REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2005 Name of Author CANTY S H

COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS

Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).

B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.

C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.

D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.

☑ This copy has been deposited in the Library of UCL

☐ This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganisation of the actin cytoskeleton

Sarah H. Cant

MRC Laboratory for Molecular Cell Biology and Cell Biology Unit,
University College London

Submitted for the degree of Doctor of Philosophy

June 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

G protein-coupled receptor kinase 2 (GRK2) phosphorylates and desensitises activated G protein-coupled receptors (GPCRs). In this thesis, I identify ezrin, a member of the ezrin-radixin-moesin (ERM) family of actin/plasma membrane cross-linkers, as a novel non-GPCR substrate of GRK2. GRK2 phosphorylates ezrin within a 53 amino acid region that encompasses the phosphorylation site required for ezrin activation, threonine-567. Two lines of evidence indicate that GRK2 acts as an effector of activated GPCRs through phosphorylation of ERM proteins. Firstly, in Hep2 cells muscarinic M1 receptor (M1MR) activation promotes cytoskeletal reorganisation and membrane ruffling. This ruffling response is ERM-dependent and is accompanied by ERM protein phosphorylation. Inhibition of GRK2, but not Rho kinase, protein kinase C or cAMP-dependent protein kinase, prevents ERM protein phosphorylation and membrane ruffling. Secondly, agonist-induced internalisation of the β_2-adrenergic receptor (β_2AR) and M1MR is accompanied by ERM protein phosphorylation and localisation of phosphorylated ERM proteins to receptor-containing endosomal vesicles. The Na^+/H^+ exchanger regulatory factor, however, is not required for co-localisation of phosphorylated ERM proteins to the β_2AR. Inhibition of ezrin function impedes β_2AR internalisation, further linking GPCR activation, GRK activity and ezrin function. However, ERM protein phosphorylation does not occur following M2 muscarinic receptor (M2MR) activation and ERM proteins do not colocalise with internalised M2MR, suggesting that ERM proteins may be required for the internalisation of a subset of GPCRs. Overall, my results indicate that GRK2 serves not only to attenuate but also to transduce some GPCR-mediated signals.

To Julie,

for being outstanding.

And without whose endless support and time this would not have been possible.

Thank you.

And to:

Other members of the LMCB

(especially Alison Lloyd, Mark Marsh, Anne Ridley and Nathalie Signoret)

for their advice and time.

Members of the Pitcher lab, past and present;

(especially Laura and Mark)

my fellow LMCB graduate students;

(especially Ann, Bessi, Charly, Sarah and Tim)

my friends

(especially Angela, Anjali, Anna, Charlie, Hazel, Helen, Ian, James, Marica, Michelle, Naomi, Penny, Rachel and everyone at the University of London Sub Aqua Club and London School of Samba)

and my family

(especially Mum, Dad, Rob, Grannie and Grandpa R, and Grandma and Grandpa A)

for their unrelenting love, support, advice, understanding and encouragement.

Abbreviations used in this thesis

αAR:	α-adrenergic receptor
β_2AR:	β_2-adrenergic receptor
βARK:	β-adrenergic receptor kinase
βARs:	β-adrenergic receptors
Abp1:	actin binding protein 1
Ach:	acetylcholine
ACK:	activated Cdc42-associated tyrosine kinase
ADP:	adenosine diphosphate
Arf:	ADP-ribosylation factor
ASK1:	apoptosis signal-regulating kinase 1
ATP:	adenosine triphosphate
$AT_{1A}R$:	angiotensin 1A receptor
BM:	binding medium
Ca^{2+}:	calcium
cAMP:	cyclic adenosine monophosphate
CBP:	calcium-binding protein
CCPs:	clathrin coated pits
CCV:	clathrin-coated vesicle
C-ERMAD:	carboxy-ERM association domain
cGMP:	cyclic guanosine monophosphate
DMEM:	Dulbecco's Modified Eagle Medium
DNA:	deoxyribonucleic acid
EEA1:	Early endosome antigen 1
EGF:	epidermal growth factor
ERK:	extracellular signal-regulated kinase
ERM:	ezrin-radixin-moesin
ET_BR:	endothelin B receptor
EVH1:	Enabled/VASP-1 domain
F-actin:	filamentous actin
FCS:	foetal calf serum
FERM:	4.1, ezrin, radixin, moesin

G protein:	GTP-binding protein
G-actin:	globular actin
GAP:	GTPase activating protein
GDI:	guanine nucleotide dissociation inhibitor
GEF:	guanine nucleotide exchange factor
GIT:	GRK interactor
GPCR:	G protein-coupled receptor
GRK:	G protein-coupled receptor kinase
GST:	glutathione S transferase
GTP:	guanosine triphosphate
Hip1R:	Hungtingtin interacting protein 1R
hKOR:	human κ opioid receptor
Iso:	isoproterenol
JNK3:	c-Jun amino-terminal kinase 3
LAMP1:	lysosomal-associated membrane protein 1
LDL:	low density lipoprotein
LPA:	lysophosphatidic acid
M1MR:	M1 muscarinic receptor
M2MR:	M2 muscarinic receptor
M3MR:	M3 muscarinic receptor
MAPK:	mitogen-activated protein kinase
MEK1:	MAPK/ERK kinase 1
MRCK:	myotonic dystrophy kinase-related Cdc42-binding kinase
mRNA:	messenger RNA
N-ERMAD:	amino-ERM association domain
NHERF:	Na^+/H^+ exchanger regulatory factor
NSF:	*N*-ethylmaleimide-sensitive factor
P:	phosphate
PAR2:	proteinase-activated receptor 2
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PDEγ:	γ subunit of the type 6 retinal cGMP phosphodiesterase
PDZ:	PSD-95, Dlg, ZO-1 homology
pERM:	threonine-phosphorylated ERM proteins

PH:	pleckstrin homology
PIP:	phosphatidylinositol phosphate
PIP_2:	phosphatidylinositol 4,5-bisphosphate
PKA:	cAMP-dependent protein kinase
PKC:	protein kinase C
PMSF:	phenylmethanesulfonyl fluoride
PTB:	phosphotyrosine-binding domain
RGS:	regulators of G protein signalling
RNA:	ribonucleic acid
RNAi:	RNA interference
ROS:	rod outer segments
RT:	room temperature
SDS:	sodium dodecyl sulphate
SDS-PAGE:	SDS polyacrylamide gel electrophoresis
SH3:	Src homology 3
TEMED:	N, N, N′, N′-tetramethyl-ethylenediamine
TPβ:	thromboxane A2 receptor
VIP_1R:	vasoactive intestinal peptide 1 receptor
VSVG:	vesicular stomatitis virus glycoprotein
WASP:	Wiscott-Aldrich syndrome protein

Table of contents

Index of figures

Index of tables

1 Introduction

G protein-coupled receptor kinase 2 (GRK2) is the most extensively characterised member of the G protein-coupled receptor kinases (GRKs), a family of serine/threonine kinases that specifically phosphorylate agonist-occupied G protein-coupled receptors (GPCRs) (Penela, et al., 2003). Typically, such a phosphorylation event leads to the binding of cytosolic βarrestin proteins to the receptor, which cause the receptor to become uncoupled from its associated effector G protein and initiate the internalisation of the receptor via clathrin-mediated endocytosis (Shenoy and Lefkowitz, 2003). In this way, GRK-mediated phosphorylation of GPCRs is important in controlling their signalling activity as well as the number at the cell surface.

Agonist-occupied GPCRs allosterically activate GRKs and can enhance their phosphorylation of non-receptor substrates (Freeman et al., 2002; Haga et al., 1998; Pitcher et al., 1998a; Penn et al., 2000). In the case of GRK2, non-receptor substrates identified to date include tubulin, phosducin, ribosomal protein P2, synucleins, the inhibitory gamma subunit of the type 6 retinal cyclic guanosine monophosphate (cGMP) phosphodiesterase and the β-subunit of the epithelial Na^+ channel (Carman et al., 1998; Dinudom et al., 2004; Freeman et al., 2002; Haga et al., 1998; Pitcher et al., 1998a; Pronin et al., 2000; Ruiz-Gomez et al., 2000; Wan et al., 2001). βarrestins have also been shown to act as molecular scaffolds for kinase relays downstream of GPCRs (Shenoy and Lefkowitz, 2003, see below). These findings have led to the hypothesis that ligand binding not only instigates receptor desensitisation by GRK2, but may also promote other, novel GPCR-mediated signalling pathways downstream of the activated GRK.

The actin cytoskeleton has previously been shown to play an important role in receptor endocytosis and vesicle trafficking (da Costa et al., 2003). In this thesis, I identify the cortical actin/plasma membrane cross-linker ezrin as a novel substrate of GRK2 and examine its role in GPCR-mediated actin cytoskeleton rearrangements and receptor endocytosis. The introduction will give a general overview on GPCRs and the structure and functions of GRKs (Sections 1.1 and 1.2). It will also describe the role of the actin cytoskeleton in receptor-mediated endocytosis and the structure

and functions of the ezrin-radixin-moesin family of actin cross-linker proteins (Sections 1.3 – 1.6).

1.1 GPCRs

G protein-coupled receptors are a family of receptor proteins encoded by over 800 genes in the human genome, whose members share similar amino acid sequences and a characteristic tertiary architecture of seven transmembrane-spanning-domains (Pierce et al., 2002). The determination of the three-dimensional crystal structure of the light-activated visual GPCR, rhodopsin, revealed a highly organised bundle of seven integral membrane α-helices linked by extracellular and cytoplasmic loops and it is thought that other GPCRs will display a comparable configuration (Figure 1.1A) (Palczewski et al., 2000). Many types of ligands, such as hormones, chemokines, neurotransmitters, ions, odorants and photons of light, have been shown to bind to and activate various GPCRs, implicating these receptors in diverse physiological functions including endocrine regulation, behaviour and sensory recognition (Gainetdinov et al., 2004). Furthermore, the majority of pharmaceutical drugs manufactured today target GPCRs either directly or indirectly.

GPCRs can be grouped into three families, A, B and C, based on sequence similarity. The families share the overall structure of seven transmembrane helices, with over 25% sequence homology in this core region, as well as shared motifs and conserved amino acids. The largest group is family A, to which most GPCR types belong, including rhodopsin and the olfactory receptors. Family B receptors include those for hormones such as calcitonin, parathyroid hormone and the gastrointestinal peptide hormone family. Members of family C have large extracellular amino termini that are required for agonist binding and receptor activation. They include some taste receptors, the $GABA_B$ receptor and the metabotropic glutamate receptor family (Pierce et al., 2002).

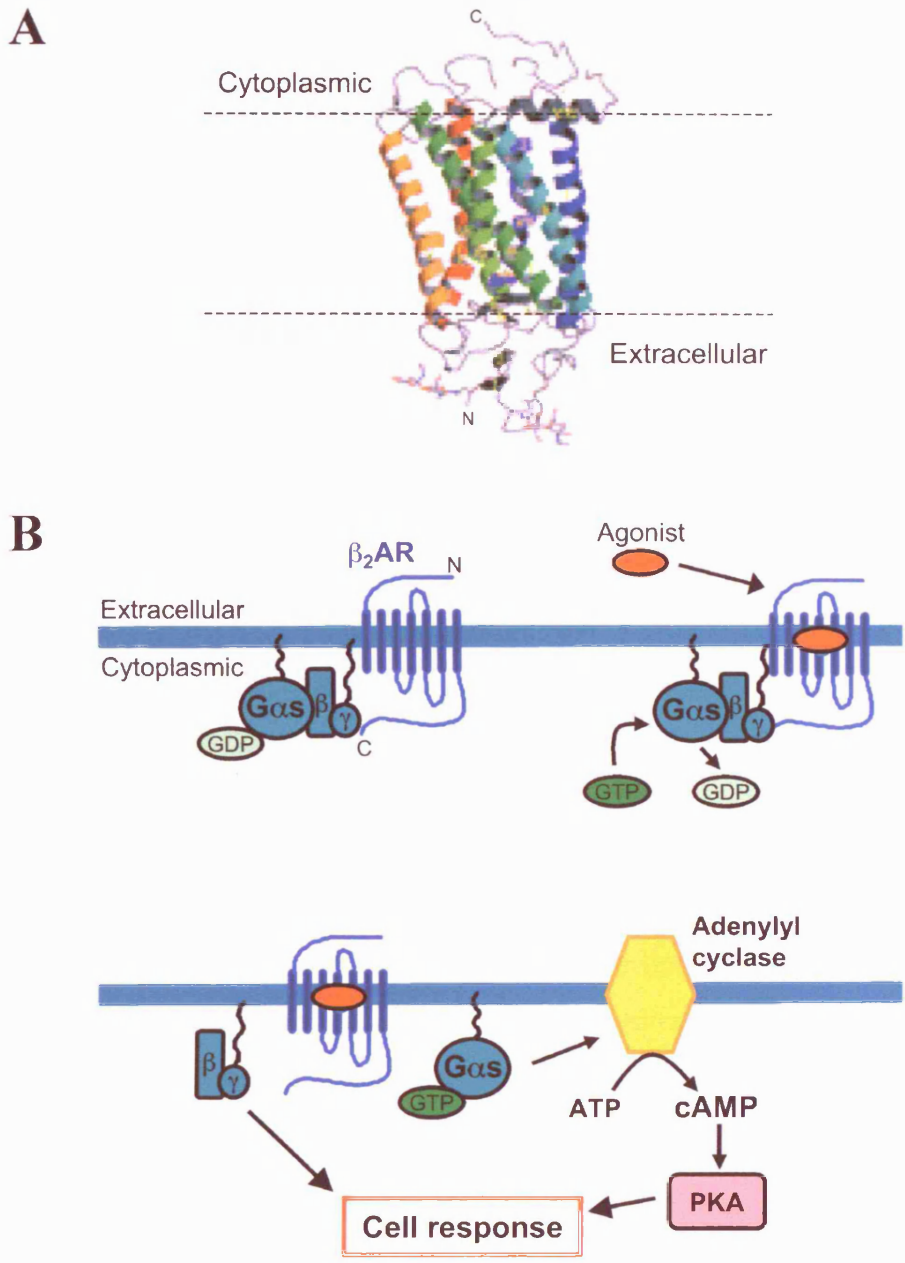

Figure 1.1. Classical structure and signalling of GPCRs. **A.** Crystal structure of rhodopsin determined to 2.8Å. The dotted lines indicate the approximate position of the plasma membrane. Adapted from Palczewski et al., 2000. **B.** Classical β_2AR signalling. In the absence of agonist, β_2AR is in a low-affinity conformation. Agonist binding leads to a transient high-affinity receptor/agonist/G protein complex. The activated receptor stimulates the dissociation of GDP from the Gαs-subunit and its replacement with GTP. This activates the Gαs-subunit and causes it to detach from the βγ dimer. Both GTP-Gαs and βγ regulate separate downstream intracellular effectors. One such effector is adenylyl cyclase, which on activation by GTP-Gαs increases cytoplasmic cAMP levels, thus activating PKA. β_2AR: β_2-adrenergic receptor. C: carboxyl terminus. N: amino terminus. PKA: cAMP-dependent protein kinase.

Most GPCRs primarily signal via activation of associated heterotrimeric GTP-binding proteins (G proteins) comprising of α, β and γ subunits (Figure 1.1B). Fifteen α-subunits have been identified and have been grouped into four gene families, Gαs, Gαi, Gαq and Gα12, all of which contain a guanine nucleotide-binding site and can hydrolyse GTP. The β- and γ-subunits form a tightly linked dimer consisting of any combination of four β-subunits and fourteen γ-subunits cloned to date (Gainetdinov et al., 2004). A fifth β-subunit, Gβ5, does not associate with γ-subunits, but instead binds to RGS proteins (Witherow and Slepak, 2003). In general, heterotrimeric G protein complexes are categorised by the class of α-subunit bound, i.e. Gs refers to a G protein heterotrimer that contains Gαs.

In the absence of agonist, a GPCR and its associated G protein are considered to be in a low affinity state whereby the α-, β- and γ-subunits of the G protein interact to form a heterotrimeric complex and GDP is bound to the α-subunit. Agonist-occupancy of the GPCR activates the receptor and leads to a transient high affinity agonist/receptor/G protein complex. The activated receptor stimulates the dissociation of GDP from the α-subunit and its replacement with GTP. This binding of GTP activates the α-subunit and may cause it to detach from the βγ dimer: subsequently both GTP-α and βγ regulate separate downstream intracellular effectors involved in various second messenger signalling pathways and ion channel modulation (Figure 1.1B) (Pierce et al., 2002).

The four families of α-subunits couple to the regulation of different effectors. Gs subunits stimulate cAMP production by adenylyl cyclase, whereas Gi proteins inhibit adenylyl cyclases; Gq α-subunits activate phospholipase C-β (PLCβ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate and diacylglycerol and leads to an increase in intracellular Ca^{2+}; and G12 proteins stimulate guanine nucleotide exchange factors for the Rho family of small GTPases (Cabrera-Vera et al., 2003). The βγ subunits have been demonstrated to regulate adenylyl cyclases, PLCβ, potassium channels, phosphatidylinositol 3-kinases, G protein-coupled receptor kinases and secretory granule exocytosis via binding to the SNARE complex (Blackmer et al., 2005; Cabrera-Vera et al., 2003). Following receptor and G protein activation, regulators of G protein signalling (RGS proteins)

act to promote the hydrolysis of GTP by the α-subunits (Sierra et al., 2000). GDP-α reassociates with the βγ dimer and signalling to downstream effectors ceases.

A recent study has demonstrated that, following agonist stimulation of the β_2-adrenergic receptor (β_2AR), Gs appears to be trafficked into the cell via lipid rafts. The internalised Gα subunit did not colocalise with the internalised β_2AR, or endosomes and lysosomes (Allen et al., 2005). These data indicate that activated Gs, and possibly other Gα subunits, can enter the cell, where they may mediate signalling at sites distinct from the plasma membrane.

1.1.1 Mechanisms of desensitisation

Continuous activation of a GPCR leads to a reduction in its ability to signal to downstream pathways, a process known as desensitisation, often followed by internalisation of cell surface receptors. The most common immediate mechanism of regulating GPCR signalling is that of receptor phosphorylation. GPCRs such as the β_2AR, the opioid receptors and the leutinising hormone receptor have additionally been demonstrated to undergo transcriptional and translational regulation to control their expression over a longer time-frame. Expression of receptors can be regulated by diverse mechanisms, including the action of signalling molecules such as chemokines and steroid hormones, as well as chromatin remodelling and histone acetylation. Post-transcriptional modifications involve mRNA splicing and down-regulation of mRNA (Collins et al., 1991; Wei and Loh, 2002; Zhang and DuFau, 2003).

1.1.1.1 Heterologous desensitisation

Second messenger kinases such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) are activated downstream of Gs and Gq respectively (Hansen and Cassanova, 1994; Lustig at el., 1993). These kinases participate in a negative-feedback loop by directly phosphorylating GPCRs and uncoupling them from their associated G proteins. Following activation both PKA and PKC can also phosphorylate other GPCRs, even those not stimulated by the particular agonist acting on the cell, and thus regulate the response of those receptors to an activating

ligand (Gainetdinov et al., 2004). This process is known as heterologous desensitisation.

Activation of PKA via the Gs-coupled β-adrenergic receptors (βAR) leads to phosphorylation of the receptor by PKA and uncoupling from Gs. This simultaneously enhances the coupling of the βAR to Gi, which further inhibits Gs-mediated signalling and initiates activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways (Daaka et al., 1997a; Zamah et al., 2002). This mechanism is known as signal switching and serves to regulate the Gα-coupling specificity of the receptor.

1.1.1.2 Homologous desensitisation

1.1.1.2.1 Uncoupling

Homologous desensitisation is mediated by G protein-coupled receptor kinases and arrestin proteins. GRKs are activated by and phosphorylate agonist-occupied GPCRs, and are discussed in detail in Section 1.2. Phosphorylation of an activated GPCR by a GRK promotes the binding of an arrestin adaptor protein, which sterically hinders (uncouples) the interaction of the receptor with its associated G protein (Luttrell and Lefkowitz, 2002). This serves to attenuate G protein-mediated second messenger signalling (Figure 1.2).

Arrestins are a family of cytosolic proteins that recognise GRK-phosphorylated, agonist-occupied GPCRs (Lohse et al., 1992). Visual arrestins (arrestin-1, or rod arrestin, and arrestin-4, or cone arrestin) are expressed in retinal cells and regulate phototransduction by rhodopsin and cone opsin. The non-visual arrestins (arrestin-2, or βarrestin1, and arrestin-3, or βarrestin2) are ubiquitously expressed in all other tissues and modulate the homologous desensitisation of almost all the non-visual GPCRs (Gainetdinov et al., 2004; Luttrell and Lefkowitz, 2002).

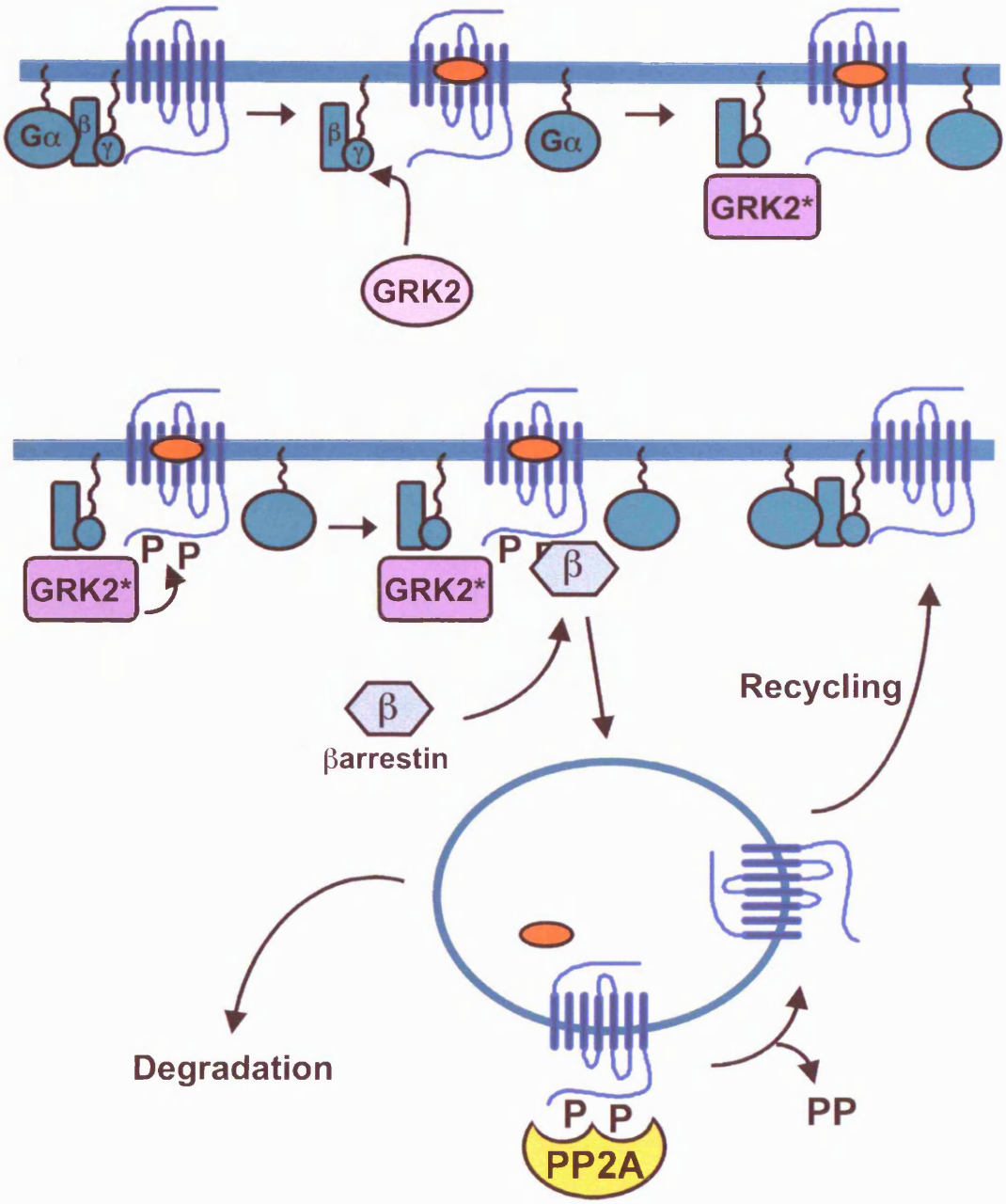

Figure 1.2. Homologous desensitisation and trafficking of GPCRs by GRK2. Agonist binding activates the GPCR and leads to the dissociation of Gα and βγ subunits. GRK2 is recruited to the agonist-occupied receptor and free βγ at the plasma membrane, where it is activated. Active GRK2 phosphorylates the GPCR carboxyl-terminus, promoting the binding of a βarrestin adaptor protein. The recruitment of βarrestin sterically hinders the interaction of the receptor with the associated G protein. The GPCR- βarrestin complex is targeted to clathrin-coated pits and internalised into acidic endosomal compartments. Here the GPCR is either dephosphorylated and recycled back to the plasma membrane or degraded. GRK2* indicates activated GRK2. P denotes a phosphate group. PP2A: protein phosphatase 2A.

1.1.2 Clathrin-mediated receptor endocytosis

In addition to mediating GPCR/G protein uncoupling, arrestins promote internalisation of phosphorylated, activated receptors via clathrin-mediated endocytosis (Goodman et al., 1996). Following agonist treatment, βarrestins can be shown to translocate from the cytosol to phosphorylated GPCRs at the plasma membrane. Here they also directly bind to phosphoinositides, the heavy chain of the coat protein clathrin and the β-subunit of the adaptor protein AP-2, which acts to enhance clathrin coat assembly and receptor recruitment to clathrin-coated pits (CCPs) (Gaidarov et al., 1999; Goodman et al., 1996; Laporte et al., 1999; Laporte et al., 2000). These interactions serve to target GPCRs to CCPs, which then undergo fission from the cell surface via the action of the GTPase dynamin (Scott et al., 2002; Zhang et al., 1996) (Figure 1.3). Internalised phosphorylated receptors are dephosphorylated and recycled back to the plasma membrane (resensitisation) or degraded (downmodulation) (Figure 1.2).

1.2 GRKs

1.2.1 Structure and function

G protein-coupled receptor kinases (GRKs) are a family of serine/threonine kinases originally identified by their ability to phosphorylate specifically agonist-occupied GPCRs. Seven mammalian GRKs have been identified to date and have been grouped into sub-families based on similarities in structure and function (Figure 1.4A). These groups are:

- *GRK1, or visual GRK, subfamily*

  Comprises GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase)

- *GRK2, or β-adrenergic receptor kinase (βARK), subfamily*

  Comprises GRK2 (βARK1) and GRK3 (βARK2)

- *GRK4 subfamily*

  Comprises GRK4, GRK5 and GRK6

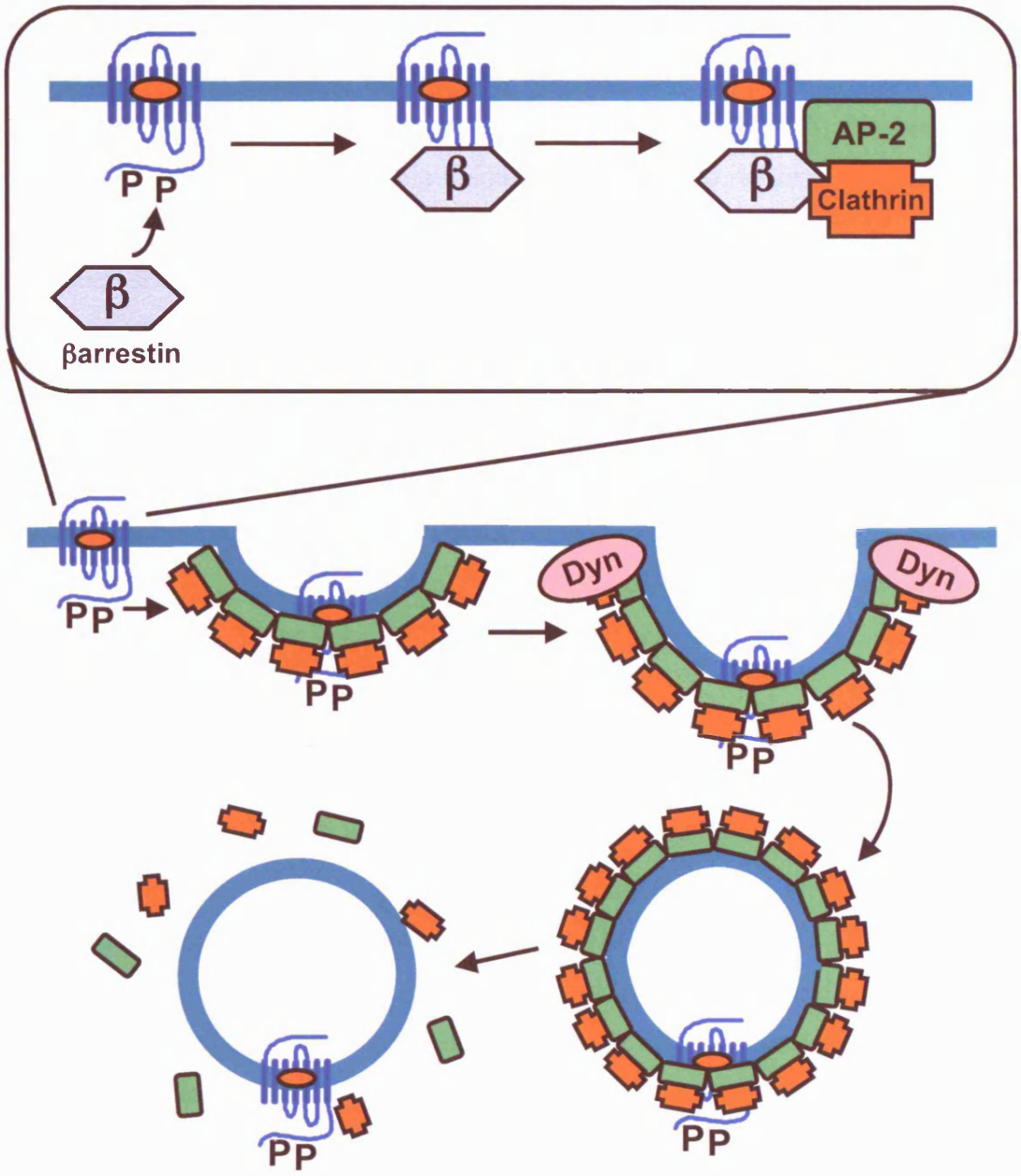

Figure 1.3. Clathrin mediated endocytosis of GPCRs. βarrestin is recruited to phosphorylated GPCRs at the plasma membrane, where it also binds to phosphoinositides, the heavy chain of the coat protein clathrin and the β-subunit of the adaptor protein AP-2. The interaction of βarrestin with AP-2 serves to promote the recruitment of phosphorylated GPCRs to clathrin coated pits (CCPs) where AP-2 enhances clathrin coat assembly. The CCPs then undergo fission from the cell surface via the action of the GTPase dynamin, forming clathrin coated vesicles. The vesicles are subsequently uncoated in the cytosol where they fuse with the early endosome. Dyn: dynamin. P denotes a phosphate group.

Among all of the mammalian GRKs sequence homology is 53-93%, with 87% similarity between GRK1 members, 84% similarity between GRK2 members and 70% similarity between GRK4 members (Hisatomi et al., 1998; Pitcher at al., 1998b). GRKs -2, -3, -5 and -6 are ubiquitously expressed, whereas GRK-1 and -7 are restricted to retinal cells and GRK4 is mainly found in the testes, although it has also been shown to function in neurons (Penela et al., 2003; Perroy et al., 2003). Furthermore, GRKs -4 and -6 undergo alternative splicing. GRK4 has four splice variants with differentially spliced exons in the amino- and carboxyl-termini and GRK6 has three carboxyl-terminal splice variants (Willets et al., 2003). Homologues exist in both *Drosophila melanogaster* and *Caenorhabditis elegans*. The *Drosophila* GPRK1 has 65% homology to bovine GRK2, *Drosophila* GPRK2 has 50% homology to bovine GRK5, and the equivalent *C. elegans* proteins show 51% and 53% homology to bovine GRK2 and GRK5 respectively (Cassill et al., 1991; Pitcher et al., 1998b).

GRKs share a highly conserved, centrally located catalytic domain of approximately 263-266 amino acids that is related to PKC and PKA families (Pitcher et al., 1998b). The 185 amino acid amino-terminal domains of the GRKs are structurally similar and contain a region of homology to regulators of G protein signalling proteins (RGS domain) as well as sites required for regulation of kinase activity and localisation. It had been speculated that the amino-terminus may be important for receptor recognition, mainly due to the similarity in its size and structure amongst the GRKs, however the recently determined crystal structure of GRK2 indicates that a receptor may dock onto the kinase domain (Figure 1.4B) (Lodowski et al., 2003).

Conversely, the carboxyl-terminal regions of the GRKs show variety in structure and length (Figure 1.4A). Through either post-translational modifications or regions that bind lipids or membrane proteins, they function in the targeting and translocation of the kinases to the plasma membrane. The carboxyl-termini of the GRK1 subfamily are 100 amino acids in length and are isoprenylated, while those of the GRK2 subfamily are 230 amino acids long and contain a pleckstrin homology (PH) domain that mediates binding to phospholipids and $G\beta\gamma$. Members of the GRK4 subfamily have a carboxyl-terminus of approximately 130 amino acids: GRKs -4 and -6 are

palmitoylated and GRK5 has a polybasic region of positively charged amino acids that bind phospholipids (Penela et al., 2003; Willets et al., 2003).

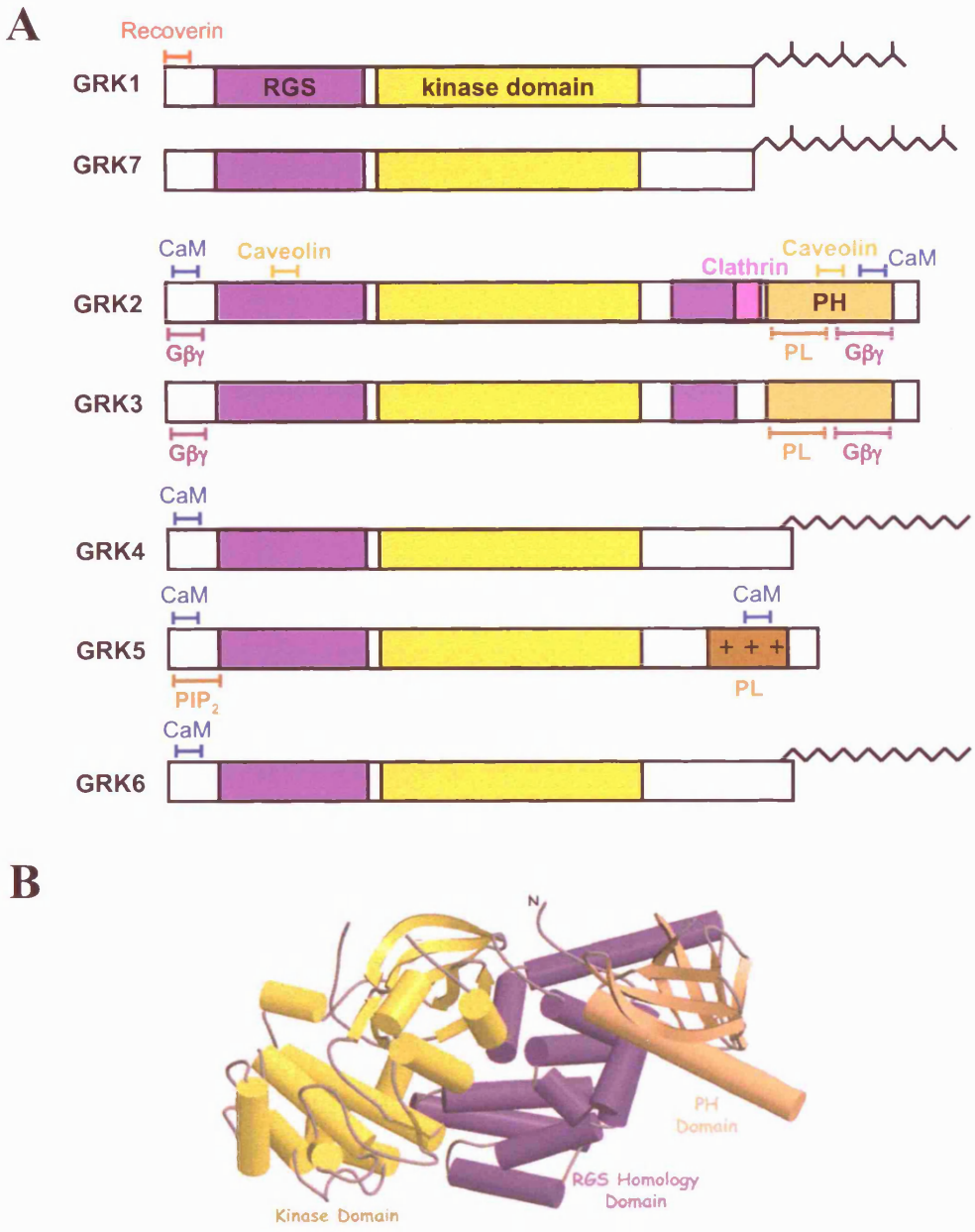

Figure 1.4. GRK structure. **A.** Cartoon depicting the structural organisation and key regulatory sites of the GRKs. Sites of protein and lipid binding are indicated above and below each kinase. The lipid modifications depicted are: farnesylation (GRK1), geranylgeranylation (GRK7) and palmitoylation (GRK4 and-6). CaM: calmodulin. Clathrin: clathrin box. PH: pleckstrin homology domain. PIP_2: phosphatidylinositol 4,5-bisphosphate. PL: phospholipid binding. RGS: regulator of G-protein signalling homology domain. + indicates positively charged amino acids. **B.** Crystal structure of GRK2, adapted from Lodowski et al., 2003. N: amino terminus.

In general, the catalytic activity of GRKs towards both receptor and non-receptor substrates is enhanced by agonist-occupied GPCRs (Freeman et al., 2002; Haga et al., 1998; Pitcher et al., 1998a; Penn et al., 2000). Although there is no known consensus sequence for substrates, GRKs have been demonstrated to preferentially phosphorylate activated GPCRs on either the third intracellular loop or the cytoplasmic carboxyl-terminal tail; often a pair of acidic amino acids are located at the amino-terminal side of the phosphorylation site (Pitcher et al., 1998b). Recently, the sites of GRK2 interaction on the α_{2A}-adrenergic receptor were located to the second and third intracellular loops, specifically a number of basic residues within three discrete regions on the third loop. Mutation of these residues inhibited both GRK2-mediated phosphorylation of the receptor and receptor-mediated activation of GRK2 (Pao and Benovic, 2005).

In order to phosphorylate a receptor the GRK must first be targeted to the plasma membrane, where it is allosterically activated by the agonist-occupied receptor. With over 800 GPCRs encoded in the genome and only seven GRKs, most of which have an overlapping expression profile, the activity of the GRKs must be strictly regulated within the cell. Consequently, a number of different mechanisms to control the activity, sub-cellular localisation and expression levels of the GRKs have arisen (Kohout and Lefkowitz, 2003).

1.2.2 Regulation of activity and localisation

1.2.2.1 GRK1 subfamily

Due to the covalent carboxyl-terminal farnesylation of GRK1 and the predicted geranylgeranyl modification of GRK7, these kinases can associate with the plasma membrane on light activation of the visual GPCRs, rhodopsin or cone opsin (Kohout and Lefkowitz, 2003; Pitcher et al., 1998b). The activity of GRK1 is modulated by recoverin, a calcium-binding protein (CBP) with a similar tissue distribution (Pitcher et al., 1998b). Myristoylated recoverin associates with membranes, where it binds to the amino-terminus of GRK1 in a Ca^{2+}-dependent manner (IC_{50} = 0.8μM) and inhibits its activity, presumably by hindering the interaction of the kinase with rhodopsin (Sallese et al., 2000a). Since levels of Ca^{2+} in the photoreceptor cell are high in the dark, it is predicted that recoverin would inhibit GRK1 activity until the

cell is illuminated, leading to a reduction in the concentration of free Ca^{2+}, disassociation of recoverin from GRK1 and phosphorylation of rhodopsin (Kohout and Lefkowitz, 2003). GRK1 also undergoes autophosphorylation at serine and threonine residues within the amino- and carboxyl-termini, which impairs the ability of the kinase to bind to phosphorylated, light-activated rhodopsin (Pitcher et al., 1998b). This has been suggested to serve as a mechanism to release GRK1 from rhodopsin following desensitisation.

1.2.2.2 GRK2 subfamily

GRK2 and GRK3 are primarily cytoplasmic and briefly translocate to the plasma membrane on agonist-occupancy of a GPCR. This is thought to be predominantly due to the release of the Gβγ dimer from Gα following heterotrimeric G protein activation. GRKs –2 and –3 bind to Gβγ at a site overlapping the PH domain in the carboxyl-terminus and possibly also via the amino-terminus (Carman et al., 2000; Eichmann et al., 2003). This interaction is required for localisation of GRK2 at lipid vesicles and phosphorylation of agonist-occupied GPCRs *in vitro* (Pitcher et al., 1995; Pitcher et al., 1998b). Additionally, GRK2 was shown to require PIP_2 and other acidic phospholipids for activity: GRK2 was unable to phosphorylate receptors reconstituted in 100% phosphatidylcholine vesicles, but the presence of acidic phospholipids stimulated its activity (DebBurman et al., 1995; DebBurman et al., 1996; Pitcher et al., 1995). PIP_2 and Gβγ were demonstrated to act synergistically to activate GRK2 by simultaneously binding to the PH domain (DebBurman et al., 1996; Pitcher et al., 1995). The association was also shown to occur in intact cells following GPCR stimulation, which induced translocation of GRK2 to the plasma membrane where it co-immunoprecipitated with Gβγ (Daaka et al., 1997b). This study also demonstrated the specificity of GRK2 and GRK3 for different Gβ subunits: Gβ1 and Gβ2 were able to bind to both GRK2 and -3, whereas Gβ3 preferentially bound GRK3 (Daaka et al., 1997b). These results agree with an earlier *in vitro* study, which further indicated that GRK2 might preferentially interact with Gγ2 over Gγ3 (Muller et al., 1993).

GRK2 and GRK3 can also be recruited to the plasma membrane via association with GTP-bound, active Gq/11 at their amino-terminal RGS domain. RGS domain-

containing proteins modulate heterotrimeric G protein signal pathways by acting as GTPase activating proteins (GAPs) for Gα subunits to promote GTP hydrolysis and terminate signalling (Sierra et al., 2000). GRKs –2 and –3 specifically bind Gq/11 (Carman et al., 1999a) and the residues within the GRK2 RGS domain that are important for this interaction were recently identified as arginine-106, phenylalanine-109, aspartate-110, methionine-114, lysine-115, glutamate-116, leucine-117 and valine-137 (Sterne-Marr et al., 2003). Mutation of these amino acids inhibited translocation of GRK2 to the plasma membrane but did not affect kinase activity (Sterne-Marr et al., 2003). The GRK2 binding site of Gq has also recently been characterised and is localised to a helical region that contains overlapping sites for binding to both GRK2 and other RGS proteins (Day et al., 2004).

Although GRK2 has only weak GAP activity, it has been demonstrated to selectively inhibit Gq-mediated stimulation of inositol triphosphate production by PLCβ (Carman et al., 1999a; Sallese et al., 2000b). This was not as a result of GPCR desensitisation since the RGS domain alone or a catalytically inactive mutant ($GRK2_{K220R}$) were also able to terminate Gq signalling (Carman et al., 1999a; Sallese et al., 2000b). That the RGS domain of GRK2 can sequester Gq and inhibit inositol triphosphate production by a number of Gq-coupled receptors has led to the speculation that GRK2 may also mediate phosphorylation-independent desensitisation of GPCRs (Pao and Benovic, 2002).

The cellular localisation of GRK2 is also regulated by endocytic coat proteins. GRK2 associates with caveolin-rich membrane fractions and contains a caveolin–binding motif within its carboxyl-terminal PH domain as well as at the amino-terminus (Carman et al., 1999b). Caveolin-1 and –3 were demonstrated to significantly inhibit the catalytic activity of GRK2 (Carman et al., 1999b), suggesting that caveolin may function to restrict inactive GRK2 to specific membrane domains in order to either suppress its activity or to localise it to a particular target prior to activation.

Additionally, a clathrin box has been identified in the carboxyl-tail of GRK2 that allows the interaction of GRK2 with clathrin heavy chain (Shiina et al., 2001) and GRK2 has been shown to be associated with internalised GPCRs (Ruiz-Gomez and

Mayor Jr., 1997; Schulz et al., 2002). Although the functional significance of this association is unknown, it is possible that GRK2 could directly mediate the internalisation of a subset of GPCRs (Claing et al., 2000; Shiina et al., 2001). The endothelin B (ET_BR), vasoactive intestinal peptide 1 (VIP_1R), and angiotensin 1A ($AT_{1A}R$) GPCRs have all been shown to recruit GRK2 and to internalise via a βarrestin-independent mechanism, suggesting that GRK2 may act as a clathrin adapter in this process (Claing et al., 2000; Freedman et al., 1997; Oppermann et al., 1996; Shetzline et al., 2002). GRK2 also brings GRK-interactor (GIT) proteins to βarrestin-dependent GPCRs at the plasma membrane (Claing et al., 2000; Premont et al., 1998). GIT proteins act as GAPs towards ADP-ribosylation factors (Arfs), small GTPases believed to function in the recruitment of coat proteins onto membranes (Donaldson, 2003), suggesting a second mechanism for GRK2 to modulate GPCR internalisation via the clathrin pathway (Premont et al., 1998; Premont et al., 2000).

Both PKC and PKA phosphorylate GRK2 and regulate its kinase activity. PKC phosphorylation enhances GRK2 activity towards GPCRs and the phosphorylation site has been identified as serine-29, although a carboxyl-terminal site may also exist (Chuang et al., 1995; Krasel et al., 2000; Winstel et al., 1996). A recent study has suggested that this phosphorylation relieves the tonic inhibition of GRK2 by calmodulin and allows GRK2 to translocate to the plasma membrane (Krasel et al., 2000). PKA in complex with the A kinase-anchoring protein AKAP79, which targets PKA to both the plasma membrane and the β_2AR, promotes increased receptor phosphorylation by GRK2. Phosphorylation of serine-685 by PKA was shown to enhance the binding of GRK2 to Gβγ at the plasma membrane (Cong et al., 2001a).

GPCR stimulation can lead to activation of signalling pathways such as the ERK/MAPK cascades (Pierce et al., 2002). Following agonist occupancy of β_2AR, activation of this cascade is facilitated by the interaction of the non-receptor tyrosine kinase c-Src with βarrestin, which recruits c-Src to the plasma membrane (Luttrell et al., 1999; Miller et al., 2000). c-Src can phosphorylate GRK2 to increase its catalytic activity (Fan et al., 2001; Sarnago et al., 1999). The serine/threonine kinase ERK1 has also been demonstrated to phosphorylate GRK2 *in vitro*, reducing its catalytic activity for both receptor and soluble substrates (Elorza et al., 2000; Pitcher at al.,

1999). Phosphorylation occurs at serine-670, which lies within the Gβγ-binding domain (Pitcher et al., 1999), and could cause GRK2 to be released from Gβγ and dissociate from the GPCR at the plasma membrane. Since GRK2 and ERK1 have been shown to associate following β_2AR stimulation (Elorza et al., 2000), it has been suggested that ERK1 may regulate receptor desensitisation by rapidly "switching off" GRK2 phosphorylation of β_2AR after agonist binding (Kohout and Lefkowitz, 2003).

In addition to regulation of subcellular localisation, direct association with a number of proteins can modulate the kinase activity of GRK2 subfamily members. Binding of the CBP calmodulin to sites at both the amino- and carboxyl-termini of GRK2 inhibited the ability of GRK2 to phosphorylate rhodopsin, albeit at high concentrations of calmodulin ($IC_{50} \approx 2\mu M$) (Pronin et al., 1997). A second CBP, neuronal calcium sensor-1, has also been shown to interact with GRK2 and impair the desensitisation of the D2 dopamine receptor (Kabbani et al., 2002). The actin binding protein α-actinin is able to inhibit GRK2 activity (Freeman et al., 2000) and even intramolecular interactions have been implicated in modulation of this kinase (Sarnago et al., 2003). It is predicted that the amino- and carboxyl-termini of GRK2 associate, leading to an inactive conformation that is also unable to translocate to the plasma membrane. Binding to phospholipids and proteins such as Gβγ or Gq could cause release of the amino- and carboxyl-termini and membrane localisation (Sarnago et al., 2003).

1.2.2.3 GRK4 subfamily

Members of the GRK4 subfamily are predominantly associated with the plasma membrane, either via palmitoylation of the carboxyl tail (GRKs –4 and –6) or, in the case of GRK5, through association of positively charged amino acids at the carboxyl-terminal with phospholipids (Kohout and Lefkowitz, 2003). GRK5 has also been shown to bind PIP_2 at a site in the amino-terminus that is conserved in all GRK4 subfamily members (Pitcher at al., 1996). Phosphorylation of β_2AR by GRK5 was increased specifically in the presence of PIP_2 due to enhanced membrane localisation of the kinase (Pitcher et al., 1996). Although GRK4 subfamily members have RGS domains, their binding partners have not been identified.

Like GRK2, GRK5 activity is downregulated by calmodulin, although GRK5 is more sensitive to calmodulin and associates with it at much lower concentrations ($IC_{50} \approx 50nM$) (Pronin et al., 1997). Calmodulin binding to sites at the amino- and carboxyl-termini leads to autophosphorylation of the kinase and inhibition of the interaction with both receptor and phospholipid (Pronin et al., 1997; Pronin et al., 1998). The increased sensitivity of GRK5 to calmodulin implies that it would be less likely to desensitise Gq-coupled receptors than GRK2.

The activity of GRK5 is also modulated by phosphorylation. Following non-specific phospholipid stimulation, GRK5 undergoes autophosphorylation at sites distinct to those phosphorylated on calmodulin binding (Kunapuli et al., 1994). Autophosphorylation is thought to promote the association of GRK5 with agonist-occupied GPCRs. GRK5 is also a substrate of PKC although, in contrast to GRK2, its catalytic activity *in vitro* is inhibited following PKC-mediated phosphorylation (Pronin and Benovic, 1997).

The activity of GRK4 subfamily members can additionally be regulated by protein-protein interactions. Actin and the actin binding proteins α-actinin and spectrin inhibit the kinase activity of GRK5 against both GPCR and soluble substrates. Interestingly, calmodulin binding relieves the actin- and α-actinin-mediated inhibition of soluble substrate phosphorylation, whereas PIP_2 binding reduces the α-actinin-mediated inhibition towards receptor substrates (Freeman et al., 1998; Freeman et al., 2000). The co-ordinated regulation of GRK5 by these proteins and lipids could determine its substrate specificity under different cellular conditions.

1.2.3 Regulation of expression

GRKs are also tightly regulated at the level of their transcription and their stability within the cell. In vascular cells and lymphocytes, the expression of GRK2 mRNA is controlled by a variety of signal transduction pathways, although the precise mechanisms involved are unknown (Penela et al., 2003). Post-transcriptionally, GRK2 is polyubiquitinated and degraded by the proteasome an hour or so after

expression in a pathway that requires βarrestin and c-Src as well as GRK2 kinase activity (Penela et al., 2001a; Penela et al., 2003).

1.2.4 Receptor specificity and physiological roles

Identification of the particular GPCR targets phosphorylated by specific GRKs was originally achieved through the use of *in vitro* or tissue culture studies. Such work assessed GRK substrate specificity by utilising techniques including: purified GRKs acting on either rod outer segment preparations or purified receptors reconstituted into phospholipid vesicles; over-expression of GRKs and GPCRs in cultured cells; and inhibition of GRKs *in vitro* using antibodies or dominant negative GRK mutants (Pitcher et al., 1998b). Although these results gave some idea of the GRK(s) responsible for the desensitisation of an individual receptor, the development of mutant mice that lack different GRK isoforms has increased our knowledge of the physiological targets of a particular GRK and the role it plays in a particular tissue.

Mice lacking GRK1 expression display a prolonged response of retinal rod and cone cells to light and a decreased recovery rate. The light-stimulated desensitisation of rhodopsin is reduced (Lyubarsky et al., 2000). The importance of GRK1 in vision has been further demonstrated by the discovery that a congenital night blindness can be caused by null mutations in the gene encoding GRK1 (Yamamoto et al., 1997).

Transgenic mice homozygous for the GRK2 deletion ($\beta ARK1^{-/-}$) have been generated (Jaber et al., 1996). These mice died during gestation and did not survive beyond embryonic day E15.5, displaying cardiac abnormalities (Jaber et al., 1996). GRK2 was also shown to modulate myocardial contractile function in $\beta ARK1^{+/-}$ mice over-expressing the carboxyl terminus of GRK2 (βARKct), which inhibits endogenous GRK2 activity by competing for Gβγ binding (Rockman et al., 1998). $\beta ARK1^{+/-}$ /βARKct mice develop normally but have only 25% of wildtype GRK2 activity and show enhanced myocardial contractility in response to β-adrenergic receptor stimulation (Rockman et al., 1998). These studies indicate that GRK2 predominantly acts in the heart and modulates its function.

Despite its homology to GRK2, deletion of GRK3 in transgenic mice leads to viable animals. The mice have an increase in the muscarinic receptor-mediated airway response to muscarinic agonist stimulation and decreased desensitisation of odorant receptors (Peppel et al., 1997; Walker et al., 1999). $GRK3^{-/-}$ mice also show a reduced tolerance to the opioid fentanyl, suggesting that GRK3 may regulate the opioid receptor response to high efficacy opioids (Terman et al., 2004). Thus, although GRK3 shares sequence similarities with GRK2, its physiological function and receptor targets appear to be very different.

Mice lacking GRK5 are viable and have no developmental abnormalities, although they exhibit an enhanced behavioural sensitivity to muscarinic receptor stimulation and show reduced muscarinic receptor desensitisation in the brain (Gainetdinov et al., 1999). These data indicate that the M2 and M3 muscarinic receptors are the principal targets of GRK5 in the brain. A second study of these knockout mice indicated that GRK5 regulates the M2 muscarinic receptor (M2MR) in the airway but not the heart (Walker et al., 2004).

GRK6$^{-/-}$ mice show increased sensitivity to psychostimulant drugs such as amphetamine and cocaine and augmented signalling of dopamine D2 and D3 receptors (Gainetdinov et al., 2003). A second study demonstrated that *GRK6*$^{-/-}$ mice have an amplified inflammatory response following BLT1 receptor stimulation (Kavelaars et al., 2003). GRK6 may also play a role in regulation of chemotaxis since, *in vitro*, lymphocytes from *GRK6*$^{-/-}$ mice exhibit decreased motility in response to activation of the chemokine receptor CXCR4 while neutrophils from the same mice have enhanced chemotaxis following BLT1 or CXCR4 stimulation (Fong et al., 2002; Kavelaars et al., 2003; Vroon et al., 2004).

Overall it appears that, although most GRKs are ubiquitously expressed and appear to phosphorylate a wide range of GPCRs *in vitro*, the subcellular localisation, kinase activity and physiological function of an individual GRK is tightly regulated and its *in vivo* receptor targets restricted. However, it is has also been shown that a particular receptor may not be regulated by the same GRK in all tissues, such as is the case with the M2MR. Further studies investigating tissue-specific GRK knockouts or stimulating alternative GPCRs in each GRK-deficient mouse model

should show which GRKs are active in a particular tissue or in response to different physiological conditions.

Recent research using RNA interference (RNAi) has indicated that the $AT_{1A}R$ can be phosphorylated by GRK2, -3, -5 and -6 (Kim et al., 2005). Phosphorylation by GRK2 or –3 leads to βarrestin recruitment and receptor internalisation, whereas phosphorylation by GRK5 or -6 is required for βarrestin-mediated ERK activation (Kim et al., 2005), see also Section 1.2.6.1. It therefore appears that the phosphorylation of the carboxyl tail of $AT_{1A}R$ by different GRKs can lead to distinct cellular outcomes. Such a mechanism of differential signalling regulation via phosphorylation by different GRKs may apply to other GPCRs, although further research is needed to identify any such receptors.

1.2.5 GRKs and disease

GPCRs regulate a large variety of vital functions within the human body. Therefore it is likely that, due to the role GRKs play in regulating GPCR function, disorders in GRK-mediated activity will contribute to or cause disease. Many studies have investigated the function of GRKs in heart disease. Muscarinic and adrenergic GPCRs are responsible for regulating homeostasis in the cardiovascular system and it appears that increases in GRK expression and activity are associated with different cardiac disorders (Rockman et al., 2002).

The desensitisation of β-adrenergic receptors (βARs) in response to persistent agonist signalling leads to a loss of βAR number and activity and results in chronic heart failure (Petrofski and Koch, 2003). Studies in animal models indicate that this is due to increased GRK2 (and possibly GRK5) expression and activity (Petrofski and Koch, 2003). Although the exact mechanisms involved are currently unknown, treatment with βAR blockers has been shown to reduce GRK2 expression and function (Koch and Rockman, 1999). Expression of the GRK2 inhibitor βARKct in mouse and rabbit models of human heart failure prevented progressive deterioration of the heart and was shown to improve survival synergistically with β-blocker treatment (Rockman et al., 2002). Increased GRK2 levels are also associated with myocardial ischaemia, hypertension, and pressure overload ventricular hypertrophy

(Koch and Rockman, 1999). It is possible that inhibition of GRK2 with βARKct could be a powerful new therapy for all of these heart disorders, although effective methods of gene therapy to transfer the inhibitor to affected cells have yet to be developed.

Changes in GRK expression levels are also associated with a number of other diseases. Increased GRK2 activity is concomitant with chronic morphine treatment and may contribute to opiate addiction and tolerance (Ozaita et al., 1998; Terwilliger at al., 1994), whereas decreases in GRK2, as well as GRK6, function are seen in rheumatoid arthritis (Lombardi et al., 1999). GRK2 expression is significantly reduced in leukocytes from patients with multiple sclerosis (Vroon et al., 2005). Alterations in GRK2 and GRK5 expression have also been demonstrated in differentiated thyroid carcinoma and hypothyroidism (Metaye et al., 2002; Penela et al., 2001b).

1.2.6 Signalling roles of the receptor desensitisation machinery

Although GRKs and βarrestins were originally identified and characterised as essential mediators of GPCR desensitisation, it now appears that they, along with proteins such as GIT, also function as effectors in signalling pathways downstream of GPCRs.

1.2.6.1 βarrestin and ERK

As well as acting in GPCR desensitisation and internalisation, βarrestins have been shown to play a role in initiating signals following GPCR stimulation. They can function as adapters for the non-receptor tyrosine kinase c-Src and scaffolds for MAPKs, thus initiating ERK/MAPK cascades that regulate processes including gene expression, cell growth and apoptosis (Figure 1.5) (Shenoy and Lefkowitz, 2003). Upon agonist occupancy of β_2AR, βarrestin binds c-Src and recruits it to the plasma membrane, where it phosphorylates dynamin and promotes clathrin-mediated receptor endocytosis (Ahn et al., 1999). βarrestin-mediated recruitment of c-Src following stimulation of a GPCR also leads to ERK1/2 activation via the small G protein Ras, the MAPK kinase kinase Raf-1 and MEK1 (MAPK/ERK kinase 1) (DeFea et al., 2000; Luttrell et al., 1999; Luttrell et al., 2001). βarrestin acts as a

scaffold, forming a complex with Raf-1, MEK1 and ERK (DeFea et al;., 2000; Luttrell et al., 2001). Stimulation of GPCRs that transiently associate with βarrestins (class A receptors) cause nuclear translocation of ERK and activation of mitogenic pathways; class B receptors, which remain associated with βarrestins in endosomal compartments, lead to retention of ERK activity in the cytosol and a decrease in the mitogenic response, possibly inducing alternative ERK signalling pathways such as cell migration (Ge et al., 2003; Shenoy and Lefkowitz, 2003).

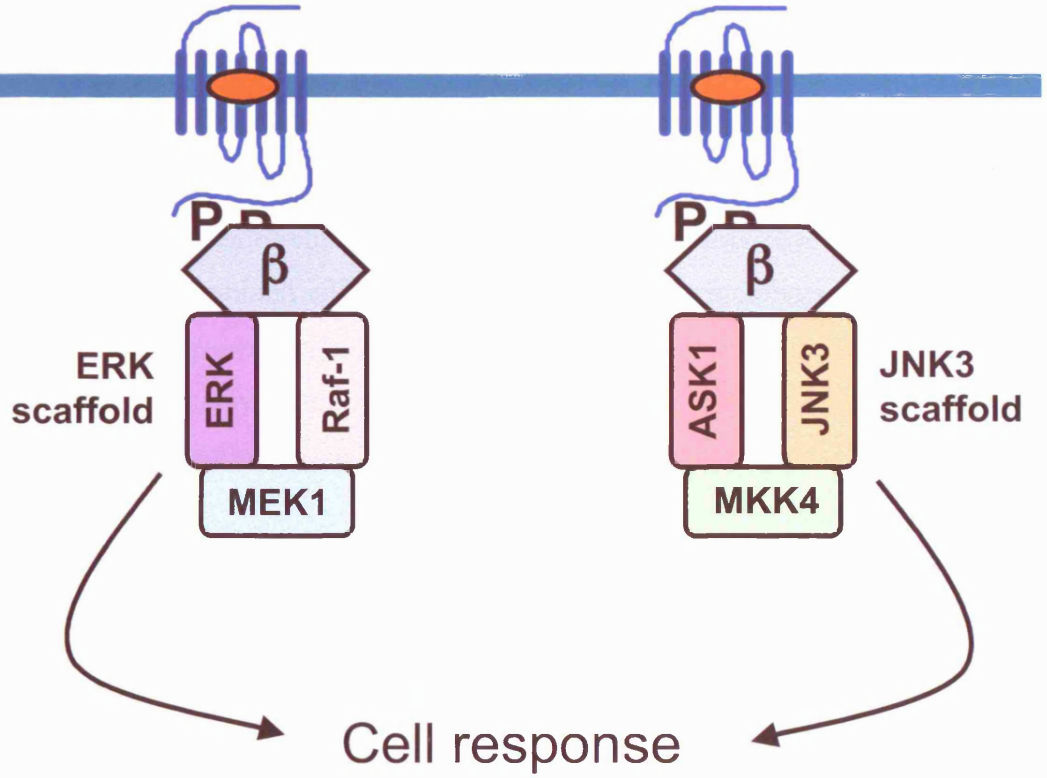

Figure 1.5. βarrestin scaffolding of MAPK cascades. Following agonist binding to the GPCR and its GRK-dependent phosphorylation, βarrestin is recruited to the receptor, bringing it with components of a MAPK cascade. Association with βarrestin facilitates the activation of the kinases in the cascade, ultimately leading to activation of either ERK or JNK3. β: βarrestin. P denotes a phosphate group.

βarrestins can also scaffold components of the MAPK-related JNK3 (c-Jun amino-terminal kinase 3). On $AT_{1A}R$ stimulation, βarrestin2 forms a complex with the MAPK kinase kinase ASK1 (apoptosis signal-regulating kinase 1), the MAPK kinase MKK4 and JNK3, resulting in JNK3 activation (McDonald et al., 2000). The active JNK3 is retained in the cytosol, although the purpose for this is currently unknown.

Recent research has shown that βarrestins are also important in processes such as chemotaxis, apoptosis and even regulation of transcription (Gao et al., 2004; Shenoy and Lefkowitz, 2003).

1.2.6.2 GIT

As discussed above, GIT1 was originally identified as a GAP for Arfs 1 and 6, with a role in mediating GPCR endocytosis (Claing et al., 2000). As well as being recruited to the plasma membrane via its interaction with GRK2 (Premont et al., 1998), GIT1 has been shown to bind to the focal adhesion adapter paxillin, and a complex of the Rac/Cdc42 GEF PIX and the p21 GTPase-activated protein kinase, PAK (Premont et al., 2000). A recent study has indicated that c-Src-phosphorylated GIT1 is associated with MEK1 and is required for both $AT_{1A}R$- and epidermal growth factor (EGF) tyrosine kinase receptor-stimulated ERK activation, possibly at focal adhesions (Yin et al., 2004). Combined with reports that GIT1 family members are important for focal adhesion turnover and cell motility (Turner et al., 1999; West et al., 2001; Zhao et al., 2000), these interactions suggest that, following GPCR activation, GIT1 may regulate the cytoskeleton as well as receptor internalisation.

1.2.7 Non-receptor substrates of GRKs

A number of non-receptor substrates have been identified for GRKs, indicating that they too may have additional roles within the cell. Tubulin was the first to be characterised. It has been shown to associate with the carboxyl-terminus of GRK2, which can phosphorylate tubulin with a K_m of 1-3μM (Carman et al., 1998, Haga et al., 1998; Pitcher et al., 1998a). Furthermore, stimulation of GPCRs *in vivo* and *in vitro* promotes both the GRK2/tubulin interaction and the phosphorylation reaction (Haga et al., 1998; Pitcher et al., 1998a). GRKs –2, -3 and –5 have also been demonstrated to phosphorylate phosducin and phosducin-like protein, cytosolic proteins that regulate heterotrimeric G protein signalling by binding Gβγ (Ruiz-Gomez et al., 2000). Phosphorylation by GRK2 reduces the binding of phosducin to Gβγ, suggesting that GRKs may regulate Gβγ signalling following GPCR activation (Ruiz-Gomez et al., 2000).

Synucleins, small lipid-binding proteins predominantly expressed in the brain, are phosphorylated by all GRKs (Pronin et al., 2000). Phosphorylation is enhanced in the presence of phospholipids and Gβγ (GRK2) or phospholipids alone (GRK5), implying that GRKs may phosphorylate synucleins following GPCR activation. GRK phosphorylation hinders synuclein-mediated inhibition of phospholipase D2, an enzyme that stimulates endocytic vesicle formation via production of phosphatidic acid. Since synucleins associate with synaptic vesicles, it is possible that GRKs could modulate receptor endocytosis through interactions with these proteins (Pronin et al., 2000).

GRK2 was also shown to phosphorylate the inhibitory γ subunit of the type 6 retinal cGMP phosphodiesterase (PDEγ) (Wan et al., 2001). Phosphorylation is induced following both thrombin G protein-coupled receptor and EGF receptor stimulation and leads to augmented activation of p42/p44 MAPK and association of PDEγ with dynamin II (Wan et al., 2001). This study also indicated that GRK2 is essential for EGF-mediated p42/p44 MAPK stimulation. Further evidence suggests that GRK2 may act downstream of the EGF receptor, since treatment of cells with EGF leads to association of GRK2 with a complex of PDEγ and the MAPK activator c-Src (Wan et al., 2003).

The ribosomal protein P2 has been identified as a GRK2 substrate (Freeman et al., 2002). Phosphorylation by GRK2 promotes translational activity in purified 60S ribosomal subunits and is enhanced following β_2AR stimulation (Freeman et al., 2002). A more recent study has shown that GRK2 can also phosphorylate the β-subunit of epithelial Na^+ channels, causing them to become insensitive to regulation by ubiquitin protein ligases (Dinudom et al., 2004). These studies establish GRKs as potential mediators of an array of signalling pathways additional to those leading to GPCR desensitisation. Activation of GRKs through agonist occupation of GPCRs could lead to cytoskeletal rearrangement, modulation of protein translation and activity, facilitation of endocytosis, and participation in signalling pathways such as those downstream of Gβγ and c-Src.

1.3 A role for the actin cytoskeleton in GPCR desensitisation

1.3.1 Actin structure

The actin cytoskeleton of eukaryotic cells consists of a network of microfilaments that extend throughout the cytoplasm, just beneath the plasma membrane, forming the cell cortex. Rather than acting as a static scaffold, this microfilament network provides a highly adaptable and dynamic structure that undergoes constant assembly and disassembly. Filamentous actin (F-actin) is a two-stranded helical polymer created following nuclear hydrolysis of globular actin monomers (G-actin). A dynamic equilibrium exists between F- and G-actin, with soluble G-actin monomers being added to the fast growing barbed ends of microfilaments and disassembly occurring at the slow growing pointed ends (Figure 1.6A) (reviewed in Pollard and Borisy, 2003).

Arp2/3 is a 200kDa complex made up of the actin related proteins Arp-2 and -3 and five novel subunits designated ARPC1-5 (May, 2001). It acts as the central control of actin filament nucleation and branching by overcoming the energetic barrier involved in the formation of new actin filaments from G-actin (Figure 1.6A) (May, 2001). Arp2/3 is activated by members of the WASP (Wiscott-Aldrich syndrome protein) family of scaffolding proteins (Mullins, 2000). WASP family proteins are modular molecules able to bind to PIP_2, the small GTPase Cdc42, actin and Arp2/3 (Figure 1.6B). In the presence of Cdc42 and PIP_2, WASP is activated and can stimulate the Arp2/3 complex to initiate actin polymerisation (Rohatgi et al., 1999). WASP proteins contain a proline-rich region and are regulated by an array of SH3 (Src homology 3) domain-containing proteins, including intersectin and syndapins (da Costa et al., 2003, see below). The actin binding proteins Abp1 (actin binding protein 1) and cortactin have been identified as alternative activators of Arp2/3 in yeast and mammalian cells respectively (Goode et al., 2001; Weaver et al., 2001).

The actin network is involved in many essential cell functions such as maintenance of cell shape, motility, attachment to the extracellular matrix and cytokinesis. Microfilaments form cellular structures, such as lamellipodia (thin cytoplasmic sheets containing arrays of microfilaments), filopodia (narrow membrane protrusions

rich in F-actin bundles) and stress fibres (contractile actin filaments), and can undergo remodelling in response to both extracellular and intracellular signals. This reorganisation of actin filaments is highly regulated by the Rho family of small GTPases (Burridge and Wennerberg, 2004). By utilising the ability of the actin cytoskeleton to undergo dynamic changes via F-actin assembly and disassembly, a cell can react quickly to any change in conditions.

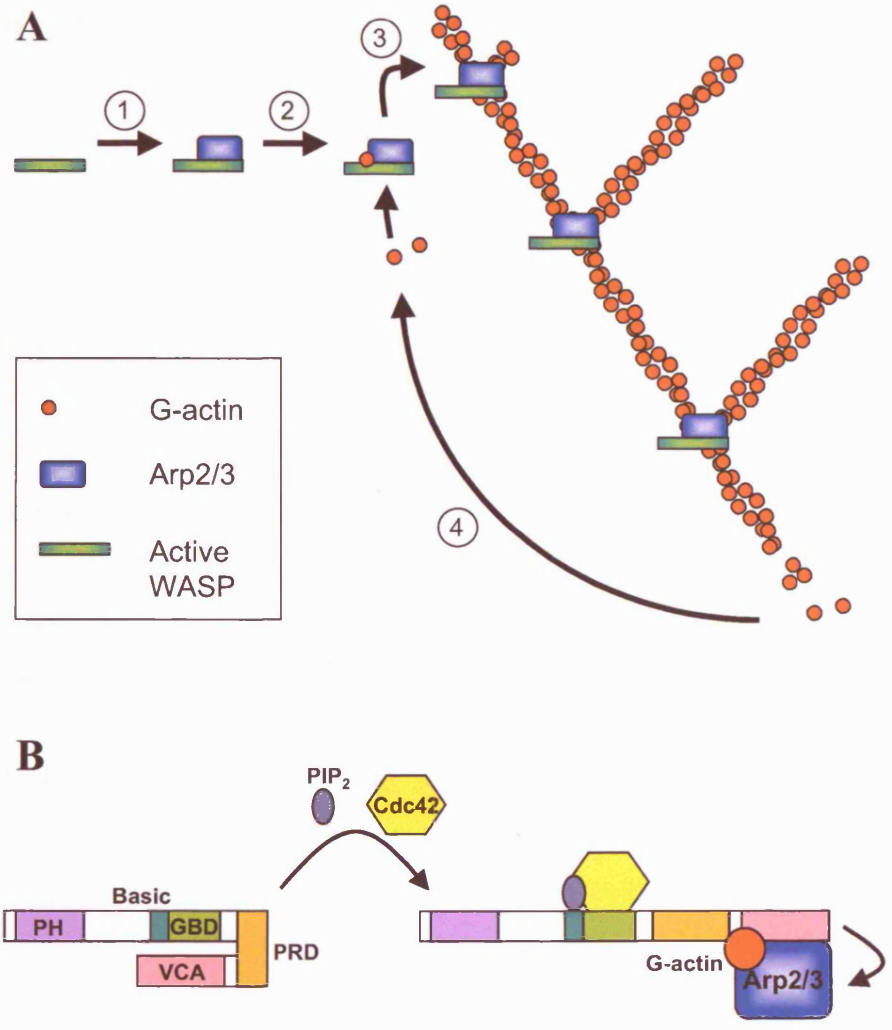

Figure 1.6. Actin filament nucleation by Arp2/3 and WASP. **A.** Active WASP binds Arp2/3 (1), and a G-actin monomer (2). This facilitates actin nucleation at the barbed end, elongating and branching F-actin (3). At the pointed end of F-actin, actin monomers dissociate and are returned to a pool available for F-actin nucleation (4). **B.** Proposed WASP activation mechanism. WASP is auto-inhibited through binding of its GTPase binding domain (GBD) to its carboxyl terminus. Binding of Cdc42 and PIP_2 to the GBD and basic domains leads to unmasking of the verproline-cofilin-acidic (VCA) domain. The free VCA domain binds to Arp2/3 and G-actin, facilitating Arp2/3-mediated actin nucleation. PH: pleckstrin homology. PRD: proline-rich domain.

1.3.2 Actin and clathrin-mediated endocytosis

The main steps of clathrin-mediated endocytosis, following cargo selection, are those of plasma membrane invagination and coat formation, vesicle fission, transport of the vesicle from the membrane, uncoating and fusion to endosomes (Figure 1.3). All of these stages require remodelling of actin and the cell cortex. Data from yeast indicate that, rather than playing a passive role, the actin cytoskeleton is directly involved in endocytosis. Approximately one third of the proteins implicated in internalisation in *Sacchoromyces cerevisiae* participate in actin assembly and many of these have mammalian homologues (Engqvist-Goldstein and Drubin, 2003).

Several mammalian proteins have been identified that associate with components of both the actin cytoskeleton and the endocytic machinery (Figure 1.7). Some of these proteins have been shown to interact directly with the clathrin coat. A splice variant of the actin-based motor protein myosin VI associates with the adaptor protein AP-2 and is enriched in clathrin-coated vesicle (CCV) preparations. The localisation of this variant with CCVs was dependent on a large insert in the tail domain, over-expression of which inhibited transferrin endocytosis (Buss et al., 2001). Recent research has also demonstrated that, in hippocampal neurons, myosin VI exists in a complex with AP-2 and AMPA-type glutamate receptors and that internalisation of AMPA-type receptors is inhibited in myosin VI-deficient neurons (Osterweil et al., 2005). Since myosin VI is known to move towards the pointed ends of microfilaments, which are oriented away from the plasma membrane, it is hypothesised that myosin VI could provide the force for membrane invagination or transporting CCVs towards the cell body (Buss et al., 2001).

Huntingtin interacting protein 1R (Hip1R) is an F-actin-binding protein that co-localises and co-purifies with CCVs (Engqvist-Goldstein et al., 1999). The yeast homologue, Sla2p, is essential for both actin assembly and endocytosis (Engqvist-Goldstein and Drubin, 2003). In mammalian cells, Hip1R is believed to associate with the clathrin light chain as over-expression of the clathrin hub domain, which competes for binding to the light chain, caused Hip1R to dissociate from CCVs (Bennett et al., 2001). Furthermore, RNAi silencing studies have indicated that Hip1R regulates the interaction between the endocytic machinery and the actin cytoskeleton (Engqvist-Goldstein et al., 2004).

The adaptor protein intersectin is an SH3 domain-containing protein that can associate with both CCV coat components, such as AP-2 and clathrin, and other proteins associated with endocytosis, including dynamin and synaptojanin (da Costa et al., 2003). Although these interactions indicate a role for intersectin as a scaffolding molecule in endocytosis, a splice variant, intersectin-l, has also been shown to mediate actin nucleation via WASP and Cdc42 (Hussain et al., 2001, see below). Disruption of the association between intersectin and Cdc42 inhibited clathrin-mediated endocytosis of the T cell antigen receptor (McGavin et al., 2001).

Dynamin can also bind many other SH3 domain-containing proteins that are involved in actin remodelling (da Costa et al., 2003; Schafer, 2002). Dynamin is believed to primarily function as a mechano-enzyme mediating fission of a CCV from the plasma membrane, however it has also been associated with actin comet tails generated by the *Listeria monocytogenes* pathogen in host cells and regulation of the actin cytoskeleton in lamellipodia (Lee and De Camilli, 2002; McNiven et al., 2000). It is thought that it mediates actin assembly via its interactions with proteins such as Abp1 and cortactin, which activate Arp2/3, and syndapin, a WASP-binding adaptor protein that regulates actin cytoskeleton remodelling during endocytosis (da Costa et al., 2003; Engqvist-Goldstein and Drubin, 2003, see below). A study using evanescent field microscopy to visualise late stages in CCV formation demonstrated that the recruitment of dynamin to vesicles just prior to their translocation was accompanied by a transient burst of actin assembly (Merrifield et al., 2002). More recently, research using RNAi to suppress cortactin expression has indicated that both binding to Arp2/3 and actin polymerisation are necessary for cortactin to associate with dynamin (Zhu et al., 2005). This suggests that, following the recruitment of dynamin to a ligand-bound receptor, actin polymerisation by Arp2/3 promotes the association of cortactin with dynamin. The action of cortactin would further increase actin polymerisation via Arp2/3 and may serve to drive the fission of CCVs (Zhu et al., 2005).

An additional protein that has been implicated in both clathrin-mediated endocytosis and actin remodelling is Arf6. Arfs are believed to function in the recruitment of coat proteins onto membranes, the activation of lipid-modifying enzymes and

remodelling of the actin cytoskeleton (Donaldson, 2003). Although active Arf6 has not been shown to directly recruit coat proteins to membranes, it is localised at the plasma membrane and has been demonstrated to stimulate PIP_2 production through activation of phosphatidylinositol phosphate (PIP) 5-kinase, both directly and via phospholipase D (Cavenagh et al., 1996; Honda et al., 1999; Melendez et al., 2001). A localised concentration of PIP_2 at the plasma membrane would lead to clathrin recruitment and could facilitate dynamin-induced vesicle fission (Takei and Haucke, 2001).

Both Arf6 and its guanine nucleotide exchange factor, ARNO, bind to βarrestin2 and the cycle of Arf6 GTP binding and hydrolysis is required for β_2AR internalisation (Shenoy and Lefkowitz, 2003). Arf6 has been shown to be essential for the endocytosis of a number of GPCRs, regardless of whether they internalise via a clathrin-dependent or –independent process (Houndolo et al., 2004). It is now well established that Arf6 modulates actin microfilaments in processes such as formation of membrane protrusions, membrane ruffling and migration (Donaldson, 2003), and it may serve as a further link between GPCR endocytosis and the actin cytoskeleton.

Although it is still unclear as to the exact role(s) of actin in mammalian clathrin-mediated endocytosis, one study has advanced understanding of how actin is coupled to endocytosis in yeast (Kaksonen et al., 2003). The study used real-time fluorescence microscopy to localise and track GFP-tagged proteins during the early stages of CCV formation. The results showed that the WASP homologue Las17p is recruited at an early stage to actin "patches" at sites of endocytosis followed by the Hip1R homologue Sla2p, the yeast homologue of the mammalian endocytic adaptor Eps15 (Pan1p) and a second adaptor protein (Sla1p). Actin, Abp1 and the Arp2/3 complex are then brought to the patch and slow movement of the structure is seen. The early components dissociate and the patch is seen to move rapidly towards the interior of the cell. Additionally, actin nucleation was demonstrated at the endocytic site (Kaksonen et al., 2003).

In yeast Las17p, Pan1p and Abp1 can activate Arp2/3, implying that actin assembly may be stimulated sequentially during endocytosis as these proteins are recruited. Since homologues exist in mammalian cells, a similar process may occur. Recent

research has demonstrated that phosphorylation of Pan1p by the Prk1p kinase serves to inhibit Arp2/3-mediated actin polymerisation on endocytic vesicles (Toshima et al., 2005). This is thought to promote vesicle fusing with early endosomes as it has been shown that actin polymerisation must cease following internalisation to allow vesicle fusion (Sekiya-Kawaski et al., 2003).

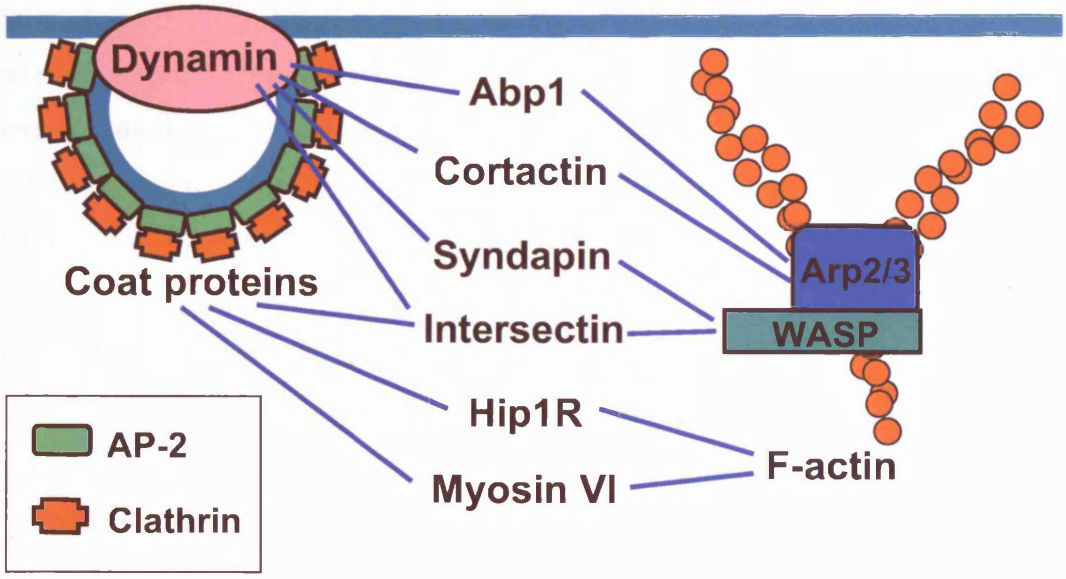

Figure 1.7. Summary of the protein associations between components of the endocytic machinery and the actin cytoskeleton. Blue lines between proteins indicate specific interactions. Dynamin is thought to regulate actin assembly via Abp1 and cortactin, which activate Arp2/3, and syndapin, which binds and regulates WASP. Intersectin can associated with AP-2, clathrin and dynamin, and also mediates actin nucleation via WASP. Hip1Ris an F-actin binding protein that co-purifies with clathrin coated vesicles and has been shown to regulate the interaction between the endocytic machinery and the actin cytoskeleton. Myosin VI associates with AP-2 and may act to facilitate membrane invagination or transport of vesicles towards the cell body. Figure adapted from Schafer, 2002.

The effect of actin-perturbing drugs on the clathrin-mediated endocytosis of transferrin in mammalian cells has also been investigated. The uptake of transferrin in a number of different cell lines grown on a variety of substrates and treated with either latrunculin A, cytochalasin D or jasplakinolide was examined (Fujimoto et al., 2000). The report concluded that the requirement for actin in internalisation depended on cell type and growth conditions. These conclusions, together with the inconsistent results yielded from other studies using these drugs, have led to the notion that while the actin cytoskeleton does not play an obligate role in the

internalisation of the transferrin receptor via clathrin-mediated endocytosis in mammalian cells, it may facilitate and regulate the process (Fujimoto et al., 2000; Schafer, 2002). More recent research using fluorescence microscopy has demonstrated that disruption of the F-actin assembly/disassembly cycle by actin-perturbing drugs inhibits clathrin-mediated endocytic events in mammalian cells (Yarar et al., 2005). The assembly and disassembly of F-actin is required for CCP formation, the constriction of CCVs from the plasma membrane and CCV internalisation. However, actin disruption inhibited the internalisation of approximately 80% of the CCVs and 45% of total cell surface transferrin receptor, indicating that mammalian cells may have alternative actin- and clathrin-independent endocytosis pathways (Yarar et al., 2005).

1.3.3 Rho GTPase family

The Rho family consists of 23 ubiquitous small GTPases that act as molecular switches to control cellular functions through regulation of the actin cytoskeleton, downstream of extracellular cues (Wherlock and Mellor 2002). Within the cell, these GTPases cycle between inactive (GDP-bound) and active (GTP-bound) conformations that serve as "off" and "on" states in intracellular signalling pathways (Figure 1.8). The GTP-bound proteins activate downstream effectors until hydrolysis of the GTP to GDP switches the signal "off".

The GTP/GDP cycling of small GTPases is highly controlled (Figure 1.8). GEFs (guanine nucleotide exchange factors) promote GDP release and GTP binding, while GAPs (GTPase-activating proteins) stimulate the hydrolysis of GTP. Both Rho GAPs and Rho GEFs are subject to regulation and can act upstream and downstream of a number of signalling pathways (Moon and Zheng, 2002; Schmidt and Hall, 2002). Rho proteins require prenylation of a carboxyl-terminal CAAX motif for membrane targeting and function. Inactive Rho GTPases are thought to exist in a complex with Rho GDIs (guanine nucleotide dissociation inhibitors), which inhibit GDP dissociation. GDIs have also been shown to extract Rho proteins from membranes, masking the prenylated tail and leading to their sequestration in the cytosol (Olofsson, 1999). Proteins such as the plasma membrane/actin cross-linker radixin (see Section 1.6) have been demonstrated to bind to RhoGDI and cause the displacement of Rho (Takahashi et al., 1997), suggesting that GDIs could deliver

inactive Rho proteins to particular sites for activation by GEFs, possibly as components of specific signalling complexes (Olofsson, 1999; Sasaki and Takai, 1998).

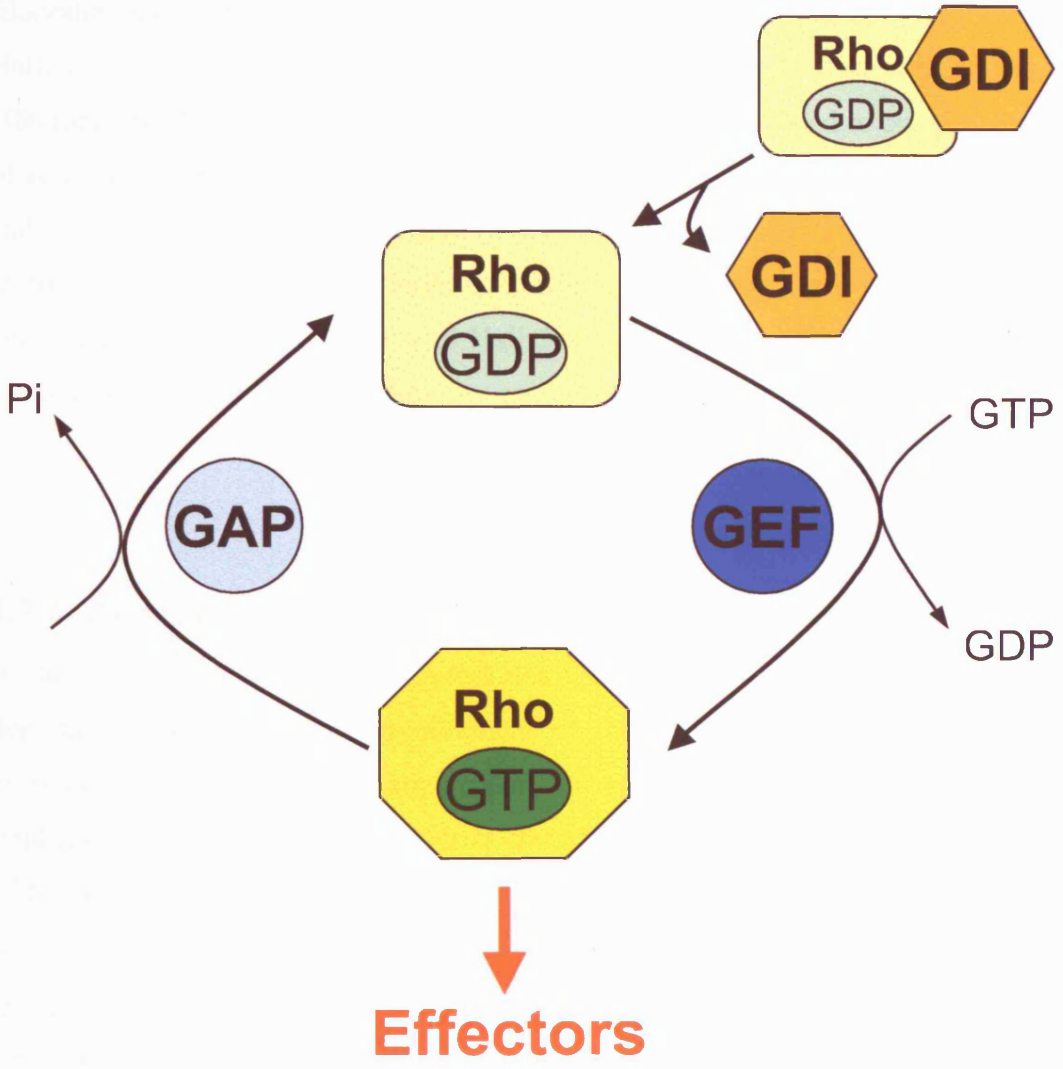

Figure 1.8. The Rho GTPase activation cycle. Within the cell, Rho GTPases cycle between inactive GDP-bound and active GTP-bound conformations. This cycle is highly controlled by GEFs (guanine nucleotide exchange factors), which promote GDP release and GTP binding, and GAPs (GTPase-activating proteins), which stimulate the hydrolysis of GTP. Inactive Rho GTPases are thought to exist in a complex with Rho GDIs (guanine nucleotide dissociation inhibitors), which inhibit GDP dissociation. The GTP-bound proteins activate downstream effectors until hydrolysis of the GTP to GDP switches the signal "off".

1.3.3.1 Rho and actin cytoskeleton regulation

The most extensively characterised members of the Rho GTPase family are Rho, Rac and Cdc42. Initial studies with constitutively active and dominant negative forms indicated that they functioned to regulate remodelling of the actin cytoskeleton. Rho was implicated in stress fibre formation, Rac induced lamellipodia, Cdc42 promoted filopodia, and all three were involved in adhesion complex formation (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley and Hall, 1992b). Over 40 Rho family effectors have been identified and Rho GTPases have been found to act in a variety of actin-dependent processes (including migration, morphogenesis, cytokinesis and endocytosis), transducing extracellular cues by linking membrane receptors to actin remodelling in a co-ordinated fashion (Burridge and Wennerberg, 2004; Qualmann and Mellor, 2003; Raftopoulou and Hall 2004). Rho proteins also regulate other pathways such as those leading to cell polarity, cell cycle progression and transcription factor regulation (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002).

1.3.4 Rho GTPases in clathrin-mediated endocytosis

In the same way that the actin cytoskeleton is not strictly essential for clathrin-dependent endocytosis, the involvement of the Rho family of GTPases is not absolutely required, although they appear to regulate internalisation. Early studies implicated RhoA and Rac in transferrin clathrin-mediated endocytosis as expression of constitutively active mutants inhibited receptor internalisation and CCV budding (Lamaze et al., 1996). GTP-bound Rac has been demonstrated to interact with synaptojanin 2 and recruit it to the plasma membrane (Malecz et al., 2000). Following vesicle internalisation synaptojanin 2 hydrolyses PIP_2, leading to vesicle uncoating and endosome formation (Cremona et al., 1999). Since Rac can also stimulate PIP_2 production by PIP 5-kinases (Ren and Schwartz, 1998), it is possible that Rac controls the rate of both CCV coating, at localised sites of PIP_2 at the plasma membrane (Takei and Haucke, 2001) and uncoating, via activation of synaptojanin 2.

The Rac GEF Sos1 has been shown to bind to syndapins, endocytic adapter proteins that associate with synaptojanin (da Costa et al., 2003; Wasiak et al., 2001). Syndapins are also WASP binding partners and may promote Rac translocation and

activation at the plasma membrane via interactions with Sos1, leading to localised PIP_2 synthesis and WASP activation (Wasiak et al., 2001). A further connection between Rac and endocytosis may be via the Arp2/3 activator Abp1, which can bind both dynamin and F-actin (Kessels et al., 2001) and which localises to sites of *de novo* F-actin assembly following Rac activation (Kessels et al., 2000).

Cdc42 may play a role in clathrin-mediated endocytosis by means of its interaction with the endocytic adaptor protein intersectin. A splice variant of intersectin, intersectin-l, contains a Dbl-homology domain that imparts a GEF activity towards Cdc42. Binding of WASP to intersectin-l upregulates its GEF function, promoting GTP-bound Cdc42 and leading to actin remodelling via WASP activation (Hussain et al., 2001). Expression of a mutant intersectin-l that lacked the Dbl-homology domain reduced Cdc42 activation following T cell antigen receptor stimulation and inhibited receptor internalisation (McGavin et al., 2001). Furthermore, studies have shown that the Cdc42 effector ACK (activated Cdc42-associated tyrosine kinase) may be involved in directing the trafficking of endocytosed receptors to the lysosome through its interaction with sorting nexin 9, which has been demonstrated to be regulate dynamin activity during clathrin mediated endocytosis (Qualmann and Mellor, 2003, Soulet et al., 2005).

Rho has also been implicated in the regulation of traffic through early and late endosomes. RhoD is involved in early endosome motility, which it impedes through recruitment of a Diaphanous-family protein, hDia2C, and subsequent tethering of endosomes along actin filaments (Gasman et al., 2003). A second Rho-type protein, RhoB, is localised to the cytosolic surface of endosomes where it controls the trafficking of the epidermal growth factor receptor to the lysosome via its effector PRK1 kinase (Gampel et al., 1999). Consequently, Rho family GTPases may be involved in the regulation of clathrin-mediated internalisation during both vesicle formation at the plasma membrane and sorting through endocytic compartments.

1.4 Additional pathways of receptor endocytosis

Internalisation of the ET_BR and VIP_1R receptors has been demonstrated to be independent of βarrestins and dependent on dynamin, suggesting that they internalise

via caveolae pathway (Claing et al., 2000). A number of other GPCRs and constituent molecules of downstream signal cascades have been localised to caveolae, although it is unclear as to whether these findings implicate caveolae in GPCR internalisation or if they act as lipid rafts to compartmentalise particular signalling pathways. Additionally, the M2MR and $AT_{1A}R$ appear to internalise in a βarrestin- and dynamin-independent process that may not require clathrin or caveolin (Claing et al., 2000; Delaney et al., 2002; Roseberry and Hosey, 2001; Zhang et al., 1996). Internalisation of the M2MR is regulated by Arf6 (See Section 1.3.2), which has also been shown to regulate the clathrin-mediated endocytosis of the β_2 adrenergic and luteinising hormone/chorionic gonadotropin receptors (Claing et al., 2001; Delaney et al., 2002; Mukherjee et al., 2002). Interestingly, internalised M2MR colocalises with early endosomes derived from clathrin-dependent endocytic pathway, indicating that two distinct mechanisms of GPCR internalisation may converge (Delaney et al., 2002).

1.5 Trafficking

Following internalisation into endocytic vesicles, GPCRs can be divided into two groups: those that are promptly recycled back to the cell surface and those that are more readily degraded in the lysosome (von Zastrow, 2003). The sorting of receptors to either the recycling or lysosomal pathways occurs in an early endosomal compartment and it appears that the information required for this decision is located in the cytoplasmic carboxyl tails of the receptors. Studies utilising chimeric receptors have shown that these sorting signals are transplantable and can mediate the recycling of a normally degraded GPCR and vice versa (Gage et al., 2001; Trejo and Coughlin, 1999).

One protein that may be involved in recognising such a signal is the Na^+/H^+ exchanger regulatory factor (NHERF, or EBP50). This PDZ (PSD-95, Dlg, ZO-1 homology) domain-containing protein can bind to the very carboxyl terminus of the β_2AR and is also indirectly associated with the actin cytoskeleton via its interactions with ezrin-radixin-moesin (ERM) proteins (Hall et al., 1998a; Hall et al., 1998b; Reczek et al., 1997, see Section 1.6). Disruption of the interaction of NHERF with either β_2AR or ERM proteins caused mis-sorting of the usually recycled receptor to

lysosomes (Cao et al., 1999). NHERF has also been shown to regulate the trafficking of the human κ opioid receptor (hKOR) as over-expression of NHERF inhibited agonist-induced hKOR downmodulation, presumably by increasing receptor recycling (Li et al., 2002).

Additionally, the *N*-ethylmaleimide-sensitive factor (NSF) has been demonstrated to recognise an overlapping region of the β_2AR tail as NHERF and modulate β_2AR recycling (Cong et al., 2001b). NSF can also bind to the carboxyl tail of the D1 dopamine receptor, which undergoes agonist-induced endocytosis and recycling (Heydorn et al., 2004). It is unclear, however, whether NSF is necessary for β_2AR internalisation, as a recent study has demonstrated that PDZ ligands within the carboxyl-termini of GPCRs are sufficient to promote receptor recycling independently of NSF (Gage et al., 2005). Of the three GPCR PDZ ligands investigated in the study, one did not bind to NHERF but still promoted efficient receptor recycling (Gage et al., 2005). Since β-finger motifs believed to function as internal PDZ ligands have been identified within the carboxyl tails of a number of GPCRs that are recycled to the plasma membrane following endocytosis (Paasche et al., 2005), it is possible that PDZ ligands represent general recycling sorting signals within GPCR carboxyl tails.

GPCRs that are degraded after agonist-induced internalisation also interact with sorting proteins. Sorting nexin 1 has been shown to associate with the protease-activated receptor-1 GPCR in an interaction required for targeting the receptor to lysosomes (Wang et al., 2002). The D5 dopamine receptor can bind sorting nexin 1 via its carboxyl tail and, although little is known about the trafficking of D5, this interaction may mediate its fate following endocytosis (Heydorn et al., 2004).

Ubiquitination has also been shown to act as a signal for internalisation and GPCR trafficking. The β_2AR, along with βarrestin, was demonstrated to be rapidly ubiquitinated on agonist occupancy (Shenoy et al., 2001). Ubiquitination requires the interaction of βarrestin with an E3 ubiquitin ligase, MDM2, which catalyses the covalent attachment of ubiquitin to lysine residues of the substrate. A β_2AR receptor mutant that could not be ubiquitinated was internalised but not degraded efficiently

and ubiquitination of βarrestin was shown to be necessary for receptor internalisation (Shenoy et al., 2001). Ubiquitination was also shown to be important for lysosomal degradation of the CXCR4 chemokine receptor (Marchese and Benovic, 2002). Transmembrane proteins that are ubiquitinated are sorted into multivesicular endosomes via a succession of proteins including the ubiquitin-binding protein Hrs/Vps27, from where they are trafficked to the lysosome (reviewed in Raiborg et al., 2003).

βarrestins may have a further role in modulating the recycling of internalised GPCRs. Some receptors, such as the β_2AR, D1 dopamine receptor, endothelin A receptor and μ opioid receptor, recycle rapidly (class A receptors) while others, such as the $AT_{1A}R$, vasopressin 2 receptor and substance-P receptor, recycle slowly (class B receptors). The association of βarrestins with class A receptors is transient and only occurs at the plasma membrane. In contrast, βarrestins have a more prolonged interaction with class B receptors and are trafficked with them to endosomal vesicles (Pierce et al., 2002).

Hence, desensitisation and internalisation are important processes for regulating the signalling capabilities of GPCRs. Many different mechanisms exist to control endocytosis and subsequent sorting of receptors back to the plasma membrane or to a degradation pathway. The variety of proteins involved and the large number of GPCRs expressed suggests that particular subsets of receptors may employ different methods of regulation, many of which have yet to be identified.

1.6 ERM proteins

Within this thesis, I shall show that ezrin is a novel substrate of GRK2. Ezrin is a member of the ezrin-radixin-moesin (ERM) family of cross-linking proteins that regulate the structure and function of the actin cortex, principally at specific plasma membrane domains (reviewed in Bretscher et al., 2002). Their primary role is that of linking plasma membrane proteins or associated proteins to F-actin. Ezrin was originally purified from the cytoskeleton of intestinal epithelial cell microvilli and ERM members were subsequently found to be enriched in a number of other membrane structures, including membrane ruffles and adherens junctions (Bretscher,

1989; Tsukita et al., 1989). They are thought to function in cell motility, adhesion and morphogenesis; growth signal transduction; and regulation of membrane transport and membrane protein localisation.

The vertebrate ERM proteins have a molecular weight of approximately 80 kDa and show a high level (approximately 80%) of amino acid and structural conservation (Bretscher et al., 2002). Homologues exist in *C. elegans*, *Drosophila*, tapeworms and *Lytechinus variegates* (sea urchin), although none have yet been identified in *Sacchoromyces cerevisiae*, suggesting that they are unique to multicellular organisms. Studies of the single *Drosophila* ERM protein Dmoesin indicate that it has a critical role in organising cell polarity, cell adhesion and the actin cytoskeleton during development (reviewed in Polesello and Payre, 2004). Elucidation of the distinct roles of the three mammalian ERM members has proved more difficult since the structural similarity and overlapping tissue expression implies that they will show functional redundancy. This has been demonstrated in targeted gene inactivation studies in mice: moesin knockouts have no apparent defects, even in the absence of ezrin and radixin upregulation, and radixin knockouts show only liver malfunction due to the absence of apical microvilli in bile canaliculi (Doi et al., 1999; Kikuchi et al., 2002). Recently, however, the generation of ezrin-null mice has indicated that ezrin is essential for the organisation of the apical surface of the intestinal epithelium (Saotome et al., 2004; Tamura et al., 2005). Mice lacking ezrin were born at submendelian ratios, failed to thrive and died before weaning. It is not yet known whether other ezrin-rich tissues were affected.

1.6.1 Structure

ERM proteins consist of two structural domains, the amino- and carboxy-ERM association domains (N- and C-ERMADs), connected by a coiled-coil (α-helical) region (Figure 1.9A). The N-ERMAD comprises a FERM (4.1, ezrin, radixin, moesin) domain. Proteins that contain FERM domains are characterised as part of the Protein 4.1 superfamily, members of which generally associate with the plasma membrane and cytoskeleton (Chishti et al., 1998). The globular FERM domains of ezrin, radixin and moesin are highly conserved and are composed of three distinct subdomains, F1, F2 and F3 (Figure 1.9B). F1 is structurally homologous to ubiquitin, F2 shares similarities with acyl-CoA binding proteins and F3 is related to

PH, Enabled/VASP-1 (EVH1) and phosphotyrosine-binding (PTB) domains (Pearson et al., 2000). FERM domains serve as protein- and lipid-binding sites, which in the case of ERM proteins enable them to interact with both plasma membrane-associated proteins (Finnerty et al., 2004; Hamada et al., 2003) and PIP_2 (Barret et al., 2000; Niggli et al., 1995).

The C-ERMAD consists of a β-sheet and six α-helices, four of which form an actin-binding domain within the last 34 residues of the carboxy-terminus (Pearson et al., 2000). In the cytosol, ERM proteins adopt an inactive conformation wherein intramolecular or intermolecular interactions between the N- and C-ERMADs mask the membrane- and actin-binding sites (Gary and Bretscher, 1995; Magendantz et al., 1995). Although ERM protein homo- and heterodimers have been identified (Gary and Bretscher, 1993), most cytosolic ezrin exists in a monomeric form in which its N- and C-ERMADs are associated (Berryman et al., 1995; Bretscher et al., 1995). This conformation is considered dormant since it masks the binding sites for molecules such as Rho GDI (Takahashi et al., 1997) or other ERM molecules (Gary and Bretscher, 1995) at the N-ERMAD and actin at the C-ERMAD (Figure 1.9C) (Algrain et al., 1993; Gary and Bretscher, 1995).

The crystal structure of inactive moesin reveals that the C-ERMAD forms an extended conformation that binds to the FERM domain, masking both a large proportion of the surface of the FERM domain and the carboxy-terminal actin-binding region (Pearson et al., 2000). Low-angle rotary-shadowing electron microscopy of inactive and active forms of radixin has shown that the inactive protein adopts a closed, globular structure and that the active protein assumes an extended form with two globular structures connected by a straight filamentous domain (Ishikawa et al., 2001). Further studies have demonstrated that the adhesion molecule I-CAM-2 binds to the radixin FERM domain in the same site as a β-strand of the C-ERMAD in dormant radixin (Hamada et al., 2003) and that the NHERF binding site is masked by the C-ERMAD in inactive ezrin and moesin (Finnerty et al., 2004; Reczek and Bretscher, 1998). These results endorse the hypothesis that the intramolecular association of the N- and C-ERMADs is responsible for the inactivation of ERM proteins.

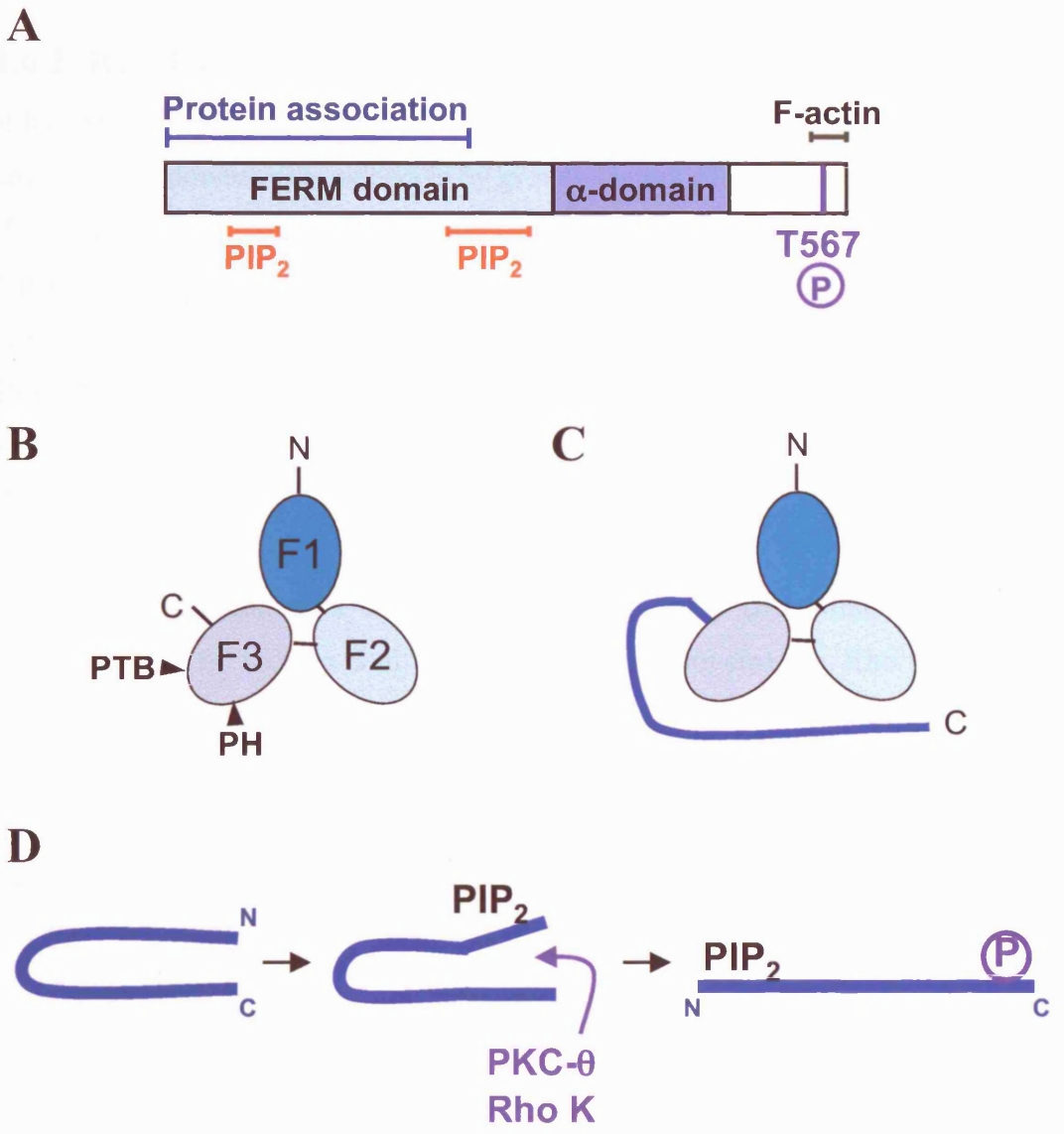

Figure 1.9. ERM protein structure and activation. **A.** Cartoon depicting the structural organisation and key regulatory sites of ERM proteins. Sites of protein and lipid binding are indicated above and below. P indicates the phosphorylation site for activation, threonine-567. α-domain: α-helical region. FERM: 4.1, ezrin, radixin, moesin domain. T567: threonine-567. **B.** Cartoon depicting the structural organisation of the globular subdomains (F1 - F3) of the FERM domain. The regions with structural similarity to pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains are indicated. **C.** Cartoon depicting the dormant, inactive, structure of ERM proteins. Cytosolic ezrin exists in a monomeric form in which its N- and C-ERMADs are associated, masking the binding sites for molecules at the N-ERMAD and the C-ERMAD. **D.** Cartoon depicting conformational activation of ERM proteins. PIP_2 binding to the FERM domain is thought to open the dormant structure. This allows kinases such as Rho kinase (Rho K) and protein kinase C (PKC-θ) to phosphorylate the regulatory threonine, fully activating the ERM protein. C: carboxyl-terminus. N: amino-terminus . PIP_2: phosphatidylinositol 4,5-bisphosphate.

1.6.2 Regulation of conformation and activity

It has been shown that various stimuli activate ERM members. Activation of Rho and resulting downstream pathways by growth factors such as lysophosphatidic acid (LPA) promotes ezrin-enriched microvillus formation (Matsui et al., 1999). Furthermore, EGF treatment of human carcinoma A-431 cells leads to ezrin oligomerisation and membrane ruffling, to which ezrin is localised (Berryman et al., 1995; Bretscher, 1989). The formation of ezrin oligomers suggested that EGF stimulation was responsible for overcoming the closed, inactive conformation (Berryman et al., 1995).

The phosphorylation of a carboxy-terminal threonine (threonine-567 in ezrin; threonine-564 in radixin and threonine-558 in moesin) by Rho kinase was demonstrated following LPA stimulation of Swiss 3T3 cells (Matsui et al., 1998) and osteopontin treatment of osteoclasts (Chellaiah et al., 2003). This phosphorylation was shown to inhibit the binding of the carboxyl-terminus of radixin to its amino-terminus, but did not affect the carboxyl-terminus binding to actin (Matsui et al., 1998). Further studies established that protein kinase C-θ (PKC-θ) and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) could phosphorylate moesin at threonine-558 (Nakamura et al., 2000; Pietromonaco et al., 1998). PKC-θ phosphorylation unmasked the NHERF and F-actin binding sites of ezrin and moesin (Simons et al., 1998) and active MRCK was required for ERM protein activation and filopodia formation in NIH 3T3 cells (Nakamura et al., 2000). Additionally, PKC-α-induced cell migration was demonstrated to correlate with phosphorylation of ezrin at threonine-567 (Ng et al., 2001).

Analysis of the role of threonine-phosphorylated ERM proteins was performed using T567D ezrin to mimic the phosphorylated state and T567A ezrin, a non-phosphorylatable mutant (Gautreau et al., 2000). T567A ezrin was poorly associated with the actin cytoskeleton but could form oligomers at the plasma membrane. Conversely, T567D ezrin was shown to be linked to the cytoskeleton and to trigger the formation of structures such as microvilli, lamellipodia and membrane ruffles, to which it was localised. Moreover, T567D ezrin was a monomer at the plasma

membrane, suggesting that phosphorylation at threonine-567 prevented N- and C-ERMAD association and was the active form of the protein (Gautreau et al., 2000). These results agreed with an earlier study in which threonine-phosphorylated ERM proteins were specifically localised to the plasma membrane in A-431 cells following EGF treatment (Yonemura et al., 1999).

Lipids have also been shown to play a role in ERM protein activation. Moesin phosphorylated at threonine-558 associates with F-actin only in the presence of poly-phosphatidylinositides (Nakamura et al., 1999). A PIP_2-binding domain had previously been identified within the N-ERMAD of ezrin (Niggli et al., 1995) and was later more precisely localised to lysine residues at positions 63, 64, 253, 254, 262 and 263 (Barret et al., 2000). A K253N, K254N, K262N, K263N ezrin mutant expressed in cells was cytosolic, although it still retained its ability to bind F-actin *in vitro* (Barret et al., 2000). Furthermore, injection of the PIP_2-binding drug neomycin into MDCK cells caused translocation of ezrin from the plasma membrane to the cytosol with associated loss of microvilli (Yonemura et al., 2002).

Examination of the crystal structure of inactive moesin indicates that threonine-558 is both in contact with the FERM domain and exposed to solvent. Phosphorylation of this residue would weaken the electrostatic attraction between the positively charged region of the C-ERMAD helix and the negatively charged face of the FERM domain. Sterically, the phosphate group cannot be accommodated by the closely packed side-chains of surrounding residues and would thus lead to conformational rearrangement (Pearson et al., 2000). The structural relevance of PIP_2 binding in ERM protein activation is less clear. Lysine residues 253, 254, 262 and 263 are located on the surface of the FERM F3 lobe and it has been postulated that negatively-charged phospholipids may bind to this positively charged face and interfere with the binding of a carboxyl-terminal helix (Barret et al., 2000; Pearson et al., 2000). A more recent study has determined that the ability to bind PIP_2 is required for the phosphorylation of threonine-567 and conformational activation of ezrin (Figure 1.9D). PIP_2, along with threonine-567 phosphorylation, was also shown to be required for the correct apical membrane distribution of ezrin and its morphogenic effects (Fievet et al., 2004).

Within a cellular context, however, regulation of ERM protein activity appears to be complex and involves phosphorylation at additional sites. CDK5 phosphorylation of threonine-235 augments ezrin activation and membrane localisation in senescent osteosarcoma cells, although this event requires prior phosphorylation of threonine-567 (Yang and Hinds, 2003). Phosphorylation of tyrosines-145 and -353 of ezrin occurs following signalling via growth factor receptors in epidermoid carcinoma cells or Lck activation in T cells (Autero et al., 2003; Crepaldi et al., 1997; Krieg and Hunter, 1992). Mutation of these tyrosine residues to phenylalanines did not affect the localisation of ezrin at microvilli but did inhibit hepatocyte growth factor-mediated motility and morphogenesis in epithelial cells (Crepaldi et al., 1997). Recently, c-Src-mediated phosphorylation of open, active ezrin at tyrosine-145 was demonstrated to be necessary for growth factor-induced cell spreading and proliferation (Srivastava et al., 2005). Finally, phosphorylation of serine-66 in ezrin by PKA has been shown to be essential for cytoskeletal remodelling during parietal cell activation. This phosphorylation was not required to target ezrin to the plasma membrane, but was important in regulating parietal cell membrane morphology in response to histamine (Zhou et al., 2003). It therefore seems that, following conformational activation, further phosphorylation events are likely to be important for regulating specific ERM-mediated cytoskeletal reorganisation in response to particular stimuli.

1.6.3 Cross-linking the plasma membrane to the actin cytoskeleton

The active, elongated conformation of an ERM protein has both its amino-terminal PIP_2- and protein-binding domains and carboxyl-terminal F-actin-association domain exposed, satisfying the structural requirements of a membrane/actin cross-linker. The FERM domain of the ERM members has been shown to interact directly with the cytoplasmic tails of transmembrane adhesion molecules including I-CAM-1, -2 and -3, CD43 and CD44 and the Na^+/H^+ exchanger NHE1 (reviewed in Bretscher et al., 2002). Expression of the transmembrane and cytoplasmic domains of ERM-binding membrane proteins such as CD43, CD44 and I-CAM-2 induced microvilli formation and ERM protein phosphorylation dependent on ERM binding (Yonemura

et al., 1999). This data suggests that ezrin is directly responsible for the actin reorganisation that follows ligand binding to adhesion receptors.

The scaffolding protein NHERF also binds to the ERM FERM domain, providing an indirect link to transmembrane proteins such as β_2AR and the platelet-derived growth factor receptor (Hall et al., 1998a; Hall et al., 1998b; Maudsley et al., 2000; Reczek et al., 1997). NHERF has been implicated in the regulation of GPCR trafficking, the control of the Na^+/H^+ exchanger NHE3, and the apical membrane targeting of specific proteins, all of which exploit the ability of NHERF to bind to F-actin via ERM proteins (reviewed in Voltz et al., 2001 and discussed in Section 1.5). The binding sites of both NHERF and I-CAM-2 have been identified within the FERM domains of moesin and radixin respectively (Finnerty et al., 2004; Hamada et al., 2003). These appear to be distinct, suggesting that adhesion receptors and scaffolding molecules may bind to ERM proteins simultaneously.

1.6.4 Role of ERM in downstream signalling pathways

Additionally to their direct involvement in cell surface morphogenesis following stimulation, ERM proteins are also implicated in signal-transduction pathways via interactions with cytosolic signalling molecules. The growth factor response pathways mediated by Rho require ERM protein activation to bring about cytoskeletal modifications such as stress fibre and focal adhesion formation (Hirao et al., 1996; Mackay et al., 1997). ERM proteins might also be downstream of Rac and Cdc42. Ezrin, radixin and moesin were identified as effectors of Rac required for actin polymerisation at the cell periphery (Mackay et al., 1997), and the Cdc42-effector MRCK has been shown to phosphorylate moesin at threonine-558 and may regulate ERM activation downstream of Cdc42 signalling (Nakamura et al., 2000).

The involvement of ERM proteins in growth factor signal transduction implies that they may also be important for cell survival. Following growth factor signalling, ezrin is phosphorylated at tyrosine-145 and tyrosine-353 (Autero et al., 2003; Crepaldi et al., 1997; Krieg and Hunter, 1992). Phosphorylation of tyrosine-353 was demonstrated to be essential for epithelial cell survival as it is required for ezrin to interact with PI 3-kinase and activate the Akt survival pathway (Gautreau et al., 1999).

ERM proteins have also been demonstrated to function downstream of PKC. Following EGF stimulation of A-431 cells, membrane ruffling and colocalisation of ezrin and CD44 to the plasma membrane appear to depend on PKC-α (Miyata et al., 1989; Stapleton et al., 2002). Recent studies indicate that both PKC-α and PKC-θ can phosphorylate ERM proteins at the regulatory threonine (Ng et al., 2001; Pietromonaco et al., 1998) and both kinases may regulate the interaction between CD44 and ezrin required for chemotaxis (Legg et al., 2002; Stapleton et al., 2002).

In summary, members of the ERM family serve to cross-link F-actin to the plasma membrane, functioning to implement cortical cytoskeleton alterations in response to a variety of signals. Their regulated conformational activation and array of binding partners enable them to act as effectors in numerous signalling pathways, co-ordinating membrane morphogenesis, cell adhesion, motility, trafficking and cell survival.

1.7 Aim of this thesis

GRK2 was originally characterised as a GPCR-specific serine/threonine kinase, phosphorylating agonist-occupied receptors to initiate their homologous desensitisation and internalisation. It has recently become clear that components of the GPCR desensitisation machinery, such as βarrestin and GIT1, have additional roles in promoting novel signalling pathways downstream of GPCRs. Since a number of non-receptor substrates have been identified for GRK2, it appears likely that this and other GRKs can act as GPCR signal effectors.

My aim in this thesis was to investigate whether the actin/plasma membrane cross-linker ezrin was a novel substrate for GRK2. Furthermore, I wanted to establish what role ezrin played in GPCR-mediated cytoskeletal rearrangements and receptor endocytosis. The majority of these analyses used HEK293 and Hep2 cell systems, although some *in vitro* work was also performed. Chapter 2 outlines the methods used in this thesis.

Chapter 3 investigates whether ezrin is a substrate of GRK2. Its findings demonstrate that GRK2 can phosphorylate ezrin *in vitro*. Phosphorylation occurs within a carboxyl-terminal 53 amino acid stretch that encompasses the phosphorylation site required for ezrin activation, threonine-567. It also describes my attempts to demonstrate either directly or indirectly that threonine-567 of ezrin is the site of GRK2 phosphorylation.

Chapter 4 demonstrates that agonist treatment of M1MR-expressing Hep2 cells induces cytoskeletal reorganisation in the form of membrane ruffles to which active, phosphorylated ERM proteins are localised. These ruffles require both functional ezrin and active GRK2 at the plasma membrane, and it is probable that GRK2 is the kinase directly responsible for phosphorylating and activating ERM proteins in this cell system. These results indirectly demonstrate that GRK2 is likely to phosphorylate ezrin at threonine-567.

Chapter 5 describes experiments that show that ezrin may play an important role in GPCR internalisation, although this function may be limited to a subset of these

receptors. Phosphorylated ERM proteins (pERM) are observed co-localised with internalised GPCRs at endosomal compartments. Ezrin is demonstrated to be required for β_2AR endocytosis, but not the internalisation of the transferrin receptor, suggesting that it is not required for clathrin-mediated endocytosis *per se*. 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 up with MilliQ deionised water unless specified. Buffer ingredients, cDNAs and antibodies referred to in the body of this Chapter are listed in tables 2.1, 2.2 and 2.3. Ezrin and GRK2 constructs used in this thesis are also illustrated in Figure 2.1.

2.1 Cell Culture

2.1.1 Cell lines

HEK293 cells stably expressing His6-tagged β_2AR (HEKβ2 cells, a generous gift of Dr Stefano Marullo, Institut Cochin, Paris) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum (FCS), 100IU penicillin and 100μg/ml streptomycin and 2mg/ml geneticin (Gibco BRL). COS, Hep2 and HEK293 cells were maintained in DMEM containing 10% FCS, 100IU penicillin and 100μg/ml streptomycin. 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 (or approximately $1x10^6$ cells per 9cm dish).

2.1.2 Cell thawing

To recover cells stored in liquid nitrogen, aliquots were rapidly thawed at 37 C and the cells diluted in 10 ml culture medium. Cells were pelleted by centrifugation (1000 rpm for 3 minutes) and the pellet was resuspended in 10 ml pre-warmed culture medium. Cells were maintained in a humidified incubator at 37 C, 5% CO_2. If HEKβ2 cells were thawed, geneticin was not added to the culture medium until the cells had first reached confluency.

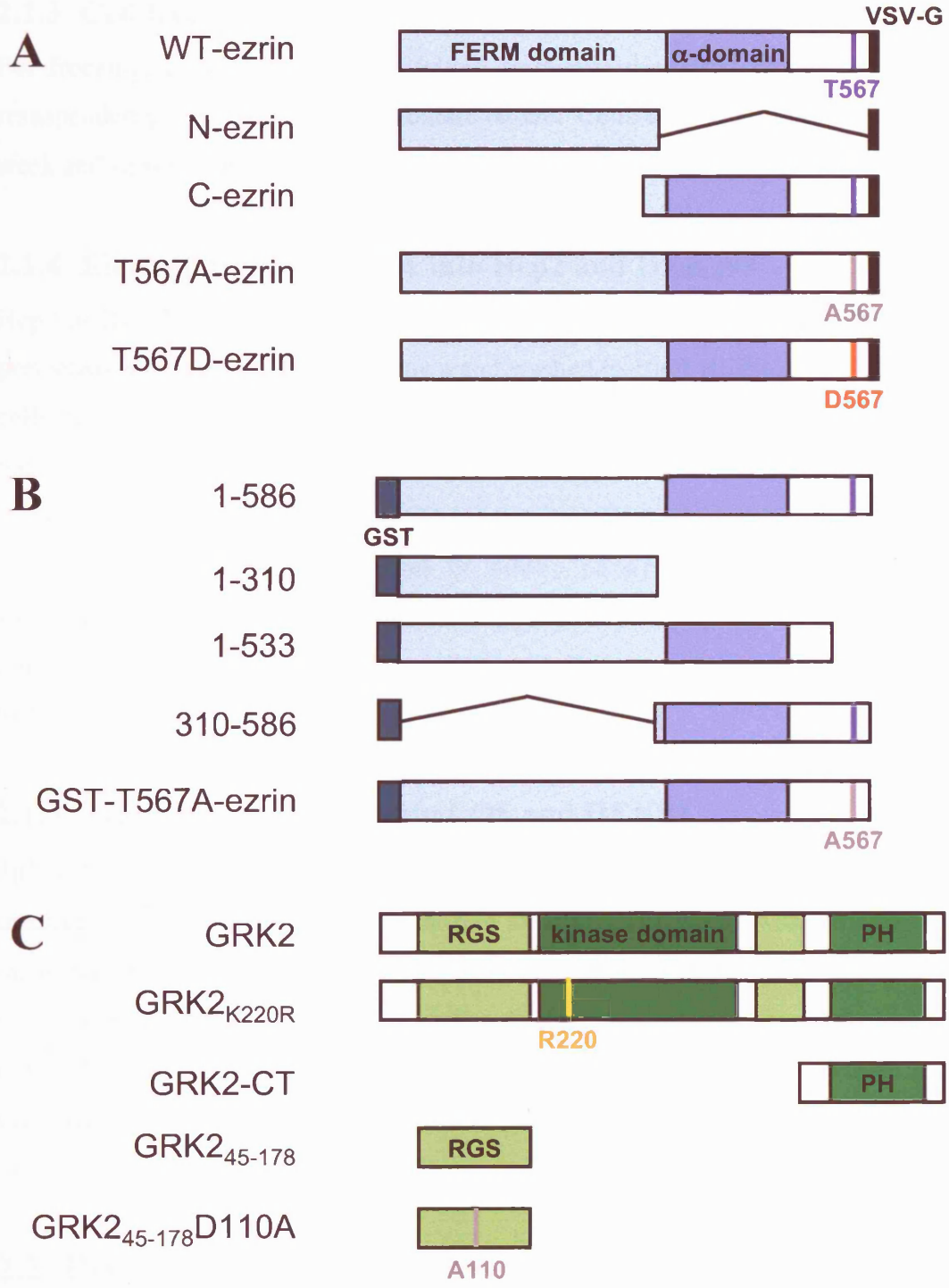

Figure 2.1. Ezrin and GRK2 cDNA constructs used in this thesis. **A.** Human ezrin-VSV-G constructs (Algrain et al., 1993; Gautreau et al., 2000). **B.** Human GST-ezrin constructs (Roy et al., 1997). **C.** Bovine GRK2 constructs (Freedman et al., 1995; Pierce et al., 2001; Sterne-Marr et al., 2003). A110: alanine-110. A567: alanine-567. D567: aspartate-567. FERM: 4.1, ezrin, radixin, moesin. GST: glutathione-S-transferase. PH: pleckstrin homology domain. R220: arginine-220. RGS: regulators of G protein signalling proteins. VSV-G: vesicular stomatitis virus glycoprotein.

2.1.3 Cell freezing

For freezing, $2x10^6$ pelleted cells (taken from plates of 50% confluency) were resuspended in 1ml cold freezing medium on ice. Cells were frozen at –70 C for 1 week and subsequently transferred to liquid nitrogen.

2.1.4 Electroporation of DNA into Hep2 and HEK293

Hep2 or HEK293 cells at approximately 75% confluence that had been plated 24h previously were trypsinised, spun down and washed in 10ml HEBS buffer. Washed cells were recovered by centrifugation and resuspended in 250µl HEBS and transferred to $0.4cm^3$ electroporation cuvettes. 1µg DNA was added to the cuvettes and the cells mixed gently by hand. Using the Gene Electropulser II (BioRad), cells were electroporated with 2 pulses of 400V, 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 and HEKβ2

3µl of GeneJuice Transfection Reagent (Novagen) for every 1µg of DNA to be transfected was added to 150µl serum-free DMEM. The media was vortexed and incubated at RT for 5 minutes. DNA was added to the media, mixed gently by hand and incubated at RT for 15 minutes. The media/DNA mixture was then dropped onto 70% confluent cells that had been plated 24h previously in 10% DMEM. The cell media was mixed by gentle agitation and the cells were left to express the transfected construct(s) for up to 48h.

2.2 DNA manipulation

2.2.1 cDNA Constructs

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

2.2.2 Generation of GST-T567A-ezrin construct

In order to generate a glutathione S transferase (GST)-tagged ezrin construct with threonine-567 mutated to alanine, the T567A-ezrin cDNA was amplified from pCB6-Ezrin-T567A-VSVG (Figure 2.1A) using the technique of polymerase chain reaction (PCR). The PCR primers were designed so that the resulting T567A-ezrin cDNA had a 5′ EcoRI site and a 3′ XhoI site. The cDNA was then inserted into the pGEX-5X-1 vector (Amersham Biosciences) downstream of and in frame with the GST moiety.

2.2.3 PCR

In order to amplify T567A-ezrin cDNA from pCB6-Ezrin-T567A-VSVG, reactions were set up as follows:

10μl	10x PCR buffer
10μl	10x HF2 dNTP Mix
2μl	3μM 5′ primer
2μl	3μM 3′ primer
2μl	enzyme
10μl	template
64μl	ddH_2O

using the Advantage-HF2 PCR kit (BD Biosciences). The primers used were:

5′ primer 5′-TTGTTGGAATTCATGCCGAAACCAATCAATGTCC-3′
(EcoRI)

3′ primer 5′-TTGTTGCTCGAGTTACAGGGCCTCGAACTCGTC-3′.
(XhoI)

The PCR was performed in a PTC-2000 Peltier Thermal Cycler (MJ Research) for 30 cycles under the following conditions:

94 C	10 seconds;
59 C	30 seconds;
68 C	2 minutes.

5μl of the PCR product was run in a 1% agarose gel containing ethidium bromide along with the 1kb Plus DNA ladder (Invitrogen) and visualised using the Gene Genius UV lightbox (Syngene) to confirm that the PCR was successful and had

generated DNA of the expected size. The amplified DNA was purified from the remainder of the reaction using the QIAquick PCR purification kit (QIAGEN).

2.2.4 Restriction digestion

All of the amplified T567A-ezrin and 2μg of the pGEX-5X-1 vector were digested with EcoRI and XhoI (Promega) at 37 C for 1h in separate reactions containing:

7μl 10x Buffer D

1μl EcoRI

1μl XhoI

xμl DNA

Made up to 70μl with H_2O.

Digested T567A-ezrin was purified from the reaction using the QIAquick PCR purification kit. The restriction digest reaction containing pGEX-5X-1 was run on a 1% agarose gel containing ethidium bromide with the 1kb Plus DNA ladder. Digested pEX-5X-1 was cut from the gel under UV illumination and purified from the agarose using the QIAquick gel extraction kit (QIAGEN).

2.2.5 Ligation

The digested T567A-ezrin cDNA was ligated into the digested pGEX-5X-1 vector at 16 C overnight under the following conditions:

1.5μl 10x ligation buffer (Promega)

1μl ligase (Promega)

5μl T567A-ezrin DNA

1μl pGEX-5X-1 DNA

6.5μl ddH_2O.

5μl of the reaction was used to transform DH5α *Escherichia coli.*

2.2.6 Bacterial transformation and plasmid DNA extraction

XL1-Blue (Stratagene) or DH5α ultracompetent *Escherichia coli* were thawed on ice and mixed by hand. 100μl were aliquoted into a pre-chilled 1.5ml Eppendorf tube and 20ng of DNA 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 250rpm. Bacteria were then plated onto Luria Broth (LB) agar plates containing 100μg/ml ampicillin or 25μg/ml kanamycin 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 kanamycin overnight at 37 C with shaking at 250rpm. The next day, bacteria were pelleted and the plasmid DNA was extracted using either a QIAprep Spin Miniprep or Maxiprep kit (QIAGEN) respectively. To test whether the plasmid contained an insert of the right size, a restriction digest was performed using 5 units of the relevant restriction enzyme (Promega) for 1h at 37 C. Digested products were analysed on a 1% agarose 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).

2.2.7 Sequencing

Sequencing was carried out by MWG Biotech UK (Milton Keynes). Primers used to sequence GST-T567A-ezrin in pGEX-5X-1 in stages along the 5′ to 3′ direction were: pGex forward (MWG Biotech UK); 5′-GTACTGCCCCCCTGAGACTG-3′; 5′-GTGAAATCAGGAACATCTCT-3′ and 5′-CAAAGAAGGCAGAGAGAGAG-3′. pGex reverse (MWG Biotech UK) was used to sequence in the 3′ to 5′ direction. pGEX-2T-$ez_{310\text{-}586}$ was sequenced using identical primers. The sequences obtained were compared to the published sequence of ezrin using the Gene Jockey II Sequence Processor program (Biosoft).

Sequencing of the PCR product of 310-586 verified that the region surrounding threonine-567 was error free and that the majority of the sequence matched that deposited in GenBank (GenBank identification NM_003379, Figure 3.4). An adenosine to thymidine substitution had occurred at base 1275. This was a conservative substitution and did not lead to change in the peptide sequence at residue 425 (ACA to ACT, i.e. threonine to threonine). A base reversal was detected at bases 1593 and 1594 (CG to GC), causing a change in the peptide sequence at

residue 532 (CTC GTG to CTG CTG, i.e. leucine valine to leucine leucine). This constitutes a conservative substitution of the amino acid sequence, as both valine and leucine are aliphatic amino acids. The valine to leucine substitution at residue 532 present in the 310-586 construct also occurred in GST-T567A-ezrin. Comparison of sequences of human ezrin deposited in SwissProt demonstrated that two sequences (A34400 and P15311) contained a valine at position 532, whereas one sequence (NP_003370) had a leucine at 532. This may therefore represent a natural single nucleotide polymorphism of human ezrin and is unlikely to affect its function.

2.3 Immunofluorescence techniques

2.3.1 Membrane ruffling assay and immunofluorescence

Hep2 cells were grown to 60-70% confluency on glass coverslips prior to transfection with 0.5μg HA-M1MR cDNA by electroporation. Cells were then treated with 100μM acetylcholine for 5 minutes or 200ng/ml EGF for 3 minutes at 37 C to induce membrane ruffling. Following treatment, cells were fixed in 10% trichloroacetic acid for 15 minutes at 4 C and washed twice for 10 minutes in quench buffer at RT. Permeabilisation buffer was subsequently used to block and permeabilise fixed cells.

Primary antibody was diluted in permeabilisation buffer and incubations were performed for 1h at RT. Cells were washed three times with permeabilisation buffer and incubated with secondary antibody diluted in permeabilisation buffer for 1h at RT. The antibody 297S was used to visualise pERM, except where Hep2 cells were treated with the PKA inhibitor Rp-8-Br-CAMPS (see Section 2.3.1.1), when a commercial anti-pERM antibody was used (Cell Signalling Technology). Where used, 1U Alexa Fluor 594-conjugated phalloidin (594-phalloidin, Molecular Probes) was diluted in 200μl permeabilisation buffer per coverslip and added to the coverslips for 20 minutes prior to washing. After 3 washes in permeabilisation buffer followed by one wash in PBS and one wash in distilled water, coverslips were mounted on slides in mountant.

2.3.1.1 Treatment with kinase inhibitors

Where used, cells were treated with 10μM Y27632, 20μM GF109203X or 1mM Rp-8-Br-CAMPS (BIOLOG) for 1 hour at 37 C prior to membrane ruffling assays.

2.3.2 Endocytosis assay

HEK293 or Hep2 cells were grown to 60-70% confluency on glass coverslips prior to transfection with receptor cDNA by electroporation. Cells were incubated in primary antibody diluted in serum free DMEM for 1h on ice. After washing three times in cold serum free DMEM, the cells were re-heated to 37 C and drugs added to induce receptor internalisation. Acetylcholine was used at a concentration of 100μM for 20 minutes and (-)-isoproterenol was used at a concentration of 50μM for 15 minutes or 30 minutes.

Following treatment, cells were fixed in 10% trichloroacetic acid for 15 minutes at 4 C (anti-ezrin or 297S antibodies) or 4% paraformaldehyde for 15 minutes at RT (all other antibodies) and washed twice for 10 minutes in quench buffer at RT. Permeabilisation buffer was subsequently used to block and permeabilise fixed cells. Secondary antibody incubations were performed for 1h at RT. Cells were subsequently washed with permeabilisation buffer and mounted on slides in mountant.

Where used, cells on coverslips were incubated in 100μM of the metal chelator Desferal at 4 C for 30 minutes before incubation in 200nM Alexa Fluor 647-conjugated transferrin (647-transferrin, Molecular Probes) in binding media (BM) for 30 minutes at 4 C. The cells were then immediately placed in 200nM transferrin-647 in BM at 37 C, with or without 50μM (-)-isoproterenol. Coverslips were removed after 0 and 30 minutes and washed three times in ice cold BM. Cells were fixed in 4% paraformaldehyde (in PBS) for 15 minutes at RT and then permeabilised and labelled with antibody as above.

2.3.3 Confocal microscopy

Confocal images were taken at RT within 24h of immunofluorescent labelling of the cells. Nikon Plan Apo 40x and 60x oil immersion lenses and a Bio-Rad 1024 MRC

confocal were used with a Nikon microscope and Bio Rad Lasersharp 2000 software to acquire the images. Images were optimised for contrast in Adobe Photoshop but no further manipulations were made.

2.3.4 Flow Cytometry and FACS® Analysis

HEKβ2 cells at approximately 50% confluency were transfected using GeneJuice Transfection Reagent with either 5μg dynamin K44A, N-ezrin or C-ezrin (Figure 2.1A) at a ratio of 2:1 with pDsRed-Express (BD Biosciences Clontech). 24h post-transfection cells were detached in PBS containing 10mM EDTA, washed in ice-cold BM and resuspended at $5x10^6$cells/ml in cold BM containing 40μM (-)-isoproterenol. Immediately, six aliquots of 100μl ($5x10^5$ cells) were washed in cold BM and kept on ice. The remaining cells were incubated at 37°C for 30 minutes, after which time a further six aliquots were taken and treated as above. The rest of the cells were cooled on ice, centrifuged (1500rpm, 5 minutes, at 4 C), washed three times in cold BM, resuspended in prewarmed BM containing 100μM (-)-alprenolol and incubated for 30 minutes at 37°C. Six aliquots of cells were pelleted by centrifugation and washed in cold BM, as before.

All cells were labelled with primary antibody in wash buffer for 1h on ice. The cells were then washed twice in cold wash buffer and fixed in 1% FCS/1% paraformaldehyde (TAAB)/PBS overnight at 4 C. The fixative was removed by washing once in cold wash buffer and the cells were incubated with an AlexaFluor-488-conjugated secondary antibody in wash buffer for 1h on ice. Finally, the cells were washed three times in wash buffer. Cells expressing pDsRed-Express were assumed to also express the dynamin or ezrin mutants. These cells were gated and 10000 pDsRed-Express expressing cells analysed for His6-tagged β_2AR expression using a FACS®Calibur flow cytometer (Becton Dickinson) and Cell-Quest software (Becton Dickinson).

2.4 Other techniques

2.4.1 Purification of GST-ezrin fusion proteins

GST-ezrin fusion proteins (Figure 2.1B) were expressed in BL21 *E. coli* (Invitrogen), as described above. Single colonies of bacteria were picked and grown

in 100ml LB medium containing 100μg/ml ampicillin (LB/ampicillin) at 37 C, 250rpm overnight. The cultures were diluted 1:10 in LB/ampicillin and grown at 37 C with shaking at 250rpm until $A_{550} \geq 0.5$. IPTG was added subsequently to the cultures at a final concentration of 1mM to induce fusion protein expression for an additional 4h. Cultures were centrifuged at 2500g for 10 minutes at 4 C, bacterial pellets resuspended in 35ml cold PBS containing the protease inhibitors 40μg/ml phenylmethanesulfonyl fluoride (PMSF) and 1mM benzamidine and subject to sonication for 2 x 2 minutes. Triton X-100 was added to a final concentration of 1% and the lysates centrifuged at 40000g for 30 minutes at 4 C. 1ml glutathione agarose was added to the clarified supernatants, which were incubated on a rotor mixer at 4 C for 2h. The agarose was washed three times with cold 50mM Tris pH 8.0 /1% Tween/1% Triton X-100 and once with cold 50mM Tris pH8.0. Where required, GST-fusion proteins were eluted from the agarose with 10-50mM glutathione in 50mM Tris pH8.0 containing protease inhibitors, dialysed against 50mM Tris pH8.0 containing protease inhibitors and concentrated in Centriprep YM-30 centrifugal filter devices (Amicon).

2.4.2 In vitro phosphorylation assays

GRK2 purified from Sf9 cells expressing recombinant baculovirus encoding the GRK was a generous gift of Dr. Robert Lefkowitz. All phosphorylation reactions were carried out with purified GST-ezrin fusion proteins (1μM) or GST (4μM,) and GRK2 (0.2μM) in phosphorylation reaction buffer in the presence of 60μM [γ^{32}P]ATP (15000cpm/pmole) (Amersham Pharmacia Biotech), for 1 h at 37 °C in a total reaction volume of 25μl. Where used, 0.5mg/ml purified and sonicated lipids (PIP_2 and phosphatidylcholine in PBS) and purified Gβγ from bovine brain (Dr. Robert Lefkowitz) were also included. Either 10% PIP_2 and 2.125μM Gβγ or 20% PIP_2 and 8.7μM Gβγ were used, with essentially equivalent results. Reactions were incubated on ice for 15 minutes prior to addition of [γ^{32}P]ATP in the presence or absence of 0.6μg purified bovine rod outer segments (ROS). Where ROS were used, the reactions were incubated under bright electric light. If larger quantities of phosphorylated ezrin constructs were required, reactions were carried out as described and then pooled. The reactions were terminated by addition of SDS reducing buffer and samples were resolved by SDS-PAGE (see Section 2.4.7).

To detect proteins, the gel was immersed 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 subsequently destained using Coomassie de-stain until proteins were visible. The gel was then placed on 2 sheets of filter paper, covered with Saran wrap and dried using a Model 583 Gel Dryer (BioRad) at 80 C for 90 minutes before being exposed to film (Biomax ML, Kodak) at -70 C overnight. Film was developed using an Agfa automatic film processor.

In order to determine the stochiometry of the phosphorylation reaction, known amounts of the phosphorylation reaction buffer containing [γ^{32}P]ATP were spotted onto a piece of filter paper and allowed to dry. These were exposed, along with the dried gel, to a phosphorimager screen for 30 minutes at RT. The amount of ATP incorporated into each ezrin construct was determined by comparison with the standard ATP concentrations using a Molecular Imager FX (BioRad) and the Quantity One version 4.2.1 program (BioRad).

2.4.3 Protein extraction and determination of concentration

A 9cm tissue culture dish of confluent cells was washed in ice cold PBS. 500µl ice cold lysis buffer was added to the cells and the cells scraped with a rubber cell scraper before being transferred to a pre-chilled 1.5ml Eppendorf tube and incubated on a rotor shaker for 30 minutes at 4 C. The cells were then centrifuged at 12000 rpm for 15 minutes at 4 C and the supernatant transferred to a new 1.5ml tube.

Protein concentration was determined using the BioRad protein assay. BioRad Protein Assay Dye Reagent Concentrate (BioRad) was diluted 1:5 in water and filtered. 2µl 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 BSA.

2.4.4 Immunoprecipitation

Clarified cell lysates containing 1mg total protein were taken and 2μg primary antibody added. The lysates were incubated at 4 C for 45 minutes on a rotor shaker. 50μl protein A/G sepharose was then added and the lysates incubated at 4 C on a rotor shaker for a further 45 minutes. The sepharose gel was washed three times with lysis buffer and 50μl SDS reducing buffer added. The immunoprecipitated proteins were then subject to SDS-PAGE and western blotting. An aliquot of each clarified lysate was also subject to a binding control in which the incubation with primary antibody was omitted prior to incubation with protein A/G sepharose.

2.4.5 Assay to determine if GRK2-mediated phosphorylation of ezrin regulates ezrin function

COS cells at approximately 50% confluency were transfected using Gene Juice. 48h post-transfection cells were washed in cold PBS, lysed on ice in cold lysis buffer and incubated for 30 minutes at 4 C on a rotor mixer. The lysates were centrifuged at 13000rpm for 15 minutes at 4 C and the supernatants transferred to clean tubes. The samples were diluted 10-fold in lysis buffer and 20μg lysate added to 50μl of a 50% slurry of GST-1-310 ezrin bound to glutathione agarose (3.7μg protein) or 50μl of a 50% slurry of glutathione agarose alone that had been pre-washed with total cell lysate. Reactions were incubated at 4 C for 1h on a rotor mixer. The agarose beads were washed 3 times in cold lysis buffer then 50μl SDS sample buffer added and the beads boiled at 90 C for 10 minutes to dissociate any bound protein. Resin-bound proteins were subject to SDS-PAGE and western blotting.

2.4.6 Determination of relative amount of endogenous ezrin in different cell lines

For total protein extraction to determine the relative quantities of ezrin in different cell lines (Hep2, HEK293 and HEKβ2), a 9cm tissue culture dish of each confluent line was taken, trypsinised and resuspended in 5ml ice cold PBS. The total number of cells was determined using a haemocytometer. The cells were pelleted and resuspended in 1ml gel running buffer. $1x10^6$ cells from each line were subject to SDS-PAGE and western blotting. In order to determine the relative amounts of

protein precipitated, the developed film was scanned using a GS-710 Calibrated Imaging Densitometer (BioRad) and the optical density of each band calculated using Quantity One version 4.2.1 software, with background subtracted.

2.4.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Laemmli method (Laemmli, 1970) with a discontinuous SDS-polyacrylamide gel and the Hoefer Scientific Instruments (HSI) vertical slab gel unit SE 600 gel system. A 10% SDS-polyacrylamide resolving gel was cast between two glass plates by polymerising 10% (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% sodium dodecyl sulphate (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 immersed in gel running buffer. The appropriate volume of 6x SDS reducing buffer was added to known concentrations of samples prior to heating at 95 C for 10 minutes. Samples were immediately cooled on ice, briefly centrifuged to collect all droplets and then loaded onto the gel. Rainbow Coloured Protein molecular weight markers (14.3 - 220kD, Amersham Biosciences) were also loaded onto the gel. The gel was subject to a constant voltage of 200-300V for 90 minutes.

2.4.8 Western blotting and immunodetection

The proteins separated on the 10% SDS-polyacrylamide gel 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 0.8A per cm^2 of nitrocellulose membrane applied for 2h. 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 either 1h at RT or overnight at 4 C with constant agitation. 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 3x 5 minute washes in blocking buffer, a horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer was added to the immunoblots for 1h at RT. The blots were washed in TTBS for 3x 5 minutes.

0.125ml total of ECL reagent per cm^2 of nitrocellulose membrane was added to the protein side of the blot and incubated at RT for 1 minute (ECL Western Blotting Detection Reagents or ECL Advance Western Blotting Detection Kit, depending on antibody used. Amersham Biosciences). After developing the blot was wrapped in cling film. 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. The optical density of each band was calculated as detailed in section 2.4.6.

2.4.8.1 Stripping antibodies from nitrocellulose membranes

Nitrocellulose membranes were submerged in pre-warmed stripping buffer and incubated in a sealed container in a 50 C water bath for 30 minutes with occasional agitation. The membranes were then washed twice in an excess volume of PBS-T for 10 minutes at RT on a shaker. Incubation in blocking buffer for 1h at RT was performed before immunoblotting with an alternative antibody, as detailed in Section 2.4.8.

2.5 Antibodies

Table 2.3 lists antibodies used in this thesis, their concentrations and suppliers. Commercial antibodies were stored at 4 C or -20 C, according to manufacturers' instructions. Non-commercial antibodies were stored at 4 C.

2.6 Statistical analysis

Results were analysed using the student's two-sample T-test to test 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

Binding medium (BM)	1x RPMI 10mM Hepes 0.2% BSA pH 7.0
Blocking buffer	2% skimmed milk powder 0.1% Tween-20 made up in PBS
Coomassie de-stain	40% MeOH 10% acetic acid
Coomassie stain	40% MeOH 10% acetic acid 0.05% Brilliant Blue R
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 with NaOH

Lysis buffer	250mM NaCl 50mM HEPES pH7.2 50mM NaF 10mM $NaPPO_4$ 2mM EDTA 1mM benzamidine 50nM calyculin A 10% glycerol 0.5% NP-40 40μg/ml PMSF
PBS-T	0.1% Tween-20 made up in PBS
Mountant	90% glycerol 3% N-propyl-galate made up in PBS Stored at 4 C, away from light
Permeabilisation buffer	1% BSA 0.2% saponin made up in PBS
Phosphorylation reaction buffer	20mM Tris-HCl pH 7.5 5mM $MgCl_2$ 2mM EDTA 2mM DTT
Quench buffer	0.37% glycine 0.27% NH_4Cl 0.05% azide made up in PBS

6x SDS reducing buffer	25mM Tris pH6.5 10% glycerol 8% SDS 5% β-mercaptoethanol a few grains of Brilliant Blue G
Stripping buffer	100mM β-mercaptoethanol 62.5mM Tris HCl pH6.7 2% SDS
Transfer buffer	0.05M Tris 0.04M glycine 0.01M SDS 20% MeOH
TTBS	50mM NaCl 20mM Tris 0.2% Tween-20
Wash buffer (for FACS)	1% FCS 0.02% azide made up in PBS

Table 2.2 cDNA constructs

cDNA	Construct name	Supplier	Reference or catalogue number
pGEX-2T-ez$_{1-586}$ pGEX-2T-ez$_{1-533}$ pGEX-2T-ez$_{310-586}$ pGEX-2T-ez$_{1-310}$	1-586 1-533 310-586 1-310	Dr. Christian Roy, Université Montpellier	Roy et al., 1997
pCB6-Ezrin-VSVG pCB6-Ezrin-Nter-VSVG pCB6-Ezrin-Cter-VSVG	WT-ezrin N-ezrin C-ezrin	Dr. Monique Arpin, Institut Curie, Paris	Algrain et al., 1993
pCB6-Ezrin-T567A-VSVG pCB6-Ezrin-T567D-VSVG	T567A ezrin T567D ezrin	Dr. Monique Arpin, Institut Curie, Paris	Gautreau et al., 2000
pEGB-βARK-CT	GRK2-CT	Dr. Robert Lefkowitz, Howard Hughes Medical Institute, NC	Pierce et al., 2001
pBC12BI-βARK1 pBC12BI-βARK1-K220R	GRK2 GRK2$_{K220R}$	Dr. Robert Lefkowitz	Freedman et al., 1995
pEGFP-GRK2-(45-178)-GFP pEGFP-D110A-GRK2-(45-178)-GFP	GRK2$_{45-178}$ GRK2$_{45-178}$-D110A	Dr. Rachel Sterne-Marr	Sterne-Marr et al., 2003
pSV2-neo-FLAG-β_2AR pSV2-neo-FLAG-β_2AR(L413A)	β_2AR β_2AR$_{L413A}$	Dr. Robert Lefkowitz	Hall et al., 1998b
pRK5-HA-M1MR pRK5-HA-M2MR	M1MR M2MR	Dr. Robert Lefkowitz	Claing et al., 2000

pUHD10-3-ele1	$Dynamin_{K44A}$	Dr. Robert Lefkowitz	Claing et al., 2000
pGEX-5X-1	pGEX-5X-1	Amersham Biosciences	27-4584-01
pDsRed-Express	pDsRed-Express	BD Biosciences Clontech	632412

Table 2.3 Antibody reagents

Antigen	Antibody name	Species and isotype	Dilution	Supplier
Immunoprecipitation				
Primary				
Ezrin	Anti-ezrin	Rabbit	2μg/ml	Upstate Biotechnology
GRK2	Anti-GRK2/3	Mouse IgG2aκ	2μg/ml	Upstate Biotechnology
Immunofluorescence				
Primary				
EEA1	EEA1 antibody	Rabbit	1:2000	Abcam
Ezrin	Anti-ezrin	Rabbit	1:250	Upstate Biotechnology
FLAG	M2	Mouse IgG1	1:400	Sigma
GRK2	Anti-GRK2/3	Mouse IgG2aκ	1:300	Upstate Biotechnology
HA	Anti-HA	Rat	1:100	Roche
His6	Anti-His6	Mouse IgG2a	1:200	BD Biosciences

LAMP1	Anti-human LAMP1	Rabbit	1:750	Dr. Sven Carlsson, Umea University, Sweden[1]
pERM	297S	Rat	Undiluted	Dr. Shigenobu Yonemura, RIKEN Center for Developmental Biology, Japan[2]
pERM	Anti-pERM	Rabbit	1:50	Cell Signalling Technology
VSVG	Anti-VSVG, clone P5D4	Mouse IgG1	1:2000	Sigma
Secondary				
Mouse IgG	Alexa Fluor 488 anti-mouse	Donkey	1:800	Molecular Probes
	Alexa Fluor 594 anti-mouse	Donkey	1:800	Molecular Probes
	Alexa Fluor 647 anti-mouse	Donkey	1:800	Molecular Probes
Rabbit IgG	Alexa Fluor 488 anti-rabbit	Donkey	1:800	Molecular Probes

[1] Carlsson et al., 1988
[2] Hayashi et al., 1999; Matsui et al., 1998

Rabbit IgG	Alexa Fluor 594 anti-rabbit	Donkey	1:800	Molecular Probes
Rat IgG	Alexa Fluor 488 anti-rat	Donkey	1:800	Molecular Probes
Rat IgG	Alexa Fluor 594 anti-rat	Donkey	1:800	Molecular Probes
Directly conjugated molecules				
Alexa Fluor 594-conjugated phalloidin			1U/200µl	Molecular Probes
Alexa Fluor 647-conjugated transferrin			200nM	Molecular Probes
Flow cytometry				
Primary				
His6	Anti-His6	Mouse IgG2a	1:100	BD Biosciences
Secondary				
Mouse IgG	Alexa Fluor 488 anti-mouse	Donkey	1:800	Molecular Probes
Western blotting and immunodetection				
Primary				
Ezrin	Anti-ezrin	Rabbit	1:500	Upstate Biotechnology
GRK2	Anti-GRK2/3	Mouse IgG2aκ	1:300	Upstate Biotechnology

β-tubulin	Anti-β-tubulin (H-235)	Rabbit	1:200	Santa Cruz Biotechnology, Inc.
Secondary (peroxidase linked)				
Mouse IgG	Anti-mouse IgG	Sheep	1:2500	Amersham Biosciences
Rabbit IgG	Anti-rabbit IgG	Donkey	1:2500	Amersham Biosciences

3 Ezrin is a novel substrate of GRK2

GRK2 phosphorylates a number of non-GPCR substrates, including tubulin, phosducin, synucleins, PDEγ, ribosomal protein P2 and the β-subunit of the epithelial Na^+ channel (Carman et al., 1998; Dinudom et al., 2004; Wan et al., 2001; Freeman et al., 2002; Haga et al., 1998; Pitcher et al., 1998a; Pronin et al., 2000; Ruiz-Gomez et al., 2000). This indicates that GRK2 may potentially act as a mediator of signalling pathways other than those leading to GPCR desensitisation. Phosphorylation of PDEγ has been shown to be required for EGF-mediated MAPK activation (Wan et al., 2001), indicating that GRK2-mediated phosphorylation of at least one non-receptor substrate has been shown to regulate downstream signalling events in a cellular setting.

In an attempt to identify novel non-GPCR substrates for GRK2, Dr. Julie Pitcher transfected HEK293 cells with either the β_2AR, or the β_2AR and GRK2. The β_2AR regulates a variety of biological functions, such as the control of heart rate, and vascular and respiratory smooth muscle relaxation (Benovic, 2002; Rockman et al., 2002). It is coupled to both Gs and Gi, classically stimulating cAMP production via adenylyl cyclase, activating PKA and some cAMP-gated ion channels (Benovic, 2002). GRK2 has been shown to phosphorylate β_2AR following agonist stimulation both *in vitro* and *in vivo* (Choi et al., 1997; Fredericks et al., 1996).

The transfected HEK293 cells were labelled with [^{32}P]orthophosphate and either left untreated or treated with the β-adrenergic receptor agonist isoproterenol. Cells were subsequently lysed and ^{32}P-labelled proteins subjected to two-dimensional gel electrophoresis and autoradiography. Proteins whose ^{32}P-content increased upon expression of GRK2 in an agonist-dependent fashion were excised and identified using MALDI-TOF mass spectrometry. One potential GRK2 substrate identified in this fashion was the actin-binding protein ezrin (data not shown).

In this Chapter, I describe experiments designed to test the hypothesis that ezrin was a substrate of GRK2 *in vitro* and to identify the site of phosphorylation. Furthermore, I intended to design and execute an assay to determine whether GRK2 phosphorylation led to ezrin activation.

3.1 Ezrin is a substrate of GRK2 *in vitro*

To determine if ezrin serves as a GRK2 substrate, I expressed and purified human GST-ezrin constructs from *E. coli* (Figures 2.1B, 3.1A and 3.2A) and subjected them to *in vitro* phosphorylation by purified GRK2, as described in the methods. The full-length GST-ezrin construct (residues 1-586) failed to serve as a substrate for GRK2 (Figure 3.1B). However, addition of Gβγ and lipid vesicles consisting of phosphatidylcholine and PIP_2 to the reaction led to efficient phosphorylation of 1-586 by GRK2 (Figure 3.1B). The stochiometry of the phosphorylation was calculated to be between 0.15 and 0.3 pmoles ATP incorporated per pmole of 1-586.

The carboxy-terminal PH domain of GRK2 binds PIP_2 and Gβγ in a coordinated fashion to promote membrane localisation of the kinase (Pitcher et al., 1995). PIP_2 also binds the amino-terminus of ezrin; this interaction has been shown to facilitate phosphorylation of ezrin at threonine-567, by kinases such as Rho kinase- and PKC, and leads to ezrin activation (Fievetet al., 2004; Niggli et al., 1995). The PIP_2 and Gβγ-dependence of GRK2-mediated ezrin phosphorylation thus presumably reflects the co-localisation of both kinase and substrate to the surface of lipid vesicles and PIP_2-dependent changes in ezrin conformation.

3.2 Ezrin interacts with GRK2 and modulates its interaction with rhodopsin

I sought to investigate whether ezrin, in addition to serving as a GRK2 substrate, could modulate GRK2 activity. Purified rod outer segment (ROS) membrane preparations, which consist of approximately 90% rhodopsin, represent a convenient *in vitro* substrate for monitoring GRK2 activity. GRK2 phosphorylates specifically agonist-occupied (illuminated) rhodopsin (Gan et al., 2000). When purified ROS are incubated with GRK2 and ATP under a bright light, they are phosphorylated (Figure 3.1C). Interestingly, adding increasing concentrations of up to 1μM 1-586 to the reaction augmented the phosphorylation of ROS by GRK2, such that addition of 1μM 1-586 increased ROS phosphorylation over six-fold (Figure 3.1C). These results suggest that ezrin not only acts as a substrate for GRK2 but, by interacting with GRK2, serves to enhance its action against rhodopsin, either by directly

activating it or by promoting the membrane localisation of GRK2. The latter explanation is most probable since, in the absence of Gβγ in the purified ROS, the binding of ezrin to PIP_2 within the ROS membrane could enhance the targeting of GRK2 to the plasma membrane, where it would be activated by light-stimulated rhodopsin.

3.3 GRK2 phosphorylates ezrin between residues 533 and 586

I utilised GST-ezrin constructs with different domain deletions in an attempt to identify the region of ezrin that contains the GRK2 phosphorylation site(s). A GST fusion protein encompassing residues 310-586 of ezrin was a substrate of GRK2, localising the GRK2 phosphorylation site to the carboxy-terminus of the protein (Figure 3.2B). The reaction stochiometry was between 0.11 and 0.4 pmoles ATP incorporated per pmole 310-586, similar to that observed with the full-length construct.

Notably, addition of Gβγ and PIP_2-containing lipid vesicles inhibited GRK2-mediated phosphorylation of this protein (Figure 3.2B). These results are consistent with the PIP_2 and Gβγ-dependence observed for GRK2-mediated phosphorylation of full-length ezrin. GST-310-586 lacks the auto inhibitory amino-terminal PIP_2 binding domain of ezrin; phosphorylation sites encompassed within this carboxy-terminal domain would thus be predicted to be accessible in the absence of PIP_2. Since in the presence of Gβγ GRK2 binds PIP_2-containing vesicles (Pitcher, et al., 1996), their addition would be predicted to sequester GRK2 away from its non-PIP_2 binding GST-310-586 substrate, thereby inhibiting GRK2-mediated 310-586 phosphorylation.

An ezrin construct lacking the carboxy-terminus (residues 1-533) was poorly phosphorylated by GRK2, both in the presence and absence of Gβγ and lipid (Figure 3.2B).

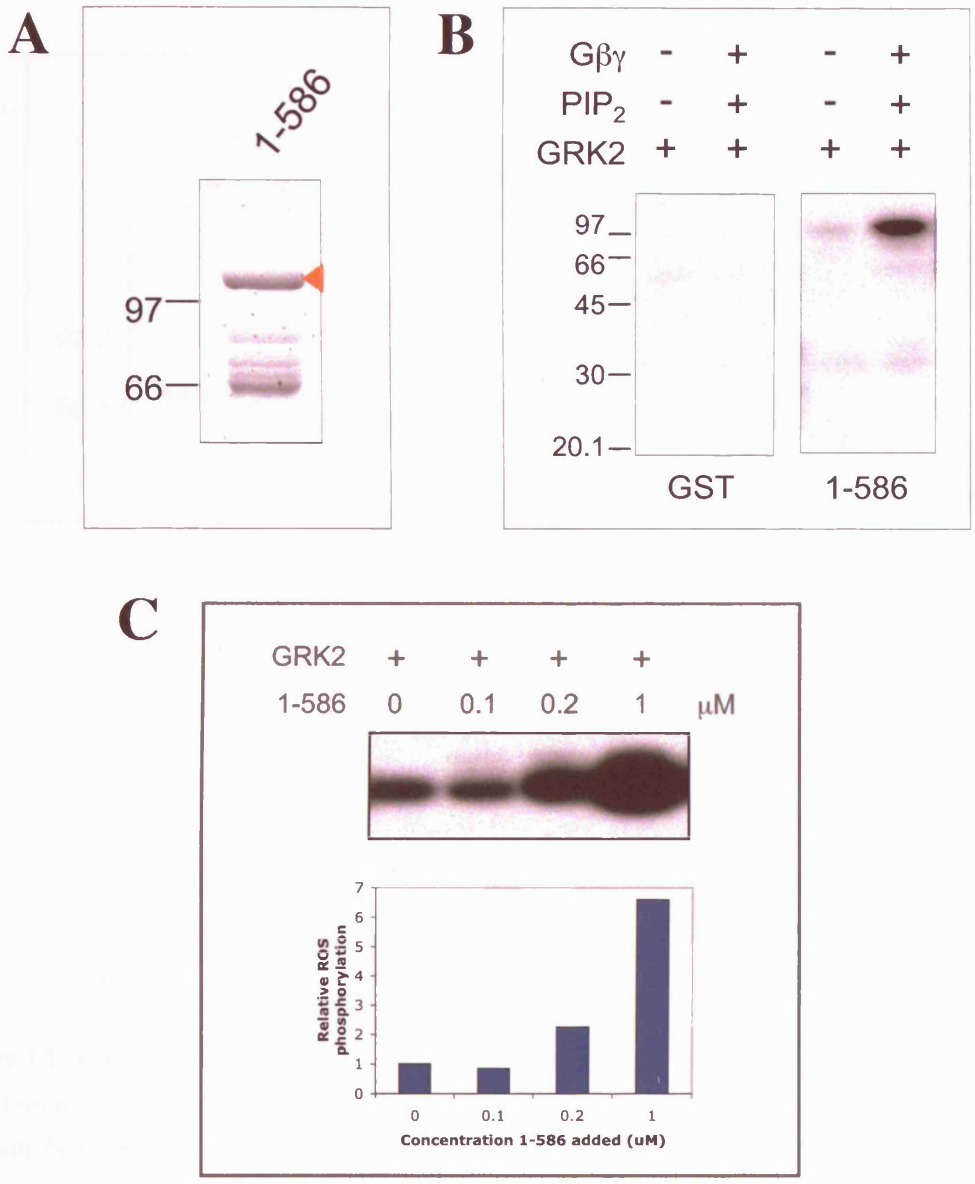

Figure 3.1. Ezrin is a substrate of GRK2 *in vitro* and promotes GRK2-mediated rhodopsin phosphorylation. **A**. A GST-fusion construct corresponding to full-length ezrin (1-586) was over-expressed in *E. coli* and purified as detailed in Chapter 2. The construct was subject to SDS-PAGE and Coomassie staining. Red arrow head indicates the full length protein construct, other bands are degradation products. **B**. Purified full-length ezrin (1μM) was phosphorylated in the presence of 0.2μM GRK2 and in the absence or presence of Gβγ and PIP_2. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. No phosphorylation of the GST moiety alone (GST) was observed. **C.** Rod outer segments (ROS) containing rhodopsin also serve as GRK2 substrates (lane 1). Adding increasing concentrations of full-length ezrin (1-586) up to 1μM augmented GRK2 phosphorylation of ROS (lanes 2-4). The optical densities of the phosphorylated ROS bands were quantified and the relative values, compared to 0 μM 1-586, calculated. Positions of protein molecular mass standards are indicated at the left of the images. Bands shown in boxes are from gels imaged at the same exposure, but with irrelevant lanes removed.

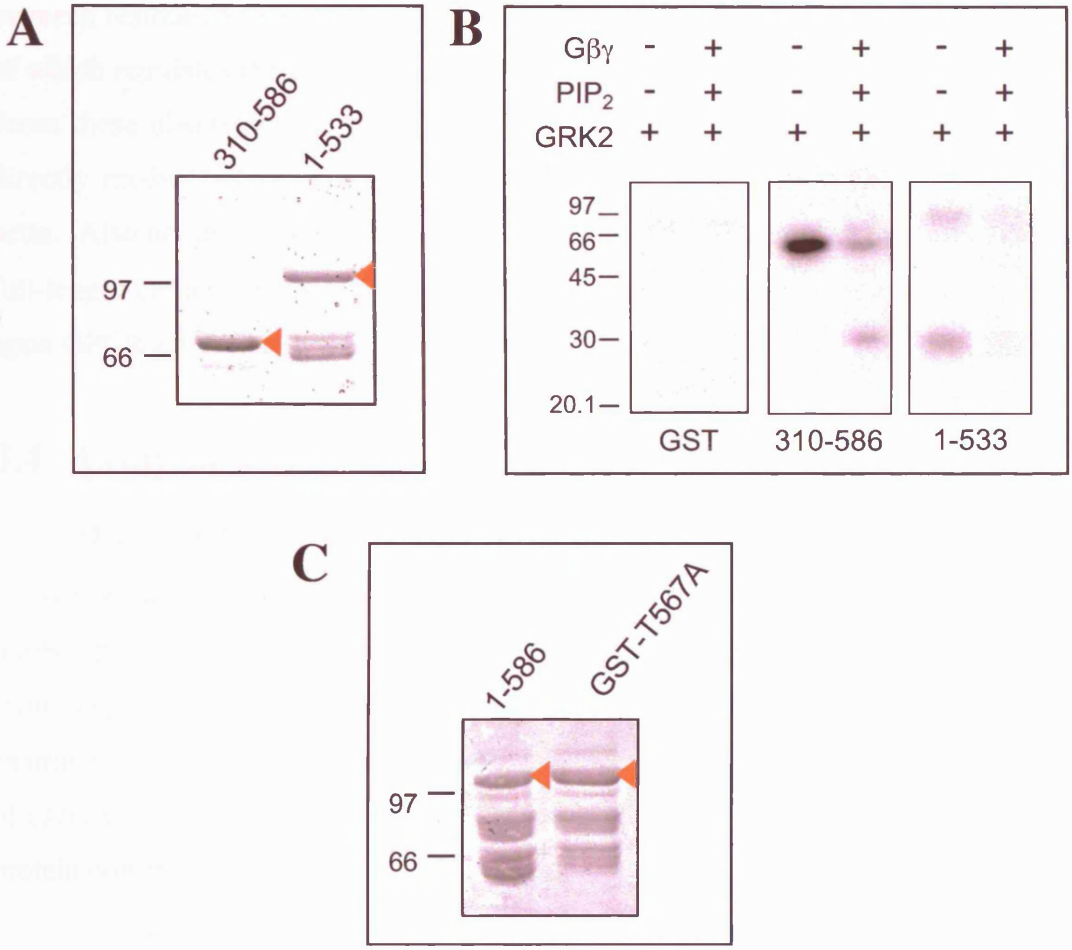

Figure 3.2. GRK2 phosphorylates the carboxyl-terminus of ezrin between residues 533 and 586. **A.** GST-fusion constructs corresponding to the catboxyl-terminus of ezrin (310-586) and a carboxyl-terminus deletion (1-533) were over-expressed in *E. coli* and purified as detailed in Chapter 2. The constructs were subject to SDS-PAGE and Coomassie staining. **B.** Purified proteins (1μM) were phosphorylated in the presence of 0.2μM GRK2 and in the absence or presence of Gβγ and PIP_2. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. No phosphorylation of the GST moiety alone (GST) was observed. **C.** A full-length ezrin construct in which threonine-567 had been mutated to alanine was cloned into the pGEX-5X-1 vector (GST-T567A). The resulting mutant GST-fusion protein was over-expressed in *E.*coli, purified and subject to SDS-PAGE and Coomassie staining, along with the full-length, wildtype GST-ezrin construct 1-586. Positions of protein molecular mass standards are indicated at the left of the images. Bands shown in boxes are from gels imaged at the same exposure, but with irrelevant lanes removed. Red arrow heads indicate the full length protein constructs, other bands in each lane are degradation products.

These results indicate that GRK2 phosphorylates the carboxy-terminus of ezrin, between residues 533 and 586. This region includes threonine-567, phosphorylation of which regulates the cross-linking activity of ERM proteins (Matsui et al., 1998). From these observations I speculated that GRK2 may, like Rho kinase and PKC, directly modulate the ability of ezrin to cross-link plasma membrane proteins to actin. Also notable was the Gβγ-dependence of GRK2-mediated phosphorylation of full-length ezrin, which suggest that this phosphorylation event might be dependent upon GPCR activation in a cellular context.

3.4 Assay to determine if GRK2-mediated phosphorylation of ezrin regulates ezrin function

Phosphorylation of ERM proteins at threonine-567 (or equivalent) has been shown to inhibit the association of the carboxyl-terminus of the protein with its amino-terminus, leading to ERM activation (Matsui et al., 1998). I therefore sought to examine whether GRK2-mediated phosphorylation of the carboxyl-terminal domain of ezrin would similarly inhibit its association with an amino-terminal ezrin fusion protein construct.

COS cells were transfected with the VSVG-tagged carboxyl-terminal domain of human ezrin (C-ezrin; residues 280-585). After 48 hours, the cells were lysed and the lysate incubated with glutathione agarose beads to which a GST-tagged amino-terminal construct of ezrin (residues 1-310) had been pre-bound. Following washing, bound protein was dissociated from the agarose beads and subject to SDS-PAGE and western blotting. C-ezrin bound to the 1-310 construct was detected using an anti-ezrin primary antibody that recognises residues 479-498 within the carboxyl-terminus of human ezrin (Upstate Biotechnology). Lysates of COS cells over-expressing C-ezrin and wildtype GRK2 or C-ezrin and a catalytically inactive GRK2 mutant ($GRK2_{K220R}$) were similarly processed.

In agreement with published observations (Matsui et al., 1998), the carboxyl-terminal ezrin construct associated with the amino-terminal construct and could be specifically purified from the COS cell lysate (Figure 3.3). It was postulated that the C-ezrin construct co-expressed with GRK2 would be phosphorylated. If GRK2

phosphorylation leads to ezrin activation, the association of C-ezrin with GST-1-310 would be predicted to be impaired. Conversely, co-expression of C-ezrin with the catalytically inactive mutant of GRK2 ($GRK2_{K220R}$) would not be predicted to affect the phosphorylation status of C-ezrin or the ability of the amino- and carboxyl-termini of ezrin to interact.

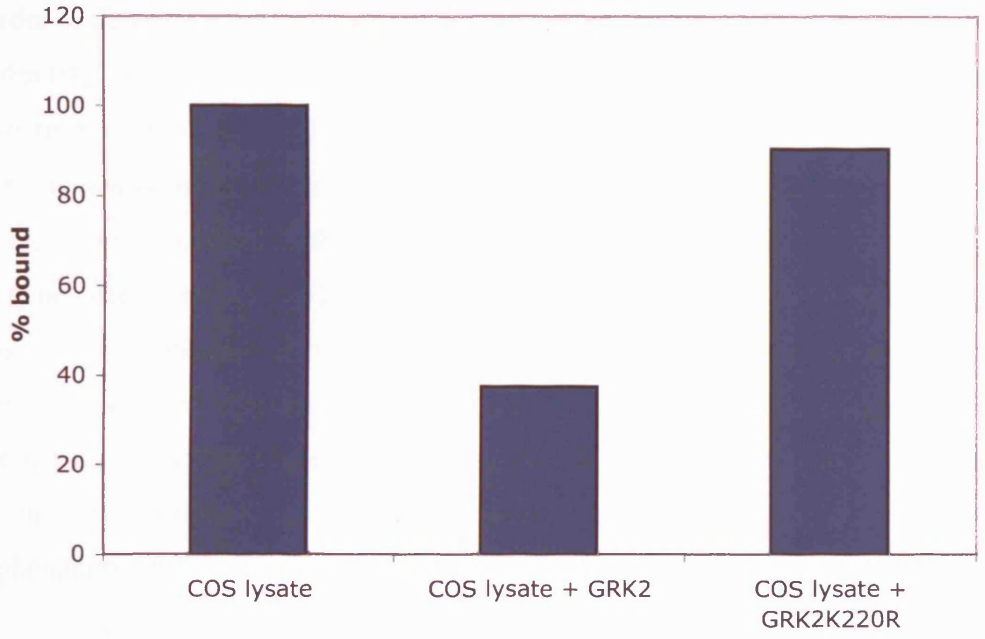

Figure 3.3. GRK2-mediated phosphorylation of ezrin inhibits the association of the amino- and carboxyl-termini of ezrin. COS cells were transfected with C-ezrin (residues 280-585). The lysate was incubated with glutathione agarose beads to which the 1-310 GST-ezrin construct had been pre-bound. Following washing, bound protein was dissociated from the agarose beads and subject to SDS-PAGE and western blotting. Any C-ezrin that had bound to the 1-310 construct was detected using an anti-ezrin primary antibody (COS lysate). This was repeated with lysates of COS cells over-expressing C-ezrin and wildtype GRK2 (COS lysate + GRK2) or C-ezrin and the catalytically inactive GRK2 mutant $GRK2_{K220R}$ (COS lysate + GRK2K220R). The mean amount of C-ezrin bound to 1-310 in the absence of GRK2 over-expression was considered to be 100% and the mean amounts of C-ezrin bound in pulldown assays in the presence of GRK2 over-expression were standardised accordingly (n=2).

Co-expression of GRK2 with C-ezrin reduced the relative amount (when compared to the total expressed in the cell lysate) of C-ezrin binding to 1-310 by 62.6%, whereas expression of $GRK2_{K220R}$ inhibited the C-ezrin/1-310 association by only 9.5% (Figure 3.3). This data suggests that GRK2 activity can inhibit the association

of the amino- and carboxyl-termini of ezrin, indicating that GRK2 phosphorylation serves to activate ezrin. These results suggest that threonine-567 may represent the site of GRK2-mediated ezrin phosphorylation.

3.5 Identification of the GRK2 phosphorylation site of ezrin

In order to determine the exact site(s) of GRK2 phosphorylation of ezrin, I attempted to identify which residues of GST-310-586 ezrin were phosphorylated after incubation with GRK2. Following confirmation of sequence, a total of 20μg 310-586 protein was subject to *in vitro* phosphorylation as described in Section 2.4.2 and the pooled reactions were subject to SDS-PAGE and Coomassie staining of the gel. The band corresponding to 310-586 was excised from the gel and sent to Dr. Richard Cook at the DeBakey Centre for Research, Texas, USA. Following in-gel trypsin digestion, peptides derived from the digest were eluted from the gel slice and enriched for phosphopeptides using an iron chelate matrix. Electrospray MALDI-TOF mass spectrometry was subsequently used in an attempt to separate and identify the phosphopeptides.

Although some phosphopeptides were recovered using this procedure, they were present in such small quantities as to make their identification by MALDI-TOF untenable. Examination of the peptide sequence of 310-586 (Figure 3.4) reveals that the carboxyl terminus of ezrin is arginine- and lysine-rich, especially in the region surrounding the candidate phosphorylation site, threonine-567. Trypsin digestion of such a phosphorylated peptide would be predicted to lead to the generation of very small phosphopeptides (Figure 3.4) that would not be likely to be detected in the mass spectrometry procedure.

Although it could have been possible to repeat this procedure with alternative proteolytic strategies, in order to attempt to generate larger phosphopeptides, I decided on an alternative strategy to determine if threonine-567 represents the site of GRK2-mediated ezrin phosphorylation. I therefore undertook a different approach. Using a PCR-based method, I cloned a human ezrin construct in which the threonine at position 567 had been mutated to alanine from pCB6-Ezrin-T567A-VSVG into the

pGEX-5X-1 vector (GST-T567A-ezrin, Figures 2.1B and 3.2C). Sequencing confirmed that the GST-T567A construct was downstream of and in frame with the GST moiety of the pGEX vector and that there was no longer a VSVG tag at the 3′ end. It also verified that threonine-567 had been mutated to alanine-567.

```
1    MPKPINVRVT TMDAELEFAI QPNTTGKQLF DQVVKTIGLR
41   EVWYFGLHYV DNKGFPTWLK LDKKVSAQEV RKENPLQFKF
81   RAKFYPEDVA EELIQDITQK LFFLQVKEGI LSDEIYCPPE
121  TAVLLGSYAV QAKFGDYNKE VHKSGYLSSE RLIPQRVMDQ
161  HKLTRDQWED RIQVWHAEHR GMLKDNAMLE YLKIAQDLEM
201  YGINYFEIKN KKGTDLWLGV DALGLNIYEK DDKLTPKIGF
241  PWSEIRNISF NDKKFVIKPI DKKAPDFVFY APRLRINKRI
281  LQLCMGNHEL YMRRRKPDTI EVQQMKAQAR EEKHQKQLER
321  QQLETEKKRR ETVEREKEQM MREKEELMLR LQDYEEKTKK
361  AERELSEQIQ RALQLEEERK RAQEEAERLE ADRMAALRAK
401  EELERQAVDQ IKSQEQLAAE LAEYTAKIAL LEEARRRKED
441  EVEEWQHRAK EAQDDLVKTK EELHLVMTAP PPPPPPVYEP
481  VSYHVQESLQ DEGAEPTGYS AELSSEGIRD DRNEEKRITE
521  AEKNERVQRQ LLTLSSELSQ ARDENKRTHN DIIHNENMRQ
561  GHDKYKTLRQ IRQGNTKQRI DEFEAL
```

Figure 3.4. Amino acid sequence of ezrin (accession number: NP_003370). Residues included in construct 310-586 are underlined. Those residues that encompass the GRK2 phosphorylation site, i.e. those that are found in construct 310-586 but not construct 1-533, are in blue. Threonine-567 is in red. Arginine and lysine residues in the vicinity of threonine-567 are highlighted in cyan.

I intended to use this mutant protein in *in vitro* phosphorylation assays to determine whether GRK2 phosphorylation of full-length ezrin would be inhibited in the absence of threonine-567. Unfortunately, due to time constraints, it was not possible

to complete these experiments. However, I have verified that this construct contains the threonine to alanine substitution at residue 567 and it should serve as a useful tool for future investigations. Indeed, it has subsequently been used by Dr. Julie Pitcher in *in vitro* phosphorylation assays to demonstrate that it is not phosphorylated by GRK2 (Cant and Pitcher, 2005).

3.6 Summary

In summary, this Chapter provides evidence that ezrin is a substrate of GRK2 *in vitro*, supporting an earlier finding by Dr. Julie Pitcher that GRK2 phosphorylation of ezrin occurs in cultured mammalian cells. GRK2 phosphorylates full-length ezrin only when PIP_2-containing lipid vesicles and $G\beta\gamma$ are present, suggesting that this event would be dependent on GPCR activation in a cellular setting. This finding agrees with the result of Dr. Pitcher's initial investigation where GRK2-mediated phosphorylation of ezrin was enhanced following agonist occupancy of the β_2AR.

Phosphorylation of ezrin by GRK2 occurs within a carboxyl-terminal 53 amino acid stretch that encompasses the phosphorylation site required for ezrin activation, threonine-567. Attempts to demonstrate either directly or indirectly that threonine-567 was the site of GRK2 phosphorylation *in vitro* were unsuccessful within the time constraints of this thesis. However, Dr. Julie Pitcher has subsequently demonstrated that the GST-T567A-ezrin construct is not phosphorylated by GRK2, suggesting that threonine-567 does indeed represent the site of GRK2-mediated phosphorylation (Cant and Pitcher, 2005).

Interestingly, ezrin appears to increase the activity of GRK2 on ROS *in vitro*, indicating that GRK2 and ezrin may interact. Ezrin might act to enhance the action of GRK2 against rhodopsin by either directly activating it or by promoting the membrane localisation of GRK2. Although the latter explanation is most probable, it would be interesting to investigate whether ezrin has any effect on the GRK2-mediated phosphorylation of ROS in the presence of $G\beta\gamma$.

The implications of the results presented in this and the following two Chapters will be discussed in detail in Chapter 6.

4 Ezrin is required for M1MR-mediated ruffling

As described in the introduction to this thesis, it has previously been shown that ERM proteins are involved in the biogenesis of lamellipodia and filopodia, structures generated during cell migration (Bretscher, 1989; Yonemura et al., 1999). Directed cell migration, or chemotaxis, is a process that occurs throughout an organism's lifespan, from embryonic development to wound repair and the leukocytic immune response. The motile cell becomes highly polarised, extending lamellipodia and filopodia in the direction of migration (Small et al., 2002). These protuberances attach to the underlying strata via adhesion complexes that link to the actin cytoskeleton to stabilise the structures and provide the traction force needed to propel the cell forward. The complexes are rapidly detached at the back of the cell as the cell body contracts and advances and the rear retracts (Horwitz and Webb, 2003).

4.1 The role of the actin cytoskeleton in migration

Lamellipodia and filopodia are sites of actin polymerisation where regulators of microfilament remodelling, such as Arp2/3, WASP, Abp1 and cortactin are localised. As the cell moves forward, its cell body is pulled in the direction of migration. This contractile force is generated through the cross-linking of actin filaments by myosin motor proteins. Disassembly of microfilaments at the rear of the cell is thought to be mediated by the actin depolymerising factor/cofilin protein family (Pollard and Borisy, 2003). The Arp2/3 activator Abp1 colocalises with Arp2/3 and F-actin at lamellipodia in response to growth factor stimulation of mammalian cells (Goode et al., 2001; Kessels et al., 2001). Cortactin, which can also bind actin and activate Arp2/3, is believed to stabilise the actin network of the lamellipodium as it can inhibit actin debranching (Weaver et al., 2001).

4.1.1 Membrane ruffles are compartments of actin reorganisation

Membrane ruffles have been identified as lamellipodia that "ruffle" as they curl away from the substrate (Small et al., 2002). A recent study has demonstrated that membrane ruffles consist of densely packed actin filaments that differ from the actin cytoskeleton that makes up the underlying cell lamella (Borm et al., 2004). The

researchers demonstrated that ruffles are distinct compartments of actin reorganisation that are enriched in ezrin and filamin. They hypothesised that membrane ruffles are formed when a migrating cell fails to establish firm adhesions to its substrate, causing lamellipodia to retract towards the cell body (Borm et al., 2004).

4.1.2 GPCR-mediated cell migration and ruffling

GPCRS such as the sphingosine-1-phosphate receptors, proteinase-activated receptor 2 (PAR2), the μ-opioid receptor and the chemokine receptors CCR2, CXCR1, CXCR2 and CXCR4 induce membrane ruffling and cell migration on agonist activation (D'Apuzzo et al., 1997; Ge et al., 2004; Jones et al., 2003; Richardson et al., 2003; Takayama and Ueda, 2005; Wang et al., 1999). GRK2 has been shown to be important in regulating the CCR2-mediated response to chemoattractants in leukocytes (Aragay et al., 1998) and is highly expressed in undifferentiated, multipotent migratory cells during early embryonic mouse development (Sefton et al., 2000). Furthermore, neutrophils and splenocytes derived from GRK6-deficient mice have altered chemotactic responses (Fong et al., 2002; Kavelaars et al., 2003; Vroon et al., 2004). These results suggest that GRKs also play key roles in regulating migratory responses to GPCR activation.

Other components of the GPCR desensitisation machinery may also contribute to cell motility. βarrestin2-deficient splenocytes have impaired chemotactic responses and βarrestin scaffolded to ERK has been shown to be required for migration following PAR2 activation (Fong et al., 2002; Ge et al., 2004). βarrestin2 has also been demonstrated to play an essential role in mediating the migration of both $AT_{1A}R$-expressing cells in response to both angiotensin II and lysophosphatidic acid (Hunton et al., 2005) and CXCR4-expressing cells in response to stromal cell-derived factor 1α, via the p38/MAPK signalling pathway (Sun et al., 2002). Finally, as discussed in Chapter 1 (Section 1.2.6.2), GIT family proteins may be required for focal adhesion dynamics and cell motility (Turner et al., 1999; West et al., 2001; Zhao et al., 2000).

4.2 M1MR-expressing Hep2 cells are a model for membrane ruffling

Hep2 cells over-expressing the HA-tagged M1 muscarinic receptor (M1MR) ruffle in response to agonist (acetylcholine) treatment. The M1MR is widely expressed in the forebrain and appears to regulate processes such as learning and memory (Wess, 2004). It is coupled to Gq (Offermanns et al., 1996), increasing intracellular levels of the second messenger Ca^{2+} and therefore activating PKC and Ca^{2+}-gated ion channels on agonist stimulation. GRK2 has been demonstrated to phosphorylate M1MR *in vitro* (Haga et al., 1996).

48 hours following transient transfection with HA-tagged M1MR, Hep2 cells were treated with the M1MR agonist acetylcholine for 5 minutes. Following fixation and receptor staining, the cells were observed to demonstrate rapid and transient membrane ruffling around their periphery (Figure 4.1), followed by M1MR internalisation (Figure 4.1). Approximately 81±6% of cells ruffle. I used this model system to investigate the potential functional significance of Gβγ-dependent GRK2-mediated ezrin phosphorylation in GPCR-mediated cytoskeletal reorganisation.

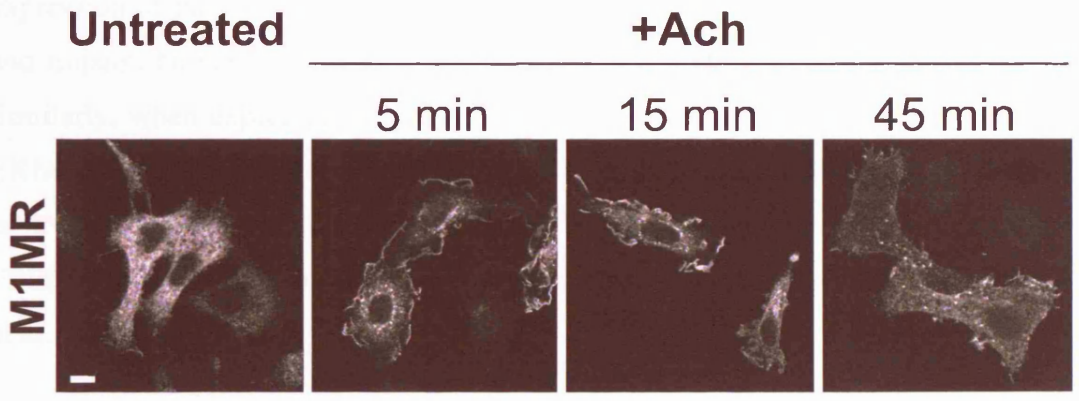

Figure 4.1. M1MR-expressing Hep2 cells are a model for membrane ruffling. Hep2 cells transiently expressing M1MR were treated with the muscarinic receptor agonist acetylcholine (Ach) for 5, 15 and 45 minutes. Cells were fixed and the M1MR visualised as described in Chapter 2. Scale bar represents 20μm.

4.3 Ezrin is required for M1MR-mediated membrane ruffling

To investigate a potential role for ezrin in M1MR-mediated membrane ruffling, I examined the subcellular localisation of endogenous ezrin in M1MR-expressing Hep2 cells. Treatment of these cells with acetylcholine for 5 minutes induced membrane ruffles to which endogenous ezrin was localised (Figure 4.2A). Using an antibody (297S) that specifically recognises ERM proteins phosphorylated at the regulatory threonine residue (pERM) (Hayashi et al., 1999; Matsui et al., 1998) it was observed that the cellular levels of pERM increase following agonist stimulation and that activated ERM proteins localise to ruffles (Figure 4.2A). These results demonstrate that M1MR activation promotes ERM protein phosphorylation and suggest that activated ERM proteins may play a role in ruffle formation.

To determine if activated ezrin is required for ruffling formation I co-transfected Hep2 cells with the M1MR and a VSVG-tagged full-length human ezrin construct (WT-ezrin) or the VSVG-tagged amino-terminal FERM domain of human ezrin (N-ezrin; residues 1-309). Expression of the FERM domain of ezrin has been shown to inhibit ezrin function in a number of model systems. In LLC-PK1 epithelial cells, expression of the FERM domain of ezrin causes redistribution of endogenous ezrin and impairs human growth factor-mediated cell migration (Crepaldi et al., 1997). Similarly, when expressed in NIH3T3 fibroblasts, the FERM domains of all three ERM proteins interferes with the cellular architecture, inhibiting filopodial protrusion and retraction (Amieva et al., 1999). Finally, in mice, expression of the FERM domain of ezrin inhibits ezrin-mediated breast carcinoma metastasis (Elliott et al., 2005).

Expression of full-length ezrin has no effect on agonist-dependent M1MR-mediated ruffling and the expressed protein, like the endogenous, localises to ruffles (WT-ezrin, Figures 4.2B and C). However, Hep2 cells co-transfected with M1MR and the FERM domain of ezrin (N-ezrin) show an approximate 64±9% reduction in ruffle formation following agonist stimulation (N-ezrin, Figures 4.2B and C). These results are consistent with an obligate role for activated, phosphorylated, ERM proteins in M1MR-mediated membrane ruffle formation in this system.

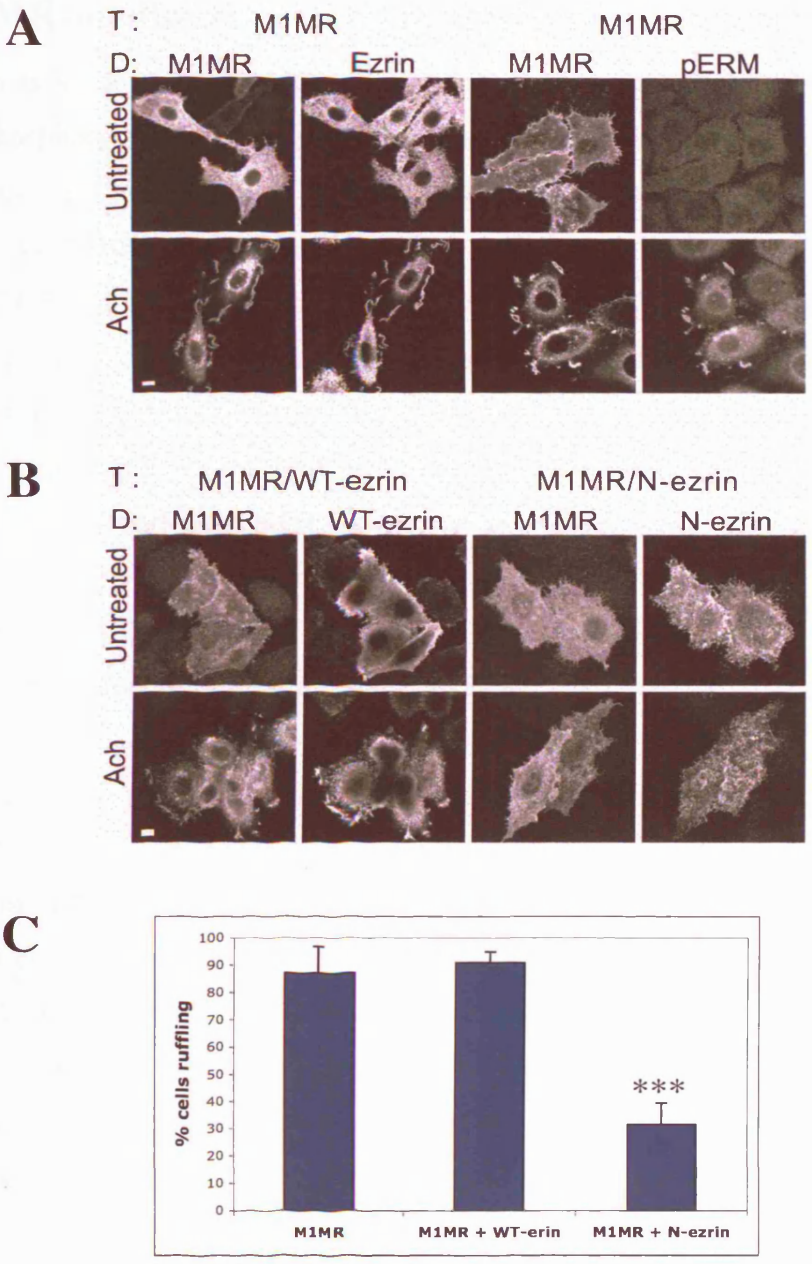

Figure 4.2. Ezrin is required for M1MR-mediated membrane ruffling. **A.** The cellular distribution of endogenous ezrin and pERM in Hep2 cells over-expressing M1MR was visualised by immunofluorescence 48 hours post-transfection. Where indicated, cells were treated with 100μM acetylcholine for five minutes prior to fixation (Ach). **B.** Hep2 cells were transfected with M1MR and a wildtype ezrin construct (WT-ezrin) or M1MR and the FERM domain of ezrin (N-ezrin). 48 hours post-transfection cells were treated with agonist, 100μM acetylcholine, for 5 minutes (Ach) and the effect on membrane dynamics visualised by immunofluorescence. T denotes the cDNA constructs transfected. D denotes the proteins detected. Scale bars represent 20μm. **C.** The effect of different ezrin mutants on M1MR-mediated ruffling was assessed by exposing the cells to 5 minutes of acetylcholine treatment and counting the number of ruffling cells that expressed both M1MR and the mutant construct. Data indicate the mean number of ruffling cells. Error bars represent S.D. of mean data collected from multiple experiments ($n \geq 3$). *** indicates $p<0.001$.

4.4 M1MR-mediated ruffling requires GRK2 activity

Since ezrin is a substrate of GRK2, I investigated a potential role for GRK2-mediated phosphorylation events in this model of cytoskeletal reorganisation. Hep2 cells were co-transfected with M1MR and either the carboxy-terminus of GRK2 (GRK2-CT, or βARKct) or the catalytically inactive mutant kinase ($GRK2_{K220R}$). Both GRK2-CT and $GRK2_{K220R}$ contain the $G\beta\gamma/PIP_2$ binding PH domain of GRK2 and their expression inhibits agonist-dependent translocation of endogenous GRK2 to activated GPCRs (Daaka et al., 1997b). Either construct would thus be anticipated to inhibit receptor-activated GRK2-mediated phosphorylation events. Cells co-expressing M1MR and GRK2-CT or M1MR and $GRK2_{K220R}$ show diminished membrane ruffling by approximately 90±5% ($n \geq 3$, $p<0.001$) and 81±14% ($n \geq 3$, $p<0.001$) respectively compared to those expressing M1MR alone, following acetylcholine treatment (Figures 4.3A and B and 4.4).

I also co-transfected Hep2 cells with either M1MR and a GFP-tagged RGS domain of GRK2 ($GRK2_{45-178}$), or M1MR and a GFP-tagged Gq binding-defective mutant RGS domain ($GRK2_{45-178}$D110A). When expressed, $GRK2_{45-178}$ associates with GTP-bound Gq at the plasma membrane, whereas $GRK2_{45-178}$D110A is unable to bind Gq and so remains localised in the cytosol (Sterne-Marr et al., 2003). $GRK2_{45-178}$ would therefore be expected to inhibit the translocation of endogenous GRK2 to activated GPCRs on agonist treatment, while expression of $GRK2_{45-178}$D110A would not be expected to have an effect. Following agonist treatment, cells co-expressing M1MR and $GRK2_{45-178}$ show reduced ruffling by approximately 75±9% ($n \geq 3$, $p<0.001$) compared to those expressing M1MR alone (Figures 4.3C and 4.4). The number of cells co-expressing M1MR and $GRK2_{45-178}$D110A that show ruffling after acetylcholine treatment does not significantly differ from those expressing M1MR alone ($n \geq 3$) (Figures 4.3D and 4.4). These results imply that, in addition to ezrin, active GRK2 is required at the plasma membrane for the formation of membrane ruffles in Hep2 cells expressing M1MR.

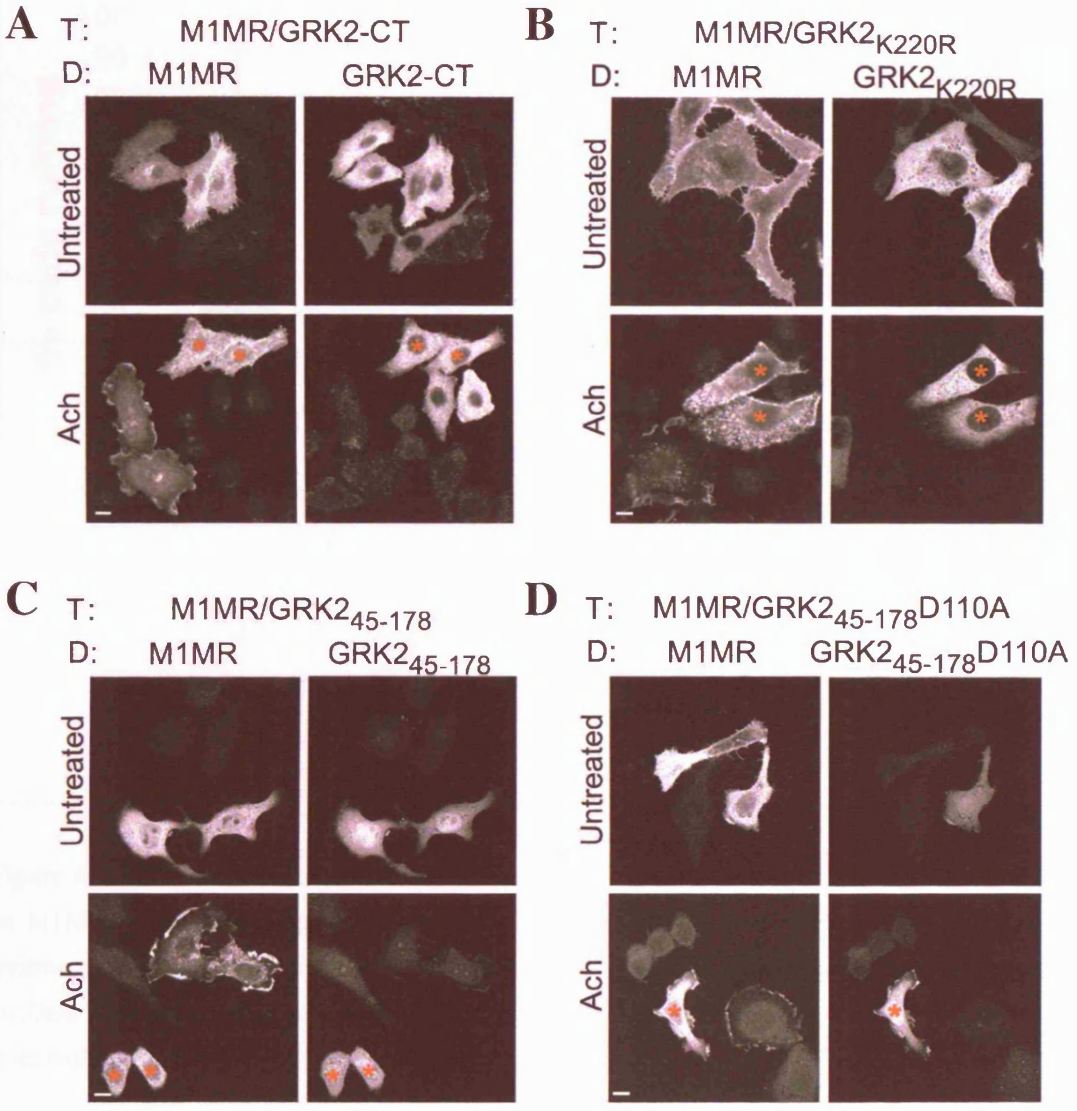

Figure 4.3. GRK2 is required for M1MR-mediated membrane ruffling. Hep2 cells were transfected with M1MR and (**A**) the carboxy-terminus of GRK2 (M1MR/GRK2-CT), (**B**) M1MR and catalytically inactive GRK2 (M1MR/$GRK2_{K220R}$), (**C**) M1MR and the GFP-tagged RGS domain of GRK2 (M1MR/$GRK2_{45-178}$), or (**D**) M1MR and the GFP-tagged Gq binding-defective mutant GRK2 RGS domain ($GRK2_{45-178}$D110A). 48 hours after transfection, the cells were treated with 100μM acetylcholine (Ach) for 5 minutes. The cells were visualised by immunofluorescence. Asterisks indicate treated cells expressing both M1MR and the GRK2 construct. T denotes cDNA constructs transfected. D denotes proteins detected. Scale bar represents 20μm.

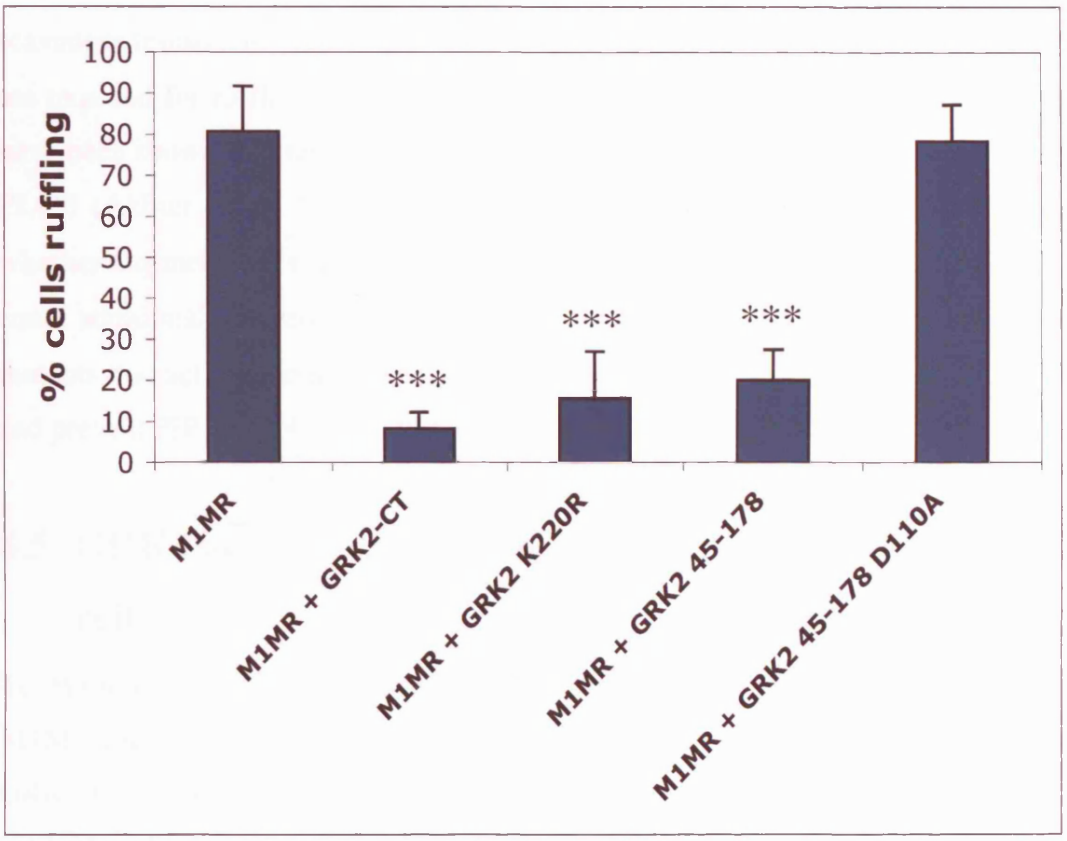

Figure 4.4. GRK2 mutants inhibit M1MR-mediated ruffling. The effect of different GRK2 mutants on M1MR-mediated ruffling was assessed by exposing the cells to 5 minutes of acetylcholine treatment and counting the number of ruffling cells that expressed both M1MR and the mutant kinase. 101Data indicate the mean number of ruffling cells. Error bars represent S.D. of mean data collected from multiple experiments ($n \geq 3$). *** indicates $p<0.001$.

However, GRKs are also likely to regulate GPCR signalling in a phosphorylation-independent fashion. Since GRKs bind to Gβγ and, where expressed, Gq-GTP, they may also mediate GPCR desensitisation by inhibiting signalling pathways downstream of Gβγ and Gq (Pao and Benovic, 2002). Although GRK2 mutants such as GRK2-CT, $GRK2_{K220R}$ and $GRK2_{45\text{-}178}$ would be expected to interfere with the translocation of wildtype GRK2 to the plasma membrane, they could also terminate signalling via Gβγ (GRK2-CT and $GRK2_{K220R}$) and/or Gq ($GRK2_{K220R}$ and $GRK2_{45\text{-}178}$) (Carmen et al., 1999a; Rockman et al., 1998; Sallese et al., 200b).

In order to determine the exact roles of GRK2, Gβγ and Gq in M1MR-mediated membrane ruffling, further experimental approaches are required. Acetylcholine stimulation of M1MR-expressing Hep2 cells transfected with the functional Gβγ

scavenger transducin (Federman et al., 1992) would establish whether Gβγ subunits are required for ruffling. The expression of carboxyl-terminal Gq peptides, which have been shown to interfere with endogenous Gq binding to GPCRs and activating PLCβ (Akhter et al., 1998), in cells co-transfected with M1MR would indicate whether Gq-mediated activation of PLCβ is required for membrane ruffling. This could additionally be investigated with the use of xanthine nucleotide-binding Gαq mutants that act in a dominant negative manner to inhibit coupling of GPCRs to Gq and prevent PIP_2 hydrolysis by PLCβ (Goel et al., 2004; Yu et al., 2000).

4.5 GRK2 activity is required for ERM activation in Hep2 cells

To examine the relationship between GRK2 activity and ERM phosphorylation in M1MR-mediated membrane ruffling, I co-transfected Hep2 cells with M1MR and GRK2-CT, agonist treated, fixed and detected endogenous pERM using the 297S antibody. In cells expressing M1MR alone, pERM were observed after agonist treatment and was localised to the ensuing ruffles (Figures 4.2A and 4.5). In cells expressing both M1MR and GRK2-CT no ruffling was observed and pERM could not be detected, even after receptor stimulation (Figure 4.5). The fact that endogenous GRK2 activity is required for the detection of active ERM species in the cell suggests that it is GRK2 that is responsible for the phosphorylation of ERM proteins. Furthermore, since the antibody utilised specifically recognises pERM, GRK2 is implicated as the kinase responsible for phosphorylating ERM proteins at this functionally relevant site. These results cannot discount an alternative hypothesis that GRK2 phosphorylates and activates an additional kinase that is itself responsible for the phosphorylation and activation of ERM proteins at membrane ruffles. However, when considered in conjunction with the *in vitro* results presented in Chapter 3, the data are highly suggestive that GRK2 directly phosphorylates and activates ERM proteins following M1MR activation in Hep2 cells.

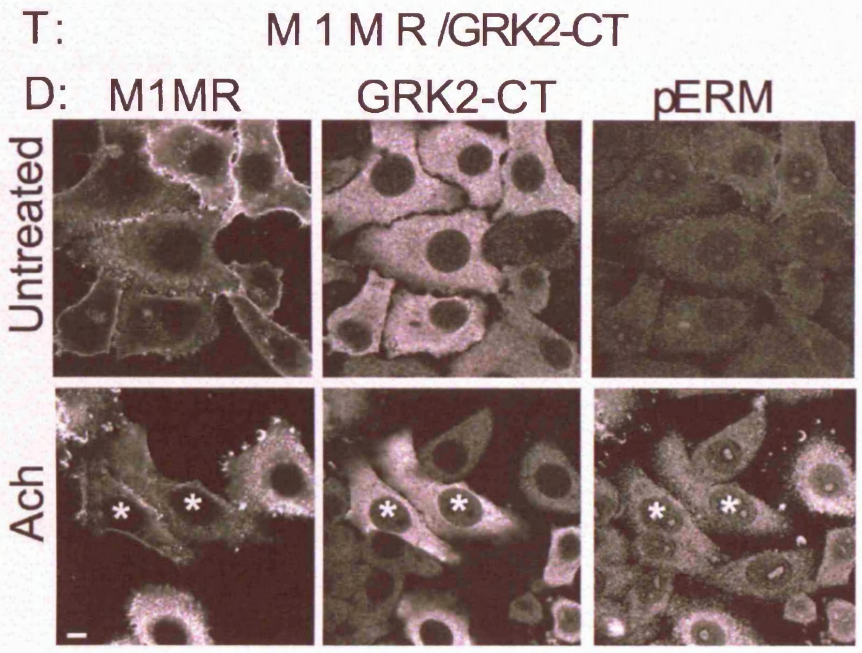

Figure 4.5. GRK2 activity is required for ERM activation in Hep2 cells. Hep2 were transfected with M1MR and GRK2-CT and incubated for 48 hours. The cells were subsequently treated with acetylcholine for 5 minutes (Ach) and M1MR, GRK2-CT and endogenous pERM detected by immunofluorescence. Asterisks indicate cells co-expressing M1MR and GRK2-CT. T denotes the cDNA constructs transfected. D denotes the proteins detected. Scale bars represent 20μm.

4.6 M1MR-mediated membrane ruffling in Hep2 cells is not Rho kinase, PKC or PKA dependent

Earlier studies have shown that ezrin can be phosphorylated at threonine-567 by PKC, Rho kinase and PKA (Matsui, et al. 1998; Ng et al., 2001; Simons et al., 1998; Zhou et al., 2003). I examined whether these kinases were responsible for the phosphorylation of ERM proteins in our model system. Hep2 cells over-expressing M1MR were pre-treated with the Rho kinase inhibitor Y27632 (Figure 4.6A), the PKC inhibitor GF109203X (Figure 4.6B) or the PKA inhibitor Rp-8-Br-CAMPS (Figure 4.6C) prior to agonist stimulation. pERM were subsequently detected in these treated cells using 297S (cells treated with Y27632 and GF109203X) or the Cell Signalling Technology anti-pERM antibody (cells treated with Rp-8-Br-CAMPS). None of the inhibitors prevent ruffle formation following agonist treatment, nor do they inhibit ERM phosphorylation or localisation of pERM to the ruffles ($n \geq 3$). These results suggest that the M1MR-dependent phosphorylation of

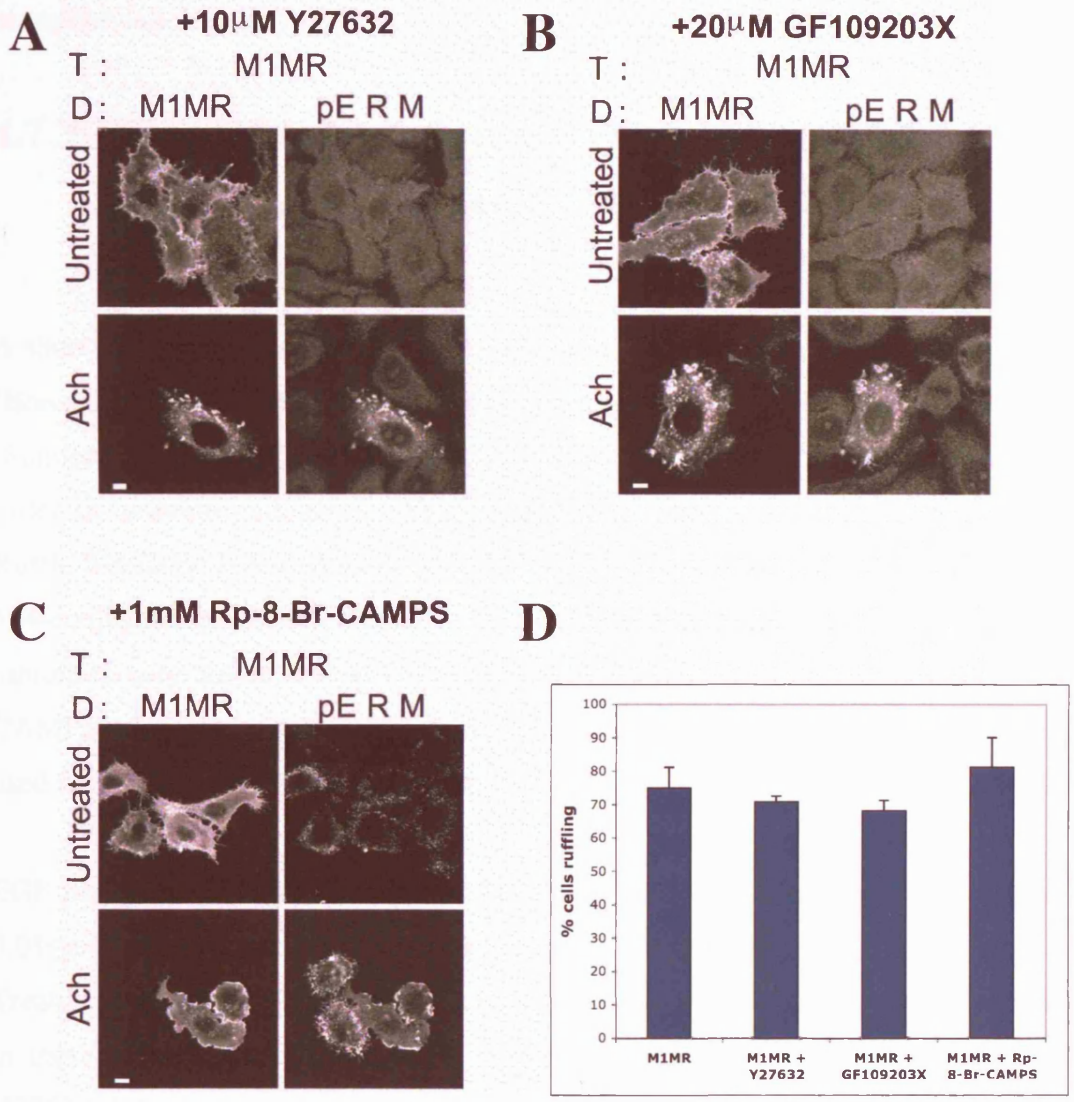

Figure 4.6. M1MR-mediated ruffling in Hep2 is not Rho kinase, PKC or PKA dependent. 48 hours following transfection, Hep2 cells overexpressing M1MR were treated with either the Rho kinase inhibitor (**A**: Y27632, 10μM), the PKC inhibitor (**B**: GF109203X, 20μM), or the PKA inhibitor (**C**: Rp-8-Br-CAMPS, 1mM) for 1 hour. Cells were then subject to 100μM acetylcholine for 5 minutes prior to fixation (Ach). M1MR and endogenous pERM were visualised by immunofluorescence using either the 297S antibody (**A** and **B**) or a commercial anti-pERM antibody (**C**). T denotes the cDNA constructs transfected. D denotes the proteins detected. Scale bar represents 20μm. **D.** The effect of the different kinase inhibitors on M1MR-mediated ruffling was assessed by counting the number of ruffling cells following inhibitor treatment and addition of agonist for 5 minutes. Data indicate the mean number of ruffling cells. Error bars represent S.D. of mean data collected from multiple experiments (n≥3).

ERM proteins observed in Hep2 cells is not mediated by Rho kinase, PKC or PKA. This further supports my contention that GRK2 is responsible for mediating this phosphorylation event.

4.7 EGF-mediated membrane ruffling in Hep2 cells is dependent on PKC and Rho kinase and independent of PKA and GRK2

A short (2-5 minutes) treatment with EGF induces membrane ruffles in A-431 cells (Bretscher, 1989). Ezrin is recruited to these ruffles in a PKC-dependent manner (Stapleton et al., 2002). I therefore treated Hep2 cells with EGF for 3 minutes in order to activate endogenous EGF receptors and stimulate membrane ruffling. Ruffle formation occurred and was visualised by staining the cells with Alexa Fluor 594-conjugated phalloidin (Figure 4.7A). To demonstrate the efficacy of the kinase inhibitors used above, I treated the cells with Y27632, GF109203X and Rp-8-Br-CAMPS prior to stimulation with EGF. The treatment protocol was identical to that used for M1MR-expressing Hep2 cells.

EGF-mediated ruffling is diminished by 50±6% in cells treated with Y27632 (n=3, $0.01 \leq p < 0.05$) and 66±6% in cells treated with GF109203X (n=3, $0.001 \leq p < 0.01$). Treatment with Rp-8-Br-CAMPS does not significantly affect EGF-induced ruffling in these cells (Figure 4.7A and C). Although a recent study demonstrated that Y27632 did not suppress EGF-induced membrane ruffling in human lens epithelial cells (Maddala et al., 2003), it appears that Rho kinase is necessary for ruffling downstream of EGF in our model system. These results show that both Y27632 and GF109203X are effective inhibitors under the conditions used and confirm that neither PKC nor Rho kinase are required for M1MR-mediated ruffling. Due to the lack of a positive control for Rp-8-Br-CAMPS efficacy, a role for PKA in our M1MR model cannot be ruled out, although it is unlikely as no role for PKA in membrane ruffling has yet been determined.

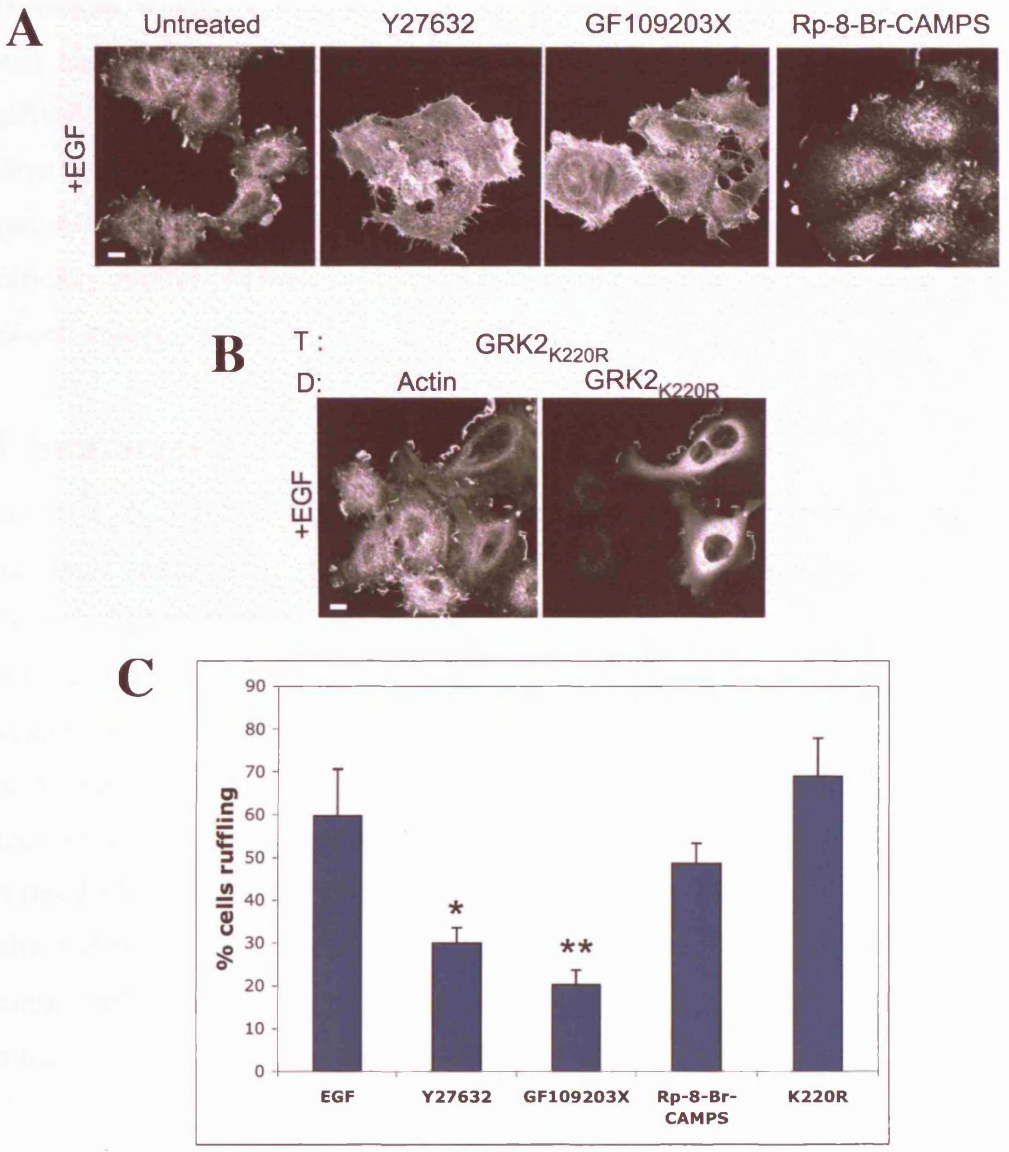

Figure 4.7. EGF-mediated membrane ruffling in Hep2 cells is dependent on PKC and Rho kinase and independent of PKA and GRK2. **A.** Hep2 cells were treated with EGF for 3 minutes to stimulate ruffling. Ruffle formation was visualised by staining the cells with Alexa Fluor 594-conjugated phalloidin. EGF-mediated ruffling is diminished in cells treated with Y27632 and GF109203X. Treatment with Rp-8-Br-CAMPS does not significantly affect EGF-induced ruffling. **B.** Hep2 cells were transfected with the kinase-dead mutant $GRK2_{K220R}$. 48 hours later, cells were treated with EGF for 3 minutes to induce ruffling and ruffles were visualised with Alexa Fluor 594-conjugated phalloidin (Actin). Cells expressing $GRK2_{K220R}$ were identified by staining with an anti-GRK2 antibody ($GRK2_{K220R}$). Expression of $GRK2_{K220R}$ does not affect EGF-mediated ruffling in Hep2 cells. T denotes cDNA constructs transfected. D denotes proteins detected. Scale bars represent 20μm. **C.** The effect of the different kinase inhibitors or $GRK2_{K220R}$ expression on EGF-mediated ruffling was assessed by exposing the cells to 3 minutes of EGF treatment and counting the number of ruffling cells. Data indicate the mean number of ruffling cells. Error bars represent S.D. of mean data collected from multiple experiments ($n \geq 3$). * indicates $0.01 \leq p < 0.05$, ** indicates $0.001 \leq p < 0.01$.

To determine whether GRK2 was involved in EGF-mediated membrane ruffling, I treated Hep2 cells transfected with $GRK2_{K220R}$ with EGF for 3 minutes and visualised the ruffles using phalloidin-594. The expression of the catalytically inactive GRK2 mutant has no effect on EGF-stimulated ruffling in Hep2 cells (n=3) (Figure 4.7B and C). These results indicate that expression of the $GRK2_{K220R}$ mutant specifically inhibits M1MR-mediated ERM-dependent membrane ruffling in this Hep2 cell model system.

4.8 Summary

In summary, the results in this Chapter demonstrate that agonist treatment of M1MR-expressing Hep2 cells induces cytoskeletal reorganisation in the form of membrane ruffles to which active, phosphorylated ERM proteins are localised. These ruffles require both functional ezrin and active GRK2 at the plasma membrane, but are not dependent on Rho kinase, PKC or PKA. It is probable that GRK2 is the kinase directly responsible for phosphorylating and activating ERM proteins in this cell system, although I have not formally ruled out the possibility that GRK2 indirectly activates ERM proteins via phosphorylation of an as yet identified kinase. These results indirectly demonstrate that GRK2 is likely to phosphorylate ezrin at threonine-567, as postulated in Chapter 3, since this would lead to the observed activation of ezrin and its detection by 297S.

The action of GRK2 in membrane ruffling appears to be specific to the M1 muscarinic receptor, since I have demonstrated that expression of a catalytically inactive GRK2 mutant does not affect EGF-mediated ruffling in Hep2 cells. These results suggest that GRK2 acts downstream of M1MR to promote actin cytoskeletal rearrangements and might also act downstream of other GPCRs, such as CCR2, to mediate cytoskeletal reorganisation prior to chemotaxis. Therefore, in addition to modulating the number and activity of GPCRs at the cell surface, and thus a cell's chemotactic response following receptor stimulation (Aragay et al., 1998), GRK2 may actually play a direct role in this important process.

5 Ezrin and receptor endocytosis

Although the actin cytoskeleton is presumed to regulate clathrin-mediated endocytosis, few studies have investigated whether it is involved in GPCR internalisation and trafficking. Disruption of the cortical actin cytoskeleton with latrunculin has been demonstrated to inhibit the internalisation of the thromboxane A2 (TPβ), bombesin and endothelin A receptors (Laroche et al., 2005; Lunn et al., 2000), and cytochalasin D treatment has been shown to disrupt the trafficking of the CXCR1 and CXCR2 receptors to the plasma membrane following endocytosis (Zaslaver et al., 2001). It is therefore possible that actin regulates both GPCR internalisation and recycling.

While there is no evidence that GPCRs can bind actin directly, it is possible that they indirectly associate with actin via binding to adapter proteins. NHERF can bind to the very carboxyl terminus of the β_2AR and is indirectly associated with the actin cytoskeleton via its association with ERM proteins (Hall et al., 1998a; Hall et al., 1998b; Reczek et al., 1997). Disruption of either of these interactions causes mis-sorting of the β_2AR to lysosomes, rather than recycling to the plasma membrane (Cao et al., 1999). Over-expression of NHERF has also been shown to inhibit agonist-induced hKOR downmodulation, presumably by increasing its rate of recycling (Li et al., 2002). The carboxyl-tail of the μ opioid receptor was recently demonstrated to bind to the actin binding protein filamin (Onoprishvili et al., 2003). This interaction was shown to be required for μ opioid receptor trafficking, as agonist-induced down-regulation of the receptor is inhibited in cells that do not express filamin (Onoprishvili et al., 2003). This study, however, did not distinguish between a reduction in the rate of receptor internalisation and an increase in the rate of receptor recycling to the plasma membrane. Filamin has also been shown to interact with the carboxyl-terminal tail of the calcitonin receptor and its expression necessary for both the decreased degradation of the internalised calcitonin receptor and increased recycling of the receptor back to the plasma membrane (Seck et al., 2003). The calcium sensing receptor and the dopamine D2 and D3 receptors have also been reported to interact with filamin, suggesting that it may play a role in the endocytic sorting and recycling of a number of GPCRs (Seck et al., 2003).

ERM proteins serve as actin/plasma membrane cross-linkers and may play a role in GPCR trafficking via NHERF (Cao et al., 1999). As I have shown that pERM colocalise with M1MR at the plasma membrane following agonist occupancy and are required for M1MR-dependent cytoskeletal reorganisation (see Chapter 4), I investigated whether ERM proteins are involved in GPCR internalisation.

5.1 HEKβ2 cells express high levels of endogenous ezrin

In order to determine the relative levels of endogenous ezrin in different cell lines, I extracted total cellular proteins from confluent plates of Hep2 cells, HEK293 cells and HEK293 cells stably expressing His_6-tagged β_2AR (HEKβ2). $1x10^6$ cells from each line were subject to SDS-PAGE and western blotting and the relative amount of ezrin detected using an anti-ezrin antibody (Figure 5.1A). The blots were then stripped and re-probed with an anti-β-tubulin antibody to determine the relative amounts of total protein loaded onto the gels (Figure 5.1A).

The optical densities of the ezrin and tubulin bands on the developed films were determined using a densitometer. The relative levels of ezrin expression in each cell line were calculated by expressing the optical density of the ezrin band as a percentage of the optical density of the tubulin band and then standardising relative to that expressed in Hep2 cells. This indicated that HEKβ2 cells express the most ezrin, relative to Hep2 cells (Figure 5.1B). Interestingly, HEKβ2 cells express approximately three-fold more ezrin than the parental HEK293 cell line, suggesting that the stable over-expression of β_2AR may lead to increased ezrin expression.

5.2 pERM colocalise with internalised β_2AR and M1MR

To investigate a potential role for ezrin in GPCR internalisation, I examined by confocal immunomicroscopy the subcellular localisation of endogenous pERM following receptor activation and endocytosis. HEKβ2 cells were treated with (-)-isoproterenol for 15 min to induce receptor internalisation (Figure 5.2A). This treatment increased endogenous pERM levels (detected using 297S), which colocalised with β_2AR at internalised vesicles. The colocalisation of pERM with internalised receptor was also observed in HEK293 cells transiently over-expressing FLAG-tagged β_2AR (Figure 5.2B).

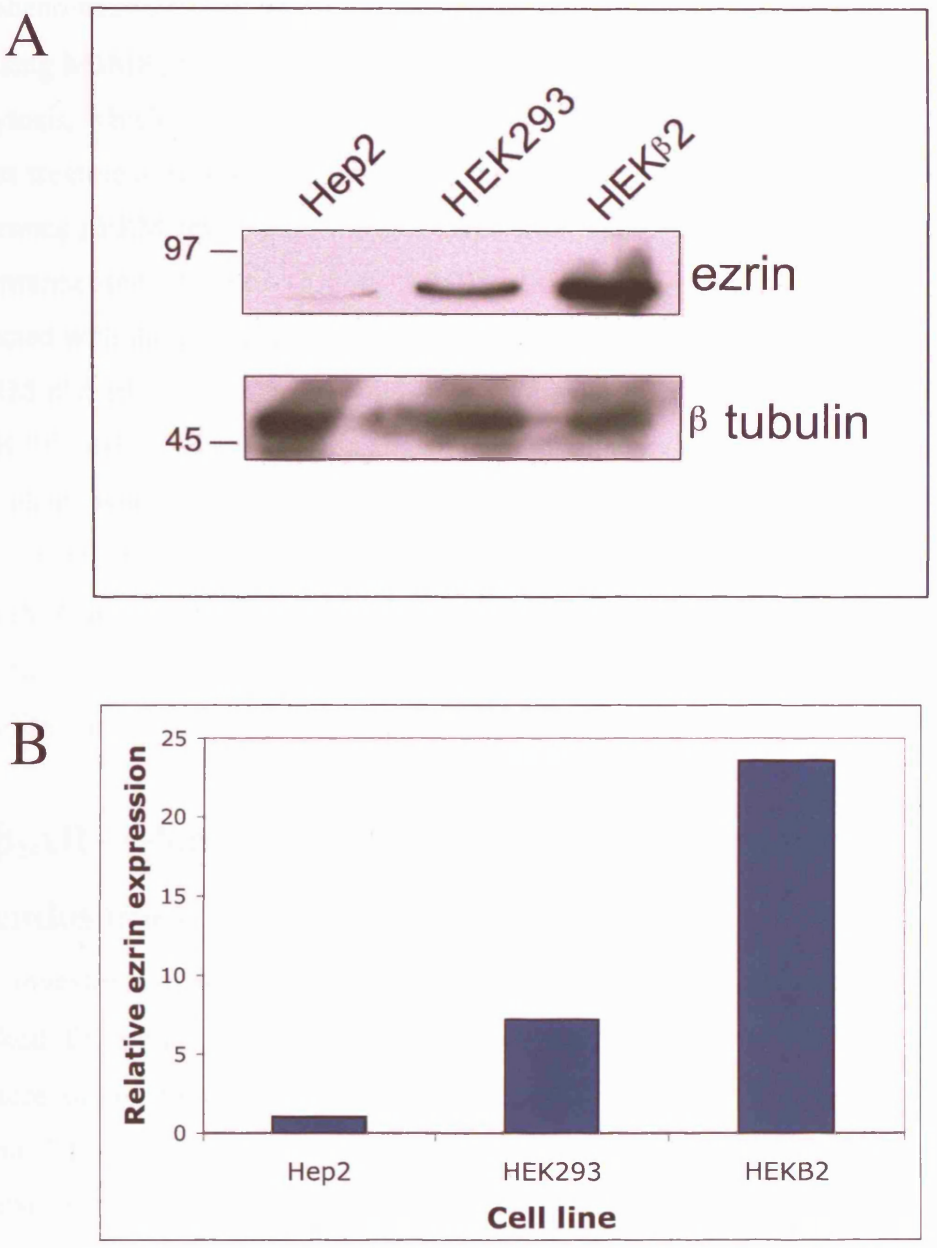

Figure 5.1. Ezrin expression in different cell lines used in this thesis. **A.** Total cellular proteins were extracted from confluent plates of Hep2, HEK293 and HEK293 cells stably expressing His_6-tagged β_2AR (HEKβ2). $1x10^6$ cells from each line were subject to SDS-PAGE and western blotting and the relative amount of ezrin detected using an anti-ezrin antibody (ezrin). The blots were stripped and re-probed with an anti-β-tubulin antibody to determine the relative amounts of total protein loaded onto the gels (β tubulin). Positions of protein molecular mass standards are indicated at the left of the images. **B**. The optical densities of the ezrin and tubulin bands on the developed films were determined using a densitometer. The relative levels of ezrin expression in each cell line were calculated by expressing the optical density of the ezrin band as a percentage of the optical density of the tubulin band and standardising relative to the amount of ezrin expressed in Hep2 cells (n=1).

This phenomenon is not restricted to β_2AR or HEK293 cells. In Hep2 cells expressing M1MR, prolonged acetylcholine treatment (20 min) results in receptor endocytosis, which occurred following transient membrane ruffling (Figure 5.2C). Agonist treatment of M1MR transfected Hep2 cells was associated with increased endogenous pERM levels and, in these cells also, active ERM proteins colocalise with internalised receptor (Figure 5.2C). In marked contrast, in Hep2 cells transfected with the HA-tagged M2 muscarinic receptor (M2MR), internalisation but not ERM phosphorylation is detected following agonist-stimulation (Figure 5.2D). In HEK293 cells M2MR has been shown to internalise in a GRK2- and β-arrestin-independent fashion (Claing et al., 2000; Pals-Rylaarsdam et al., 1995; Vogler et al., 1999). Since GRK activity is required for βarrestin recruitment, and both the β_2AR and M1MR are internalised via a βarrestin-dependent mechanism (Claing et al., 2002), these data suggest that GRK activity may be required for pERM and GPCR co-localisation at internalised vesicles.

5.3 β_2AR co-localises with the transferrin receptor in early endosomes

I then investigated the nature of the internalised vesicles to which β_2AR was trafficked following agonist activation. HEKβ2 cells were treated with (-)-isoproterenol for 15 minutes and cells were stained with antibodies against the endosomal marker early endosome antigen 1 (EEA1) (Mu et al., 1995) and the lysosomal marker lysosomal-associated membrane protein 1 (LAMP1) (Carlsson et al., 1988). After 15 minutes of agonist treatment β_2AR could be observed within internalised vesicles, the majority of which were co-labelled with EEA1 (Figure 5.3A). These vesicles do not contain the LAMP1 protein (Figure 5.3A).

HEKβ2 cells were then incubated with Alexa Fluor 647-conjugated transferrin (647-transferrin) for 30 minutes on ice, followed by an immediate incubation at 37°C for 30 minutes with both 647-transferrin and (-)-isoproterenol. The transferrin receptor is constitutively expressed in human cell lines and is trafficked to early endosomal compartments following transferrin binding (Dautry-Varsat, 1986). After 30 minutes of agonist treatment, the β_2AR co-localises with the transferrin receptor in internal vesicles that do not label with LAMP1 (Figure 5.3B). It therefore appears that the

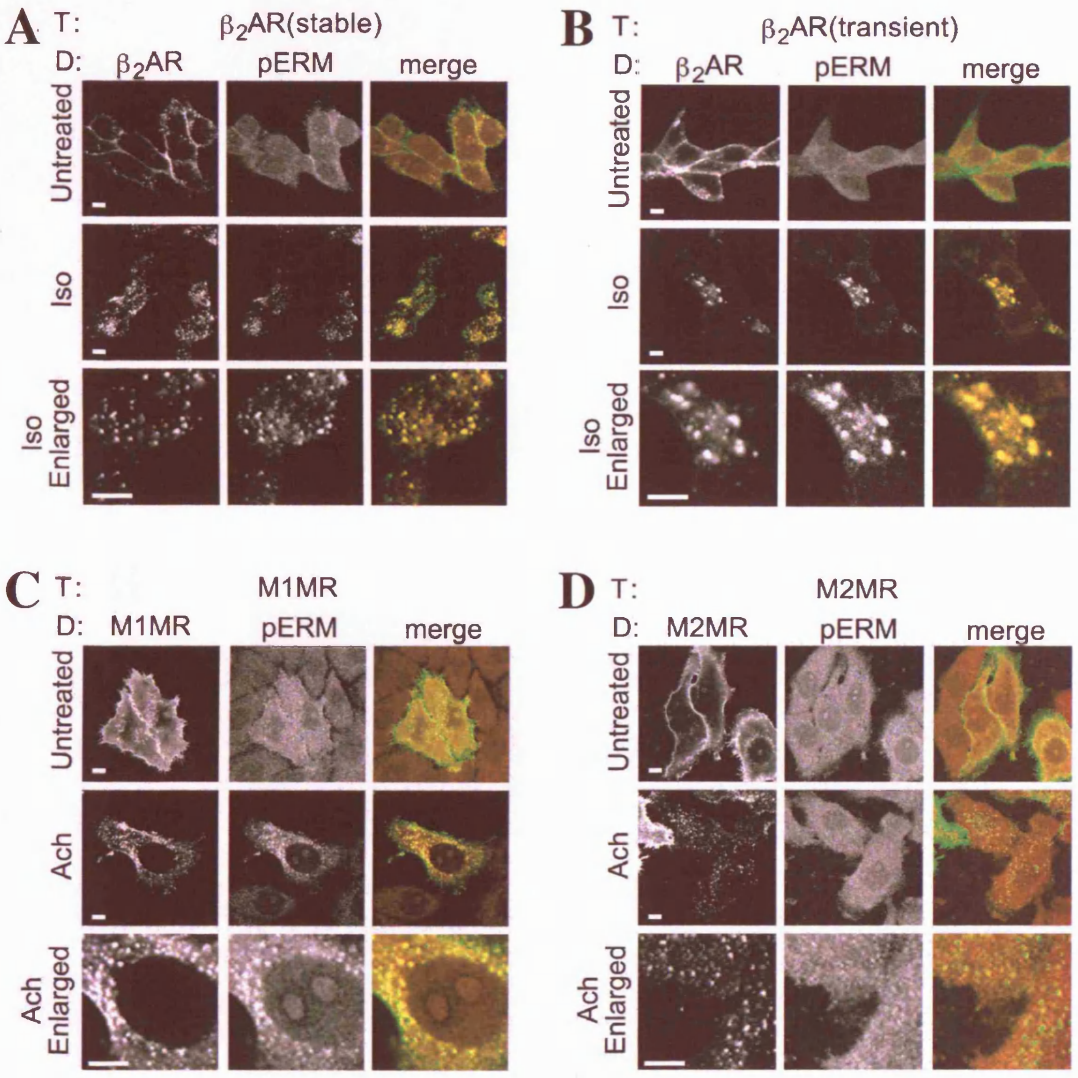

Figure 5.2. pERM proteins co-localise with internalised β_2AR and M1MR, but not M2MR. **A & B.** HEK293 cells stably expressing β_2AR (**A**) or transiently expressing β_2AR (**B**) were treated with 50μM isoproterenol (Iso) for 20 minutes. β_2AR and endogenous pERM were detected using immunofluorescence. **C & D.** Hep2 cells were transfected with M1MR (**B**) and M2MR (**C**) and incubated for 48 hours. The cells were then subject to 100μM acetylcholine for 20 minutes (Ach) and receptor and endogenous pERM visualised by immunofluorescence. 297S, a rat anti-pERM antibody was used for visualisation of endogenous pERM. T denotes cDNA constructs transfected. D denotes proteins detected. Scale bars represent 20μm.

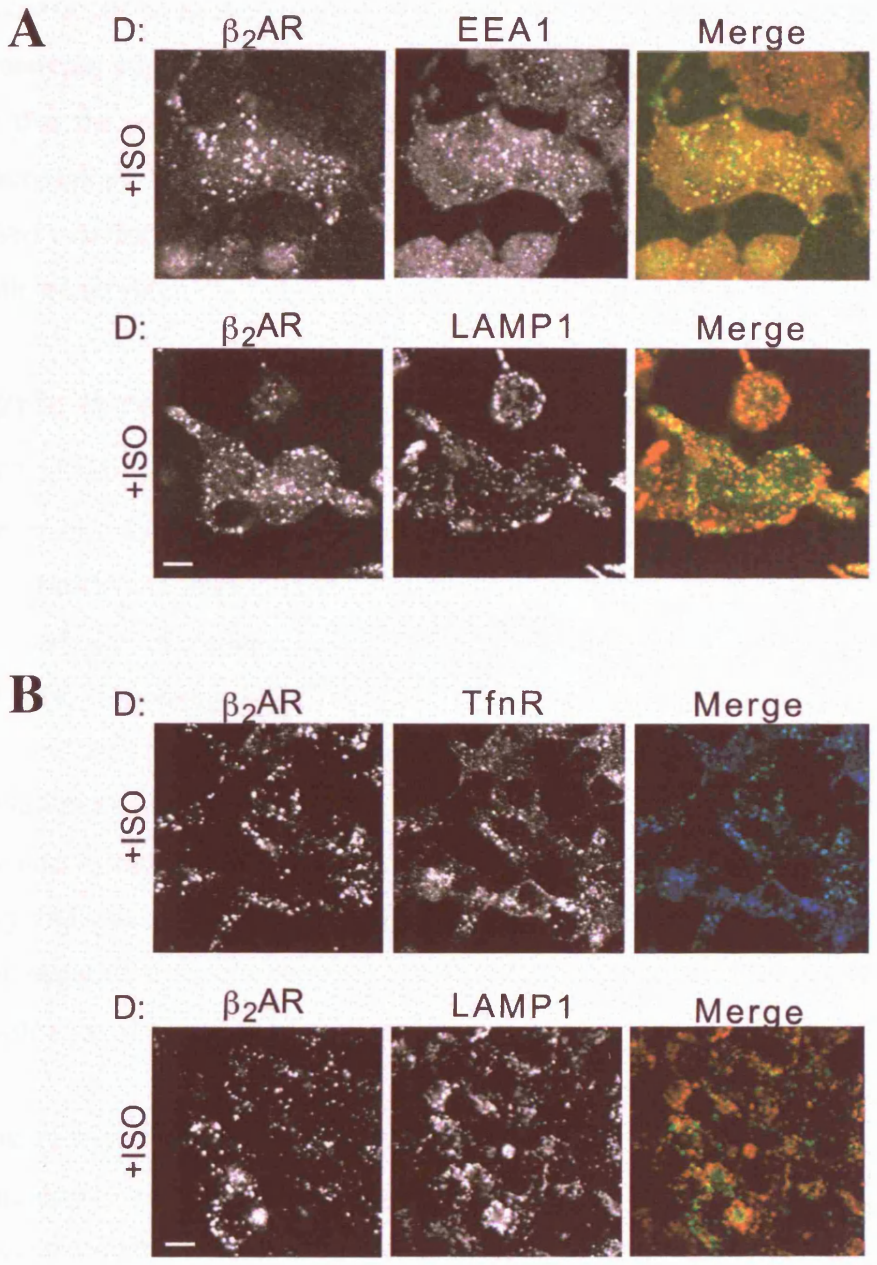

Figure 5.3. β_2AR co-localises with the transferrin receptor in early endosomes. **A.** HEKβ2 cells were treated with (-)-isoproterenol (ISO) for 15 minutes and then stained with antibodies against the endosomal marker EEA1 and the lysosomal marker LAMP1. β_2AR (green) could be observed within internalised vesicles, the majority of which were co-labelled with EEA1 (red), (colocalisation appears yellow). These vesicles do not contain the LAMP1 protein (red). **B.** HEKβ2 cells were incubated with Alexa Fluor 647-conjugated transferrin for 30 minutes on ice, followed by an immediate incubation at 37°C for 30 minutes with both 647-transferrin and ISO. The transferrin receptor is constitutively expressed in human cell lines and is trafficked to early endosomal compartments following transferrin binding (TfnR). After 30 minutes of agonist treatment, the β_2AR (green) co-localises with the transferrin receptor (blue) in internal vesicles (colocalisation appears cyan), but does not colocalise with LAMP1 (red). D denotes the proteins detected. Scale bars represent 20μm.

β_2AR is trafficked to EEA1-positive recycling endosomal compartments following (-)-isoproterenol stimulation. The absence of co-localisation of β_2AR and LAMP1 suggests that the receptor is not directed to lysosomes after up to 30 minutes of agonist activation. The co-localisation of pERM and the agonist-occupied β_2AR at internalised vesicles suggests that activated ezrin is trafficked to the early endosome along with the receptor.

5.4 Ezrin is required for β_2AR endocytosis

Since pERM co-localise with internalised β_2AR, I sought to examine whether activated ezrin plays a role in regulating the agonist-dependent trafficking of this receptor. Mock transfected HEKβ2 cells were subject to 30 minutes of agonist treatment followed immediately by 30 minutes of antagonist (alprenolol) treatment. Samples were removed prior to treatment (untreated), after agonist treatment (ISO (30 min)) and after agonist removal and subsequent antagonist treatment (ALP (30 min)). Plasma membrane-located β_2AR was labelled with an anti-His_6 monoclonal antibody and a fluorescently labelled secondary antibody and quantified by flow cytometry analysis. Results were compared to those of HEKβ2 cells expressing a dominant negative dynamin construct (dynamin K44A), the dominant negative N-ezrin construct or the carboxyl-terminal C-ezrin construct.

Following agonist treatment of mock transfected cells, levels of β_2AR at the plasma membrane decreased by approximately 32% (Figure 5.4, ISO (30min)). Removal of agonist and treatment with antagonist resulted in the delivery of both internalised and newly synthesised receptor to the plasma membrane. In cells transfected with dynamin K44A, which is known to inhibit endocytosis (van der Bliek et al., 1993), less than 5% of total β_2AR at the cell surface was internalised. Expression of N-ezrin inhibited β_2AR internalisation such that only 11% of cell surface receptor was endocytosed following isoproterenol treatment. Similarly, cells expressing C-ezrin internalised less than 20% of the surface β_2AR. Neither the mutant dynamin construct nor the ezrin deletion constructs affected delivery of β_2AR to the plasma membrane after removal of agonist.

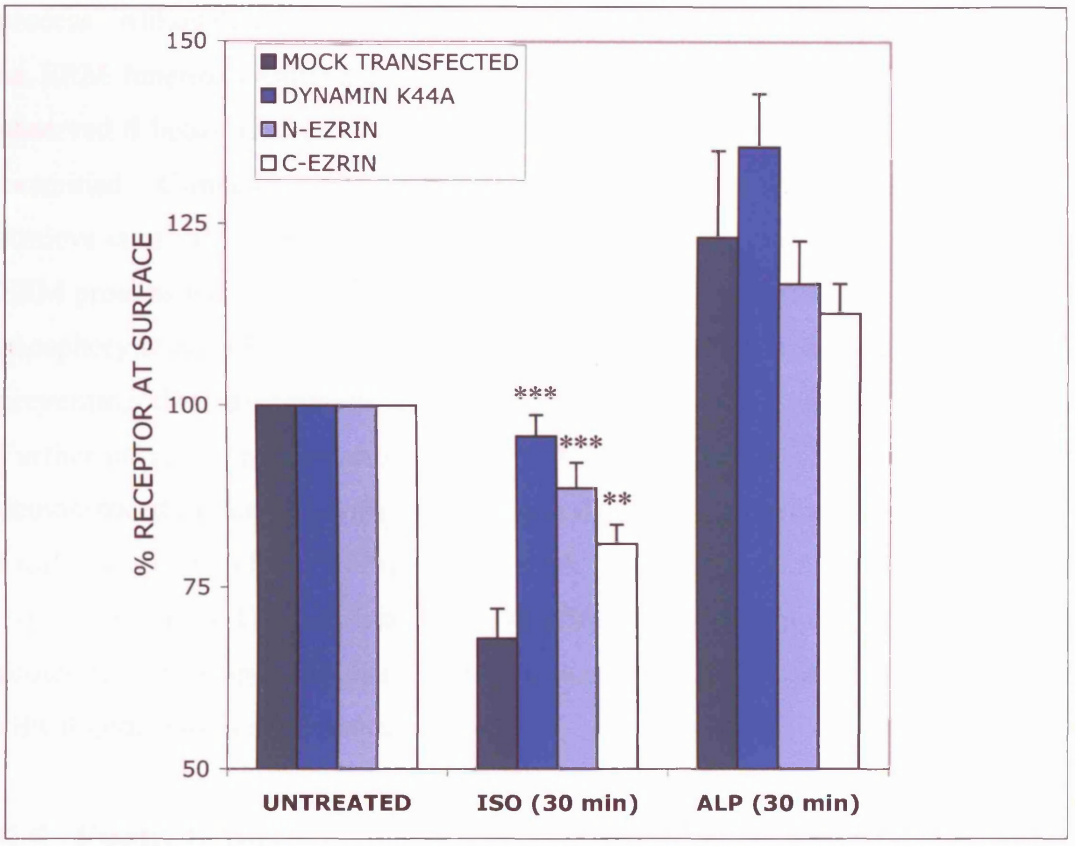

Figure 5.4. Ezrin is required for β_2AR endocytosis. Fluorescence flow cytometry was used to estimate internalisation of β_2AR in HEK293 cells stably expressing the His6-tagged receptor (HEKβ2 cells). Cells were incubated with 40μM isoproterenol for 30 min, washed, and then incubated with 100μM alprenolol for 30 minutes to stop further endocytosis. Receptors present in the plasma membrane prior to treatment (untreated), after isoproterenol treatment (Iso (30min)) and after alprenolol treatment (Alp (30min)) were labelled with an anti-His6 monoclonal antibody. Following fixation, the primary antibody was detected with a fluorophore-conjugated secondary antibody and quantified by flow cytometry. This was repeated for HEKβ2 cells overexpressing a dominant negative dynamin construct (dynamin K44A), the FERM domain of ezrin (N-ezrin) or the carboxyl-terminus of ezrin (C-ezrin). Data indicate the mean surface receptor fluorescence of cells. Error bars represent S.D. of mean fluorescence data collected from multiple samples of a representative experiment (n=4). *** indicates $p<0.001$. ** indicates $0.001 \leq p < 0.01$.

It appears that not only does phosphorylated, active ezrin colocalise with internalised β_2AR at early endosomes, it is also required for agonist-induced β_2AR endocytosis. Expression of the FERM domain of ezrin has been shown to inhibit ERM function in a number of model systems and is thought to compete with endogenous ERM proteins for plasma membrane binding sites (Algrain et al., 1993; Amieva et al., 1999; Crepaldi et al., 1997; Elliott et al., 2005). My results are consistent with this hypothesis, as expression of N-ezrin inhibits what I believe to be an ezrin-dependent

process. Although over-expression of C-moesin has been observed to have no effect on ERM function (Amieva et al., 1999), transfected cells in this study were only observed 6 hours after electroporation and just the effect on cell morphology was examined. C-moesin and C-ezrin bind to actin filaments (Algrain et al., 1993; Amieva et al., 1999) and over-expression may inhibit the interaction of endogenous ERM proteins with actin, compete with endogenous ERM proteins for threonine-567 phosphorylation or bind to the amino-termini of endogenous activated ERM proteins, preventing their association with the plasma membranes and binding partners. Furthermore, over-expression of the carboxyl-terminus of radixin has been demonstrated to interfere with cytokinesis at the cleavage furrow, to which ERM are localised (Henry et al., 1995). More work is required to investigate how over-expression of the ERM carboxyl-termini affects the function of endogenous ERM proteins, as it is apparent that C-ezrin has an inhibitory effect on ezrin-dependent GPCR endocytosis in my model system.

5.5 Ezrin is not required for endocytosis of the transferrin receptor

If ezrin is required for clathrin-mediated endocytosis *per se*, expression of ezrin deletion- and point-mutants would be anticipated to inhibit the internalisation of the transferrin receptor, which is known to internalise via CCPs (Dautry-Varsat, 1986). To investigate this hypothesis, I transfected HEKβ2 cells with WT-ezrin, N-ezrin or C-ezrin. The cells were incubated with 647-transferrin for 30 minutes on ice and then immediately incubated at 37°C for 30 minutes in the presence of 647-transferrin. Following fixation and permeabilisation, the cells were labelled with an anti-VSVG antibody to detect expression of the ezrin constructs. Immunofluorescence data suggests that expression of dominant negative ezrin or the carboxyl-terminal domain of ezrin has no observable effects on transferrin receptor internalisation when compared to cells over-expressing wildtype ezrin (Figure 5.5).

These results indicate that ezrin is required for the endocytosis of a subset of GPCRs, rather than for clathrin-mediated internalisation in general. Quantifying the extent of the down-modulation of transferrin receptors in cells expressing the mutant ezrin constructs would have more robustly demonstrated the specificity of ezrin for GPCR

endocytosis. However, it was impractical to use flow cytometry to analyse transferrin receptor internalisation since it was not possible to label surface transferrin receptors using 647-transferrin, which causes immediate endocytosis of the receptors on binding, and anti-transferrin receptor antibodies did not label the receptor efficiently.

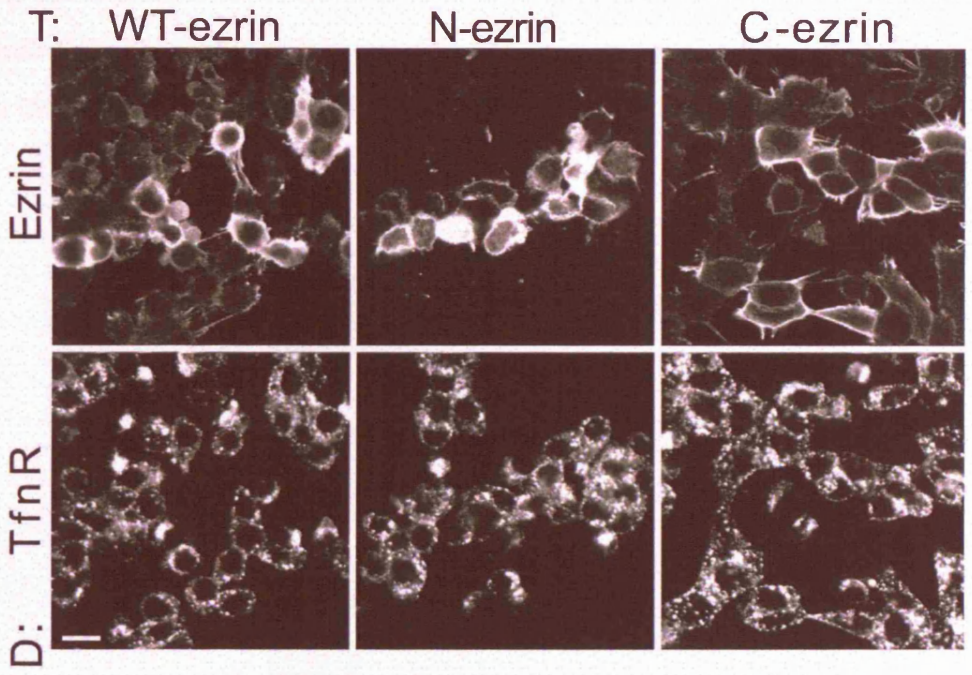

Figure 5.5. Ezrin is not required for endocytosis of the transferrin receptor. HEK293 cells were transfected with either wildtype ezrin (WT-ezrin), the FERM domain of ezrin (N-ezrin) or the carboxyl-terminus of ezrin (C-ezrin). The cells were incubated with 647-transferrin for 30 minutes on ice and then at 37°C for 30 minutes. Following fixation and permeabilisation, transferrin receptor (TfnR) and expressed ezrin constructs (Ezrin) were visualised by immunofluorescence. Expression of N-ezrin or C-ezrin have no observable effects on transferrin receptor internalisation when compared to cells over-expressing WT-ezrin. T denotes the cDNA constructs transfected. D denotes the proteins detected. Scale bar represents 20μm.

5.6 Co-localisation of pERM with β_2AR does not require NHERF

Previous studies have shown that the Na^+/H^+ exchanger regulatory factor (NHERF) can bind to the carboxyl-terminus of the β_2AR via its PDZ domain (Hall et al., 1998a), and to the FERM domain of ERM proteins (Reczek et al., 1997; Reczek and

Bretscher, 1998). I investigated whether NHERF was responsible for the co-localisation of pERM with internalised β_2AR. HEK293 cells were transiently transfected with either FLAG-tagged wildtype β_2AR or a mutant FLAG-tagged β_2AR that cannot bind NHERF (β_2AR_{L413A}). Cells were subject to 15 minutes of isoproterenol treatment, fixed, permeabilised and stained for receptor and endogenous pERM with 297S. As in Figure 5.2B, activation of transiently expressed β_2AR led to receptor internalisation and colocalisation with activated ERM proteins (Figure 5.6). Similarly, pERM could be seen co-localised with β_2AR_{L413A} at internal vesicles, indicating that NHERF is not required for this interaction.

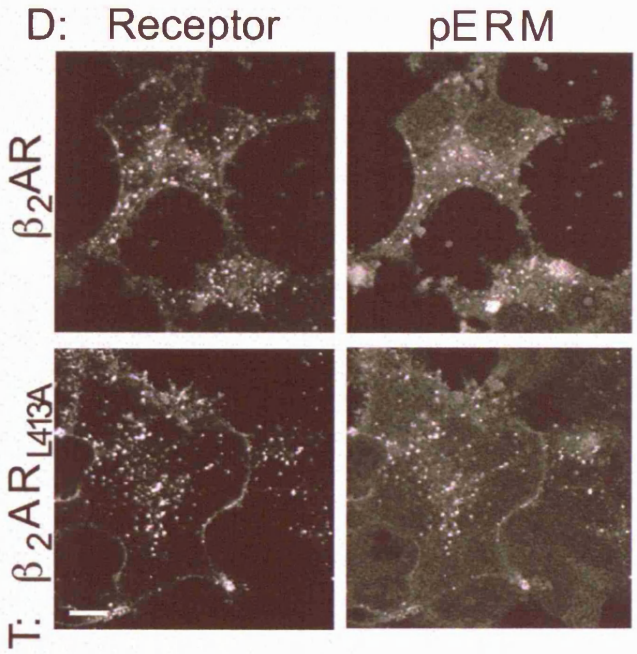

Figure 5.6. NHERF is not required to link pERM to β_2AR. HEK293 cells were transfected with either the wildtype β_2AR or a mutant β_2AR that cannot bind NHERF (β_2AR_{L413A}). Cells were subject to 15 minutes of isoproterenol treatment, fixed and permeabilised. Receptor and endogenous pERM were visualised by immunofluorescence. T denotes the cDNA constructs transfected. D denotes the proteins detected. Scale bar represents 20μm.

5.7 Ezrin co-immunoprecipitates with GRK2

The results in this and the previous Chapter show that ezrin is in close proximity to agonist-occupied GPCRs at the plasma membrane immediately after receptor stimulation and then within endocytic vesicles. I have demonstrated that NHERF is

not required for the co-localisation of ezrin with the β_2AR, however ezrin may be conveyed to GPCRs via interactions with other proteins, such as components of the desensitisation machinery. Since the results in Chapter 3 suggest that ezrin may interact with GRK2, I sought to investigate whether ezrin can associate with GRK2.

COS cells were transfected with either GRK2, GRK2 and a VSVG-tagged human ezrin mutant construct in which threonine-567 had been substituted with alanine (T567A-ezrin) or GRK2 and a VSVG-tagged human ezrin mutant construct in which threonine-567 had been substituted with aspartate (T567D-ezrin). T567D-ezrin has been shown to mimic ezrin phosphorylated at threonine-567, whereas T567A-ezrin is a non-phosphorylatable mutant (Gautreau et al., 2000). 48 hours after transfection, GRK2 was immunoprecipitated and subject to SDS-PAGE and Western blotting. Any ezrin bound to the GRK2 was detected using an anti-ezrin antibody. Faint bands were observed in the immunoprecipitated lanes that were not present in the control lanes, indicating that GRK2 can associate with both T567A- and T567D-ezrin (Figure 5.7A). Samples from the same lysates were subject to immunoprecipitation of ezrin and any bound GRK2 was detected. Again, a small amount of both over-expressed T567A- and T567D-ezrin could be detected bound to GRK2, but not to sepharose alone (Figure 5.7B).

The *in vitro* ROS phosphorylation assays described in Chapter 3 suggest that ezrin can interact with GRK2. The results communicated here are preliminary evidence that suggests that ezrin may associate with GRK2 in a manner that does not appear to depend on either the phosphorylation (i.e. activation) state of ezrin or agonist activation of a GPCR. Further investigation, such as *in vitro* binding assays, is required in order to characterise this putative interaction and to ensure that it is not an artefact of immunoprecipitation. GRK2 has been demonstrated to colocalise with internalised β_2AR at endosomal vesicles (Ruiz-Gomez and Mayor Jr., 1997). If robustly proven to interact with ERM proteins, GRK2 could act to link ezrin to a GPCR following endocytosis, which would account for the colocalisation of ezrin and β_2AR on internalised vesicles. Although this hypothesis is consistent with the data from the *in vitro* phosphorylation assays, where GRK2 was demonstrated to phosphorylate full-length ezrin at PIP_2-containing vesicles, I have not proved that GRK2 colocalises with internalised β_2AR and pERM in this experimental system.

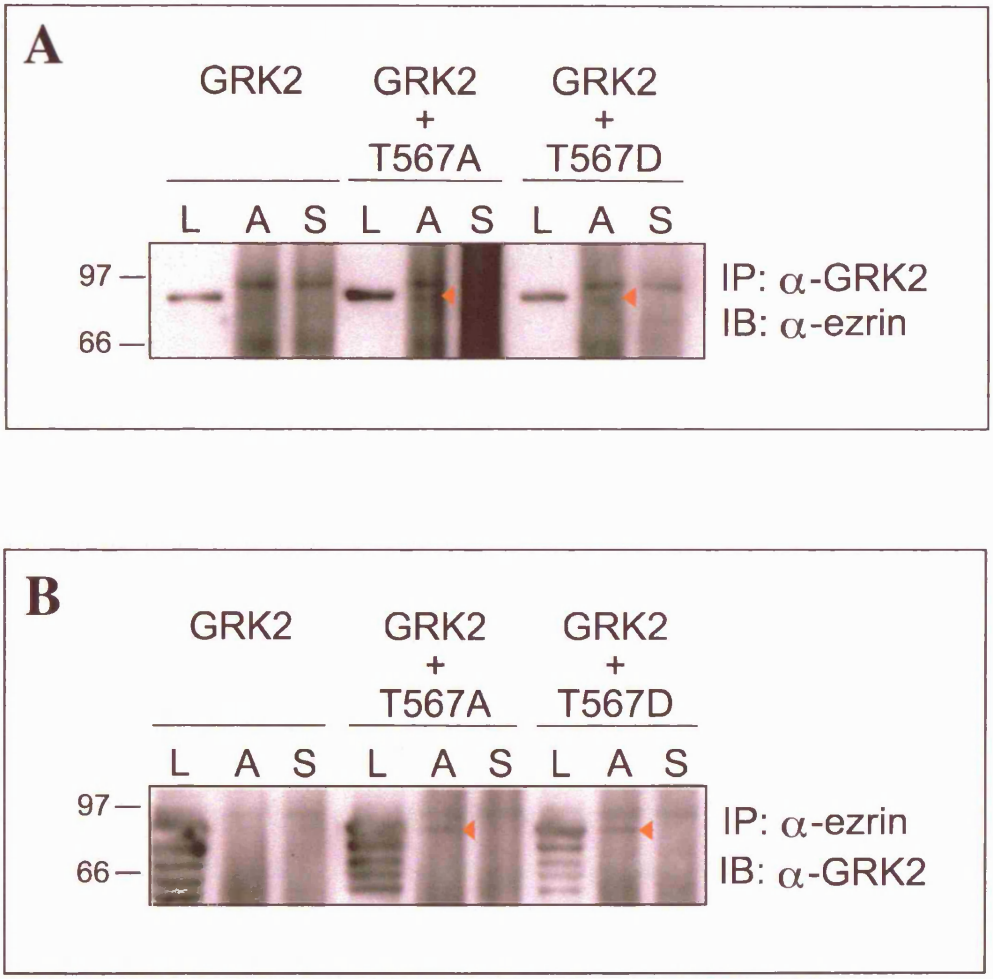

Figure 5.7. Ezrin interacts with GRK2. COS cells were transfected with either GRK2, GRK2 and T567A-ezrin (GRK2 + T567A), or GRK2 and T567D-ezrin (GRK2 + T567D). **A.** 48 hours post-transfection, GRK2 was immunoprecipitated (IP) and subject to SDS-PAGE and Western blotting. Any ezrin bound to the GRK2 was detected using an anti-ezrin antibody (IB). GRK2 was found to be associated with both T567A- and T567D-ezrin (red arrow heads). **B.** Samples from the same lysates were subject to immunoprecipitation (IP) of ezrin and any bound GRK2 was detected (IB). Again, both over-expressed T567A- and T567D-ezrin could be detected bound to GRK2 (red arrow heads). Positions of protein molecular mass standards are indicated at the left of the images. L: total cell lysate. A: immunoprecipitation with antibody and sepharose beads. S: immunoprecipitation with sepharose beads only.

5.8 Summary

In summary, this Chapter demonstrates that ezrin may be required for GPCR internalisation, although this role may be limited to a subset of these receptors. Following agonist treatment, active, phosphorylated ERM proteins co-localise with both the β_2AR and the M1MR at internalised vesicles that appear to be early endosomes. This co-localisation may depend on the nature of the endocytic mechanism employed by a given GPCR: pERM are not seen with the internalised M2MR, which has been shown to endocytose via a GRK- and βarrestin-independent mechanism (Claing et al., 2002).

Ezrin is required for β_2AR endocytosis, but expression of either the dominant negative FERM domain or the carboxyl-terminal domain of ezrin had no observable effect on the endocytosis of the transferrin receptor, suggesting that it is not required for clathrin-mediated endocytosis *per se*. Although NHERF has previously been shown to bind both β_2AR and ERM proteins and to have a role in β_2AR recycling, it does not appear to be necessary for the interaction of pERM with the β_2AR following internalisation. Interestingly, my preliminary data in this Chapter suggests that ezrin may associate with GRK2. If these findings are verified through further investigation, they would support the data from Chapter 3 that indicate that ezrin and GRK2 may interact, since increasing concentrations of ezrin augments GRK2-mediated rhodopsin phosphorylation, possibly by enhancing the membrane localisation of GRK2 in the absence of Gβγ.

It could therefore be speculated that GRK2 might associate with ERM proteins, which may promote the localisation of GRK2 to the plasma membrane. Once at the plasma membrane, agonist-occupation of GPCRs would activate GRK2 and lead to the phosphorylation of ERM proteins. Such a mechanism would explain the specific co-localisation of pERM with activated GPCRs that require GRK for endocytosis, such as β_2AR and M1MR, but not M2MR.

Although no studies have yet shown that GRK2 and M1MR colocalise following internalisation, GRK2 has been shown to colocalise with δ-opioid receptors following endocytosis (Schulz et al., 2002). Should future research indicate that

ezrin can interact with GRK2, it would also be interesting to investigate whether pERM colocalise with internalised δ-opioid receptors, which have been demonstrated to internalise via a GRK-dependent mechanism (Schulz et al., 2002). However, further enquiry is also needed to determine whether pERM associate with other binding partners in order to co-localise with GPCRs at early endosomes, or if ezrin can directly associate with a subset of GPCRs. Overall, the results presented in this Chapter indicate that ERM proteins may act as important connectors between some GPCRs and the actin cytoskeleton during receptor-mediated endocytosis.

6 Discussion

In this thesis, I have identified ezrin as a novel substrate of GRK2 and as a downstream effector of GPCR signalling. My data increase the list of known non-receptor GRK2 substrates, which already includes tubulin, phosducin, ribosomal protein P2, synucleins, the inhibitory gamma subunit of the type 6 retinal cGMP phosphodiesterase and the β-subunit of the epithelial Na^+ channel (Carman et al., 1998; Dinudom et al., 2004; Wan et al., 2001; Freeman et al., 2002; Haga et al., 1998; Pitcher et al., 1998a; Pronin et al., 2000; Ruiz-Gomez et al., 2000). These findings also corroborate the hypothesis that GRK2 may have additional functions within the cell, besides phosphorylation of agonist-occupied GPCRs.

6.1 Ezrin is a novel substrate of GRK2

The ability of GRK2 to phosphorylate ezrin was initially discovered by Dr. Julie Pitcher using a cellular assay. I subsequently confirmed this observation *in vitro*, as detailed in Chapter 3. Notably, GRK2-mediated phosphorylation of full-length ezrin is PIP_2 and Gβγ-dependent. This is consistent with previous findings that show PIP_2-mediated changes in ezrin conformation may facilitate phosphorylation at threonine-567 (Fievet et al., 2004; Nakamura et al., 1999). The PIP_2- and Gβγ-dependence of GRK2-mediated phosphorylation additionally suggests that this phosphorylation event might occur in an agonist- and GPCR-dependent fashion.

Using GST-ezrin fusion proteins, the GRK2 phosphorylation site within ezrin was localised to a carboxyl-terminal region of 53 amino acids (residues 533 to 586) that encompassed threonine-567. The phosphorylation of threonine-567 has been shown to be important for ezrin activation and is thought to disrupt its inactive conformation such that the plasma membrane and actin binding domains are exposed (Pearson et al., 2000). However, a number of other serine and threonine residues are also contained within this region (Figure 3.1) and further analysis was necessary to identify the exact phosphorylation site.

In order to determine if indeed threonine-567 represented the site of GRK2-mediated ezrin phosphorylation, I created a GST-ezrin fusion protein in which threonine-567

was mutated to alanine (GST-T567A ezrin). Although I did not have the opportunity to utilise this fusion protein in *in vitro* phosphorylation assays during the course of my thesis, Dr. Julie Pitcher has recently demonstrated that this construct cannot be phosphorylated by GRK2 in either the presence or absence of PIP_2 and $G\beta\gamma$. This finding directly confirms what the membrane ruffling data in Chapter 4 of this thesis implies: that threonine-567 represents the principle site of GRK2-mediated ezrin phosphorylation. This result, along with much of the work contained in this thesis, has recently been published (Cant and Pitcher, 2005).

6.2 GRK2 mediates cytoskeletal reorganisation downstream of GPCRs via phosphorylation of ERM proteins

6.2.1.1 ERM proteins are required for M1MR-mediated ruffling in Hep2 cells

The data presented in Chapters 4 and 5 suggest that GRK2 activity in a cellular context leads to ERM protein activation. Stimulation of Hep2 cells overexpressing M1MR and of HEK293 cells stably and transiently expressing β_2AR leads to an increase in endogenous ERM proteins specifically phosphorylated at threonine-567 (ezrin), -564 (radixin) or -558 (moesin). In the Hep2 cell system, brief exposure to agonist causes rapid and transient membrane ruffling that requires both active ezrin and GRK2. These observations are similar to those seen following stimulation of the adenosine 2b receptor, which colocalises with ezrin on agonist occupancy (Sitaraman et al., 2002).

Cytoskeletal reorganization and the association of ezrin with plasma membrane and actin filaments have previously been found to be regulated by the Rho family of small GTPases (Aspenstrom et al., 2004; Nakamura et al., 2000; Okamoto et al., 1999) and to involve phosphorylation of ezrin by either Rho kinase or PKC, depending on the cellular context (Matsui et al., 1998; Simons et al., 1998). PKA-mediated ezrin phosphorylation has also been shown to be important for the cytoskeletal remodelling associated with acid secretion in gastric parietal cells (Zhou et al., 2003). In this thesis I have shown that in Hep2 cells expressing M1MR, acetylcholine treatment leads to membrane ruffling and ERM protein activation even

in the presence of the Rho kinase inhibitor Y27632, the PKC inhibitor GF109203X or the PKA inhibitor Rp-8-Br-CAMPS. That GRK2, but not Rho kinase, PKC or PKA, inhibition ablates M1MR-mediated ERM protein phosphorylation and ruffling suggests that GRK2 is responsible for the activation of ERM proteins and consequent actin filament/cell surface remodelling observed in this system.

6.2.1.2 Factors influencing GPCR-mediated membrane ruffling

6.2.1.2.1 Gα subunits

Stimulation of M1MR expressed in Hep2 cells leads to GRK2-mediated phosphorylation of ERM proteins and membrane ruffling. Interestingly, although agonist stimulation of β_2AR expressed in HEK293 cells also leads to ERM protein activation, it does not cause membrane ruffling. It would therefore appear that the GRK2-mediated activation of ERM proteins via M1MR and β_2AR results in different functional outcomes.

The differing cellular responses brought about by ERM activation following agonist occupancy of M1MR in Hep2 cells and β_2AR in HEK293 cells could reflect the different complement of proteins expressed by each cell line. However, stimulation of β_2AR expressed in Hep2 cells does not lead to membrane ruffling, even though an increase in ERM protein phosphorylation is observed (Dr. Julie Pitcher, unpublished observations). This implies that ERM proteins are involved in different signalling pathways downstream of M1MR and β_2AR and that GRK2-mediated ERM phosphorylation, although required, is not sufficient for membrane ruffling.

That GRK2-mediated membrane ruffling in Hep2 cells is M1MR-dependent but not β_2AR-dependent could be a reflection of the Gα subtype associated with the receptors. M1MR is coupled to Gq, whereas β_2AR is coupled to Gs and Gi (Daaka et al., 1997a; Offermanns et al., 1994; Zamah et al., 2002). GRK2 binds Gq via its RGS domain and inhibits the Gq-mediated stimulation of PIP_2 hydrolysis by PLCβ (Carman et al., 1999a; Sallese et al., 2000b). A localised increase in PIP_2 concentration would not only augment ERM protein activation by GRK2 (Fievet et al., 2004), but could also increase actin polymerisation at the plasma membrane (Ren and Schwartz, 1998). Gq-coupled receptors such as M1MR, AT_{2A}R and the TPβ

receptor have been shown to induce actin reorganisation (Barnes et al., 2005; Dutt et al., 2002; Laroche et al., 2005), and bombesin signalling via Gq leads to Arf6 and Rac-dependent membrane ruffling in CHO cells (Boshans et al., 2000).

The activation of Gq-coupled receptors would therefore be expected to bring about membrane ruffling, regardless of the cell line the receptors are expressed in. However, stimulation of HEK293 cells expressing M1MR does not lead to ruffling, although ERM protein phosphorylation occurs (Dr. Julie Pitcher, unpublished observations). These observations indicate that the functional outcome of GRK2-mediated ERM protein phosphorylation downstream of GPCRs may not only depend on the Gα subunit coupled to the receptor, but is likely to be cell line-specific as well.

6.2.1.2.2 GRKs

GPCRs including CCR2 (Jones et al., 2003) and CXCR4 (D'Apuzzo et al., 1997) induce cytoskeletal reorganisation in the form of membrane ruffling and cell migration on agonist activation. Both CCR2 and CXCR4 have been shown to undergo GRK2-dependent endocytosis following ligand binding (Aragay et al., 1998; Orsini et al., 1999). In addition, studies have demonstrated that neutrophils and splenocytes derived from GRK6-deficient mice have altered chemotactic responses (Fong et al., 2002; Kavelaars et al., 2003; Vroon et al., 2004), indicating that GRK6 may be involved in regulating the cytoskeletal remodelling required for their migration. In conjunction with the results in this thesis, these data suggest that GRKs may have a general role in regulation of the actin cytoskeleton in processes such as cell motility. It would be therefore be interesting to investigate whether ezrin or other ERM proteins serve as substrates for other GRK family members. The identification of ERM proteins as general GRK substrates could indicate a common pathway for actin filament modulation downstream of activated GPCRs.

6.2.2 The actin cytoskeleton and GPCR endocytosis

Many studies have demonstrated that the actin cytoskeleton has a role in clathrin-mediated endocytosis, as discussed in Chapter 1. Studies with actin perturbing drugs suggest that, while actin may not be absolutely essential for the clathrin-mediated internalisation of receptors, it probably facilitates and regulates the process

(Fujimoto et al., 2000). More recent research has indicated that mammalian cells may have alternative actin- and clathrin-independent endocytosis pathways through which receptors can internalise following actin disruption (Yarar et al., 2005).

Drug-induced disruption of the actin cytoskeleton has demonstrated that actin polymerisation is involved in the internalisation of the bombesin, endothelin A and TPβ receptors (Laroche et al., 2005; Lunn et al., 2000). However, the inhibition of clathrin-mediated TPβ receptor endocytosis can be overcome by over-expression of βarrestin2. Over-expression of βarrestin1 does not support TPβ internalisation following actin disruption, even though both βarrestin1 and –2 mediate endocytosis of the receptor in the presence of an intact actin cytoskeleton (Laroche et al., 2005). This suggests that both actin-dependent and –independent mechanisms of clathrin-mediated GPCR sequestration exist.

6.2.3 Ezrin is required for clathrin-mediated endocytosis of a subset of GPCRs

In this thesis I demonstrate that in HEK293 cells stably expressing β_2AR and Hep2 cells expressing M1MR, prolonged agonist stimulation causes receptor endocytosis paralleled by the colocalisation of pERM with receptor at endosomal vesicles. Flow cytometry analysis to quantify surface β_2AR following isoproterenol treatment demonstrates that expression of either the dominant negative N-ezrin or the carboxyl-terminal C-ezrin fusion proteins inhibit receptor downmodulation to a similar extent as expression of the dominant negative dynamin K44A protein. Ezrin does not appear to be necessary for clathrin-mediated endocytosis *per se* since neither N-ezrin nor C-ezrin inhibited transferrin receptor internalisation in immunofluorescence assays.

Recently, the association of the low-density lipoprotein (LDL) receptor with ezrin has been shown to be necessary for its clathrin-mediated endocytosis (Smith et al., 2004). pERM colocalise with LDL particles at the plasma membrane and, although active ERM proteins do not associate with internalised LDL, expression of N-ezrin significantly inhibits LDL uptake (Smith et al., 2004). This suggests that other, non-

G protein-coupled, receptors may also utilise a similar ezrin- and clathrin-dependent endocytosis pathway.

Although my data suggest that ezrin may also have an important function in GPCR internalisation, this role may be limited to receptors that internalise in a GRK2- and βarrestin-dependent manner. M2MR endocytosis is not correlated with ERM protein activation and pERM are not associated with internalised M2 receptor. In HEK293 cells M2MR has been shown to undergo endocytosis in a βarrestin-independent fashion and does not appear to internalise via typical clathrin- or caveolin-dependent pathways in HeLa or HEK293 (Claing et al., 2000; Delaney et al., 2002; Pals-Rylaarsdam et al., 1995; Roseberry and Hosey, 2001; Vogler et al., 1999). In contrast, both β_2AR and M1MR are sequestered via a βarrestin- and therefore GRK-dependent pathway in these cells (Claing et al., 2000). The routes of M1MR and M2MR internalisation in this Hep2 cell system have yet to be confirmed as being dependent and independent of βarrestin/GRK respectively, however my results indicate that activated ezrin probably plays an obligate role in the internalisation of a subset of GPCRs.

Much is known about GPCR internalisation and the different mechanisms involved. Rapid sequestration of receptors such as β_2AR is characterised by GRK phosphorylation leading to βarrestin binding and endocytosis via clathrin-coated pits; other GPCRs may internalise via caveolae or even in a GRK-independent manner (Chini and Parenti, 2004; von Zastrow, 2003). My results suggest that a requirement for activated ERM proteins may be an additional distinguishing feature of these endocytic pathways and may correlate with GRK dependence. In order to further identify and characterise the GPCRs that internalise via an ERM protein-dependent route, endocytosis assays would need to be performed in cells that do not express ERM proteins. Ablation of ERM protein expression could be achieved either via an RNAi-based approach, or by using cells derived from ezrin, radixin or moesin knockout mice (Doi et al., 1999; Kikuchi et al., 2002; Saotome et al., 2004; Tamura et al., 2005). Since ERM proteins appear to be functionally redundant in most cell types (Doi et al., 1999; Kikuchi et al., 2002; Saotome et al., 2004; Tamura et al., 2005), it is likely that the contribution of ERM proteins to GPCR endocytosis can only be examined in cells that do not express any of the three ERM family members.

6.2.4 Ezrin colocalises with agonist-occupied GPCRs

6.2.4.1 Potential ezrin binding partners

Further investigation is required to establish how ezrin associates with GPCRs following agonist occupation. The ERM-binding protein NHERF is known to bind to the carboxy-termini of certain receptors, including the β_2AR, and has been shown to be involved in sorting β_2AR between the recycling endosome and lysosome following endocytosis (Cao et al., 1999). NHERF has also been shown to regulate the trafficking of the human κ opioid receptor and the parathyroid hormone receptor (Li et al., 2002; Sneddon et al., 2003). Additionally, the NHERF homologue E3KARP forms a complex with ezrin and the adenosine 2b receptor following stimulation of T84 cells with adenosine (Sitaraman et al., 2002).

My data indicate that NHERF is not required to link ERM proteins to a receptor following stimulation since pERM colocalise with both a mutant β_2AR that is unable to bind NHERF, and to M1MR, which does not contain the carboxyl-terminal motif recognised by NHERF (Hall et al., 1998b). However, it is possible that, like the integral membrane glycoprotein podocalyxin, β_2AR may be able to bind ERM proteins both directly and via NHERF (Schmieder et al., 2004). Alternatively other, as yet unidentified, PDZ domain-containing proteins could link ERM proteins to GPCRs. PDZ ligands essential for internalisation have been identified within the carboxyl tails of a number of GPCRs, although the PDZ domain proteins that bind them have not been characterised (Gage et al., 2005; Paasche et al., 2005). Further studies will be necessary to identify such proteins and investigate whether they act as ERM protein binding partners.

pERM localisation could be dependent on other vesicle-associated proteins. For example, a recent study demonstrated that Arf6 colocalises with ezrin at the plasma membrane (Macia et al., 2004). Arf6 is thought to regulate vesicle formation in endocytosis and interacts with βarrestin, suggesting that βarrestin may be responsible for the recruitment of Arf6 (Claing et al., 2001), and hence possibly ERM proteins, to agonist-occupied GPCRs.

6.2.4.2 Ezrin may interact with GRK2

Although I have not investigated whether βarrestin or Arf6 associate with ERM proteins, my data suggests that ezrin may interact with GRK2. As demonstrated in Chapter 3, increasing the concentration of full-length ezrin fusion protein in a GRK2 phosphorylation assay augments GRK2-mediated phosphorylation of rhodopsin in ROS. This result suggests that ezrin, in addition to serving as a GRK2 substrate, can interact with GRK2. The immunoprecipitation data from Chapter 5 appear to show that GRK2 can associate with ezrin, although these results are preliminary and further investigation is required to confirm this.

It could thus be speculated that ERM proteins might promote the translocation of GRK2 to the plasma membrane via their interaction with PIP_2. At the plasma membrane, GRK2 would be targeted to agonist-occupied GPCRs via binding with Gβγ and/or Gq, leading to GRK2 activation and ERM phosphorylation. As GRK2 has been demonstrated to remain associated with internalised β_2AR and δ-opioid receptors (Ruiz-Gomez and Mayor Jr., 1997; Schulz et al., 2002), GRK2 could be suggested to act as the link between pERM and an internalised GPCR at the early endosome.

Should further studies, involving for example *in vitro* binding assays, indicate that GRK2 and ezrin can associate, it would also be interesting to map the sites of interaction for both proteins. Although the binding sites of kinases such as PKC and Rho kinase have not yet been determined, ezrin has been shown to interact with a number of membrane-associated and cytosolic proteins via its FERM domain (Finnerty et al., 2004; Hamada et al., 2003; Hirao et al., 1996; Manchanda et al., 2005; Takahashi et al., 1997). Should this domain prove important for this putative association, it would be interesting to determine if GRK2, and even other GRKs, can interact with other ERM family members or FERM-domain containing proteins and whether these would also serve as GRK substrates. FERM domains are found in many membrane-associated cytoskeletal and signalling proteins (Bretscher et al., 2002) and could function as general linkers between GRKs and the cytoskeleton or other signalling pathways.

Thus, in much the same manner as GRK2 is responsible for recruiting GIT to GPCRs at the plasma membrane (Claing et al., 2000; Premont et al., 1998), GRK2 may also bring ERM proteins to activated GPCRs. However, unlike its interaction with GIT, GRK2 would not just passively target ERM proteins to GPCRs, but also serve to activate them. If this hypothesis proves correct, it should be noted that for receptors, such as the LDL receptor, that internalise in an ezrin- and clathrin-dependent but GRK-independent pathway, other factors such as Arf6 might be responsible for recruiting ezrin, and other non-GRK kinases may bring about ezrin activation. It is also possible that ERM proteins can directly bind to a number of receptors, such as the LDL receptor and a subset of GPCRs, and that it may form part of a complex with receptors as well as proteins involved in both endocytosis and actin cytoskeletal rearrangements. Both of these prospects require further investigation.

6.2.5 ERM proteins may play a role in numerous GPCR trafficking pathways

This thesis has shown that ERM proteins are involved in the endocytosis of a number of GPCRs. ERM proteins may also play a role in other GPCR trafficking pathways. The requirement of NHERF for β_2AR recycling has been demonstrated to depend on the interaction of NHERF with ERM proteins: disruption of its ERM binding domain caused mis-sorting of receptors to the lysosome (Cao et al., 1999). Furthermore, ERM proteins were shown to play a role in the tethering and fusion of vesicles delivering rhodopsin to the ROS (Deretic et al., 2003). These results indicate that, as well as regulating the internalisation of a subset of GPCRs, ERM proteins may have a more general role mediating GPCR trafficking. Although my data suggest that ezrin is not involved in the delivery of β_2AR to the plasma membrane, it would be interesting to examine the role of ERM proteins in β_2AR recycling in more detail. This would be possible by using antibody feeding assays prior to flow cytometry to analyse the recycling of internalised β_2AR back to the plasma membrane in HEK cells over-expressing either N- or C-ezrin.

6.3 GRKs as co-coordinators of cytoskeletal reorganisation downstream of GPCRs

In this thesis I demonstrate that the GRK2-mediated phosphorylation of ERM proteins is required for the cytoskeletal reorganisation associated with β_2AR internalisation and M1MR-dependent membrane ruffling in Hep2 cells. Previous studies have shown that GRK2 activity can indirectly influence this actin remodelling via the recruitment of βarrestin to phosphorylated, agonist-occupied GPCRs. βarrestin recruits both c-Src to the plasma membrane, where it activates dynamin, and Arf6 (Ahn et al., 1999; Shenoy and Lefkowitz, 2003).

The large GTPase dynamin was originally characterised as a mechano-enzyme catalysing the fission of CCVs following endocytosis. It has also been shown to regulate actin filament dynamics at the plasma membrane via its interactions with actin-regulatory proteins, as reviewed in Chapter 1. In this way, dynamin acts to integrate both endocytosis and cytoskeletal reorganisation at the plasma membrane and probably functions to regulate actin polymerisation so that vesicle internalisation can proceed. Interestingly, following stimulation of migration in fibroblasts, dynamin is recruited to membrane ruffles at the leading edge and may regulate the cytoskeletal rearrangements required to alter cell morphology at the lamellipodia of motile cells (McNiven et al., 2000).

Likewise, Arf6 has been shown to have a dual role in both endocytosis and cell morphology remodelling. The cycle of Arf6 GTP binding and hydrolysis is required for β_2AR internalisation (Shenoy and Lefkowitz, 2003), possibly because it stimulates PIP_2 production, which would recruit clathrin and dynamin to CCVs (Cavenagh et al., 1996; Honda et al., 1999; Melendez et al., 2001; Takei and Haucke, 2001). Arf6 is also localised to and required for the formation of membrane ruffles (Honda et al., 1999; Song et al., 1998), where it is recruited following both EGF stimulation and signalling via the Gq-coupled bombesin receptor (Boshans et al., 2000; Honda et al., 1999).

My data suggest that GRK2 can also directly influence actin remodelling, via the phosphorylation and activation of ERM proteins. GRK2-mediated ERM activation

at agonist-occupied GPCRs directly regulates cytoskeletal reorganisation and receptor internalisation. In this way, GRK2 acts as a general coordinator of cytoskeletal remodelling downstream of GPCRs, which it regulates both directly (via ERM proteins) and indirectly (via βarrestin, dynamin and Arf6).

Although more investigation is required to ascertain the exact pathways downstream of GRK2-mediated ERM protein activation that lead to cytoskeletal reorganisation, an initial general model can be suggested (Figure 6.1). Agonist binding to a GPCR, such as M1MR or β_2AR, leads to colocalisation of GRK2 and an ERM protein with the receptor at the plasma membrane. GRK2 is activated and phosphorylates the GPCR carboxyl-terminus, promoting the binding of βarrestin. βarrestin binds to Arf6 (Shenoy and Lefkowitz, 2003), bringing it to the plasma membrane. Rac is trafficked to the plasma membrane along with Arf6 (Boshans et al., 2000; Radhakrishna et al., 1999), where both stimulate the increased production of PIP_2 (Honda et al., 1999; Ren and Schwartz, 1998; Takei and Haucke, 2001). The action of Rac would lead to morphological changes such as the formation of ruffles and lamellipodia (Nobes and Hall, 1995; Ridley and Hall, 1992a; Ridley and Hall, 1992b) and the increase in PIP_2 could regulate a number of actin binding proteins, leading to actin polymerisation (Ren and Schwartz, 1998).

The presence of a localised concentration of PIP_2 would also facilitate ERM protein phosphorylation by GRK2 and augment ERM protein activation (Fievet et al., 2004). Activated ERM proteins would act to cross-link the GPCR complex to the actin cytoskeleton. ERM proteins may also directly regulate actin organisation. Expression of the radixin FERM domain has been shown to displace Rho from RhoGDI, enabling Rho activation through the exchange of GDP for GTP (Takahashi et al., 1997). In addition to this, moesin has been demonstrated to inhibit WASP-mediated actin polymerisation both *in vitro* and *in vivo* via binding WASP at its FERM domain (Manchanda et al., 2005). Therefore, ERM proteins may exert a more direct effect on actin reorganisation by regulating the activity of both Rho and WASP.

The recruitment of βarrestin to a GPCR not only brings Arf6 to the plasma membrane, but also targets the receptor to preformed CCPs. This is followed by

dynamin recruitment and the fission of the CCV from the plasma membrane (Figure 1.3). Whilst dynamin could serve to mediate vesicle fission through its regulation of the actin cytoskeleton, the actin cross-linking action of ERM proteins at the CCV may aid vesicle trafficking through remodelling of the underlying cortical actin. For a subset of GPCRs, ERM proteins may be added to the known sequence of protein interactions required for CCV formation and internalisation, in much the same way as an interaction with GIT is essential for those GPCRs that internalise via a βarrestin- and clathrin-dependent pathway (Claing et al., 2000).

How ERM proteins co-ordinate actin reorganisation downstream of the M1MR in Hep2 cells to bring about first membrane ruffling and then endocytosis, compared to their role in mediating the cytoskeletal remodelling required only for β_2AR internalisation in HEK293 cells needs further investigation. Components of the GPCR desensitisation machinery such as GRKs, βarrestins and GIT have been shown to have roles in GPCR-mediated chemotaxis (as discussed in Chapter 4), suggesting that ruffling and cell migration may be closely linked to receptor endocytosis. Since chemotaxis depends on a cell distinguishing and responding to a ligand gradient, this must involve different degrees of receptor activation at different locations on the cell surface as it moves through the gradient. The dynamic control of GPCR signalling would be achieved by rapid turning on and off of receptor signalling, presumably via the desensitisation machinery. The involvement of ERM proteins in both processes would link them to the reorganisation of the cytoskeleton obligatory for chemotaxis and beneficial for receptor-mediated endocytosis.

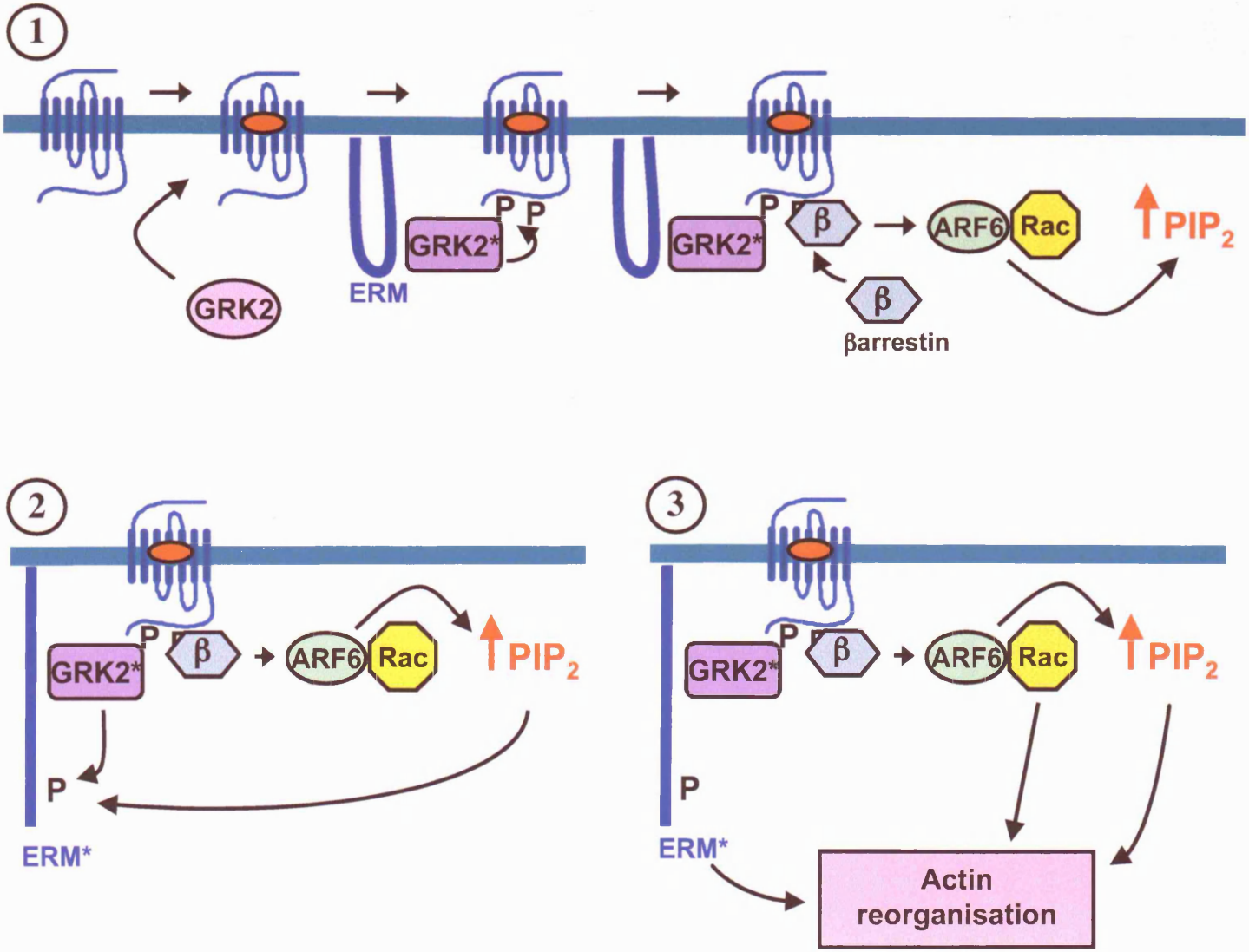
1
GRK2
ERM
GRK2*
P
P
GRK2*
P
β
ARF6
Rac
PIP2
β
βarrestin
2
GRK2*
P
β
ARF6
Rac
PIP2
P
ERM*
3
GRK2*
P
β
ARF6
Rac
PIP2
P
ERM*
Actin
reorganisation

Figure 6.1. Proposed model of the role of GRK2-mediated ERM protein phosphorylation in cytoskeletal reorganisation. **1.** Agonist binding to a GPCR leads to colocalisation of GRK2 and an ERM protein with the receptor at the plasma membrane. GRK2 is activated and phosphorylates the GPCR carboxyl-terminus, promoting the binding of βarrestin. This brings Arf6 and Rac to the plasma membrane, where both stimulate the increased production of PIP_2. **2.** The localised concentration of PIP_2 facilitates ERM protein phosphorylation by GRK2 and leads to ERM protein activation. **3.** Activated ERM proteins act to cross-link the GRK2/GPCR complex to the actin cytoskeleton and may also influence actin organisation via WASP or RhoGDI. The action of Rac leads to actin-mediated morphological changes such as the formation of ruffles and lamellipodia, and an increase in PIP_2 at the membrane regulates a number of actin binding proteins and thereby influences actin polymerisation. The resulting cytoskeletal remodelling could lead to membrane ruffling and/or GPCR internalisation.

It is not currently known at what stage an ERM protein would translocate to a GPCR at the plasma membrane, or with which protein(s) it would associate. Therefore, for simplicity, neither details of the translocation of ERM proteins to the plasma membrane nor any ERM interactions have been included in this diagram.

* indicates activated proteins. P denotes a phosphate group.

6.4 GRK2 may regulate other ERM protein-dependent functions

As well as regulating ERM protein activation at the plasma membrane following GPCR stimulation, GRK2 could have a wider role in mediating ERM protein functions in other cellular processes. pERM have been localised to the cleavage furrow during cytokinesis (Tsukita and Yonemura, 1999) and, although their role during cytokinesis is not well understood, expression of the carboxyl-terminus of radixin has been shown to interfere with this process (Henry et al., 1995). Depletion of known cleavage furrow kinases Aurora B and Rho kinase does not inhibit ERM protein phosphorylation at the cleavage furrow, indicating the presence of another kinase (Yokoyama et al., 2005). Since GRK2 activity appears to be necessary for the regulation of mitosis and cytokinesis (Dr. Julie Pitcher, unpublished observations), it is tempting to speculate that GRK2 could be the kinase responsible for phosphorylating ERM proteins at the cleavage furrow.

6.5 Cellular screening techniques to identify novel substrates of GRK2

The data included in this thesis validates the use of a cellular screening technique to identify novel substrates for GRK2. Agonist treatment of HEK293 cells over-expressing β_2AR and GRK2 in the presence of [^{32}P]orthophosphate led to the identification of a number of potential GRK2 substrates that were subsequently identified using MALDI-TOF mass spectrometry. During the course of my studies, I corroborated one of these findings by demonstrating that ezrin was both a substrate and an effector of GRK2. Such a screen is therefore a useful method to initially identify novel GRK2 substrates. With advances in both mass spectrometry and the purification of phosphorylated proteins using chelate matrices, the sensitivity of this screen could be improved.

6.6 Experimental weaknesses of this thesis

Throughout this thesis I have described experiments conducted in immortalised cell lines over-expressing particular proteins of interest. Transfection and over-

expression of proteins might induce signalling pathways that do not normally occur in a physiological environment. With this in mind, it might be more physiologically relevant to work with primary cells or cells that have not been transformed to determine the signalling pathways activated in the absence of overexpression. Unfortunately this is not always experimentally feasible since the propagation of primary cells is more challenging, they are often difficult to transfect and it may be impossible to observe endogenous, untagged proteins for which no suitable antibody exists.

A further complexity involved with using immortalised cell lines such as HEK293 and Hep2 is that they express endogenous kinases able to phosphorylate GPCRs. This can be helpful as it allows transfected receptors to be phosphorylated by the endogenous machinery on agonist-occupancy. However, it can make investigations to decipher the exact contributions of individual components, such as kinases, difficult. Inhibiting the action of one kinase, for example a particular GRK, may not completely abolish GPCR phosphorylation and desensitisation since other endogenous GRK subtypes, or kinases such as casein kinase 1α, may still contribute in these cell lines (Tobin, 2002).

Moreover, kinases may have more than one action within the cell, which can complicate the interpretation of results. The recruitment of GRK2 to agonist-occupied GPCRs is concurrent with GRK2 binding to G$\beta\gamma$ and, where associated, Gq (Carman et al., 1999a; Carman et al., 2000; Eichmann et al., 2003). It is therefore likely that GRK2 can also mediate phosphorylation-independent desensitisation of GPCRs by inhibiting signalling pathways downstream of G$\beta\gamma$ and Gq (Pao and Benovic, 2002). Expression of either the GRK2 RGS domain alone or a catalytically inactive GRK2 mutant ($GRK2_{K220R}$) can terminate Gq signalling and both the carboxyl-terminal domain of GRK2 and $GRK2_{K220R}$ can interfere with G$\beta\gamma$-mediated processes, even though none of these constructs can phosphorylate GPCRs or other GRK2 substrates (Carman et al., 1999a; Rockman et al., 1998; Sallese et al., 2000b). Whilst the singular use of any one of these mutants may not completely inhibit all GRK2-dependent effects, the sequential expression of all three within a model system should facilitate the determination of the respective contributions of GRK2-

mediated phosphorylation and Gq- and Gβγ-sequestration within a particular pathway.

The effect of Gq- and Gβγ-sequestration can also be further investigated with the use of heterotrimeric G protein-derived constructs. Transducin is a Gβγ scavenger that can be transiently expressed in mammalian cells (Federman et al., 1992). Gq-derived constructs, such as carboxyl-terminal Gq peptides or xanthine nucleotide-binding Gq mutants, can also be transfected into cells to interfere with endogenous Gq signalling (Akhter et al., 1998; Yu et al., 2000). The sequential expression of each of these constructs in acetylcholine-stimulated M1MR-expressing Hep2 cells should inhibit the activities of Gβγ and Gq respectively and thus further clarify the functions of GRK2 in M1MR-mediated ruffling.

However, it should be noted that both Gβγ and Gq are required for localisation of GRK2 with agonist-occupied GPCRs at the plasma membrane. Therefore, the above approaches would also inhibit GRK2 activity at the plasma membrane and so would not determine whether signalling via Gβγ/Gq, or signalling via GRK2 is required. An alternative approach to determining the function of a protein, such as GRK2, is to use cells and transgenic animals in which the protein has been knocked out. However, the deletion of certain proteins can prove lethal in animal models and might restrict the viability of cell lines. This is the case with GRK2: *βARK1*$^{-/-}$ mice are not viable (Jaber et al., 1996) and no cell lines currently exist in which GRK2 has been deleted. In addition, knocking out key proteins in regulatory and signalling pathways may affect the expression of other proteins within the cell, possibly hindering the characterisation of the role of an individual protein. With these limitations in mind it may be necessary to follow a number of approaches, based both *in vitro* and *in vivo* and using different systems and cell lines, in order to validate any protein function or signalling pathway under investigation. The utilisation of RNAi to ablate expression of GRK2, or any other particular protein, may be one such approach and may prove to be the most effective method to further characterise the role of GRK2 in a number of ERM protein-dependent processes.

6.7 Further experimental approaches

It would be interesting to undertake further experiments in order to determine in more detail the role of ERM proteins in GPCR-mediated membrane ruffling and receptor-mediated endocytosis. For example, investigations could be carried out to identify the GPCRs that internalise in an ERM-dependent manner by repeating the flow cytometry-based endocytosis assay from Chapter 5 with a number of different receptors. A range of GPCRs could also be individually transfected into Hep2 cells to establish which promote agonist-dependent membrane ruffling. Such experiments could also be performed in cells derived from ERM knock out mice (Doi et al., 1999; Saotome et al., 2004; Tamura et al., 2005) to determine the extent of the contribution made by ERM proteins in both processes.

The results of these assays may also indicate the signalling pathway components that are important for ERM-dependent membrane ruffling and endocytosis, such as whether Gq is required for GPCR-mediated ruffling. Since M1MR but not β_2AR mediates ruffling in Hep2 cells (Dr. Julie Pitcher, unpublished observations), it would be interesting to determine whether other Gq-coupled receptors can promote ruffling. Gq activates PLCβ following agonist binding to its associated GPCR (Cabrera-Vera et al., 2003); GRK2 can sequester and inhibit Gq-mediated stimulation of PIP_2 hydrolysis by PLCβ (Carman et al., 1999a; Sallese et al., 2000b). It would therefore also be interesting to investigate the requirement for PIP_2 in this model of membrane ruffling. This could be achieved by transfecting Hep2 cells simultaneously with PLC$\beta_2\Delta$, a carboxyl-terminal deletion mutant that cannot be activated by Gq, and constitutively active Rac2(12V), which stimulates the activity of PLC$\beta_2\Delta$ (Illenberger et al., 2003), and ascertaining whether M1MR-mediated ruffling occurs in the presence and absence of active GRK2. Considering the results described in Chapter 4.4, it is likely that both GRK2 activity and the inhibition of PLCβ will be necessary for M1MR-mediated membrane ruffling.

Similarly, identifying which GPCRs require ERM proteins for internalisation may aid further characterisation of the pathways and proteins involved in ERM-dependent GPCR endocytosis. The data presented in this thesis suggest that the role of ezrin in GPCR internalisation may be limited to a subset of receptors that includes β_2AR and

M1MR, but not M2MR. As discussed earlier, sequestration of receptors such as β_2AR and M1MR is characterised by GRK phosphorylation, βarrestin binding and then endocytosis via clathrin-coated pits, whereas GPCRs such as ET_BR and M2MR may internalise via caveolae or GRK-independent pathways respectively (Chini and Parenti, 2004; Claing et al., 2000; von Zastrow, 2003). It would be interesting to determine whether the involvement of ERM proteins with the endocytosis of specific GPCRs does indeed correlate with their GRK dependence, and whether other components such as βarrestin, dynamin, clathrin and GIT are also involved. Once the ERM-dependent GPCRs have been identified, the involvement of the above proteins in their internalisation can be determined by expression of βarrestin1 V53D and βarrestin1 S412D to inhibit βarrestin-dependent pathways; transfection of dynamin K44A to inhibit dynamin-dependent endocytosis; over-expression of GIT1 to inhibit GIT-dependent internalisation; and pre-treatment of cells with the clathrin cage stabiliser monodansylcadaverine to block clathrin-mediated endocytosis (Claing et al., 2000).

Furthermore, the data presented in Chapter 5.4 indicates that ezrin is required for β_2AR internalisation, but not trafficking to the plasma membrane. However, disruption of the interaction of β_2AR with NHERF, or NHERF with ERM proteins was shown to cause mis-sorting of the usually recycled receptor to lysosomes (Cao et al., 1999). Although I have demonstrated that pERM colocalise with internalised β_2AR that cannot bind NHERF, the role of ERM proteins in GPCR recycling in my system should be further examined. Antibody feeding assays prior to flow cytometry could be used to analyse the delivery of internalised β_2AR back to the plasma membrane in HEKβ2 cells in both the absence and presence of N- or C-ezrin.

Both membrane ruffling and receptor-mediated endocytosis are complicated cellular pathways that require components of the GPCR desensitisation machinery and actin cytoskeleton. Although I have shown that GRK2-mediated phosphorylation of ERM proteins is required for these processes, further investigation is required to identify the ERM binding partners. The preliminary data in Chapters 3 and 5 suggests that GRK2 can associate with ezrin. These data need to be confirmed using *in vitro* pull-down assays, and other possible binding partners such as GPCRs or Arf6 identified.

PKC-α has been shown to colocalise with ezrin at plasma membrane protrusions and a complex of PKC, ezrin and the transmembrane adhesion molecule CD44 is required for cell motility in response to phorbol esters (Legg et al., 2002; Ng et al., 2001). It is therefore possible that complexes containing ezrin with kinases, possibly including GRK2, and transmembrane proteins, such as GPCRs, may be important for kinase-mediated, ERM-dependent cytoskeletal reorganisation events. The formation of such a complex can be investigated using *in vitro* binding assays and cellular immunoprecipitation assays following M1MR and β_2AR activation.

6.8 Concluding remarks

In conclusion, GRK2 has long been considered to have an involvement in signalling pathways downstream of GPCRs besides receptor desensitisation. Here I present evidence that a non-receptor substrate of GRK2 has an effector function following GPCR activation. I show that ezrin is a novel substrate of GRK2, that ERM proteins are activated following receptor stimulation, and that GRK2-mediated ERM protein phosphorylation is required for GPCR-mediated cytoskeletal rearrangements as well as internalisation of a subset of receptors. In recent years βarrestins, key regulators of GPCR signalling, have been shown to have additional roles as scaffold and adaptor proteins in pathways downstream of GPCRs (Ahn et al., 2003). Similarly it is becoming clear that GRK2, with its growing list of non-receptor substrates, has a more diverse role within the cell than previously appreciated.

7 References

Ahn, S., S. Maudsley, L. M. Luttrell, R. J. Lefkowitz and Y. Daaka (1999). "Src-mediated tyrosine phosphorylation of dynamin is required for beta2-adrenergic receptor internalization and mitogen-activated protein kinase signaling." *J Biol Chem* **274**(3): 1185-1188.

Ahn, S., C. D. Nelson, T. R. Garrison, W. E. Miller and R. J. Lefkowitz (2003). "Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference." *Proc Natl Acad Sci U S A* **100**(4): 1740-1744.

Akhter, S. A., L. M. Luttrell, A. A. Rockman, G. Iaccarino, R. J. Lefkowitz and W. J. Koch (1998). "Targeting the Receptor-Gq Interface to Inhibit in Vivo Pressure Overload Myocardial Hypertrophy." *Science* **280**(5363): 574-575.

Algrain, M., O. Turunen, A. Vaheri, D. Louvard and M. Arpin (1993). "Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker." *J Cell Biol* **120**(1): 129-139.

Allen, J. A., J. Z. Yu, R. J. Donati and M. M. Rasenick (2005). "β-Adrenergic Receptor Stimulation Promotes Gαs Internalization through Lipid Rafts: A Study in Living Cells." *Mol Pharmacol* **67**(5): 1493-1504.

Amieva, M. R., P. Litman, L. Huang, E. Ichimaru and H. Furthmayr (1999). "Disruption of dynamic cell surface architecture of NIH3T3 fibroblasts by the N-terminal domains of moesin and ezrin: in vivo imaging with GFP fusion proteins." *J Cell Sci* **112 (Pt 1)**: 111-125.

Aragay, A. M., M. Mellado, J. M. Frade, A. M. Martin, M. C. Jimenez-Sainz, A. C. Martinez and F. Mayor, Jr. (1998). "Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2." *Proc Natl Acad Sci U S A* **95**(6): 2985-2990.

Aspenstrom, P., A. Fransson and J. Saras (2004). "Rho GTPases have diverse effects

on the organization of the actin filament system." *Biochem J* **377**(Pt 2): 327-337.

Autero, M., L. Heiska, L. Ronnstrand, A. Vaheri, C. G. Gahmberg and O. Carpen (2003). "Ezrin is a substrate for Lck in T cells." *FEBS Lett* **535**(1-3): 82-86.

Barnes, W. G., E. Reiter, J. D. Violin, X. R. Ren, G. Milligan and R. J. Lefkowitz (2005). "beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation." *J Biol Chem* **280**(9): 8041-8050.

Barret, C., C. Roy, P. Montcourrier, P. Mangeat and V. Niggli (2000). "Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP(2)) binding site in the NH(2)-terminal domain of ezrin correlates with its altered cellular distribution." *J Cell Biol* **151**(5): 1067-1080.

Bennett, E. M., C. Y. Chen, A. E. Engqvist-Goldstein, D. G. Drubin and F. M. Brodsky (2001). "Clathrin hub expression dissociates the actin-binding protein Hip1R from coated pits and disrupts their alignment with the actin cytoskeleton." *Traffic* **2**(11): 851-858.

Benovic, J. L. (2002). "Novel beta2-adrenergic receptor signaling pathways." *J Allergy Clin Immunol* **110**(6 Suppl): S229-235.

Berryman, M., R. Gary and A. Bretscher (1995). "Ezrin oligomers are major cytoskeletal components of placental microvilli: a proposal for their involvement in cortical morphogenesis." *J Cell Biol* **131**(5): 1231-1242.

Bishop, A. L. and A. Hall (2000). "Rho GTPases and their effector proteins." *Biochem J* **348 Pt 2**: 241-255.

Blackmer, T., E. C. Larsen, C. Bartleson, J. A. Kowalchyk, E. J. Yoon, A. M. Preininger, S. Alford, H. E. Hamm and T. F. Martin (2005). "G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis." *Nat Neurosci* **8**(4): 421-425.

Borm, B., R. P. Requardt, V. Herzog and G. Kirfel (2005). "Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization." *Exp Cell Res* **302**(1): 83-95.

Boshans, R. L., S. Szanto, L. van Aelst and C. D'Souza-Schorey (2000). "ADP-ribosylation factor 6 regulates actin cytoskeleton remodeling in coordination with Rac1 and RhoA." *Mol Cell Biol* **20**(10): 3685-3694.

Bretscher, A. (1989). "Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor." *J Cell Biol* **108**(3): 921-930.

Bretscher, A., K. Edwards and R. G. Fehon (2002). "ERM proteins and merlin: integrators at the cell cortex." *Nat Rev Mol Cell Biol* **3**(8): 586-599.

Bretscher, A., R. Gary and M. Berryman (1995). "Soluble ezrin purified from placenta exists as stable monomers and elongated dimers with masked C-terminal ezrin-radixin-moesin association domains." *Biochemistry* **34**(51): 16830-16837.

Burridge, K. and K. Wennerberg (2004). "Rho and Rac take center stage." *Cell* **116**(2): 167-179.

Buss, F., S. D. Arden, M. Lindsay, J. P. Luzio and J. Kendrick-Jones (2001). "Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis." *Embo J* **20**(14): 3676-3684.

Cabrera-Vera, T. M., J. Vanhauwe, T. O. Thomas, M. Medkova, A. Preininger, M. R. Mazzoni and H. E. Hamm (2003). "Insights into G protein structure, function, and regulation." *Endocr Rev* **24**(6): 765-781.

Cant, S. H. and J. A. Pitcher (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.

Cao, T. T., H. W. Deacon, D. Reczek, A. Bretscher and M. von Zastrow (1999). "A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor." *Nature* **401**(6750): 286-290.

Carlsson, S. R., J. Roth, F. Piller and M. Fukuda (1988). "Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan." *J Biol Chem* **263**(35): 18911-18919.

Carman, C. V., L. S. Barak, C. Chen, L. Y. Liu-Chen, J. J. Onorato, S. P. Kennedy, M. G. Caron and J. L. Benovic (2000). "Mutational analysis of Gbetagamma and phospholipid interaction with G protein-coupled receptor kinase 2." *J Biol Chem* **275**(14): 10443-10452.

Carman, C. V., J. L. Parent, P. W. Day, A. N. Pronin, P. M. Sternweis, P. B. Wedegaertner, A. G. Gilman, J. L. Benovic and T. Kozasa (1999a). "Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2." *J Biol Chem* **274**(48): 34483-34492.

Carman, C. V., M. P. Lisanti and J. L. Benovic (1999b). "Regulation of G protein-coupled receptor kinases by caveolin." *J Biol Chem* **274**(13): 8858-8864.

Carman, C. V., T. Som, C. M. Kim and J. L. Benovic (1998). "Binding and phosphorylation of tubulin by G protein-coupled receptor kinases." *J Biol Chem* **273**(32): 20308-20316.

Cassill, J. A., M. Whitney, C. A. Joazeiro, A. Becker and C. S. Zuker (1991). "Isolation of Drosophila genes encoding G protein-coupled receptor kinases." *Proc Natl Acad Sci U S A* **88**(24): 11067-11070.

Cavenagh, M. M., J. A. Whitney, K. Carroll, C. Zhang, A. L. Boman, A. G. Rosenwald, I. Mellman and R. A. Kahn (1996). "Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane." *J Biol Chem* **271**(36): 21767-21774.

Chini, B. and M. Parenti (2004). "G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there?" *J Mol Endocrinol* **32**(2): 325-338.

Chishti, A. H., A. C. Kim, S. M. Marfatia, M. Lutchman, M. Hanspal, H. Jindal, S. C. Liu, P. S. Low, G. A. Rouleau, N. Mohandas, J. A. Chasis, J. G. Conboy, P. Gascard, Y. Takakuwa, S. C. Huang, E. J. Benz, Jr., A. Bretscher, R. G. Fehon, J. F. Gusella, V. Ramesh, F. Solomon, V. T. Marchesi, S. Tsukita, K. B. Hoover and et al. (1998). "The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane." *Trends Biochem Sci* **23**(8): 281-282.

Choi, D. J., W. J. Koch, J. J. Hunter and H. A. Rockman (1997). "Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase." *J Biol Chem* **272**(27): 17223-17229.

Chuang, T. T., H. LeVine, 3rd and A. De Blasi (1995). "Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C." *J Biol Chem* **270**(31): 18660-18665.

Claing, A., W. Chen, W. E. Miller, N. Vitale, J. Moss, R. T. Premont and R. J. Lefkowitz (2001). "beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis." *J Biol Chem* **276**(45): 42509-42513.

Claing, A., S. A. Laporte, M. G. Caron and R. J. Lefkowitz (2002). "Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins." *Prog Neurobiol* **66**(2): 61-79.

Claing, A., S. J. Perry, M. Achiriloaie, J. K. Walker, J. P. Albanesi, R. J. Lefkowitz and R. T. Premont (2000). "Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity." *Proc Natl Acad Sci U S A* **97**(3): 1119-1124.

Collins, S., M. G. Caron and R. J. Lefkowitz (1991). "Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression." *Annu Rev*

Physiol **53**: 497-508.

Cong, M., S. J. Perry, L. A. Hu, P. I. Hanson, A. Claing and R. J. Lefkowitz (2001). "Binding of the beta2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling." *J Biol Chem* **276**(48): 45145-45152.

Cong, M., S. J. Perry, F. T. Lin, I. D. Fraser, L. A. Hu, W. Chen, J. A. Pitcher, J. D. Scott and R. J. Lefkowitz (2001). "Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79." *J Biol Chem* **276**(18): 15192-15199.

Cremona, O., G. Di Paolo, M. R. Wenk, A. Luthi, W. T. Kim, K. Takei, L. Daniell, Y. Nemoto, S. B. Shears, R. A. Flavell, D. A. McCormick and P. De Camilli (1999). "Essential role of phosphoinositide metabolism in synaptic vesicle recycling." *Cell* **99**(2): 179-188.

Crepaldi, T., A. Gautreau, P. M. Comoglio, D. Louvard and M. Arpin (1997). "Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells." *J Cell Biol* **138**(2): 423-434.

D'Apuzzo, M., A. Rolink, M. Loetscher, J. A. Hoxie, I. Clark-Lewis, F. Melchers, M. Baggiolini and B. Moser (1997). "The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4." *Eur J Immunol* **27**(7): 1788-1793.

da Costa, S. R., C. T. Okamoto and S. F. Hamm-Alvarez (2003). "Actin microfilaments et al.--the many components, effectors and regulators of epithelial cell endocytosis." *Adv Drug Deliv Rev* **55**(11): 1359-1383.

Daaka, Y., L. M. Luttrell and R. J. Lefkowitz (1997a). "Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A." *Nature* **390**(6655): 88-91.

Daaka, Y., J. A. Pitcher, M. Richardson, R. H. Stoffel, J. D. Robishaw and R. J.

Lefkowitz (1997b). "Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases." *Proc Natl Acad Sci U S A* **94**(6): 2180-2185.

Dautry-Varsat, A. (1986). "Receptor-mediated endocytosis: the intracellular journey of transferrin and its receptor." *Biochimie* **68**(3): 375-381.

Day, P. W., J. J. Tesmer, R. Sterne-Marr, L. C. Freeman, J. L. Benovic and P. B. Wedegaertner (2004). "Characterization of the GRK2 binding site of Galphaq." *J Biol Chem* **279**(51): 53643-53652.

DebBurman, S. K., J. Ptasienski, J. L. Benovic and M. M. Hosey (1996). "G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein betagamma subunits." *J Biol Chem* **271**(37): 22552-22562.

DebBurman, S. K., J. Ptasienski, E. Boetticher, J. W. Lomasney, J. L. Benovic and M. M. Hosey (1995). "Lipid-mediated regulation of G protein-coupled receptor kinases 2 and 3." *J Biol Chem* **270**(11): 5742-5747.

DeFea, K. A., J. Zalevsky, M. S. Thoma, O. Dery, R. D. Mullins and N. W. Bunnett (2000). "beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2." *J Cell Biol* **148**(6): 1267-1281.

Delaney, K. A., M. M. Murph, L. M. Brown and H. Radhakrishna (2002). "Transfer of M2 muscarinic acetylcholine receptors to clathrin-derived early endosomes following clathrin-independent endocytosis." *J Biol Chem* **277**(36): 33439-33446.

Deretic, D., V. Traverso, N. Parkins, F. Jackson, E. B. Rodriguez de Turco and N. Ransom (2004). "Phosphoinositides, ezrin/moesin, and rac1 regulate fusion of rhodopsin transport carriers in retinal photoreceptors." *Mol Biol Cell* **15**(1): 359-370.

Dinudom, A., A. B. Fotia, R. J. Lefkowitz, J. A. Young, S. Kumar and D. I. Cook (2004). "The kinase Grk2 regulates Nedd4/Nedd4-2-dependent control of epithelial

Na+ channels." *Proc Natl Acad Sci U S A* **101**(32): 11886-11890.

Doi, Y., M. Itoh, S. Yonemura, S. Ishihara, H. Takano, T. Noda and S. Tsukita (1999). "Normal development of mice and unimpaired cell adhesion/cell motility/actin-based cytoskeleton without compensatory up-regulation of ezrin or radixin in moesin gene knockout." *J Biol Chem* **274**(4): 2315-2321.

Donaldson, J. G. (2003). "Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane." *J Biol Chem* **278**(43): 41573-41576.

Dutt, P., L. Kjoller, M. Giel, A. Hall and D. Toksoz (2002). "Activated Galphaq family members induce Rho GTPase activation and Rho-dependent actin filament assembly." *FEBS Lett* **531**(3): 565-569.

Eichmann, T., K. Lorenz, M. Hoffmann, J. Brockmann, C. Krasel, M. J. Lohse and U. Quitterer (2003). "The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site." *J Biol Chem* **278**(10): 8052-8057.

Elliott, B., J. Meens, S. SenGupta, D. Louvard and M. Arpin (2005). "The membrane cytoskeletal crosslinker ezrin is required for metastasis of breast carcinoma cells." *Breast Cancer Research* **7**(3): R365-R373.

Elorza, A., S. Sarnago and F. Mayor, Jr. (2000). "Agonist-dependent modulation of G protein-coupled receptor kinase 2 by mitogen-activated protein kinases." *Mol Pharmacol* **57**(4): 778-783.

Engqvist-Goldstein, A. E. and D. G. Drubin (2003). "Actin assembly and endocytosis: from yeast to mammals." *Annu Rev Cell Dev Biol* **19**: 287-332.

Engqvist-Goldstein, A. E., M. M. Kessels, V. S. Chopra, M. R. Hayden and D. G. Drubin (1999). "An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles." *J Cell Biol* **147**(7): 1503-1518.

Engqvist-Goldstein, A. E., C. X. Zhang, S. Carreno, C. Barroso, J. E. Heuser and D. G. Drubin (2004). "RNAi-mediated Hip1R silencing results in stable association between the endocytic machinery and the actin assembly machinery." *Mol Biol Cell* **15**(4): 1666-1679.

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

Etienne-Manneville, S. and A. Hall (2002). "Rho GTPases in cell biology." *Nature* **420**(6916): 629-635.

Fan, G., E. Shumay, C. C. Malbon and H. Wang (2001). "c-Src tyrosine kinase binds the beta 2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization." *J Biol Chem* **276**(16): 13240-13247.

Federman, A. D., B. R. Conklin, K. A. Schrader, R. R. Reed and H. R. Bourne (1992). "Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits." *Nature* **356**: 159-161.

Fievet, B. T., A. Gautreau, C. Roy, L. Del Maestro, P. Mangeat, D. Louvard and M. Arpin (2004). "Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin." *J Cell Biol* **164**(5): 653-659.

Finnerty, C. M., D. Chambers, J. Ingraffea, H. R. Faber, P. A. Karplus and A. Bretscher (2004). "The EBP50-moesin interaction involves a binding site regulated by direct masking on the FERM domain." *J Cell Sci* **117**(Pt 8): 1547-1552.

Fong, A. M., R. T. Premont, R. M. Richardson, Y. R. Yu, R. J. Lefkowitz and D. D. Patel (2002). "Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice." *Proc Natl Acad Sci U S A* **99**(11): 7478-7483.

Fredericks, Z. L., J. A. Pitcher and R. J. Lefkowitz (1996). "Identification of the G protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic

receptor." *J Biol Chem* **271**(23): 13796-13803.

Freedman, N. J., A. S. Ament, M. Oppermann, R. H. Stoffel, S. T. Exum and R. J. Lefkowitz (1997). "Phosphorylation and desensitization of human endothelin A and B receptors. Evidence for G protein-coupled receptor kinase specificity." *J Biol Chem* **272**(28): 17734-17743.

Freedman, N. J., S. B. Liggett, D. E. Drachman, G. Pei, M. G. Caron and R. J. Lefkowitz (1995). "Phosphorylation and desensitization of the human beta 1-adrenergic receptor. Involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase." *J Biol Chem* **270**(30): 17953-17961.

Freeman, J. L., E. M. De La Cruz, T. D. Pollard, R. J. Lefkowitz and J. A. Pitcher (1998). "Regulation of G protein-coupled receptor kinase 5 (GRK5) by actin." *J Biol Chem* **273**(32): 20653-20657.

Freeman, J. L., P. Gonzalo, J. A. Pitcher, A. Claing, J. P. Lavergne, J. P. Reboud and R. J. Lefkowitz (2002). "Beta 2-adrenergic receptor stimulated, G protein-coupled receptor kinase 2 mediated, phosphorylation of ribosomal protein P2." *Biochemistry* **41**(42): 12850-12857.

Freeman, J. L., J. A. Pitcher, X. Li, V. Bennett and R. J. Lefkowitz (2000). "alpha-Actinin is a potent regulator of G protein-coupled receptor kinase activity and substrate specificity in vitro." *FEBS Lett* **473**(3): 280-284.

Fujimoto, L. M., R. Roth, J. E. Heuser and S. L. Schmid (2000). "Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells." *Traffic* **1**(2): 161-171.

Gage, R. M., K. A. Kim, T. T. Cao and M. von Zastrow (2001). "A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors." *J Biol Chem* **276**(48): 44712-44720.

Gage, R. M., E. A. Matveeva, S. W. Whiteheart and M. von Zastrow (2005). "Type I

PDZ ligands are sufficient to promote rapid recycling of G Protein-coupled receptors independent of binding to N-ethylmaleimide-sensitive factor." *J Biol Chem* **280**(5): 3305-3313.

Gaidarov, I., J. G. Krupnick, J. R. Falck, J. L. Benovic and J. H. Keen (1999). "Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding." *Embo J* **18**(4): 871-881.

Gainetdinov, R. R., L. M. Bohn, T. D. Sotnikova, M. Cyr, A. Laakso, A. D. Macrae, G. E. Torres, K. M. Kim, R. J. Lefkowitz, M. G. Caron and R. T. Premont (2003). "Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice." *Neuron* **38**(2): 291-303.

Gainetdinov, R. R., L. M. Bohn, J. K. Walker, S. A. Laporte, A. D. Macrae, M. G. Caron, R. J. Lefkowitz and R. T. Premont (1999). "Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice." *Neuron* **24**(4): 1029-1036.

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

Gampel, A., P. J. Parker and H. Mellor (1999). "Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB." *Curr Biol* **9**(17): 955-958.

Gan, X. Q., J. Y. Wang, Q. H. Yang, Z. Li, F. Liu, G. Pei and L. Li (2000). "Interaction between the conserved region in the C-terminal domain of GRK2 and rhodopsin is necessary for GRK2 to catalyze receptor phosphorylation." *J Biol Chem* **275**(12): 8469-8474.

Gao, H., Y. Sun, Y. Wu, B. Luan, Y. Wang, B. Qu and G. Pei (2004). "Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways." *Mol Cell* **14**(3): 303-317.

Gary, R. and A. Bretscher (1993). "Heterotypic and homotypic associations between ezrin and moesin, two putative membrane-cytoskeletal linking proteins." *Proc Natl Acad Sci U S A* **90**(22): 10846-10850.

Gary, R. and A. Bretscher (1995). "Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site." *Mol Biol Cell* **6**(8): 1061-1075.

Gasman, S., Y. Kalaidzidis and M. Zerial (2003). "RhoD regulates endosome dynamics through Diaphanous-related Formin and Src tyrosine kinase." *Nat Cell Biol* **5**(3): 195-204.

Gautreau, A., D. Louvard and M. Arpin (2000). "Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane." *J Cell Biol* **150**(1): 193-203.

Gautreau, A., P. Poullet, D. Louvard and M. Arpin (1999). "Ezrin, a plasma membrane-microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway." *Proc Natl Acad Sci U S A* **96**(13): 7300-7305.

Ge, L., Y. Ly, M. Hollenberg and K. DeFea (2003). "A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis." *J Biol Chem* **278**(36): 34418-34426.

Ge, L., S. K. Shenoy, R. J. Lefkowitz and K. DeFea (2004). "Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2." *J Biol Chem* **279**(53): 55419-55424.

Goel, R., P. J. Phillips-Mason, A. Gardner, D. M. Raben and J. J. Baldassare (2004). "α-Thrombin-mediated Phosphatidylinositol 3-kinase Activation Through Release of Gβγ Dimers from Gαq and Gα12." *J Biol Chem* **279**(8): 6701-6710.

Goode, B. L., A. A. Rodal, G. Barnes and D. G. Drubin (2001). "Activation of the Arp2/3 complex by the actin filament binding protein Abp1p." *J Cell Biol* **153**(3): 627-634.

Goodman, O. B., Jr., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen and J. L. Benovic (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." *Nature* **383**(6599): 447-450.

Haga, K., K. Kameyama, T. Haga, U. Kikkawa, K. Shiozaki and H. Uchiyama (1996). "Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C." *J Biol Chem* **271**(5): 2776-2782.

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

Hall, R. A., L. S. Ostedgaard, R. T. Premont, J. T. Blitzer, N. Rahman, M. J. Welsh and R. J. Lefkowitz (1998a). "A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins." *Proc Natl Acad Sci U S A* **95**(15): 8496-8501.

Hall, R. A., R. T. Premont, C. W. Chow, J. T. Blitzer, J. A. Pitcher, A. Claing, R. H. Stoffel, L. S. Barak, S. Shenolikar, E. J. Weinman, S. Grinstein and R. J. Lefkowitz (1998b). "The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange." *Nature* **392**(6676): 626-630.

Hamada, K., T. Shimizu, S. Yonemura, S. Tsukita and T. Hakoshima (2003). "Structural basis of adhesion-molecule recognition by ERM proteins revealed by the crystal structure of the radixin-ICAM-2 complex." *Embo J* **22**(3): 502-514.

Hansen, S. H. and J. E. Casanova (1994). "Gs alpha stimulates transcytosis and

apical secretion in MDCK cells through cAMP and protein kinase A." *J Cell Biol* **126**(3): 677-687.

Hayashi, K., S. Yonemura, T. Matsui and S. Tsukita (1999). "Immunofluorescence detection of ezrin/radixin/moesin (ERM) proteins with their carboxyl-terminal threonine phosphorylated in cultured cells and tissues." *J Cell Sci* **112 (Pt 8)**: 1149-1158.

Henry, M. D., C. Gonzalez Agosti and F. Solomon (1995). "Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains." *J Cell Biol* **129**(4): 1007-1022.

Heydorn, A., B. P. Sondergaard, N. Hadrup, B. Holst, C. R. Haft and T. W. Schwartz (2004). "Distinct in vitro interaction pattern of dopamine receptor subtypes with adaptor proteins involved in post-endocytotic receptor targeting." *FEBS Lett* **556**(1-3): 276-280.

Hirao, M., N. Sato, T. Kondo, S. Yonemura, M. Monden, T. Sasaki, Y. Takai and S. Tsukita (1996). "Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway." *J Cell Biol* **135**(1): 37-51.

Hisatomi, O., S. Matsuda, T. Satoh, S. Kotaka, Y. Imanishi and F. Tokunaga (1998). "A novel subtype of G-protein-coupled receptor kinase, GRK7, in teleost cone photoreceptors." *FEBS Lett* **424**(3): 159-164.

Honda, A., M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A. J. Morris, M. A. Frohman and Y. Kanaho (1999). "Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation." *Cell* **99**(5): 521-532.

Horwitz, R. and D. Webb (2003). "Cell migration." *Curr Biol* **13**(19): R756-759.

Houndolo, T., P. L. Boulay and A. Claing (2005). "G protein-coupled receptor

endocytosis in ADP-ribosylation factor 6-depleted cells." *J Biol Chem* **280**(7): 5598-5604.

Hunton, D. L., W. G. Barnes, J. Kim, X. R. Ren, J. D. Violin, E. Reiter, G. Milligan, D. D. Patel and R. J. Lefkowitz (2005). "Beta-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis." *Mol Pharmacol* **67**(4): 1229-1236.

Hussain, N. K., S. Jenna, M. Glogauer, C. C. Quinn, S. Wasiak, M. Guipponi, S. E. Antonarakis, B. K. Kay, T. P. Stossel, N. Lamarche-Vane and P. S. McPherson (2001). "Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP." *Nat Cell Biol* **3**(10): 927-932.

Illenberger, D., C. Walliser, J. Strobel, O. Gutman, H. Niv, V. Gaidzik, Y. Kloog, P. Gierschik and Y. I. Henis (2003). "Rac2 Regulation of Phospholipase C-β_2 Activity and Mode of Membrane Interactions in Intact Cells." *J Biol Chem* **278**(10): 8645-8652.

Ishikawa, H., A. Tamura, T. Matsui, H. Sasaki, T. Hakoshima and S. Tsukita (2001). "Structural conversion between open and closed forms of radixin: low-angle shadowing electron microscopy." *J Mol Biol* **310**(5): 973-978.

Jaber, M., W. J. Koch, H. Rockman, B. Smith, R. A. Bond, K. K. Sulik, J. Ross, Jr., R. J. Lefkowitz, M. G. Caron and B. Giros (1996). "Essential role of beta-adrenergic receptor kinase 1 in cardiac development and function." *Proc Natl Acad Sci U S A* **93**(23): 12974-12979.

Jones, G. E., E. Prigmore, R. Calvez, C. Hogan, G. A. Dunn, E. Hirsch, M. P. Wymann and A. J. Ridley (2003). "Requirement for PI 3-kinase gamma in macrophage migration to MCP-1 and CSF-1." *Exp Cell Res* **290**(1): 120-131.

Kabbani, N., L. Negyessy, R. Lin, P. Goldman-Rakic and R. Levenson (2002). "Interaction with neuronal calcium sensor NCS-1 mediates desensitization of the D2 dopamine receptor." *J Neurosci* **22**(19): 8476-8486.

Kaksonen, M., Y. Sun and D. G. Drubin (2003). "A pathway for association of receptors, adaptors, and actin during endocytic internalization." *Cell* **115**(4): 475-487.

Kavelaars, A., A. Vroon, R. P. Raatgever, A. M. Fong, R. T. Premont, D. D. Patel, R. J. Lefkowitz and C. J. Heijnen (2003). "Increased acute inflammation, leukotriene B4-induced chemotaxis, and signaling in mice deficient for G protein-coupled receptor kinase 6." *J Immunol* **171**(11): 6128-6134.

Kessels, M. M., A. E. Engqvist-Goldstein, D. G. Drubin and B. Qualmann (2001). "Mammalian Abp1, a signal-responsive F-actin-binding protein, links the actin cytoskeleton to endocytosis via the GTPase dynamin." *J Cell Biol* **153**(2): 351-366.

Kikuchi, S., M. Hata, K. Fukumoto, Y. Yamane, T. Matsui, A. Tamura, S. Yonemura, H. Yamagishi, D. Keppler and S. Tsukita (2002). "Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes." *Nat Genet* **31**(3): 320-325.

Kim, J., S. Ahn, X. R. Ren, E. J. Whalen, E. Reiter, H. Wei and R. J. Lefkowitz (2005). "Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling." *Proc Natl Acad Sci U S A* **102**(5): 1442-1447.

Koch, W. J. and H. A. Rockman (1999). "Exploring the role of the beta-adrenergic receptor kinase in cardiac disease using gene-targeted mice." *Trends Cardiovasc Med* **9**(3-4): 77-81.

Kohout, T. A. and R. J. Lefkowitz (2003). "Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization." *Mol Pharmacol* **63**(1): 9-18.

Krasel, C., S. Dammeier, R. Winstel, J. Brockmann, H. Mischak and M. J. Lohse (2001). "Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin." *J Biol Chem* **276**(3): 1911-1915.

Krieg, J. and T. Hunter (1992). "Identification of the two major epidermal growth factor-induced tyrosine phosphorylation sites in the microvillar core protein ezrin." *J Biol Chem* **267**(27): 19258-19265.

Kunapuli, P., J. J. Onorato, M. M. Hosey and J. L. Benovic (1994). "Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5." *J Biol Chem* **269**(2): 1099-1105.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* **227**(5259): 680-685.

Lamaze, C., T. H. Chuang, L. J. Terlecky, G. M. Bokoch and S. L. Schmid (1996). "Regulation of receptor-mediated endocytosis by Rho and Rac." *Nature* **382**(6587): 177-179.

Laporte, S. A., R. H. Oakley, J. A. Holt, L. S. Barak and M. G. Caron (2000). "The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits." *J Biol Chem* **275**(30): 23120-23126.

Laporte, S. A., R. H. Oakley, J. Zhang, J. A. Holt, S. S. Ferguson, M. G. Caron and L. S. Barak (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis." *Proc Natl Acad Sci U S A* **96**(7): 3712-3717.

Laroche, G., M. D. Rochdi, S. A. Laporte and J. L. Parent (2005). "Involvement of actin in agonist-induced endocytosis of the G protein-coupled receptor for thromboxane A2: Overcoming of actin disruption by arrestin-3 but not arrestin-2." *J Biol Chem.*

Lee, E. and P. De Camilli (2002). "Dynamin at actin tails." *Proc Natl Acad Sci U S A* **99**(1): 161-166.

Legg, J. W., C. A. Lewis, M. Parsons, T. Ng and C. M. Isacke (2002). "A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility." *Nat Cell Biol* **4**(6): 399-407.

Li, J. G., C. Chen and L. Y. Liu-Chen (2002). "Ezrin-radixin-moesin-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate." *J Biol Chem* **277**(30): 27545-27552.

Lodowski, D. T., J. A. Pitcher, W. D. Capel, R. J. Lefkowitz and J. J. Tesmer (2003). "Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma." *Science* **300**(5623): 1256-1262.

Lohse, M. J., S. Andexinger, J. Pitcher, S. Trukawinski, J. Codina, J. P. Faure, M. G. Caron and R. J. Lefkowitz (1992). "Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems." *J Biol Chem* **267**(12): 8558-8564.

Lombardi, M. S., A. Kavelaars, M. Schedlowski, J. W. Bijlsma, K. L. Okihara, M. Van de Pol, S. Ochsmann, C. Pawlak, R. E. Schmidt and C. J. Heijnen (1999). "Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis." *Faseb J* **13**(6): 715-725.

Lunn, J. A., H. Wong, E. Rozengurt and J. H. Walsh (2000). "Requirement of cortical actin organization for bombesin, endothelin, and EGF receptor internalization." *Am J Physiol Cell Physiol* **279**(6): C2019-2027.

Lustig, K. D., B. R. Conklin, P. Herzmark, R. Taussig and H. R. Bourne (1993). "Type II adenylylcyclase integrates coincident signals from Gs, Gi, and Gq." *J Biol Chem* **268**(19): 13900-13905.

Luttrell, L. M., S. S. Ferguson, Y. Daaka, W. E. Miller, S. Maudsley, G. J. Della

Rocca, F. Lin, H. Kawakatsu, K. Owada, D. K. Luttrell, M. G. Caron and R. J. Lefkowitz (1999). "Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes." *Science* **283**(5402): 655-661.

Luttrell, L. M. and R. J. Lefkowitz (2002). "The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals." *J Cell Sci* **115**(Pt 3): 455-465.

Luttrell, L. M., F. L. Roudabush, E. W. Choy, W. E. Miller, M. E. Field, K. L. Pierce and R. J. Lefkowitz (2001). "Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds." *Proc Natl Acad Sci U S A* **98**(5): 2449-2454.

Lyubarsky, A. L., C. Chen, M. I. Simon and E. N. Pugh, Jr. (2000). "Mice lacking G-protein receptor kinase 1 have profoundly slowed recovery of cone-driven retinal responses." *J Neurosci* **20**(6): 2209-2217.

Macia, E., F. Luton, M. Partisani, J. Cherfils, P. Chardin and M. Franco (2004). "The GDP-bound form of Arf6 is located at the plasma membrane." *J Cell Sci* **117**(Pt 11): 2389-2398.

Mackay, D. J., F. Esch, H. Furthmayr and A. Hall (1997). "Rho- and rac-dependent assembly of focal adhesion complexes and actin filaments in permeabilized fibroblasts: an essential role for ezrin/radixin/moesin proteins." *J Cell Biol* **138**(4): 927-938.

Maddala, R., V. N. Reddy, D. L. Epstein and V. Rao (2003). "Growth factor induced activation of Rho and Rac GTPases and actin cytoskeletal reorganization in human lens epithelial cells." *Mol Vis* **9**: 329-336.

Magendantz, M., M. D. Henry, A. Lander and F. Solomon (1995). "Interdomain interactions of radixin in vitro." *J Biol Chem* **270**(43): 25324-25327.

Malecz, N., P. C. McCabe, C. Spaargaren, R. Qiu, Y. Chuang and M. Symons

(2000). "Synaptojanin 2, a novel Rac1 effector that regulates clathrin-mediated endocytosis." *Curr Biol* **10**(21): 1383-1386.

Manchanda, N., A. Lyubimova, H. Y. Ho, M. F. James, J. F. Gusella, N. Ramesh, S. B. Snapper and V. Ramesh (2005). "The NF2 tumor suppressor Merlin and the ERM proteins interact with N-WASP and regulate its actin polymerization function." *J Biol Chem* **280**(13): 12517-12522.

Marchese, A. and J. L. Benovic (2001). "Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting." *J Biol Chem* **276**(49): 45509-45512.

Matsui, T., M. Maeda, Y. Doi, S. Yonemura, M. Amano, K. Kaibuchi and S. Tsukita (1998). "Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association." *J Cell Biol* **140**(3): 647-657.

Matsui, T., S. Yonemura and S. Tsukita (1999). "Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases." *Curr Biol* **9**(21): 1259-1262.

Maudsley, S., A. M. Zamah, N. Rahman, J. T. Blitzer, L. M. Luttrell, R. J. Lefkowitz and R. A. Hall (2000). "Platelet-derived growth factor receptor association with Na(+)/H(+) exchanger regulatory factor potentiates receptor activity." *Mol Cell Biol* **20**(22): 8352-8363.

May, R. C. (2001). "The Arp2/3 complex: a central regulator of the actin cytoskeleton." *Cell Mol Life Sci* **58**(11): 1607-1626.

McGavin, M. K., K. Badour, L. A. Hardy, T. J. Kubiseski, J. Zhang and K. A. Siminovitch (2001). "The intersectin 2 adaptor links Wiskott Aldrich Syndrome protein (WASp)-mediated actin polymerization to T cell antigen receptor endocytosis." *J Exp Med* **194**(12): 1777-1787.

McNiven, M. A., L. Kim, E. W. Krueger, J. D. Orth, H. Cao and T. W. Wong (2000). "Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape." *J Cell Biol* **151**(1): 187-198.

Melendez, A. J., M. M. Harnett and J. M. Allen (2001). "Crosstalk between ARF6 and protein kinase Calpha in Fc(gamma)RI-mediated activation of phospholipase D1." *Curr Biol* **11**(11): 869-874.

Merrifield, C. J., M. E. Feldman, L. Wan and W. Almers (2002). "Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits." *Nat Cell Biol* **4**(9): 691-698.

Metaye, T., E. Menet, J. Guilhot and J. L. Kraimps (2002). "Expression and activity of g protein-coupled receptor kinases in differentiated thyroid carcinoma." *J Clin Endocrinol Metab* **87**(7): 3279-3286.

Miller, W. E., S. Maudsley, S. Ahn, K. D. Khan, L. M. Luttrell and R. J. Lefkowitz (2000). "beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis." *J Biol Chem* **275**(15): 11312-11319.

Miyata, Y., E. Nishida, S. Koyasu, I. Yahara and H. Sakai (1989). "Protein kinase C-dependent and -independent pathways in the growth factor-induced cytoskeletal reorganization." *J Biol Chem* **264**(26): 15565-15568.

Moon, S. Y. and Y. Zheng (2003). "Rho GTPase-activating proteins in cell regulation." *Trends Cell Biol* **13**(1): 13-22.

Mu, F. T., J. M. Callaghan, O. Steele-Mortimer, H. Stenmark, R. G. Parton, P. L. Campbell, J. McCluskey, J. P. Yeo, E. P. Tock and B. H. Toh (1995). "EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif." *J Biol Chem* **270**(22): 13503-13511.

Mukherjee, S., V. V. Gurevich, A. Preninger, H. E. Hamm, M. F. Bader, A. T. Fazleabas, L. Birnbaumer and M. Hunzicker-Dunn (2002). "Aspartic acid 564 in the third cytoplasmic loop of the luteinizing hormone/choriogonadotropin receptor is crucial for phosphorylation-independent interaction with arrestin2." *J Biol Chem* **277**(20): 17916-17927.

Muller, S., M. Hekman and M. J. Lohse (1993). "Specific enhancement of beta-adrenergic receptor kinase activity by defined G-protein beta and gamma subunits." *Proc Natl Acad Sci U S A* **90**(22): 10439-10443.

Mullins, R. D. (2000). "How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures." *Curr Opin Cell Biol* **12**(1): 91-96.

Nakamura, F., L. Huang, K. Pestonjamasp, E. J. Luna and H. Furthmayr (1999). "Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides." *Mol Biol Cell* **10**(8): 2669-2685.

Nakamura, N., N. Oshiro, Y. Fukata, M. Amano, M. Fukata, S. Kuroda, Y. Matsuura, T. Leung, L. Lim and K. Kaibuchi (2000). "Phosphorylation of ERM proteins at filopodia induced by Cdc42." *Genes Cells* **5**(7): 571-581.

Ng, T., M. Parsons, W. E. Hughes, J. Monypenny, D. Zicha, A. Gautreau, M. Arpin, S. Gschmeissner, P. J. Verveer, P. I. Bastiaens and P. J. Parker (2001). "Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility." *Embo J* **20**(11): 2723-2741.

Niggli, V., C. Andreoli, C. Roy and P. Mangeat (1995). "Identification of a phosphatidylinositol-4,5-bisphosphate-binding domain in the N-terminal region of ezrin." *FEBS Lett* **376**(3): 172-176.

Nobes, C. D. and A. Hall (1995). "Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia." *Cell* **81**(1): 53-62.

Offermanns, S., T. Wieland, D. Homann, J. Sandmann, E. Bombien, K. Spicher, G. Schultz and K. H. Jakobs (1994). "Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11." *Mol Pharmacol* **45**(5): 890-898.

Okamoto, I., Y. Kawano, M. Matsumoto, M. Suga, K. Kaibuchi, M. Ando and H. Saya (1999). "Regulated CD44 cleavage under the control of protein kinase C, calcium influx, and the Rho family of small G proteins." *J Biol Chem* **274**(36): 25525-25534.

Olofsson, B. (1999). "Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling." *Cell Signal* **11**(8): 545-554.

Onoprishvili, I., M. L. Andria, H. K. Kramer, N. Ancevska-Taneva, J. M. Hiller and E. J. Simon (2003). "Interaction between the mu opioid receptor and filamin A is involved in receptor regulation and trafficking." *Mol Pharmacol* **64**(5): 1092-1100.

Oppermann, M., M. Diverse-Pierluissi, M. H. Drazner, S. L. Dyer, N. J. Freedman, K. C. Peppel and R. J. Lefkowitz (1996). "Monoclonal antibodies reveal receptor specificity among G-protein-coupled receptor kinases." *Proc Natl Acad Sci U S A* **93**(15): 7649-7654.

Orsini, M. J., J. L. Parent, S. J. Mundell, J. L. Benovic and A. Marchese (1999). "Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization." *J Biol Chem* **274**(43): 31076-31086.

Osterweil, E., D. G. Wells and M. S. Mooseker (2005). "A role for myosin VI in postsynaptic structure and glutamate receptor endocytosis." *J Cell Biol* **168**(2): 329-338.

Ozaita, A., P. V. Escriba, P. Ventayol, C. Murga, F. Mayor, Jr. and J. A. Garcia-Sevilla (1998). "Regulation of G protein-coupled receptor kinase 2 in brains of opiate-treated rats and human opiate addicts." *J Neurochem* **70**(3): 1249-1257.

Paasche, J. D., T. Attramadal, K. Kristiansen, M. P. Oksvold, H. K. Johansen, H. S. Huitfeldt, S. G. Dahl and H. Attramadal (2005). "Subtype-Specific Sorting of the ETA Endothelin Receptor by a Novel Endocytic Recycling Signal for G Protein-Coupled Receptors." *Mol Pharmacol* **67**(5): 1581-1590.

Palczewski, K., T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto and M. Miyano (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." *Science* **289**(5480): 739-745.

Pals-Rylaarsdam, R., Y. Xu, P. Witt-Enderby, J. L. Benovic and M. M. Hosey (1995). "Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms." *J Biol Chem* **270**(48): 29004-29011.

Pao, C. S. and J. L. Benovic (2002). "Phosphorylation-independent desensitization of G protein-coupled receptors?" *Sci STKE* **2002**(153): PE42.

Pao, C. S. and J. L. Benovic (2005). "Structure/function analysis of alpha2A-adrenergic receptor interaction with G protein-coupled receptor kinase 2." *J Biol Chem* **280**(12): 11052-11058.

Pearson, M. A., D. Reczek, A. Bretscher and P. A. Karplus (2000). "Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain." *Cell* **101**(3): 259-270.

Penela, P., A. Elorza, S. Sarnago and F. Mayor, Jr. (2001a). "Beta-arrestin- and c-Src-dependent degradation of G-protein-coupled receptor kinase 2." *Embo J* **20**(18): 5129-5138.

Penela, P., M. Barradas, M. Alvarez-Dolado, A. Munoz and F. Mayor, Jr. (2001b). "Effect of hypothyroidism on G protein-coupled receptor kinase 2 expression levels in rat liver, lung, and heart." *Endocrinology* **142**(3): 987-991.

Penela, P., C. Ribas and F. Mayor, Jr. (2003). "Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases." *Cell Signal* **15**(11): 973-981.

Penn, R. B., A. N. Pronin and J. L. Benovic (2000). "Regulation of G protein-coupled receptor kinases." *Trends Cardiovasc Med* **10**(2): 81-89.

Peppel, K., I. Boekhoff, P. McDonald, H. Breer, M. G. Caron and R. J. Lefkowitz (1997). "G protein-coupled receptor kinase 3 (GRK3) gene disruption leads to loss of odorant receptor desensitization." *J Biol Chem* **272**(41): 25425-25428.

Perroy, J., L. Adam, R. Qanbar, S. Chenier and M. Bouvier (2003). "Phosphorylation-independent desensitization of GABA(B) receptor by GRK4." *Embo J* **22**(15): 3816-3824.

Petrofski, J. A. and W. J. Koch (2003). "The beta-adrenergic receptor kinase in heart failure." *J Mol Cell Cardiol* **35**(10): 1167-1174.

Pierce, K. L., R. T. Premont and R. J. Lefkowitz (2002). "Seven-transmembrane receptors." *Nat Rev Mol Cell Biol* **3**(9): 639-650.

Pierce, K. L., A. Tohgo, S. Ahn, M. E. Field, L. M. Luttrell and R. J. Lefkowitz (2001). "Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding." *J Biol Chem* **276**(25): 23155-23160.

Pietromonaco, S. F., P. C. Simons, A. Altman and L. Elias (1998). "Protein kinase C-theta phosphorylation of moesin in the actin-binding sequence." *J Biol Chem* **273**(13): 7594-7603.

Pitcher, J. A., Z. L. Fredericks, W. C. Stone, R. T. Premont, R. H. Stoffel, W. J. Koch and R. J. Lefkowitz (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." *J Biol Chem* **271**(40): 24907-24913.

Pitcher, J. A., R. A. Hall, Y. Daaka, J. Zhang, S. S. Ferguson, S. Hester, S. Miller, M. G. Caron, R. J. Lefkowitz and L. S. Barak (1998a). "The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin." *J Biol Chem* **273**(20): 12316-12324.

Pitcher, J. A., N. J. Freedman and R. J. Lefkowitz (1998b). "G protein-coupled receptor kinases." *Annu Rev Biochem* **67**: 653-692.

Pitcher, J. A., J. J. Tesmer, J. L. Freeman, W. D. Capel, W. C. Stone and R. J. Lefkowitz (1999). "Feedback inhibition of G protein-coupled receptor kinase 2 (GRK2) activity by extracellular signal-regulated kinases." *J Biol Chem* **274**(49): 34531-34534.

Pitcher, J. A., K. Touhara, E. S. Payne and R. J. Lefkowitz (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**(20): 11707-11710.

Polesello, C. and F. Payre (2004). "Small is beautiful: what flies tell us about ERM protein function in development." *Trends Cell Biol* **14**(6): 294-302.

Pollard, T. D. and G. G. Borisy (2003). "Cellular motility driven by assembly and disassembly of actin filaments." *Cell* **112**(4): 453-465.

Premont, R. T., A. Claing, N. Vitale, J. L. Freeman, J. A. Pitcher, W. A. Patton, J. Moss, M. Vaughan and R. J. Lefkowitz (1998). "beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein." *Proc Natl Acad Sci U S A* **95**(24): 14082-14087.

Premont, R. T., A. Claing, N. Vitale, S. J. Perry and R. J. Lefkowitz (2000). "The

GIT family of ADP-ribosylation factor GTPase-activating proteins. Functional diversity of GIT2 through alternative splicing." *J Biol Chem* **275**(29): 22373-22380.

Pronin, A. N. and J. L. Benovic (1997). "Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C." *J Biol Chem* **272**(6): 3806-3812.

Pronin, A. N., C. V. Carman and J. L. Benovic (1998). "Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation." *J Biol Chem* **273**(47): 31510-31518.

Pronin, A. N., A. J. Morris, A. Surguchov and J. L. Benovic (2000). "Synucleins are a novel class of substrates for G protein-coupled receptor kinases." *J Biol Chem* **275**(34): 26515-26522.

Pronin, A. N., D. K. Satpaev, V. Z. Slepak and J. L. Benovic (1997). "Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain." *J Biol Chem* **272**(29): 18273-18280.

Qualmann, B. and H. Mellor (2003). "Regulation of endocytic traffic by Rho GTPases." *Biochem J* **371**(Pt 2): 233-241.

Radhakrishna, H., O. Al-Awar, Z. Khachikian and J. G. Donaldson (1999). "ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements." *J Cell Sci* **112 (Pt 6)**: 855-866.

Raftopoulou, M. and A. Hall (2004). "Cell migration: Rho GTPases lead the way." *Dev Biol* **265**(1): 23-32.

Raiborg, C., T. E. Rusten and H. Stenmark (2003). "Protein sorting into multivesicular endosomes." *Curr Opin Cell Biol* **15**(4): 446-455.

Reczek, D., M. Berryman and A. Bretscher (1997). "Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family." *J Cell Biol* **139**(1): 169-179.

Reczek, D. and A. Bretscher (1998). "The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule." *J Biol Chem* **273**(29): 18452-18458.

Ren, X. D. and M. A. Schwartz (1998). "Regulation of inositol lipid kinases by Rho and Rac." *Curr Opin Genet Dev* **8**(1): 63-67.

Richardson, R. M., R. J. Marjoram, L. S. Barak and R. Snyderman (2003). "Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation." *J Immunol* **170**(6): 2904-2911.

Ridley, A. J. and A. Hall (1992a). "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors." *Cell* **70**(3): 389-399.

Ridley, A. J. and A. Hall (1992b). "Distinct patterns of actin organization regulated by the small GTP-binding proteins Rac and Rho." *Cold Spring Harb Symp Quant Biol* **57**: 661-671.

Rockman, H. A., D. J. Choi, S. A. Akhter, M. Jaber, B. Giros, R. J. Lefkowitz, M. G. Caron and W. J. Koch (1998). "Control of myocardial contractile function by the level of beta-adrenergic receptor kinase 1 in gene-targeted mice." *J Biol Chem* **273**(29): 18180-18184.

Rockman, H. A., W. J. Koch and R. J. Lefkowitz (2002). "Seven-transmembrane-spanning receptors and heart function." *Nature* **415**(6868): 206-212.

Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa and M. W. Kirschner (1999). "The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly." *Cell* **97**(2): 221-231.

Roseberry, A. G. and M. M. Hosey (2001). "Internalization of the M2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is

independent of clathrin and caveolae." *J Cell Sci* **114**(Pt 4): 739-746.

Roy, C., M. Martin and P. Mangeat (1997). "A dual involvement of the amino-terminal domain of ezrin in F- and G-actin binding." *J Biol Chem* **272**(32): 20088-20095.

Ruiz-Gomez, A., J. Humrich, C. Murga, U. Quitterer, M. J. Lohse and F. Mayor, Jr. (2000). "Phosphorylation of phosducin and phosducin-like protein by G protein-coupled receptor kinase 2." *J Biol Chem* **275**(38): 29724-29730.

Ruiz-Gomez, A. and F. Mayor, Jr. (1997). "Beta-adrenergic receptor kinase (GRK2) colocalizes with beta-adrenergic receptors during agonist-induced receptor internalization." *J Biol Chem* **272**(15): 9601-9604.

Sallese, M., L. Iacovelli, A. Cumashi, L. Capobianco, L. Cuomo and A. De Blasi (2000a). "Regulation of G protein-coupled receptor kinase subtypes by calcium sensor proteins." *Biochim Biophys Acta* **1498**(2-3): 112-121.

Sallese, M., S. Mariggio, E. D'Urbano, L. Iacovelli and A. De Blasi (2000b). "Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq." *Mol Pharmacol* **57**(4): 826-831.

Saotome, I., M. Curto and A. I. McClatchey (2004). "Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine." *Dev Cell* **6**(6): 855-864.

Sarnago, S., A. Elorza and F. Mayor, Jr. (1999). "Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase." *J Biol Chem* **274**(48): 34411-34416.

Sarnago, S., R. Roca, A. de Blasi, A. Valencia, F. Mayor, Jr. and C. Murga (2003). "Involvement of intramolecular interactions in the regulation of G protein-coupled receptor kinase 2." *Mol Pharmacol* **64**(3): 629-639.

Sasaki, T. and Y. Takai (1998). "The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control." *Biochem Biophys Res Commun* **245**(3): 641-645.

Schafer, D. A. (2002). "Coupling actin dynamics and membrane dynamics during endocytosis." *Curr Opin Cell Biol* **14**(1): 76-81.

Schmidt, A. and A. Hall (2002). "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." *Genes Dev* **16**(13): 1587-1609.

Schmieder, S., M. Nagai, R. A. Orlando, T. Takeda and M. G. Farquhar (2004). "Podocalyxin activates RhoA and induces actin reorganization through NHERF1 and Ezrin in MDCK cells." *J Am Soc Nephrol* **15**(9): 2289-2298.

Schulz, R., A. Wehmeyer and K. Schulz (2002). "Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3." *J Pharmacol Exp Ther* **300**(2): 376-384.

Scott, M. G., A. Benmerah, O. Muntaner and S. Marullo (2002). "Recruitment of activated G protein-coupled receptors to pre-existing clathrin-coated pits in living cells." *J Biol Chem* **277**(5): 3552-3559.

Seck, T., R. Baron and W. C. Horne (2003). "Binding of filamin to the C-terminal tail of the calcitonin receptor controls recycling." *J Biol Chem* **278**(12): 10408-10416.

Sefton, M., M. J. Blanco, P. Penela, F. Mayor and M. A. Nieto (2000). "Expression of the G protein-coupled receptor kinase 2 during early mouse embryogenesis." *Mech Dev* **98**(1-2): 127-131.

Sekiya-Kawasaki, M., A. C. Groen, M. J. Cope, M. Kaksonen, H. A. Watson, C. Zhang, K. M. Shokat, B. Wendland, K. L. McDonald, J. M. McCaffery and D. G. Drubin (2003). "Dynamic phosphoregulation of the cortical actin cytoskeleton and

endocytic machinery revealed by real-time chemical genetic analysis." *J Cell Biol* **162**(5): 765-772.

Shenoy, S. K. and R. J. Lefkowitz (2003). "Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling." *Biochem J* **375**(Pt 3): 503-515.

Shenoy, S. K., P. H. McDonald, T. A. Kohout and R. J. Lefkowitz (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." *Science* **294**(5545): 1307-1313.

Shetzline, M. A., J. K. Walker, K. J. Valenzano and R. T. Premont (2002). "Vasoactive intestinal polypeptide type-1 receptor regulation. Desensitization, phosphorylation, and sequestration." *J Biol Chem* **277**(28): 25519-25526.

Shiina, T., K. Arai, S. Tanabe, N. Yoshida, T. Haga, T. Nagao and H. Kurose (2001). "Clathrin box in G protein-coupled receptor kinase 2." *J Biol Chem* **276**(35): 33019-33026.

Sierra, D. A., S. Popov and T. M. Wilkie (2000). "Regulators of G-protein signaling in receptor complexes." *Trends Cardiovasc Med* **10**(6): 263-268.

Simons, P. C., S. F. Pietromonaco, D. Reczek, A. Bretscher and L. Elias (1998). "C-terminal threonine phosphorylation activates ERM proteins to link the cell's cortical lipid bilayer to the cytoskeleton." *Biochem Biophys Res Commun* **253**(3): 561-565.

Sitaraman, S. V., L. Wang, M. Wong, M. Bruewer, M. Hobert, C. H. Yun, D. Merlin and J. L. Madara (2002). "The adenosine 2b receptor is recruited to the plasma membrane and associates with E3KARP and Ezrin upon agonist stimulation." *J Biol Chem* **277**(36): 33188-33195.

Small, J. V., T. Stradal, E. Vignal and K. Rottner (2002). "The lamellipodium: where motility begins." *Trends Cell Biol* **12**(3): 112-120.

Smith, P. M., A. Cowan and B. A. White (2004). "The low-density lipoprotein receptor is regulated by estrogen and forms a functional complex with the estrogen-regulated protein ezrin in pituitary GH3 somatolactotropes." *Endocrinology* **145**(7): 3075-3083.

Sneddon, W. B., C. A. Syme, A. Bisello, C. E. Magyar, M. D. Rochdi, J. L. Parent, E. J. Weinman, A. B. Abou-Samra and P. A. Friedman (2003). "Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50)." *J Biol Chem* **278**(44): 43787-43796.

Soulet, F., D. Yarar, M. Leonard and S. L. Schmid (2005). "SNX9 Regulates Dynamin Assembly and Is Required for Efficient Clathrin-mediated Endocytosis." *Mol Biol Cell* **16**(4): 2058-2067.

Srivastava, J., B. E. Elliott, D. Louvard and M. Arpin (2005). "Src-dependent ezrin phosphorylation in adhesion-mediated signaling." *Mol Biol Cell* **16**(3): 1481-1490.

Stapleton, G., A. Malliri and B. W. Ozanne (2002). "Downregulated AP-1 activity is associated with inhibition of Protein-Kinase-C-dependent CD44 and ezrin localisation and upregulation of PKC theta in A431 cells." *J Cell Sci* **115**(Pt 13): 2713-2724.

Sterne-Marr, R., J. J. Tesmer, P. W. Day, R. P. Stracquatanio, J. A. Cilente, K. E. O'Connor, A. N. Pronin, J. L. Benovic and P. B. Wedegaertner (2003). "G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signaling homology domain for binding G alpha subunits." *J Biol Chem* **278**(8): 6050-6058.

Sun, Y., Z. Cheng, L. Ma and G. Pei (2002). "Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation." *J Biol Chem* **277**(51): 49212-49219.

Takahashi, K., T. Sasaki, A. Mammoto, K. Takaishi, T. Kameyama, S. Tsukita and Y. Takai (1997). "Direct interaction of the Rho GDP dissociation inhibitor with

ezrin/radixin/moesin initiates the activation of the Rho small G protein." *J Biol Chem* **272**(37): 23371-23375.

Takayama, N. and H. Ueda (2005). "Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia." *J Neurosci* **25**(2): 430-435.

Takei, K. and V. Haucke (2001). "Clathrin-mediated endocytosis: membrane factors pull the trigger." *Trends Cell Biol* **11**(9): 385-391.

Tamura, A., S. Kikuchi, M. Hata, T. Katsuno, T. Matsui, H. Hayashi, Y. Suzuki, T. Noda and S. Tsukita (2005). "Achlorhydria by ezrin knockdown: defects in the formation/expansion of apical canaliculi in gastric parietal cells." *J Cell Biol* **169**(1): 21-28.

Terman, G. W., W. Jin, Y. P. Cheong, J. Lowe, M. G. Caron, R. J. Lefkowitz and C. Chavkin (2004). "G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal." *Br J Pharmacol* **141**(1): 55-64.

Terwilliger, R. Z., J. Ortiz, X. Guitart and E. J. Nestler (1994). "Chronic morphine administration increases beta-adrenergic receptor kinase (beta ARK) levels in the rat locus coeruleus." *J Neurochem* **63**(5): 1983-1986.

Tobin, A. B. (2002). "Are we beta-ARKing up the wrong tree? Casein kinase 1 alpha provides an additional pathway for GPCR phosphorylation." *Trends Pharmacol Sci* **23**(7): 337-343.

Toshima, J., J. Y. Toshima, A. C. Martin and D. G. Drubin (2005). "Phosphoregulation of Arp2/3-dependent actin assembly during receptor-mediated endocytosis." *Nat Cell Biol* **7**(3): 246-254.

Trejo, J. and S. R. Coughlin (1999). "The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling." *J Biol Chem* **274**(4): 2216-2224.

Tsukita, S. and Y. Hieda (1989). "A new 82-kD barbed end-capping protein (radixin) localized in the cell-to-cell adherens junction: purification and characterization." *J Cell Biol* **108**(6): 2369-2382.

Tsukita, S. and S. Yonemura (1999). "Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins." *J Biol Chem* **274**(49): 34507-34510.

Turner, C. E., M. C. Brown, J. A. Perrotta, M. C. Riedy, S. N. Nikolopoulos, A. R. McDonald, S. Bagrodia, S. Thomas and P. S. Leventhal (1999). "Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling." *J Cell Biol* **145**(4): 851-863.

van der Bliek, A. M., T. E. Redelmeier, H. Damke, E. J. Tisdale, E. M. Meyerowitz and S. L. Schmid (1993). "Mutations in human dynamin block an intermediate stage in coated vesicle formation." *J Cell Biol* **122**(3): 553-563.

Vogler, O., B. Nolte, M. Voss, M. Schmidt, K. H. Jakobs and C. J. van Koppen (1999). "Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin." *J Biol Chem* **274**(18): 12333-12338.

Voltz, J. W., E. J. Weinman and S. Shenolikar (2001). "Expanding the role of NHERF, a PDZ-domain containing protein adapter, to growth regulation." *Oncogene* **20**(44): 6309-6314.

von Zastrow, M. (2003). "Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway." *Life Sci* **74**(2-3): 217-224.

Vroon, A., C. J. Heijnen, R. Raatgever, I. P. Touw, R. E. Ploemacher, R. T. Premont and A. Kavelaars (2004). "GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and impaired responsiveness to G-CSF in vivo." *J Leukoc Biol* **75**(4): 698-704.

Vroon, A., A. Kavelaars, V. Limmroth, M. S. Lombardi, M. U. Goebel, A. M. Van Dam, M. G. Caron, M. Schedlowski and C. J. Heijnen (2005). "G protein-coupled

receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis." *J Immunol* **174**(7): 4400-4406.

Walker, J. K., R. R. Gainetdinov, D. S. Feldman, P. K. McFawn, M. G. Caron, R. J. Lefkowitz, R. T. Premont and J. T. Fisher (2004). "G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation." *Am J Physiol Lung Cell Mol Physiol* **286**(2): L312-319.

Walker, J. K., K. Peppel, R. J. Lefkowitz, M. G. Caron and J. T. Fisher (1999). "Altered airway and cardiac responses in mice lacking G protein-coupled receptor kinase 3." *Am J Physiol* **276**(4 Pt 2): R1214-1221.

Wan, K. F., B. S. Sambi, M. Frame, R. Tate and N. J. Pyne (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." *J Biol Chem* **276**(41): 37802-37808.

Wan, K. F., B. S. Sambi, R. Tate, C. Waters and N. J. Pyne (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." *J Biol Chem* **278**(20): 18658-18663.

Wang, F., J. R. Van Brocklyn, J. P. Hobson, S. Movafagh, Z. Zukowska-Grojec, S. Milstien and S. Spiegel (1999). "Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis." *J Biol Chem* **274**(50): 35343-35350.

Wang, Y., Y. Zhou, K. Szabo, C. R. Haft and J. Trejo (2002). "Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1." *Mol Biol Cell* **13**(6): 1965-1976.

Wasiak, S., C. C. Quinn, B. Ritter, E. de Heuvel, D. Baranes, M. Plomann and P. S.

McPherson (2001). "The Ras/Rac guanine nucleotide exchange factor mammalian Son-of-sevenless interacts with PACSIN 1/syndapin I, a regulator of endocytosis and the actin cytoskeleton." *J Biol Chem* **276**(28): 26622-26628.

Weaver, A. M., A. V. Karginov, A. W. Kinley, S. A. Weed, Y. Li, J. T. Parsons and J. A. Cooper (2001). "Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation." *Curr Biol* **11**(5): 370-374.

Wei, L. N. and H. H. Loh (2002). "Regulation of opioid receptor expression." *Curr Opin Pharmacol* **2**(1): 69-75.

Wess, J. (2004). "Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications." *Annu Rev Pharmacol Toxicol* **44**: 423-450.

West, K. A., H. Zhang, M. C. Brown, S. N. Nikolopoulos, M. C. Riedy, A. F. Horwitz and C. E. Turner (2001). "The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL)." *J Cell Biol* **154**(1): 161-176.

Wherlock, M. and H. Mellor (2002). "The Rho GTPase family: a Racs to Wrchs story." *J Cell Sci* **115**(Pt 2): 239-240.

Willets, J. M., R. A. Challiss and S. R. Nahorski (2003). "Non-visual GRKs: are we seeing the whole picture?" *Trends Pharmacol Sci* **24**(12): 626-633.

Winstel, R., S. Freund, C. Krasel, E. Hoppe and M. J. Lohse (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**(5): 2105-2109.

Witherow, D. S. and V. Z. Slepak (2003). "A novel kind of G protein heterodimer: the G beta5-RGS complex." *Receptors Channels* **9**(3): 205-212.

Yamamoto, S., K. C. Sippel, E. L. Berson and T. P. Dryja (1997). "Defects in the rhodopsin kinase gene in the Oguchi form of stationary night blindness." *Nat Genet*

15(2): 175-178.

Yang, H. S. and P. W. Hinds (2003). "Increased ezrin expression and activation by CDK5 coincident with acquisition of the senescent phenotype." *Mol Cell* **11**(5): 1163-1176.

Yarar, D., C. M. Waterman-Storer and S. L. Schmid (2005). "A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis." *Mol Biol Cell* **16**(2): 964-975.

Yin, G., J. Haendeler, C. Yan and B. C. Berk (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**(2): 875-885.

Yokoyama, T., H. Goto, I. Izawa, H. Mizutani and M. Inagaki (2005). "Aurora-B and Rho-kinase/ROCK, the two cleavage furrow kinases, independently regulate the progression of cytokinesis: possible existence of a novel cleavage furrow kinase phosphorylates ezrin/radixin/moesin (ERM)." *Genes Cells* **10**(2): 127-137.

Yonemura, S., T. Matsui and S. Tsukita (2002). "Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo." *J Cell Sci* **115**(Pt 12): 2569-2580.

Yonemura, S. and S. Tsukita (1999). "Direct involvement of ezrin/radixin/moesin (ERM)-binding membrane proteins in the organization of microvilli in collaboration with activated ERM proteins." *J Cell Biol* **145**(7): 1497-1509.

Yu, B., L. Gu and M. I. Simon (2000). "Inhibition of Subsets of G Protein-coupled Receptors by Empty Mutants of G Protein α Subunits in Go, G11 and G16." *J Biol Chem* **275**(1): 71-76.

Zamah, A. M., M. Delahunty, L. M. Luttrell and R. J. Lefkowitz (2002). "Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system." *J Biol Chem*

277(34): 31249-31256.

Zaslaver, A., R. Feniger-Barish and A. Ben-Baruch (2001). "Actin filaments are involved in the regulation of trafficking of two closely related chemokine receptors, CXCR1 and CXCR2." *J Immunol* **166**(2): 1272-1284.

Zhang, J., S. S. Ferguson, L. S. Barak, L. Menard and M. G. Caron (1996). "Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization." *J Biol Chem* **271**(31): 18302-18305.

Zhang, Y. and M. L. Dufau (2003). "Dual mechanisms of regulation of transcription of luteinizing hormone receptor gene by nuclear orphan receptors and histone deacetylase complexes." *J Steroid Biochem Mol Biol* **85**(2-5): 401-414.

Zhao, Z. S., E. Manser, T. H. Loo and L. Lim (2000). "Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly." *Mol Cell Biol* **20**(17): 6354-6363.

Zhou, R., X. Cao, C. Watson, Y. Miao, Z. Guo, J. G. Forte and X. Yao (2003). "Characterization of protein kinase A-mediated phosphorylation of ezrin in gastric parietal cell activation." *J Biol Chem* **278**(37): 35651-35659.

Zhu, J., K. Zhou, J. J. Hao, J. Liu, N. Smith and X. Zhan (2005). "Regulation of cortactin/dynamin interaction by actin polymerization during the fission of clathrin-coated pits." *J Cell Sci* **118**(Pt 4): 807-817.