were then washed as before.

Sufficient ECL reagent was added to cover the protein side of the blot and incubated at RT for 1 minute with vigorous shaking (ECL Western Blotting Detection Reagents, Amersham Biosciences). Bound antibody was detected by exposing the immunoblot to film (Biomax ML, Kodak) for the required time and the film was developed using an Agfa automatic film processor and quantified using a densitometer (BioRad).

2.4.4. Purification of GRK5 & 6

GRK5 and GRK6 were overexpressed in baculovirus-infected SF9 cells by Dr Julie Pitcher. Cell pellets were thawed, supplemented with fresh protease inhibitors (40μg/ml phenylmethanesulfonyl fluoride (PMSF), 1mM benzamidine), and homogenised with 10 strokes of a tightglass Dounce homogeniser on ice. All subsequent manipulations were performed at 4°C, and all buffers contained protease

inhibitors as above. The homogenate was spun at 17,000 rpm (JA-17 rotor, Beckman Coulter Avanti J-25 centrifuge) for 20 min. The resulting pellet was re-homogenised with 50 ml of purification buffer with 20 mM NaCl and spun as before. The two supernatants were pooled and passed through a 10-ml column of S-Sepharose (Pharmacia) at a flow rate of 1 ml/min. Most proteins failed to bind to the resin. The column was washed with 50 ml of purification buffer with 20 mM NaCl and eluted with a linear 100-ml gradient of 20-750 mM NaCl in purification buffer. Fractions were assessed for GRK5 or 6 content by coomassie staining of 10% SDS-PAGE gels or by Western blotting and those fractions containing GRK5 or 6 and the fewest protein contaminants were pooled. Pooled fractions (20 ml) were diluted with purification buffer to below 100 mM NaCl, and applied to a 10-ml column of heparin-Sepharose (Pharmacia) at 1 ml/min. The column was washed with 50 ml of purification buffer with 150 mM NaCl and eluted with a linear 100-ml gradient of 15-1500 mM NaCl in purification buffer with 0.02% Triton X-100. Fractions containing purified GRK5 or 6 as assessed by SDS-PAGE gel coomassie staining were pooled and concentrated to 1 ml in a Centriprep 30 spin concentrator (Amicon). Purified GRKs were stored at 4°C or at –20°C in 50% glycerol. Final purity of purified GRKs was assessed by staining the proteins in a SDS-PAGE gel with coomassie stain as described in Section 2.4.2.

2.4.5. DNA-binding assay

Cos-7 cells expressing GRKs were lysed in 300µl cold DNA-binding buffer and lysates clarified by centrifugation at 13,000 rpm (Heltich bench top centrifuge Mikro 20) for 8 min. Protein concentration of the clarified lysates was determined using the

BioRad protein assay. Cell lysate was diluted 1:40 in BioRad protein assay reagent (previously diluted 1:5 in water). The A_{595} of samples was measured using an Ultraspec 2000 spectrophotometer and the protein concentration of samples determined by comparison to a standard curve obtained by measuring the A_{595} of known amounts of BSA. Cell lysates (~10µg) or purified GRK (150ng, see Section 2.4.4) were incubated with 25µl of native DNA-cellulose (~10µg DNA, Amersham), single stranded DNA-cellulose (~10µg DNA, Sigma), poly(A)-sepharose 4B (Amersham) or cellulose (Sigmacell Cellulose type 50, Sigma) in 100µl cold DNA-binding buffer for 1h at 4°C. For competition assays, GRKs 5 or 6 were pre-incubated with the stated amounts of DNA (sonicated, calf thymus, Amersham) or ribonucleic acid (RNA, calf liver type IV, Sigma) for 30 min on ice and then added to 25µl native DNA-cellulose. Following incubation the resin was washed four times with 1ml binding buffer (at 4°C) and the amount of GRK retained on the resin determined by Western blot analysis and immunodetection as described in Section 2.4.3. Autoradiographs were subsequently quantified using a densitometer (BioRad) Significance was determined by performing a t-test.

2.4.6. Purification of rod outer segments (ROS)

ROS were prepared from dark adapted bovine retinas. 50 retinas were suspended in 50 ml of ROS buffer A, homogenised on ice and centrifuged at 4,000 rpm (JA-17 rotor, Beckman Coulter Avanti J-25 centrifuge) for 5 min. The supernatant, containing the ROS, was diluted with 2 volumes of 10mM Tris-acetate, pH 7.4, and centrifuged as above. The crude ROS pellets were resuspended in ~30 ml of ROS

buffer B (4°C), homogenised, and then further purified on a sucrose gradient (See ROS sucrose gradient in buffers table 2.1) by centrifugation in a SW-28 swinging bucket rotor at 26,000 rpm (Beckman Coulter Optima LE-80K Ultracentrifuge). The interface between the 0.84 and 1.00M sucrose layers was collected and diluted 1:1 with ROS buffer A without sucrose, and sedimented at 17,000 rpm (JA-17 rotor, Beckman Coulter Avanti J-25 centrifuge) for 30 min. This pellet was resuspended in 10ml ROS buffer A without sucrose and centrifuged as before. The pellet was resuspended in 10ml ROS buffer A, centrifuged at 23,000 rpm (JA-25.5 rotor, Beckman Coulter Avanti J-25 centrifuge), and the recovered ROS resuspended in 15ml 4M urea in 10mM Hepes (pH7.0) and left on ice for 20 min to remove endogenous GRK1. The ROS were then diluted with four volumes of ROS buffer C and centrifuged as before. The pelleted ROS were resuspended in 10mM Hepes (pH7.0) and re-centrifuged as before. The final pellet of purified ROS were resuspended in 10ml 10mM Hepes (pH7.0) and homogenised. ROS protein concentration was determined using the BioRad protein assay as described in Section 2.4.5 and diluted to 2mg/ml with 10mM Hepes (pH7.0). ROS were aliquoted (50µl) under safe light, wrapped in foil and stored in liquid nitrogen. ROS are approximately 90% pure at the end of this procedure.

2.4.7. GRK5 kinase assays

Kinase assays were performed in kinase assay buffer containing 50µM adenosine triphosphate (ATP) (~6000cpm/pmol) in a total volume of 25µl. Purified rod outer segments (ROS, 1µg), or purified tubulin (Cytoskeleton, 10µg) were included as GRK substrates as indicated.

When measuring DNA/RNA-mediated inhibition of GRK5 or GRK5ΔNLS activity transfected Cos-7 cell lysates were used as the source of GRK5. Cos-7 cells were transfected with 2μg of the appropriate cDNA. 24h post-transfection, cells were lysed in 1ml of kinase assay lysis buffer by homogenisation (two 15sec pulses, Branson Sonifier 450, setting 6) and lysates clarified by centrifugation at 13,000 rpm (Heltich bench top centrifuge Mikro 20) for 10min. Equivalent rhodopsin kinase activities of GRK5 and GRK5ΔNLS were used in the assays, corresponding to respectively, ~0.6 and ~1.0mg of total protein. For each assay condition the kinase activity present in an equivalent amount of protein from a mock-transfected lysate was determined and subtracted from that obtained with the GRK5 expressing lysate.

The activity comparison of GRK5, GRK5ΔNLS, GRK5NTPB, GRK5CTPB and GRK5K215R was performed using partially purified transfected Cos-7 cell extracts. GRK5NTPB is expressed at approximately 20% of the level of the other GRK5 constructs and detecting GRK5NTPB-mediated tubulin phosphorylation above a background of endogenous kinases proved difficult without this enrichment step. Cells transfected with the appropriate cDNA were lysed as described above. Lysates (~2mg of protein) were diluted 2-fold in kinase assay dilutant buffer and loaded onto 0.5ml S-Sepharose columns by gravity flow. The columns were washed sequentially with 2.5ml of kinase assay dilutant buffer, kinase assay dilutant buffer + 400mM NaCl and kinase assay dilutant buffer + 1M NaCl. Wild type GRK5 and mutant kinases eluted in the 1M NaCl wash were subsequently desalted and concentrated in Nanosep 30K centrifugal devices (Pall Life Sciences). In total three separate transfections and three S-Sepharose columns were run for each enzyme. Before

assaying activity the individual purifications were pooled and the amount of GRK5/unit volume quantified by western blot analysis. The amount of total protein assayed was adjusted such that equivalent amounts of each kinase were used in the reactions. This ranged between 200-300ng of protein with the exception of GRK5NTPB expressing lysates where approximately 5 times more protein was used. Assays using equivalent amounts of protein derived from a partially purified mock-transfected lysate were used to determine endogenous kinase activity, which was subsequently subtracted from the relevant kinase expressing lysate activity value.

Where purified GRK was used in kinase assays, 75ng of purified GRK5 was used in each assay. All reactions were incubated at 30°C for 10min. When rhodopsin was used as a substrate samples were illuminated during this incubation period. Reactions were stopped by addition of an equal volume of SDS reducing buffer, and electrophoresed on 10% SDS-PAGE gels. The dried gels were analysed and quantified using a phosphorimager (BioRad) and significance was determined by performing a Student's t-test.

2.4.8. NHERF-1 overlays

His/S-tagged full length Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) was expressed in bacteria (BL-21, Stratagene) and purified using a His-tag purification kit (Novagen). GRK6A and GRK6A-flag were expressed in Cos-7 cells and lysates made as described for the DNA-binding assay. The lysates (25 μg/lane) were run on 10% SDS-PAGE gels and subject to western blot analysis. The blots were overlaid with 100nM fusion protein in buffer (2% milk, 0.1% Tween 20, 10 mM HEPES, pH 7.4, 50 mM NaCl) for 1 h at RT. All subsequent steps were performed in Western

blot blocking buffer. The blots were washed three times, incubated for 1h at room temperature with a horseradish peroxidase-conjugated anti-S-Tag antibody (Novagen), washed three more times, and visualised via chemiluminescence.

2.5. Antibodies & Tagged-Conjugates

Tables 2.3-2.6 list antibodies and tagged-conjugates used in this thesis, their concentrations and suppliers. Commercial antibodies were stored at 4°C, –20°C or –80°C according to manufacturers' instructions. Non-commercial antibodies were stored at -80°C.

2.6. Statistical analysis

Results were analysed using the student's two-sample T-test to determine whether measurements made on two populations were different from each other (Ennos, 1999). The null hypothesis proposed that the two populations were the same in every case.

Table 2.1: Buffers

Blocking buffer (Western blot)	4% skimmed milk powder made up in TTBS
Blocking buffer (rat cardiac myocytes)	5% calf serum made up in wash buffer
Coomassie de-stain	40% MeOH 10% acetic acid
Coomassie stain	40% MeOH 10% acetic acid 0.05% Brilliant Blue R
Digestion buffer	100ml isolation buffer 250mg collagenase type II CLS2 (Worthington) 25mg pancreatin from porcine pancreas
DNA-binding buffer	10mM Hepes pH7.4 1mM $MgCl_2$ 0.1% Triton X-100 3mM DTT 0.1M NaCl 0.5mM EDTA 40µg/ml PMSF 1mM benzamidine
Freezing medium	20% FCS 10% DMSO made up in DMEM
Gel running buffer	2M glycine 0.25M Tris 0.03M SDS
HEBS buffer	137mM NaCl 20mM Hepes 6mM D-glucose 5mM KCl 0.7mM Na_2HPO_4 pH adjusted to pH7.05

Isolation buffer	116mM NaCl
	20mM Hepes
	0.77mM NaH_2PO_4
	5.5mM glucose
	5.4mM KCl
	0.4mM $MgSO_4$
	pH adjusted to pH7.35
Kinase assay buffer	20mM Tris-HCl, pH 7.5
	2.0mM EDTA
	10mM $MgCl_2$
	1mM DTT
Kinase assay lysis buffer	20mM Hepes, pH7.2
	250mM NaCl
	10mM EDTA
	0.02% Triton X-100
	40μg/ml PMSF
	1mM benzamidine
Kinase assay dilutant buffer	20mM Hepes pH7.2
	2mM EDTA
	40μg/ml PMSF
	1mM benzamidine
Lysis buffer (cell lysates)	150mM NaCl
	50mM Tris pH8
	2mM EDTA
	1mM benzamidine
	1% Triton X-100
	10% glycerol
	40μg/ml PMSF
Mountant	90% glycerol
	3% N-propyl-galate
	made up in PBS
	Stored at 4°C, away from light
Immunofluorescence	1% BSA
permeabilisation/block/wash	0.2% saponin
buffer (cell lines)	made up in PBS

Immunofluorescence permeabilisation buffer (rat cardiac myocytes)	0.5% Triton X-100 made up in PBS
Purification buffer	20 mM HEPES 2 mM EDTA pH 7.2
Quench buffer	0.37% glycine 0.27% NH_4Cl 0.05% azide made up in PBS
ROS buffer A	34% sucrose (w/w) 65 mM NaCl 2 mM $MgCl_2$ 10 mM Tris-acetate buffer, pH 7.4 0.1mM EDTA 500μM PMSF 1μM Leupeptin 1μM Pepstatin
ROS buffer B	0.77M sucrose 1 mM $MgCl_2$ 10mM Tris-acetate, pH 7.4
ROS buffer C	10mM Hepes, pH 7.0 1mM $MgCl_2$
ROS sucrose gradient	10ml: 0.84 M sucrose 2mM $MgCl_2$ 10mM Tris acetate, pH 7.4 0.1mM EDTA 15ml: 1M sucrose 2mM $MgCl_2$ 10mM Tris acetate, pH 7.4 0.1mM EDTA

SDS reducing buffer	25mM Tris pH6.5 10% glycerol 8% SDS 5% β-mercaptoethanol a few grains of Brilliant Blue G
TBE	890mM Tris 890mM boric acid 20mM EDTA pH 8.3
Transfer buffer	0.05M Tris 0.04M glycine 0.01M SDS 20% MeOH
TTBS	50mM NaCl 20mM Tris 0.05% Tween-20
Wash buffer (rat cardiac myocytes)	0.5% BSA 0.1% glycine made up in PBS

Table 2.2: cDNA constructs

Construct name	cDNA	Supplier	Reference
GRK5	GRK5-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Pitcher et al., 1996)
GRK5NTPB	GRK5NTPB-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Pitcher et al., 1996)
GRK5CTPB	GRK5CTPB-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Pitcher et al., 1996)
GRK5K215R	GRK5K215R-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	
GRK5AP	GRK5ST/AA-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Kunapuli et al., 1994)
GRK5ΔNLS	GRK5ΔNLS-pRK5	Constructed by Laura Johnson.	(Johnson et al., 2004)
GRK5ΔNES	GRK5ΔNES-pRK5	Constructed by Laura Johnson.	
GRK2	GRK2-pRK5	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Pitcher et al., 1999)
GRK3	GRK3-pcDNA1	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US.	(Menard et al., 1996)

Construct name	cDNA	Supplier	Reference
GRK4α, β, γ, δ	GRK4-α, -β, -γ, -δ-pRK5	Dr Richard Premont, Duke University, US.	(Premont et al., 1996)
GRK4-αΔNLS, -βΔNLS, -γΔNLS, -δΔNLS	GRK4αΔNLS, βΔNLS, γΔNLS, δΔNLS pRK5	Constructed by Laura Johnson.	
GRK6-A, -B, -C	GRK6-A, -B, -C-pBK(Δ)	Dr Mario Tiberi, Universite de Montreal, Canada.	
GRK6-AΔNLS, -BΔNLS, -CΔNLS	GRK6A-ΔNLS, -BΔNLS, -CΔNLS -pBK(Δ)	Constructed by Laura Johnson.	
GRK6A-Flag	GRK6A-pBK(Δ)-Flag	Dr Mario Tiberi, Universite de Montreal, Canada.	
GRK6A-3C3A	GRK6A-3C3A-pCMV5	Dr Richard Premont, Duke University.	
I39E, F527D, I39E/I165E, I165E/F527D	GRK6-I39E, -I165E, -F527D, -I39E/I165E, -I165E/F527D – pcDNA3	Prof. John Tesmer, University of Texas	(Lodowski et al., 2006)
S-tag-NHERF-1	6xHis/S-tag-NHERF-1-pET30a	Dr Robert Lefkowitz, HHMI/Duke University Medical Center, US. & Dr Randy Hall, Emory University School of Medicine, US.	(Weinman et al., 1998)
CaMBP/m	CaMBP/m-pEYFP	Dr Marcia A. Kaetzel, University of Cincinnati, US; Prof John Dedman, University of Cincinnati, US; Prof David Sacks, Harvard Medical School, US.	(Li et al., 2003)

Construct name	cDNA	Supplier	Reference
CaMBP/n	CaMBP/n-pEYFP	Dr Marcia A. Kaetzel, University of Cincinnati, US; Prof John Dedman, University of Cincinnati, US; Prof David Sacks, Harvard Medical School, US.	(Wang et al., 1995)

Table 2.3: Primary antibodies used in immunofluorescence

Antigen	Antibody Name	Species & Isotype	Dilution	Supplier
GRK2	Anti-GRK2/3	Mouse IgG2aκ	1:300	Upstate Biotechnology
GRK5	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
	Anti-GRK5 C20	Rabbit IgG	1:50	Santa Cruz
	Anti-GRK5 C20 blocking peptide	N/A	10X more than antibody	Santa Cruz
GRK4	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
GRK6	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
α-Actinin	Anti-α-actinin	Mouse IgG1	1:200	Sigma
Muscarinic-M3 receptor	Anti-M3	Rabbit IgG	1:50	Dr Andrew Tobin, University of Leicester
Acetyl-histone H3	Anti-AcH3	Rabbit IgG	1:300	Upstate Biotechnology
Acetyl-histone H4	Anti-AcH4	Rabbit IgG	1:300	Upstate Biotechnology
Phospho-histone H3	Anti-P-H3	Rabbit IgG	1:300	Upstate Biotechnology

Table 2.4: Secondary antibodies used in immunofluorescence

Antigen	Antibody Name	Species & Isotype	Dilution	Supplier
Mouse IgG	Alexa Fluor 488 anti-mouse	Donkey	1:700	Molecular Probes
	Alexa Fluor 594 anti-mouse	Donkey	1:700	Molecular Probes
Rabbit IgG	Alexa Fluor 488 anti-rabbit	Donkey	1:700	Molecular Probes
	Alexa Fluor 594 anti-rabbit	Donkey	1:700	Molecular Probes

Table 2.5: Primary antibodies/tagged conjugates used in western blotting and immunodetection

Antigen	Antibody/Tagged Conjugate Name	Species & Isotype	Dilution	Supplier
GRK2	Anti-GRK2/3	Mouse IgG2aκ	1:300	Upstate Biotechnology
GRK5	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
	Anti-GRK5 C20	Rabbit IgG	1:500	Santa Cruz
GRK4	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
	Anti-GRK4 (H64)	Rabbit IgG	1:500	Santa Cruz
GRK6	Anti-GRK4-6	Mouse IgG1κ	1:300	Upstate Biotechnology
	Anti-GRK6 (C20)	Rabbit IgG	1:500	Santa Cruz
S-protein	S-protein-HRP	N/A	1:5000	Novagen

Table 2.6: Secondary antibodies used in western blotting and immunodetection

Antigen	Antibody Name	Species & Isotype	Dilution	Supplier
Mouse IgG	Anti-Mouse IgG	Sheep	1:2000	Amersham Biosciences
Rabbit IgG	Anti-Rabbit IgG	Sheep	1:2000	Amersham Biosciences

Table 2.7: Putative nuclear localisation sequences

GRK	Predicted localisation Seq.	Species	Protein Sequence and location	DNA sequence and location
GRK5	NLS	Bovine	WT: 388-**RKEKVKR**E-395 Mutant: 388-**AAEAVAA**E-395	WT: 1162-**CGC AAG** GAG **AAG** GTG **AAG CGG** GAG-1185 Mutant: 1162- **GCT GCT** GAG **GCT** GTG **GCT GCT** GAG-1185
	NES	Bovine	WT: 259-**LCLVLTI**-265 Mutant: 259-**ACAAA**TI-265	WT: 775-**CTG** TGT **TTG GTC CTG** ACC **ATC**-795 Mutant: 775- **GCG** TGT **GCA GCT GCT** ACC **GCC**-795
GRK4	NLS	Human	WT: QK**KRIKK**RK Mutant: QK**ARIAA**RK α,γ: 219-227 β,δ: 187-195	WT: CAA AAA **AAA** AGA ATA **AAG AAG** AGG AAA Mutant: CAA AAA **GCT** AGA ATA **GCT GCT** AGG AAA α,γ: 655-681 β,δ: 558-585
	NES	Human	WT: **LCLVLTI** α,γ: 260-266 β,δ: 228-234	Mutant not constructed
GRK6	NLS	Human	WT: 387-QR**KKK**IKRE-395 Mutant: 387-QR**AAA**IKRE-395	WT: 1159-CAG AGG **AAG AAG AAG** ATC AAG CGG GAG-1185 Mutant: 1159-CAG AGG **GCA GCT GCT** ATC AAG CGG GAG-1185
	NES	Human	WT: 259-**LCLVL**T-264	Mutant not constructed

Table 2.8: Custom sequencing primers

Primers synthesised by Invitrogen were designed to sequence both the sense (F) and anti-sense (R) strands in stages from 5' to 3' in order to check that mutagenesis was as desired.

Target sequence	Primer Name (F-sense, R-antisense)	Primer Sequence and location (5'-3' on sense strand)
GRK5 (bovine)	GRK5seqF1	^{1}ATGGAGCTGGAAAACATCGT^{20}
	GRK5seqF2	^{400}CTTCAGAAACCCTGCAAAGA^{419}
	GRK5seqF3	^{814}AAGTTCCACATTTACAACAT^{833}
	GRK5seqF4	^{1201}GTGTTGGAGACAGAGGAGGT^{1220}
	GRK5seqF5	^{1556}AGTGCTTTAAGGAGCTGAAC^{1575}
	GRK5seqR1	^{1754}TGAGGTGGCCTTCGTCGATC^{1773}
	GRK5seqR2	^{1270}GAGTGGTTTCTACGTTTCGT^{1289}
	GRK5seqR3	^{910}GTACTCTTGTAACACATGTC^{833}
	GRK5seqR4	^{439}GTCAGACAGGTGCTGATGGA^{458}
	GRK5seqR5	^{232}CTCAGGATGTAAGTCAAGGA^{251}

Target sequence	**Primer Name (F-sense, R-antisense)**	**Primer Sequence and location (5'-3' on sense strand)**
GRK4 (human)	HGRK4DSEQ1F	ATGGAGCTCGAGAACATCGT (α, β, γ, δ: 1-20)
	HGRK4DSEQ2F	CATATTTTTCTCAGTTTTTACAATGGAA (α, γ: 497-524, β, δ: 401-428)
	HGRK4DSEQ3F	CTTGGAAGATTTACAGAGGGAAAG (α, γ: 897-920, β, δ: 801-824)
	HGRK4DSEQ4F	CTGGGCTGCAGGGG (α, γ: 1297-1214, β, δ: 1201-1214)
	HGRK4DSEQ1R	CACTGTCTTACATCTAACCCTGAC (α, γ: 376-399, β, δ: 280-303)
	HGRK4DSEQ2R	CACGAACCACGAGTGGTAAT (α, γ: 780-799, β, δ: 684-703)
	HGRK4DSEQ3R	TTACCCTCCTCCAGCTAGT (α, γ: 1181-1199, β, δ: 1085-1103)
	HGRK4DSEQ4R	CTTGGGTTCGTTACGACT (α: 1720-1737, β: 1085-1103, γ: 1181-1199, δ: 1486-1503)
GRK6 (human)	MGRK6ASEQF1	^{1}ATGGAGCTCGAGAACATCGT^{20}
	HGRK6ASEQF2	^{401}AGCAGGGTCCCTGCAAA^{417}
	HGRK6ASEQF3	^{801}CGGGGGCGACCTCA^{814}
	HGRK6ASEQF4	^{1201}GTGAAGGAGGTCCCCGA^{1217}
	MGRK6ASEQF5	^{1601}CCCCAGACCTGGACTGGA^{1618}
	MGRK6ASEQR5 (A & B only)	GGGGTGGGCGGAGATC (A: 1715-1731, B: 1718-1733)
	MGRK6CSEQR5 (C only)	^{1664}AGAAGTCAGCGGTTTCCACT^{1683}
	HGRK6ASEQR1	^{113} TGTAGTCGGTCACGCTTCT^{131}
	HGRK6ASEQR3	^{915}CGCGTAGCACATGTCCC^{931}
	HGRK6ASEQR4	^{1316} GGGCGCTCCACTTCCT^{1331}

Table 2.9: MWG supplied primers

MWG supplied primers were used to check cloning junctions.

Target sequence	**Primer Name**	**Primer Sequence and location (5'-3' on sense strand)**
pcDNA3.1™ 3.1/*myc*-His(-)A, B (Invitrogen)	T7 promoter/ priming site (5' Cloning Junction)	[863]TAA TAC GAC TCA CTA TAG GG[882]
	BGH reverse priming site (3' Cloning Junction)	[1113]TAG AAG GCA CAG TCG AGG[1130]

3. GRK5 contains a DNA-binding nuclear localisation sequence and nuclear export sequence

The role of GRKs in regulating desensitisation of GPCRs is well understood, however evidence has emerged to suggest that in addition to this established role GRKs also have additional cellular functions (summarised in Section 1.3). Following the observation made by Dr. Julie Pitcher that the GRK4 subfamily, but not the GRK2 subfamily, are present in the nucleus in HEp2 cells, I investigated the possibility that the GRK4 subfamily of GRKs might have nuclear functions. GRK5 represents the most intensively studied, and thus, best characterised member of the GRK4 subfamily. I thus sought to determine if nuclear localisation was a universal feature of GRK5 in a range of cell lines and to identify motifs within the kinase that may account for its subcellular localisation. I additionally describe experiments aimed at establishing whether the nuclear localisation of GRK5 is regulated and to explore potential functional consequences of this localisation.

3.1. Endogenous and overexpressed GRK5 is present in the nucleus of various cell lines

Overexpression of GRK5 in a variety of mammalian cell lines (Figure 3.1A); HEp2 (a), HeLa (b), HEK-293 (c) and Cos-7 (d), and GRK2 in HEp2 (e), reveals distinct cellular distributions for these two related kinases. GRK2, a member of the GRK2 subfamily, is cytoplasmic and is excluded from the nucleus in HEp2 cells (Figure

3.1A(e)) whereas GRK5, a member of the GRK4 subfamily, is ubiquitously distributed throughout the cell, including the nucleus (Figure 3.1A).

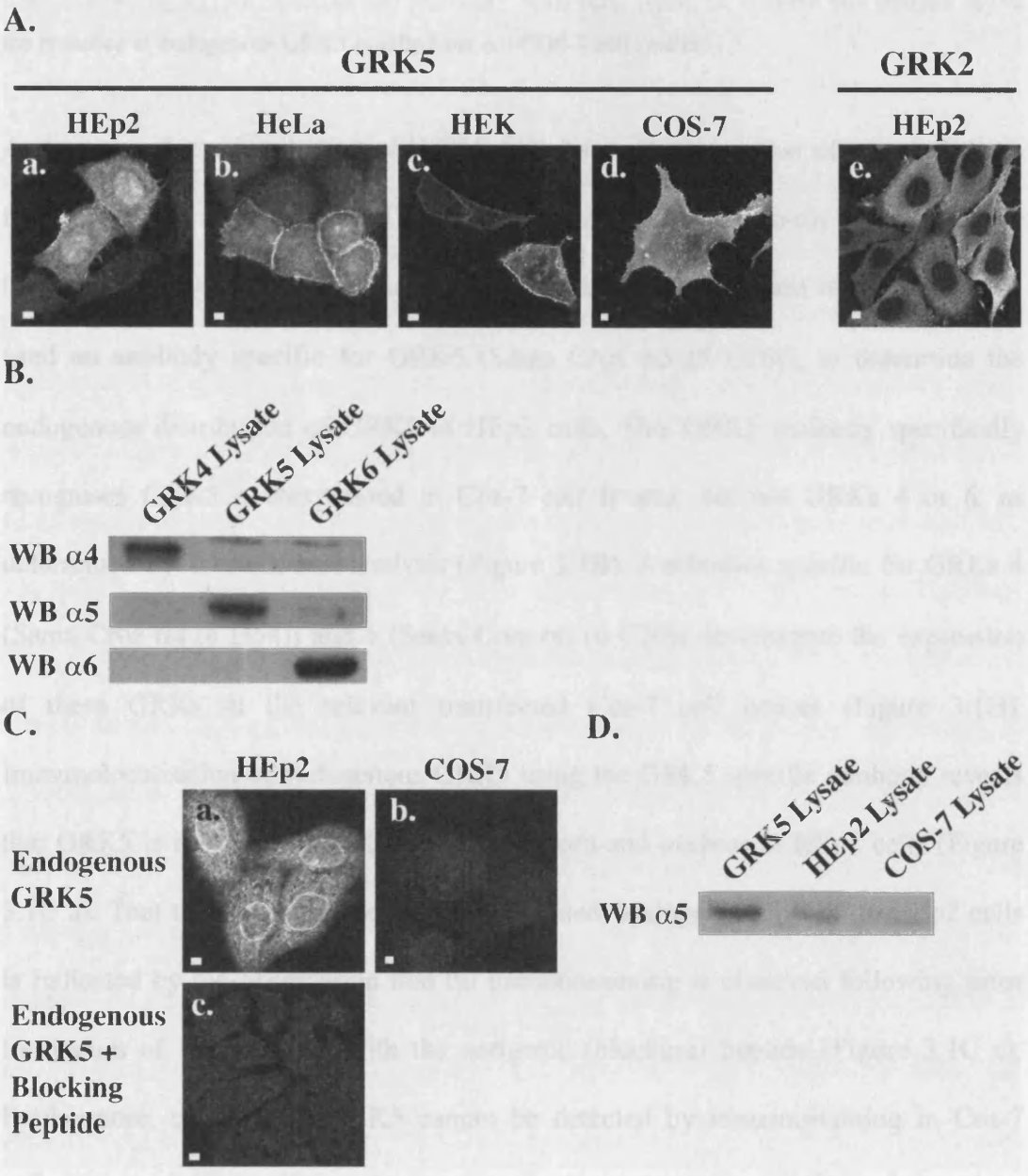


Figure 3.1. GRK5 is localised in the nucleus of cells.

A. Confocal microscopy of GRK5 transiently transfected into a. HEp2, b. HeLa, c. HEK and d. Cos-7 cell lines and e. GRK2 in HEp2 cells. Scale bars, 10μM. Background staining of untransfected cells can be seen in panels a, b and c. B. Western blot showing that the anti-GRK5 antibody (Santa Cruz α5 (5 C20)) specifically detects GRK5 overexpressed in Cos-7 cells. The presence of expressed GRK4

and 6 in the appropriate cell lysates is also demonstrated. C. Endogenous GRK5 can be detected (see Materials and Methods) in a. HEp2 cells but not b. Cos-7 cells. The immunofluorescence observed in HEp2 cells (a) can be blocked by addition of the GRK5 antibody blocking peptide during immunolabelling (c) (see Materials and Methods). Scale bars, 10μM. D. Western blot analysis shows the presence of endogenous GRK5 in HEp2 but not COS-7 cell lysates.

A similar uniform distribution of GRK5 was observed in a number of other cell lines including HeLa, HEK-293 and Cos-7 cells (Figure 3.1A panels b-d). To confirm that the presence of GRK5 in the nucleus is not an artifact of transient overexpression, I used an antibody specific for GRK5 (Santa Cruz α5 (5 C20)), to determine the endogenous distribution of GRK5 in HEp2 cells. This GRK5 antibody specifically recognises GRK5 overexpressed in Cos-7 cell lysates, but not GRKs 4 or 6, as determined by Western blot analysis (Figure 3.1B). Antibodies specific for GRKs 4 (Santa Cruz α4 (4 H64)) and 6 (Santa Cruz α6 (6 C20)) demonstrate the expression of these GRKs in the relevant transfected Cos-7 cell lysates (Figure 3.1B). Immunolocalisation of endogenous GRK5 using the GRK5 specific antibody reveals that GRK5 is localised throughout the cytoplasm and nucleus in HEp2 cells (Figure 3.1C a). That this antibody specifically recognises endogenous GRK5 in HEp2 cells is indicated by the observation that no immunostaining is observed following prior incubation of the antibody with the antigenic (blocking) peptide (Figure 3.1C c). Furthermore, endogenous GRK5 cannot be detected by immunostaining in Cos-7 cells (Figure 3.1C b). Using the same antibody, endogenous GRK5 is detected by Western blot in HEp2 cell lysates but not in Cos-7 cell lysates (Figure 3.1D). These observations indicate that the immunofluorescence observed in HEp2 cells using the anti-GRK5 antibody (Santa Cruz α5 (5 C20)) does indeed represent the distribution

of endogenous GRK5. This data reveals that GRK5 is present in the nucleus of a range of commonly used immortalised cell lines.

3.2. GRK5 contains nuclear localisation and export sequences

Nuclear import and export of macromolecules has been studied in great detail. Molecules larger than 40kDa in weight require active import in order to enter the nucleus (Tran and Wente, 2006). Active import/export of macromolecules is regulated by nuclear import receptors called importins and export receptors called exportins (Tran and Wente, 2006). Importins and exportins bind to NLS and NESs respectively in cargo molecules and then this complex is transported through the NPC via a Ran GTPase-dependent mechanism (Moroianu, 1999; Quimby and Corbett, 2001). Since GRK5 is a 65kDa protein it seemed unlikely that GRK5 was present in the nucleus of cells on the basis of simple diffusion across the nuclear membrane. In an attempt to account for the differential cellular distribution of the GRK2 and 4 subfamilies, we searched for potential NLSs in the GRKs using PredictNLS. This database contains NLS consensus sequences from reported nuclear proteins and predicts NLSs by comparing protein sequences to known consensus NLSs (Cokol et al., 2000). Using PredictNLS a putative NLS was identified between residues 388-395 in the catalytic domain of GRK5 (^{388}RKEKVKRE^{395}, Figure 3.2A underlined residues) but not GRK2 (Cokol et al., 2000). I mutated basic amino acids in the putative NLS of GRK5 to alanine residues (^{388}AAEAVAAE^{395}, Figure 3.2A bold residues and shown as a schematic in Figure 3.2B), this results in the nuclear exclusion of the mutant kinase (GRK5ΔNLS) when expressed in HEp2 cells

(compare Figure 3.2C panels a & b). The cytoplasmic distribution of the GRK5ΔNLS mutant suggests that GRK5 contains a functional NLS and points towards potentially distinct functions of the 2 and 4 subfamilies of GRKs.

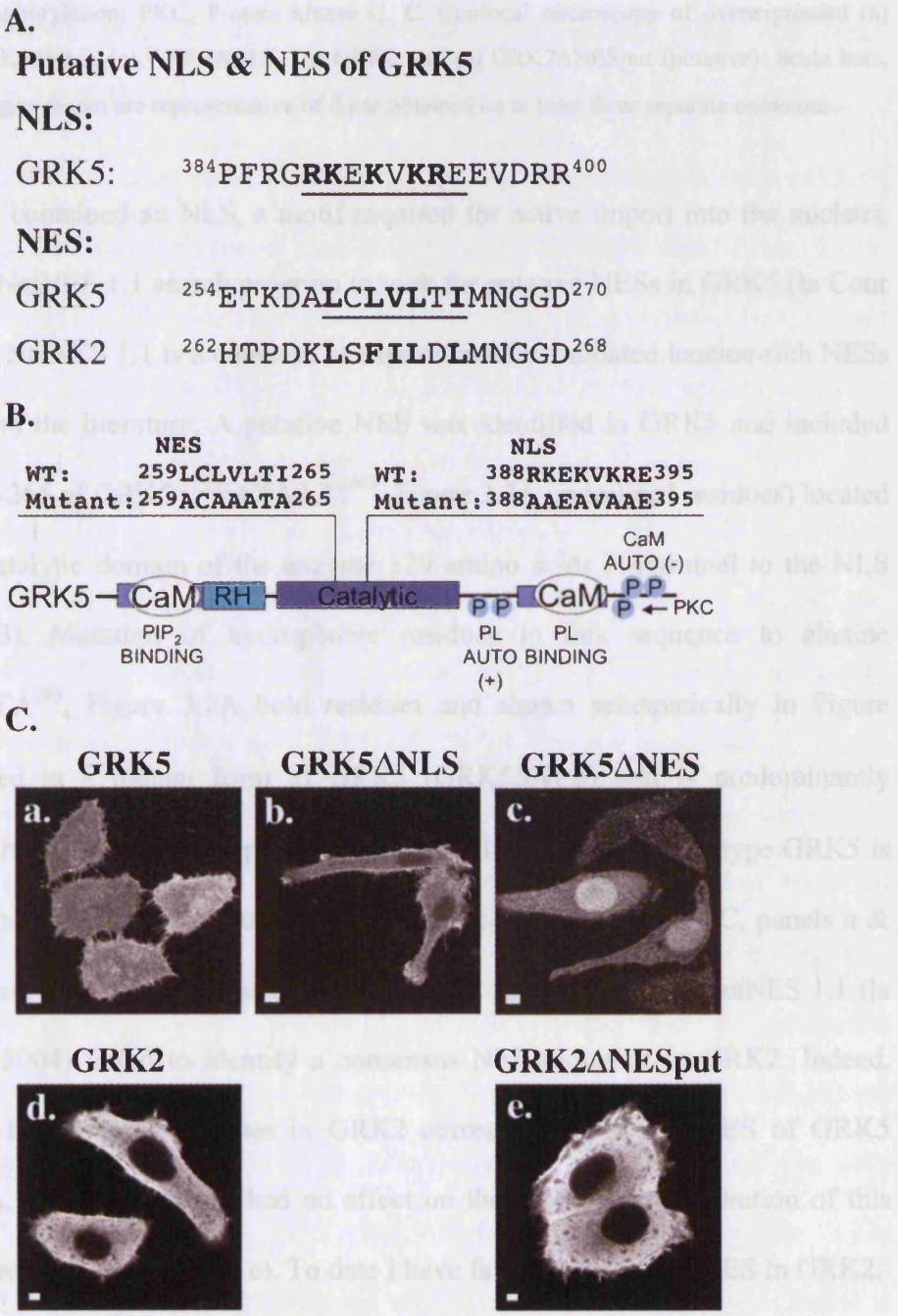


Figure 3.2. GRK5 contains a functional NLS and NES.

A. Sequence (underlined) of the putative NLS and NES of GRK5 together with the homologous sequence in GRK2. Residues mutated in mutant enzymes are indicated in bold. Numbers indicate amino acid location. B. Domain structure of GRK5 showing the location and amino acid sequence of the putative NLS and NES (Adapted from Penela et al., 2003). CaM, Calmodulin binding domain; RH, Regulator of G-protein signalling (RGS) homology domain; PL, phospholipid; P, Phosphorylation site; Auto, autophosphorylation; PKC, Protein kinase C. C. Confocal microscopy of overexpressed (a) GRK5, (b) GRK5ΔNLS, (c) GRK5ΔNES, (d) GRK2 and (e) GRK2ΔNESput (putative). Scale bars, 10μM. The images shown are representative of those obtained on at least three separate occasions.

Since GRK5 contained an NLS, a motif required for active import into the nucleus, we used the NetNES 1.1 search program to look for putative NESs in GRK5 (la Cour et al., 2004). NetNES 1.1 is a database of experimentally validated leucine-rich NESs collected from the literature. A putative NES was identified in GRK5 and included residues 259-265 of GRK5 (^{259}LCLVLTI^{265}, Figure 3.2A underlined residues) located within the catalytic domain of the enzyme 129 amino acids N-terminal to the NLS (Figure 3.2B). Mutation of hydrophobic residues in this sequence to alanine (^{259}ACAAATA^{265}, Figure 3.2A bold residues and shown schematically in Figure 3.2B) resulted in a mutant form of GRK5 (GRK5ΔNES) that is predominantly confined to the nucleus when expressed in HEp2 cells. In contrast wildtype GRK5 is distributed throughout the nucleus and cytoplasm (compare Figure 3.2C, panels a & c). These observations imply that GRK5 contains a functional NES. NetNES 1.1 (la Cour et al., 2004) failed to identify a consensus NES sequence in GRK2. Indeed, mutation of hydrophobic residues in GRK2 corresponding to the NES of GRK5 (Figure 3.2A, residues in bold) had no effect on the subcellular distribution of this kinase (Figure 3.2C, panel d and e). To date I have failed to identify a NES in GRK2.

Together these results suggest that possession of an NLS and NES distinguishes

GRK5 from GRK2 and may indicate distinct cellular functions for these two GRKs. These findings are somewhat surprising since no nuclear substrates or functions for the GRKs have been identified to date.

3.3. Calcium ionophore treatment promotes the nuclear export of GRK5

As described in Section 1.4 of the introduction, GRKs are subject to a wide range of regulatory mechanisms that control their activity and subcellular distribution. I next wanted to determine if the nuclear localisation of GRK5 is modulated by extrinsic signals. Initially, I examined if GRK5 subcellular distribution was altered when co-overexpressed in HEp2 cells with the β_2AR (data not shown). I also subjected HEp2 cells expressing GRK5 to serum starvation (data not shown), PMA treatment (data not shown) or Ca^{2+} ionophore (A23187) treatment. Of these extrinsic signals, only treatment with Ca^{2+} ionophore resulted in a change in GRK5 subcellular localisation as compared to its basal distribution. A23187 is a cell permeable mobile ion-carrier that forms stable complexes with divalent cations and therefore causes an increase in intracellular Ca^{2+} levels. A23187 treatment of cells stimulated nuclear export of GRK5 (compare Figure 3.3A, a & b and c & d). Both transfected and endogenous GRK5 display similar subcellular distributions and Ca^{2+} ionophore-dependent nuclear export. Quantification of nuclear export reveals that 93±1.9% of GRK5 transfected cells have nuclear GRK5 (Figure 3.3B). After treatment with Ca^{2+} ionophore only 10±3% of the transfected cells contain GRK5 in the nucleus (Figure 3.3B). Taken together these results indicate that both transfected and endogenous GRK5 is exported from the nucleus in a Ca^{2+}-dependent fashion. As expected, ionophore

treatment of HEp2 cells expressing GRK5ΔNLS or GRK5ΔNES (Figure 3.3C) does not alter the localisation of these mutants confirming that GRK5 does contain both a functional NLS and NES. Since the GRK5ΔNES mutant is still predominantly nuclear after a rise in intracellular Ca^{2+}, this suggests that Ca^{2+} is involved in promoting nuclear export of GRK5 via its NES. I will discuss this further in Chapter 5.

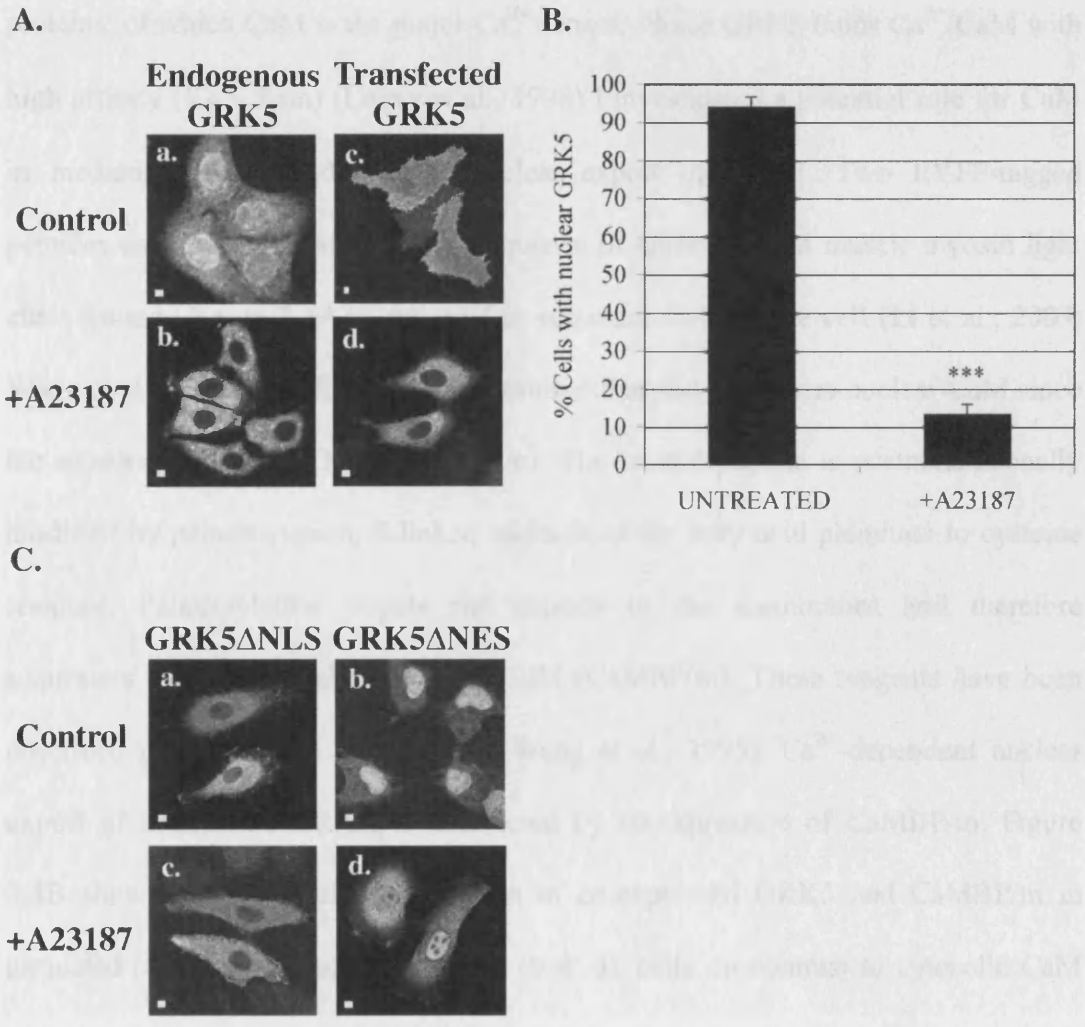


Figure 3.3. GRK5 exhibits calcium-dependent nuclear export.

A. Effect of calcium ionophore A23187 treatment on the cellular distribution of endogenous and transfected GRK5 in HEp2 cells. HEp2 cells were either untreated (a and c) or treated with the calcium

ionophore A23187 prior to fixation as described in the Materials and Methods. Scale bars, 10μM. B. Quantification of the percentage of cells with nuclear transfected GRK5 as shown in A with and without ionophore treatment. ***, $P<0.001$. C. GRK5ΔNLS and GRK5ΔNES cellular distribution following calcium ionophore A23187 treatment. Scale bars, 10μM. The images shown are representative of those obtained on at least three separate occasions.

3.4. The nuclear export of GRK5 is calmodulin-dependent

The cellular actions of Ca^{2+} are largely mediated through a family of Ca^{2+} binding proteins, of which CaM is the major Ca^{2+} sensor. Since GRK5 binds Ca^{2+}/CaM with high affinity (K_D ~ 8nm) (Levay et al., 1998) I investigated a potential role for CaM in mediating the Ca^{2+}-dependent nuclear export of GRK5. Two EYFP-tagged peptides encoding the CaM-binding sequence of rabbit skeletal muscle myosin light chain kinase (Figure 3.4A) were used to sequester CaM in the cell (Li et al., 2003; Wang et al., 1995). The first peptide is unmodified and sequesters nuclear CaM since the sequence contains a NLS (CaMBP/n). The second peptide is posttranslationally modified by palmitoylation, S-linked addition of the fatty acid palmitate to cysteine residues. Palmitoylation targets the peptide to the membranes and therefore sequesters cytosolic/membrane bound CaM (CaMBP/m). These reagents have been described previously (Li et al., 2003; Wang et al., 1995). Ca^{2+}-dependent nuclear export of transfected GRK5, is unaffected by co-expression of CaMBP/m. Figure 3.4B shows the subcellular distribution of co-expressed GRK5 and CaMBP/m in untreated (a & c), or ionophore treated (b & d), cells. In contrast to cytosolic CaM sequestration, expression of CaMBP/n, and thereby sequestration of nuclear CaM, inhibits ionophore-dependent nuclear export of GRK5 (Figure 3.4C, a & b show, respectively, the distribution of GRK5 in untreated and ionophore treated cells and c

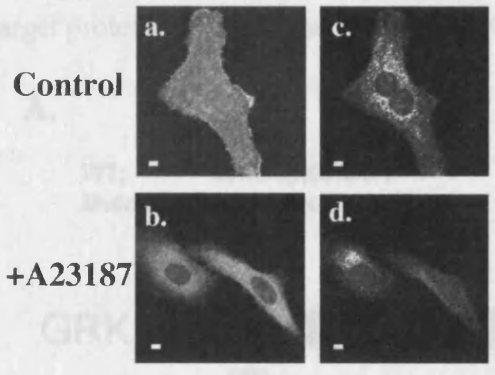
& d CaMBP/n expression in these same cells). These results indicate that Ca^{2+}/CaM mediates Ca^{2+} ionophore-dependent nuclear export of GRK5 and suggests that the interaction of nuclear Ca^{2+}/CaM with nuclear located GRK5 is required for this process.

A.

Protein Sequence of Ca^{2+}/CaM Sequestrants:

CaMBP/m: MLCCMRRTKQVEKNDEDQKI-EYFP-KRRWKKNFIAVSAANRFKK
CaMBP/n: EYFP-KRRWKKNFIAVSAANRFKK

B.



C.

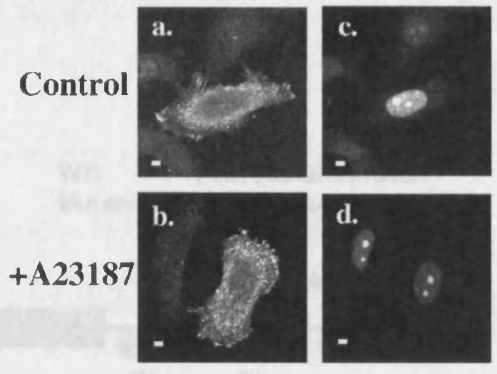


Figure 3.4. The nuclear export of GRK5 is regulated by nuclear Ca^{2+}/CaM.

A. Protein sequence of the Ca^{2+}/CaM sequestrants used in B. CaMBP/m; The N-terminal 20 amino acids of neuromodulin (which contains a signal for posttranslational palmitoylation of cysteines 3 and 4 that targets the fusion protein to membranes) is fused to yellow-green fluorescent variant of the enhanced green fluorescent protein (EYFP) and Rabbit skeletal myosin light chain kinase (MLCK) CaM binding sequence. CaMBP/n; EYFP fused to unmodified rabbit skeletal MLCK CaM binding peptide which contains a NLS. B. Sequestration of cytosolic CaM does not affect the Ca^{2+}-dependent nuclear export of GRK5. HEp2 cells were cotransfected with GRK5 and YFP-tagged CaMBP/m. Twenty-four hours post transfection, cells were either left untreated (a and c) or treated with A23187 (b and d), and expressed proteins were visualised as described in Materials and Methods. C. Sequestration of nuclear CaM inhibits Ca^{2+}-dependent nuclear export of GRK5. HEp2 cells cotransfected with GRK5 and YFP-tagged CaMBP/n were treated as described for panel B. Panels a and c show the distribution of, respectively, GRK5 and CaMBP/n in untreated cells, and panels b and d show the distribution of GRK5 and CaMBP/n in ionophore-treated cells. Scale bars, 10μM. The

images shown are representative of those obtained on at least three separate occasions.

3.5. Binding of Ca^{2+}/CaM to the amino-terminal calmodulin-binding site of GRK5 is required for nuclear export

Two CaM binding sites have been identified in GRK5 located between residues 20-39 in the N-terminus and residues 540-578 in the C-terminus of the kinase (Figure 3.5A, Levay et al., 1998). These regions of the kinase contain clusters of basic and hydrophobic residues characteristic of CaM-binding sites identified in other CaM target proteins (Rhoads and Friedberg, 1997).

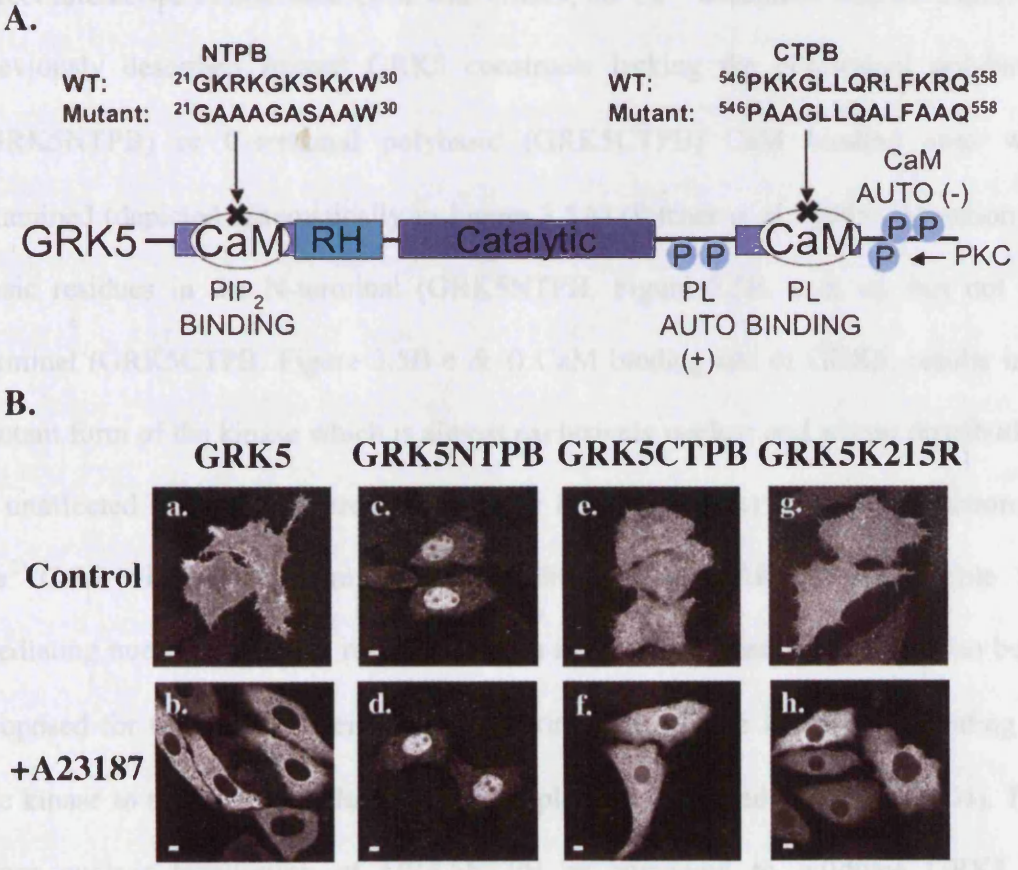


Figure 3.5. The nuclear export of GRK5 requires the interaction of Ca^{2+}/CaM with the N-

terminal CaM-binding site of the kinase.

A. Schematic showing the domain structure of GRK5. GRK5 contains two Ca^{2+}/CaM binding sites, one at its N-terminus (residues 20-39) and one at its C-terminus (residues 540-578). Mutants of these two Ca^{2+}/CaM binding sites GRK5NTPB (N-terminal polybasic) and GRK5CTPB (C-terminal polybasic) have been described previously (Pitcher et al., 1996) (Schematic adapted from Penela et al., 2003). CaM, Calmodulin binding domain; RH, Regulator of G-protein signalling (RGS) homology domain; PL, phospholipid; P, Phosphorylation site; Auto, autophosphorylation; PKC, Protein kinase C. B. The cellular distribution of (a) GRK5, (c) GRK5NTPB, (e) GRK5CTPB, and (g) GRK5K215R (kinase dead) expressed in HEp2 cells was visualised by fluorescence confocal microscopy as described in Materials and Methods. Where indicated, cells were treated with calcium ionophore (A23187) prior to fixation (b, d, f, and h). Scale bars, 10μM. The images shown are representative of those obtained on at least three separate occasions.

To determine if Ca^{2+}/CaM-mediated nuclear export of GRK5 is dependent on the direct interaction of activated CaM with GRK5, the Ca^{2+}-dependent nuclear export of previously described mutant GRK5 constructs lacking the N-terminal polybasic (GRK5NTPB) or C-terminal polybasic (GRK5CTPB) CaM binding sites was examined (depicted schematically in Figure 3.5A) (Pitcher et al., 1996). Mutation of basic residues in the N-terminal (GRK5NTPB, Figure 3.5B, c & d), but not C-terminal (GRK5CTPB, Figure 3.5B e & f) CaM binding site of GRK5, results in a mutant form of the kinase which is almost exclusively nuclear and whose distribution is unaffected by ionophore treatment. These findings suggest that the interaction of Ca^{2+}/CaM with the N-terminal CaM-binding site of GRK5 is responsible for mediating nuclear export. A role for CaM in mediating nuclear export has also been proposed for Ca^{2+}/CaM dependent protein kinase I-α where it promotes binding of the kinase to the CRM1 nuclear export complex *in vitro* (Stedman et al., 2004). The more nuclear localisation of GRK5NTPB as compared to wildtype GRK5 in untreated cells suggests, that under basal conditions, endogenous Ca^{2+}/CaM binding

to this site may contribute to the ubiquitous distribution of the wildtype kinase (compare Figure 3.5B a & c). However, it is also possible that the ubiquitous distribution of overexpressed GRK5 is determined by other molecules which also bind to the N-terminal CaM binding region of GRK5. These include PIP_2, actin, and the actin binding protein, α-actinin (Freeman et al., 1998; Freeman et al., 2000; Pitcher et al., 1996). Since all four ligands bind to the same region of GRK5 it is conceivable that not only CaM, but also PIP_2, actin and α-actinin might retain GRK5 in the cytoplasm. Since GRK5NTPB is mutated in this region, it cannot bind these molecules and therefore be anchored in the cytosol, restricting its localisation to the nucleus. To rule out the possibility that GRK5 nuclear export is kinase dependent or requires a specific 3D conformation, which is disrupted by mutations in the NTPB domain, the ability of GRK5, GRK5ΔNLS, GRK5NTPB, GRK5CTPB and GRK5K215R to phosphorylate light activated rhodopsin (Figure 3.6A) and tubulin (Figure 3.6B) was assessed using partially purified Cos-7 cell lysates expressing these kinases. As shown in Figure 3.6A & B, GRK5NTPB, although approximately 50% less active than wildtype GRK5, has a similar activity against both substrates as GRK5CTPB, a mutant form of the kinase which exhibits ionophore-dependent nuclear export. These results suggest that impaired CaM-binding, rather than impaired kinase activity, most likely explains the inability of GRK5NTPB to exit the nucleus in a Ca^{2+}-dependent fashion (Figure 3.5B, c & d). Indeed, the catalytically inactive mutant of GRK5 (GRK5K215R) (Figure 3.6A & B) is exported from the nucleus following ionophore treatment (Figure 3.5B, g & h), demonstrating that Ca^{2+}/CaM-dependent nuclear export of GRK5 does not require kinase activity.

Furthermore, the observation that GRK5ΔNLS has comparable kinase activity to wildtype GRK5 (Figure 3.6 A & B) suggests that the nuclear exclusion of this mutant enzyme (Figure 3.2C, c) is not simply a consequence of mutation-induced gross structural abnormalities in the NLS.

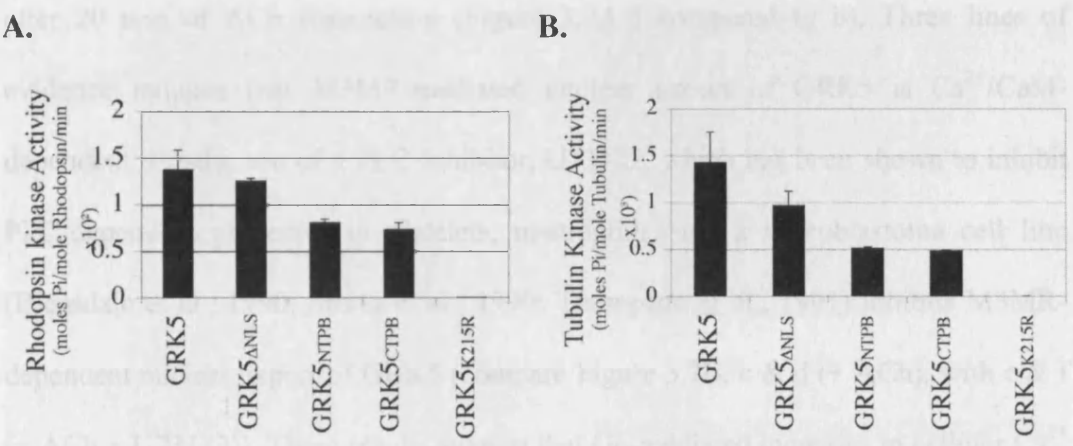


Figure 3.6. Rhodopsin and tubulin kinase activities of wild-type and mutant GRK5s.

Partially purified Cos-7 cell lysates expressing GRK5, GRK5ΔNLS, GRK5NTPB, GRK5CTPB, and GRK5K215R were assayed for their ability to phosphorylate (A) the GPCR substrate rhodopsin and (B) the soluble substrate tubulin as described in Materials and Methods. The results shown for each enzyme represent the average activities ± standard errors of the means for three separate transfections and partial purifications that were subsequently pooled and assayed in triplicate. Assays were performed using equivalent amounts of GRK5 protein as determined by quantitation of Western blots, using a densitometer.

3.6. Activation of a Gq-coupled receptor promotes the nuclear export of GRK5

Given that Ca^{2+}/CaM regulates GRK5 sub-cellular localisation, I hypothesised that GPCRs that increase intracellular levels of Ca^{2+} may promote the nuclear export of GRK5. To this end, the distribution of GRK5 was examined in CHO cells stably expressing the Gq-coupled M3MR (CHO-M3MR) and transiently expressing GRK5

(Figure 3.7). Agonist stimulation of M3MR with ACh (100μM) promotes the rapid and reversible nuclear export of GRK5 (Figure 3.7A, left hand panels show M3MR and right hand panels GRK5 staining). Within 0.5 min of ACh treatment, GRK5 is exported from the nucleus (Figure 3.7A d compared to b) but re-enters the nucleus after 20 min of ACh stimulation (Figure 3.7A j compared to b). Three lines of evidence indicate that M3MR-mediated nuclear export of GRK5 is Ca^{2+}/CaM-dependent. Firstly, use of a PLC inhibitor, U73122, which has been shown to inhibit PLC-dependent processes in platelets, neutrophils, and a neuroblastoma cell line (Bleasdale et al., 1990; Smith et al., 1990; Thompson et al., 1991) inhibits M3MR-dependent nuclear export of GRK5 (compare Figure 3.7B, c & d (+ ACh), with e & f (+ ACh + U73122)). These results suggest that Gq-mediated increases in cellular Ca^{2+} levels are important for this process. Secondly, co-expression of GRK5 and the nuclear CaM sequestrant (CaMBP/n) in this M3MR expressing cell line prevents agonist-dependent GRK5 redistribution (Figure 3.7B g, h & i). Thirdly, GRK5NTPB, a mutant form of the kinase lacking the N-terminal CaM-binding site, fails to exit the nucleus following M3MR activation (Figure 3.7B, j & k). Together these results indicate that activation of a Gq-coupled GPCR can induce the cytoplasmic redistribution of GRK5 via a mechanism that requires the interaction of Ca^{2+}/CaM with the N-terminal CaM-binding site of GRK5.

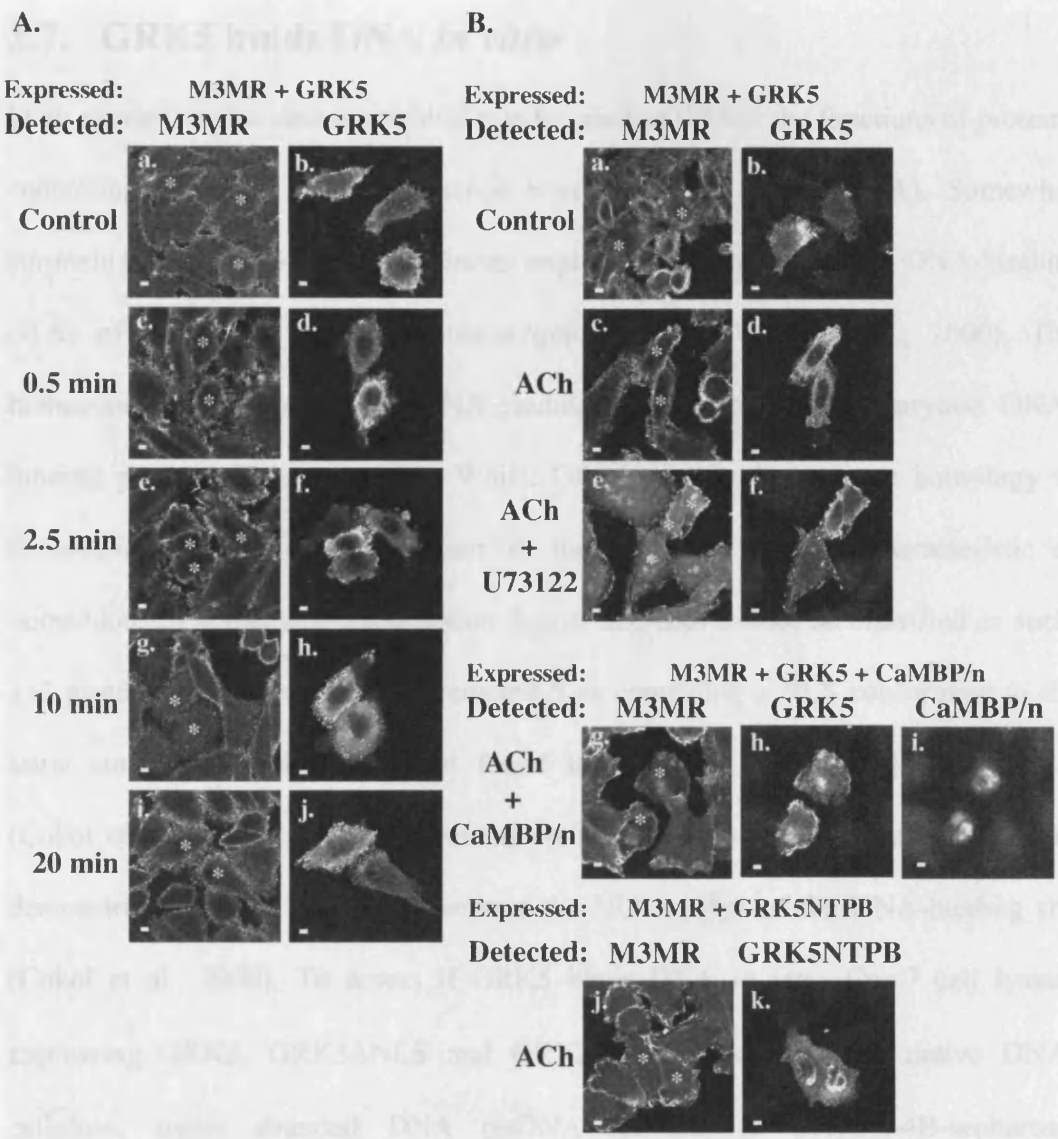


Figure 3.7. Muscarinic-M3 receptor activation promotes the nuclear export of GRK5.

A. CHO-M3MR cells were transfected with GRK5 and treated with ACh for the indicated times (min). M3MR and GRK5 in coexpressing cells were visualised by immunofluorescence. Asterisks indicate cotransfected cells. Scale bars, 10 μM. B. CHO-M3MR cells were transfected with GRK5 (a to f), GRK5, and CaMBP/n (g, h, and i) or GRK5NTPB (j and k). Transfected cells were subsequently left untreated (Control) or treated with ACh for 5 min, and GRK5 and M3MR were detected as previously described. Where indicated (U73122), cells were pretreated with 1 μM U73122 for 1 h prior to agonist treatment. Asterisks indicate cotransfected cells. Scale bars, 10μm.

3.7. GRK5 binds DNA *in vitro*

In an attempt to elucidate a potential role for nuclear GRK5, the functions of proteins containing NLSs of similar sequence were examined (Figure 3.8A). Somewhat surprisingly, the NLS of GRK5 shares sequence homology with the DNA-binding NLSs of homeobox containing transcription factors (Cokol et al., 2000). The homeodomain is a conserved DNA-binding domain found in eukaryotic DNA-binding proteins (Billeter, 1996). Whilst GRK5's NLS shows some homology to homeodomains, it does not contain all the conserved residues characteristic of homeodomain-containing transcription factors and thus cannot be classified as such. 132 proteins were identified by PredictNLS as containing a NLS conforming to the same consensus sequence as that found in GRK5 (R[R/K]x[K/R]x[R/K]$_2$[D/E]) (Cokol et al., 2000). All these proteins contained a homeodomain and of the 119 demonstrated to bind DNA, 116 contain the NLS as part of the DNA-binding site (Cokol et al., 2000). To assess if GRK5 binds DNA *in vitro* Cos-7 cell lysates expressing GRK5, GRK5ΔNLS and GRK2 were incubated with native DNA-cellulose, single stranded DNA (ssDNA)-cellulose or poly(A)-4B-sepharose. Following extensive washing, the amount of GRK retained on the resin was determined by western blot analysis as shown in Figure 3.8B. The blot was scanned on a densitometer and the amount of GRK bound (B1-3, Figure 3.8B) relative to the load (L, Figure 3.8B) was determined.

A.

*** Predicted GRK5 consensus NLS**		R[RK]X[KR]X[RK{2,}?[DE]
NLS-containing proteins conforming to consensus		132
No. proteins containing NLS consensus which bind DNA		119
No. protein DNA binding which bind via NLS		116
Function of consensus containing proteins	**Homeobox-containing transcription factors**	119
	Transcriptional co/activators & co/repressors	12

*** Format for NLS motifs:**		
[KR]	read	K or R
G{3}	read	GGG
x{3,5}	read	between 3 and 5 x
where 'x' stands for 'any amino acid'		

B.

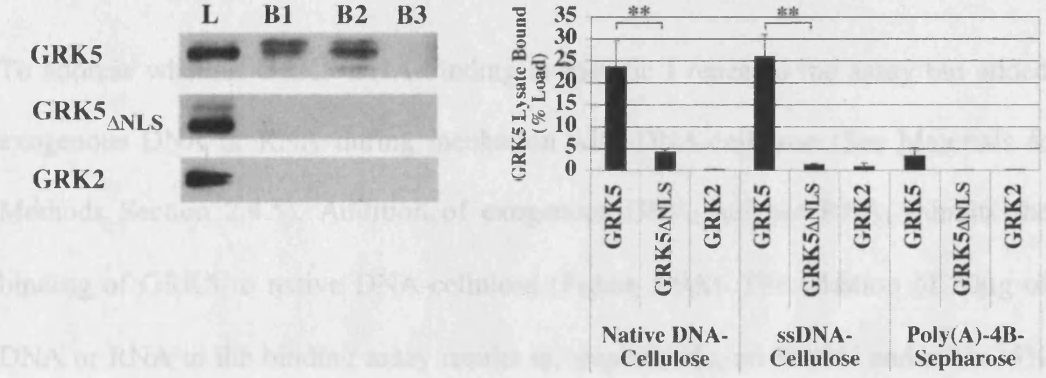


Figure 3.8. The NLS of GRK5 binds DNA *in vitro*.

A. Table detailing the putative NLS in GRK5. Data on proteins containing this same consensus NLS are given. B. GRK5, but not GRK5ΔNLS or GRK2, binds DNA *in vitro*. Lysates of cells expressing GRK5, GRK5ΔNLS, or GRK2 were incubated with native DNA-cellulose, ssDNA-cellulose, or poly(A)-4B-Sepharose, and after extensive washing the amount of bound GRK was determined by Western blot analysis. A representative Western blot of one such experiment is shown. L, 25% of total lysate loaded; B1, native DNA-cellulose-bound GRK; B2, ssDNA cellulose-bound GRK; B3, poly(A)-4B-Sepharose-bound GRK. Quantification of Western blot analyses from multiple experiments is represented graphically. The *y-axis* represents the percentage of loaded GRK5 that remained bound to the resin. The data shown represent the mean values ± standard errors of the mean for at least three experiments. **, $P<0.01$.

The blot reveals that GRK5, but not GRK5ΔNLS or GRK2, binds native and ssDNA-cellulose *in vitro* (Figure 3.8B). Quantification of a minimum of three separate experiments indicates that approximately 25% of the applied GRK5 was retained on both native and single stranded DNA cellulose (Figure 3.8B). In marked contrast, very little GRK5ΔNLS and GRK2 bound these resins (~4% of the load, Figure 3.8B). No significant binding to poly(A)-4B-sepharose was detected for any of the kinases (Figure 3.8B) suggesting that a non-specific ionic interaction is unlikely to account for the binding of GRK5 to DNA.

To address whether GRK5 DNA-binding is specific I repeated the assay but added exogenous DNA or RNA during incubation with DNA-cellulose (See Materials & Methods Section 2.4.5). Addition of exogenous DNA, but not RNA, inhibits the binding of GRK5 to native DNA-cellulose (Figure 3.9A). The addition of 20μg of DNA or RNA to the binding assay results in, respectively, an 80±8% and a 22±15% reduction in binding of GRK5 to immobilised DNA ($p<0.01$). No detectable binding of GRK5 to the cellulose support was observed under these conditions (Figure 3.9A), suggesting that the interaction of GRK5 with DNA is specific. Together, the results shown in Figure 3.8B and 3.9A indicate that GRK5 binds specifically to DNA *in vitro* and that the NLS of GRK5 is required for this interaction. Since cell lysates expressing GRK5 were used for these experiments, however, I could not exclude the possibility that the binding of GRK5 to DNA is indirect.

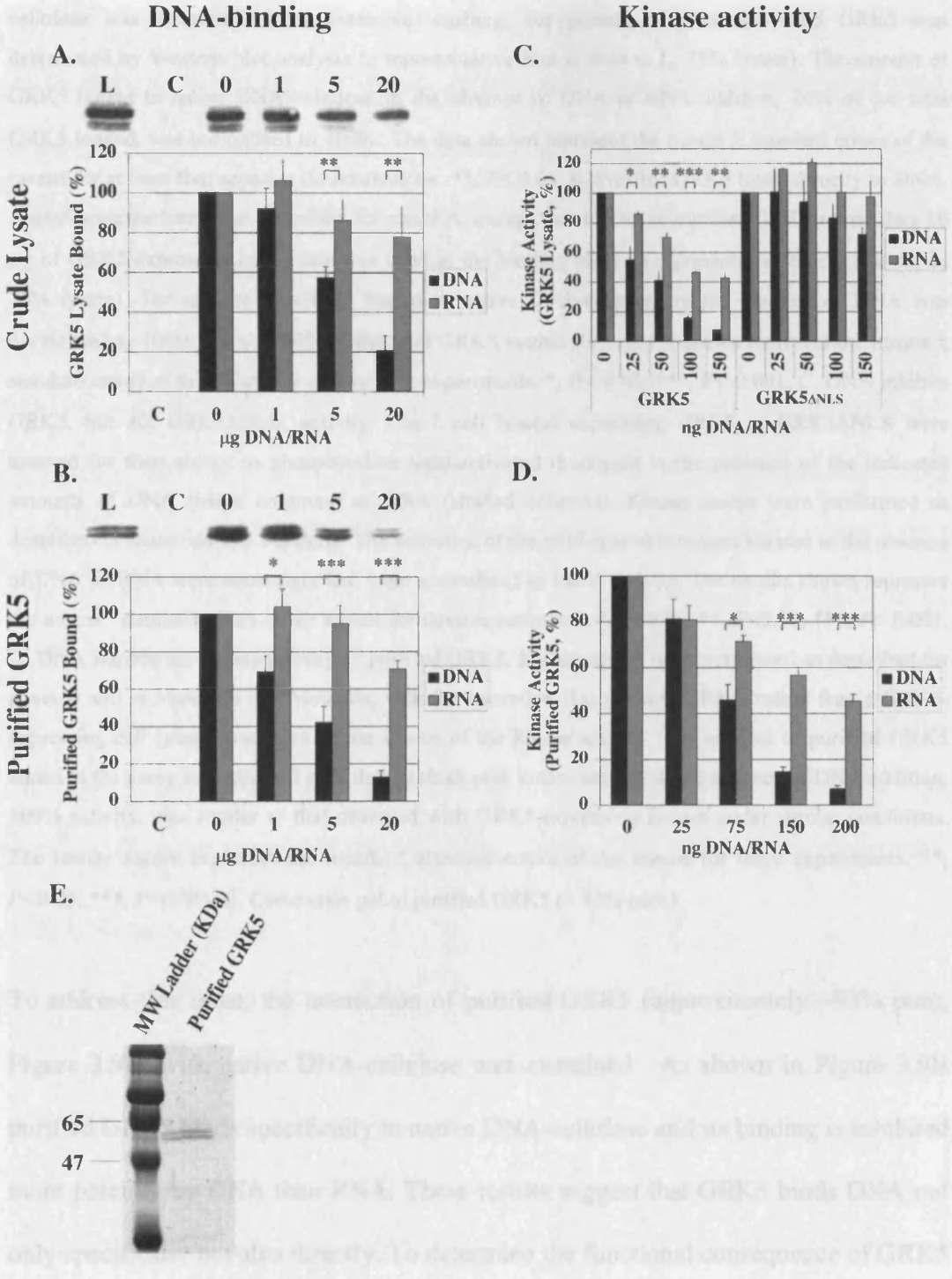


Figure 3.9. GRK5 DNA binding is specific and direct.

A. DNA, but not RNA, inhibits the interaction of GRK5 with native DNA-cellulose. Lysates (10µg) of Cos-7 cells overexpressing GRK5 were incubated with native DNA-cellulose, following a 30-min preincubation with the indicated amounts of DNA or RNA. Additionally, the interaction of GRK5 with

cellulose was examined. After extensive washing, the amount of resin-associated GRK5 was determined by Western blot analysis (a representative blot is shown; L, 25% lysate). The amount of GRK5 bound to native DNA-cellulose in the absence of DNA or RNA addition, 24% of the total GRK5 loaded, was normalised to 100%. The data shown represent the means ± standard errors of the means for at least four separate determinations. **, $P< 0.01$. B. Purified GRK5 binds directly to DNA. Assays were performed as described for panel A, except that 150 ng of purified GRK5 rather than 10 μg of GRK5-expressing cell lysate was used in the binding assay (a representative blot is shown; L, 25% lysate). The amount of GRK5 bound to native DNA-cellulose in the absence of DNA was normalised to 100% and was 40% of the total GRK5 loaded. The results shown represent the means ± standard errors of the means for at least five experiments. *, $P< 0.05$; ***, $P< 0.001$. C. DNA inhibits GRK5, but not GRK5ΔNLS, activity. Cos-7 cell lysates expressing GRK5 or GRK5ΔNLS were assayed for their ability to phosphorylate light-activated rhodopsin in the presence of the indicated amounts of DNA (black columns) or RNA (shaded columns). Kinase assays were performed as described in Materials and Methods. The activities of the wild-type and mutant kinases in the absence of DNA or RNA were equivalent and were normalised to 100% activity. The results shown represent the means standard errors of the means for three experiments. *, $P<0.05$; **, $P<0.01$; ***, $P< 0.001$. D. DNA inhibits the kinase activity of purified GRK5. Kinase assays were performed as described for panel B and in Materials and Methods, with the exception that purified GRK5, rather than a GRK5-expressing cell lysate, was used as the source of the kinase activity. The amount of purified GRK5 added to the assay was adjusted such that its rhodopsin kinase activity in the absence of DNA addition, 100% activity, was similar to that observed with GRK5-expressing lysates under similar conditions. The results shown represent the means ± standard errors of the means for three experiments. **, $P<0.05$; ***, $P<0.001$. E. Coomassie gel of purified GRK5 (~ 93% pure).

To address this issue, the interaction of purified GRK5 (approximately ~93% pure, Figure 3.9E) with native DNA-cellulose was examined. As shown in Figure 3.9B purified GRK5 binds specifically to native DNA-cellulose and its binding is inhibited more potently by DNA than RNA. These results suggest that GRK5 binds DNA not only specifically but also directly. To determine the functional consequence of GRK5 DNA-binding Cos-7 cell lysates expressing GRK5 or GRK5ΔNLS were assessed for their ability to phosphorylate light activated rhodopsin in the presence of increasing concentrations of DNA or RNA. As shown in Figure 3.9C, when assayed at

equivalent rhodopsin kinase activities, GRK5 is more potently inhibited by DNA than its mutant counterpart (Figure 3.9C, black columns). The addition of 150ng of DNA inhibits GRK5 activity by 93±0.2% and GRK5ΔNLS activity by 28±5% ($p<0.001$), results which suggest that DNA-binding is mediated by the NLS of GRK5. RNA is a much less effective inhibitor of GRK5 activity than DNA, 150ng of RNA inhibits GRK5 activity by 58±4% (Figure 3.9C, left hand side, shaded columns). GRK5ΔNLS activity is unaffected by RNA addition over the range of concentrations used (Figure 3.9C, right hand side, shaded columns). Consistent with the results obtained using lysates expressing GRK5, DNA potently inhibits the kinase activity of purified GRK5 (Figure 3.9D, black columns). RNA proved a much less effective inhibitor of the purified enzyme, the addition of 200ng of DNA or RNA resulting in, respectively, a 93±1% and a 55±3% inhibition of kinase activity ($p<0.001$) (Figure 3.9D, shaded columns). Overall these results demonstrate that GRK5 binds directly to DNA *in vitro* via its NLS and provocatively suggest hitherto unsuspected functions for this enzyme.

3.8. Summary

In summary, the data in this Chapter provide evidence that both overexpressed GRK5 in a range of tissue culture cells lines and endogenous GRK5 in HEp2 cells is located in the nucleus. GRK5 nuclear localisation appears to be regulated by the presence of both a functional NLS and NES. Mutation of these motifs results in kinases that are exclusively cytosolic or nuclear respectively.

GRK5 nuclear localisation is regulated by nuclear Ca^{2+}/CaM binding to the N-terminus of the kinase. Application of Ca^{2+} ionophore does not induce nuclear export of a mutant form of GRK5 that cannot bind to CaM at its N-terminus. Furthermore,

Ca^{2+} ionophore treatment does not alter the cellular distribution of the NLS and NES mutants of GRK5 confirming the functionality of these motifs. The M3MR, activation of which increases intracellular Ca^{2+} levels via Gq, also promotes Ca^{2+}-dependent nuclear export of GRK5. This can be inhibited by inhibiting PLC activation, downstream of Gq, or by sequestering nuclear CaM.

The literature suggests that the M3MR is not a substrate for GRK5-mediated phosphorylation in cells (Tsuga et al., 1998; Willets et al., 2002; Willets et al., 2003b). Consistent with this, I did not observe agonist-induced M3MR internalisation when co-expressed with GRK5 in CHO cells (Section 3.6 and Figure 3.7A left hand panels). Despite this, activation of the M3MR induces GRK5 nuclear export, presumably negatively regulating its nuclear function rather than activating its GPCR kinase function. This might suggest that GRK5 not only functions as a GPCR kinase but also has novel nuclear functions.

Sequence homology of the NLS of GRK5 suggests that this particular motif might bind DNA. Evidence supporting this is that GRK5 binds DNA *in vitro* via its NLS motif. GRK5 DNA-binding appears to be direct and specific since DNA-binding of both purified GRK5 and GRK5 in cell lysates is inhibited more potently by addition of DNA than RNA. GRK5 DNA-binding via its NLS appears to inhibit kinase activity suggesting again that GRK5 DNA-binding is direct and specific, and that GRK5 might well be catalytically inactive when bound to DNA in the nucleus. This data tentatively points towards a nuclear role for GRK5, perhaps in the regulation of transcription.

The implications of the results presented in this and the following Chapters will be

discussed in more detail in Chapter 6.

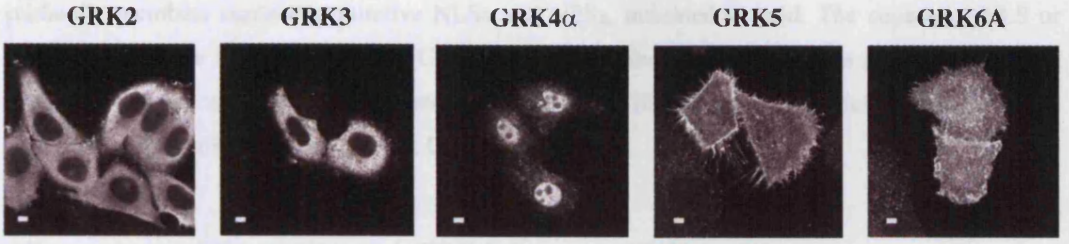
4. The presence of nuclear localisation and nuclear export sequences is a common feature of the GRK4 subfamily members

In the last Chapter I described the discovery of nuclear localisation and export sequences in GRK5, in this Chapter I investigate whether possession of an NLS and NES is specific to GRK5 or if it is a common feature of the GRK4 subfamily of GRKs. The ability of GRK4α, β, γ and δ; GRK5 and GRK6A, B and C to bind DNA *in vitro* and the regulatory mechanisms controlling their nuclear localisation are also investigated.

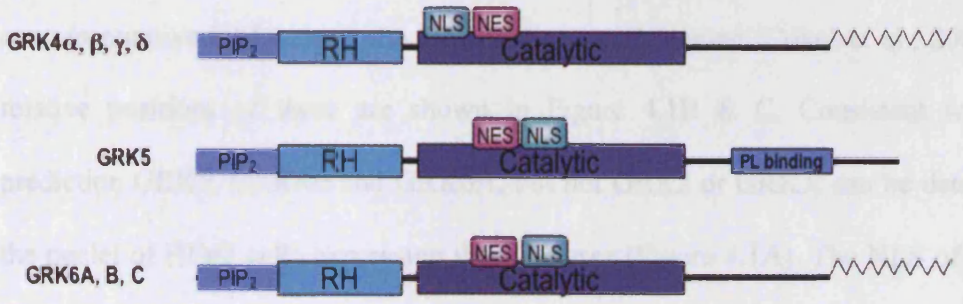
4.1. GRKs of the GRK1 and GRK4 subfamilies contain putative NLSs

As described in Chapter 3, GRK5 can be detected in the nucleus of HEp2 cells when assessed by indirect immunofluorescence (Figure 4.1A). GRK5 contains a NLS, located between amino acids 388 and 395 (^{388}RKEKVKRE^{395}) and a leucine rich NES, located between amino acids 259 and 265 (^{259}LCLVLTI^{265}), which regulate the nuclear localisation of this kinase (Johnson et al., 2004). There are three GRK subfamilies (Figure 1.1). The GRK1 subfamily contains GRK1 and GRK7. The GRK2 subfamily contains GRKs 2 and GRK3. The third subfamily and the focus of this Chapter, is the GRK4 subfamily, which contains three kinases, GRK4, GRK5 and GRK6. Four splice variants of human GRK4 (α, β, γ, δ) and three of GRK6 (A, B and C) have been identified (Figure 4.1B).

A.



B.



C.

Putative NLS of the GRKs

GRK1:	^{216}YACKKLNK**KRLKKRK**GYQGA^{235}
NLS Consensus	KXLKKRK
GRK7:	^{217}YAC**KKLDKKRLKKK**GGEKMA^{236}
NLS Consensus	KKLXKKXXKKK
GRK4α/γ:	^{213}YACKKL**QKKRIKKRK**GEAMA^{232}
NLS Consensus	QKXXXKKRK
GRK4β/δ:	^{181}YACKKL**QKKRIKKRK**GEAMA^{200}
NLS Consensus	QKXXXKKRK
GRK5:	^{381}GQSPFRG**RKEKVKRE**EVDRR^{400}
NLS Consensus	RKXKXKRE
GRK6:	^{381}GQSPFQ**QRKKKIKRE**EVERL^{400}
NLS Consensus	QRKXKXKRE

Putative NES of the GRK4 subfamily

GRK5:	^{254}ETKDA**LCLVLTI**MNGGD^{270}
GRK4α:	^{255}ETKDA**LCLVLTI**MNGGD^{271}
GRK6A:	^{254}ETKDA**LCLVLTI**MNGGD^{270}

Figure 4.1. Members of the GRK1 and -4, but not GRK2, subfamilies contain putative NLSs.

A. The subcellular localisation of GRK family members when overexpressed in HEp2 cells as determined by fluorescence confocal microscopy. Scale bars, 10μM. B. Schematic representation of the GRK4 subfamily members and their splice variants. Relative localisation of putative NLSs and

NESs are shown (Adapted from Penela et al., 2003). C. Regions of the catalytic domains of GRK subfamily members containing putative NLSs and NESs, indicated in bold. The consensus NLS or NES sequences are shown below each GRK sequence. Amino acid numbering is indicated. Note the NLS of GRKs 4, 1 and 7 are more N-terminal than that of GRKs 5 and 6. The putative NES is located in the same region of all members of the GRK4 subfamily.

All members of the GRK1 and GRK4, but not GRK2, subfamilies are predicted to contain putative NLSs using the NLSPredict search engine (Cokol et al., 2000), the relative positions of these are shown in Figure 4.1B & C. Consistent with this prediction GRK5, GRK4α and GRK6A, but not GRK2 or GRK3, can be detected in the nuclei of HEp2 cells expressing these kinases (Figure 4.1A). The NLS of GRK5, located towards the C-terminus of the catalytic domain of the enzyme, is essentially identical in position (Figure 4.1B) and sequence (Figure 1.4C) to the putative NLS of GRK6, suggesting that GRK6 may also contain a functional DNA-binding NLS. In contrast, the predicted NLS of GRK4 is distinct from that of GRKs 5 and 6, and is located within the N-terminus of the catalytic domain (Figure 4.1B & C). The NLS of GRK4 is homologous to the predicted NLS of the GRK1 subfamily rather than the NLS of GRKs 5 and 6 (Figure 4.1B). The putative NLS of GRK4, like that of GRK1 and 7, is not predicted to bind DNA (Cokol et al., 2000). Human GRK4α shows 64% and 65% homology to human GRKs 5 and 6A respectively. Interestingly, human GRK4α shows 45% and 44% homology to human GRKs 1 and 7 respectively. Whilst GRK4α clearly has more sequence homology to GRKs 5 and 6 it is not completely dissimilar from the GRK1 subfamily in terms of homology and features such as the sequence of its NLS. This might suggest a rationale for GRK4, the most divergent member of the GRK4 subfamily, being grouped with the GRK1 subfamily. The fact

that GRK2 and 3 are excluded from the nucleus, and do not contain a NLS, suggests divergent functions for the GRK2 and GRK1/4 subfamilies.

Furthermore, the NES of GRK5 is conserved in all the GRK4 subfamily members, GRKs 4, 5 and 6 (Figure 4.1B & C) suggesting that the possession of a NES is a common feature of this subfamily of GRKs. NetNES 1.1 predicts that GRK7 contains a Leucine at residue 332 in its sequence that may form part of a NES (la Cour et al., 2003). The corresponding sequence in GRK1 is dissimilar from GRK7 and NetNES 1.1 does not predict that this sequence would contain a NES.

4.2. Nuclear localisation is a common feature of the GRK4 subfamily of GRKs.

To determine if the putative NLS of GRKs 4 and 6 is functional, I mutated basic amino acids present in these motifs to alanine residues (Figure 4.2A). This resulted in the nuclear exclusion of all the splice variants of GRK4 and GRK6 when transfected into HEp2 cells and visualised by immunofluorescence (Figure 4.2B, compare wildtype to ΔNLS mutants). These data confirm that all members of the GRK4 subfamily contain functional NLSs. Confirmation that the putative NES of GRKs 4 and 6 is functional remains to be established.

A.

GRK4α/γ:	^{219}QKKRIKKRK^{227}
ΔNLS Mutant	^{219}QK**A**RI**AA**RK^{227}
GRK4β/δ:	^{187}QKKRIKKRK^{195}
ΔNLS Mutant	^{187}QK**A**RI**AA**RK^{195}
GRK5:	^{388}RKEKVKRE^{395}
ΔNLS Mutant	^{388}**AA**E**A**V**AA**E^{395}
GRK6A, B & C:	^{387}QRKKKIKRE^{395}
ΔNLS Mutant	^{387}QR**AAA**IKRE^{395}

B.

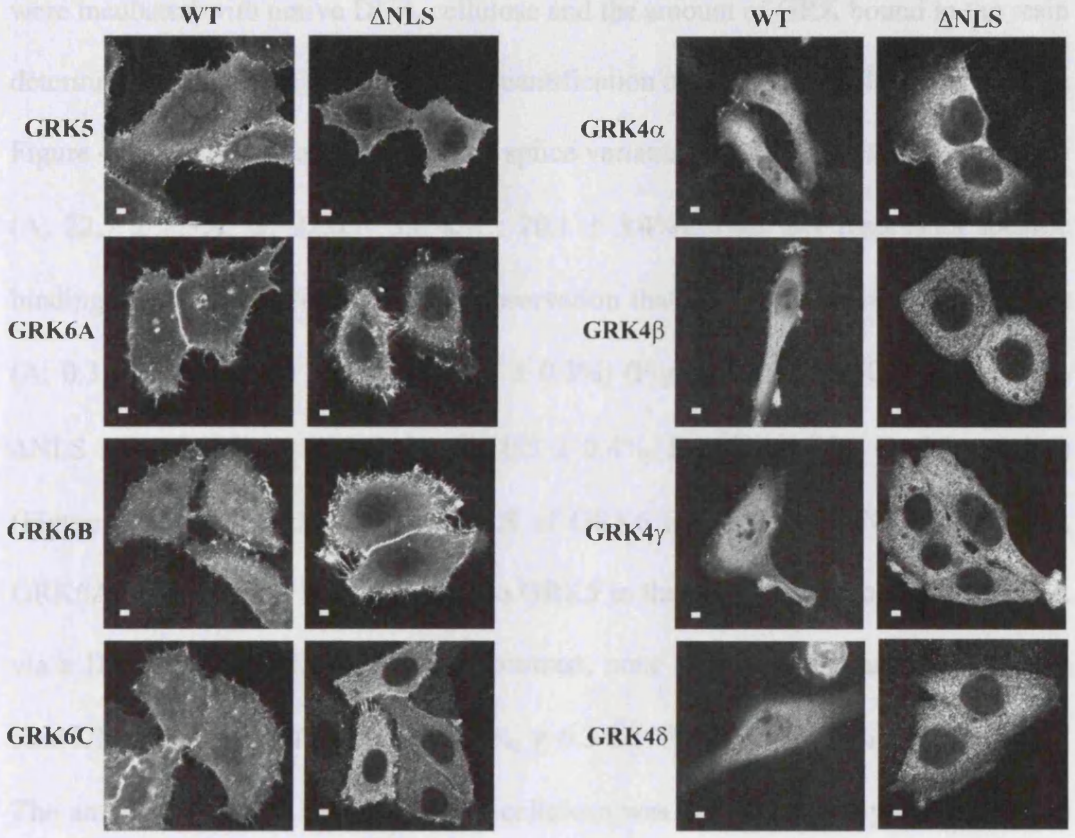


Figure 4.2. The GRK4 subfamily contain functional NLSs.

A. Regions of the catalytic domains of GRKs 4, 5 and 6 containing putative NLSs as predicted by PredictNLS (Cokol et al., 2000). The position of amino acids mutated to Alanine in GRKΔNLS mutants (indicted in bold) is shown. B. The putative NLSs of the GRK4 subfamily members are functional. GRK5, GRK6A, B and C, and GRK4α, β, γ and δ, together with their respective ΔNLS mutants were expressed in HEp2 cells and visualised by indirect immunofluorescence as described in

Materials and Methods. Scale bars 10μM. The images shown are representative of those obtained on at least three separate occasions.

4.3. GRK5 and GRK6, but not, GRK4 bind DNA *in vitro*

GRK5 binds DNA *in vitro* and DNA-binding is mediated by its NLS (Chapter 3 and Johnson et al., 2004). To determine if the other GRK4 subfamily members contain DNA-binding NLSs, Cos-7 cell lysates expressing wildtype or ΔNLS mutant kinases, were incubated with native DNA-cellulose and the amount of GRK bound to the resin determined by Western blot analysis. Quantification of the Western blots, as shown in Figure 4.3A, reveals that all the GRK6 splice variants bind to DNA-cellulose *in vitro* (A; 22.7 ± 3.5%, B; 22.6 ± 3.8%, C; 20.1 ± 3.4%). That this represents specific binding to DNA is indicated by the observation that GRK6 does not bind cellulose (A; 0.3 ± 0.1%, B; 1.1 ± 0.6%, C; 0.2 ± 0.2%) (Figure 4.3A). GRK6A-, B- and C-ΔNLS mutants fail to bind DNA (A; 0.5 ± 0.4%, B; 3.2 ± 1.8%, C; 0.3 ± 0.1%) (Figure 4.3A), suggesting that the NLS of GRK6 is part of its DNA-binding site. GRK6A, B and C thus appear similar to GRK5 in that they bind specifically to DNA via a DNA-binding NLS. In marked contrast, none of the splice variants of GRK4 bind DNA (α; 5.6 ± 1.4%, β; 2.7 ± 0.4%, γ; 6.3 ± 1.7%, δ; 1.7 ± 1.5%) (Figure 4.3A). The amount of GRK4 bound to DNA-cellulose was not significantly different from that bound by the cellulose support. The observation that GRKs 5 and 6, but not 4, bind DNA *in vitro* suggests distinct nuclear functions for these enzymes.

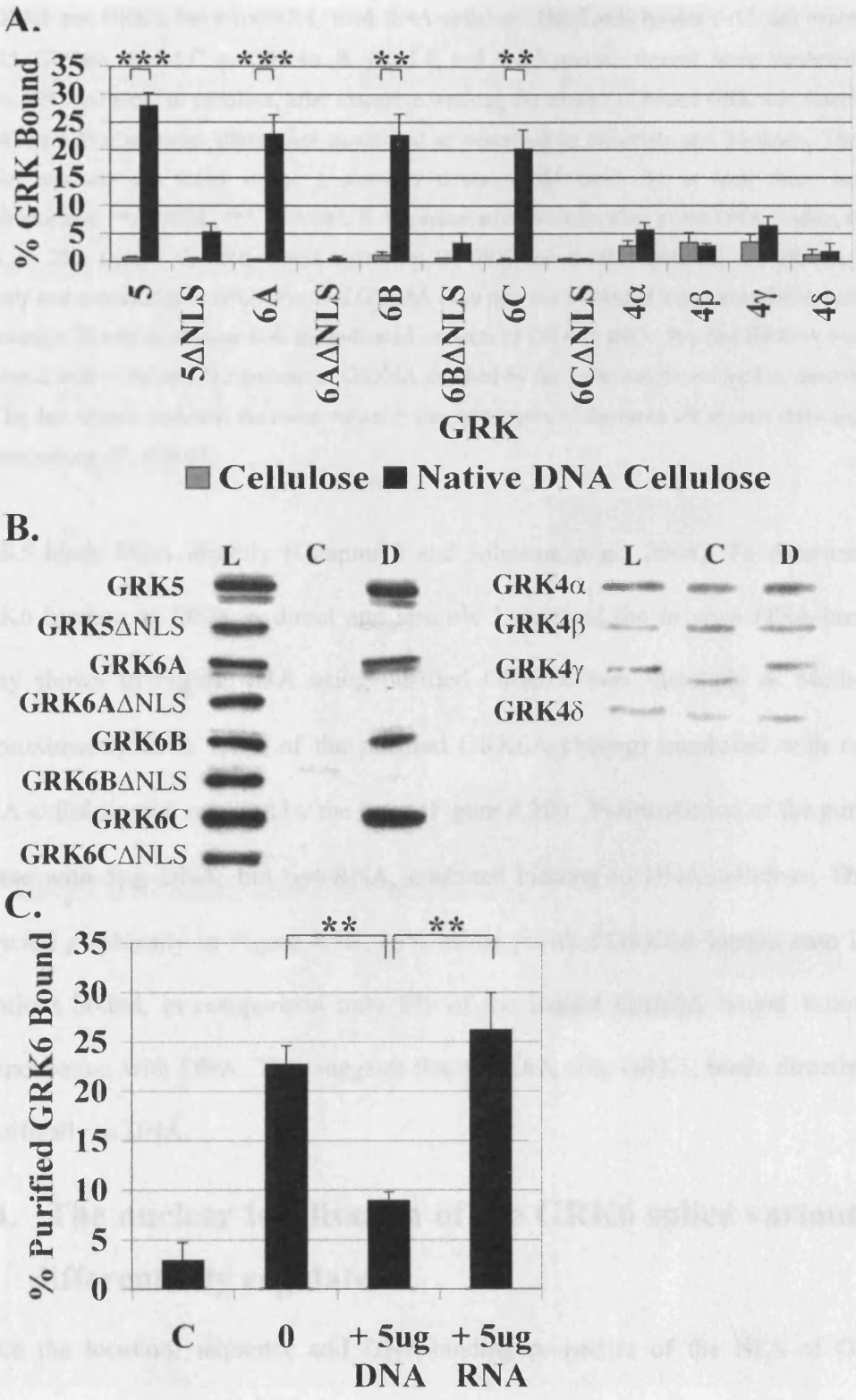


Figure 4.3. GRKs 5 and 6 bind DNA *in vitro*.

A. GRK5 and GRK6, but not GRK4, bind DNA cellulose. Cos-7 cell lysates (~15 μg) expressing GRK5, GRK6A, B and C, or GRK4α, β, γ and δ, and ΔNLS mutants thereof, were incubated with native DNA-cellulose or cellulose, after extensive washing the amount of bound GRK was determined by Western blot analysis. Blots were quantified as described in Materials and Methods. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. **, P<0.01; ***, P<0.001. B. Representative Western blots of the DNA binding shown in A. L, 25% Lysate; C, GRK bound Cellulose; D, GRK bound DNA cellulose. C. GRK6A binds directly and specifically to DNA. Purified GRK6A (150 ng) was incubated with native DNA-cellulose following a 30 min incubation with the indicated amounts of DNA or RNA. Purified GRK6A was also incubated with cellulose. The amount of GRK6A retained by the resin was determined as described in A. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. **, P<0.01.

GRK5 binds DNA directly (Chapter 3 and Johnson et al., 2004). To determine if GRK6 binding to DNA is direct and specific I repeated the *in vitro* DNA-binding assay shown in Figure 4.3A using purified GRK6A (see Materials & Methods). Approximately 23 ± 1.7% of the purified GRK6A (150ng) incubated with native DNA-cellulose was retained by the resin (Figure 4.3B). Preincubation of the purified kinase with 5μg DNA, but not RNA, inhibited binding to DNA-cellulose. This is depicted graphically in Figure 4.3B, 23% of the purified GRK6A loaded onto DNA cellulose bound, in comparison only 9% of the loaded GRK6A bound following preincubation with DNA. This suggests that GRK6A, like GRK5, binds directly and specifically to DNA.

4.4. The nuclear localisation of the GRK6 splice variants is differentially regulated

Since the location, sequence and DNA-binding properties of the NLS of GRK6 closely resembles that of GRK5 I investigated if the nuclear localisation of these

kinases was similarly regulated. Nuclear export of GRK5 requires CaM binding to the N-terminal CaM-binding domain of the kinase (between residues 20-39) (Johnson et al., 2004). The N-terminal CaM binding site is conserved in GRK6A, B and C but has an approximately 3-fold lower affinity for CaM than GRK5 (Pronin et al., 1997). To establish if GRK6 nuclear export is regulated by Ca^{2+}, HEp2 cells transiently transfected with GRK5, 6A, 6B or 6C were treated with Ca^{2+} ionophore, A23187 (25μM, 15min), and the subcellular distribution of the kinases assessed by indirect immunofluorescence (Figure 4.4A).

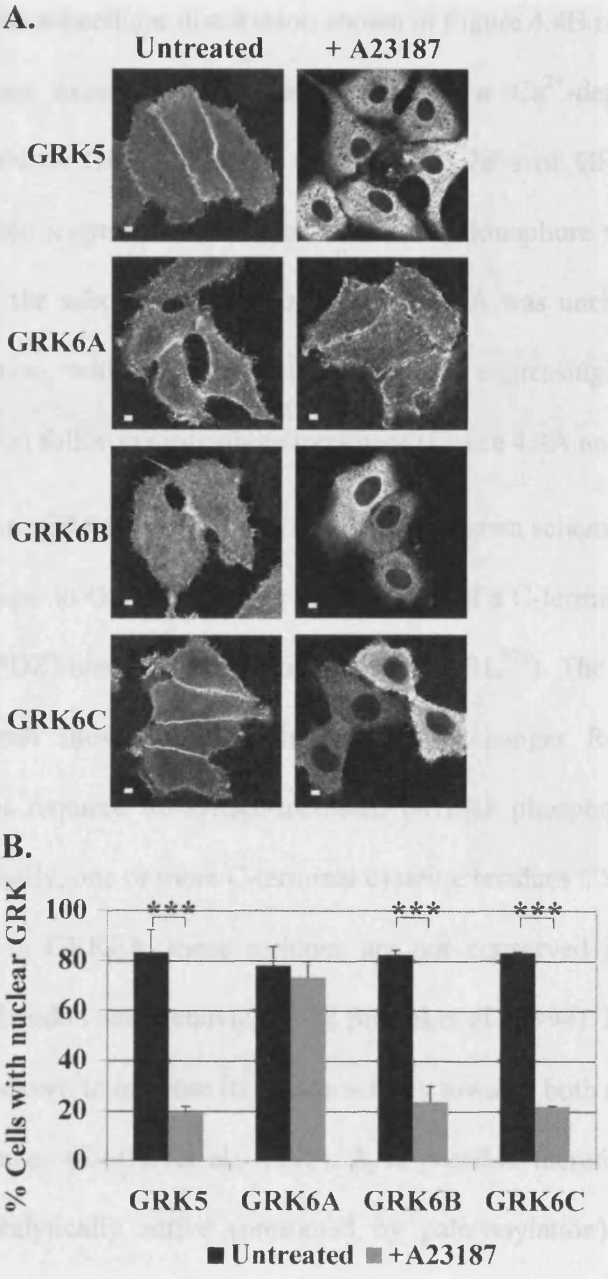


Figure 4.4. Differential regulation of the GRK6 splice variants by calcium.

A. HEp2 cells overexpressing GRK5, 6A, 6B or 6C were left untreated or treated with the calcium ionophore A23187 (+ A23187; 25μM, 15min). The subcellular distribution of the transfected GRKs was subsequently visualised by indirect immunofluorescence. Scale bars, 10μM. The images shown are representative of those obtained on at least three separate occasions.B. Quantification of A performed as described in Material and Methods. The data shown represent the mean values ± standard errors of the mean for at least three separate experiments. ***, $P<0.001$.

Quantification of the subcellular distribution shown in Figure 4.4B reveals that GRKs 5, 6B and 6C are exported from the nucleus in a Ca^{2+}-dependent fashion. Approximately 90% of GRK5, 76% of GRK6B and 78% of GRK6C expressing HEp2 cells exhibited a cytosolic distribution following ionophore treatment (Figure 4.4B). In contrast, the subcellular distribution of GRK6A was unchanged following ionophore application, with only 27% of the GRK6A expressing cells showing a cytosolic distribution following ionophore treatment (Figure 4.4A and B).

GRK6A differs from GRK6B and C at its C-terminus, shown schematically in Figure 4.5A. Features unique to GRK6A include the presence of a C-terminal PSD-95, Dlg, ZO-1 homology (PDZ)-binding motif (amino acids ^{574}TRL^{576}). The PDZ of GRK6A has previously been shown to bind the Na^+/H^+ Exchanger Regulatory Factor (NHERF-1), and is required for GRK6-mediated NHERF phosphorylation (Hall et al., 1999). Additionally, one or more C-terminal cysteine residues C561, 562, and 565 are palmitoylated in GRK6A, these residues are not conserved in GRK6B or C (Figure 4.5A and Loudon and Benovic, 1997; Stoffel et al., 1994). Palmitoylation of GRK6A has been shown to increase its kinase activity towards both membrane bound and soluble substrates (Stoffel et al., 1998). It is possible therefore that GRK6A requires to be catalytically active (promoted by palmitoylation) in order to be exported from the nucleus.

To assess the role of the PDZ-binding motif in inhibiting Ca^{2+}-dependent nuclear export of GRK6A I used a C-terminal flag tagged version of GRK6A. To confirm the disruption of the PDZ-binding domain of GRK6A by addition of the flag tag I demonstrated the inability of this mutant form of the enzyme to interact with S-tag-

NHERF-1 as assessed by an overlay assay (Figure 4.5B & C) (Hall et al., 1999) As shown in Figure 4.5C, GRK6A and GRK6A-flag lysates were subject to Western blot analysis and purified S-antigen-NHERF-1, shown in Figure 4.5B, overlaid on the blot (See Materials & Methods).

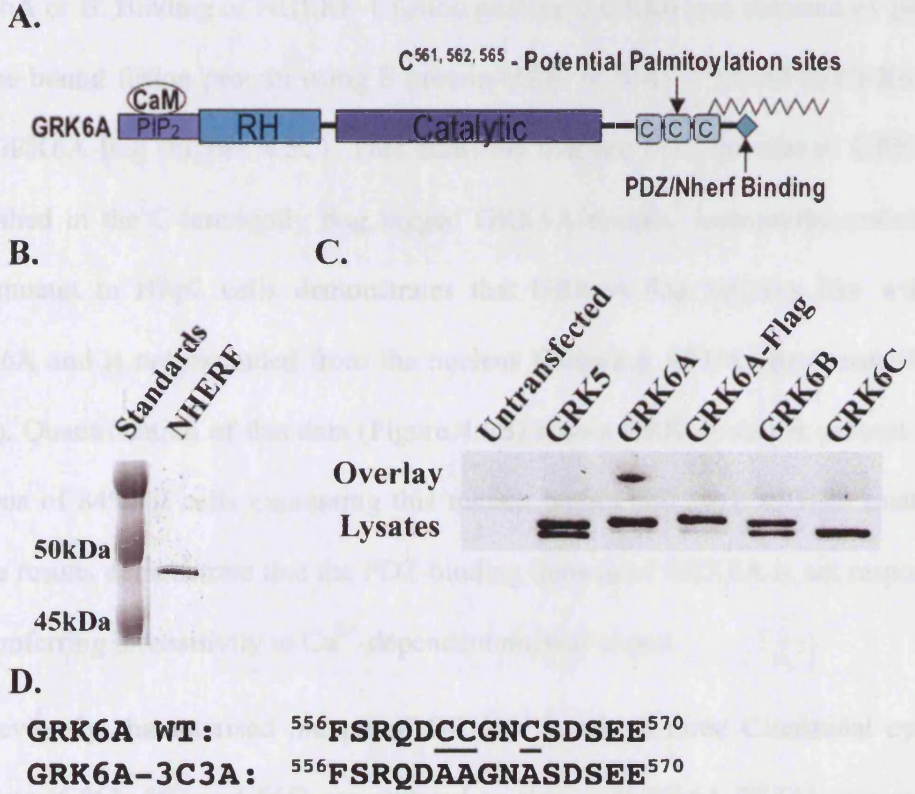


Figure 4.5 GRK6A but not GRK6A-flag binds to NHERF.

A. Schematic representation of GRK6A (Adapted from Penela et al., 2003). GRK6A differs from splice variants B and C at its C-terminus, it contains three potential palmitoylation sites (cysteines 561, 562, 565) and a NHERF-binding PDZ interacting domain (Hall et al., 1999; Stoffel et al., 1994). B. Coomassie gel of Purified 6-His/S-tag NHERF. C. Overlay western blot. Cos-7 cell lysates expressing the indicated GRKs were subject to SDS-PAGE and Western blot and purified 6-His/S-tag NHERF overlaid onto the blot. NHERF binds only to GRK6A and not GRK6-Flag or other GRK controls. D. The amino acid sequence of the C-terminal of GRK6A. Potential sites of palmitoylation are underlined. The C-terminal sequence of the non-palmitoylated mutant form of GRK6A (GRK6A-3C3A) is also shown.

GRK6B as detected by Western blot in cell lysates appears as a doublet whereas GRK6A and C appear as single bands (Figure 4.5C). A similar observation was made by Hall *et al* (Hall et al., 1999). These results suggest, either, that GRK6B is differentially post-translationally modified or is more susceptible to proteolysis than GRK6A or B. Binding of NHERF-1 fusion protein to GRK6 was detected by probing for the bound fusion protein using S-protein-HRP. NHERF-1 bound to GRK6A but not GRK6A-flag (Figure 4.5C). This confirms that the PDZ domain of GRK6A is abolished in the C-terminally flag tagged GRK6A mutant. Immunofluorescence of this mutant in HEp2 cells demonstrates that GRK6A-flag behaves like wildtype GRK6A and is not excluded from the nucleus following A23187 treatment (Figure 4.6A). Quantification of this data (Figure 4.6B) shows GRK6A-flag is present in the nucleus of 84% of cells expressing this mutant before and after A23187 treatment. These results demonstrate that the PDZ-binding domain of GRK6A is not responsible for conferring insensitivity to Ca^{2+}-dependent nuclear export.

A previously characterised mutant of GRK6A in which three C-terminal cysteine residues (C561, 562 and 565) are mutated to alanine (GRK6A-3C3A), was used to determine if palmitoylation of the kinase explains its refractory nature to ionophore treatment (Figure 4.5A) (Stoffel et al., 1998; Stoffel et al., 1994). Immunofluorescence reveals that this mutant of GRK6 is excluded from the nucleus even in the absence of ionophore treatment (Figure 4.6B and C) and A23187 treatment has no effect on its subcellular distribution (Figure 4.6B and C). This was a surprising result since inhibition of palmitoylation has previously been reported to promote the nuclear localisation of R7 binding protein and scramblase I (Song et al.,

2006a; Wiedmer et al., 2003).

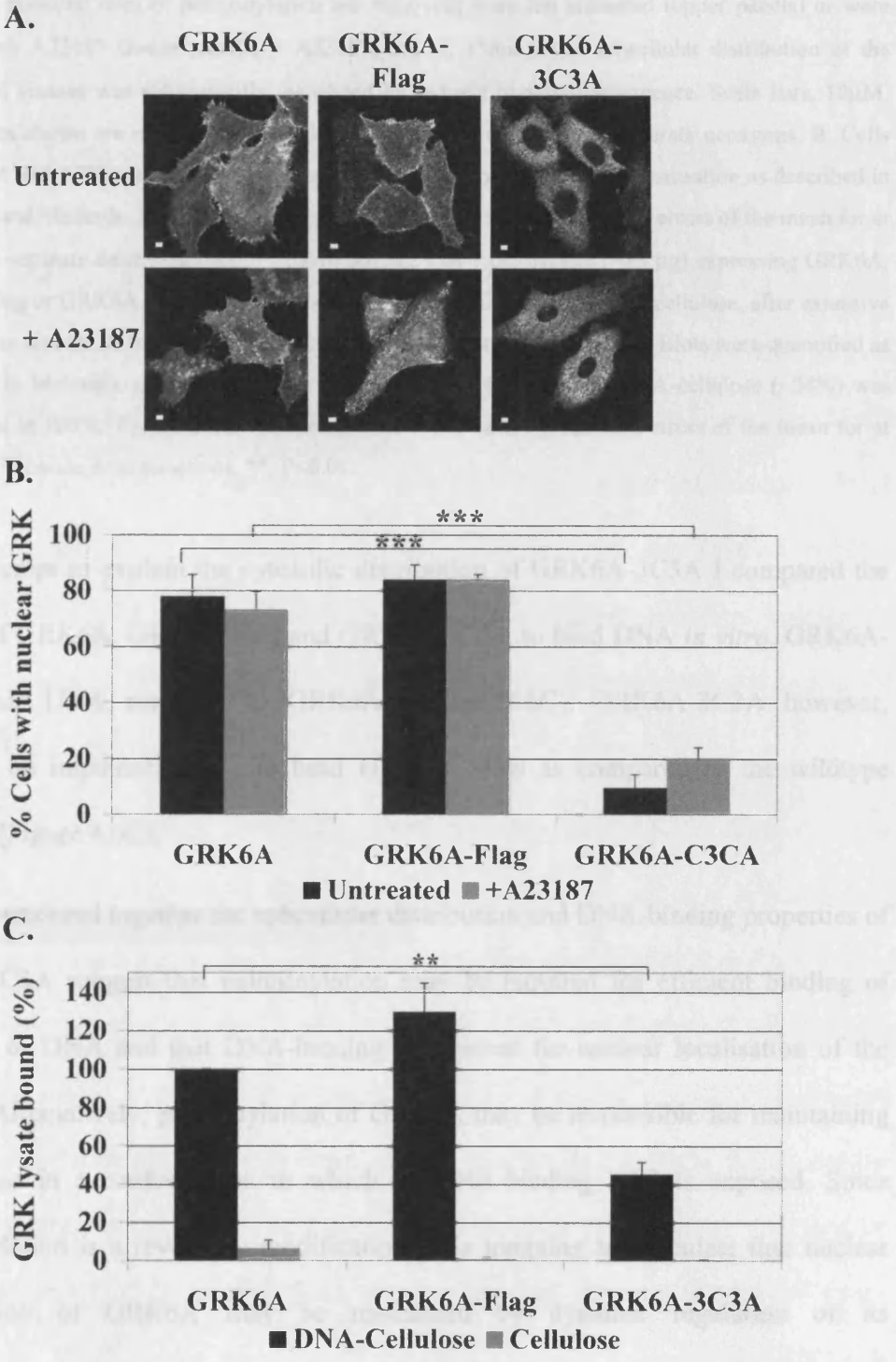


Figure 4.6. Palmitoylation of GRK6A is required for nuclear localisation and DNA-binding.

A. Mutation of all potential sites of palmitoylation results in the nuclear exclusion of GRK6A. HEp2 cells expressing GRK6A, GRKA6-flag (a mutant lacking the PDZ-binding domain) or GRK6A-3C3A (in which potential sites of palmitoylation are removed) were left untreated (upper panels) or were treated with A23187 (lower panels, + A23187; 25μM, 15min). The subcellular distribution of the transfected kinases was subsequently visualised by indirect immunofluorescence. Scale bars, 10μM. The images shown are representative of those obtained on at least three separate occasions. B. Cells transfected with GRK6A, or mutants thereof, were scored for their nuclear localisation as described in Materials and Methods. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. ***, $P<0.001$. C. Cos-7 cell lysates (~15 μg) expressing GRK6A, GRK6A-flag or GRK6A-3C3A were incubated with native DNA-cellulose or cellulose, after extensive washing the amount of bound GRK was determined by Western blot analysis. Blots were quantified as described in Materials and Methods. The amount of GRK6A bound to DNA-cellulose (~24%) was normalised to 100%. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. **, $P<0.01$.

In an attempt to explain the cytosolic distribution of GRK6A-3C3A I compared the ability of GRK6A, GRK6A-flag and GRK6A-3C3A to bind DNA *in vitro*. GRK6A-flag binds DNA similarly to GRK6A (Figure 4.6C). GRK6A-3C3A however, displays an impaired ability to bind DNA *in vitro* as compared to the wildtype enzyme (Figure 4.6C).

When considered together the subcellular distribution and DNA-binding properties of GRK6-3C3A suggest that palmitoylation may be required for efficient binding of GRK6A to DNA and that DNA-binding is required for nuclear localisation of the kinase. Alternatively, palmitoylation of GRK6A may be responsible for maintaining the kinase in a conformation in which its DNA-binding NLS is exposed. Since palmitoylation is a reversible modification, it is tempting to speculate that nuclear localisation of GRK6A may be modulated by dynamic regulation of its palmitoylation status. That the nuclear localisation of the splice variants of GRK6 is differentially regulated raises the possibility that distinct extracellular cues modulate

their putative nuclear functions.

4.5. Summary

The data in this Chapter hints towards novel roles of the GRK4 subfamily in the nucleus. In Chapter 3 I reported that GRK5 contains a DNA-binding NLS. I have now shown that the two other members of the GRK4 subfamily, GRK4 and GRK6, also contain functional NLSs. The NLS of GRK4 is different in sequence and location to that of GRKs 5 and 6 suggesting that this kinase is the most divergent of the group and is actually more similar in terms of NLS identity to the GRK1 subfamily. GRK4 and 6 also possess a putative NES, with the same sequence and location as the NES of GRK5. I have not confirmed that this putative NES is functional in GRKs 4 and 6 but since all GRK4 subfamily members contain an identical sequence I would predict that possession of a functional NES is a conserved feature of this GRK subfamily.

Interestingly, the GRK4 subfamily members show differential DNA-binding properties, perhaps suggesting that GRKs 5 and 6 (which bind DNA *in vitro*) may have different nuclear functions to GRK4 (which does not bind DNA *in vitro*). Since GRK6 shares the most sequence homology with GRK5, I investigated whether this kinase was regulated by Ca^{2+} in a similar fashion. Interestingly, GRK6B and C are subject to nuclear export in the presence of Ca^{2+} in a similar fashion to GRK5, but the nuclear localisation of GRK6A appears to be refractory to this regulatory mechanism. The C-terminal PDZ motif of GRK6A does not appear to account for this difference between the GRK6 splice variants. Palmitoylation at the C-terminus of GRK6A is however required for nuclear localisation. These data indicate that the GRK4

subfamily of kinases contain NLSs but appear to be differentially regulated. This may suggest that these kinases might have roles in the nucleus distinct from their classical involvement in GPCR desensitisation.

5. GRK5 DNA-binding ability and kinase activity is required for inhibition of hypertrophy in myocytes

In this Chapter, I identify the structural determinants of GRK5 required for DNA-binding *in vitro* and nuclear localisation in cells. Using isolated rat neonatal myocytes, I demonstrate that GRK5 overexpression inhibits the hypertrophic phenotype observed following αAR or PLC activation. GRK5-mediated inhibition of hypertrophy requires both the DNA-binding ability and kinase activity of GRK5 suggesting a direct role for this enzyme in regulating gene transcription.

5.1. Structural determinants of GRK5 required for nuclear localisation and DNA-binding

GRK5 contains a DNA-binding NLS and is present in the nucleus of HEp2 cells both endogenously and when transiently overexpressed (Chapter 3 and Johnson et al., 2004). Whether GRK5 binds DNA in a cellular setting and the functional consequences of this interaction have, however, not been explored. I reasoned that a clear demonstration of a nuclear function for GRK5 would rely on the use of mutant forms of the enzyme that have distinct subcellular localisations and differ in their DNA-binding properties.

5.1.1. GRK5 nuclear localisation is dependent on the NLS and NES motifs

The identification of both a NLS and NES in GRK5 (discussed in Chapter 3) suggests that its nuclear localisation may be regulated. In Chapter 3 I examined, using confocal immunofluorescence, the subcellular localisation of a range of GRK5 mutants, including GRK5ΔNLS, GRK5ΔNES, GRK5NTPB, GRK5CTPB and GRK5K215R, before and after calcium ionophore treatment (A23187). This data revealed GRK5 structural domains that contribute to the regulation of its nuclear localisation. A schematic of the mutant constructs of GRK5 used (Figure 5.1A) and confocal immunofluoresence of these mutants (Figure 5.1B) is recapitulated here in this chapter for purposes of clarity.

I introduce here one further GRK5 mutant which has not been previously discussed in this thesis, GRK5AP. Inhibition of GRK5 autophosphorylation (GRK5AP) (Figure 5.1A) does not seem to alter the subcellular distribution of GRK5 compared to the wildtype kinase, being uniformly distributed throughout the cell including the nucleus (Figure 5.1B compare panel g and a). This suggests that whilst regulation of kinase activity and GRK5 autophosphorylation status are important in governing GRK5 activity in the cell, these modifications do not contribute directly to modifying its subcellular localisation.

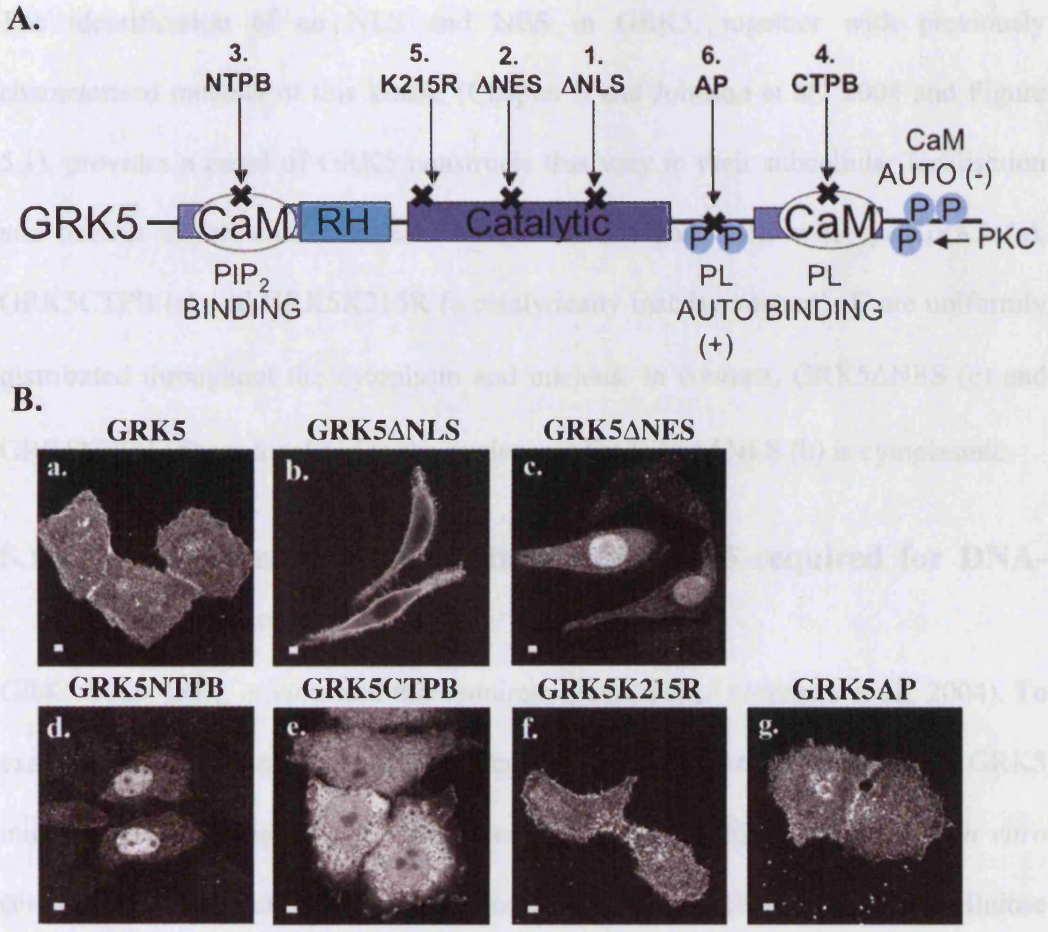


Figure 5.1. Structural determinants of GRK5 required for calcium-mediated nuclear export.

A. Schematic representation of GRK5 mutant constructs shown by immunofluorescence in panel B (Adapted from Penela et al., 2003). 1. ΔNLS, mutation of basic residues in the NLS (residues 388-395) to alanine; 2. ΔNES, mutation of hydrophobic residues in the NES (259-265) to alanine; 3. NTPB (N-terminal polybasic), mutation of basic residues between residue 22 and 29 to alanine in the N-terminal PIP_2 and CaM binding sites of GRK5; 4. CTPB (C-terminal polybasic), mutation of basic residues between residues 547 and 557 to alanine in the C-terminal phospholipid and CaM binding region of GRK5; 5. K215R, kinase dead GRK5 mutant; 6, AP, autophosphorylation deficient GRK5 resulting from mutations S484A and T485A. CaM, Calmodulin binding domain; RH, Regulator of G-protein signalling (RGS) homology domain; PL, phospholipid; P, Phosphorylation site; Auto, autophosphorylation; PKC, Protein kinase C. B. Confocal microscopy showing cellular distribution of GRK5 and GRK5 mutants thereof, overexpressed in HEp2 cells. Scale bars are 10μM. The images shown are representative of those obtained on at least three separate occasions.

The identification of an NLS and NES in GRK5, together with previously characterised mutants of this kinase (Chapter 3 and Johnson et al., 2004 and Figure 5.1), provides a panel of GRK5 constructs that vary in their subcellular localisation and nuclear export characteristics. As shown in Figure 5.1B wildtype GRK5 (a), GRK5CTPB (e) and GRK5K215R (a catalytically inactive mutant) (f) are uniformly distributed throughout the cytoplasm and nucleus. In contrast, GRK5ΔNES (c) and GRK5NTPB (d) are localised to the nucleus whilst GRK5ΔNLS (b) is cytoplasmic.

5.1.2. The structural determinants of GRK5 required for DNA-binding *in vitro*

GRK5 binds DNA *in vitro* and this requires an intact NLS (Johnson et al., 2004). To examine if other structural features regulate DNA-binding, the panel of GRK5 mutants shown in Figure 5.1, were assessed for their ability to bind DNA *in vitro* (Figure 5.2). Consistent with previous findings wildtype GRK5 bound DNA-cellulose (27.8±2.3% of the load bound) but not the cellulose support (0.7±0.2%) (Johnson et al., 2004). GRK5ΔNLS failed to bind DNA (5.2±1.5%), confirming that an intact NLS is required for this interaction (Johnson et al., 2004). Analysis of the DNA-binding properties of the other GRK5 mutants shown in Figure 5.2 reveals that the DNA-binding ability of GRK5ΔNES (22±6.9%) is identical to that of the wildtype enzyme (Figure 5.2, ΔNES) while that of GRK5NTPB (6±2.5%) (Figure 5.2, NTPB) is similar to that of GRK5ΔNLS. Thus despite having a similar nuclear localisation, GRK5ΔNES and GRK5NTPB exhibit very different DNA-binding abilities. The inability of GRK5NTPB to bind DNA suggests that, like the NLS, this region of the kinase may be in direct contact with DNA. Presumably these two regions form part,

or all, of the DNA-binding domain of GRK5.

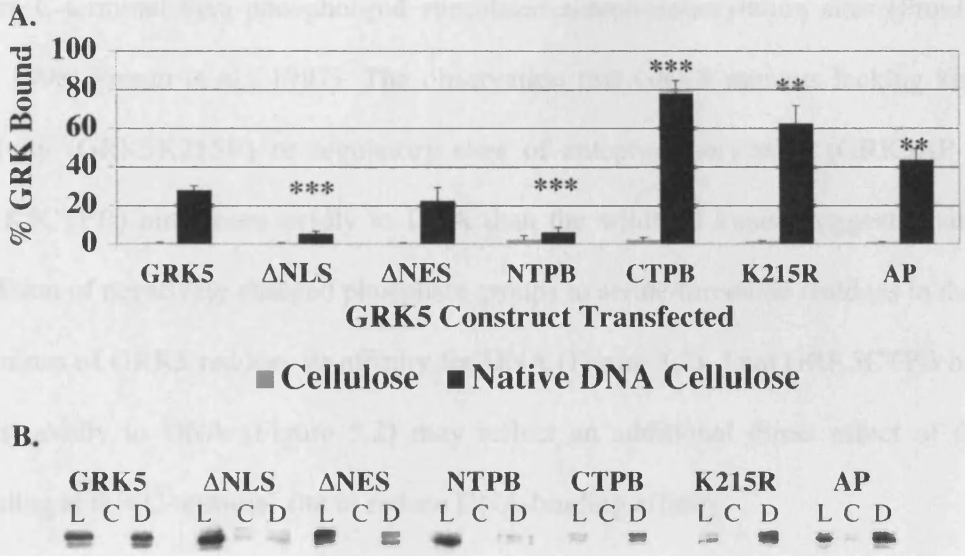


Figure 5.2. Structural determinants of GRK5 required for DNA-binding *in vitro*.

GRK5 DNA-binding *in vitro* requires an intact NLS and N-terminal CaM binding site. A. DNA-binding assays, described in Materials and Methods, were performed on Cos-7 cell lysates overexpressing GRK5, or the mutant GRK5 constructs indicated. The results shown represent the mean values ± standard errors of the mean for at least three separate determinations. Statistical significance is determined relative to wildtype GRK5. **, $P<0.01$; ***, $P<0.001$. B. Representative western blots of mutant GRK5 DNA binding. L, GRK Lysate (25%); C, GRK bound to Cellulose; D, GRK bound to DNA-Cellulose.

GRK5CTPB, GRK5K215R and GRK5AP bind DNA more avidly than their wildtype counterpart, respectively, 78±6.6%, 62±9.3% and 44±5.9% of the load bound (Figure 5.2). GRK5 undergoes phospholipid stimulated autophosphorylation (Figure 5.1A) at serine 484 and threonine 485, the residues mutated in the GRK5AP mutant (Kunapuli et al., 1994). Additionally, the binding of CaM to the C-terminal CaM binding site of GRK5 stimulates autophosphorylation at sites distinct from these 'classical' phospholipid stimulated autophosphorylation sites (Pronin et al., 1997). Although the sites phosphorylated in the presence of CaM remain to be definitively mapped they

reside in the C-terminus of the kinase between residues 579 and 585, i.e. at a site more C-terminal than phospholipid stimulated autophosphorylation sites (Pronin et al., 1998; Pronin et al., 1997). The observation that GRK5 mutants lacking kinase activity (GRK5K215R) or regulatory sites of autophosphorylation (GRK5AP and GRK5CTPB) bind more avidly to DNA than the wildtype kinase suggests that the addition of negatively charged phosphate groups to serine/threonine residues in the C-terminus of GRK5 reduces its affinity for DNA (Figure 5.2). That GRK5CTPB binds most avidly to DNA (Figure 5.2) may reflect an additional direct effect of CaM binding at this C-terminal site to reduce DNA-binding affinity.

5.1.3. Model of regulation of GRK5 nuclear localisation and DNA-binding

Analysis of the subcellular localisation and DNA-binding properties of the seven GRK5 mutants used in this thesis suggests a potential model, depicted schematically in Figure 5.3, whereby GRK5 nuclear localisation may be regulated.

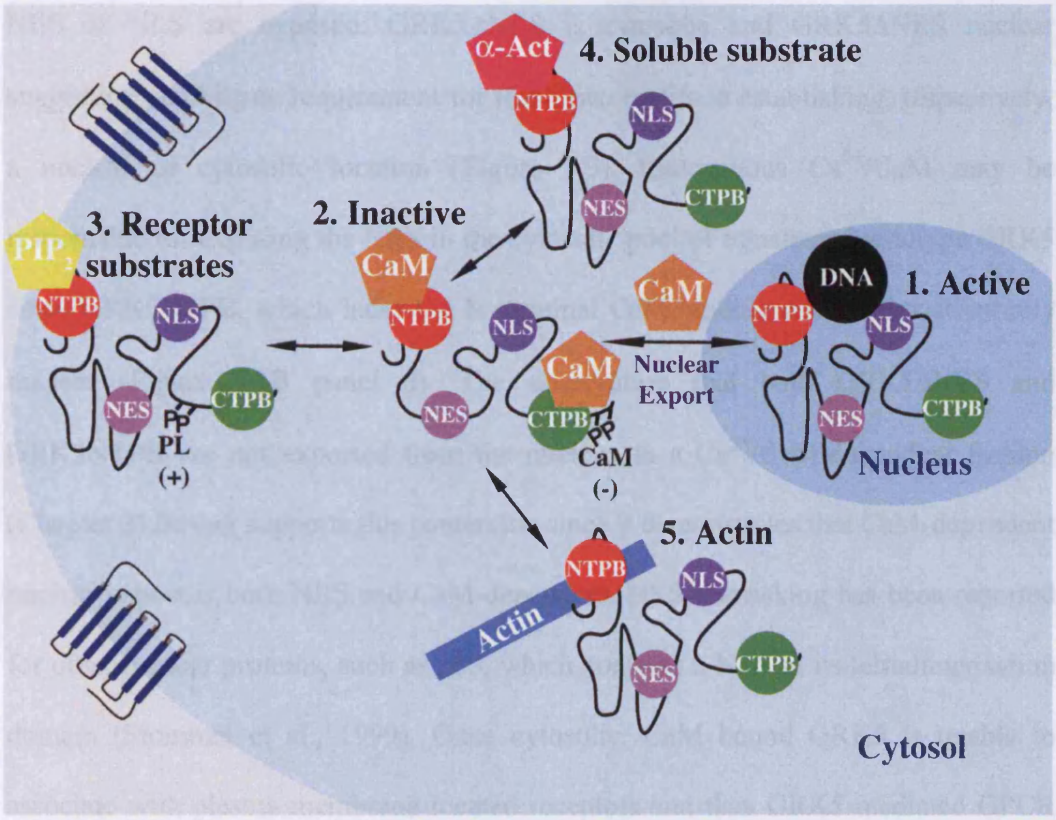


Figure 5.3. Model illustrating potential mechanisms regulating DNA-binding and nuclear export of GRK5.

1. GRK5 resides in the nucleus, free of CaM, in a conformation in which its NLS is exposed and NES hidden. DNA-binding is mediated, at least in part by its NLS and N-terminal CaM binding domain (NTPB). **2.** CaM binding to the N-terminus of GRK5 exposes the NES of GRK5 facilitating its nuclear export. CaM-binding to the C-terminal CaM-binding site of GRK5 induces autophosphorylation of the kinase at multiple sites within its C-terminus, although not directly promoting nuclear export, facilitates dissociation of GRK5 from DNA. If CaM remains bound to GRK5 in the cytoplasm, CaM will inhibit GRK5 receptor association and phosphorylation. GRK5 can associate with a number of molecules which compete with CaM to bind to the N-terminus of GRK5 that may promote its cytosolic retention: **3.** PIP_2, which promotes receptor phosphorylation **4.** α-actinin (α-act), which promotes phosphorylation of soluble substrates and **5.** actin, which inhibits GRK5 catalytic activity.

The wildtype kinase is distributed throughout the cytoplasm and nucleus of transfected cells suggesting two populations of GRK5 in which, respectively, the

NES or NLS are exposed. GRK5ΔNLS is cytosolic and GRK5ΔNES nuclear suggesting an obligate requirement for these two motifs in establishing, respectively, a nuclear or cytosolic location (Figure 5.3). Endogenous Ca^{2+}/CaM may be responsible for exposing the NES in the cytosolic pool of transfected wildtype GRK5 since GRK5NTPB, which lacks the N-terminal CaM binding site, is almost entirely nuclear (Figure 5.1B panel d). The observation that both GRK5ΔNES and GRK5NTPB are not exported from the nucleus in a Ca^{2+}/CaM-dependent fashion (Chapter 3) further supports this contention since it demonstrates that CaM-dependent nuclear export is both NES and CaM-dependent. NES unmasking has been reported for other nuclear proteins, such as p53, which contains a NES in its tetradimerisation domain (Stommel et al., 1999). Once cytosolic, CaM bound GRK5 is unable to associate with plasma membrane located receptors and thus GRK5-mediated GPCR desensitisation is inhibited. Even in the absence of Ca^{2+}/CaM GRK5 may be retained in the cytosol by binding to other ligands that associate with the N-terminal polybasic domain including PIP_2/α-actinin/actin (Figure 5.3). The functional consequences of these binding events are be predicted to be complex, resulting in forms of the kinase with different substrate specificities (illustrated in Figure 5.3).

The nuclear pool of wildtype GRK5 is postulated to be free of CaM. In the nucleus GRK5 may be bound to DNA. DNA-binding requires both an intact NLS and N-terminal CaM binding site since mutation of either of the sites prevents DNA-binding *in vitro* (Figure 5.2). Binding of CaM to the N-terminal CaM binding site of GRK5 may thus serve, not only to expose the NES of GRK5, but may also contribute directly to its dissociation from DNA. GRK5K215R, GRK5AP and GRK5CTPB all

exhibit an enhanced DNA-binding ability *in vitro* as compared to the wildtype enzyme. This suggests that phosphate addition, and potentially CaM binding, to the C-terminus of the kinase contributes to reduce its affinity for DNA. These modifications do not, however, result in NES exposure since these mutant forms of the enzyme show a subcellular distribution identical to that of wildtype GRK5 (Figure 5.1B).

Overall these studies suggest a complex mechanism for regulating the conformation of GRK5 via interaction with regulatory proteins (CaM) or by post-translational modifications (autophosphorylation). Altering conformation is postulated to modulate NLS/NES exposure thereby affecting nuclear localisation and DNA-binding properties of GRK5. aPKCλ (Perander et al., 2001), and MAPK-activated protein kinase 2 (MK2) (Engel et al., 1998) also contain both a NLS and NES. In comparison to GRK5 however, which does not necessarily need to be catalytically active in order to be present in or exported from the nucleus, aPKCλ and MK2 phosphorylation-dependent activation is required to expose their NLS/NES and alter their nuclear localisation. Active aPKCλ enters, whilst active MK2 exits, the nucleus (Engel et al., 1998; Perander et al., 2001).

5.2. GRK5 activity and the ability to bind DNA are required for inhibition of cardiac hypertrophy *ex vivo*

Determining the DNA-binding properties of wildtype and mutant GRK5 constructs (shown in Figure 5.1) provides valuable tools for investigating potential nuclear functions of GRK5 in a cellular setting. For example, both wildtype and catalytically inactive GRK5 (GRK5K215R) are located throughout the cell and both bind DNA,

whereas the ΔNLS and N-terminal CaM binding (NTPB) mutants of GRK5 do not bind DNA but are cytosolic and nuclear in distribution, respectively.

5.2.1. GRK5 DNA-binding and kinase activity is required to rescue phenylephrine-induced cardiac hypertrophy

Adaptation of the heart to pressure or stress results in cardiac hypertrophy, where myocytes increase in cell size, enhance protein synthesis and heighten organisation of the sarcomere (Reviewed in Frey et al., 2004). Initiation of the fetal gene program is observed since genes required for induction of hypertrophy are typically those expressed during development (Frey et al., 2004). Although hypertrophy initially compensates for cardiac stress it is eventually linked to an increased risk of HF (Frey et al., 2004). In transgenic mice, cardiac specific overexpression of a constitutively active mutant of the α_{1B}AR induces cardiac hypertrophy (Eckhart et al., 2000). Hypertrophy is however inhibited in mice expressing both the mutant receptor and GRK5. Coupling of the $\alpha 1_B$AR to Gq is not affected under these conditions, suggesting that GRK5 is not simply acting to desensitise the receptor (Eckhart et al., 2000). I hypothesised that this GRK5-mediated inhibition of hypertrophy reflects a novel, potentially nuclear, function of GRK5 and sought to recapitulate this observation *ex vivo*.

Application of PE to rat neonatal cardiac myocytes in culture activates ARs and induces cardiac hypertrophy. This is evident by an increase in cell size and increased organisation of α-actinin as shown in Figure 5.4A. As a measure of hypertrophy I determined cell area (μM^2). Treatment of cardiac myocytes for 48h with 200μM PE results in an increase in cell area from approximately 530μM^2 to 1180μM^2, a 2.2 fold

increase (Figure 5.4A and B).

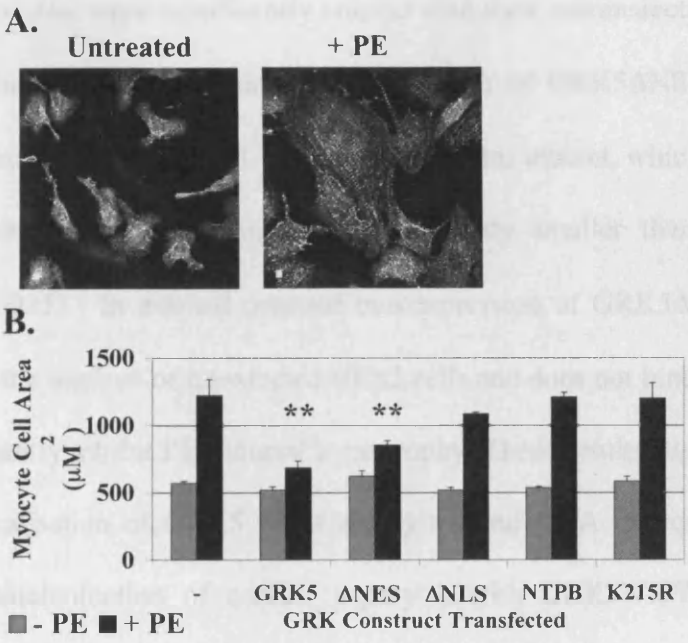


Figure 5.4. GRK5 overexpression rescues cardiac hypertrophy in myocytes.

A. Induction of cardiac hypertrophy *ex vivo* induces myocyte cell growth and reorganisation of α-actinin. Rat neonatal myocytes plated on coverslips were left untreated (untreated) or were treated with PE (200μM, 48h) (+ PE). α-Actinin organisation was subsequently visualised by indirect immunofluorescence. Scale bars are 10μM. B. The DNA-binding ability of GRK5 and its kinase activity are required for GRK5-mediated inhibition of hypertrophy. The extent of PE (200μM, 48h) induced hypertrophy in rat neonatal myocytes transfected with various GRK5 constructs was determined by measuring cell area (μM^2) as described in the methods. GRK5, the wildtype enzyme; ΔNES, GRK5ΔNES; ΔNLS, GRK5ΔNLS; NTPB, GRK5 N-terminal CaM binding site mutant; K215R, catalytically inactive GRK5. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. Statistical significance is determined relative to values obtained for wildtype GRK5 + PE. **, $P<0.01$.

Nucleofection and expression of GRK5 did not alter cell area per se (P=0.3), but did

inhibit PE-dependent hypertrophy ($P<0.01$, Figure 5.4B). PE treated, GRK5 expressing myocytes, were significantly smaller than their untransfected counterparts ($P=0.0029$). Similarly, nucleofection and expression of GRK5ΔNES rescued PE-induced hypertrophy (Figure 5.4B). Cells expressing this mutant, which is exclusively nuclear and binds DNA *in vitro*, were significantly smaller than untransfected controls ($P=0.0035$). In marked contrast overexpression of GRK5ΔNLS, which is excluded from the nucleus of transfected HEp2 cells and does not bind DNA *in vitro*, fails to significantly inhibit PE-induced hypertrophy. These results suggest that either the nuclear localisation of GRK5 or its ability to bind DNA is required to inhibit hypertrophy. Nucleofection of cardiac myocytes with GRK5NTPB was used to distinguish these two possibilities. GRK5NTPB, a mutant enzyme that lacks the N-terminal CaM-binding site of GRK5, is present in the nucleus of HEp2 cells but, like GRK5ΔNLS, fails to bind DNA *in vitro*. GRK5NTPB expression did not inhibit PE-induced hypertrophy. This observation suggests that GRK5-dependent inhibition of PE-induced hypertrophy requires the DNA-binding ability of GRK5. The kinase activity of GRK5ΔNLS and GRK5NTPB is similar to that of wildtype GRK5, when assessed *in vitro* using rhodopsin as a substrate (Johnson et al., 2004). This suggests that GRK5-dependent rescue of hypertrophy is a bona fide affect of DNA-binding and is likely not explained by reduced kinase activity of the non-DNA-binding GRK5 mutants. GRK5CTPB binds to DNA with a higher affinity thann wild type GRK5 as assessed by the *in vitro* DNA binding assay (Section 5.1.2 and Figure 5.2). I thus hypothesised that this mutant would, like its wild type counterpart, inhibit PE-induced hypertrophy *ex vivo*. However, expression of GRK5CTPB in myocytes resulted in

cell death, although the significance of this observation is not known, it may indicate an important functional role for the DNA-binding ability of GRK5 *ex vivo*. Notably however, expression of GRK5K215R, a catalytically inactive mutant of GRK5, failed to rescue PE-induced hypertrophy (Figure 5.4B). Together these results point to a role for both the DNA-binding ability of GRK5 and its kinase activity in regulating gene expression during hypertrophy.

As GRK5 is exported from the nucleus following activation of the Gq-coupled M3MR (Chapter 3 and Johnson et al., 2004) it is tempting to postulate that Gq coupled αAR activation may similarly affect GRK5 and thus act to inhibit nuclear functions of this enzyme. βarr-1 has recently been reported to translocate to the nucleus upon δ-opioid receptor activation and promote gene transcription by recruiting the histone acetylase p300 to the promoter of specific genes (Kang et al., 2005). Perhaps Nuclear GRK5 might act to repress gene transcription. Following Gq-coupled GPCR activation and nuclear export of GRK5 this repression may be relieved. A consequence of overexpressing GRK5 in cardiac myocytes may be restoration of the DNA-binding and kinase-dependent inhibitory effect of GRK5 on gene transcription.

Although GRK5ΔNLS and GRK5NTPB are active kinases *in vitro* the possibility remains that they are not active in a cellular setting. I thus sought to confirm that the activity requirement for GRK5-mediated inhibition of cardiac hypertrophy represented a novel function of this enzyme rather than desensitisation of ARs and termination of the hypertrophic signal. To address this problem, I took two approaches, firstly, to show that GRK5 can rescue hypertrophy in myocytes induced

by receptors which are not known to be substrates for GRK5 *in vivo* and secondly, and perhaps more convincingly, by activating hypertrophy downstream of GPCRs using a PLC activator, *m*-3M3FBS.

5.2.2. GRK5 rescues hypertrophy induced by EGF and AngII

Since the kinase activity as well as DNA-binding ability of GRK5 is required to rescue hypertrophy in rat neonatal cardiac myocytes, I sought to confirm that this rescue was not simply due to GRK5-mediated desensitisation of the α_{1B}AR and therefore termination of hypertrophic signalling. AngII stimulation is known to increase cardiac myocyte cell size by transactivating the EGFR which in turn activates MAPKs (Shah and Catt, 2003). Overexpression of GRK5 in the hearts of mice does not alter the contractile response to AngII stimulation suggesting that GRK5 does not contribute to desensitisation of ATRs in cardiac tissue *in vivo* (Rockman et al., 1996).

The tyrosine kinase receptor, EGFR, as is typical for this class of receptor, is desensitised by degradation by the proteasome following ubiquitination. To date, GRK2, but not GRK5, has been shown to bind to and phosphorylate this receptor tyrosine kinase, a non-GPCR substrate. The functional significance of this event is unknown since GRK2 phosphorylation does not appear to induce desensitisation of this receptor (Freedman et al., 2002; Gao et al., 2005). Stimulation of the EGFR results in a hypertrophic response mediated by p38 stimulation of STAT5 transcription (Rebsamen et al., 2000).

Activation of hypertrophy via the AT_{1A}R/EGFR pathway is thus unlikely to be

rescued by GRK5-mediated receptor desensitisation. Inducing hypertrophy in myocytes using EGF or AngII results in an increase in cell area of approximately 42±11% and 48±3% respectively in control cells (Figure 5.5). As was observed with the induction of hypertrophy using PE, GRK5 expression inhibits both EGF- (167mM, 48h) and AngII- (200nM, 48h) dependent hypertrophy (Figure 5.5). This suggests that GRK5 mediated rescue of the increase in cell area is due to its DNA-binding ability and not its ability to desensitise plasma membrane located receptors. Presumably kinase activity is required to phosphorylate a nuclear substrate and not a receptor. This hypothesis is supported by the observation that GRK5ΔNLS is present in the cytosol but not the nucleus and exhibits wildtype catalytic activity, cannot rescue PE-induced hypertrophy.

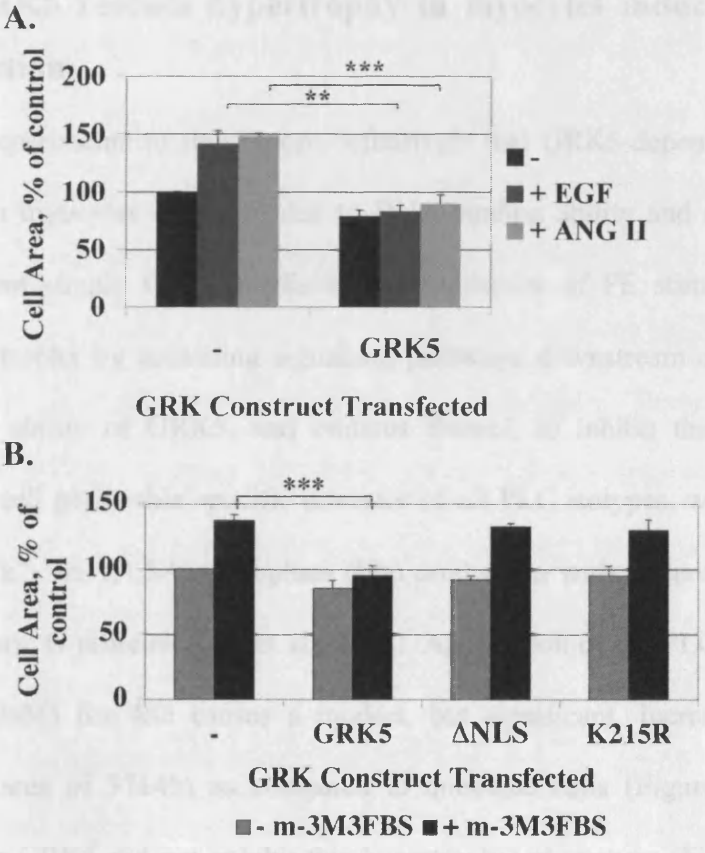


Figure 5.5. GRK5-dependent phosphorylation of GPCRs is not required for rescue of hypertrophy.

A. GRK5 overexpression rescues hypertrophy induced by receptors that are unlikely to represent substrates for this kinase *in vivo*. Induction of hypertrophy in rat neonatal cardiac myocytes in culture by EGF (167mM, 48h) or AngII (200nM, 48h) caused cell area to increase by approximately 42% and 48% respectively. Overexpression of GRK5 prevented this induction of hypertrophy. The data shown represent the mean values ± standard errors of the mean for at least three experiments. Control cell area was normalised to 100% and other values calculated as a percentage of this size. *P* values compared to untreated control cells. **, $P<0.01$; ***, $P<0.001$. B. GRK5 overexpression rescues hypertrophy induced downstream of GPCRs. GRK5 inhibits hypertrophy induced by activation of signalling pathways downstream of GPCRs. The PLC activator *m*-3M3FBS (100nM, 48h) was used to induce hypertrophy in isolated myocytes and the effect of GRK5 or GRK5 mutant expression on cell area quantified. The data shown represent the mean values ± standard errors of the mean for at least three experiments. Untransfected unstimulated myocyte cell area was normalised to 100% and other values calculated as a percentage of this value. Statistical significance is determined relative to values obtained for wildtype GRK5 + *m*-3M3FBS. ***, $P<0.001$.

5.2.3. GRK5 rescues hypertrophy in myocytes induced by PLC activation

I conducted experiments to show more definitively that GRK5-dependent rescue of hypertrophy in myocytes is indeed due to DNA-binding ability and nuclear kinase activity and not simply GRK5-mediated desensitisation of PE stimulated ARs. I induced hypertrophy by activating signalling pathways downstream of GPCRs and examined the ability of GRK5, and mutants thereof, to inhibit this process. *M*-3M3FBS is a cell permeable specific activator of all PLC isotypes, which increases intracellular Ca^{2+} via 1,4,5-trisphosphate (IP_3) production without having any effect on heterotrimeric G proteins (Bae et al., 2003). Application of the PLC activator, *m*-3M3FBS (100nM) for 48h causes a modest, but significant, increase in cardiac myocyte cell area of 37±4% as compared to untreated cells (Figure 5.5B). Cells overexpressing GRK5 did not exhibit this hypertrophic phenotype (Figure 5.5B). In

contrast, and consistent with results obtained using PE, GRK5ΔNLS or GRK5K215R expression did not inhibit *m*-3M3FBS-induced hypertrophy (Figure 5.5B, ΔNLS and K215R). That a kinase deficient mutant of GRK5 fails to inhibit *m*-3M3FBS-induced hypertrophy strongly suggests that phosphorylation of non-GPCR nuclear substrates is required for GRK5-mediated inhibition of hypertrophy. Combined with the data from Section 5.2.2, I believe this demonstrates that GRK5-dependent inhibition of hypertrophy is due to its DNA-binding ability and catalytic activity and is not simply due to its ability to desensitise ARs.

5.3. Summary

In this Chapter I have made a step towards understanding how GRK5 nuclear localisation and DNA-binding ability is regulated by structural determinants. This data allows me to propose a model of how the DNA-binding ability and nuclear localisation of GRK5 may be regulated. The NLS and NES of GRK5 control nuclear and cytosolic localisation of the kinase. GRK5 DNA-binding ability depends upon an intact NLS and N-terminal CaM binding region. It may be that CaM binding to the N-terminus of GRK5 sterically hinders DNA-binding and exposes the NES of GRK5 since nuclear export of the kinase is NES and CaM dependent. Phosphate addition at the C-terminus of GRK5 inhibits DNA-binding since a kinase dead mutant of GRK5 (GRK5K215R) and an autophosphorylation (GRK5AP) or C-terminal CaM binding mutant (GRK5CTPB) all show an increased affinity for DNA *in vitro* as compared to the wildtype enzyme. CaM binding at the C-terminus of GRK5, apart from stimulating inhibitory autophosphorylation may also serve to directly inhibit DNA-binding. However, GRK5 catalytic activity is not required for nuclear export and

appears to solely affect the ability of GRK5 to bind DNA.

In order to attempt to understand the functional significance of GRK5 nuclear localisation I recapitulated *ex vivo* published work that reported GRK5-mediated rescue of hypertrophy *in vivo* (Eckhart et al., 2000). In rat neonatal cardiac myocytes, PE-induced cardiac hypertrophy is prevented by overexpression of GRK5 and this effect requires both the DNA-binding ability and kinase activity of this enzyme. GRK5 overexpression also rescued hypertrophy induced by AngII and EGF, agonists which activate receptors likely to be refractory to GRK5-mediated desensitisation events. Furthermore, GRK5 expression prevents hypertrophy induced by a PLC activator which stimulates signalling pathways downstream of GPCRs and heterotrimeric G proteins. The molecular mechanisms whereby GRK5 inhibits myocyte hypertrophy *ex vivo* represents an exciting area of future research.

6. Discussion

GRKs phosphorylate and desensitise activated GPCRs and thereby play an important role in regulating intracellular signal transduction cascades. The identification of non-receptor substrates for these kinases, suggests that they may play a more diverse, and direct, role in regulating signalling than previously appreciated (Penela et al., 2006). In this thesis, I have shown that the GRK4 subfamily of GRKs are not only membrane bound/cytosolic kinases but are also present in the nucleus. GRKs 4, 5 and 6 possess functional nuclear localisation signals for nuclear import, and by homology with GRK5 all are predicted to contain NESs. GRKs 5 and 6, but not 4, bind DNA *in vitro* suggesting that they may have differential nuclear functions. The nuclear localisation of GRK5 and 6A is differentially regulated by Ca^{2+}/CaM indicating that a potentially common function of these GRKs may be subject to distinct regulatory mechanisms. I have also shown *ex vivo* that GRK5 overexpression inhibits cardiac hypertrophy, and that this requires both the DNA-binding ability and kinase activity of GRK5.

6.1. GRK5 is a nuclear protein

In Chapter 3 I demonstrate that GRK5, a member of the GRK4 subfamily of GRKs is a nuclear protein and contains a DNA-binding NLS. Furthermore, nuclear localisation of GRK5 is regulated in a GPCR-dependent fashion. Agonist-occupancy of the M3MR, which elevates intracellular Ca^{2+} levels and activates the Ca^{2+} sensor protein, CaM, promotes NES-dependent nuclear export of GRK5. The binding of activated CaM to the N-terminus of GRK5 is required for this export event. It is

possible, since α-actinin and phospholipids also bind to the same site as CaM that these molecules regulate GRK5 DNA-binding and nuclear export. However, since CaM is abundantly expressed in the nucleus, particularly in heart, and since I have shown that CaM sequestrants inhibit nuclear export this seems unlikely (Chin and Means, 2000; Maier et al., 2006). By regulating the nuclear localisation of GRK5, and thus, the ability of the kinase to bind DNA in cells, Gq-coupled GPCRs could inhibit potential nuclear functions of this kinase.

6.1.1. GRK5 contains both a NLS and NES

Using two search engines which predict NLS and NES sequences in proteins, I identified a monopartite NLS, which has homology to the NLS found in DNA-binding proteins such as the homeobox-containing proteins, along with a Leucine rich NES (Cokol et al., 2000; la Cour et al., 2004). Homeobox-containing proteins are DNA-binding transcription factors which all contain a DNA-binding homeodomain and regulate transcription of a multiplicity of genes during development (Billeter, 1996). The homeodomain containing transcription factor *Csx/Nkx2-5* is highly expressed in the heart during murine embryogenesis and throughout adulthood (Komuro and Izumo, 1993). *Csx/Nkx2-5* deficient mice showed a reduction in expression of *ANF* and *MLC2v* genes, amongst others, indicating that *Csx/Nkx2-5* plays a crucial role in regulating transcription of cardiac-specific genes essential for cardiac differentiation (Tanaka et al., 1999). Whilst GRK5 and 6 knockout mice develop to adulthood (Gainetdinov et al., 2003; Gainetdinov et al., 1999), it would be interesting to determine if a GRK5/6 double knockout mouse showed any signs of irregular cardiac development.

In a similar fashion to the GRK 2 and 4 subfamilies, the two ubiquitously expressed members of the arrestin family (βarr-1 and 2) also show distinct subcellular localisations when expressed in a variety of cell lines (Benmerah et al., 2003). βarr-1, like the GRK4 subfamily, is uniformly distributed throughout the cell and is found both in the cytoplasm and nucleus. In contrast βarr-2, like the GRK2 subfamily of GRKs, is cytosolic. βarr-2, but not βarr-1, contains an NES and shuttles through the nucleus. The nucleocytoplasmic shuttling of βarr-2 results in the cytoplasmic retention of nuclear βarr-2-binding partners, such as Mdm2 and activated JNK, thereby indirectly regulating gene transcription (Benmerah et al., 2003; Lefkowitz and Whalen, 2004). Like GRK5, βarr-2, other proteins required for receptor endocytosis such as epsin1, eps15 and clathrin assembly lymphoid myeloid leukaemia (CALM) exhibit nucleocytoplasmic shuttling (Benmerah et al., 2003). Eps15 exhibits transcriptional activity in GAL4 reporter assays, suggesting a potential role for this protein in regulating gene expression (Benmerah et al., 2003). Many membrane localised proteins which function in receptor desensitisation and internalisation thus appear to have multifunctional cellular roles including nuclear signalling. Like the majority of proteins that contain an NES, the GRK4 subfamily NES does not conform to the classical NES consensus sequence (L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI]) (Kutay and Guttinger, 2005; la Cour et al., 2004). The critical residues conform but the spacing of these residues differs from the classical NES consensus. Only one 'x' residue rather than 2 or 3 residues spaces the critical residues. Although to date I have failed to identify an NES in GRK2 the ability of this kinase to shuttle through the nucleus warrants further investigation.

6.1.2. GRK5 nuclear export is regulated by CaM

In Chapter 3, transfected and endogenous GRK5 was detected in the nuclei of HEp2 cells using fluorescent confocal microscopy (Figure 3.1). Similarly, Yi and colleagues have reported endogenous GRK5 in the nuclei of rat cardiac myocytes (Yi et al., 2002). Treatment of these cells with 12-o-tetradecanoylphorbol-13-acetate (TPA), a strong PKC activator, results in a more pronounced nuclear localisation of the endogenous kinase (Yi et al., 2002). GRK5 is itself a PKC substrate (Pronin and Benovic, 1997) and PKC-mediated GRK5 phosphorylation inhibits GRK5 receptor phosphorylation. I attempted to recapitulate this result by transiently overexpressing GRK5 in HEp2 cells however in this model system TPA treatment did not reproducibly induce nuclear accumulation of GRK5. This warrants further investigation in rat neonatal cardiac myocytes. However, Yi and colleagues (Yi et al., 2002), utilised six-month old adult rats in their studies and perhaps, since GRK5 has homology with homeodomain containing TFs which are known to be crucial for cardiac development, GRK5 has a different nuclear function(s) during heart development and maturation compared to that in terminally differentiated myocytes during adulthood. Conversely, in Chapter 3 the binding of Ca^{2+}/CaM to the N-terminal CaM-binding site of GRK5 is demonstrated to result in nuclear export of the kinase (Figure 3.5B). Combined, these observations suggest, that multiple regulatory mechanisms control the intracellular, and specifically nuclear, localisation of GRK5. Similarly to TPA-mediated PKC phosphorylation of GRK5, the binding of Ca^{2+}/CaM to this kinase potently inhibits GRK5-mediated GPCR phosphorylation (IC_{50}~50nM) (Sallese et al., 2000a). However, Ca^{2+}/CaM binding, in contrast to PKC

phosphorylation of GRK5, specifically prevents the interaction of GRK5 with phospholipids and activated receptors without directly affecting catalytic activity (Sallese et al., 2000a). Of the two identified CaM-binding sites in GRK5, the C-terminal site appears principally responsible for Ca^{2+}/CaM-mediated inhibition of membrane located substrate phosphorylation. Deletion of the C-terminal CaM binding site attenuates CaM-mediated inhibition of GRK5 phosphorylation of ROS *in vitro*. Whereas, mutation of the N-terminal site has no effect on CaM-mediated inhibition of GRK5 catalytic activity (Levay et al., 1998; Pronin et al., 1997). My thesis suggests an alternative role for the N-terminal CaM-binding site of GRK5; CaM-dependent nuclear export of the kinase (Chapter 3).

Mutation of this N-terminal CaM binding site (GRK5NTPB) results in nuclear retention of the mutant GRK5 (Chapter 3, Figure 3.5B). This indicates that CaM binding at the N-terminus of the kinase may retain GRK5 in the cytosol. Notably however, the N-terminal CaM-binding site of GRK5 is coincident with binding sites for PIP_2 and actin/α-actinin and the interaction of these ligands with GRK5 is likely to be competitive (Freeman et al., 1998). Indeed, a role for PIP_2, actin or α-actinin in retaining GRK5 in the cytosol seems likely since sequestration of membrane/cytosolic CaM does not result in the nuclear import of wildtype GRK5 (Chapter 3, Figure 3.4).

6.1.3. GRK5 nuclear export is regulated by the Gq-coupled M3-Muscarinic receptor

As described previously, Ca^{2+}/CaM-binding to GRK5 inhibits GRK5-mediated GPCR phosphorylation by inhibiting membrane localisation of the kinase.

Additionally, Ca^{2+}/CaM-binding stimulates autophosphorylation of GRK5 inhibiting kinase activity and further contributing to impaired receptor phosphorylation (Pronin and Benovic, 1997). The effects of CaM-binding on GRK5-mediated GPCR phosphorylation coupled with the observation that PKC-mediated GRK5 phosphorylation directly inhibits the catalytic activity of the kinase (Pronin and Benovic, 1997) suggests that agonist-occupied Gq-coupled receptors, and thus the M3MR, represent poor substrates for this kinase. Three lines of evidence are consistent with this hypothesis. Firstly, overexpression of catalytically inactive GRK6, but not GRK5, inhibits M3MR receptor desensitisation induced by endogenous GRKs in human neuroblastoma SH-SY5Y cells (Willets et al., 2003b). Secondly, mice lacking the GRK5 gene exhibit no difference in M3MR-mediated agonist-induced contraction of airway smooth muscle, when compared to wildtype littermates (Walker et al., 2004). Thirdly, overexpression of GRK5 in CHO cells stably expressing the M3MR fails to promote agonist-dependent internalisation of the receptor (Figure 3.7A, left hand panels). Although apparently not a GRK5 substrate the M3MR does, however, play a role in regulating the subcellular localisation of GRK5. Following activation of the M3MR GRK5 is translocated to the cytosol (Figure 3A); potential nuclear functions of this kinase may thus be negatively regulated by this receptor or perhaps less specifically Gq-coupled receptors.

6.2. The GRK4 subfamily are all localised to the nucleus

Since GRK5, a GRK4 subfamily member, but not GRK2, a GRK2 subfamily member is present in the nucleus of a range of tissue culture cells when transiently overexpressed, I sought to determine if this was a common shared feature of the

GRK4 subfamily. The data in Chapter 4 revealed unsuspected diversity within the GRK4 subfamily as well as highlighting the differences and similarities between the GRK subfamilies. Perhaps even questioning the rationale for grouping the GRKs into their current subfamilies.

6.2.1. The GRK4 subfamily all contain nuclear localisation sequences

In Chapter 4 I show that all members of the GRK4 subfamily contain functional nuclear localisation signals and that the NLS of GRK6 like that of GRK5, but not 4, bind DNA via this sequence. This data may suggest nuclear function(s) for GRK4 distinct from that of GRKs 5 and 6. GRK4 is the most divergent member of the GRK4 subfamily (Premont et al., 1999). In addition, the NLS of GRK4 is most similar in sequence and location to predicted putative NLSs in GRK1 and GRK7 rather than the DNA-binding NLSs of GRKs 5 and 6 (Figure 4.1) (Cokol et al., 2000).

Of the 4 splice variants of GRK4 only GRK4α has been shown capable of phosphorylating receptor substrates *in vitro* (ROS and β_2ARs). Interestingly, a fourth splice variant of GRK6 has been identified in mouse, GRK6D, which is catalytically inactive and entirely nuclear in localisation (Vatter et al., 2005). The GRK2 subfamily does not contain putative NLSs, as assessed by the PredictNLS search engine, suggesting that these kinases may be the only GRK family members without nuclear functions.

Data from Chapter 4 highlights both a structural diversity between the GRK2 and GRK4 subfamilies of GRKs and a functional diversity within the GRK4 subfamily. Additionally, it raises the provocative possibility that the GRK4 subfamily of GRKs

has signalling roles in the nucleus distinct from their characterised role of mediating GPCR desensitisation at the plasma membrane. Recent evidence has suggested that GPCRs are located in the nucleus (Gobeil et al., 2006), raising the intriguing possibility that since the GRK4 subfamily members are localized to the nucleus that they could also be regulating the activity of nuclear GPCRs as part of their nuclear function(s).

6.2.2. Differential localisation and DNA-binding properties of the GRK4 subfamily

In Chapter 3 I discovered that the nuclear export of GRK5 is promoted by Ca^{2+}, in particular by CaM, a ubiquitous Ca^{2+} sensor known to be a regulator of GRK function both at the membrane and in the cytosol. I examined whether the nuclear localisation of the other GRK4 subfamily members was also regulated by Ca^{2+}. Like GRK5, nuclear export of GRK6B and GRK6C is Ca^{2+}-dependent however GRK6A appears to be refractory to this regulatory mechanism (Chapter 4.4). This observation suggests that, like GRK5, the nuclear localisation of GRK6B and C could be promoted by activation of a Gαq-coupled GPCR, although this has yet to be demonstrated. CaM activation fails to promote nuclear export of GRK6A even though GRK6A-mediated rhodopsin phosphorylation is inhibited by CaM (Pronin et al., 1997). Investigating the features of GRK6A responsible for conferring insensitivity to Ca^{2+}-dependent nuclear export suggests a role for palmitoylation of the kinase in this process. Mutation of three C-terminal cysteine residues, previously shown to encompass the palmitoylation site(s) of GRK6A (GRK6A-3C3A), leads to the nuclear exclusion of the mutant enzyme (Chapter 4.4). GRK6A-3C3A also displays a reduced ability to bind DNA as

compared to wildtype GRK6A. This is a somewhat surprising finding since similar mutations in other membrane associated proteins, including the R7 family-binding protein and phospholipid scramblase I, are associated with their release from the plasma membrane and nuclear accumulation (Song et al., 2006a; Wiedmer et al., 2003). Additionally, palmitoylated GRK6A has previously been reported to be associated with membranes (Stoffel et al., 1994). In these studies, however, the methodology used to prepare the membrane fraction would be anticipated to result in nuclei contamination (Stoffel et al., 1994). A potential explanation of my results is a conformational dependence of GRK6A on palmitoylation i.e. palmitoylation may be responsible for maintaining GRK6A in a conformation in which its DNA-binding NLS is exposed and NES hidden. Consistent with a role for palmitoylation in regulating GRK6A conformation is the observation that a non-palmitoylatable mutant of GRK6A has impaired kinase activity, against both receptor and soluble substrates, as compared to its wildtype counterpart (Stoffel et al., 1998; Stoffel et al., 1994). However, since palmitoylation of GRK6A has been shown to increase its catalytic activity (Stoffel et al., 1998), perhaps GRK6A requires kinase activity in order to be exported from the nucleus. This is not true for GRK5 since GRK5NTPB is catalytically active, exclusively nuclear, but incapable of nuclear export (Chapter 3). That the nuclear export of the splice variants of GRK6 is differentially regulated suggests that a potentially common nuclear function for these enzymes may be affected differently upon activation of a specific GPCR.

Mechanisms regulating the nuclear localisation of GRK4 splice variants remain to be investigated. In HEp2 cells the wildtype, but interestingly not the ΔNLS, versions of

these kinases are relatively poorly expressed making such studies difficult to quantify. Differences between the effects of ionophore treatment on the nuclear localisation of GRK4α, β, γ and δ might, however, be anticipated. GRK4α, by homology with GRKs 5 and 6 is predicted to contain both N and C-terminal CaM-binding sites, and has been shown to bind directly to CaM *in vitro* (Sallese et al., 1997). In contrast, GRK4β is predicted to contain just the N-terminal CaM binding site and GRK4γ the C-terminal CaM binding site. GRK4δ is not predicted to bind CaM. If, as is the case with GRK5, CaM-binding to the N-terminal CaM-binding site causes the nuclear export of these enzymes then GRK4 α and β, but not γ and δ, may be sensitive to ionophore treatment. Therefore, it might be envisaged that whilst CaM might promote nuclear export of GRK4α and β, other regulatory mechanisms might regulate the other splice variants or indeed all of them.

6.3. Nuclear functions for the GRK4 subfamily?

The principle question raised by this thesis is what, if any, nuclear function is performed by the GRK4 subfamily of GRKs? I discuss in more detail putative functions of GRK5 in the nucleus in Section 6.4, but first I will address potential nuclear functions for GRKs 4 and 6.

6.3.1. Potential nuclear functions for GRK4

GRK4 exists as four alternatively spliced variants, α, β, γ and δ, and represents the least studied member of the GRK4 subfamily of GRKs. Since GRK4 shows limited tissue distribution in brain, kidney and testes/spermatocytes (Premont et al., 1996; Sallese et al., 1994; Sallese et al., 1997; Virlon et al., 1998), work on GRK4 has

focused largely on examining the ability of GRK4 to phosphorylate and mediate desensitisation of GPCRs coexpressed in these tissues. Relatively few substrates for GRK4 have been identified, certainly in a physiological setting. GRK4α, but not β, γ and δ, has been shown to phosphorylate rhodopsin *in vitro* (Sallese et al., 1997). All GRK4 splice variants however exhibit, to varying extents, desensitisation of the rat luteinizing hormone/chorionic gonadatrophin receptor in HEK293 cells as determined by a decrease in cAMP (cyclic adenosine monophosphate) signalling (Premont et al., 1996). GRK4α overexpression also causes desensitisation of the metabotropic glutamate receptor I (Sallese et al., 2000b), the Ca^{2+}-sensing receptor (Pi et al., 2005) and the phosphorylation independent desensitisation of the GABA B receptor (Perroy et al., 2003) in HEK293 and cerebellar granule cells. Purified GRK4α has been shown to phosphorylate purified β_2AR *in vitro* (Premont et al., 1996). GRK4δ may have a role in desensitizing human M2MR as identified in Cos-7 and BHK21 cells (Tsuga et al., 1998). GRK4γ has been shown to contribute to development of hypertension (Felder et al., 2002). A SNP identified in human GRK4γ results in a ligand-independent increase in catalytic activity of this splice variant in CHO cells and in a transgenic mice overexpressing the GRK4γ SNP (Table 1.1). This leads to increased desensitisation of the D1R in proximal tubule cells in the kidney resulting in defective sodium excretion from the body and consequently development of hypertension (Felder et al., 2002). A role for D1R in regulating the nuclear localisation of GRK4 in specific cell types such as neurons, kidney tubule cells and sperm thus warrants investigation.

The consensus NLS of GRK4 has been identified in ten nuclear proteins (Cokol et al.,

2000) including histone H2B from a variety of species including human, p53 in fish and heat shock factor protein in yeast. This consensus NLS is also found in the cell growth regulating nucleolar protein found in immature spermatocytes in mouse testis, a putative oncoprotein which promotes cell growth (Su et al., 1993). I might therefore tentatively suggest a role for GRK4 in cell growth or in cell cycle control. Indeed, a kinase RNAi screen in HeLa cervical carcinoma cells identified GRK4 as one kinase responsible for promoting cell survival (MacKeigan et al., 2005).

6.3.2. Potential nuclear functions for GRK6

The consensus NLS of GRKs 5 and 6 is found in a large number of sequence-specific homeobox-containing transcription factors and transcriptional DNA-binding co-factors in a range eukaryotes (Cokol et al., 2000), potentially suggesting these GRKs regulate transcription.

GRK6A, which contains a C-terminal PDZ-binding domain, binds to and phosphorylates the PDZ containing protein NHERF-1. NHERF-1 is an adaptor protein that was originally identified as a regulator of the Na^+/H^+ exchanger (Weinman et al., 1995). GRK6A phosphorylation of NHERF-1 maintains this adaptor in a hyperphosphorylated state which has been suggested to promote its oligomerisation and thus allows it to act as a cofactor to inhibit Na^+/H^+ exchange (Hall et al., 1999; Lau and Hall, 2001). In contrast cyclin-dependent kinase 2 phosphorylates NHERF-1 during mitosis and inhibits its oligomerisation and heteroligomerisation with NHERF-2 as well as with other binding partners (He et al., 2001). Cyclin-dependent kinase-mediated NHERF-1 phosphorylation thus presumably inhibits its function as a Na^+/H^+ exchanger regulatory co-factor. In the

hepatocellular carcinoma (HCC) cell line, NHERF-1 has been localised to the nucleus (Shibata et al., 2003). Furthermore, in HCC and a colorectal cancer cell line NHERF-1 increases β-catenin and T-cell factor dependent transcription (Shibata et al., 2003). NHERF-1 has also been reported to be required for cystic fibrosis transmembrane conductance regulator-dependent expression of the chemokine RANTES (Estell et al., 2003). Thus it appears that NHERF-1 participates in both cytosolic and nuclear signalling pathways. An NLS has not been identified in NHERF-1, perhaps GRK6A by binding to the PDZ domain of NHERF-1, regulates its nuclear localisation and function(s). This requires further investigation.

The crystal structure of GRK6A has recently been solved and suggests that this kinase may exist as a dimer (Lodowski et al., 2006). This is in contrast to the crystal structure elucidated for GRK2 that suggests a monomeric structure for this kinase (Lodowski et al., 2003a). The relative positions of the DNA-binding domains of GRK6A, the NLS (Chapter 4) and, by homology with GRK5 (Chapter 5), the N-terminal CaM binding region of GRK6 are shown in Figure 6.1. Lodowski and colleagues report that the region containing the NLS of GRK6, located between the αF-αG loop (Figure 6.1), blocks the peptide binding channel of GRK6, which accommodates protein residues phosphorylated by this kinase (Lodowski et al., 2006). This suggests that exposure of the NLS of GRK6 may be regulated by substrate binding. Exposure of the NLS of GRK6 would allow DNA binding. When GRK6 is a dimer, the two NLSs are located at the extremity of the protein (Figure 6.1) and might allow the dimer to wrap itself around DNA. Mutations of GRK6A, predicted to inhibit dimerisation, had no effect on GRK6A-mediated phosphorylation

of rhodopsin *in vitro* (Lodowski et al., 2006). It is thus possible that dimerisation is required for other, perhaps nuclear, functions of GRK6A. DNA-binding of many transcription factors/co-regulators is dimerisation-dependent (Kohler et al., 1999; Lee, 1992). It would thus be of interest to examine whether the dimerisation of GRK6A is required for DNA-binding (discussed further in Section 6.6.1). Potentially palmitoylation of GRK6A may promote the nuclear localisation of this kinase and its interaction with DNA by regulating its dimerisation status. It has been reported that lipid modification, in particular, palmitoylation is required for efficient oligomerisation of sphingosine kinase type Ib, Post-synaptic density protein 95 and VIP21-caveolin (Christopherson et al., 2003; Kihara et al., 2006; Monier et al., 1996). Determination of the crystal structures of GRK5 and GRK4 that, respectively, do and don't bind DNA might elucidate whether the ability to dimerise is a feature specific to the DNA-binding GRKs (GRKs 5 and 6) or is a common feature of the GRK4 subfamily of GRKs.

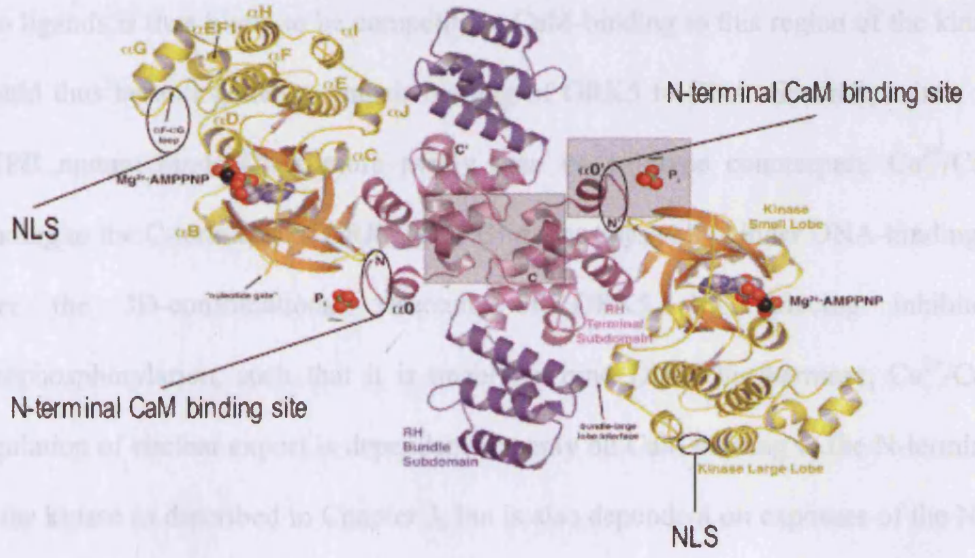


Figure 6.1. Crystal Structure of GRK6 homodimer.

GRK6 forms a homodimer at the surface of the RH domain. The relative positions of the proposed DNA binding regions of GRK6, the NLS and N-terminal CaM binding region (by homology to GRK5), are indicated. Mg^{2+}·AMPPNP is bound within each active site. Nitrogen atoms are coloured blue, oxygen is red, phosphate is green and magnesium black. Carbon atoms are coloured according to the domain in which they are found except for those in AMPPNP that are grey (Reproduced from Lodowski et al., 2006).

6.4. GRK5 inhibits hypertrophy in cardiac myocytes

6.4.1. Structural determinants of GRK5 regulate its DNA-binding and nuclear export

I used a panel of GRK5 mutants in Chapter 5 to determine what function(s) GRK5 might have in the nucleus. Characterisation of these mutants also revealed information about how GRK5 may bind to DNA, at least *in vitro* and, how DNA-binding ability and nuclear export of GRK5 may be regulated by CaM.

Ca^{2+}/CaM binding to GRK5 is postulated to inhibit DNA-binding in two ways. Firstly, the NTPB domain of GRK5 binds both DNA and CaM, the binding of these two ligands is thus likely to be competitive. CaM-binding to this region of the kinase would thus be anticipated to impair binding of GRK5 to DNA. Secondly, since the CTPB mutant binds DNA more avidly than its wildtype counterpart, Ca^{2+}/CaM binding to the C-terminus of GRK5 (CTPB) might physically hinder DNA-binding or alter the 3D-confomational structure of GRK5, by inducing inhibitory autophosphorylation, such that it is unable to bind DNA. Furthermore, Ca^{2+}/CaM regulation of nuclear export is dependent not only on CaM binding to the N-terminus of the kinase as described in Chapter 3, but is also dependent on exposure of the NES (Chapter 5.1.1). Perhaps CaM binding to GRK5, which inhibits DNA-binding also

results in exposure of its NES promoting its nuclear export. CaM and other Ca^{2+} sensing proteins, such as Calreticulin, have been shown to contribute to the nuclear export of other DNA-binding nuclear proteins. Calreticulin, a Ca^{2+} binding protein originally identified in the lumen of the endoplasmic reticulum (ER), binds directly to the DNA-binding domain of glucocorticoid receptors (DeFranco, 2001). This interaction inhibits DNA-binding and mediates nuclear export of the nuclear hormone receptor (DeFranco, 2001). In a similar vein, Ca^{2+}/CaM binds to the DNA-binding domain of members of the basic-helix-loop-helix (bHLH) family of transcription factors to inhibit their interaction with DNA (Corneliussen et al., 1994; Hermann et al., 1998; Onions et al., 1997; Onions et al., 2000; Saarikettu et al., 2004). Regulation of nuclear-protein export by Ca^{2+} signalling thus appears to be a common mechanism whereby TF activity might be inhibited. As discussed in Chapter 5, upon nuclear export of GRK5, a range of molecules including phospholipids (e.g. PIP_2), actin/α-actinin and CaM could compete for binding to the NTPB of GRK5, regulating its catalytic activity towards either membrane bound/receptor (PIP_2) or soluble substrates (actin/α-actinin and CaM).

In order to attempt to address the question of what function(s) GRK5 may be carrying out in the nucleus, I turned to the literature in an attempt to identify leads that might suggest putative nuclear roles for this kinase. Notably, there is some evidence linking GRK5 expression to changes in gene expression (Eckhart et al., 2000). Cardiac hypertrophy, the physiological response of the heart to an increased work load, is associated with transcriptional activation of genes encoding embryonic markers, including ANF, α-skeletal actin and β-MHC (reviewed in Chien, 1999). Transgenic

mice expressing a constitutively activated mutant of the α_{1B}AR in the heart exhibit myocardial hypertrophy and have elevated DAG content and ventricular ANF expression (Eckhart et al., 2000; Milano et al., 1994). Interestingly, concomitant expression of GRK5 did not affect myocardial DAG content but did significantly attenuate constitutively active mutant-α_{1B}AR-induced hypertrophy and ANF expression (Eckhart et al., 2000). Thus the cardiac overexpression of GRK5 whilst not affecting α_{1B}AR/Gq-coupling does apparently inhibit cardiac gene transcription. It is tempting to speculate that the ability of GRK5 to bind DNA may explain its differential effects on receptor function and gene transcription in this model system.

Endogenous GRK5 displays a more nuclear localisation in cardiac myocytes derived from spontaneously hypertensive heart failure (SHHF) rats than in age-matched control animals (Yi et al., 2002). The SHHF rat represents a genetic model for cardiac hypertrophy and the more nuclear localisation of GRK5 in these cells may reflect either, a role for GRK5 in hypertrophic gene expression, or alternatively, an adaptive response to hypertrophy. This second explanation would serve to reconcile the results obtained using SHHF rats with those obtained using transgenic mice expressing constitutively active α_{1B}AR in the heart, where co-expression of GRK5 inhibits gene expression. Since βarr-1 translocates to the nucleus upon δ-opioid receptor activation and is found in complex with the histone acetyltransferase p300 (Kang et al., 2005), it is tempting to speculate that GRK5 is in a similar signalling complex temporally and spatially regulating transcription by modifying chromatin. In the case of GRK5, however, GRK5-dependent changes in chromatin structure may be anticipated to inhibit gene transcription. Interestingly, βarr-1 does not contain a NLS or NES yet

has been shown to have a nuclear function (Kang et al., 2005). Perhaps GRK2 which also does not contain a NLS or NES, as far as I have been able to assess to date, might also function in the nucleus, gaining import by binding to a protein which contains nuclear import/export sequences. Since GRK2 is known to be involved in progression of heart disease this would be a new and interesting line of enquiry.

6.4.2. GRK5 nuclear catalytic activity and DNA-binding ability are required for rescue of cardiac hypertrophy

In Chapter 5, I recapitulated the published finding that GRK5 can rescue PE-induced cardiac hypertrophy in rat myocytes *ex vivo* by overexpressing GRK5 or GRK5ΔNES, both of which are present in the nucleus and can bind DNA *in vitro* (Eckhart et al., 2000). Moreover, I went on to demonstrate that this rescue requires kinase activity and the ability of GRK5 to bind DNA. Importantly, I demonstrate that GRK5 inhibits hypertrophy induced by the activation of signalling pathways downstream of the GPCR, since GRK5 inhibits hypertrophy induced by a PLC activator. This data suggests that it is not GRK5-dependent desensitisation of GPCRs that is responsible for GRK5-mediated inhibition of hypertrophy. Kinase activity however, appears to be essential as a kinase dead mutant of GRK5 cannot rescue hypertrophy. The DNA-binding ability of GRK5 is also required to rescue hypertrophy since the ΔNLS mutant of GRK5, which does not bind DNA *in vitro,* does not inhibit hypertrophy progression *ex vivo* (Chapter 3). Similarly, overexpression of GRK5NTPB, which is catalytically active (although reduced compared to wildtype) but does not bind DNA *in vitro* (Chapter 3), cannot rescue hypertrophy. Unexpectedly perhaps, it seems that GRK5 kinase activity and DNA-

binding ability are both necessary for impeding advancement of hypertrophy in myocytes. The potential nuclear substrates of GRK5 and functional consequences of GRK5 DNA-binding are not yet understood, but merit further investigation.

Cardiac hypertrophy frequently deteriorates into congestive HF and a wealth of literature implicates a role for the GRKs in the development of HF phenotypes. Both GRK5 and GRK2 are upregulated in animal models of HF, and elevated GRK2 levels are associated with congestive HF in humans (Penela et al., 2006). Coronary artery ligation in rats reveals, however, that whereas myocardial GRK2 levels increase early after induction of myocardial infarction, GRK5 upregulation occurs at a later stage (Vinge et al., 2001). Furthermore, immunohistochemical analysis of myocardial tissue reveals that GRK2 is predominantly found in endothelial cells whereas GRK5 is found mainly in myocytes (Vinge et al., 2001). This suggests that GRKs 2 and 5 may be required for different events in the myocardial remodelling process leading to HF. Consistent with this hypothesis the subcellular distribution of GRK2 and 5 in myocytes from SHHF rats as they progress, with age, from concentric to eccentric hypertrophy and to congestive HF, is very different (Yi et al., 2005). GRK2 becomes progressively localised to the intercalated discs whilst GRK5 redistributes from the cytoplasm to the nucleus (Yi et al., 2005). These observations are consistent with the data reported here showing that GRK5, but not GRK2, contains a NES and a DNA-binding NLS. Notably, the nuclear redistribution of GRK5 observed in the myocytes from SHHF rats precedes myocyte lengthening and shows a similar time course to ANF expression. Thus, these authors postulated a role for GRK5 in positively regulating hypertrophic gene transcription (Yi et al., 2005). In this thesis GRK5

overexpression inhibits PE-induced hypertrophy of rat myocytes *ex vivo*. If GRK5 does indeed positively regulate transcription of specific genes then the inhibition of hypertrophy observed here may be explained by mislocalisation of GRK5-interacting transcription factors/co-regulators upon kinase overexpression. Alternatively, the nuclear translocation of GRK5 observed during progression to HF in the SHHF rats may reflect an adaptive response to inappropriate gene expression. In this case overexpression of GRK5 in rat myocytes would be anticipated to inhibit hypertrophy, by increasing the amount of nuclear GRK5 and transcriptional repression. I anticipate that use of the GRK5 mutants characterised in this study in isolated rat myocytes may ultimately lead to an understanding of the molecular events associated with GRK5-mediated inhibition of hypertrophy.

6.5. Experimental weaknesses of this thesis

In Chapters 3 and 4 I relied on the use of immortalised cell lines overexpressing wildtype and mutant proteins of interest. These systems, while quick and easy to use to address specific scientific questions do not always accurately reflect the subcellular localisation of proteins in physiological relevant systems. Immortalised cell lines were however useful for initially identifying that the GRK4 subfamily localised to the nucleus. I was also able to determine that endogenous GRK5 is localised to the nucleus of HEp2 cells. Further work is required to determine if endogenous GRK4 subfamily members display a nuclear localisation in other cell types. GRK5 is highly expressed in cardiac tissue (Yi et al., 2002), whilst GRK6 is present in lymphocytes (Fong et al., 2002), GRK4 shows a limited expression pattern, being expressed predominantly in the testes, kidney and brain (Felder et al., 2002; Premont et al.,

1996; Sallese et al., 1994). Due to a lack of time and access to suitable tissue-samples, I did not pursue this avenue further although it is important to show nuclear localisation of the GRK4 subfamily members in their physiologically relevant settings. The use of more physiologically relevant primary cells or tissue specific derived cell lines would also facilitate studies designed to investigate the regulation of the nuclear localization of GRK4 subfamily members and determine if Ca^{2+} signalling is a *bona fide* mechanism for regulating their subcellular localisation.

Due to low expression of GRK4 when transfected in HEp2 cells I was unable to investigate the regulatory mechanisms controlling nuclear localisation of this kinase. It might be easier to determine how GRK4 is regulated using cells expressing this kinase and its splice variants endogenously, e.g. in neurons. Interestingly, the ΔNLS mutants of the GRK4 splice variants expressed well in HEp2 cells suggesting that nuclear localised GRK4 when overexpressed may lead to cell death.

To determine nuclear functions for GRK5 I did use experimentally relevant primary cells expressing endogenous ARs, rat neonatal cardiac myocytes. However, again I used overexpression of mutant constructs to investigate the mechanism of GRK5-mediated rescue of hypertrophy. Whilst this was useful for determining that GRK5 requires nuclear kinase activity and DNA-binding capability to inhibit PE-induced hypertrophy in myocytes *ex vivo*, RNAi of endogenous GRK5, or myocytes isolated from GRK5 knockout mice might yield additional insights into the role of GRK5 in regulating progression to a hypertrophic phenotype.

I did not address directly whether GRK5-mediated phosphorylation and therefore desensitisation of ARs in myocytes contributes to the mechanism via which GRK5

overexpression prevents development of hypertrophy. Following PE activation of ARs in myocytes I could monitor PIP_2 hydrolysis by measuring the amount of IP_3 produced to establish if Gq-coupled signalling is attenuated when GRK5 and mutants thereof are overexpressed. If IP_3 production is not decreased in myocytes expressing GRK5 compared to untransfected cells, then we can be confident that GRK5 is preventing hypertrophy via a nuclear mechanism rather than via desensitisation of ARs.

6.6. Future Directions

6.6.1. GRK6

It would be very interesting to carry out experiments to determine in more detail how the nuclear localization of GRK6 is regulated. I characterised in detail the mechanism of regulation of GRK5 nuclear localisation and DNA-binding by the Ca^{2+} sensor, CaM. I would hypothesise that since GRKs 6B and C are exported from the nucleus after application of Ca^{2+} ionophore that their regulation may be similar to GRK5 and involve CaM binding to the N-terminal NTPB. More interestingly though, GRK6A nuclear export does not appear to be regulated by Ca^{2+} and therefore is unlikely to be mediated by CaM. Since one of the defining features of GRK6A compared to it other splice variants is a C-terminal PDZ-binding motif at its C-terminus, it would be interesting to determine if GRK6A has any unique nuclear binding partners compared to GRKs 6B and C. This might be achieved using a Yeast two-hybrid system, or by immunoprecipitating GRK6 splice variants from the nucleus and identifying binding partners by mass spectrometry. An alternative approach would be to use fusion proteins of the GRK6 splice variants over which nuclear lysates have been passed and

again determine novel binding partners for GRK6A by mass spectrometry. This might give clues regarding the nuclear function of GRK6A. Performing these experiments using GRK6A-flag, which has no functional PDZ motif, would distinguish nuclear binding partners that interact with GRK6A via a PDZ interaction.

It would be interesting to determine if GRK6A is present in a complex with NHERF-1 in the HCC cell line since these molecules have been localised to the nucleus and shown to increase transcription mediated by β-catenin and the T-cell transcription factor in these cells (Shibata et al., 2003). Moreover, using luciferase based transcription assays the ability of wildtype GRK6A (which binds DNA *in vitro*) or GRK6A-3C3A (which binds DNA poorly) to regulate NHERF-1-dependent gene transcription could be assessed (Hall et al., 1999; Shibata et al., 2003). Overexpressing GRK6A, GRK6AΔNLS and GRK6A-flag (ablated PDZ domain) with NHERF-1 might reveal whether GRK6A regulates NHERF-1 nuclear functions by mediating NHERF-1 nuclear localisation since NHERF itself does not contain an NLS.

Overexpression of GRK6 in myocytes to determine if this GRK4 subfamily member can prevent development of myocyte hypertrophy similarly to GRK5 would be worthwhile if only to establish if there is functional redundancy within the GRK4 subfamily. Since mouse GRK6D, a fourth mouse specific GRK6 splice variant, is nuclear and catalytically inactive, determining if overexpression of this splice variant in mouse cardiac myocytes can inhibit hypertrophy might indicate if this kinase has a nuclear function even in the absence of kinase activity. Since GRK5 appears to require both kinase activity and DNA-binding ability for rescue of hypertrophy, use

of GRK6D, a naturally occurring splice variant, might be useful to dissect GRK6 nuclear functions which require DNA-binding ability versus those which require kinase activity.

The crystal structure of GRK6 suggests this kinase exists as a dimer (Lodowski et al., 2006). It would be interesting to determine if dimerisation, which is not required for GPCR phosphorylation, is actually required for GRK6 DNA-binding. Preliminary data using mutants of GRK6 in which dimerisation is predicted to be impaired suggests that indeed dimerisation may be required for DNA binding (Figure 6.2, DNA-binding carried out as described in Materials & Methods). In these mutants basic amino acids in the dimerisation domain are mutated to negatively charged, acidic amino acids. Thus I cannot exclude the possibility that these mutants fail to bind DNA simply because it too is a negatively charged molecule. Generation of mutants which inhibit dimerisation but do not change the overall local charge of the dimerisation site might address this question more convincingly. Since many transcription factors act as dimers in order to 'clamp' round DNA (Lee, 1992), this may not be such an inconceivable hypothesis.

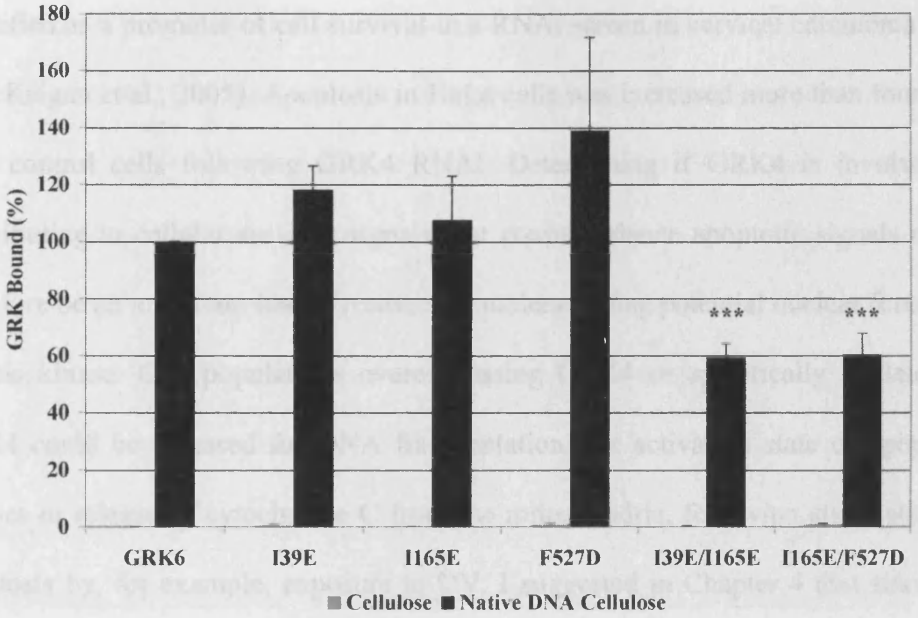


Figure 6.2. GRK6 double dimerisation mutants (I39E/I165E and I156/F527D), but not single dimerisation mutants, show a reduced affinity for DNA *in vitro*.

Lysates of cells expressing GRK6 and mutants thereof (~15µg) were incubated with native DNA-cellulose or ssDNA-cellulose (data not shown) and after extensive washing the amount of bound GRK was determined by Western blot analysis as described in the Materials & Methods. The amount of GRK6A bound to DNA-cellulose (~20%) was normalised to 100%. The data shown represent the mean values ± standard errors of the mean for at least three separate determinations. ***, $P<0.001$.

6.6.2. GRK4

GRK4 has been shown to desensitise certain neuronal metabotropic GPCRs. Confocal microscopy of the cellular localisation of GRK4 before and after stimulation of the metabotropic glutamate or GABA B receptor in neurons might therefore reveal mechanisms whereby the nuclear localisation of this enzyme is regulated. Since the NLS of GRK4 is non-DNA-binding but found in a number of proteins involved in cell growth, it would also be interesting to carry out assays to determine if GRK4 overexpression or RNAi alters cell growth or cell cycle progression. GRK4 was

identified as a promoter of cell survival in a RNAi screen in cervical carcinoma cells (MacKeigan et al., 2005). Apoptosis in HeLa cells was increased more than four-fold over control cells following GRK4 RNAi. Determining if GRK4 is involved in contributing to cellular survival signals that counterbalance apoptotic signals might therefore be an important line of research in understanding potential nuclear functions of this kinase. Cell populations overexpressing GRK4 or specifically depleted in GRK4 could be assessed for DNA fragmentation, the activation state of apoptotic kinases or release of cytochrome C from the mitochondria, following stimulation of apoptosis by, for example, exposure to UV. I suggested in Chapter 4 that since the NLS of GRK4 and the GRK1 subfamily share homology and are not predicted to bind DNA, that GRK4 might best be grouped with the GRK1 subfamily. Lack of GRK1 (rhodopsin kinase) in mice results in apoptotic retinal degeneration (Chen et al., 1999). It would therefore be interesting to determine if both the GRK1 subfamily and GRK4 function to regulate apoptosis.

6.6.3. GRK5

Since GRK5 is the best characterised member of the GRK4 subfamily, investigation of its potential role in inhibiting cardiac hypertrophy would be predicted to advance more rapidly than studies aimed at elucidating nuclear functions of GRKs 4 and 6.

Following on from experiments I described in Chapter 5, it would be exciting to investigate the molecular mechanisms underlying the requirement for the DNA-binding ability and kinase activity of GRK5 for inhibition of hypertrophy. Nuclear substrates of GRK5 may be identified by transfecting cells with wildtype and catalytically inactive GRK5 and subsequently labelling with [^{32}P] orthophosphate.

Two-dimensional gel electrophoresis and autoradiography of lysates of the nuclei of these cells may reveal proteins which are specifically phosphorylated following GRK5 overexpression. Identification by Mass spectrometry of potential nuclear substrates of GRK5 may suggest nuclear signalling pathways regulated by this kinase. Initially, these experiments could be performed in cultured neonatal cardiomyocytes (H9C2 cells) as transfection of these cells is more efficient than transfection of cardiac myocytes.

To address the role of GRK5 in the inhibition of cardiac hypertrophy *ex vivo* the DNA-binding specificity of GRK5 needs to be assessed. I tried unsuccessfully to identify a DNA-binding site for GRK5 using CASTing (cyclic amplification and selection of targets) (Wright and Funk, 1993) and a modified chromatin immunoprecipitation (ChIP) technique (Weinmann and Farnham, 2002; Wells and Farnham, 2002) in CHO cells. CASTing involves mixing purified GRK5 with a library of degenerate oligonucleotides flanked at each end by a defined primer sequence for PCR amplification. Immunoprecipitation of GRK5, isolation of DNA fragments and sequencing of these amplified fragments could in theory reveal, after several rounds, the preferred DNA-binding consensus sequence of GRK5. This sequence could be blasted against promoter regions of the human genome to determine possible target genes that GRK5 might regulate. It would be interesting to see if any of the genes which are upregulated or downregulated during cardiac hypertrophy are identified from such a blast search. An alternative approach used was a modified ChIP technique which involves cross-linking GRK5 to chromatin in cells, sonicating the nuclei and purifying, by immunoprecipitation, GRK5 linked to DNA.

Reversal of cross-links reveals, in theory, DNA fragments which can be sequenced to determine the GRK5 DNA-binding consensus sequence but in addition, any protein binding partners of GRK5 in the nucleus may also be identified by Mass spectrometry. The additional benefit of this approach is that identification of nuclear binding partners of GRK5 might indicate the nuclear role of GRK5. Although initial attempts using this technique failed, in hindsight it would have been better to carry out these experiments in myocytes. Another approach would be to carry out a 'classical' ChIP protocol, and immunoprecipitate GRK5 and probe for genes which are known to be upregulated in cardiac hypertrophy such as ANF, *c-fos* or β-MHC.

It might be useful to take a candidate approach to predict nuclear binding partners of GRK5. βarr-1 is known to bind to the HAT p300 and recruit it to certain promoters where it acetylates chromatin and promotes initiation of transcription (Kang et al., 2005). Since GRK5 overexpression in myocytes inhibits hypertrophy perhaps GRK5 suppresses hypertrophic transcription by regulating similar molecules (HDACs or HATs). I carried out some preliminary experiments to attempt to understand if GRK5 is involved in chromatin remodelling (Figure 6.3). I investigated histone acetylation states in Cos-7 cells expressing wildtype GRK5 or GRK5K215R (catalytically inactive GRK5). Transfected and untransfected cells were scored (positive or negative) for immunofluorescently labelled acetylated histone H3, H4 or phosphorylated histone H3, the results are represented graphically in Figure 6.3.

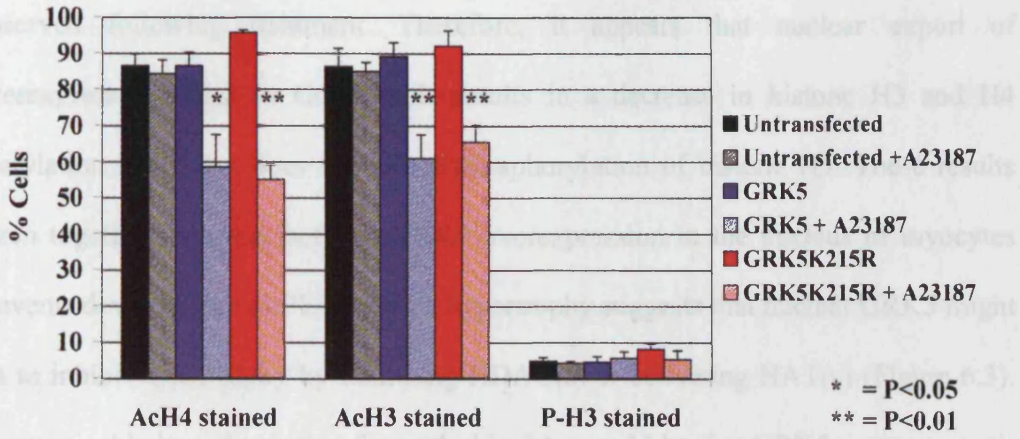


Figure 6.3. Nuclear export of GRK5 inhibits acetylation of histones H3 and H4.

Cos-7 cells were transiently transfected with wildtype GRK5 or GRK5K215R and either left untreated or treated with calcium ionophore, A23187 (25μM, 15 min). Cells expressing GRK5 constructs or untransfected cells were subject to immunofluorescent labelling as described in the Materials & Methods. Transfected and untransfected cells were scored (positive or negative) for acetylated histones H3 and H4 or phosphorylated histone H3. Nuclear export of wildtype GRK5 following A23187 treatment, results in a significant reduction in acetylation of histone H3 and H4 compared to untransfected cells. The same result was observed with catalytically inactive GRK5 (K215R) suggesting that GRK5 kinase activity is not required for this effect. No significant alteration in phosphorylated histone H3 was observed between untransfected and transfected cells with or without A23187 treatment. *, P<0.05; **, P<0.01.

Following Ca^{2+} ionophore treatment and nuclear export of wildtype GRK5, I observed a 28% decrease in both acetyl-H3 and –H4 staining compared to untreated cells (compare solid blue bars with crossed blue bars). A significant decrease in acetyl-H3 or –H4 staining was not observed in untransfected cells following A23187 treatment (compare black bars with black crossed bars). Importantly, the same effect was observed in cells transfected with GRK5K215R; 41% fewer cells showed aceyl-H3 staining and 27% fewer cells showed acetyl-H4 staining compared to untreated cells (compare red bars to crossed red bars) suggesting that this effect is not mediated by GRK5 kinase activity. No change in the phosphorylation status of histone H3 was

observed following treatment. Therefore, it appears that nuclear export of overexpressed GRK5 in Cos-7 cells results in a decrease in histone H3 and H4 acetylation levels but does not affect phosphorylation of histone H3. These results taken together with the fact that GRK5 overexpression in the nucleus of myocytes prevents development of PE-mediated hypertrophy suggests that nuclear GRK5 might act to inhibit hypertrophy by inhibiting HDAC(s) or activating HAT(s) (Figure 6.3). A conceivable hypothesis that fits with this data would be that GRK5 promotes anti-hypertrophic gene transcription, such as the α-MHC gene, which is downregulated during hypertrophy.

An alternative approach to examine the role of GRK5 in cardiac dysfunction would be to generate GRK5ΔNLS and GRK5ΔNES knock-in mice to determine if these mice exhibit resistance or susceptibility to the development of hypertrophy and heart disease. Any such phenotype might aid in unraveling the molecular mechanism(s) whereby GRK5 modulates myocyte function. Completion of these experiments would further our understanding of the mechanisms regulating GRK4 subfamily nuclear localisation and function(s) in the nucleus.

6.7. Concluding remarks

In conclusion, in this thesis I present evidence that strengthens the emerging concept that GRKs act not only to desensitise GPCRs but are also involved in downstream signalling events. The list of non-receptor substrates for GRKs is growing rapidly but this study provides evidence for novel properties of the GRK4 subfamily including regulated nuclear localisation and DNA-binding. These studies have also revealed diversity among the GRK4 subfamily members and also between the GRK2 and 4

subfamilies. I have showed that GRKs 5 and 6 bind DNA *in vitro* and further work is required to understand the mechanism and relevance of this finding in a cellular setting. Importantly, I have shown that GRK5 may play a role in regulating cardiac hypertrophy since it appears to represses such a phenotype in cardiac myocytes *ex vivo*. This finding suggests that GRK5 may represent a therapeutic target for the management of cardiac hypertrophy and heart disease.

7. References

Agell, N., Aligue, R., Alemany, V., Castro, A., Jaime, M., Pujol, M. J., Rius, E., Serratosa, J., Taules, M., and Bachs, O. (1998). New nuclear functions for calmodulin. Cell Calcium *23*, 115-121.

Akazawa, H., and Komuro, I. (2003). Roles of cardiac transcription factors in cardiac hypertrophy. Circ Res *92*, 1079-1088.

Akhter, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., and Koch, W. J. (1998). Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. Science *280*, 574-577.

Antonsson, A., Hughes, K., Edin, S., and Grundstrom, T. (2003). Regulation of c-Rel nuclear localization by binding of Ca2+/calmodulin. Mol Cell Biol *23*, 1418-1427.

Antos, C. L., McKinsey, T. A., Dreitz, M., Hollingsworth, L. M., Zhang, C. L., Schreiber, K., Rindt, H., Gorczynski, R. J., and Olson, E. N. (2003). Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors. J Biol Chem *278*, 28930-28937.

Antos, C. L., McKinsey, T. A., Frey, N., Kutschke, W., McAnally, J., Shelton, J. M., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002). Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A *99*, 907-912.

Backs, J., and Olson, E. N. (2006). Control of cardiac growth by histone acetylation/deacetylation. Circ Res *98*, 15-24.

Bae, Y. S., Lee, T. G., Park, J. C., Hur, J. H., Kim, Y., Heo, K., Kwak, J. Y., Suh, P. G., and Ryu, S. H. (2003). Identification of a compound that directly stimulates phospholipase C activity. Mol Pharmacol *63*, 1043-1050.

Baker, A. J., Redfern, C. H., Harwood, M. D., Simpson, P. C., and Conklin, B. R. (2001). Abnormal contraction caused by expression of G(i)-coupled receptor in transgenic model of dilated cardiomyopathy. Am J Physiol Heart Circ Physiol *280*, H1653-1659.

Barrett, T. B., Hauger, R. L., Kennedy, J. L., Sadovnick, A. D., Remick, R. A., Keck, P. E., McElroy, S. L., Alexander, M., Shaw, S. H., and Kelsoe, J. R. (2003). Evidence that a single nucleotide polymorphism in the promoter of the G protein receptor kinase 3 gene is associated with bipolar disorder. Mol Psychiatry *8*, 546-557.

Benmerah, A., Scott, M., Poupon, V., and Marullo, S. (2003). Nuclear functions for plasma membrane-associated proteins? Traffic *4*, 503-511.

Bers, D. M., and Guo, T. (2005). Calcium signaling in cardiac ventricular myocytes. Ann N Y Acad Sci *1047*, 86-98.

Billeter, M. (1996). Homeodomain-type DNA recognition. Prog Biophys Mol Biol *66*, 211-225.

Bisognano, J. D., Weinberger, H. D., Bohlmeyer, T. J., Pende, A., Raynolds, M. V., Sastravaha, A., Roden, R., Asano, K., Blaxall, B. C., Wu, S. C., *et al.* (2000). Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. J Mol Cell Cardiol *32*, 817-830.

Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J., and Bunting, S. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J Pharmacol Exp Ther *255*, 756-768.

Booz, G. W., Day, J. N., and Baker, K. M. (2002). Interplay between the cardiac renin angiotensin system and JAK-STAT signaling: role in cardiac hypertrophy, ischemia/reperfusion dysfunction, and heart failure. J Mol Cell Cardiol *34*, 1443-

1453.

Boulikas, T. (1993). Nuclear localization signals (NLS). Crit Rev Eukaryot Gene Expr *3*, 193-227.

Bowling, N., Walsh, R. A., Song, G., Estridge, T., Sandusky, G. E., Fouts, R. L., Mintze, K., Pickard, T., Roden, R., Bristow, M. R., *et al.* (1999). Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart. Circulation *99*, 384-391.

Braz, J. C., Bueno, O. F., Liang, Q., Wilkins, B. J., Dai, Y. S., Parsons, S., Braunwart, J., Glascock, B. J., Klevitsky, R., Kimball, T. F., *et al.* (2003). Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. J Clin Invest *111*, 1475-1486.

Bueno, O. F., De Windt, L. J., Lim, H. W., Tymitz, K. M., Witt, S. A., Kimball, T. R., and Molkentin, J. D. (2001). The dual-specificity phosphatase MKP-1 limits the cardiac hypertrophic response in vitro and in vivo. Circ Res *88*, 88-96.

Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Klevitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C. F., *et al.* (2000). The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. Embo J *19*, 6341-6350.

Cant, S. H., and Pitcher, J. A. (2005). G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol Biol Cell *16*, 3088-3099.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Carman, C. V., Parent, J. L., Day, P. W., Pronin, A. N., Sternweis, P. M.,

Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999). Selective Regulation of Galpha q/11 by an RGS Domain in the G Protein-coupled Receptor Kinase, GRK2. Journal of Biological Chemistry *274*, 34483-34492.

Carman, C. V., Som, T., Kim, C. M., and Benovic, J. L. (1998). Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. Journal of Biological Chemistry *273*, 20308-20316.

Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004). Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol *24*, 8467-8476.

Chen, C. K., Burns, M. E., Spencer, M., Niemi, G. A., Chen, J., Hurley, J. B., Baylor, D. A., and Simon, M. I. (1999). Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. Proc Natl Acad Sci U S A *96*, 3718-3722.

Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995). Ca(2+)-dependent interaction of recoverin with rhodopsin kinase. J Biol Chem *270*, 18060-18066.

Chen, E. P., Bittner, H. B., Akhter, S. A., Koch, W. J., and Davis, R. D. (2001). Myocardial function in hearts with transgenic overexpression of the G protein-coupled receptor kinase 5. *71*, 1320-1324.

Chen, L., and Feany, M. B. (2005). Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. Nat Neurosci *8*, 657-663.

Chien, K. R. (1999). Stress pathways and heart failure. Cell *98*, 555-558.

Chin, D., and Means, A. R. (2000). Calmodulin: a prototypical calcium sensor. Trends Cell Biol *10*, 322-328.

Choukroun, G., Hajjar, R., Kyriakis, J. M., Bonventre, J. V., Rosenzweig, A., and Force, T. (1998). Role of the stress-activated protein kinases in endothelin-induced cardiomyocyte hypertrophy. J Clin Invest *102*, 1311-1320.

Christopherson, K. S., Sweeney, N. T., Craven, S. E., Kang, R., El-Husseini Ael, D., and Bredt, D. S. (2003). Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks. J Cell Sci *116*, 3213-3219.

Chuang, T. T., LeVine, H., 3rd, and De Blasi, A. (1995). Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. J Biol Chem *270*, 18660-18665.

Chuang, T. T., Paolucci, L., and De Blasi, A. (1996). Inhibition of G protein-coupled receptor kinase subtypes by Ca2+/calmodulin. Journal of Biological Chemistry *271*, 28691-28696.

Cideciyan, A. V., Zhao, X., Nielsen, L., Khani, S. C., Jacobson, S. G., and Palczewski, K. (1998). Null mutation in the rhodopsin kinase gene slows recovery kinetics of rod and cone phototransduction in man. Proc Natl Acad Sci U S A *95*, 328-333.

Cobelens, P. M., Kavelaars, A., Heijnen, C. J., Ribas, C., Mayor, F., Jr., and Penela, P. (2006). Hydrogen peroxide impairs GRK2 translation via a calpain-dependent and cdk1-mediated pathway. Cell Signal.

Cokol, M., Nair, R., and Rost, B. (2000). Finding nuclear localization signals. EMBO reports *1*, 411-415.

Corneliussen, B., Holm, M., Waltersson, Y., Onions, J., Hallberg, B., Thornell, A., and Grundstrom, T. (1994). Calcium/calmodulin inhibition of basic-helix-loop-helix transcription factor domains. Nature *368*, 760-764.

Crabtree, G. R., and Olson, E. N. (2002). NFAT signaling: choreographing the social lives of cells. Cell *109 Suppl*, S67-79.

D'Angelo, D. D., Sakata, Y., Lorenz, J. N., Boivin, G. P., Walsh, R. A., Liggett, S. B., and Dorn, G. W., 2nd (1997). Transgenic Galphaq overexpression induces cardiac contractile failure in mice. Proc Natl Acad Sci U S A *94*, 8121-8126.

Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature *390*, 88-91.

DeFranco, D. B. (2001). Nuclear export: DNA-binding domains find a surprising partner. Curr Biol *11*, R1036-1037.

Delmas, P., Abogadie, F. C., Buckley, N. J., and Brown, D. A. (2000). Calcium channel gating and modulation by transmitters depend on cellular compartmentalization. Nat Neurosci *3*, 670-678.

Dinudom, A., Fotia, A. B., Lefkowitz, R. J., Young, J. A., Kumar, S., and Cook, D. I. (2004). The kinase GRK2 regulates Nedd4/Nedd4-2-dependent control of epithelial Na+ channels. Proc Natl Acad Sci U S A *101*, 11886-11890.

Dzimiri, N., Muiya, P., Andres, E., and Al-Halees, Z. (2004). Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases. Eur J Pharmacol *489*, 167-177.

Eckhart, A. D., Duncan, S. J., Penn, R. B., Benovic, J. L., Lefkowitz, R. J., and Koch, W. J. (2000). Hybrid transgenic mice reveal in vivo specificity of G protein-coupled receptor kinases in the heart. Circulation Research *86*, 43-50.

Eckhart, A. D., Ozaki, T., Tevaearai, H., Rockman, H. A., and Koch, W. J. (2002). Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in

transgenic mice attenuates beta-adrenergic receptor signaling and increases resting blood pressure. Mol Pharmacol *61*, 749-758.

Engel, K., Kotlyarov, A., and Gaestel, M. (1998). Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. Embo J *17*, 3363-3371.

Engelhardt, S., Hein, L., Keller, U., Klambt, K., and Lohse, M. J. (2002). Inhibition of Na(+)-H(+) exchange prevents hypertrophy, fibrosis, and heart failure in beta(1)-adrenergic receptor transgenic mice. Circ Res *90*, 814-819.

Ennos, R. (1999). Statistical and Data Handling Skills in Biology, Longman).

Ertley, R. N., Bazinet, R. P., Lee, H. J., Rapoport, S. I., and Rao, J. S. (2006). Chronic Treatment with Mood Stabilizers Increases Membrane GRK3 in Rat Frontal Cortex. Biol Psychiatry.

Eschenhagen, T., Mende, U., Nose, M., Schmitz, W., Scholz, H., Haverich, A., Hirt, S., Doring, V., Kalmar, P., Hoppner, W., and et al. (1992). Increased messenger RNA level of the inhibitory G protein alpha subunit Gi alpha-2 in human end-stage heart failure. Circ Res *70*, 688-696.

Esposito, G., Prasad, S. V., Rapacciuolo, A., Mao, L., Koch, W. J., and Rockman, H. A. (2001). Cardiac overexpression of a G(q) inhibitor blocks induction of extracellular signal-regulated kinase and c-Jun NH(2)-terminal kinase activity in in vivo pressure overload. Circulation *103*, 1453-1458.

Estell, K., Braunstein, G., Tucker, T., Varga, K., Collawn, J. F., and Schwiebert, L. M. (2003). Plasma Membrane CFTR Regulates RANTES Expression via Its C-Terminal PDZ-Interacting Motif. Molecular and Cellular Biology *23*, 594-606.

Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994). cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein

kinase pathway in COS-7 cells. J Biol Chem *269*, 7851-7854.

Felder, R. A., Sanada, H., Xu, J., Yu, P. Y., Wang, Z., Watanabe, H., Asico, L. D., Wang, W., Zheng, S., Yamaguchi, I., *et al.* (2002). G protein-coupled receptor kinase 4 gene variants in human essential hypertension. Proc Natl Acad Sci U S A *99*, 3872-3877.

Ferguson, G. D., and Storm, D. R. (2004). Why calcium-stimulated adenylyl cyclases? Physiology (Bethesda) *19*, 271-276.

Ferguson, S. S. G. (2001). Evolving Concepts in G Protein-Coupled Receptor Endocytosis: The Role in Receptor Desensitization and Signaling. Pharmacological Reviews *53*, 1-24.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475-483.

Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002). Defective lymphocyte chemotaxis in beta -arrestin2- and GRK6-deficient mice. Proceedings of the National Academy of Sciences *99*, 7478-7483.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Frank, M., Thumer, L., Lohse, M. J., and Bunemann, M. (2005). G Protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. J Biol Chem *280*, 24584-24590.

Freedman, N. J., Kim, L. K., Murray, J. P., Exum, S. T., Brian, L., Wu, J. H., and Peppel, K. (2002). Phosphorylation of the platelet-derived growth factor receptor-beta and epidermal growth factor receptor by G protein-coupled receptor kinase-2.

Mechanisms for selectivity of desensitization. J Biol Chem *277*, 48261-48269.

Freeman, J. L., Cruz, E. M., Pollard, T. D., Lefkowitz, R. J., and Pitcher, J. A. (1998). Regulation of G protein-coupled receptor kinase 5 (GRK5) by actin. Journal of Biological Chemistry *273*, 20653-20657.

Freeman, J. L., Gonzalo, P., Pitcher, J. A., Claing, A., Lavergne, J. P., Reboud, J. P., and Lefkowitz, R. J. (2002). Beta 2-adrenergic receptor stimulated, G protein-coupled receptor kinase 2 mediated, phosphorylation of ribosomal protein P2. Biochemistry *41*, 12850-12857.

Freeman, J. L. R., Pitcher, J. A., Li, X., Bennett, V., and Lefkowitz, R. J. (2000). [alpha]-Actinin is a potent regulator of G protein-coupled receptor kinase activity and substrate specificity in vitro. *473*, 280-284.

Frey, N., Katus, H. A., Olson, E. N., and Hill, J. A. (2004). Hypertrophy of the heart: a new therapeutic target? Circulation *109*, 1580-1589.

Frey, N., and Olson, E. N. (2003). Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol *65*, 45-79.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-311.

Gainetdinov, R. R., Bohn, L. M., Sotnikova, T. D., Cyr, M., Laakso, A., Macrae, A. D., Torres, G. E., Kim, K. M., Lefkowitz, R. J., Caron, M. G., and Premont, R. T. (2003). Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice. Neuron *38*, 291-303.

Gainetdinov, R. R., Bohn, L. M., Walker, J. K., Laporte, S. A., Macrae, A. D., Caron, M. G., Lefkowitz, R. J., and Premont, R. T. (1999). Muscarinic supersensitivity and

impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. Neuron *24*, 1029-1036.

Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2004). Desensitization of G protein-coupled receptors and neuronal functions. Annu Rev Neurosci *27*, 107-144.

Gao, H., Sun, Y., Wu, Y., Luan, B., Wang, Y., Qu, B., and Pei, G. (2004). Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. Mol Cell *14*, 303-317.

Gao, J., Li, J., Chen, Y., and Ma, L. (2005). Activation of tyrosine kinase of EGFR induces Gbetagamma-dependent GRK-EGFR complex formation. FEBS Lett *579*, 122-126.

Geng, Y. J., Ishikawa, Y., Vatner, D. E., Wagner, T. E., Bishop, S. P., Vatner, S. F., and Homcy, C. J. (1999). Apoptosis of cardiac myocytes in Gsalpha transgenic mice. Circ Res *84*, 34-42.

Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. Annu Rev Biochem *56*, 615-649.

Gobeil, F., Fortier, A., Zhu, T., Bossolasco, M., Leduc, M., Grandbois, M., Heveker, N., Bkaily, G., Chemtob, S., and Barbaz, D. (2006). G-protein-coupled receptors signalling at the cell nucleus: an emerging paradigm. Can J Physiol Pharmacol *84*, 287-297.

Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature *383*, 447-450.

Gorlich, D., Henklein, P., Laskey, R. A., and Hartmann, E. (1996a). A 41 amino acid

motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. Embo J *15*, 1810-1817.

Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996b). Identification of different roles for RanGDP and RanGTP in nuclear protein import. Embo J *15*, 5584-5594.

Gusterson, R., Brar, B., Faulkes, D., Giordano, A., Chrivia, J., and Latchman, D. (2002). The transcriptional co-activators CBP and p300 are activated via phenylephrine through the p42/p44 MAPK cascade. J Biol Chem *277*, 2517-2524.

Gusterson, R. J., Jazrawi, E., Adcock, I. M., and Latchman, D. S. (2003). The transcriptional co-activators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. J Biol Chem *278*, 6838-6847.

Haendeler, J., Yin, G., Hojo, Y., Saito, Y., Melaragno, M., Yan, C., Sharma, V. K., Heller, M., Aebersold, R., and Berk, B. C. (2003). GIT1 mediates Src-dependent activation of phospholipase Cgamma by angiotensin II and epidermal growth factor. J Biol Chem *278*, 49936-49944.

Haga, K., Ogawa, H., Haga, T., and Murofushi, H. (1998). GTP-binding-protein-coupled receptor kinase 2 (GRK2) binds and phosphorylates tubulin. Eur J Biochem *255*, 363-368.

Haga, K., Tsuga, H., and Haga, T. (1997). Ca2+-dependent inhibition of G protein-coupled receptor kinase 2 by calmodulin. Biochemistry *36*, 1315-1321.

Hall, R. A., Spurney, R. F., Premont, R. T., Rahman, N., Blitzer, J. T., Pitcher, J. A., and Lefkowitz, R. J. (1999). G Protein-coupled receptor kinase 6A phosphorylates the Na+/H+ exchanger regulatory factor via a PDZ domain-mediated interaction. Journal of Biological Chemistry *274*, 24328-24334.

Harding, V. B., Jones, L. R., Lefkowitz, R. J., Koch, W. J., and Rockman, H. A. (2001). Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. Proc Natl Acad Sci U S A *98*, 5809-5814.

Harreman, M. T., Kline, T. M., Milford, H. G., Harben, M. B., Hodel, A. E., and Corbett, A. H. (2004). Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals. J Biol Chem *279*, 20613-20621.

Harrison, B. C., Roberts, C. R., Hood, D. B., Sweeney, M., Gould, J. M., Bush, E. W., and McKinsey, T. A. (2004). The CRM1 nuclear export receptor controls pathological cardiac gene expression. Mol Cell Biol *24*, 10636-10649.

Hata, J. A., Williams, M. L., Schroder, J. N., Lima, B., Keys, J. R., Blaxall, B. C., Petrofski, J. A., Jakoi, A., Milano, C. A., and Koch, W. J. (2006). Lymphocyte levels of GRK2 (betaARK1) mirror changes in the LVAD-supported failing human heart: lower GRK2 associated with improved beta-adrenergic signaling after mechanical unloading. J Card Fail *12*, 360-368.

Hattori, R., Maulik, N., Otani, H., Zhu, L., Cordis, G., Engelman, R. M., Siddiqui, M. A., and Das, D. K. (2001). Role of STAT3 in ischemic preconditioning. J Mol Cell Cardiol *33*, 1929-1936.

He, J., Lau, A. G., Yaffe, M. B., and Hall, R. A. (2001). Phosphorylation and Cell Cycle-dependent Regulation of Na+/H+ Exchanger Regulatory Factor-1 by Cdc2 Kinase. Journal of Biological Chemistry *276*, 41559-41565.

Hermann, S., Saarikettu, J., Onions, J., Hughes, K., and Grundstrom, T. (1998). Calcium regulation of basic helix-loop-helix transcription factors. Cell Calcium *23*, 135-142.

Hirotani, S., Otsu, K., Nishida, K., Higuchi, Y., Morita, T., Nakayama, H.,

Yamaguchi, O., Mano, T., Matsumura, Y., Ueno, H., *et al.* (2002). Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. Circulation *105*, 509-515.

Ho, J., Cocolakis, E., Dumas, V. M., Posner, B. I., Laporte, S. A., and Lebrun, J. J. (2005). The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. Embo J *24*, 3247-3258.

Hoefen, R. J., and Berk, B. C. (2006). The multifunctional GIT family of proteins. J Cell Sci *119*, 1469-1475.

Hoshijima, M., and Chien, K. R. (2002). Mixed signals in heart failure: cancer rules. J Clin Invest *109*, 849-855.

Huang, C. L., Jan, Y. N., and Jan, L. Y. (1997). Binding of the G protein betagamma subunit to multiple regions of G protein-gated inward-rectifying K+ channels. FEBS Lett *405*, 291-298.

Iaccarino, G., Barbato, E., Cipolletta, E., De Amicis, V., Margulies, K. B., Leosco, D., Trimarco, B., and Koch, W. J. (2005). Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. Eur Heart J.

Iaccarino, G., Rockman, H. A., Shotwell, K. F., Tomhave, E. D., and Koch, W. J. (1998a). Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs. Am J Physiol *275*, H1298-1306.

Iaccarino, G., Tomhave, E. D., Lefkowitz, R. J., and Koch, W. J. (1998b). Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by beta-adrenergic receptor stimulation and blockade. Circulation *98*, 1783-1789.

Iacovelli, L., Sallese, M., Mariggio, S., and Blasi, A. D. (1999). Regulation of G-protein-coupled receptor kinase subtypes by calcium sensor proteins. The FASEB

Journal *13*, 1-8.

Ichikawa, K. I., Hidai, C., Okuda, C., Kimata, S. I., Matsuoka, R., Hosoda, S., Quertermous, T., and Kawana, M. (1996). Endogenous endothelin-1 mediates cardiac hypertrophy and switching of myosin heavy chain gene expression in rat ventricular myocardium. J Am Coll Cardiol *27*, 1286-1291.

Ikura, M., Osawa, M., and Ames, J. B. (2002). The role of calcium-binding proteins in the control of transcription: structure to function. Bioessays *24*, 625-636.

Iwata, A., Maruyama, M., Kanazawa, I., and Nukina, N. (2001a). alpha-Synuclein affects the MAPK pathway and accelerates cell death. J Biol Chem *276*, 45320-45329.

Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001b). alpha-Synuclein forms a complex with transcription factor Elk-1. J Neurochem *77*, 239-252.

Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross, J., Jr., Lefkowitz, R. J., Caron, M. G., and Giros, B. (1996). Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function. Proc Natl Acad Sci U S A *93*, 12974-12979.

Jacoby, E., Bouhelal, R., Gerspacher, M., and Seuwen, K. (2006). The 7 TM G-protein-coupled receptor target family. ChemMedChem *1*, 761-782.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Johnson, G. L., and Lapadat, R. (2002). Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science *298*, 1911-1912.

Johnson, J. A., and Lima, J. J. (2003). Drug receptor/effector polymorphisms and pharmacogenetics: current status and challenges. Pharmacogenetics *13*, 525-534.

Johnson, L. R., Scott, M. G., and Pitcher, J. A. (2004). G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. Mol Cell Biol *24*, 10169-10179.

Kammermeier, P. J., Ruiz-Velasco, V., and Ikeda, S. R. (2000). A voltage-independent calcium current inhibitory pathway activated by muscarinic agonists in rat sympathetic neurons requires both Galpha q/11 and Gbeta gamma. J Neurosci *20*, 5623-5629.

Kang, J., Shi, Y., Xiang, B., Qu, B., Su, W., Zhu, M., Zhang, M., Bao, G., Wang, F., Zhang, X., *et al.* (2005). A nuclear function of beta-arrestin1 in GPCR signaling: regulation of histone acetylation and gene transcription. Cell *123*, 833-847.

Kariya, K., Karns, L. R., and Simpson, P. C. (1994). An enhancer core element mediates stimulation of the rat beta-myosin heavy chain promoter by an alpha 1-adrenergic agonist and activated beta-protein kinase C in hypertrophy of cardiac myocytes. J Biol Chem *269*, 3775-3782.

Keys, J. R., and Koch, W. J. (2004). The adrenergic pathway and heart failure. Recent Prog Horm Res *59*, 13-30.

Khani, S. C., Nielsen, L., and Vogt, T. M. (1998). Biochemical evidence for pathogenicity of rhodopsin kinase mutations correlated with the oguchi form of congenital stationary night blindness. Proc Natl Acad Sci U S A *95*, 2824-2827.

Kihara, A., Anada, Y., and Igarashi, Y. (2006). Mouse sphingosine kinase isoforms SPHK1a and SPHK1b differ in enzymatic traits including stability, localization, modification, and oligomerization. J Biol Chem *281*, 4532-4539.

Klenchin, V. A., Calvert, P. D., and Bownds, M. D. (1995). Inhibition of rhodopsin kinase by recoverin. Further evidence for a negative feedback system in phototransduction. J Biol Chem *270*, 16147-16152.

Koch, W. J., Lefkowitz, R. J., and Rockman, H. A. (2000). Functional consequences of altering myocardial adrenergic receptor signaling. Annu Rev Physiol *62*, 237-260.

Koch, W. J., and Rockman, H. A. (1999). Exploring the role of the beta-adrenergic receptor kinase in cardiac disease using gene-targeted mice. Trends Cardiovasc Med *9*, 77-81.

Koch, W. J., Rockman, H. A., Samama, P., Hamilton, R. A., Bond, R. A., Milano, C. A., and Lefkowitz, R. J. (1995). Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. Science *268*, 1350-1353.

Kohler, J. J., Metallo, S. J., Schneider, T. L., and Schepartz, A. (1999). DNA specificity enhanced by sequential binding of protein monomers. Proc Natl Acad Sci U S A *96*, 11735-11739.

Kohout, T. A., and Lefkowitz, R. J. (2003). Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. Mol Pharmacol *63*, 9-18.

Komuro, I., and Izumo, S. (1993). Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci U S A *90*, 8145-8149.

Krasel, C., Dammeier, S., Winstel, R., Brockmann, J., Mischak, H., and Lohse, M. J. (2001). Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. J Biol Chem *276*, 1911-1915.

Kunapuli, P., Gurevich, V. V., and Benovic, J. L. (1994). Phospholipid-stimulated autophosphorylation activates the G protein- coupled receptor kinase GRK5. Journal

of Biological Chemistry *269*, 10209-10212.

Kunisada, K., Negoro, S., Tone, E., Funamoto, M., Osugi, T., Yamada, S., Okabe, M., Kishimoto, T., and Yamauchi-Takihara, K. (2000). Signal transducer and activator of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. Proc Natl Acad Sci U S A *97*, 315-319.

Kunkel, M. T., and Peralta, E. G. (1995). Identification of domains conferring G protein regulation on inward rectifier potassium channels. Cell *83*, 443-449.

Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, 1061-1071.

Kutay, U., and Guttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol *15*, 121-124.

la Cour, T., Gupta, R., Rapacki, K., Skriver, K., Poulsen, F. M., and Brunak, S. (2003). NESbase version 1.0: a database of nuclear export signals. Nucleic Acids Res *31*, 393-396.

la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K., and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. Protein Eng Des Sel *17*, 527-536.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999). The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc Natl Acad Sci U S A *96*, 3712-

3717.

Lau, A. G., and Hall, R. A. (2001). Oligomerization of NHERF-1 and NHERF-2 PDZ domains: differential regulation by association with receptor carboxyl-termini and by phosphorylation. Biochemistry *40*, 8572-8580.

Lee, K. A. (1992). Dimeric transcription factor families: it takes two to tango but who decides on partners and the venue? J Cell Sci *103 (Pt 1)*, 9-14.

Lefkowitz, R. J. (2004). Historical review: a brief history and personal retrospective of seven-transmembrane receptors. Trends Pharmacol Sci *25*, 413-422.

Lefkowitz, R. J., and Shenoy, S. K. (2005). Transduction of receptor signals by beta-arrestins. Science *308*, 512-517.

Lefkowitz, R. J., and Whalen, E. J. (2004). beta-arrestins: traffic cops of cell signaling. Curr Opin Cell Biol *16*, 162-168.

Leitch, J. W., McElduff, P., Dobson, A., and Heller, R. (1998). Outcome with calcium channel antagonists after myocardial infarction: a community-based study. J Am Coll Cardiol *31*, 111-117.

Levay, K., Cabrera, J. L., Satpaev, D. K., and Slepak, V. Z. (1999). Gbeta5 prevents the RGS7-Galphao interaction through binding to a distinct Ggamma-like domain found in RGS7 and other RGS proteins. Proc Natl Acad Sci U S A *96*, 2503-2507.

Levay, K., Satpaev, D. K., Pronin, A. N., Benovic, J. L., and Slepak, V. Z. (1998). Localization of the sites for Ca2+-binding proteins on G protein-coupled receptor kinases. Biochemistry *37*, 13650-13659.

Levine, J. D., Coderre, T. J., Helms, C., and Basbaum, A. I. (1988). Beta 2-adrenergic mechanisms in experimental arthritis. Proc Natl Acad Sci U S A *85*, 4553-4556.

Li, L., Li, Z., and Sacks, D. B. (2003). Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. Journal of Biological Chemistry *278*, 1195-1200.

Liang, Q., and Molkentin, J. D. (2003). Redefining the roles of p38 and JNK signaling in cardiac hypertrophy: dichotomy between cultured myocytes and animal models. J Mol Cell Cardiol *35*, 1385-1394.

Liu, Y. H., Yang, X. P., Sharov, V. G., Nass, O., Sabbah, H. N., Peterson, E., and Carretero, O. A. (1997). Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. J Clin Invest *99*, 1926-1935.

Lloyd-Jones, D. M., Larson, M. G., Leip, E. P., Beiser, A., D'Agostino, R. B., Kannel, W. B., Murabito, J. M., Vasan, R. S., Benjamin, E. J., and Levy, D. (2002). Lifetime risk for developing congestive heart failure: the Framingham Heart Study. Circulation *106*, 3068-3072.

Lodowski, D. T., Barnhill, J. F., Pitcher, J. A., Capel, W. D., Lefkowitz, R. J., and Tesmer, J. J. (2003a). Purification, crystallization and preliminary X-ray diffraction studies of a complex between G protein-coupled receptor kinase 2 and Gbeta1gamma2. Acta Crystallogr D Biol Crystallogr *59*, 936-939.

Lodowski, D. T., Pitcher, J. A., Capel, W. D., Lefkowitz, R. J., and Tesmer, J. J. (2003b). Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma. Science *300*, 1256-1262.

Lodowski, D. T., Tesmer, V. M., Benovic, J. L., and Tesmer, J. J. (2006). The structure of G protein-coupled receptor kinase 6 defines a second lineage of GRKS. J Biol Chem.

Lombardi, M. S., Kavelaars, A., Schedlowski, M., Bijlsma, J. W., Okihara, K. L.,

Van de Pol, M., Ochsmann, S., Pawlak, C., Schmidt, R. E., and Heijnen, C. J. (1999). Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis. Faseb J *13*, 715-725.

Loudon, R. P., and Benovic, J. L. (1997). Altered activity of palmitoylation-deficient and isoprenylated forms of the G protein-coupled receptor kinase GRK6. J Biol Chem *272*, 27422-27427.

Luttrell, L. M., and Lefkowitz, R. J. (2002). The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. Journal of Cell Science *115*, 455-465.

Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001). Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proceedings of the National Academy of Science USA *98*, 2449-2454.

Lyubarsky, A. L., Chen, C., Simon, M. I., and Pugh, E. N., Jr. (2000). Mice lacking G-protein receptor kinase 1 have profoundly slowed recovery of cone-driven retinal responses. J Neurosci *20*, 2209-2217.

MacKeigan, J. P., Murphy, L. O., and Blenis, J. (2005). Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat Cell Biol *7*, 591-600.

Maier, L. S., Ziolo, M. T., Bossuyt, J., Persechini, A., Mestril, R., and Bers, D. M. (2006). Dynamic changes in free Ca-calmodulin levels in adult cardiac myocytes. J Mol Cell Cardiol.

Mascareno, E., Dhar, M., and Siddiqui, M. A. (1998). Signal transduction and activator of transcription (STAT) protein-dependent activation of angiotensinogen promoter: a cellular signal for hypertrophy in cardiac muscle. Proc Natl Acad Sci U S

A *95*, 5590-5594.

Mathew, J., Sleight, P., Lonn, E., Johnstone, D., Pogue, J., Yi, Q., Bosch, J., Sussex, B., Probstfield, J., and Yusuf, S. (2001). Reduction of cardiovascular risk by regression of electrocardiographic markers of left ventricular hypertrophy by the angiotensin-converting enzyme inhibitor ramipril. Circulation *104*, 1615-1621.

McCudden, C. R., Hains, M. D., Kimple, R. J., Siderovski, D. P., and Willard, F. S. (2005). G-protein signaling: back to the future. Cell Mol Life Sci *62*, 551-577.

McCullar, J. S., Larsen, S. A., Millimaki, R. A., and Filtz, T. M. (2003). Calmodulin Is a Phospholipase C-{beta} Interacting Protein. Journal of Biological Chemistry *278*, 33708-33713.

McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000). Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science *290*, 1574-1577.

McKinsey, T. A., and Olson, E. N. (2004). Cardiac histone acetylation--therapeutic opportunities abound. Trends Genet *20*, 206-213.

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001). Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev *11*, 497-504.

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem Sci *27*, 40-47.

Menard, L., Ferguson, S. S., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A. M., Lefkowitz, R. J., and Caron, M. G. (1996). Members of the G protein-coupled receptor kinase family that phosphorylate the beta2-adrenergic receptor facilitate sequestration. Biochemistry *35*, 4155-4160.

Metaye, T., Gibelin, H., Perdrisot, R., and Kraimps, J. L. (2005). Pathophysiological roles of G-protein-coupled receptor kinases. Cell Signal *17*, 917-928.

Metaye, T., Menet, E., Guilhot, J., and Kraimps, J. L. (2002). Expression and activity of g protein-coupled receptor kinases in differentiated thyroid carcinoma. J Clin Endocrinol Metab *87*, 3279-3286.

Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F., and Lefkowitz, R. J. (1994). Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. Proc Natl Acad Sci U S A *91*, 10109-10113.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell *93*, 215-228.

Monier, S., Dietzen, D. J., Hastings, W. R., Lublin, D. M., and Kurzchalia, T. V. (1996). Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. FEBS Lett *388*, 143-149.

Morano, I. (1999). Tuning the human heart molecular motors by myosin light chains. J Mol Med *77*, 544-555.

Moroianu, J. (1999). Nuclear import and export pathways. *Suppl 32-33*, 76-83.

Mylonis, I., Chachami, G., Samiotaki, M., Panayotou, G., Paraskeva, E., Kalousi, A., Georgatsou, E., Bonanou, S., and Simos, G. (2006). Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor 1a. J Biol Chem.

Naga Prasad, S. V., Barak, L. S., Rapacciuolo, A., Caron, M. G., and Rockman, H. A. (2001). Agonist-dependent Recruitment of Phosphoinositide 3-Kinase to the

Membrane by beta -Adrenergic Receptor Kinase 1. A ROLE IN RECEPTOR SEQUESTRATION. Journal of Biological Chemistry *276*, 18953-18959.

Naga Prasad, S. V., Esposito, G., Mao, L., Koch, W. J., and Rockman, H. A. (2000). Gbetagamma-dependent phosphoinositide 3-kinase activation in hearts with in vivo pressure overload hypertrophy. J Biol Chem *275*, 4693-4698.

Naga Prasad, S. V., Jayatilleke, A., Madamanchi, A., and Rockman, H. A. (2005). Protein kinase activity of phosphoinositide 3-kinase regulates beta-adrenergic receptor endocytosis. Nat Cell Biol *7*, 785-796.

Naga Prasad, S. V., Laporte, S. A., Chamberlain, D., Caron, M. G., Barak, L., and Rockman, H. A. (2002). Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. J Cell Biol *158*, 563-575.

Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000). Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem *275*, 17201-17210.

Olson, E. N., and Williams, R. S. (2000). Calcineurin signaling and muscle remodeling. Cell *101*, 689-692.

Onions, J., Hermann, S., and Grundstrom, T. (1997). Basic helix-loop-helix protein sequences determining differential inhibition by calmodulin and S-100 proteins. J Biol Chem *272*, 23930-23937.

Onions, J., Hermann, S., and Grundstrom, T. (2000). A novel type of calmodulin interaction in the inhibition of basic helix-loop-helix transcription factors. Biochemistry *39*, 4366-4374.

Oyama, N., Urasawa, K., Kaneta, S., Sakai, H., Saito, T., Takagi, C., Yoshida, I., Kitabatake, A., and Tsutsui, H. (2005). Chronic beta-adrenergic receptor stimulation enhances the expression of G-Protein coupled receptor kinases, GRK2 and GRK5, in both the heart and peripheral lymphocytes. Circ J *69*, 987-990.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., *et al.* (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. Science *289*, 739-745.

Panasyuk, G., Nemazanyy, I., Zhyvoloup, A., Bretner, M., Litchfield, D. W., Filonenko, V., and Gout, I. T. (2006). Nuclear export of S6K1 II is regulated by protein kinase CK2 phosphorylation at ser 17. J Biol Chem.

Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G., and Nemer, M. (2000). Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. Proc Natl Acad Sci U S A *97*, 931-936.

Parameswaran, N., Pao, C. S., Leonhard, K. S., Kang, D. S., Kratz, M., Ley, S. C., and Benovic, J. L. (2006). Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappa B1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. J Biol Chem.

Pemberton, L. F., and Paschal, B. M. (2005). Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic *6*, 187-198.

Penela, P., Elorza, A., Sarnago, S., and Mayor, F., Jr. (2001). Beta-arrestin- and c-Src-dependent degradation of G-protein-coupled receptor kinase 2. Embo J *20*, 5129-5138.

Penela, P., Murga, C., Ribas, C., Tutor, A. S., Peregrin, S., and Mayor, F., Jr. (2006). Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. Cardiovasc Res *69*, 46-56.

Penela, P., Ribas, C., and Mayor, J. (2003). Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. Cellular Signalling *15*, 973-981.

Penela, P., Ruiz-Gomez, A., Castano, J. G., and Mayor, F., Jr. (1998). Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. J Biol Chem *273*, 35238-35244.

Penn, R. B., and Benovic, J. L. (1994). Structure of the human gene encoding the beta-adrenergic receptor kinase. J Biol Chem *269*, 14924-14930.

Perander, M., Bjorkoy, G., and Johansen, T. (2001). Nuclear import and export signals enable rapid nucleocytoplasmic shuttling of the atypical protein kinase C lambda. J Biol Chem *276*, 13015-13024.

Perrino, C., Naga Prasad, S. V., Patel, M., Wolf, M. J., and Rockman, H. A. (2005). Targeted inhibition of beta-adrenergic receptor kinase-1-associated phosphoinositide-3 kinase activity preserves beta-adrenergic receptor signaling and prolongs survival in heart failure induced by calsequestrin overexpression. J Am Coll Cardiol *45*, 1862-1870.

Perroy, J., Adam, L., Qanbar, R., Chenier, S., and Bouvier, M. (2003). Phosphorylation-independent desensitization of GABAB receptor by GRK4. The EMBO Journal *22*, 3816-3824.

Petrich, B. G., Molkentin, J. D., and Wang, Y. (2003). Temporal activation of c-Jun N-terminal kinase in adult transgenic heart via cre-loxP-mediated DNA recombination. Faseb J *17*, 749-751.

Petrofski, J. A., and Koch, W. J. (2003). The [beta]-adrenergic receptor kinase in heart failure. *35*, 1167-1174.

Pi, M., Oakley, R. H., Gesty-Palmer, D., Cruickshank, R. D., Spurney, R. F., Luttrell, L. M., and Quarles, L. D. (2005). Beta-arrestin- and G protein receptor kinase-mediated calcium-sensing receptor desensitization. Mol Endocrinol *19*, 1078-1087.

Pierce, K. L., and Lefkowitz, R. J. (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. Nat Rev Neurosci *2*, 727-733.

Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002). Seven-Transmembrane Receptors. Nature Reviews Molecular Cell Biology *3*, 639-650.

Pitcher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J., and Lefkowitz, R. J. (1996). Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-coupled receptor kinase (GRK) activity. LOCATION, STRUCTURE, AND REGULATION OF THE PIP2 BINDING SITE DISTINGUISHES THE GRK SUBFAMILIES. Journal of Biological Chemistry *271*, 24907-24913.

Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998). G protein-coupled receptor kinases. Annual Review of Biochemistry *67*, 653-692.

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992). Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. Science *257*, 1264-1267.

Pitcher, J. A., Tesmer, J. J. G., Freeman, J. L. R., Capel, W. D., Stone, W. C., and Lefkowitz, R. J. (1999). Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases. Journal of Biological Chemistry *274*, 34531-34534.

Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995). Pleckstrin homology domain-mediated membrane association and activation of the beta-

adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. J Biol Chem *270*, 11707-11710.

Prall, O. W., Elliott, D. A., and Harvey, R. P. (2002). Developmental paradigms in heart disease: insights from tinman. Ann Med *34*, 148-156.

Premont, R. T., Claing, A., Vitale, N., Freeman, J. L., Pitcher, J. A., Patton, W. A., Moss, J., Vaughan, M., and Lefkowitz, R. J. (1998). Beta 2-adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. Proceedings of the National Academy of Sciences *95*, 14082-14087.

Premont, R. T., Macrae, A. D., Aparicio, S. A. J. R., Kendall, H. E., Welch, J. E., and Lefkowitz, R. J. (1999). The GRK4 Subfamily of G Protein-coupled Receptor Kinases. ALTERNATIVE SPLICING, GENE ORGANIZATION, AND SEQUENCE CONSERVATION. Journal of Biological Chemistry *274*, 29381-29389.

Premont, R. T., Macrae, A. D., Stoffel, R. H., Chung, N., Pitcher, J. A., Ambrose, C., Inglese, J., MacDonald, M. E., and Lefkowitz, R. J. (1996). Characterization of the G protein-coupled receptor kinase GRK4. Journal of Biological Chemistry *271*, 6403-6410.

Pronin, A. N., and Benovic, J. L. (1997). Regulation of the G Protein-coupled Receptor Kinase GRK5 by protein kinase C. Journal of Biological Chemistry *272*, 3806-3812.

Pronin, A. N., Carman, C. V., and Benovic, J. L. (1998). Structure-Function Analysis of G Protein-coupled Receptor Kinase-5. ROLE OF THE CARBOXYL TERMINUS IN KINASE REGULATION. Journal of Biological Chemistry *273*, 31510-31518.

Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000). Synucleins are a novel class of substrates for G protein-coupled receptor kinases. Journal of

Biological Chemistry *275*, 26515-26522.

Pronin, A. N., Satpaev, D. K., Slepak, V. Z., and Benovic, J. L. (1997). Regulation of G Protein-coupled Receptor Kinases by Calmodulin and Localization of the Calmodulin Binding Domain. Journal of Biological Chemistry *272*, 18273-18280.

Purcell, N. H., Tang, G., Yu, C., Mercurio, F., DiDonato, J. A., and Lin, A. (2001). Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc Natl Acad Sci U S A *98*, 6668-6673.

Quimby, B. B., and Corbett, A. H. (2001). Nuclear transport mechanisms. Cell Mol Life Sci *58*, 1766-1773.

Ramirez, M. T., Zhao, X. L., Schulman, H., and Brown, J. H. (1997). The nuclear deltaB isoform of Ca2+/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. J Biol Chem *272*, 31203-31208.

Ramos-Ruiz, R., Penela, P., Penn, R. B., and Mayor, F., Jr. (2000). Analysis of the human G protein-coupled receptor kinase 2 (GRK2) gene promoter: regulation by signal transduction systems in aortic smooth muscle cells. Circulation *101*, 2083-2089.

Rebsamen, M. C., Arrighi, J. F., Juge-Aubry, C. E., Vallotton, M. B., and Lang, U. (2000). Epidermal growth factor induces hypertrophic responses and Stat5 activation in rat ventricular cardiomyocytes. J Mol Cell Cardiol *32*, 599-610.

Reich, N. C., and Liu, L. (2006). Tracking STAT nuclear traffic. Nat Rev Immunol *6*, 602-612.

Rhoads, A. R., and Friedberg, F. (1997). Sequence motifs for calmodulin recognition. Faseb J *11*, 331-340.

Ritter, O., and Neyses, L. (2003). The molecular basis of myocardial hypertrophy and heart failure. Trends Mol Med *9*, 313-321.

Rockman, H. A., Chien, K. R., Choi, D. J., Iaccarino, G., Hunter, J. J., Ross, J., Jr., Lefkowitz, R. J., and Koch, W. J. (1998a). Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. Proc Natl Acad Sci U S A *95*, 7000-7005.

Rockman, H. A., Choi, D. J., Akhter, S. A., Jaber, M., Giros, B., Lefkowitz, R. J., Caron, M. G., and Koch, W. J. (1998b). Control of myocardial contractile function by the level of beta-adrenergic receptor kinase 1 in gene-targeted mice. J Biol Chem *273*, 18180-18184.

Rockman, H. A., Choi, D. J., Rahman, N. U., Akhter, S. A., Lefkowitz, R. J., and Koch, W. J. (1996). Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. Proceedings of the National Academy of Sciences *93*, 9954-9959.

Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002). Seven-transmembrane-spanning receptors and heart function. Nature *415*, 206-212.

Rodriguez-Vilarrupla, A., Jaumot, M., Abella, N., Canela, N., Brun, S., Diaz, C., Estanyol, J. M., Bachs, O., and Agell, N. (2005). Binding of calmodulin to the carboxy-terminal region of p21 induces nuclear accumulation via inhibition of protein kinase C-mediated phosphorylation of Ser153. Mol Cell Biol *25*, 7364-7374.

Rohrer, D. K. (1998). Physiological consequences of beta-adrenergic receptor disruption. J Mol Med *76*, 764-772.

Rohrer, D. K., Desai, K. H., Jasper, J. R., Stevens, M. E., Regula, D. P., Jr., Barsh, G. S., Bernstein, D., and Kobilka, B. K. (1996). Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. Proc Natl Acad

Sci U S A *93*, 7375-7380.

Roth, S. Y., Denu, J. M., and Allis, C. D. (2001). Histone acetyltransferases. Annu Rev Biochem *70*, 81-120.

Ruiz-Gomez, A., Humrich, J., Murga, C., Quitterer, U., Lohse, M. J., and Mayor, F., Jr. (2000). Phosphorylation of phosducin and phosducin-like protein by G protein-coupled receptor kinase 2. J Biol Chem *275*, 29724-29730.

Saarikettu, J., Sveshnikova, N., and Grundstrom, T. (2004). Calcium/calmodulin Inhibition of transcriptional activity of E-proteins by prevention of their binding to DNA. J Biol Chem.

Sabri, A., and Steinberg, S. F. (2003). Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. Mol Cell Biochem *251*, 97-101.

Sakata, Y., Hoit, B. D., Liggett, S. B., Walsh, R. A., and Dorn, G. W., 2nd (1998). Decompensation of pressure-overload hypertrophy in G alpha q-overexpressing mice. Circulation *97*, 1488-1495.

Salamanca, D. A., and Khalil, R. A. (2005). Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. Biochem Pharmacol *70*, 1537-1547.

Sallese, M., Iacovelli, L., Cumashi, A., Capobianco, L., Cuomo, L., and De Blasi, A. (2000a). Regulation of G protein-coupled receptor kinase subtypes by calcium sensor proteins. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1498*, 112-121.

Sallese, M., Lombardi, M. S., and De Blasi, A. (1994). Two isoforms of G protein-coupled receptor kinase 4 identified by molecular cloning. Biochem Biophys Res

Commun *199*, 848-854.

Sallese, M., Mariggio, S., Collodel, G., Moretti, E., Piomboni, P., Baccetti, B., and De Blasi, A. (1997). G protein-coupled receptor kinase GRK4. MOLECULAR ANALYSIS OF THE FOUR ISOFORMS AND ULTRASTRUCTURAL LOCALIZATION IN SPERMATOZOA AND GERMINAL CELLS. Journal of Biological Chemistry *272*, 10188-10195.

Sallese, M., Salvatore, L., D'Urbano, E., Sala, G., Storto, M., Launey, T., Nicoletti, F., Knopfel, T., and De Blasi, A. (2000b). The G-protein-coupled receptor kinase GRK4 mediates homologous desensitization of metabotropic glutamate receptor 1. Faseb J *14*, 2569-2580.

Santella, L., Ercolano, E., and Nusco, G. A. (2005). The cell cycle: a new entry in the field of Ca2+ signaling. Cell Mol Life Sci *62*, 2405-2413.

Schluter, K. D., Goldberg, Y., Taimor, G., Schafer, M., and Piper, H. M. (1998). Role of phosphatidylinositol 3-kinase activation in the hypertrophic growth of adult ventricular cardiomyocytes. Cardiovasc Res *40*, 174-181.

Schneider, T. D., and Stephens, R. M. (1990). Sequence logos: a new way to display consensus sequences. Nucleic Acids Res *18*, 6097-6100.

Schulz, R. A., and Yutzey, K. E. (2004). Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. Dev Biol *266*, 1-16.

Schwartz, K., Chassagne, C., and Boheler, K. R. (1993). The molecular biology of heart failure. J Am Coll Cardiol *22*, 30A-33A.

Scott, M. G. H., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, S., Marullo, S., and Benmerah, A. (2002). Differential Nucleocytoplasmic Shuttling of beta -Arrestins. CHARACTERIZATION OF A LEUCINE-RICH NUCLEAR

EXPORT SIGNAL IN beta -ARRESTIN2. Journal of Biological Chemistry *277*, 37693-37701.

Shah, B. H., and Catt, K. J. (2003). A central role of EGF receptor transactivation in angiotensin II -induced cardiac hypertrophy. Trends Pharmacol Sci *24*, 239-244.

Sharpe, N. (1996). Beta-blockers in heart failure. Future directions. Eur Heart J *17 Suppl B*, 39-42.

Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.

Shibata, T., Chuma, M., Kokubu, A., Sakamoto, M., and Hirohashi, S. (2003). EBP50, a [beta]-catenin-associating protein, enhances Wnt signaling and is over-expressed in hepatocellular carcinoma. Hepatology *38*, 178-186.

Shioi, T., Kang, P. M., Douglas, P. S., Hampe, J., Yballe, C. M., Lawitts, J., Cantley, L. C., and Izumo, S. (2000). The conserved phosphoinositide 3-kinase pathway determines heart size in mice. Embo J *19*, 2537-2548.

Sidorova, J. M., Mikesell, G. E., and Breeden, L. L. (1995). Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. Mol Biol Cell *6*, 1641-1658.

Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A., and Bleasdale, J. E. (1990). Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. J Pharmacol Exp Ther *253*, 688-697.

Snow, B. E., Krumins, A. M., Brothers, G. M., Lee, S. F., Wall, M. A., Chung, S., Mangion, J., Arya, S., Gilman, A. G., and Siderovski, D. P. (1998). A G protein

gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. Proc Natl Acad Sci U S A *95*, 13307-13312.

Song, J. H., Waataja, J. J., and Martemyanov, K. A. (2006a). Subcellular targeting of RGS9-2 is controlled by multiple molecular determinants on its membrane anchor, R7BP. J Biol Chem *281*, 15361-15369.

Song, K., Backs, J., McAnally, J., Qi, X., Gerard, R. D., Richardson, J. A., Hill, J. A., Bassel-Duby, R., and Olson, E. N. (2006b). The transcriptional coactivator CAMTA2 stimulates cardiac growth by opposing class II histone deacetylases. Cell *125*, 453-466.

Speirs, H. J., Katyk, K., Kumar, N. N., Benjafield, A. V., Wang, W. Y., and Morris, B. J. (2004). Association of G-protein-coupled receptor kinase 4 haplotypes, but not HSD3B1 or PTP1B polymorphisms, with essential hypertension. J Hypertens *22*, 931-936.

Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-1050.

Stedman, D. R., Uboha, N. V., Stedman, T. T., Nairn, A. C., and Picciotto, M. R. (2004). Cytoplasmic localization of calcium/calmodulin-dependent protein kinase I-alpha depends on a nuclear export signal in its regulatory domain. FEBS Lett *566*, 275-280.

Stephanou, A., Brar, B. K., Scarabelli, T. M., Jonassen, A. K., Yellon, D. M., Marber, M. S., Knight, R. A., and Latchman, D. S. (2000). Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. J Biol Chem *275*, 10002-10008.

Stephanou, A., Scarabelli, T. M., Brar, B. K., Nakanishi, Y., Matsumura, M., Knight, R. A., and Latchman, D. S. (2001). Induction of apoptosis and Fas receptor/Fas

ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1 transcription factor but not tyrosine 701. J Biol Chem *276*, 28340-28347.

Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell *77*, 83-93.

Stoffel, R. H., Inglese, J., Macrae, A. D., Lefkowitz, R. J., and Premont, R. T. (1998). Palmitoylation increases the kinase activity of the G protein-coupled receptor kinase, GRK6. Biochemistry *37*, 16053-16059.

Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., and Inglese, J. (1994). Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. J Biol Chem *269*, 27791-27794.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. Embo J *18*, 1660-1672.

Su, L., Hershberger, R. J., and Weissman, I. L. (1993). LYAR, a novel nucleolar protein with zinc finger DNA-binding motifs, is involved in cell growth regulation. Genes Dev *7*, 735-748.

Sugden, P. H., and Clerk, A. (1998). "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. Circ Res *83*, 345-352.

Suo, Z., Cox, A. A., Bartelli, N., Rasul, I., Festoff, B. W., Premont, R. T., and Arendash, G. W. (2006). GRK5 deficiency leads to early Alzheimer-like pathology and working memory impairment. Neurobiol Aging.

Suo, Z., Wu, M., Citron, B. A., Wong, G. T., and Festoff, B. W. (2004). Abnormality of G-protein-coupled receptor kinases at prodromal and early stages of Alzheimer's disease: an association with early beta-amyloid accumulation. J Neurosci *24*, 3444-3452.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development *126*, 1269-1280.

Tang, W. J., and Gilman, A. G. (1991). Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. Science *254*, 1500-1503.

Thiagalingam, S., Cheng, K. H., Lee, H. J., Mineva, N., Thiagalingam, A., and Ponte, J. F. (2003). Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci *983*, 84-100.

Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E., and Fisher, S. K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. J Biol Chem *266*, 23856-23862.

Tran, E. J., and Wente, S. R. (2006). Dynamic nuclear pore complexes: life on the edge. Cell *125*, 1041-1053.

Tsuga, H., Okuno, E., Kameyama, K., and Haga, T. (1998). Sequestration of human muscarinic acetylcholine receptor hm1-hm5 subtypes: effect of G protein-coupled receptor kinases GRK2, GRK4, GRK5 and GRK6. J Pharmacol Exp Ther *284*, 1218-1226.

Ungerer, M., Bohm, M., Elce, J. S., Erdmann, E., and Lohse, M. J. (1993). Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. Circulation *87*, 454-463.

Ungerer, M., Parruti, G., Bohm, M., Puzicha, M., DeBlasi, A., Erdmann, E., and Lohse, M. J. (1994). Expression of beta-arrestins and beta-adrenergic receptor kinases in the failing human heart. Circ Res *74*, 206-213.

Vakili, B. A., Okin, P. M., and Devereux, R. B. (2001). Prognostic implications of left ventricular hypertrophy. Am Heart J *141*, 334-341.

Valovka, T., Verdier, F., Cramer, R., Zhyvoloup, A., Fenton, T., Rebholz, H., Wang, M. L., Gzhegotsky, M., Lutsyk, A., Matsuka, G., *et al.* (2003). Protein kinase C phosphorylates ribosomal protein S6 kinase betaII and regulates its subcellular localization. Mol Cell Biol *23*, 852-863.

Vatter, P., Stoesser, C., Samel, I., Gierschik, P., and Moepps, B. (2005). The variable C-terminal extension of G-protein-coupled receptor kinase 6 constitutes an accessorial autoregulatory domain. Febs J *272*, 6039-6051.

Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N., and McKinsey, T. A. (2004). Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol *24*, 8374-8385.

Verdin, E., Dequiedt, F., and Kasler, H. G. (2003). Class II histone deacetylases: versatile regulators. Trends Genet *19*, 286-293.

Vinge, L. E., Oie, E., Andersson, Y., Grogaard, H. K., Andersen, G., and Attramadal, H. (2001). Myocardial distribution and regulation of GRK and beta-arrestin isoforms in congestive heart failure in rats. Am J Physiol Heart Circ Physiol *281*, H2490-2499.

Virlon, B., Firsov, D., Cheval, L., Reiter, E., Troispoux, C., Guillou, F., and Elalouf, J. M. (1998). Rat G Protein-Coupled Receptor Kinase GRK4: Identification, Functional Expression, and Differential Tissue Distribution of Two Splice Variants. Endocrinology *139*, 2784-2795.

Vroon, A., Heijnen, C. J., Lombardi, M. S., Cobelens, P. M., Mayor, F., Jr., Caron, M. G., and Kavelaars, A. (2004a). Reduced GRK2 level in T cells potentiates chemotaxis and signaling in response to CCL4. J Leukoc Biol *75*, 901-909.

Vroon, A., Heijnen, C. J., Raatgever, R., Touw, I. P., Ploemacher, R. E., Premont, R. T., and Kavelaars, A. (2004b). GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and impaired responsiveness to G-CSF in vivo. J Leukoc Biol *75*, 698-704.

Walker, J. K., Gainetdinov, R. R., Feldman, D. S., McFawn, P. K., Caron, M. G., Lefkowitz, R. J., Premont, R. T., and Fisher, J. T. (2004). G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation. Am J Physiol Lung Cell Mol Physiol *286*, L312-319.

Walker, J. K., Peppel, K., Lefkowitz, R. J., Caron, M. G., and Fisher, J. T. (1999). Altered airway and cardiac responses in mice lacking G protein-coupled receptor kinase 3. Am J Physiol *276*, R1214-1221.

Wan, K. F., Sambi, B. S., Frame, M., Tate, R., and Pyne, N. J. (2001). The Inhibitory gamma Subunit of the Type 6 Retinal Cyclic Guanosine Monophosphate Phosphodiesterase Is a Novel Intermediate Regulating p42/p44 Mitogen-activated Protein Kinase Signaling in Human Embryonic Kidney 293 Cells. Journal of Biological Chemistry *276*, 37802-37808.

Wan, K. F., Sambi, B. S., Tate, R., Waters, C., and Pyne, N. J. (2003). The inhibitory gamma subunit of the type 6 retinal cGMP phosphodiesterase functions to link c-Src and G-protein-coupled receptor kinase 2 in a signaling Unit that regulates p42/p44 mitogen-activated protein kinase by epidermal growth factor. Journal of Biological Chemistry *278*, 18658-18663.

Wang, J., Campos, B., Jamieson, G. A., Jr., Kaetzel, M. A., and Dedman, J. R. (1995). Functional elimination of calmodulin within the nucleus by targeted

expression of an inhibitor peptide. J Biol Chem *270*, 30245-30248.

Wang, P., Wu, Y., Ge, X., Ma, L., and Pei, G. (2003). Subcellular Localization of beta -Arrestins Is Determined by Their Intact N Domain and the Nuclear Export Signal at the C Terminus. Journal of Biological Chemistry *278*, 11648-11653.

Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., and Chien, K. R. (1998a). Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J Biol Chem *273*, 2161-2168.

Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., and Chien, K. R. (1998b). Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. J Biol Chem *273*, 5423-5426.

Weinman, E. J., Steplock, D., Tate, K., Hall, R. A., Spurney, R. F., and Shenolikar, S. (1998). Structure-function of recombinant Na/H exchanger regulatory factor (NHE-RF). J Clin Invest *101*, 2199-2206.

Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1995). Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane Na(+)-H+ exchanger. J Clin Invest *95*, 2143-2149.

Weinmann, A. S., and Farnham, P. J. (2002). Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. *26*, 37-47.

Wells, J., and Farnham, P. J. (2002). Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. Methods *26*, 48-56.

Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell *82*, 463-473.

Wiedmer, T., Zhao, J., Nanjundan, M., and Sims, P. J. (2003). Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane. Biochemistry *42*, 1227-1233.

Willets, J. M., Challiss, R. A., and Nahorski, S. R. (2003a). Non-visual GRKs: are we seeing the whole picture? Trends Pharmacol Sci *24*, 626-633.

Willets, J. M., Challiss, R. A. J., and Nahorski, S. R. (2002). Endogenous G Protein-coupled Receptor Kinase 6 Regulates M3 Muscarinic Acetylcholine Receptor Phosphorylation and Desensitization in Human SH-SY5Y Neuroblastoma Cells. Journal of Biological Chemistry *277*, 15523-15529.

Willets, J. M., Mistry, R., Nahorski, S. R., and Challiss, R. A. (2003b). Specificity of G protein-coupled receptor kinase 6-mediated phosphorylation and regulation of single-cell M3 muscarinic acetylcholine receptor signaling. Mol Pharmacol *64*, 1059-1068.

Williams, S. M., Ritchie, M. D., Phillips, J. A., 3rd, Dawson, E., Prince, M., Dzhura, E., Willis, A., Semenya, A., Summar, M., White, B. C., *et al.* (2004). Multilocus analysis of hypertension: a hierarchical approach. Hum Hered *57*, 28-38.

Winstel, R., Freund, S., Krasel, C., Hoppe, E., and Lohse, M. J. (1996). Protein kinase cross-talk: membrane targeting of the beta-adrenergic receptor kinase by protein kinase C. Proc Natl Acad Sci U S A *93*, 2105-2109.

Witherow, D. S., Garrison, T. R., Miller, W. E., and Lefkowitz, R. J. (2004). beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha. Proc Natl Acad Sci U S A *101*, 8603-8607.

Wright, W. E., and Funk, W. D. (1993). CASTing for multicomponent DNA-binding complexes. *18*, 77-80.

Wu, J. H., Goswami, R., Cai, X., Exum, S. T., Huang, X., Zhang, L., Brian, L., Premont, R. T., Peppel, K., and Freedman, N. J. (2006). Regulation of the platelet-derived growth factor receptor-beta by G protein-coupled receptor kinase-5 in vascular smooth muscle cells involves the phosphatase Shp2. J Biol Chem.

Xu, M., Petraschka, M., McLaughlin, J. P., Westenbroek, R. E., Caron, M. G., Lefkowitz, R. J., Czyzyk, T. A., Pintar, J. E., Terman, G. W., and Chavkin, C. (2004). Neuropathic pain activates the endogenous kappa opioid system in mouse spinal cord and induces opioid receptor tolerance. J Neurosci *24*, 4576-4584.

Xuan Nguyen, T. L., Choi, J. W., Lee, S. B., Ye, K., Woo, S. D., Lee, K. H., and Ahn, J. Y. (2006). Akt phosphorylation is essential for nuclear translocation and retention in NGF-stimulated PC12 cells. Biochem Biophys Res Commun.

Yano, M., Kim, S., Izumi, Y., Yamanaka, S., and Iwao, H. (1998). Differential activation of cardiac c-jun amino-terminal kinase and extracellular signal-regulated kinase in angiotensin II-mediated hypertension. Circ Res *83*, 752-760.

Yao, T. P., Oh, S. P., Fuchs, M., Zhou, N. D., Ch'ng, L. E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., and Eckner, R. (1998). Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell *93*, 361-372.

Yi, X. P., Gerdes, A. M., and Li, F. (2002). Myocyte redistribution of GRK2 and GRK5 in hypertensive, heart-failure-prone rats. Hypertension *39*, 1058-1063.

Yi, X. P., Zhou, J., Baker, J., Wang, X., Gerdes, A. M., and Li, F. (2005). Myocardial expression and redistribution of GRKs in hypertensive hypertrophy and failure. Anat Rec A Discov Mol Cell Evol Biol *282A*, 13-23.

Yin, G., Haendeler, J., Yan, C., and Berk, B. C. (2004). GIT1 functions as a scaffold for MEK1-extracellular signal-regulated kinase 1 and 2 activation by angiotensin II

and epidermal growth factor. Mol Cell Biol *24*, 875-885.

Yin, G., Zheng, Q., Yan, C., and Berk, B. C. (2005). GIT1 is a scaffold for ERK1/2 activation in focal adhesions. J Biol Chem *280*, 27705-27712.

Yoneda, Y. (2000). Nucleocytoplasmic protein traffic and its significance to cell function. Genes Cells *5*, 777-787.

Young, K. W., Willets, J. M., Parkinson, M. J., Bartlett, P., Spiegel, S., Nahorski, S. R., and Challiss, R. A. (2003). Ca2+/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane relocation but not activation. Cell Calcium *33*, 119-128.

Yusuf, S., Sleight, P., Pogue, J., Bosch, J., Davies, R., and Dagenais, G. (2000). Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med *342*, 145-153.

Zhang, C. L., McKinsey, T. A., Chang, S., Antos, C. L., Hill, J. A., and Olson, E. N. (2002). Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell *110*, 479-488.

Zhang, F., White, R. L., and Neufeld, K. L. (2000). Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. Proc Natl Acad Sci U S A *97*, 12577-12582.

Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K., and Xiao, R. P. (2001). Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. Proc Natl Acad Sci U S A *98*, 1607-1612.