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MEK1 binds β arrestin1 directly, influencing both its phosphorylation by ERK and the timing of its isoprenaline-stimulated internalization.

A thesis submitted to the

FACULTY OF BIOMEDICAL AND LIFE SCIENCES

for the degree of

DOCTOR OF PHILOSOPHY

by

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Neuroscience and Molecular Pharmacology
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September 2009

Declaration

I declare that the work described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

Dong Meng

September 2009

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Abstract

cAMP is a well studied second messenger that is ubiquitously expressed in mammals. It conducts its function by activating its downstream effectors: protein kinase A (PKA), exchange proteins regulated by cAMP (EPAC) and cyclic nucleotide gated ion-channels. The sole mechanism to inactivate cAMP is through degradation via cyclic-phosphodiesterases (PDEs). The PDEs, especially PDE4s, are involved in many diseases including asthma, chronic obstructive pulmonary disease (COPD) and depression. Therefore, PDEs have been a consistently popular research subject for decades and pharmaceutical companies have devoted considerable effort in developing PDE inhibitors. β -arrestin interacts with PDE4D5 and is a multifunctional protein that plays pivotal roles in signal transduction. It functions as an adaptor protein in the c-Raf/MEK/ERK cascade by interacting with c-Raf and ERK.

In this study, I have shown that (1) β -arrestin1 can bind MEK1 directly, mediated at least in part by D²⁶D²⁹ of β -arrestin1 and R⁴⁷K⁴⁸R⁴⁹ of MEK1. (2) Disruption of this association by mutagenesis or small peptides decreases the phosphorylation of Ser412 on β -arrestin1, and accelerates isoprenaline-stimulated G-protein coupled receptor (GPCR) internalization.

Dimerization of PDEs is considered important for their specificity. In this study, yeast two hybrid and co-immunoprecipitation were utilised to demonstrate that (1) PDE4D5 can form stable homodimers in both yeast and mammalian cell lines. (2) The dimerisation requires multiple interaction sites such as R173/N174/N175, E228/T229/L230, L306/M307/H308 and K323/T324/E325 (together named QUAD) in which R173/N174/N175 contributes most toward the dimerisation. (3) Association of an ion-pair R499/D463 also proved to be a necessary condition for dimer formation. (4) PDE4D5 dimerisation can be affected by challenge with the PDE inhibitor, anisomycin, and cAMP elevating agonists forskolin (Fsk) and isobutylmethylxanthine (IBMX).

RACK1, another PDE4D5 binding partner, mediates and initiates cell migration in many cell types and affects the activity of the c-Jun NH2-terminal kinase (JNK) signalling pathway, via its interaction with PKC. SUMOylation of proteins is an important method of regulation. In the current study, preliminary investigations

were undertaken to determine whether RACK1 is SUMOylated. SUMOylation of K²⁷¹ of a 25-mer peptide sequence from RACK1 was observed, yet there was no SUMOylation of RACK1 observed in HEK293 cells in the presence or absence of overexpressed E3 ligases.

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Abbreviations

7MSR	seven-membrane-spanning receptor
AC	adenylyl cyclase
ADAM	alkenyldiarylmethanes
AKAP	A kinase anchoring protein
ARF	ADP-ribosylation factor
ARNO	ARF nucleotide binding site opener
ASK	apoptosis signal-regulating kinase
ATP	adenosine trisphosphate
β_2 AR	β_2 adrenergic receptor
BSA	bovine serum albumin
$\text{Ca}^{2+}/\text{CaM}$	calcium/calmodulin
CamKII	calcium/calmodulin-dependent kinase II
cAMP	cyclic 3'5' adenosine monophosphate
CDK	cyclin-dependent kinases
COPD	chronic obstructive pulmonary disease
Cpc	cross-pathway-control
CRE	cAMP response element
CREB	cAMP response element binding protein
CXCR4	CXC chemokine receptor 4
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
DISC1	disrupted in schizophrenia 1
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOR	Delta-opioid receptor
DTT	dithiothreitol
EC	excitation-contraction
ECM	extracellular matrix
EDTA	Diaminoethanetetra-acetic acid
EGF	epidermal growth factor
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
ETAR	endothelin type A receptor
FAK	focal adhesion kinase
FCS	foetal calf serum
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide binding regulatory protein
GRK	G-protein receptor specific kinase
GST	Glutathione S-transferase
GTP	guanosine triphosphate
HEK	Human embryo kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBMX	isobutylmethylxanthine
IGF-IR	insulin-like growth factor I receptor
IL	interleukin
JNK	c-Jun NH2-terminal kinase

kb	kilobase
kDa	kiloDaulton
l	litre
LPS	lipopolysaccharide
LR	linker region
M	molar
mg	milligram
MAP	kinase mitogen-activated protein kinase
MEK	MAPK kinase
min	minute
mRNA	messenger RNA
NdeI	nuclear distribution element-like
PA	phosphatidic acid
PAS	Per-ARNT-Sim
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PGE2	prostaglandin E2
PI3K	phosphatidylinositol-3-OH kinase
PKA	protein kinase A
PKC	protein kinase C
PP2A	protein phosphatase 2A
RACK1	receptor for activated C kinase 1
RAID1	RACK1 interaction domain
RNA	ribonucleic acid
rpm	revolutions per minute
RyR	ryanodine receptor
SBE	Smad binding element
SDS	sodium dodecyl sulphate
SH2	domain Src homology 2 domain
SH3	domain Src homology 3 domain
TAE	tris/acetate/EDTA
TBS	tris buffered saline
TNFR	tumour necrosis factor receptor
Ub	ubiquitin
UCR	upstream conserved region
VSMC	vascular smooth muscle cell

1 General Introduction

1.1 Cyclic Nucleotide Phosphodiesterase

Cyclic AMP serves as a second messenger that plays an important role in cell signaling. It controls the action of a series of hormones and neurotransmitters, and impacts on cell growth, differentiation, survival and inflammatory processes. It is produced from ATP by adenylate cyclase, an integral cell membrane protein that exists as a number of isoforms. The various adenylate cyclase isoforms have distinct regulatory properties, expression patterns and localization in different parts of cell plasma membrane (Houslay 2001). There are two main ways to regulate adenylate cyclase, both of which are G-protein dependent. The first pathway depends on the activation of G_s . The activated G_s stimulates adenylate cyclase activity, which leads to an increase in cAMP levels. Cyclic AMP then activates downstream signalling factors such as protein kinase A (PKA), EPAC and cyclic nucleotide gated ion-channels (Rubin 1994). In contrast, the second pathway depends on the activation of G_i , which leads to an inhibition of adenylate cyclase activity. However, the only way to inactivate cAMP is to degrade it via the action of cAMP phosphodiesterases (PDEs) (Houslay 2001).

1.1.1 Cyclic Nucleotide Phosphodiesterase Families

There are at least 11 families of PDE which have been discovered at present (Table 1.1). These isoenzymes can hydrolyze cAMP, cGMP or both. According to their primary structures, different substrates, inhibitors and different ways of regulation, each PDE family can extend to a series of subfamilies (Houslay and Milligan 1997)

PDE family	Genes	Substrates	Selective Inhibitors	Regulation	Domain pairs
PDE1	1A,B, C	cAMP & cGMP	Nicardipine,Vinopectine	Ca/Cam,PKA/PKG	CaM binding domain
PDE2	2A	cAMP & cGMP	EHNA	Activated by cGMP	GAF
PDE3	3A,B	cAMP	Cilostimide,Milrinone	Inhibited by cGMP	
PDE4	4A,B,C,D	cAMP	Rolipram,Ro20-1724 Ariflo, RP73401	Phosphorylated by PKA, ERK	UCR
PDE5	5A	cGMP	Sildenafil	Binds cGMP Phosphorylated by PKA, PKC	GAF
PDE6	6A,B,C	cGMP		Activated by transducin	
PDE7	7A,B	cAMP			
PDE8	8A,B	cAMP			
PDE9	9A	cGMP			
PDE10	10A	cAMP and cGMP			GAF
PDE11	11A	cAMP and cGMP			GAF

Table 1. 1: PDE families, substrates, selective inhibitors and paired control domains

Each of the PDE families consists of one to four genes and, via alternative 5' mRNA splicing and different transcription initiation sites usage, a number of these genes can generate several isoforms. Due to this ability, PDE isoenzymes have a conserved central catalytic unit and variable NH₂-terminal regions and COOH-terminal regions. Alternative 3' mRNA splicing only exists in the members of the PDE1 family but 5' mRNA splicing is found in several families (Houslay 2001).

There is a general structure for all PDEs. The region of PDE catalytic units is conserved between members of the same gene family and locates in the central portion of these PDE isoforms. Essential histidine residues are pivotal to the tight binding of divalent cations such as Zn²⁺ and Mg²⁺, which are critical for catalytic activity (Houslay and Adams 2003). However, compared to the conserved structure of the catalytic region, the N-terminal region is very different and contain sequence motifs that are involved in the regulation of PDE catalytic activity and in intracellular targeting (**Figure 1.1**).

The PDE1 family has the ability to hydrolyse both cAMP and cGMP by the binding of Ca^{2+} /CaM, and provides a means of cross-talk between the cAMP and Ca^{2+} signalling systems (Xu, Hassell et al. 2000).

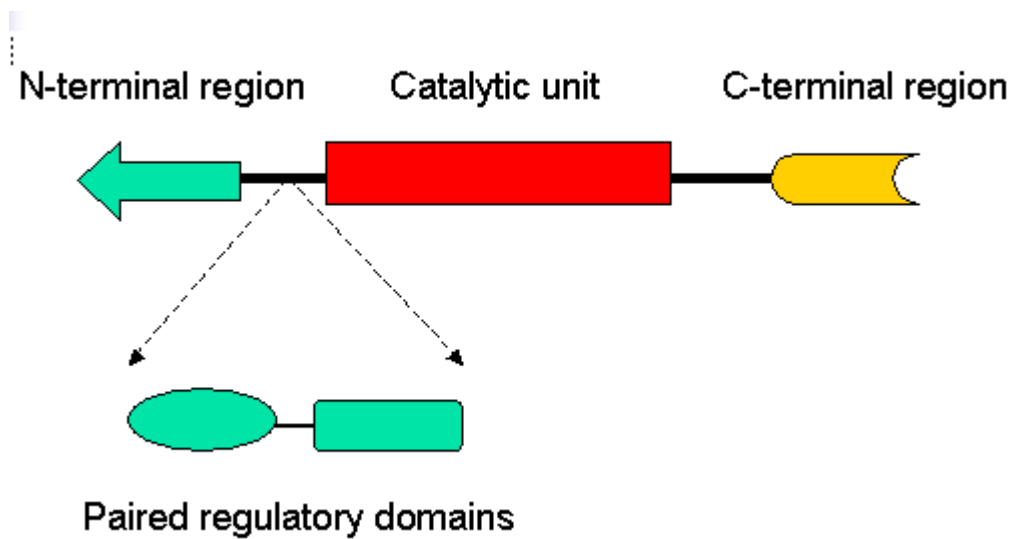


Figure 1.1: The core components of PDE.

The PDE2, PDE5, PDE6, PDE10 and PDE11 families contain two GAF domains in their N-terminal regions. GAF domains exist in a variety of proteins from plants, bacteria, and vertebrates. Although the functions of these domains in many PDEs haven't been discovered, it is considered that the domains probably play a role in regulation. For example, the GAF domains of PDE2, PDE5, and PDE6 isoenzymes bind cGMP and in the case of PDE2 this leads to enzyme activation (reviewed by Houslay, M.D., 2001)

The PDE3s are distinguished by their high specificity and affinity for cAMP hydrolysis. However, they can bind cGMP with high affinity as well, whereby cGMP is an inhibitor of the hydrolysis of cAMP. Therefore cross-talk between the NO pathway and PDE3 is considered to elicit an increase of cAMP levels due to PDE inhibition (Denninger and Marletta 1999). There are two subfamilies of PDE3, PDE3A and PDE3B. PDE3s are also generated as two different types, membrane-bound and cytosolic forms. Some groups elucidated that these enzymes are essential to the regulation of many important metabolic processes such as lipolysis and glycogenolysis, and this ability is due to an important feature of these enzymes that they are able to be activated by insulin (Degerman, Belfrage et al. 1997).

The PDE6 family, which is only expressed in the rod and cone photoreceptors of the retina, has an important role in visual signal transduction (Yarfitz and Hurley 1994). Compared to other PDE families, PDE6 is a multi-subunit protein containing two types of catalytic units, α (called PDE6A) and β (called PDE6B), and form a complex with two molecules of the inhibitory γ subunit.

PDE8 isoenzymes are cAMP-specific and contain Per-ARNT-Sim (PAS) homology domains in the N-terminal regions. PAS domains are known as their ability of sensing oxygen, redox potential, light, and some other stimuli (Gu, Hogenesch et al. 2000).

1.1.2 PDEs and their inhibitors

As PDEs are the only way to hydrolyze the secondary messengers cAMP and cGMP, they are involved in many cellular signaling pathways. Thus they are related to many diseases processes, inflammatory processes, as well as cell survival, memory and learning. Therefore, PDEs have attracted the attention of research and also PDE inhibitors have potential therapeutic functions. Despite the weak inhibitory effect, non-selective PDE inhibitors such as theophylline and papverine have been used in therapeutic treatment for over 70 years for a range of diseases (Boswell-Smith, Spina et al. 2006). However, due to the increase in identification of PDE isoforms over last 10 years and the elucidation of their functions, PDE selective drugs have begun to be used in the treatment of disease. For example, sildenafil in treating erectile dysfunction (**Table 2.2**).

PDE ISOENZYME	NO. OF ISOFORMS	SUBSTRATE	KM (μ M) CAMP	KM (μ M) GMP	TISSUE EXPRESSION	SPECIFIC INHIBITORS
1	8	Ca ²⁺ /calmodulin-stimulated	1-30	3	Heart, brain, lung, smooth muscle	KS-505a
2		cGMP-stimulated	50	50	Adrenal gland, heart, lung, liver, platelets	EHNA (MEP-1)
3	4	cGMP-inhibited, cAMP-selective	0.2	0.3	Heart, lung, liver, platelets, adipose tissue, inflammatory cells	Cilostamide Enoxamone Milrinone Siguazodan
4	20	cAMP-specific	4		Sertoli cells, kidney, brain, liver, lung, inflammatory cells	Rolipram, Roflumilast Cilomilast
5	3	cGMP-specific	150	1	Lung, platelets, vascular smooth muscle	Sildenafil, Zaprinast
6		cGMP-specific		60	Photoreceptor	Dipyridamole
7	3	cAMP-specific, high-affinity	0.2		Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes	BRL-50481
8		cAMP-selective,	0.06		Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes	none
9	4	cGMP-specific,		0.17	Kidney, liver, lung, brain	BAY 73-6691
10	2	cGMP-sensitive, cAMP-selective	0.05	3.0	Testes, brain	none
11	4	cGMP-sensitive, dual specificity	0.7	0.6	Skeletal muscle, prostate, kidney, liver, pituitary and salivary glands, testes	none

Table 1.2: PDE inhibitors

1.1.2.1 PDE3 inhibitors

As mentioned above, PDE3 has high affinity for cAMP and also for cGMP. However, according to Lugnier (Lugnier 2006) PDE3 can hydrolyze cAMP with a V_{max} that is 10 times greater than that for which it hydrolyzes cGMP. Thus cGMP acts as a competitive inhibitor of cAMP hydrolysis. PDE3 was found as a potential therapeutic target in cardiovascular disease and asthma by the means of high expression in both the vasculature and airways (reviewed by (Barnes, Chung et al. 1988)). Subsequently, PDE3 inhibitors have been shown to have the ability to relax vascular and airway smooth muscle, inhibit platelet aggregation (Barnes, Chung et al. 1988) and cause the induction of lipolysis (Manganiello, Taira et al.

1995). The remarkable effects of PDE3 inhibitors as positive inotropic agents (Nicholson, Challiss et al. 1991) gave a high possibility of designing new therapies for chronic heart disease. Indeed the PDE3 selective inhibitor, milrinone, was developed for this but was withdrawn from clinical trials because of side effects (Packer, Carver et al. 1991). Nevertheless, milrinone is still applied in acute treatment of heart failure.

1.1.2.2 PDE4 inhibitors

PDE4 isoenzymes are cAMP-specific and play an essential role in the majority of cells related to inflammatory responses (Muller, Engels et al. 1996). 30 years ago, rolipram was developed as a drug for treatment of depression (Scott, Perini et al. 1991), however, side effects of nausea and gastrointestinal disturbance ended its development (Scott, Perini et al. 1991). On the other hand, the finding that increasing the intracellular cAMP level in inflammatory cells by PDE4 inhibition inhibited their function and the wide distribution of PDE4 in these cells and the lung caused the exploration of using PDE4 selective inhibitors as potential drugs for airway disease (Torphy and Undem 1991). The first generations of PDE4 inhibitors, including rolipram, were effective at inhibiting a wide range of inflammatory cells function *in vitro* (Nielson, Vestal et al. 1990; Giembycz, Corrigan et al. 1996; Weston, Anderson et al. 1997). In this regard the PDE4 selective inhibitor, CDP840, was developed in 1997 and showed a beneficial effect in patients with asthma (Hughes, Howat et al. 1996; Harbinson, MacLeod et al. 1997). Subsequently, cilomilast and roflumilast were developed by Ted Torphy and Dr Schudt respectively (Brown 2005; Rabe, Bateman et al. 2005). Although several PDE4 selective inhibitors were found, inhibitors with better side effect profiles are still needed.

1.1.2.3 PDE5 inhibitors

PDE5 was first found in rat platelets in 1978 and proved to be a cGMP-specific isoform (Hamet and Coquil 1978). Because PDE5 inhibitors can induce vascular smooth muscle relaxation, it was considered as a potential drug target for cardiovascular disease. For this reason, sildenafil was first designed for angina treatment but its effect was disappointing. However, an interesting side effect drew the investigators' attention that penile erections were found in a number of patients (reviewed by (Boswell-Smith, Spina et al. 2006)). This led to the

change of the sildenafil research programme to investigate the drug as a treatment of erectile dysfunction (Boolell, Allen et al. 1996). Finally, sildenafil became the most popular medicine in the world market. Furthermore, two new PDE5 inhibitors, vardenafil and tadalafil, proved useful for the treatment of erectile dysfunction (Maggi, Filippi et al. 2000). Vardenafil is more potent than the other two and tadalafil is less active against PDE6 isoforms (Maggi, Filippi et al. 2000).

1.1.3 PDE4 family

The PDE4 family consists of four PDE4 subfamilies, PDE4A, 4B, 4C and 4D, of which each contain several isoforms and that make at least 20 isoenzymes of PDE4 (Houslay and Adams 2003). There is a *dunce* gene found in the fruit fly *Drosophila melanogaster* whose identification generated a probe that led to the identification of a rat PDE known as PDE4A1 or RD1 (Davis, Takayasu et al. 1989), not only the first PDE4 isoform but also the first PDE isoform to be found. PDE4 isoforms are identified by their unique N-terminal regions, which are generally encoded by a single 5' exon except PDE4C1 that is encoded by two 5' exons (Davis and Davidson 1986). There are 20+ exons encoding all the PDE4 isoenzymes and interestingly, the PDE4 exonic sequence is highly conserved between human and mouse excluding the PDE4A gene which has a different 3' coding region (Houslay and Adams 2003) This high conservation of PDE4 isoenzymes indicates that there must be a high selective pressure that exists in the genes evolution, otherwise the PDE4 gene would be lost or changed, which also indicates that PDE4 is essential for cells.

Bolger and colleagues' study (Bolger, Michaeli et al. 1993) provided a model of PDE4 structure and also a way to characterize PDE4 isoforms. They found that there were two homologous regions located between the N-terminal region and catalytic unit (Bolger, Michaeli et al. 1993). These two regions thus were named upstream conserved region 1 and 2 (UCR1 and 2). In addition, the existence of UCR1 (containing 60 amino acids) and UCR2 (containing 80 amino acids) is a unique feature of the PDE4 family. Between UCR1 and UCR2, a linkage region called linker region 1 (LR1) is inserted and also a linker region 2 between UCR2 and the catalytic region (Houslay 2001). Following the different organization of UCR1 and UCR2, PDE4 isoforms can be characterized as 4 types: long, short,

supershort and dead-short PDE4 isoforms (**Figure 1.2**). The long PDE4 isoform contains complete UCR1, UCR2 and two linker regions such as PDE4A4B; the short isoform contains a complete UCR2; and for the supershort isoforms have a truncated UCR2.

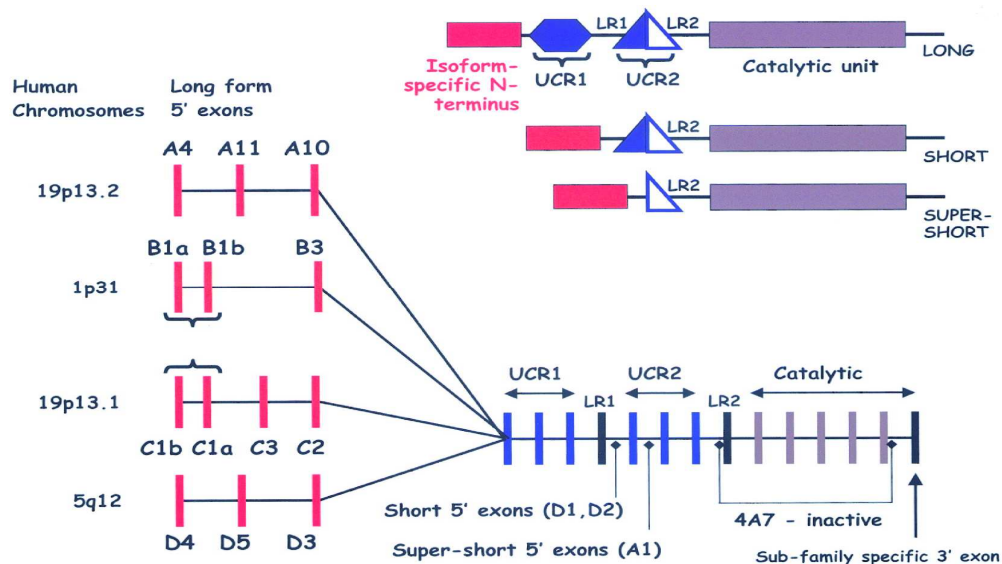


Figure 1. 2: Schematic representation of structure of PDE4 Isoforms.

The Figure shows the gene organization of four PDE4 subfamilies and their location on human chromosomes. The common structure of core regions forming the UCR1/2 and catalytic components is indicated. Certain unique N-terminal regions are encoded by two 5' exons, which are indicated as 'a' and 'b'. Isoform subcategories, generated by alternative mRNA splicing, based upon presence of absence of UCR1/2 regions, are also shown schematically. The range of species interacting with PDE4 isoforms and presumed key sites of interaction is indicated. (Reproduced from Houslay, M.D. and Adams, D.R. 2003).

The four PDE4 subfamilies all have their unique C-terminal regions; however the function of these regions still remains obscure.

A PDE4 isoform that should also be mentioned is PDE4A7, which consists of a 32 amino acid N-terminal region and a truncated catalytic unit. This is due to its unique mRNA alternative splicing at both 3' and 5' ends. The truncated catalytic unit deprives the PDE4 isoform of catalytic ability (Horton, Sullivan et al. 1995) and is called a dead-short enzyme (Houslay, Baillie et al. 2007). Despite its inactive catalytic ability, PDE4A7 is widely expressed in cells (Barber, Baillie et al. 2004; Johnston, Erdogan et al. 2004; Houslay, Baillie et al. 2007) and is considered to have some functions, although, which have not been found.

Although PDE4 isoforms shared a highly conserved catalytic unit and regulatory UCR1/2 regions, there is little redundancy among its four subfamilies. In human embryonic kidney cells stably expressing beta2 adrenergic receptors (HEKb2), PDE4B and PDE4D consist of >90% of PDE4 phosphodiesterase activity (Lynch, Baillie et al. 2005). However, using PDE4 interacting protein Barrestin to immunoprecipitate PDE4B or PDE4D, only endogenous PDE4D was detected in the pellet, but not PDE4B (Lynch, Baillie et al. 2005). Selective knockdown of PDE4D via small interference RNA (siRNA) increased isoprenaline stimulated phosphorylation of β_2 -adrenergic receptor (β_2 AR) and Gi dependent extracellular signal-regulated kinase (ERK) activation. Knockdown of PDE4B has no such effect (Wallace, Johnston et al. 2005). Nevertheless, gene knockdown experiments have shown that mouse peripheral leukocytes from PDE4B-/- mice, not PDE4D-/-, lost the ability to respond to lipopolysaccharide (LPS) induced PDE4B mRNA accumulation and decreased PDE activity. LPS induced tumor necrosis factor- α secretion by circulating leukocytes was also decreased by 90% in PDE4B-/- mice (Jin and Conti 2002).

1.1.4 UCR1 and UCR2 modules of PDE4

1.1.4.1 UCR1 and UCR2 interaction

Beard *et al* found that the hydrophobic region of UCR1 can interact with the hydrophilic N-terminal region of UCR2. They also identified two arginine residues in UCR1 (Arg98 and Arg100 in PDE4D3) and a group of negatively charged residues in UCR2 (Glu146, Glu147 and Asp149 in PDE4D3) played key roles in the interaction (Beard, Olsen et al. 2000).

Jin *et al* (Jin, Swinnen et al. 1992) showed that the deletion of UCR2 or the N-terminal region led to an increase of PDE4D catalytic activity. In addition, Beard *et al* found a similar response for PDE4A5 (Beard, Huston et al. 2002). These findings support the hypothesis that UCR2 might be a constitutive inhibitor for the catalytic unit (Jin, Swinnen et al. 1992).

1.1.4.2 PDE4 and PKA

A single serine residue found in UCR1 of all long form PDE4 isoforms plays a key role for the activation of the catalytic unit. The special residue is located at the

very N-terminal end of UCR1 and it can be phosphorylated by PKA (MacKenzie, Baillie et al. 2002). The phosphorylation increases the PDE4 activity by 60% in mammalian cells and even more (2-3 fold) in PDE4D3 and PED4A4 (MacKenzie, Baillie et al. 2002). This indicates that the upstream conserved regions regulate PDE4 activity.

Hoffmann and collaborators mutated the target serine to either an aspartate or glutamate to mimic the PKA activation and interestingly adjacent to the target serine there is a glutamate residue that attenuates PKA phosphorylation (Hoffmann, Baillie et al. 1999). However, mutation of this glutamate residue also mimics the activation by PKA phosphorylation. This phenomenon indicates that the PDE catalytic activity might be inhibited at a low level due to the interaction between target serine residue and an ion-pair. The phosphorylation of UCR1 also is considered to intervene in the interaction between UCR1 and UCR2 via a conformational change. Lim *et al* discovered an antiserum against the C-terminal portion of UCR2 activates PDE4D3 to a similar level of that activated by PKA phosphorylation. Thus the antiserum and PKA phosphorylation might regulate the catalytic activity through their disruption of the interaction between UCR2 and the catalytic unit (Lim, Pahlke et al. 1999). In addition, a higher sensitivity to stimulation by Mg^{2+} (in PDE4D3 and PDE4A4), which followed by the PKA phosphorylation is also considered to relate to the conformational change of UCR1 and UCR2.

1.1.4.3 PDE4 and ERK

The extracellular-signal-related protein kinase (ERK) MAP kinase signaling pathway is a well-investigated pathway which is essential for cell growth and survival. All PDE4 subfamilies except PDE4A contain an ERK consensus motif (Pro-Xaa-Ser-Pro) in the third subdomain of the catalytic unit (MacKenzie, Baillie et al. 2000). The serine residue of the motif can be phosphorylated by ERK. A distinct docking site that helps the specificity of the phosphorylation of the serine residue is the KIM (kinase interaction motif). There also is an FFX motif in the catalytic region of PDE4 isoforms, which plays an important role in defining the specificity for phosphorylation by ERK (Sharrocks, Yang et al. 2000).

When the target serine is exclusively phosphorylated by ERK, an inhibitory effect occurs in PDE4 long isoforms. This ERK-regulated inhibitory phosphorylation

exerts an increase of local cAMP concentration, which leads to the activation of PKA. This elicits the phosphorylation of the serine residue in the UCR1 of PDE4 long isoforms, which has been described above, and then causes an ablation of the inhibitory effect of ERK phosphorylation on PDE4 long isoforms.

Intriguingly, a reprogramming of the ERK-activated effect on the long PDE4D5 isoform was showed by Baillie *et al* in human aortic smooth-muscle cells (Baillie, MacKenzie et al. 2001). ERK in these cells leads to the autocrine generation of prostaglandin E2 (PGE2) by the activation of PKA2 and the production of arachidonic acid. The increasing concentration of PGE2 stimulates adenylate cyclase to initiate cAMP generation, and leads to PDE4D5 phosphorylation and activation by PKA.

1.1.4.4 UCR2 and Targeting

Accumulated evidence indicates that UCR2 is probably also involved in intracellular targeting. Verde and collaborators provided the primary evidence. They used two-hybrid analyses to investigate PDE4D3, and found a PDE4D3 potential binding partner, myomegalin. Myomegalin, known for its scaffold function, locates at the Golgi/centractin region and has a domain similar to that found in dynactin/centractin. By utilizing truncation studies, Verde showed there was an interaction between the extreme C-terminal 550 amino acids of myomegalin and the N-terminal region of UCR2 (Verde, Pahlke et al. 2001).

In addition, Liu and Maurice treated RASM cells with PMA and forskolin together and found this caused the release of particulate PDE4D3. This also indicates multi-site phosphorylation of PDE4D3 may lead to re-targeting and suggests that multi-site phosphorylation may have the ability to release PDE4 from anchor sites (Liu and Maurice 1999).

1.1.5 PDE4 and AKAPs

AKAPs can bind to the dimerization interface of the PKA cAMP binding site, which is called the RII regulatory subunit. The dimerization interface of R subunit forms a hydrophobic pocket that allows the insertion of an amphipathic helix of AKAPs, and this exerts a high-affinity interaction. The main functions of

AKAPs are their intracellular targeting ability and action as scaffolding protein in cells (Diviani and Scott 2001; Feliciello, Gottesman et al. 2001).

Studies show that the long PDE4D3 isoform is able to bind mAKAP (muscle-selective AKAP) (Dodge, Khouangsathiene et al. 2001) and AKAP450 (Tasken, Collas et al. 2001). mAKAP binds to the N-terminal region of PDE4D3 and AKAP450 binds to UCR2. The interaction between AKAP450 and UCR2 gives a rise to the possibility that AKAP450 may affect the PDE activity and also the phosphorylation of PDE4D3 by PKA and ERK. A possible impact is that the mAKAP localized PKA may facilitate the phosphorylation of PDE4D3. This interaction also suggests a negative feedback control pathway. The pathway starts with the increase of cAMP concentration, which leads to the activation of mAKAP localized PKA. The PDE4D3 activated by PKA phosphorylation will initiate the decrease of cAMP levels, mAKAP-bound PKA's inactivation, and dephosphorylation of PDE4D3 (Dodge, Khouangsathiene et al. 2001).

1.1.6 PDE4 and RACK1

Receptor for activated C kinase 1 (RACK1) as a scaffold protein can bind to protein kinase C, Src, integrin β -subunits and γ -aminobutyric acid receptors and the long PDE4D5 isoform (McCahill, Warwicker et al. 2002). RACK1 interacts with PDE4D5 by binding to the RAID1 (RACK1 interaction domain), a helical domain that locates at the PDE4D5 N-terminal region (Bolger, McCahill et al. 2002). However, the function of the interaction is still unknown.

1.1.7 PDE4 and SH3 domain

SRC homology 3 (SH3) domain, that binds proteins containing PXXP motifs, exist in protein-tyrosine kinases, Src, Lyn and Fyn, which act as essential controllers in a number of cellular processes. The PXXP motif was found in PDE4A4/5 and PDE4D4 isoforms and gives a mean of interaction between PDE and protein-tyrosine kinases (Pawson 1995).

Membrane-associated PDE4D5 is found in ruffles at the cell periphery and also at a discrete perinuclear region. By using deletion analysis, Huston, E et al (Huston, Beard et al. 2000) showed that a lack of the SH3-domain-interacting site on PDE4A5 released the PDE4A5 from ruffles and led to a uniform distribution of

PDE4A5 at the cell margin (Huston, Beard et al. 2000). A study performed on apoptotic cells showed that during the caspase 3, PDE4D5 lost its SH3-domain-interacting region through a cleavage at Asp⁷² (Huston, Beard et al. 2000). This raises the possibility that eliminating PDE4D5 from a functional relevant site may facilitate apoptosis. That overexpression of PDE4A5 in these cells does attenuate apoptosis indicates that the location of PDE4A5 may play an important part in cell survival. Although the mechanism remains obscure, PDE4A5 is activated through the phosphoinositide 3-kinase cell-survival pathway (MacKenzie, Yarwood et al. 1998).

Unlike PDE4A5, its human homologue, PDE4A4 contains another SH3-domain-interacting site that locates to the linker region 2 (LR2) (McPhee, Yarwood et al. 1999). A notable increase of PDE4A4's sensitivity to inhibition by rolipram has been observed by McPhee, I. and the colleagues (McPhee, Yarwood et al. 1999). According to this, it is suggested that the side effect of emesis and nausea of rolipram may be caused by the high affinity of PDE4 for rolipram inhibition in the central nervous system (Souness and Rao 1997; Torphy 1998).

PDE4D4 binds the three tyrosyl kinases at a similar level, whereas PDE4A5 showed preference of these, Lyn>Fyn>Src (Houslay 2001).

1.1.8 PDE4 subfamily

1.1.8.1 PDE4A1

PDE4A1 (U97584) was the first PDE isoform to be cloned. It is also the only purely membrane associated PDE4 isoform (Scotland and Houslay 1995; Shakur, Wilson et al. 1995; Baillie, Huston et al. 2002) and shows restricted expression in brain (Bolger, Rodgers et al. 1994; Shakur, Wilson et al. 1995). Thanks to Sullivan and his colleagues, we now know that the human isoform of PDE4A1 is located in chromosome 19p13.2, and a distinct 5'-exon encoded its specific N-terminal (Sullivan, Rena et al. 1998). Deletion of this region could generate fully active PDE4A1, but without membrane targeting ability (Shakur, Pryde et al. 1993; Huston, Houslay et al. 2006). Further studies show that the N-terminal contains two helices linked by a flexible hinge (Smith, Scotland et al. 1996). These two helices work together to control PDE4A1's distribution in cell. The helix-1 (amino acids 1-8) is pivotal for PDE4A1 intracellular targeting, especially

to the membrane and trans-Golgi stack. The Asp5 in helix-1 is the key amino acid to interact with Ca^{2+} after phospholipase-D-dependent PA (phosphatidic acid) generation (Baillie, Huston et al. 2002). The helix-2 (amino acids 14-25) contains a TAPAS1 domain which can insert into lipid bilayers (Baillie, Huston et al. 2002). This function is also regulated by Ca^{2+} interaction with Asp21. Increase Ca^{2+} level or mutation of either Asp5 or Asp21 would see elicits PDE4A1's redistribution from trans-Golgi stack to throughout the cytosol (Huston, Gall et al. 2006).

PDE4A1 was found in a number of brain tumour types, which raised the hypothesis that it may serve an important role in tumour growth and resistance (Goldhoff, Warrington et al. 2008). Indeed, a recent report from Goldhoff P. showed that overexpression of PDE4A1 in Daoy medulloblastoma and U87 glioblastoma cells could significantly shorten their in vivo doubling time. However, using rolipram along with temozolomide and conformal radiation therapy could stop tumour growth and promote tumour regression (Goldhoff, Warrington et al. 2008).

1.1.8.2 PDE4A4/PDE4A5

PDE4A4 (L20965) (Bolger, Michaeli et al. 1993) is a long isoform PDE that has been shown to undertake a dynamic redistribution in living cells independent from cAMP (Terry, Cheung et al. 2003). This process is triggered by treatment with PDE4 inhibitor Rolipram (Terry, Cheung et al. 2003). The peak of PDE4A4 foci formation occurred after 10 hours treatment of rolipram and is dose dependent. The foci formation is also reversible upon washout of rolipram. Further investigation showed that a Db-LR2-PDE4A4 construct with deletion of eight amino acids (APRPRPSQ) from LR2 decreased foci formation. The Db-LR2-PDE4A4 construct has a conformational change in PDE4A4 which disables LYN SH3 interaction to alter the kinetics of rolipram inhibition of the catalytic activity (McPhee, Yarwood et al. 1999). Three single mutations, His505Asn, His506Asn and Val475Asp, in the catalytic unit ablated the foci formation but was still sensitive to rolipram inhibition (Terry, Cheung et al. 2003).

Protein-tyrosine kinases Src, Lyn and Fyn can interact with the SH3 interacting domain PxxP (Pawson 1995) which locates at the N-terminal region of PDE4A4/5 and PDE4D4 (Oconnell, McCallum et al. 1996; Beard, O'Connell et al. 1999; MCPhee, Yarwood et al. 1999; Beard, Huston et al. 2002). This interaction is

responsible for the enzymes' intracellular targeting. The membrane-associated PDE4A5 existed at ruffles of the cell periphery and at a discrete perinuclear localization (Beard, Huston et al. 2002). Deletion or disruption of the SH3-domain-interacting site on PDE4D5 released PDE4D5 from ruffles at the cell margin, therefore causing a uniform distribution of PDE4D5 through the cell margin. In apoptotic cells, the caspase-3-mediated cleavage of PDE4A5 at Asp72 eliminates the SH3-domain-interacting site, which leads to a similar PDE4D5 redistribution from ruffles to throughout the cell margin (Huston, Beard et al. 2000). Furthermore, overexpression of PDE4A5 in apoptotic cells attenuated apoptosis, whereas overexpression of another differently targeted PDE4 isoform displayed no such effect (Huston, Beard et al. 2000). This indicates that the specific localization of PDE4A5 at the cell margin may promote cell survival by controlling compartmentalized cAMP levels (Houslay and Adams 2003). In support of this hypothesis is that PDE4A5 is activated through the phosphoinositide 3-kinase cell survival pathway, however the mechanism of this remains obscure (MacKenzie, Yarwood et al. 1998). PDE4A4 (human homologue of PDE4A5) has an extra site for interaction with SH3 domains (McPhee, Yarwood et al. 1999), which locates at the LR2. Sensitivity of PDE4A4 to inhibition by rolipram is remarkably increased after Lyn and Src bind to this site (McPhee, Yarwood et al. 1999).

In vitro purified PDE4A4 was reported to exhibit a biphasic response to Mg^{2+} in catalyzing cAMP hydrolysis (Laliberte, Liu et al. 2002). PKA dependent phosphorylation on PDE4A4 increased its sensitivity to Mg^{2+} , which leads to a 4 fold increase of PDE4A4 activity. The PDE4A4 phosphorylation also increases the enzyme sensitivity to (R)- and (S)-rolipram (Laliberte, Liu et al. 2002).

The p75 neurotrophin receptor ($p75^{NTR}$) belongs to the family of TNFR (tumour necrosis factor receptor). It is reported to be expressed in many cell types including smooth muscle cells, endothelial cells, myofibroblasts, neurons and glia after injury (Sachs and Akassoglou 2007). It has been shown to regulate disease progression by mediating a number of functions fundamental for tissue repair such as apoptosis (Syroid, Maycox et al. 2000), cellular differentiation (Passino, Adams et al. 2007), extracellular matrix remodelling (Sachs, Baillie et al. 2007), myelination (Cosgaya, Chan et al. 2002) and inhibition of neurite outgrowth (McGee and Strittmatter 2003; Domeniconi, Zampieri et al. 2005).

PDE4A4/5 can directly interact with p75^{NTR} via binding to its unique C-terminal region, and is recruited to the plasma membrane for cAMP degradation. The PDE4 dependent inhibition of plasminogen activation allows p75NTR to increase fibrin deposition after sciatic nerve injury and lung fibrosis (Sachs, Baillie et al. 2007). The full process is described in figure below (Figure 1.3).

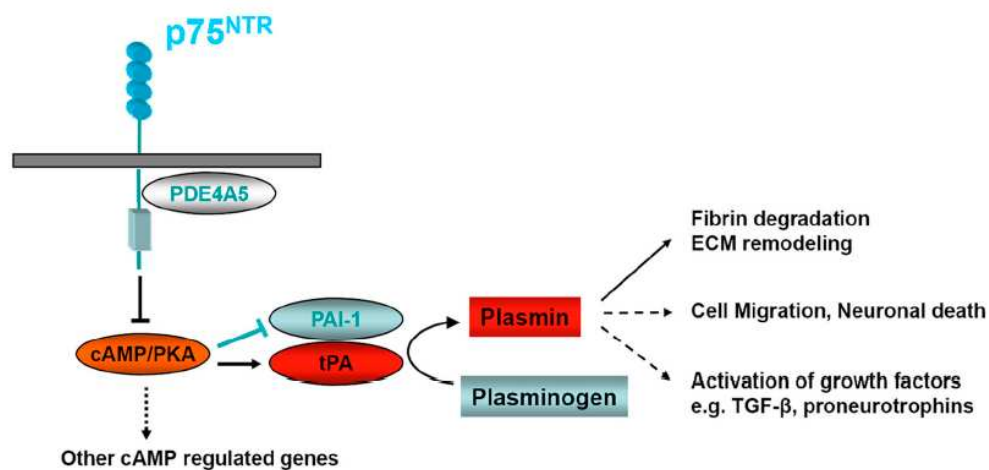


Figure 1.3: Proposed model for the role of p75NTR in the cAMP-mediated plasminogen activation. Taken from (Sachs, Baillie et al. 2007). p75NTR interacts with PDE4A4/5 resulting in degradation of cAMP and thus a reduction of PKA activity. Decrease in cAMP reduces expression of tPA and increases PAI-1, resulting in reduction of plasmin and plasmin-dependent extracellular proteolysis. Reduction of plasmin results in reduced fibrin degradation and ECM remodeling. Because plasmin can proteolytically modify nonfibrin substrates, such as growth factors and cytokines, this mechanism may be upstream of various cellular functions.

PDE4A4 is the main PDE isoform that is up-regulated in lung pathogenesis, especially COPD (chronic obstructive pulmonary disease) (Barber, Baillie et al. 2004). Therefore, it has been considered to be a suitable pharmacological target for COPD (Houslay, Schafer et al. 2005).

PDE4A5 (AAF14519), which is the rodent orthologue of PDE4A4, has a unique N-terminal region that defines its intracellular localization. Two sites of PDE4A5 N-terminal region were found to target it to the cell margin: one exists in the C-terminal portion of PDE4A5 unique region and the other one is a SH3 motif which can interact with LYN kinase. A third membrane targeting region locates at the N-terminal part of UCR2 and is believed to target PDE4A5 to the perinuclear

region (Beard, Huston et al. 2002). PDE4A5 can be cleaved by caspase-3 at 69-DAVE-72 downstream of the SH3 motif, which alters the distribution of PDE4A5 (Huston, Beard et al. 2000). Immumophilin XAP2 interaction with PDE4A5 was found to decrease activity of PDE4A5 (Bolger, Peden et al. 2003).

In rat brain, PDE4D5 was found to express in the olfactory nuclei, deep cortical layers, dentate and CA1 pyramidal layers (D'Sa, Eisch et al. 2005). Upon electroconvulsive seizure (ECS) treatment, PDE4A5 levels were elevated in the anterior cingulate and frontoparietal cortices, CA1 and dentate gyrus. PDE4A splice variants PDE4A1 and PDE4A10 were also detected in rat brain with different distribution and regulation upon stimuli treatment. Their different up-regulation may suggest a region specific response to chronic depressant-mediated cAMP increase, making them a potential target for anti-depressant therapy (D'Sa, Eisch et al. 2005).

1.1.8.3 PDE4A7

PDE4A7 (U18088) is the only PDE4 isoform that lacks catalytic activity. This is due to its novel 3' and 5' splicing, resulting in both 5' and 3' domain swaps, which gives PDE4A7 a unique 32-residue N-terminal region and a unique 14-residue region (Horton, Sullivan et al. 1995; Sullivan, Rena et al. 1998). Further investigation showed that the unique N-terminal region was able to support an active catalytic unit, whereas the C-terminal cannot (Johnston, Erdogan et al. 2004). PDE4A7 is exclusively targeted to P1 particulate fraction, whereas other active PDE4A isoforms are not. A 19-residue C-terminal region of active PDE4A splice prevents the exclusive targeting to P1 particulate fraction, whereas PDE4A7 unique C-terminal region cannot (Johnston, Erdogan et al. 2004).

1.1.8.4 PDE4A8

PDE4A8 contains a unique N-terminal region of 85 amino acids that differs from the other PDE4A long isoforms, PDE4A4, 4A10 and 4A11 (Mackenzie, Topping et al. 2008). The human isoform undergoes rapid evolutionary change in its N-terminal region, which diverges from its corresponding isoforms in rat and other mammals (Mackenzie, Topping et al. 2008). The human PDE4A8 was demonstrated to express mainly in skeletal muscle and brain, whereas other PDE4A isoforms and rat PDE4A8 displayed a different pattern. PDE4A8 is sensitive

to the PDE4 inhibitor rolipram, but less sensitive to cilomilast (Mackenzie, Topping et al. 2008). Like all other PDE4 long isoforms, PDE4A8 can be phosphorylated by protein kinase A at the conserved Ser site within UCR1, which increases PDE enzyme activity (McPhee, Cochran et al. 2001; MacKenzie, Baillie et al. 2002).

1.1.8.5 PDE4A10

PDE4A10 contains a unique 46 amino acid N-terminal region (Rena, Begg et al. 2001). It has a similar size and maximal activity to PDE4A4, but differs in many aspects such as interaction with LYN, sensitivity to inhibition by rolipram, thermostability and subcellular distribution (Rena, Begg et al. 2001). In cardiac myocytes, enhanced cAMP initiates the down-regulation of transcripts and promoter activity for PDE4A10 (McCahill, Campbell et al. 2008).

1.1.8.6 PDE4A11

PDE4A11 is a recently identified PDE4A isoform that has a unique 81 amino acids N-terminal region encoded by exon-1 (4A11) (Wallace, Johnston et al. 2005). Recombinant PDE4A11 expressed in COS-7 cells were predominantly localised around the nucleus and in membrane ruffles. It can be phosphorylated at Ser119 by PKA and can interact with beta-arrestin (Wallace, Johnston et al. 2005). However, its specific function is not clear yet.

1.1.8.7 PDE4B1

Similar to other PDE4 long isoforms, PDE4B1 can be activated by phosphorylation at the PKA consensus site within UCR1 (MacKenzie, Baillie et al. 2002). PDE4B1 along with its long isoforms such as PDE4A5 and PDE4D3 were activated by phosphatidic acid (PA), whereas the short isoforms such as PDE4A1, PDE4B2, PDE4D1 and PDE4D2 were not (Nemoz, Sette et al. 1997).

PDE4B1 can be phosphorylated at Ser659 by ERK (Baillie, MacKenzie et al. 2000). The Ser659 is located within the catalytic unit of PDE4B1; phosphorylation at this site leads to the inhibition of the enzyme activity. Such ERK phosphorylation has also been noted in PDE4D3 at Ser579 and PDE4C2 at Ser535, but not in PDE4A8

(Hoffmann, Baillie et al. 1999). However, ERK phosphorylation can activate the short variant PDE4B2 (Baillie, MacKenzie et al. 2000).

Recombinant PDE4B1 (as well as PDE4D3 and PDE4D5) was shown to interact with Nuclear distribution element-like (Ndel1; Nudel) protein, and such interaction was shown to potentiate the Ndel1-Ndel1 self-association (Collins, Murdoch et al. 2008). For PDE4D3, elevation of cAMP ablated its potentiating effect on Ndel1 self-association. However, for PDE4B1 and PDE4D5, they were resistant to the increase of cAMP (Collins, Murdoch et al. 2008).

PDE4B1, along with its PDE4 isoform PDE4B2 and PDE4B3, were found to interact with DISC1 (disrupted in schizophrenia 1) by their UCR2 domain and this association is ablated by increased cAMP level (Millar, Pickard et al. 2005). This function is described in section 1.1.8.9 below.

PDE4B1 was found to express in peripheral blood T cells (Baroja, Cieslinski et al. 1999), monocytes, and macrophages (Barber, Baillie et al. 2004).

1.1.8.8 PDE4B2

PDE4B2 has been found to be the predominant PDE isoform in monocytes and neutrophils (Wang, Wu et al. 1999). It undergoes different regulation in these two cell types (Wang, Wu et al. 1999). In monocytes, expression of the PDE4B gene is selectively induced by lipopolysaccharide (LPS) and this induction is inhibited by interleukin IL-10 and IL-4. In neutrophils, PDE4B2 is constitutively expressed and such expression is not affected by LPS or IL-10 (Wang, Wu et al. 1999). In the same report, Wang P. also found that leukocytes were the main resource for PDE4B2, indicating it to be a potential target for the design of anti-inflammatory drugs (Wang, Wu et al. 1999). Further study also showed that T cell activation was regulated by PDE4B2 through its dynamic distribution in the process of immunological synapse formation (Arp, Kirchhof et al. 2003).

Several analogues of a new non-nucleoside HIV-1 reverse transcriptase inhibitor named alkenyldiarylmethanes (ADAM), whose analogues have been identified to protect HIV-infected cells from the cytopathic effects of the virus by an unclear HIV- reverse transcriptase independent mechanism, displayed weak inhibition effects on PDE4B2 (Cullen, Cheung et al. 2008). PDE4 expression in CD4+ memory

T-cells is a necessary condition for HIV infection to occur, which makes PDE4 inhibitor potential therapeutic target for HIV treatment (Sun, Li et al. 2000).

PDE4B2 was found to be expressed in peripheral blood T cells (Baroja, Cieslinski et al. 1999).

1.1.8.9 PDE4B3

Hippocampal long-term potentiation is the most accepted cellular model for learning and memory formation (Bliss and Lomo 1973). It contains 2 phases: early-LTP (<4h) and late-LTP (>4h), and the latter phase is dependent upon protein translation and transcription. PDE4B3 was the first cAMP specific PDE found to be associated with LTP and modulated during LTP phase (Ahmed, Frey et al. 2004). It is activated through NMDA-receptors and its transcription was transiently up-regulated for 2 hours after tenanization. PDE4B3 protein expression peaks at 6 hour after LTP induction and is rapidly downregulated by 8 hours, while the cAMP level continually decreases during the LTP phase (Ahmed and Frey 2003). Further study detailed LTP specific translational (not transcriptional) regulation of PDE4B3 in hippocampal area CA1 (Ahmed, Frey et al. 2004).

PDE4B3, along with its PDE4 isoform PDE4B1 and PDE4B2, were found to interact with DISC1 (disrupted in schizophrenia 1) by their UCR2 domain and this association is ablated by increased cAMP level (Millar, Pickard et al. 2005). According to the experimental evidence, Millar *et al* (Millar, Pickard et al. 2005) proposed a mechanism that DISC1 sequesters PDE4B in resting cells and releases the activated isoform after cAMP is elevated. DISC1 is considered to be a candidate susceptibility factor for schizophrenia. The gene encoding PDE4B is disrupted by a balanced translocation in a subject diagnosed with schizophrenia and a relative with chronic psychiatric illness (Millar, Pickard et al. 2005). Therefore, PDE4B has been considered as a genetic susceptibility factor for schizophrenia (Millar, Pickard et al. 2005; Millar, Mackie et al. 2007).

1.1.8.10 PDE4B4

PDE4B4 is a recently characterised PDE4B member (Shepherd, McSorley et al. 2003). It has a unique N-terminal region with 17 amino acids and UCR1/2 regions.

It has detectable expression in liver, skeletal muscle and several regions in brain, which differs from the pattern of tissue distribution of its two long isoforms PDE4B1 and PDE4B3 (Shepherd, McSorley et al. 2003).

1.1.8.11 PDE4B5

PDE4B5 is a recently identified PDE4 variant that is brain specific (Cheung, Kan et al. 2007). It has a novel 16 amino acids N-terminal region that is identical to that of PDE4D6 which is also brain specific. It can be inhibited by PDE4 inhibitors rolipram and cilomilast, and can interact with DISC1. However, its function in brain is still poorly understood (Cheung, Kan et al. 2007).

1.1.8.12 PDE4C1/2/3

There are only three members in the PDE4C subfamily which are PDE long isoforms PDE4C1, PDE4C2 and PDE4C3 (Sullivan, Olsen et al. 1999). They are generated from different promoters and all form human. Other PDE4C splice variants PDE4C- Δ 54 and PDE4C- Δ 109 transcription is considered to be generated from a separated common promoter. PDE4Cs are ubiquitously expressed but with low concentration in lung and absent in immune system cells (Engels, Sullivan et al. 1995; Obernolte, Ratzliff et al. 1997). PDE4C- Δ 54 is unique for its specific expression in testis.

PDE4C2 and PDE4D3 can interact with PKA anchor protein AKAP450, and gate the activation of AKAP450-tethered PKA type-II localised in the perinuclear region under conditions of basal cAMP generation in resting cells (see 1.1.8.14) (McCahill, McSorley et al. 2005).

1.1.8.13 PDE4D1/2

PDE4D1 and PDE4D2 are two PDE4 short isoforms as shown below (**Figure 1.4**). The most profound finding about them is their selective expression in vascular smooth muscle cells (VSMCs) (Houslay 2005). In VSMCs, long PDE4D isoforms are not transcriptionally regulated by prolonged cAMP signalling in synthetic/activated cells, whereas short PDE4D1 and PDE4D2 are. Such an observation may indicate a reduced VSMC responsiveness to particular cAMP effects, and this may depend on the histone acetylation status of their

promoters. According to the elevated expression of PDE4D1 and PDE4D2 in synthetic/activated VSMCs, Tilley and Maurice suggested that these cells might more readily desensitize to the effects of prolonged cAMP-elevating agents than contractile/quiescent VSMCs through both increased cytosolic expression of these variants and activation of them by mitogenic stimuli via ERK. Therefore, development of PDE4D inhibitors to target specific PDE4D isoforms in contractile/quiescent and synthetic/activated VSMCs is of importance (Houslay 2005; Tilley and Maurice 2005).

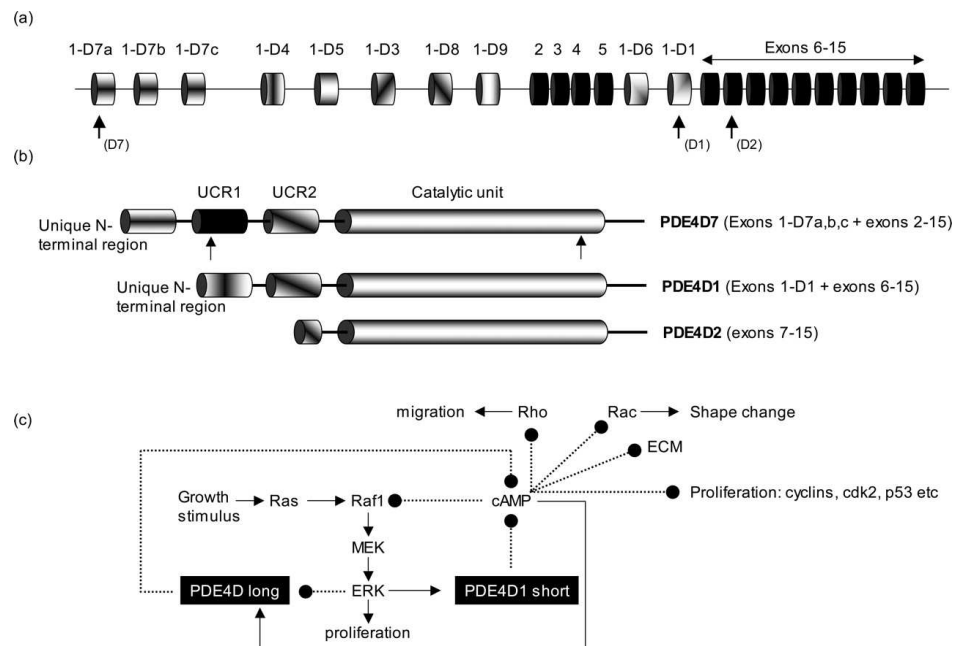


Figure 1.4. The PDE4D gene, the long PDE4D7 and supershort PDE4D1/2 products plus putative networks linking them to functions in VSMC. a, schematic of the *PDE4D* gene. Exons are numbered so as to indicate both those coding the common core PDE4D plus the unique 5' exons encoding the N-terminal regions of particular splice variants (Houslay and Adams 2003). Note that single exons encode the N-terminal regions of individual isoforms except for the "first" isoform from each PDE4 subfamily, the N-terminal region of which is encoded by multiple exons located at the extreme 5' region of the gene. The figure is a schematic and for simplicity does not indicate relative distances separating these exons to scale. The three arrows indicate the start of the PDE4D7, PDE4D1, and PDE4D2 coding regions, in order. b, schematic of the domains of the indicated PDE4D isoforms. PDE4D7 is a long isoform with both UCR1 and UCR2, PDE4D1 is a short isoform with only UCR2, and PDE4D2 is a supershort form with a truncated UCR2. PDE4D1 and PDE4D7 both have unique N-terminal regions, whereas PDE4D2 does not. Shown are the phosphorylation sites for PKA on UCR1 (arrows) and the phosphorylation site for ERK on the catalytic unit. c, schematic to show stimulatory (arrows) and inhibitory (dashed lines + circle) connections linking PDE4 long and short isoforms in VSMC. The "inhibitory" effect on cAMP exerted by PDE4 is through cAMP degradation. Although the major effector of the intracellular actions of cAMP is PKA (Tasken and Aandahl 2004), actions may also be exerted by EPAC (Bos 2003) and cyclic nucleotide-gated ion channels (Zagotta and Siegelbaum 1996). PKA is known to mediate the actions shown here on PDE4, RhoA, Raf-1, and various other actions that attenuate proliferation. Cross-talk between the ERK and cAMP pathways can occur at the level of Raf, with Raf1 providing a point of inhibition by cAMP and B-Raf a point of activation (Houslay and Kolch 2000). (Reproduced from Houslay MD, Molecular Pharmacology, 2005) (Houslay 2005)

1.1.8.14 PDE4D3

PDE4D3 is a PDE4D long isoform containing UCR1/2 regions and a unique 15 amino acids N-terminal. Similar to other long isoforms it can be activated by PKA phosphorylation at Ser54 and Ser13 after elevated cAMP (Sette and Conti 1996) (MacKenzie, Baillie et al. 2002). Such PKA activation, along with PKC stimulated activation of PDE4D3 via Raf/MEK/ERK signalling pathway, coordinated the translocation to the cytosolic fraction of vascular smooth muscle cells (Liu and Maurice 1999). However, epidermal growth factor (EGF) treatment has an inhibitory effect on PDE4D3 (and PDE4D5) activity, which is engendered by the EGF induced ERK2 phosphorylation at Ser579 of PDE4D3 and the cognate residue in PDE4D5. Such inhibition is transient and can be ablated by feedback PKA phosphorylation of PDE4D3 (Hoffmann, Baillie et al. 1999). PKA Phosphorylation of PDE4D3 at Ser13 alone increases its affinity with mAKAP, which may facilitate the recruitment of PDE4D3 and quick signal termination (Michel, Dodge et al. 2004).

Oxidative stress (treatment of cells with H₂O₂) can lead to a rapid increase of PDE4D3 activity and phosphorylation. This phosphorylation occurs at Ser579 and Ser239. ERK phosphorylation of Ser579, which locates at the extreme C-terminus of the catalytic unit, inhibits PDE4D3 activity (Baillie, MacKenzie et al. 2000). Phosphorylation of Ser239, which locates at the extreme N-terminal of the catalytic unit, occurs via an unknown kinase that is downstream of phosphatidylinositol 3-kinase (Hill, Sheppard et al. 2006). Such phosphorylation altered the inhibitory effect of ERK phosphorylation at Ser579 and leads to the increase of PDE4D3 activity. Therefore, it is supposed that phosphorylation at Ser239 attenuates interaction between UCR1 or UCR1/2 and the catalytic unit to reprogramme the functional outcome of ERK phosphorylation. This oxidative stress is considered to activate PDE4s in order to lower the cAMP level and promote inflammatory responses (Hill, Sheppard et al. 2006).

Phosphatidic acid is a second messenger which is a product of stimulation of cells by hormones and growth factor receptors in many cell types (English 1996; Exton 1997; Hodgkin, Pettitt et al. 1998) . It was found to directly bind to a 31-59 amino acids region of PDE4D3. Such binding activated PDE4D3 enzyme activity

and thus provides a new mechanism of cAMP regulation by PA (Grange, Sette et al. 2000).

PDE4D3 and PDE4D5 can interact with Barrestin and be recruited to activated β_2 adrenergic receptors (β_2 AR) (Perry, Baillie et al. 2002). In doing so, cAMP activated membrane bound protein kinase activity is restricted by accelerated degradation of cAMP and receptor desensitization (Perry, Baillie et al. 2002). This recruitment to β_2 ARs has been shown to play key roles in regulating the receptor switching its signalling from Gs to Gi in cardiac myocytes.

Phosphorylation of the β_2 AR by PKA switches its coupling from stimulatory G-protein (Gs) activation of AC to inhibitory G-protein (Gi) activation of ERK. The beta arrestins recruit PDE4 to the receptor in order to regulate the PKA activity at the membrane (Baillie, Sood et al. 2003). PDE4 inhibitor rolipram treatment significantly enhances the PKA phosphorylation of β_2 AR and β_2 AR -mediated activation of ERK1/2. This is consistent with the Gs to Gi switch model, because the ERK1/2 activation is also sensitive to both inhibitors of Gi (pertussis toxin inhibited) and PKA (H89). Thereby, the recruited PDE4 is suggested to be crucial in regulating the PKA mediated switching of β_2 AR signalling from Gs to Gi (**Figure 1.5**) (Baillie, Sood et al. 2003).

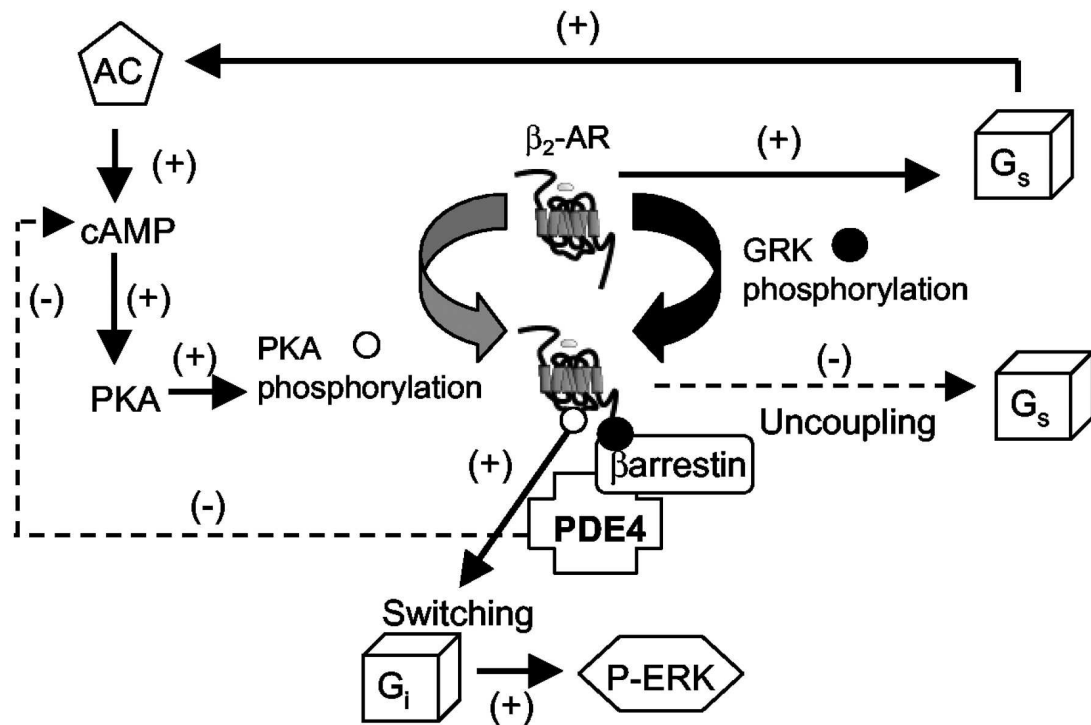


Figure 1.5: A schematic representation of the role of arrestin-recruited PDE4 in regulating the “switching” of the β_2 AR from G_s to G_i stimulation. Agonist occupancy of the β_2 AR initially leads to coupling to G_s , which causes activation of adenylyl cyclase, elevated cAMP levels, and activation of PKA, which is able to phosphorylate the β_2 AR. Concomitantly, agonist occupancy also leads to GRK-mediated phosphorylation of the β_2 AR, which allows for the recruitment of β -arrestin together with bound PDE4. PKA phosphorylation of the β_2 AR confers switching from G_s to G_i , with consequent activation of ERK1/2. However, β -arrestin-recruited PDE4 provides a negative feedback loop, the role of which is to attenuate local cAMP levels and thus the ability of membrane PKA to phosphorylate the β_2 AR. This action of β -arrestin-recruited PDE4 is uncovered by dominant negative PDE4, which replaces the active endogenous recruited PDE4 to ablate the negative feedback loop and thus accentuate switching to G_i . (reproduced from (Baillie, Sood et al. 2003))

As described for PDE4C1/2/3, PDE4D3 and PDE4C2 can interact with PKA anchor protein AKAP450, and gate the activation of AKAP450-tethered PKA type-II localised in the perinuclear region under conditions of basal cAMP generation in resting cells (Figure 1.6) (McCahill, McSorley et al. 2005).

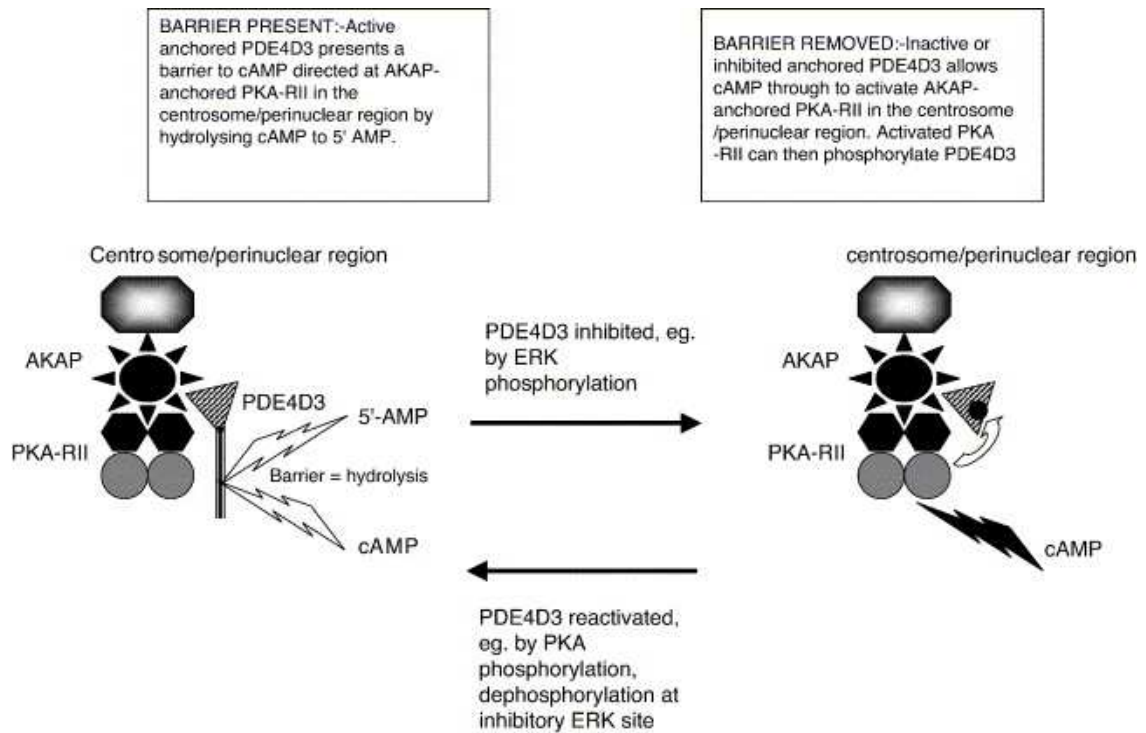


Figure 1.6: Schematic of anchored PDE4/PKA complexes.

Active anchored PDE4D3 (PDE4C2) controls the activity of AKAP-tethered PKA-RII. In resting cells the basal generation of cAMP by adenylyl cyclase is degraded by anchored PDE4. However, inhibition of anchored PDE4, such as by ERK phosphorylation or exogenously added inhibitors, now allows cAMP levels to rise in the immediate environment of tethered PKA-RII, eliciting the activation of this selected pool of PKA. Subsequent phosphorylation of the long PDE4 isoform by PKA activates the PDE4, lowering cAMP levels, deactivating PKA and resetting the system. This allows for transient activation. Taken from (McCahill, McSorley et al. 2005)

A recent study shows that PDE4D3 exists in the cardiac ryanodine receptor (RyR2)/clacium-release-channel complex, which is required for excitation-contraction (EC) coupling in heart muscle. Elimination of PDE4D from mice leads to a progressive cardiomyopathy, accelerated heart failure after myocardial infarction and cardiac arrhythmias. In human hearts, reduced PDE4D3 was found in subjects with failing hearts, which contributes to PKA-hyperphosphorylated “leaky” RyR2 channels that promote cardiac dysfunction and arrhythmias (Lehnart, Wehrens et al. 2005). Further investigation also showed that depletion of PDE4D3 in mice significantly reduced the exercise capacity of the mice (Bellinger, Reiken et al. 2008). PDE4D3, but not PDE4D5, also have been found to be recruited to the cardiac I-Ks potassium channel by the A kinase-anchoring protein Yotiao (AKAP-9) so as to regulate the cAMP level (Terrenoire, Houslay et al. 2009).

PDE4D3 can interact with Nuclear distribution element-like (Ndel1;Nudel) protein. Such interaction with Nudel enhances Nudel-Nudel self-association

(Collins, Murdoch et al. 2008). However, this potentiating effect was ablated by phosphorylation of PDE4D3 at Ser13 of its unique N-terminal region by PKA after cAMP elevation (Collins, Murdoch et al. 2008).

1.1.8.15 PDE4D4

PDE4D4 contains a SH3 binding domain in its unique N-terminal region and can bind to src, lyn and fyn with similar affinity (Beard, O'Connell et al. 1999).

Permanent epigenetic modifications of the genome by DNA methylation at CpG-rich regions (CpG islands) can regulate gene transcription by hypermethylation (silence) or hypomethylation (activate) (Momparler and Bovenzi 2000). Change of DNA methylation state has been shown to contribute to both cancer initiation and promotion (Momparler and Bovenzi 2000; Esteller 2005). A specific methylation cluster was identified in the 5'-flanking CpG island of PDE4D4 (Ho, Tang et al. 2006). This CpG region was gradually hypermethylated with aging in normal prostates, leading to the loss of PDE4D4 expression. Following studies in prostate cancer cells confirms the site-specific methylation is involved in transcriptional silencing of PDE4D4 expression and found a related hypomethylation of this gene. Because the PDE4D4 alterations in the oestrogen-exposed prostate are detectable before histopathologic changes of the gland, this suggests PDE4D4 is a potential molecular marker for prostate cancer risk assessment (Ho, Tang et al. 2006).

Dynamic cAMP changes that are restricted to a subplasma-membrane domain by PDE4 enhance endothelial barrier integrity. PDE4D4 was found to be expressed in pulmonary microvascular endothelial cells; located in plasma membrane fractions; and interacted with α II spectrin within its membrane domain (Creighton, Zhu et al. 2008). Inhibition PDE4D4 activity allows cAMP that was predominantly restricted at the membrane to enter a cytosolic domain that is rich in microtubules, where it enables PKA phosphorylation of tau at Ser214. Such phosphorylation reorganizes microtubules and promotes inter-endothelial cell gap formation (Creighton, Zhu et al. 2008).

1.1.8.16 PDE4D5

As described in the section above on PDE4D3, PDE4D5 can be recruited to activated β_2 AR by Barrestin in order to switch Gs coupling to Gi (**Figure 1.5**). However, further knock down of PDE4D3 and PDE4D5 gene by siRNA suggested PDE4D5 was the isoform that regulated the desensitization of isoprenaline-stimulated PKA phosphorylation of β_2 AR and switches the signalling to ERK (Lynch, Baillie et al. 2005). This translocation of PDE4D5 generated a spatial cAMP gradient around the membrane-bound β_2 AR, mediating receptor phosphorylation by PKA and its ability to activate ERK through Gi in cardiomyocytes (Lynch, Baillie et al. 2007).

PDE4D5 was shown to interact with both N and C-termini of Barrestin (Baillie, Adams et al. 2007). Furthermore, PDE4D5 has been shown to interact with another scaffolding protein RACK1 via its RACK1 interaction domain (RAID) (Bolger, McCahill et al. 2002). Screening scanning peptide arrays showed that RACK1 and beta-arrestin interact at overlapping sites within the unique N-terminal region of PDE4D5 and at different sites in the catalytic unit (Bolger, Baillie et al. 2006). Due to these overlapped binding sites, RACK1 competed with beta-arrestin in sequestering PDE4D5 (**Figure 1.7**). Therefore, an alteration of RACK1 interaction may affect PDE4 mediated β_2 AR coupling from Gs to Gi (Bolger, Baillie et al. 2006).

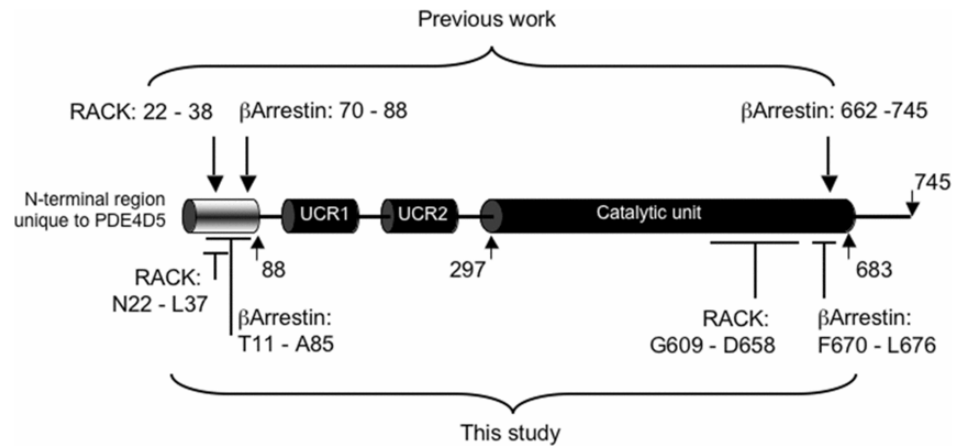


Figure 1.7: RACK1 and beta-arrestin interacting sites of PDE4D5.

A schematic of PDE4D5 with its unique N-terminal region, upstream conserved region 1 (UCR1), upstream conserved region 2 (UCR2), catalytic region and extreme C-terminal region. Indicated above the schematic are the sites of β -arrestin and RACK1 interaction identified prior to the present study. Below it are interaction sites arising from the present study. Reproduced from (Bolger, Baillie et al. 2006)

In human pulmonary artery smooth muscle cells, hypoxia causes transient up-regulation of PDE4B2 which reaches a maximum after seven days and sustained up-regulation of PDE4A10/11 and PDE4D5 in a period of 14 days (Millen, MacLean et al. 2006). The seven days of hypoxia elevated intracellular cAMP levels, PKA activity and activated ERK, but not the overall activities of PKA or PDE4 or PDE3 respectively. This may due to the ERK phosphorylation and inhibitory effect on PDE4 (Millen, MacLean et al. 2006).

PDE4D5 is also the only PDE isoform that exists in gastric smooth muscle cells (Murthy and Sriwai 2008). In smooth muscle cells, cholecystokinin (CCK) treatment increases forskolin-stimulated PDE4D5 phosphorylation and PDE4D5 activity. This enhanced phosphorylation was inhibited by U73122 (inhibitor of PI hydrolysis), bisindolylmaleimide (PKC inhibitor) and PD98059 (MEK inhibitor), but not by C3 exoenzyme (RhoA inhibitor) or Y27632 (Rho kinase inhibitor), indicating the increase of PDE4D5 phosphorylation and activity was regulated by ERK1/2 derived from sequential activation of PLC β and PKC. PP2A was shown to dephosphorylate PDE4D5 and PP2A itself was inhibited by ERK1/2. Co-immunoprecipitation suggested that PDE4D5, PKA, and PP2A bound to the common anchoring protein AKAP. Therefore, it was concluded that cAMP levels in smooth muscle are cross-regulated by contractile agonists in a mechanism: PLC- β /PKC-dependent ERK1/2 activation inhibited PP2A activity. Such

inhibition increased PDE4D5 phosphorylation and activity, which resulted in a decreased cAMP level (Das, Zhou et al. 2003; Murthy and Sriwai 2008).

In primary cardiomyocytes, mouse embryo fibroblasts and HEK293B2 cells, the beta agonist isoprenaline initiates a rapid and transient ubiquitination of PDE4D5 (Li, Baillie et al. 2009). Such ubiquitination occurs at Lys48, Lys53 and Lys78 of the PDE4D5 unique N-terminal region as well as Lys140, which locates at the UCR1 region. These ubiquitination reactions were shown to be mediated by a beta-arrestin-scaffolded pool of the E3 ligase, Mdm2. These studies indicated that PDE4D5 interacts with non-ubiquitinated pool of beta-arrestin because its binding spatially blocks the ubiquitination sites on beta-arrestin thus preventing beta-arrestin from being ubiquitinated. Ubiquitination of PDE4D5 increases the pool of PDE4D5 sequestered by beta-arrestin. Therefore ubiquitination enhances the fidelity of PDE4D5/beta-arrestin association, and decreases the fraction of PDE4D5 sequestered by the scaffolding protein, RACK1 (Li, Baillie et al. 2009).

1.1.8.17 PDE4D6 and PDE4D7

PDE4D6 is a supershort isoform and PDE4D7 is a long isoform of PDE. PDE4D6 is brain-specific and has an identical N-terminal region to PDE4B5 (Cheung, Kan et al. 2007). PDE4D7 is ubiquitously expressed and can be activated by elevated cAMP levels, which may due to the PKA phosphorylation of the conserved UCR1 region among PDE4 long isoforms. They are both sensitive to the inhibitory effect of rolipram. Their expression also have been suggested to respond to the cAMP/PKA signalling pathway (Wang, Deng et al. 2003).

PDE4D7 has been shown to interact with the light chain domains of microtubule-associated protein 1A and 1b (Kwan, Wang et al. 2003).

1.1.8.18 PDE4D8

PDE4D8 has a unique 30 amino acids N-terminal region and has similar properties as other PDE4D long isoforms (Wang, Deng et al. 2003). It is abundant in heart and skeletal muscle, but low in lung (Wang, Deng et al. 2003). It has recently been found to interact with the beta1 adrenergic receptor in a direct manner rather than a previously reported model that PDE4D5 is recruited to β_2 ARs by Barrestin (Richter, Day et al. 2008).

1.1.8.19 PDE4D9

Investigations showed that PDE4D9 was expressed in most rat tissues (Richter, Jin et al. 2005). In FRTL5 cells, PDE4D9 is the major variant that represents more than 70% of PDE activity. Therefore, the important negative feedback regulation of thyroid-receptor activation in cells is mainly mediated by PDE4D9 activation rather than PDE4D3 (Richter, Jin et al. 2005).

1.1.8.20 PDE4D10 and PDE4D11

PDE4D10 is a supershort PDE4 isoform (Chandrasekaran, Toh et al. 2008), whereas PDE4D11 is a long PDE4 isoform that was first identified in mouse brain. It has a wide distribution in many tissues including brain, liver and spleen. In mouse brain, its expression is increased in cerebellum, but decreased in hippocampus with progressive age, indicating a potential function in brain development (Lynex, Li et al. 2008).

1.2 Beta-arrestins and GPCR (β_2 adrenergic receptor)

1.2.1 Arrestins

Barrestins were first found during the purification of BARK (GRK2) (Benovic, Kuhn et al. 1987). When employing progressive purification of the BARK from bovine brain, researchers found a progressive decrease of the enzyme's capacity to desensitize the β_2 AR-mediated activation of G α s. A large amount of a new retinal protein was found to cooperate with rhodopsin kinase, a retinal enzyme, so as to end light-activated rhodopsin-mediated signalling. Furthermore, adding the new protein back to the purified BARK can largely enhance its ability for desensitization. The new protein was the first member of arrestin family that was found and so was named arrestin1 (Shenoy and Lefkowitz 2003). Intriguingly, a 200-300 fold arrestin/Gs ratio was needed compared with the rhodopsin system, which indicated there might be non-retinal original arrestin1-like proteins existing in other tissues to act in concert with GRKs to desensitize the receptors. During the period of 1990 and 1992, Lohse (Lohse, Benovic et al. 1990) and Attramadal (Attramadal, Arriza et al. 1992) found two non-retinal arrestins, respectively, with their colleagues. These two isoforms of arrestin, namely

arrestin2 and arrestin3, also referred to as β arrestin1 and β arrestin2, were ubiquitously expressed in cells and more specific to seven-membrane-spanning receptors (7MSRs) rather than rhodopsin. Finally, arrestin4 was found in retinal cones, in which it regulated colour opsins (Murakami, Yajima et al. 1993).

1.2.2 β -arrestin and desensitization

The classical role of β arrestin is its ability to desensitize GRK-phosphorylated receptors such as 7MSRs / GPCRs (Freedman and Lefkowitz 1996). The desensitization mechanism included two protein families: GRKs, which are able to phosphorylate agonist-occupied receptor molecules specifically, and the arrestins family, which can bind to the phosphorylated receptors and sterically block their interaction with heterotrimeric G proteins (Freedman and Lefkowitz 1996; Krupnick and Benovic 1998) .

The desensitization was first identified when Attramadal and the collaborators (Attramadal, Arriza et al. 1992), were testing the efficacy of purified recombinant β -arrestin1 and β -arrestin2 to blunt GTPase activity in an *in vitro* reconstituted β_2 AR/Gs model system. They found both β -arrestin1 and β -arrestin2 inhibited β_2 AR stimulated GTPase activity by up to 80% (Attramadal, Arriza et al. 1992). In addition to this, transfection of β -arrestin in cell lines with overexpression of β_2 AR generated greater desensitization. Other evidence was provided when using β -arrestin siRNA (small interfering RNA) to knock out or reduce endogenous β -arrestin expression in HEK 293 cells led to increases in cAMP concentration by stimulation of β_2 ARs (Ahn, Nelson et al. 2003). Furthermore, Mundell, S. et al characterized the G protein-coupled receptor regulation in antisense mRNA-expressing cells and observed a similar effect (Mundell, Loudon et al. 1999).

For the purpose of investigating the physiological roles of β -arrestins *in vivo*, knockout mice models have been used (Bohn, Lefkowitz et al. 1999). On one hand, double knock out of β -arrestins led to embryonic lethality while single knock out failed to obtain any abnormal phenotypes. On the other hand, stimulation of 7MSRs in these animals caused significant differences such as when challenging β -arrestin1 null mice with isoproterenol, when cardiac

responses were highly enhanced (Conner, Mathier et al. 1997). Homozygous β -arrestin2 null mice displayed prolonged and enhanced analgesia to morphine treatment caused by an impairment in μ -opioid receptor desensitization (Bohn, Lefkowitz et al. 1999). Intriguingly, Bohn, L.M. et al (Bohn, Gainetdinov et al. 2000) found β -arrestin2 knockout mice lost the ability develop tolerance to the antinociceptive effects of morphine and physical dependence on morphine unlike the wild-type controls. These suggest that the opiate tolerance might have a β -arrestin-dependent mechanism (Shenoy and Lefkowitz 2003).

It has been proved that although there is some diversity, almost all 7MSRs are controlled by β -arrestin-dependent desensitization (Kohout and Lefkowitz 2003). The 7MSRs (seven-membrane-spanning receptors) are receptor families that are expressed at the cell-surface. They are sensitive to a number of extracellular stimuli such as odour, light, peptides, lipids, hormones, neurotransmitters and chemoattractants, and they are the largest receptor family that has been discovered, including over 600 putative members exist in the human genome (Pierce, Premont et al. 2002). The 7MSRs are famous for their ability to mediate G-protein-dependent signaling by specifically binding to heterotrimeric G-proteins. Subsequently to agonist binding, 7MSRs changed to an active conformation, thereby disassociating heterotrimeric G-proteins to G_α and $G_{\beta\gamma}$ subunits. Then, the activated subunits elicit signal amplification and transduction in cells by modulating the activity of those effector molecules, including phospholipases, adenylate cyclases and ion channels.

Currently seven GRKs have been identified, GRK1 to GRK7 (Pierce, Premont et al. 2002). GRK1 and GRK7 are retinal enzymes that can phosphorylate opsins. GRK4 is mainly expressed in brain, kidney and testes. GRK2, GRK3, GRK5 and GRK6 are ubiquitously distributed, and GRK2/3 are also referred to β ARK1 (β -adrenergic receptor kinase1) and β ARK2, respectively (reviewed by (Shenoy and Lefkowitz 2003)).

1.2.3 β -arrestins and endocytosis

Endocytosis (also called sequestration) is an internalization process which removes the receptors from cell surface (Shenoy and Lefkowitz 2003). Usually,

this process happens subsequent to desensitization and it occurs for most 7MSRs (Shenoy and Lefkowitz 2003). Lefkowitz et al suggested endocytosis seemed to be necessary for dephosphorylation and resensitization of receptors rather than desensitization (Lefkowitz, Pitcher et al. 1998). Mutation studies show that the mutation of all the GRK phosphorylation sites diminishes the endocytosis of β_2 AR (Hausdorff, Campbell et al. 1991). Additionally, mutation of a highly conserved tyrosine residue (Y326A) impairs endocytosis and agonist-promoted phosphorylation (Barak, Tiberi et al. 1994).

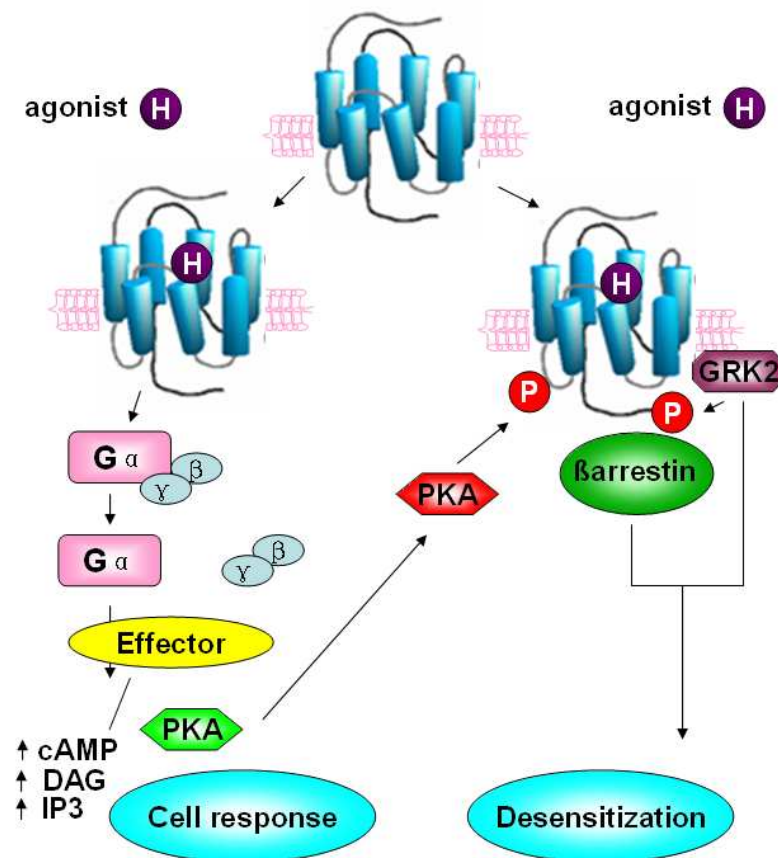


Figure 1.8: Classical role of β -arrestins: desensitisation

When an agonist binds to a 7MSR, a transient high-affinity receptor–heterotrimeric G-protein complex is formed. GDP is released from the G-protein subunits and is replaced by GTP, leading to the dissociation of G-proteins into α and $\beta\gamma$ dimers. The G-protein subunits activate several effector molecules: G α_s activates adenylate cyclases; G α_q activates phospholipase C etc. In the case of the β_2 AR, increased cAMP leads to activation of PKA, which phosphorylates the receptor as a feedback mechanism. The agonist-stimulated receptor is also a substrate for GRK-mediated phosphorylation that promotes β -arrestin binding. β -arrestins prevent further G-protein coupling and G-protein-mediated second messenger signalling. DAG, diacylglycerol; IP3, Ins(1,4,5) P_3 .

Endocytosis of 7MSRs might be mediated by clathrin-coated pits, caveolae or uncoated vesicles. The common mechanism of endocytosis is based on the interaction of 7MSRs and β -arrestins via clathrin-coated pits (Figure 1.8).

Studies (Ferguson, Downey et al. 1996) showed mutated β -arrestin could inhibit β_2 AR sequestration and this circumstance could be rescued by overexpression of β arrestin. Further study by Goodman et al. (Goodman, Krupnick et al. 1996; Goodman, Krupnick et al. 1997) showed β arrestin was able to bind a region (residues 89-100) located at the clathrin terminal domain with high affinity. Therefore, β arrestins can desensitize 7MSRs and promote endocytosis via clathrin-coated pits.

A study (Chen, ten Berge et al. 2003) shows β arrestin2 is involved in the internalization of the 7MSR Frizzled-4 after stimulation of the receptors by Wnt5A. In all previous studied endocytosis cases (Chen, ten Berge et al. 2003; Lefkowitz and Whalen 2004), β arrestin was recruited directly to a GRK-phosphorylated receptor (**Figure 1.9a**). However, in this model, β -arrestin binds to PKC-phosphorylated Dv12, an adaptor protein that can interact with Fz to mediate its canonical signaling via β arrestin (Chen, ten Berge et al. 2003).

Chen et al. (Chen, Kirkbride et al. 2003) reported that the single-membrane-spanning TGF- β type-III receptor, classified as a receptor for TGF- β , could also be internalized via binding to β arrestin2. This is the only one displaying such an ability among the tested TGF- β receptors (**Figure 1.9b**) (Chen, Kirkbride et al. 2003; Lefkowitz and Whalen 2004). Furthermore, the phosphorylation of T β RIII is necessary to its interaction with β arrestin2, and this phosphorylation is catalyzed by the TGF- β type-II receptor rather than a GRK. T β RII is a S/T kinase as well. After phosphorylation and interaction with β arrestin, T β RII and T β RIII then internalize together (Chen, Kirkbride et al. 2003).

β arrestin1 also can bind to the agonist-occupied IGF-1 (insulin-like growth factor 1) receptor and thus cause the receptor internalization (Lin, Daaka et al. 1998)(**Figure 1.9c**).

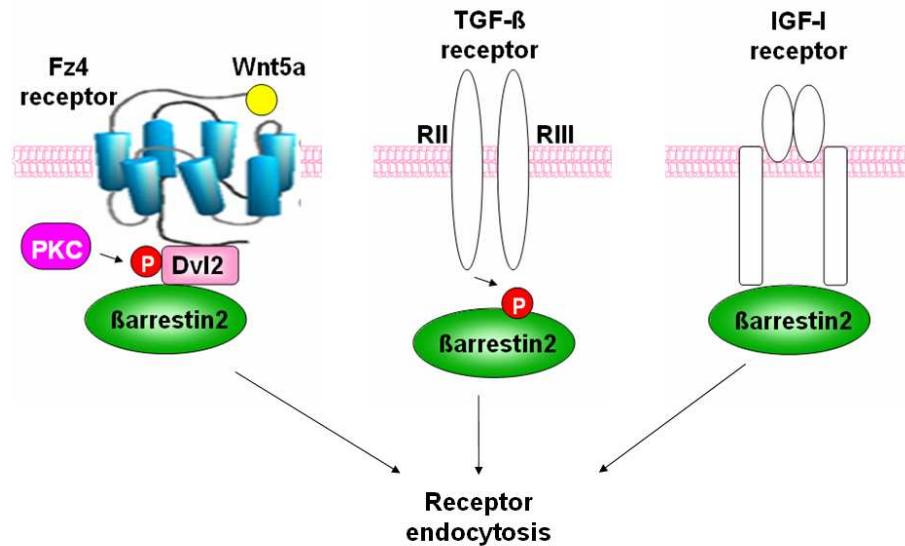


Figure 1.9: β -arrestin-mediated endocytosis of the Frizzled 4 receptor and non-7MSRs.

(Left) β -arrestin2-mediated endocytosis of the Wnt5a-stimulated Frizzled 4 (Fz4) receptor mediated by protein kinase C (PKC) phosphorylation of the intracellular β -arrestin adaptor Dishevelled 2 (Dvl2). (Middle) β -arrestin2-mediated internalization of TGF- β 1 receptor subtypes RII and RIII, facilitated by RII phosphorylation of T841 on RIII. (Right) β -arrestin-mediated internalization of the IGF1 receptor. (Lefkowitz and Whalen 2004)

1.2.4 β arrestin and ubiquitination

Ubiquitin (Ub) is known as a ubiquitous, highly conserved protein, which consists of 76 residues (Hochstrasser 1996; Hershko and Ciechanover 1998) .

Ubiquitination is a well-studied process of protein degradation in cells. The process includes three types of proteins: E1-Ub activating enzyme, E2-Ub carrier enzyme and E3-Ub ligating enzyme. After the first Ub is added to the target protein, following ubiquitination can be classified as two extended kinds, either multi-ubiquitination, Ubs attached to several different lysines, or polyubiquitination, a chain of Ubs added on the preceding Ub (Hochstrasser 1996; Hershko and Ciechanover 1998; Li, Baillie et al. 2009).

Studies (Shenoy, McDonald et al. 2001) show that ubiquitination is involved in the β arrestin- dependent internalization of 7MSRs. Such 7MSRs as β_2 AR are also ubiquitinated in an agonist dependent way. In the ubiquitination progresses, β arrestin is able to bind MDM2, an E3 ubiquitin ligase which has the ability to regulate the tumor suppressor p53 (Shenoy, McDonald et al. 2001) . The receptors such as β_2 AR will bind β arrestin after their stimulation, and then exerts MDM2-mediated ubiquitination of β arrestin (Shenoy, McDonald et al.

2001). This ubiquitination for β arrestin is believed to play a pivotal role for it to act as an adapter in the receptor internalization process. However, the precise mechanism is still unclear (Shenoy and Lefkowitz 2003).

In addition, receptor ubiquitination has been suggested (Marchese and Benovic 2001) to be crucial for the proper sorting and degradation of the internalized receptor in lysosome. This sorting function of ubiquitination has been found in HIV coreceptor CXCR4 (CXC chemokine receptor 4) (Marchese and Benovic 2001). However, the mutation of the target lysines does abolish the ubiquitination and degradation of the receptor, but not the internalization (Marchese and Benovic 2001).

1.2.5 β arrestins in receptor trafficking

Besides the ability of helping receptor internalization, β arrestin may play an important role in receptor trafficking (Lefkowitz and Whalen 2004). There are two patterns of receptor-trafficking which can be classified by their different affinity to β arrestin after β -arrestin-dependent internalization, Class A and Class B (**Figure 1.10**). Class A receptors including β_2 AR recruit β arrestin2 more efficiently than β arrestin1 and Class B receptors such as vasopressin V2 and angiotensin AT1A receptors recruit both β arrestin1 and β arrestin2 equally (Oakley, Laporte et al. 2000). After stimulation of receptors, the Class A receptors transiently bind to β arrestins and the receptors can internalize without β arrestin and recycle rapidly, whereas Class B receptors bind to the β arrestins in a more stable manner and internalize β arrestins together, recycling more slowly (Oakley, Laporte et al. 1999). This might relate to the different rates of deubiquitination of the receptor-bound β arrestins. Shenoy and Lefkowitz (Shenoy and Lefkowitz 2003) proved this hypothesis by transfecting a chimeric molecule including β -arrestin2 with ubiquitin fused in frame to the C-terminus, which can not be deubiquitinated, leading the Class A receptors to be internalized as the Class B receptors (Shenoy and Lefkowitz 2003).

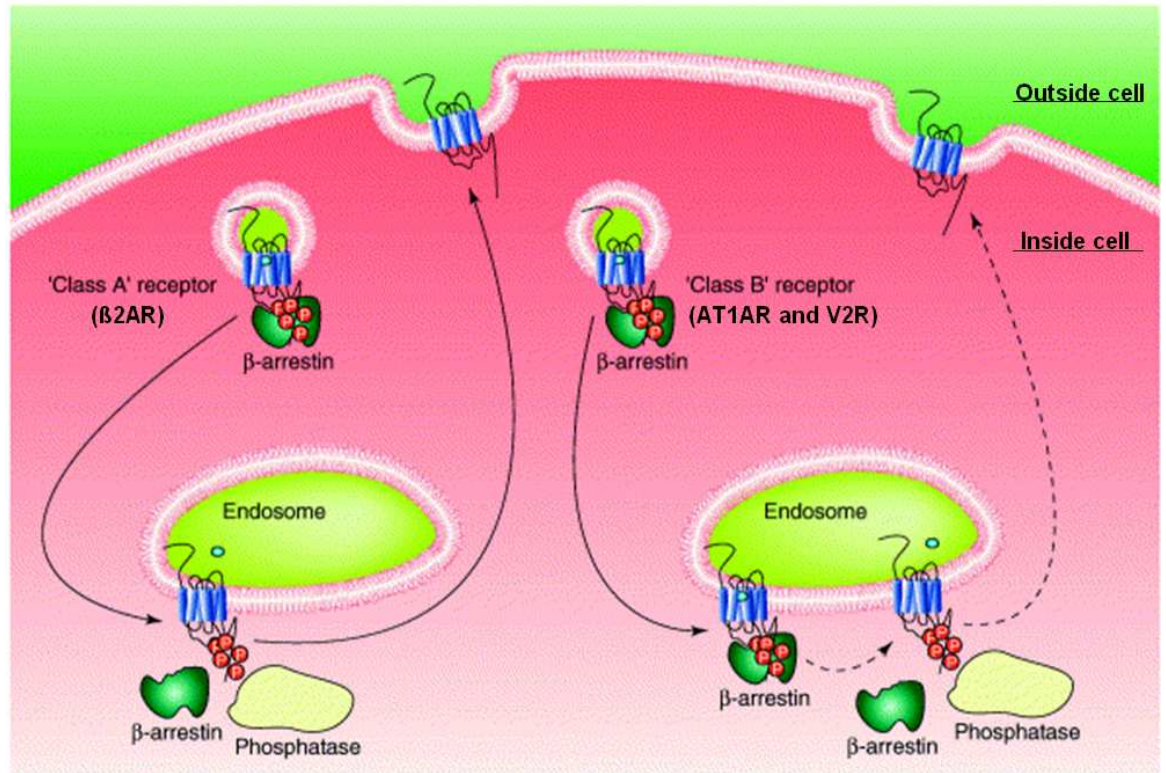


Figure 1.10: βarrestin binding and receptor trafficking properties delineate two classes of seven-transmembrane-span receptors (7MSRs).

Class A receptors, which include the β₂AR, preferentially recruit βarrestin 2, whereas Class B receptors, which include the angiotensin AT1A receptor (AT1AR) and vasopressin V2 receptor (V2R), recruit both β-arrestins with equal efficiency. After stimulation of receptors, the Class A receptors transiently bind to βarrestins and the receptors can internalize without βarrestin and recycle rapidly, whereas Class B receptors bind to the βarrestins in a more stable manner and internalize βarrestins together, recycling more slowly. (Perry and Lefkowitz 2002)

NSF (N-ethylmaleimide-sensitive fusion protein, an ATPase that plays key roles in many intracellular trafficking pathways) has been found to interact with βarrestin1 and βarrestin2, and overexpression of NSF in HEK 293 cells is able to improve βarrestin-mediated β₂AR endocytosis (McDonald, Cote et al. 1999). Besides NSF, the small GTP binding protein ARF6 (ADP-ribosylation factor 6) is also an important protein for vesicular trafficking and it also binds to βarrestin (Claing, Chen et al. 2001). Substitution of GDP by GTP is necessary for ARF6's activation (Claing, Chen et al. 2001). The activation of ARF6 can be enhanced by the GEF (guanine nucleotide exchange factor) activity of ARNO (ARF nucleotide binding site opener) which constitutively binds to βarrestin2. Intriguingly, both the GDP inactive mutant and the GTP active mutant can impair the β₂AR internalization and in contrast, overexpression of ARNO enhances the receptor internalization.

1.2.6 β arrestins and Src-family kinases

Src-family non-receptor tyrosine kinases are structurally related kinases (Kefalas, Brown et al. 1995). They are essential in various cell functional responses such as receptor endocytosis, ERK1/2 activation and exocytosis (Kefalas, Brown et al. 1995). When β arrestins bind to the kinases, they act as adaptors that regulate the recruitment of the Src-family kinases to 7MSRs, and the kinases can promote downstream cellular responses subsequently.

Most 7MSRs mediate the activation of ERK1/2 mitogen-activated protein kinases (MAPKs). Using EGF (epidermal growth factor) as an example, when transactivation of EGF via the β_2 AR, β arrestin1 plays an role as adaptor through which the kinase is recruited to the active receptor by binding to both the catalytic domains of c-Src and the SH3 (Src-homology 3) (Luttrell, Ferguson et al. 1999; Maudsley, Pierce et al. 2000; Miller, Maudsley et al. 2000) . C-Src then tyrosine phosphorylates many proteins involved in the propagation of ERK1/2 activation as soon as it has been relocated to the plasma membrane. Phosphorylation of the adaptor protein Shc by c-Src leads to the recruitment of the Ras GDP-GTP exchange factor SOS by its interaction with the adaptor Grb2, which leads to the subsequent activation of Ras, Raf-1, MEK1 and ERK1/2 (**Figure 1.11**).

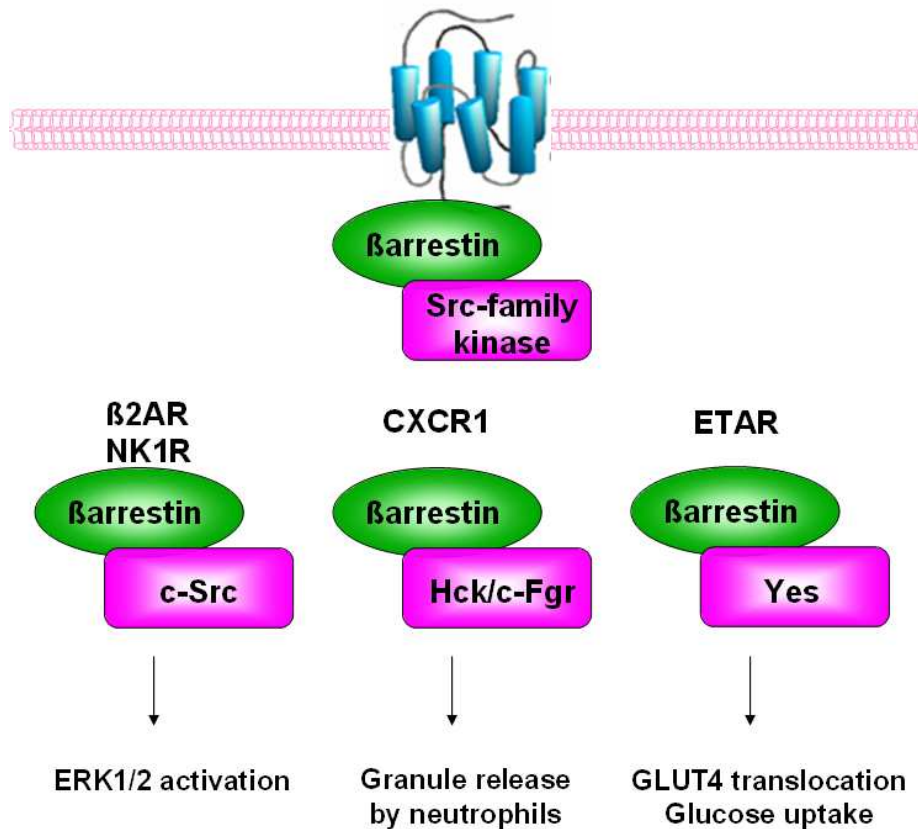


Figure 1.11: Several seven-transmembrane-span receptors (7MSRs) signal to members of the Src family of tyrosine kinases by mechanisms that depend on β -arrestin-mediated recruitment of the kinases to the 7MSRs.

c-Src can be recruited to both the β 2 adrenergic receptor (β 2AR) and the neurokinin 1 receptor (NK1R) during Erk1/2 activation. Hck and/or c-Fgr recruitment to the CXCR1 chemokine receptor 1 is necessary for the release of interleukin-8-elicited granule from neutrophils. After stimulation of adipocytes with endothelin, GLUT4 translocates to the plasma membrane (resulting in glucose uptake) by a mechanism involving the recruitment of Yes to the activated endothelin type A receptor (ETAR). (Perry and Lefkowitz 2002).

Interleukin8 (IL-8) activates the CXC-chemokine receptor 1 (CXCR1) in neutrophils, and which induces the rapid formation of complexes containing β arrestin, Hck and c-Fgr (two members of the Src-family). This elicits granule release from neutrophils (Barlic, Andrews et al. 2000).

In addition, β arrestin also has been proved necessary in the endothelin-stimulated GLUT4 translocation to plasma membrane, which results in glucose uptake via the recruitment of Yes to the activated ETAR (endothelin type A receptor) (Imamura, Huang et al. 2001).

1.2.7 β arrestin and apoptosis

Regulation of arrestin2 (the *Drosophila* functional equivalent of visual arrestin) and rhodopsin are mediated by their phosphorylation. When the flies are exposed to light, the activation of rhodopsin gives rise to the activation of Gq coupling and phospholipase C (NorpA), and then elicits signal generation (Alloway, Howard et al. 2000; Kiselev, Socolich et al. 2000). The fly 7MSRs are rapidly desensitized in the same way of that in mammalian cells. The receptors are phosphorylated first and bind to unphosphorylated arrestin2. NorpA activation initiates the activation of rhodopsin phosphatase RdgC and CamKII (calcium/calmodulin-dependent kinase II). The arrestin2 phosphorylation results in its dissociation from rhodopsin and rhodopsin dephosphorylation by RdgC, leading to the resensitization of rhodopsin. In cells with mutated non-functional NorpA or RdgC, the regulation of arrestin2 and rhodopsin phosphorylation are disturbed, which causes a formation of a stable receptor-arrestin2 complex and ends at subsequent rod-cell apoptosis. It is not too surprising that this complex formation is believed to provide the signal for apoptosis (Alloway, Howard et al. 2000; Kiselev, Socolich et al. 2000).

In HEK293 cells, β arrestins enhance the ubiquitin-dependent degradation of apoptosis signal-regulating kinase 1 (ASK1), thereby attenuating H₂O₂-induced apoptosis (Zhang, Hao et al. 2009).

1.2.8 β arrestin and MAPK kinases cascades

The MAPKs consist of a large number of members that include ERK1 (p44), ERK2 (p42), ERK5 (big MAPK or BMK), JNK1-JNK3 (stress -activated protein kinase) and p38 MAPKs (α , β , γ and σ isoforms) (Shenoy and Lefkowitz 2003). The MAPKs play pivotal roles in the regulation of variable cellular processes such as cell proliferation, gene expression and apoptosis (Shenoy and Lefkowitz 2003). MAPKs cascades are highly regulated systems, MAP2K kinases (MAP3Ks) activate MAPK kinases (MAP2Ks), which then activate MAPKs. The cascades are also complex as the same kinase may be involved in different pathways (Figure 1.12)

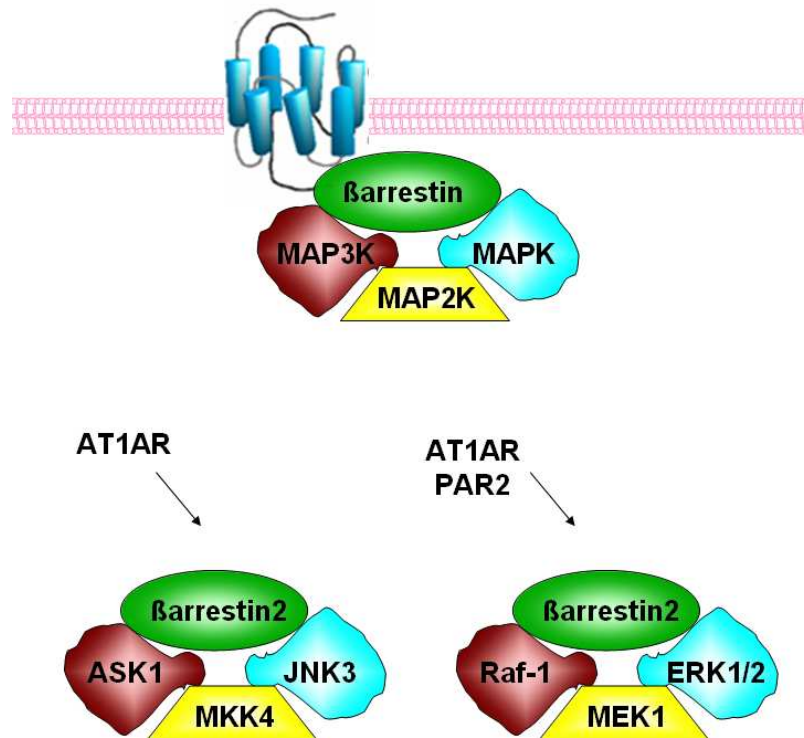


Figure 1.12: β -Arrestins act as scaffolds for mitogen-activated protein kinase (MAPK) cascades and aid in their regulation by seven-membrane-span receptors (7MSRs).

By binding to one or more of the kinases of MAPK cascades, the β -arrestins scaffold them into a signaling complex that can be recruited to and regulated by activated 7MSRs. In this manner, the pathway made up of ASK1, MKK4 and JNK3 is regulated by β arrestin2 and the angiotensin 1A receptor (AT1AR); Raf-1, MEK1 and ERK1/2 are regulated by β arrestins1 and 2, and by both the AT1AR and type-2 protease-activated receptor (PAR2). (Perry and Lefkowitz 2002)

β arrestin acts as an scaffold protein in MAPKs cascades by interacting with JNK3 and ERK1/2 MAPK modules. By yeast two-hybrid screening, JNK3 was found to bind to β arrestin. It can interact with β arrestin at the endogenous level of protein expression. Additionally, overexpression of β arrestin in HEK 293 cells can keep JNK3 in the cytosol. ASK1 activates JNK3 and forms a complex with JNK3 and β arrestin. Furthermore, β arrestin guides the formation of appropriate kinase complexes and plays a scaffolding role to help the 7MSRs mediating these proteins (Perry and Lefkowitz 2002).

β arrestins also act as 7MSR-regulated scaffold proteins for ERK1/2, which brings Raf (MAPKKK), MEK1 (MAPKK) and ERK (MAPK) together. The stimulation of G_{α_q} -coupled PAR2 results in the formation of a multi-protein signaling complex

including β arrestin, Raf-1 and ERK, cytosolic retention of phosphorylated ERK and co-localizes the receptor-arrestin- ERK complex on endocytic vesicles (Yasuda, Whitmarsh et al. 1999). Studies of uninternalized and undesensitized mutated PAR2 demonstrates that the mutant can still activate ERK by stimulation via a Ras-dependent pathway, resulting in nuclear translocation of phosphorylated ERK and mitogenic signaling (Yasuda, Whitmarsh et al. 1999). This suggests that β arrestin seems to determine different ERK activation pathways, Ras dependent or independent, via its complex with the activated PAR2. In addition, Luttrell et al. reported another G_{α_q} -coupled receptor, the $AT_{1A}R$ that is also able to induce the formation of a β arrestin/Raf/MEK/ERK complex (Luttrell, Roudabush et al. 2001).

Various data (Sun, Cheng et al. 2002) demonstrate that β arrestin is involved in the activation process of p38 MAPK through 7MSRs stimulation. This p38 activation is essential for SDF (stromal cell-derived factor)-induced chemotaxis in HEK293 cells (Sun, Cheng et al. 2002). β arrestin2-dependent p38 signaling is proved to interact with the human cytomegalus virus-encoded viral GPCR US28 (Miller, Houtz et al. 2003). Finally, β -arrestin can enhance p38 MAPK signalling of CXCR4 (Sun, Cheng et al. 2002).

1.2.9 β arrestin1 goes nuclear

The function of β arrestin in receptor endocytosis and its scaffolding role in signaling has already been uncovered (Perry and Lefkowitz 2002; Shenoy and Lefkowitz 2003; Lefkowitz and Whalen 2004) . Recent studies have shown that β arrestins and mediators of the endocytosis of 7MSRs are able to shuttle between the cytoplasm and nucleus, and intriguingly, β arrestin1 is present in both cytoplasm and the nucleus at a stable level. These data highly suggest β arrestin may play important functional roles in the nucleus.

Kang and his colleagues (Kang, Shi et al. 2005) have found a novel function of β -arrestin1 whereby the scaffolding protein acts as a cytoplasm-nucleus messenger in GPCR signalling and elucidates an epigenetic system for direct GPCR signalling from cell membrane to the nucleus via signal-dependent histone modification.

Delta-opioid receptor (DOR) is a member of the GPCR family. When it is challenged with its specific agonist DPDPE, the β arrestin1 and β arrestin2 are transferred to the cell membrane, but β arrestin 1, not β arrestin2 accumulated in nucleus. It is already known that β arrestin has a nuclear export signal, and the mutation of this signal by a single residue mutation (Q394L) eliminates β arrestin1 accumulation in nucleus.

p27 and c-fos are cycle related genes that play key roles in the regulation of cell proliferation (Kang, Shi et al. 2005). Expression of β arrestin1 notably increases p27 and protein concentration, but expression of β arrestin2 failed to do so. DPDPE stimulation significantly increases the expression of p27 and either naltridole (DOR inhibitor) or β arrestin 1 siRNA can block the DPDPE's effect. Furthermore, the inhibition of Gi/Go (pertussis toxin), PI3K (wortmannin), p38 (SB203580), JNK (SP600125) and ERK (PD98059) has no effect on the p27 transcription. These indicate that activation of DOR can mediate gene expression at a transcription level and this effect depends on β arrestin1 nuclear translocation.

Epigenetic regulation such as acetylation modification of histone is believed to be essential in the regulation of eukaryotic gene transcription. Overexpression of β arrestin 1 increases the acetylation of histone H4 and β arrestin siRNA treatment leads to decrease acetylation (Kang, Shi et al. 2005). These observations suggest that β arrestin 1 may influence the transcription of a variety of gene expression by regulating the acetylation of H4.

Overexpression of β arrestin 1 increases the level of p300 at p27 and c-fos promoters, while inhibition of β arrestin1 by siRNA decrease the accumulation of p300 (Kang, Shi et al. 2005). Additionally, DPDPE stimulation also causes the accumulation of p300 at p27 and c-fos promoters, where β arrestin 1 is temporally increased. Furthermore, p300 and β arrestin1 can co-immunoprecipitate (Kang, Shi et al. 2005). These data suggest β arrestin 1 may promote gene-specific H4 hyperacetylation by recruiting p300 to the target promoters.

As DOR is an important neurotransmitter receptor widely expressed in neural cells, Kang et al (Kang, Shi et al. 2005) tested the physiological consequence of β arrestin-dependent gene regulation in human brain neuroblastoma SK cells through DOR stimulation by DPDPK. They found that activation of the DOR in such cells promotes β arrestin1-dependent histone H4 hyperacetylation, p27 transcription and growth inhibition (Kang, Shi et al. 2005).

1.2.10 β arrestin and insulin resistance

Insulin resistance is a defect of insulin in stimulating insulin receptor signalling (Matthaei, Stumvoll et al. 2000; Taniguchi, Emanuelli et al. 2006) . It is a phenotype of type 2 diabetes, which has become more prevalent. The known insulin signalling pathway includes a series of reactions (Luan, Zhao et al. 2009). Thus, upon insulin stimulation, insulin receptor substrate proteins are recruited to and phosphorylated at the insulin receptor; resulting the activation of the phosphatidylinositol-3-OH kinase (PI₃K)- Akt pathway; the activated Akt phosphorylates its downstream effectors and transcription factors, thereby modulating insulin -induced metabolic actions (Luan, Zhao et al. 2009).

Luan *et al* (Luan, Zhao et al. 2009) found that β arrestin2 has a very low expression level in a diabetic mouse model. Silencing of β arrestin2 in transgenic mice deteriorated insulin sensitivity, whereas increased β arrestin2 expression in diabetic db/db mice restored their insulin sensitivity. This new function of β arrestin2 is due to its scaffolding role in translocating Akt and Src to the insulin receptor. Loss or dysfunction of β arrestin2 leads to the disruption of the Akt/Src/ β arrestin2 signalling complex and the disturbance of insulin signalling, thus resulting in the development of insulin resistance in type 2 diabetes (Luan, Zhao et al. 2009).

1.2.11 β 2-adrenergic receptor

Adrenergic receptors (adrenoceptors) belong to the G protein-coupled receptor superfamily (Liggett 2002). There are five subtypes of adrenergic receptors, α_1 , α_2 , β_1 , β_2 and β_3 , in which β_2 -adrenergic receptor (β_2 AR) has been extensively studied and used as a model for studying GPCR. It can be regulated by PKA and

GRK phosphorylation, and can undertake rapid desensitization upon β arrestin binding as described above. For an easy understanding, phosphorylation sites and important interaction sites of β_2 AR are labelled in Figure 1.13 (Liggett 2002). Recently, the high-resolution crystal structure of β_2 AR has been constructed (Cherezov, Rosenbaum et al. 2007), which will no doubt shed light on the structural understanding of β_2 AR as well as facilitating drug design for β_2 AR related diseases.

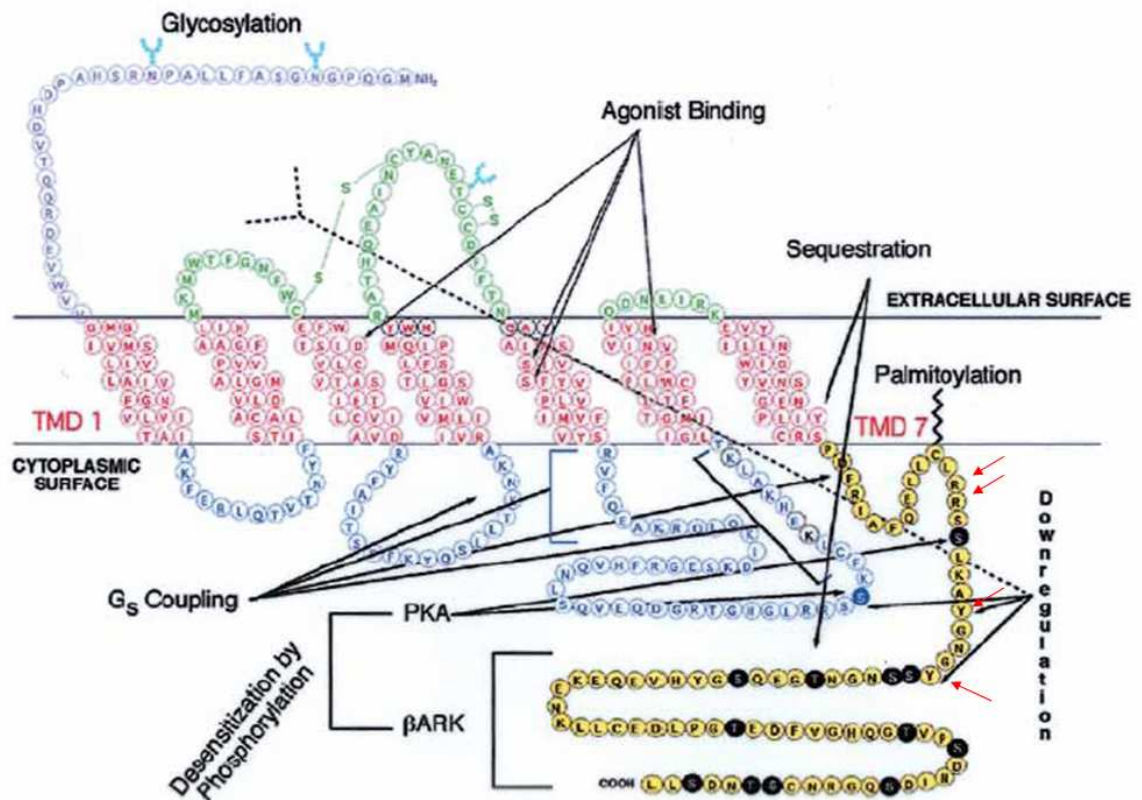


Figure 1.13: Amino acid sequence and proposed membrane topology of the human β_2 AR. (Liggett 2002)

Regions or specific domains with structural significance are labeled. Mutation of R259R260 (red arrows) or Y350Y354 (red arrows) to alanines may lead to reduced RACK1 and β arrestin translocation to the receptor. TMD 1 and TMD 7 indicate the first and seventh transmembrane- spanning domains, respectively. β ARK, β -AR kinase.

1.3 RACK1 scaffolding protein

Receptor for activated C kinase 1 (RACK1) belongs to the tryptophan-aspartate (WD) family, which is famous for its propeller-like structure (Neer, Schmidt et al. 1994). It was first identified as an intracellular receptor for protein kinase C (Mochlyrosen, Khaner et al. 1991). The identification of RACK is based on criteria

that were originally established by Ron et al. (1994) and modified by Dorn and Mochly Rosen (2002): 1) injection purified RACK in cells should block PKC mediated cell process; 2) delivery of certain peptides into cells should block the interaction between a particular PKC isozyme and RACK, and the peptides should specifically ablate a known cellular function of that isozyme; 3) delivery of peptides that trigger an interaction between a particular PKC isoform and its interacting RACK should uniquely activate that isozyme; 4) RACK should bind PKC in the presence of PKC activators (Ron and Mochlyrosen 1994; Dorn and Mochly-Rosen 2002). More and more RACK1-interacting proteins have been found in these years, extending its function in many biological processes.

1.3.1 RACKs

According to the criteria described above, three RACK isoforms were discovered: RACK1, receptor for activated C kinase 1 (also known as the receptor for activated PKC β) (Ron, Chen et al. 1994); RACK2, which binds PKC ϵ (Csukai, Chen et al. 1997); and PRKCBP1, a RACK like protein for PKC β (Fossey, Kuroda et al. 2000). RACKs have no catalytic activity and their binding sites on PKCs are isolated from their substrate binding sites (Ron, Chen et al. 1994). Various proteins apart from PKCs were found to interact with RACKs as well, leading to the hypothesis that RACKs may work as adaptor proteins (Schechtman and Mochly-Rosen 2001) (**Table 1.3**). Two functions normally seen in adaptor proteins were found in RACK as well: 1) transferring and positioning of a signalling enzyme to appropriate locations such as bringing a certain enzyme to its substrate (Jaken and Parker 2000); 2) altering the enzyme activity by direct interaction (holding PKC in an active conformation).

1.3.2 Evolutionary conservation of RACK1 genes

The RACK1 sequence is highly evolutionary conserved (McCahill, Warwicker et al. 2002). It shares 100% identity between chicken, rat and human, which implies its crucial position in regulating key signalling pathways in cellular processes. It was first cloned from a human B-lymphoblastoid cell line (H12.3) as well as from a chicken liver library (C12.3) (Guillemot, Billault et al. 1989). The human RACK1 gene (Gene Bank; GNB2L1) locates on the chromosome 5 (5q35.3) and contains 8 exons and 7 introns. RACK1 is a 36 kDa protein that has seven Trp-Asp 40 (WD40)

repeats in the pattern $X_{6-14} \text{---} [\text{GH-X}_{23-41}\text{---WD}]_{n4-8}$ where n is the number of WD repeats.

The evolutionary conserved RACK1 exists in a diverse range of eukaryotes such as plants (*Toxoplasma gondii*), fungi (*Paracoccidioides brasiliensis*), yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), insects (*Drosophila melanogaster* and *Heliothis virescens*), vertebrates (*Gallus gallus* and *Brachydanio rerio*) and mammals (*Bos taurus*, *Sus scrofa*, *Rattus norvegicus* and *Homo sapiens*) (Sklan, Podoly et al. 2006). The positioning of RACK1 WD repeats even exists in the alga *Chlamydomonas reinhardtii* which diverged from the forerunners of the plant and animal kingdoms about 600 million to 1 billion years ago (Schloss 1990). This evolutionary conservation confirms the notion that RACK1 possesses important functions in many biological systems, and this function established even before the separation of plant and animal kingdoms (Neer, Schmidt et al. 1994). Furthermore, RACK1 also is expressed ubiquitously in human tissues including liver, spleen and brain, and the promoter of RACK1 gene contains multiple transcription factor binding sites (serum response element [SRE] for example). These indicate that RACK1 has key roles in many cell types, and it may respond to various stimuli (Guillemot, Billault et al. 1989; Chou, Chou et al. 1999).

1.3.3 RACK1 binds to PKC

The first function of RACK1 that was identified relates to its ability to interact with active “conventional” protein kinase-C (PKC) such as PKC β II (Ron, Luo et al. 1995; Stebbins and Mochly-Rosen 2001) and “novel” PKCs such as PKC ϵ (Besson, Wilson et al. 2002). Conventional PKC (α , β I, β II, γ) are calcium- and diacylglycerol-dependent protein kinases which are activated upon elevated calcium and diacylglycerol that are generated by receptor-stimulated hydrolysis of plasma membrane phosphatidylinositol 4, 5-bisphosphate; whereas novel PKCs are those calcium-independent kinases (δ , ϵ , η , θ , μ) or calcium- and diacylglycerol-independent (atypical) kinases (ζ , λ) (Mellor and Parker 1998). Activation of the different PKC isoforms triggers their translocation to distinct intracellular compartments where they might play specific physiological roles.

The PKC contains four conserved regions (namely C1-C4) that are interrupted by five variable regions (namely V1-V5). Each PKC isoform consists of a catalytic unit and regulatory unit involving one or two C1 domains. The C1 domain exists in “typical” and “atypical” structure variants, where phorbol ester/DAG can bind to the “typical” but not the “atypical”. Most PKCs contain a “typical” C1 domain that is responsive to phorbol ester/DAG, whereas one exception, aPKCs, contains the “atypical” domain (Newton and Johnson 1998).

The C2 domain exists in conventional and certain modified forms of novel PKCs as well as other unrelated proteins, which are commonly of membrane associated in a Ca^{2+} dependent manner. Although the novel PKCs contains the C2 domain, they are not Ca^{2+} dependent because they lack key amino acids for Ca^{2+} binding. Unlike conventional PKCs that require Ca^{2+} to bind lipid, novel PKCs are assumed to bind anionic lipids independently.

C3 domain containing an ATP binding lobe and C4 containing the substrate binding lobe are the two catalytic domains that are present in all known PKCs. All PKCs, except the μ isoform, contain an auto-inhibitory domain (also known as pseudosubstrate domain). This domain sterically occupies the active site of PKC in order to maintain PKC in an inactive conformation. Removing this domain is necessary for activation of PKC (Newton and Johnson 1998).

RACK1 binding to PKCs is not interrupted by delivery of synthetic peptides that derived from PKC substrate and the pseudosubstrate domain, suggesting that the RACK1 binding site is distinct from the PKC substrate binding sites. Three PKC isoforms contain RACK1-homologous sequences in their C2 regions. These sequences are supposed to function as pseudo-RACK binding sites in PKCs (Ron, Chen et al. 1994).

Three C2-derived peptides, amino acids 86-198, 209-216 and 218-226 in PKC β , as well as the vesicle-specific p65 protein, which has a PKC-C2 homologous region, can bind to RACK1 (Ron, Luo et al. 1995). This implies there might be part of the RACK1 binding site on the C2 region. A peptide derived from the V5 domain (amino acids 645-650 in PKC β II) can selectively inhibit the PMA -induced translocation of PKC β II, but not PKC β I, indicating that V5 also contains part of RACK1 binding site (Stebbins and Mochly-Rosen 2001).

1.3.4 RACK1 and diseases

Enhanced RACK1 expression level was detected in colon carcinoma (Berns, Humar et al. 2000), melanoma (Lopez-Bergami, Habelhah et al. 2005), non-small-cell lung carcinoma (Berns, Humar et al. 2000), and oral squamous cell carcinoma (Wang, Jiang et al. 2008). The inhibition of RACK1 expression increased the sensibility of melanoma cells to treatment and reduced their tumorigenicity in a xenograft tumor model. Therefore, RACK1 is expected to be important in tumorigenicity (Lopez-Bergami, Habelhah et al. 2005) and may become a molecular marker for certain carcinoma.

Alterations of RACK1 expression also have been found in brain pathologies during aging. In Alzheimer's disease (AD) and some aging model animals, a lack of RACK1 was detected in post-mortem brains which contributed to the reduced activation and translocation of PKC in the aging brain. This phenomenon is suggested to be associated with the PKC related processing of the amyloid precursor protein (APP) through the action of secretases (Pascale, Fortino et al. 1996; Battaini, Pascale et al. 1997; Battaini, Pascale et al. 1999; Sanguino, Roglans et al. 2004). However, a contradicting observation showed no detectable RACK1 variation between AD brains and control brains (Shimohama, Kamiya et al. 1998). Population differences may be a reasonable explanation, but no experimental evidence has been obtained so far for this. Furthermore, RACK1 levels were significantly reduced in the cortex part of patients with Down syndrome (young patients with AD). A popular explanation for Down syndrome is that it is due to novel neuronal migration and early neurite outgrowth (Peyrl, Weitzdoerfer et al. 2002). However, this interpretation also needs to be proven.

Wang and Friedman found that the increased PKC activity in the frontal cortex of patients with bipolar disorder might due to the enhanced association between RACK1 and PKC (Wang and Friedman 2001). The anti-Parkinsonian drug Rasagiline can increase the RACK1 concentration (Bar-Am, Yogev-Falach et al. 2004).

1.3.5 Structure of RACK1

1.3.5.1 RACK1 and its β -propeller

RACK1 shares 42% identity with G protein β subunit (McCahill, Warwicker et al. 2002). A comparative model of RACK1 was constructed using bovine transducin G β as the structural template (Sondek, Bohm et al. 1996). As seen in the 3D structure, 7 blades form a conical ring of RACK1. Each blade of the propeller is generated from a WD repeat which is formed by four anti-parallel β -sheets (Sondek and Siderovski 2001; Steele, McCahill et al. 2001).

Within all WD proteins, the WD repeats are connected by various loops with different sizes. These loops are positioned above and below the of the propeller structure. Unlike the highly homologous β -sheets, these loops share little similarity, both in their sizes and properties, which distinguish each WD protein from other family members and endue them with various interacting proteins. In RACK1, the β -sheets are splayed out to form the conical shape of RACK1 and provide RACK1 with three protein interacting sites: top, bottom and circumference (**Figure 1.14**). Nevertheless, maintaining the hydrogen bond formation between β strands in the propeller provides an additional possibility for protein interactions. Thereby, proteins can interact with these potential sites simultaneously. Indeed, many proteins have been found to bind RACK1 such as C2-domain containing proteins (PKC, synaptotagmin and phospholipase Cy1) and PH-domain containing proteins (dynamin-1 and β -spectrin). RACK1 has also been proposed to bind more than one interacting partner simultaneously (Schechtman and Mochly-Rosen 2001).

1.3.5.2 Blade 6 and its lengthened loop

From the structure, an internal region that derivates from the G β structure is identified (Lambright, Sondek et al. 1996) (Lambright, Sondek et al. 1996). It locates in blade 6 of the RACK1 propeller and is distinct from G β with a longer inter- β -strand loop at the comparable region. The G β -strand preceding loop region has a SIKIWD sequence (amino acids 255-260), which is considered to be part of the PKC binding sites of RACK1 (Ron and Mochlyrosen 1995). The loop also has been assumed to form an extra β -sheet (Chen, Spiegelberg et al. 2004). The lengthened loop supports a conformational flexibility of this region; thus,

this provides the fundamental structure for the existence of many protein interaction clusters in this region (**Figure 1.14**). β -propeller models showed that the ligand-binding site also was made of these β -sheets-connecting loops (Springer 1997). For blade 6, it functions as a major docking station of RACK1, which due to its tyrosine phosphorylation followed by conformational changes. These changes might facilitate additional protein binding or exposing of hidden binding sites.

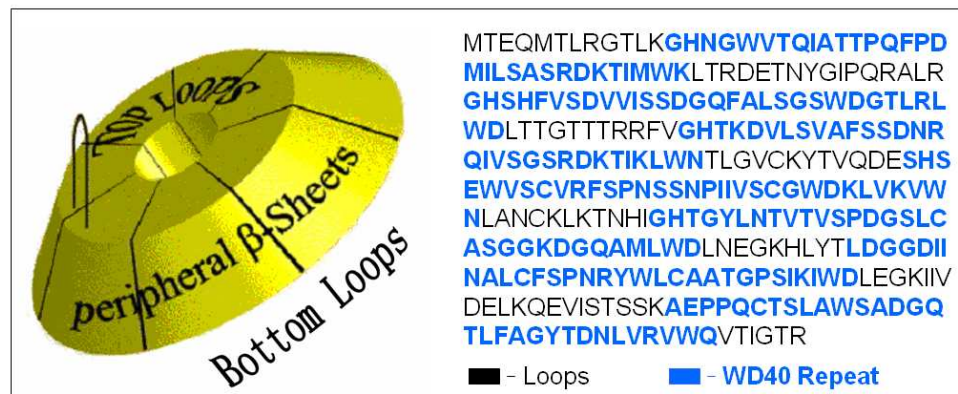


Figure 1.14: Putative interaction surfaces of RACK1.

Left: Shown is a schematic picture of the seven-blade structure characteristic of RACK1, presented as a flat-topped cone. The upward protruding loop is in the middle of two blades. The top and bottom surfaces and the peripheral β -sheets are all amenable for putative interactions. Right: Blades (blue) and loops (black) are shown in the RACK1 sequence.

1.3.5.3 RACK1 dimerization

Dimerization is common property of WD40 proteins. Indeed, RACK1 can form homo- and hetero-dimers (Kominami, Ochotorena et al. 1998; Dell, Connor et al. 2002; Chen, Spiegelberg et al. 2004). The homo-dimers are generated through its WD40 motif in a way similar to the dimer formation of F-box/WD repeats protein Pop1 and Pop2 in fission yeast (Kominami, Ochotorena et al. 1998) and the regulation by self-interaction of the WD40 repeat region apaf-1 (Hu, Ding et al. 1998). For hetero-dimerization, RACK1 can associate with its homologue GB via its WD40 repeats (Dell, Connor et al. 2002; Chen, Spiegelberg et al. 2004) as well as generates a complex with Fyn which interacts with RACK1 5-7 WD40 repeats (Tcherkasowa, Adam-Klages et al. 2002). RACK1 binds to G β y and subsequently translocates to cell membrane, indicating a scaffolding role of RACK1 for assembly of multiple protein complex.

The 7 blades structure of RACK1 also implies 6 putative forms of WD40-WD40 complex: top-to-top, top-to-bottom, top-to-side, side-to-bottom, side-to-side, and bottom-to-bottom. Therefore, specific WD40 protein may interact in unique form and allow free binding surface for additional interaction.

1.3.6 RACK1 signal transduction

1.3.6.1 PKC

RACK1 associated with PKC β II and its overlapping positioning with PKC β II have been found in CHO cells treated with PMA, in NG108-15 neuroblastoma cells and in cardiomyocytes (Ron, Jiang et al. 1999). The localization of RACK1 is cell-type dependent in both stimulated and unstimulated cells. This suggests that RACK1 may deliver active enzyme to the appropriate subcellular site rather than anchoring PKC β II in a specific position (Ron, Jiang et al. 1999; McCahill, Warwicker et al. 2002). As mentioned above, RACK1 can binds two parts of PKC β II, one is the C2 domain, the other is the V5 domain. C2- and V5 derived peptides have inhibitory effects on translocation of PKC and can disrupt several cellular functions, such as *Xenopus leavis* oocyte maturation, myocyte hypertrophy and activation of PLD (Ron, Luo et al. 1995; Thorsen, Bjorndal et al. 2000; Stebbins and Mochly-Rosen 2001).

Another PKC isoform PKC ϵ binds RACK1 with lower affinity compared to that of RACK2. In U87 glioblastoma cells, RACK1 interacts with PKC ϵ , whereas in PC12 cells, RACK1 forms a complex with PKC β II and the stress-induced variant of acetylcholinesterase AChE-R (Perry, Sklan et al. 2004).

1.3.6.2 PDE4D5

RACK1 has been shown to interact with PDE4D5 (Bolger, McCahill et al. 2002) , a key regulator in cAMP dependent signal transduction, indicating that it may involved in the regulation of pathways activated by adenylyl cyclase. Indeed, treatment with the adenylyl cyclase activator forskolin induces RACK1 translocation to the nucleus, whereas the localization of PKC β II is unaffected (Ron, Vagts et al. 2000). Furthermore, treating cells with another cAMP-elevating agent, ethanol, which increases the activity of adenylyl cyclase, therefore activating the PKA signalling pathway, also promotes the nuclear

translocation of RACK1 (Ron, Vagts et al. 2000). Such translocation can be blocked by adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer, an inhibitory analogue of cAMP that prevents the activation of PKA. Together with Dohrman's finding that ethanol also induces the translocation of PKA catalytic unit to the nucleus (Dohrman, Diamond et al. 1996), it is highly possible that RACK1 may target PDE4D5 to a fraction of PKA or certain subcellular localization in order to regulate the cAMP/PKA signalling there. Nevertheless, RACK1 can regulate PDE4D5 distribution via competing with the binding of PDE4D5 with other scaffold proteins, in particular the beta-arrestins (Bolger, Baillie et al. 2006). It has been shown (Bolger, Baillie et al. 2006) that RACK1 and beta-arrestin have overlapping binding sites on PDE4D5 N-terminal region, which makes their binding to the enzyme occur in a mutually exclusive way. Beta-arrestin was shown (Perry, Baillie et al. 2002) to recruit PDE4D5 to the activated beta2 adrenoceptor after stimulation for quick desensitization of the cAMP around the receptors and alter the receptor coupling from Gs to Gi. However, this process can be affected by elevated RACK1 levels for competing with beta-arrestin in the binding of PDE4D5 (Bolger, Baillie et al. 2006).

As for RACK1 translocating to the nucleus, it can influence gene transcription by interacting with the upstream effectors. In this regard, RACK1 was found to be a novel binding partner of Smad3 (Okano, Schnaper et al. 2006). It interacts with the linker region of Smad3 by its WD repeats 6 and 7. In human kidney epithelial cell line, knock-down of the RACK1 expression increases transcriptional activity of TGF-1-responsive promoter sequences of the Smad binding element (SBE), p3TP-Lux, and 2(I) collagen (transforming growth factor-1 [TGF-1] is crucial for renal fibrogenesis; it can stimulate phosphorylation of Smad2/3 and activate other signaling molecules). However, over expressing RACK1 negatively regulates 2(I) collagen transcriptional activity in TGF-1-stimulated cells. RACK1 has no influence on the phosphorylation of Smad3 in the linker region or the C-terminus. It reduces the Smad3 direct binding to SBE in order to modulate the transcription of 2(I) collagen (Okano, Schnaper et al. 2006).

He et al (He, Vagts et al. 2002) also found that nuclear RACK1 was involved in the induction of c-Fos mRNA and protein expression upon acute exposure of cells to ethanol. The type I receptor for PACAP (PAC1) was characterized as a

candidate downstream gene that could be altered in response to the induction of c-Fos by ethanol via RACK1 (He, Vagts et al. 2002).

1.3.6.3 Tyrosine Kinases/ Phosphatases

Apart from interaction with Ser/Thr kinases such as PKA and PKC, RACK1 also interacts with Src tyrosine kinase (Chang, Conroy et al. 1998). Two other tyrosine kinases, Lck and Fyn were identified in a following study, in which RACK1 was demonstrated to bind to the SH2 domain of Src via its WD repeat 6 (Chang, Conroy et al. 1998). *In vitro* protein kinase assay demonstrated that purified GST-RACK1 could inhibit Src activity in a concentration dependent manner (Chang, Chiang et al. 2001), whereas RACK1 has no influence on the activity of three Ser/Thr protein kinases (Chang, Conroy et al. 1998). Reduced Src activity and tyrosine phosphorylation of many protein were observed in cells overexpressing RACK1. Fibroblasts with overexpressed RACK1 grow more slowly compared to wild type cells. This slowed growth rate is due to the prolonged G0/G1 stage of the cell cycle, rather than cell death (Chang, Conroy et al. 1998). As an endogenous inhibitor, RACK1 inhibits Src, causing the inhibition of Src downstream effectors, Vav2, Rho GTPases, Stats and Myc; the subsequently suppresses cyclin D1 and cyclin-dependent kinases 2 (CDK2) and CDK4; activates CDK inhibitor p27 and retinoblastoma protein and sequesters E2F1, resulting in delayed G1/S progression (Mamidipudi, Zhang et al. 2004).

It has been shown (Yaka, Thornton et al. 2002) that Src is involved in brain functions including learning, memory, and long-term potentiation, and phosphorylated the NMDA ionotropic glutamate receptor. A Src family kinase Fyn was found to interact with RACK1, and was recruited to the ctNR2B subunit of the NMDA receptor and inhibited of its kinase activity. Thus, the recruiting and the subsequent releasing of Fyn are regulated by RACK1, leading to the phosphorylation of ctNR2B and enhancing the activity of the NMDA receptor channel (Yaka, Thornton et al. 2002).

In a yeast two-hybrid screen employing the membrane proximal catalytic region of PTP μ as bait, RACK1 was found to generate a positive interaction with PTP μ (Mourton, Hellberg et al. 2001). PTP μ is composed of an intracellular domain and an extracellular domain. The intracellular domain is responsible for its tyrosine phosphatase activity, whereas the extracellular domain is for cell adhesion

through homophilic binding. Challenging cells with phorbol esters didn't ablate the interaction of RACK1/PTP μ . However, their association was increased under high cell density, indicating that it was triggered by cell contact (Mourton, Hellberg et al. 2001). RACK1, PTP μ and PKC δ have been shown to form a complex in developing neuritis and growth cones of retinal explants (Rosdahl, Mourton et al. 2002). Inhibition of PKC activity caused the inhibition of neurite outgrowth on a PTP μ substrate, confirming that RACK1 is involved in the mediation of these cell processes (Rosdahl, Mourton et al. 2002). In subconfluent cells, RACK1 localizes mainly in the cytosol, but when cell density increases, RACK1 is mainly detected in the regions of cell-to-cell contact with PTP μ . This translocation to cell-cell contacts can be abolished by cell infection with an antisense PTP μ retrovirus (Mourton, Hellberg et al. 2001). Mourton also found that constitutively active Src could disrupt the association between RACK1 and PTP μ in a kinase independent manner, suggesting PTP μ and Src compete in binding to RACK1.

1.3.6.4 Cell development

The discovery of RACK1 homologues in the genetic model system *Drosophila melanogaster* (fruit fly) and yeast have provided us with invaluable methods for elucidating its cellular functions (Vani, Yang et al. 1997; Won, Park et al. 2001) .

In fission yeast, the RACK1 homolog Cpc2 shares 77% similarity with mammalian RACK1. It is found to interact with *Schizosaccharomyces pombe* Ran1 (Pat1), which regulates the transition between mitosis and meiosis. Activated Ran1 (Pat1) inhibits sexual differentiation in fission yeast, whereas inactivation of Ran1 (Pat1) kinase is necessary and sufficient to initiate G1 arrest, conjugation and meiosis. The mutant fission yeast lacking Cpc2 (Cpc2 Δ cells) was viable but with a delayed cell cycle. Starvation of nitrogen failed to rest the Cpc2 Δ cells in G1, which leads to defects in conjugation and meiosis (McLeod, Shor et al. 2000). All the defects in Cpc2 Δ cells can be rescued by introducing mammalian RACK1, confirming that Cpc2 is functionally homologous to mammalian RACK1. Cpc2 has no effects on Pat activity despite its physical interaction with the kinase, and fluorescent-labelling of Pat1 displays altered localization in Cpc2 Δ cells, suggesting Cpc2 may function as an anchoring protein for Pat1 (McLeod, Shor et

al. 2000). Though RACK1 can interact with PDE4D5 and is considered to be involved in cAMP signaling regulation, Cpc2 shows little effect on cAMP modulation (McLeod, Shor et al. 2000).

In *Drosophila melanogaster*, RACK1 is expressed in all developmental stages and in many tissues, especially concentrated in ovary. It has been proved to be essential at many steps of *Drosophila* development (Kadrmaz, Smith et al. 2007).

1.3.6.5 Cell movement and growth

Overexpression of RACK1 in NIH3T3 mouse fibroblasts causes a reduction in growth rate under both anchorage-dependent and independent conditions which is due to G1/S delay (Chang, Conroy et al. 1998; Hermanto, Zong et al. 2002). This delay also correlates with increased levels of the cyclin-dependent kinase inhibitors P21^{Cip/WAF1} and p27^{kip1} (Chang, Conroy et al. 1998; Hermanto, Zong et al. 2002). Furthermore, cells overexpressing RACK1 are observed having enhanced spreading, increased number of actin stress fibers, focal contacts and enhanced tyrosine phosphorylation of paxillin and focal adhesion kinase (Buensuceso, Woodside et al. 2001; Hermanto, Zong et al. 2002).

Integrins are cell surface receptors are comprised of $\alpha\beta$ -heterodimers. They mediate the binding of cells to the extracellular matrix (ECM) (Skubitz 2002). The interaction of ECM/integrins triggers signal transduction required for reorganization of the actin cytoskeleton and formation of focal adhesion complexes, leading to the activation of FAKs (Focal adhesion kinase), the Src/MAPK cascades, elevated intracellular calcium, activation of PKC, and changes in cell transcriptional activity (Humphries 1996; Yarwood and Woodgett 2001). By yeast-two-hybrid screen, RACK1 is found to interact with the catalytic β -integrin via its WD repeats 5-7, suggesting an involvement in cell adhesion and movement. Indeed, several reports show RACK1 is play important roles in both cell adhesion and movement. RACK1 has been shown to scaffold PKC ϵ to integrin β chains. Disruption of the PKC ϵ association with integrin receptors leads to reduced adhesion and cell migration (Liliental and Chang 1998; Besson, Wilson et al. 2002). In chinese hamster ovary fibroblast-like cells (CHO-K1 cells), RACK1 is localized to a subset of nascent focal complexes in areas of protrusion which contains paxillin. Cell protrusion and chemotactic migration is through RACK1 Src binding sites (Cox, Bennin et al. 2003).

Several reports have suggested that cooperation between insulin-like growth factor I receptor (IGF-IR) and integrin signaling is essential for the growth and migration of transformed cells (Goel, Breen et al. 2005; Kiely, Leahy et al. 2005). RACK1 is found to interact with the serine/threonine protein phosphatase 2A (PP2A), and this releases RACK1 immediately after stimulation using IGF-I. This dissociation of PP2A from RACK1 and the subsequent IGF-I dependent decrease in PP2A activity is not observed in cells expressing RACK1 binding deficient IGF-IR mutants. Ligation of integrins with fibronectin or Matrigel can cause IGF-I-regulated dissociation of PP2A from RACK1 as well as to recruit $\beta 1$ integrin. Furthermore, both $\beta 1$ integrin and PP2A can interact with the C-terminus of RACK1 (WD repeats 4 to 7), which implies that integrin ligation displaces PP2A from RACK1. Overexpressing RACK1 in MCF-7 cells increases their motility, which could be inhibited by the PP2A inhibitor okadaic acid. Suppressing RACK1 expression by siRNA also decreases the motility of DU145 cells. Thereby, Kiely proposed a model (**Figure 1.15**) that RACK1 increases IGF-I regulated cell migration via its mutually exclusive binding between $\beta 1$ integrin and PP2A within the complex at the IGF-IR (Kiely, O'Gorman et al. 2006).

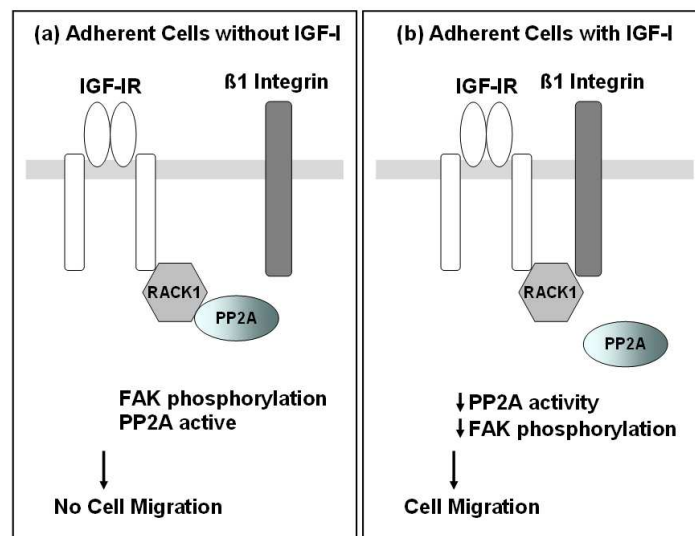


Figure 1.15: Model to illustrate actions of RACK1 in regulating recruitment of $\beta 1$ integrin and PP2A to control cell migration.

Panel (a) represents serum-starved cells where RACK1 is associated with the IGF-IR and PP2A. FAK phosphorylation and PP2A activity are high, and there is no cell migration. Panel (b) represents cells stimulated with IGF-I. PP2A is rapidly released from RACK1, and $\beta 1$ integrin is recruited to RACK1, which forms a complex that includes the IGF-IR and $\beta 1$ integrin. PP2A activity decreased transiently as well as FAK phosphorylation, and cell migration is started (Kiely, O'Gorman et al. 2006).

1.3.6.6 Intracellular Ca^{2+} regulation

Intracellular Ca^{2+} regulation is vital for nerve cell activities. Control of this process is primarily by inositol 1,4,5-trisphosphate receptors (IP_3R) (Nishiyama, Hong et al. 2000; Kandel 2001). RACK1 can modulate Ca^{2+} release from intracellular stores by increasing the binding affinity between IP_3 and IP_3R (Patterson, van Rossum et al. 2004). Release of Ca^{2+} via the action of IP_3R is vital for long-term potentiation (LTP) (Raymond and Redman 2002), implying putative RACK1 function in this neuronal network.

First of all, the complexity of LTP process, along with RACK1's scaffolding ability to interact with many protein partners, indicate some additional checkpoints provided by RACK1 interaction (Sklan, Podoly et al. 2006). Early LTP is induced by a cascade of actions: NMDA activation enables Ca^{2+} influx into the postsynaptic cell, and initiating receptor activation and association with the Src family member Fyn (Sheng and Kim 2002). RACK1 has been shown to interact with both NMDA receptors and Fyn; NMDA receptor and Fyn are essential for LTP. Therefore, RACK1 might be important in the LTP progress (Thornton, Tang et al. 2004). Elevated Ca^{2+} influx activates adenylyl cyclase, leading to the cAMP/PKA-dependent phosphorylation and activation of CREB. CREB subsequently initiates transcription of genes including neurotrophin, which induces signals that promote the survival and differentiation of neurons (Bonni, Ginty et al. 1995). RACK1 interaction with the dopamine receptor can also regulate adenylyl cyclase, providing another checkpoint (Lee, Kim et al. 2004).

Maintaining long-term L-LTP process requires different modulating input such as PKC signalling, which involves RACK1 regulation. Furthermore, L-LTP also requires mRNA translation, and RACK1 serves its part in this process (Sklan, Podoly et al. 2006).

Table 1.3: RACK1 protein partners and putative functions (Sklan, Podoly et al. 2006)

(A) Molecular mechanisms		(B) Biological processes			(C) Neuronal infect	
Signaling	Gene expression	Adhesion	Receptors and related proteins	Scaffolding protein	Viral proteins	Bacterial proteins
PKC	Type I interferon receptor β long subunit (IFN α R β L)	PTPmu	Angiotensin II receptor-associated protein (Agtrap)	β -Adrenergic receptor	HIV-1 Nef protein synthesized early after infection. Crucial for high viral loads and pathogenesis	Helicobacter pylori VacA cytotoxin
PLC γ 1	Messenger ribonucleoprotein (mRNP) complexes associated with translated mRNAs	Integrin	Beta chain of IL-5/IL-3/GM-CSF receptor	β -Spectrin	Epstein-Barr BLZF1	
PDE4D5	eIF6 translation initiation factor	Insulin-like growth factor I (IGF-IR)	FAN, an adapter protein associated with neutral sphingomyelinase activation	Synaptotagmin	Epstein-Barr A73	
G β 1 γ 1 and transducin heterotrimer G α 1 β 1 γ 1		P0 (MPZ) myelin protein	NR2B subunit of the NMDA receptor and the non-receptor protein tyrosine kinase, Fyn		Influenza A M1	
p73 α , pRB		Plectin (cytoskeletal linker protein)	Na(+)/H(+) exchange regulatory factor (NHERF1), a binding partner of CFTR		Adenoviral E1A	
p63 α			Androgen receptor (AR)		Mumps virus protein V	
Src			Dopamine transporter (DAT)		Human papillomavirus E2 protein	
p19(H-RasIDX) alternative splicing variant of c-H-ras						
p120GAP(Ras GTPase activating protein)						
Dynamin1						

1.4 SUMOylation

Small ubiquitin-like modifier (SUMO) was characterized as a reversible post-translational modification protein. The first discovered SUMOylation substrate was Ran GTPase-activating protein RanGAP1 (Matunis, Coutavas et al. 1996; Mahajan, Delphin et al. 1997). SUMO attaches to its substrate by a covalent binding. In these studies, two important features of SUMO have been revealed: SUMOylation is a reversible modification process and it can change the localization of the target protein. For example, non-sumoylated RanGAP1 is cytosolic, whereas SUMOylated RanGAP1 is translocated to the nuclear pore through interaction with nucleoporin RanBP2 (Matunis, Coutavas et al. 1996; Mahajan, Delphin et al. 1997).

SUMO proteins have a molecular size around 10 kD and have ubiquitin-like 3D structures (Bayer, Arndt et al. 1998; Mossessova and Lima 2000; Bernier-Villamor, Sampson et al. 2002). However, the SUMO is far from similar to ubiquitin. They share less than 20% amino acids identity and are distinct from their overall surface-charge distribution. SUMO protein possesses an unstructured stretch of 10 to 25 amino acids at its N-terminal region that does not exist in other ubiquitin-like proteins. These unique N-terminal sequences are responsible for the formation of SUMO chains (Geiss-Friedlander and Melchior 2007).

SUMO proteins can be found in eukaryotic organisms. So far, four SUMO proteins have been found to be expressed in humans: SUMO1-SUMO4. Among the four SUMO proteins, SUMO1/2/3 are ubiquitously expressed, whereas SUMO4 only exists in kidney, lymph node and spleen (Bernier-Villamor, Sampson et al. 2002; Guo, Li et al. 2004).

All SUMO proteins are expressed as immature precursors. The mature SUMO proteins have a universal Gly-Gly motif; whereas immature SUMO has an additional sequence containing 2-11 amino acids immediately after the Gly-Gly motif. Truncation of the C-terminal extension is necessary for mature SUMO conjugation with its substrate. SUMO2 shares 97% sequence identity with SUMO3, but only 50% amino acids identity with SUMO1.

1.4.1 Mechanism of sumoylation

Similar to ubiquitylation, it needs a three-step enzymatic cascade to form an isopeptide bond between the C-terminal Gly residue of SUMO protein and the amino group of a Lys residue in the substrate protein.

Three enzymes are involved in the sumoylation process (**Figure 1.16**). E1 activating enzyme that exists in the heterodimer ASO1-UBA2 is responsible for activation of a mature SUMO protein at its C-terminus (Johnson, Schwienhorst et al. 1997; Desterro, Rodriguez et al. 1999; Gong, Li et al. 1999). ATP is required in this step for the formation of a SUMO-adenylyl conjugate, which provides the energy for the thioester bond between the C-terminal carboxy group of SUMO and the catalytic Cys residue of UBA2 (Geiss-Friedlander and Melchior 2007). Then the SUMO is transferred to the E2 conjugating enzyme UBC9. In the last step, SUMO is transferred from UBC9 to the target substrate by the help of E3 ligases.

Three classes of E3 ligase have been identified. The first and the largest group of E3 ligase is characterized for their SP-RING motif (a RING-related sequence in the formation of $Sx_2Cx_{15}Hx_2/CSx_{17}Cx_2C$, where x is any amino acid). It includes five PIAS (1, 3, α , β and γ) in mammals, Zip3 in yeast and MMS21 (NSE2) in plants. The second class contains the vertebrate-specific nuclear pore protein RanBP2. The third group of E3 ligase is the Human Polycomb group member Pc2 (Geiss-Friedlander and Melchior 2007).

As a reversible modification, desumoylation is carried out by a specific family of proteases, which includes Ulp1 and Ulp2 in yeast and their homologues in human named sentrin-specific proteases (SEN1-3 and SENP5-7) (Li and Hochstrasser 1999). Apart from their isopeptidase function, they also control the hydrolysis of the C-terminal of newly synthesized SUMO for their maturation (Geiss-Friedlander and Melchior 2007).

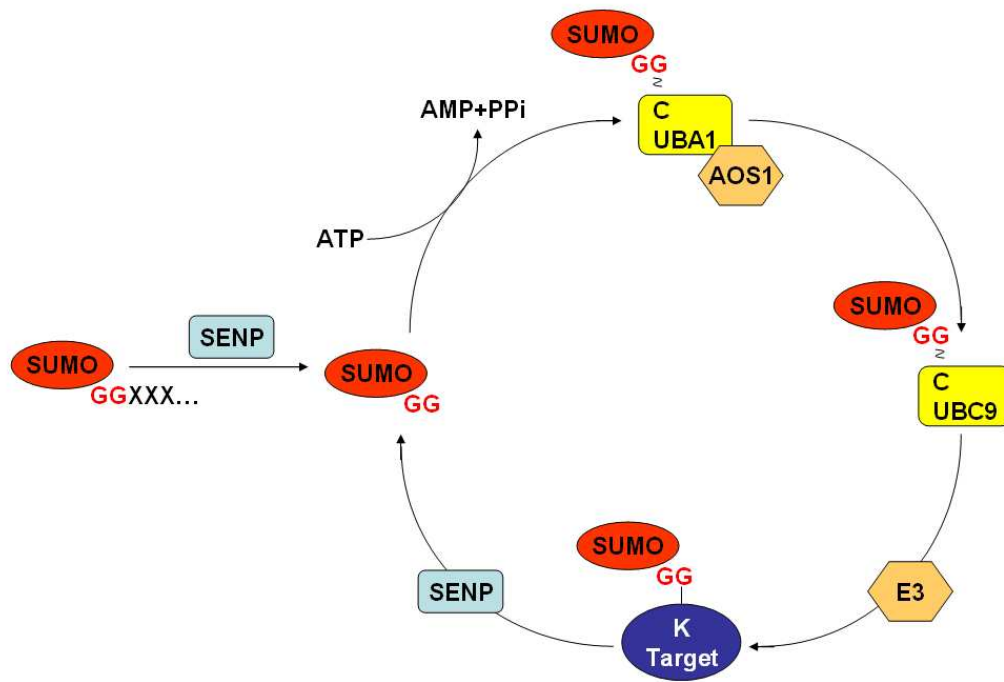


Figure 1.16: The mechanism of reversible SUMOylation.

Before the first conjugation, nascent SUMO requires to be proteolytically processed to expose its C-terminal Gly-Gly motif. This is accomplished by SUMO-specific isopeptidases (sentrin-specific proteases; SENPs), which remove 4 C-terminal amino acids from SUMO1, 11 amino acids from SUMO2 and 2 amino acids from SUMO3. Three enzymes are involved in the following sumoylation process. E1 activating enzyme that exists in the heterodimer ASO1-UBA2 is responsible for activation of a mature SUMO protein at its C-terminus. ATP is required in this step for the formation of a SUMO-adenylyl conjugate, which provides the energy for the thioester bond between the C-terminal carboxy group of SUMO and the catalytic Cys residue of UBA2. Then the SUMO is transferred to the E2 conjugating enzyme UBC9. In the last step, SUMO is transferred from UBC9 to the target substrate by the help of E3 ligases. SENP is also responsible for the reversible nature of SUMOylation.

1.4.2 SUMO binding sites

The consensus SUMO -acceptor site is ψ KxE, where ψ is an aliphatic branched amino acid and x can be any amino acid. UBC9 can directly interact with this motif: the large Lys can insert into the catalytic pocket of UBC9, and the aliphatic and acidic amino acids interact with residues on the surface of UBC9. UBC9 can only recognise the ψ KxE motif if it exists in an extended loop (RanGAP1) (Bernier-Villamor, Sampson et al. 2002), belongs to an unstructured area (ETS1) or the N terminus of SUMO2/3 (Tatham, Jaffray et al. 2001). UBC9 cannot recognise ψ KxE motifs in stable helical structures (Pichler, Knipscheer et al. 2005).

Apart from the classic consensus SUMO interacting motif ψ KxE, two extended motifs have identified for SUMO interaction (Hietakangas, Anckar et al. 2006; Yang, Galanis et al. 2006). One is ψ KxE_{exp}SP, where it has an additional phosphorylated serine and a proline. The negative charge that extended from phosphorylation of the Ser has been shown to enhance substrate-UBC9 interaction (Hietakangas, Anckar et al. 2006). Another one is the negatively charged amino-acid-dependent sunoylation motif (NDSM), in which a cluster of negatively charged amino acids has been found to be important for UBC9 interaction (Yang, Galanis et al. 2006).

K164 of proliferating cell nuclear antigen (PCNA) in *S. cerevisiae* and K14 in human E2-25K can be sumoylated as well. However, the Lys does not exist in a classic ψ KxE motif. K164 is in a hairpin turn sequence (Hoege, Pfander et al. 2002), and K14 of E2-25K is part of a α -helix (Pichler, Knipscheer et al. 2005).

1.4.3 Effects of sumoylation

Among the increasing list of SUMO substrate proteins (Geiss-Friedlander and Melchior 2007), only a limited number of them are quantitatively sumoylated either constitutively or upon stimulation. Most of the target proteins can be sumoylated in a small percentage. However, despite the small percentage of sumoylated protein, sumoylation can induce dramatic changes on the target protein and the phenotypic functional output. Although sumoylated proteins are kept at low levels, due to the rapid sumoylation and desumoylation progress, the pool of sumoylated protein can be affected in a short time.

Sumoylation is primarily considered as a nuclear effect as most of SUMO enzymes are enriched in the nucleus, and many researchers focussed on its role in DNA repair, transcription, nuclear bodies and nucleo-cytoplasmic transport (Geiss-Friedlander and Melchior 2007). Sumoylation has been shown to have a negative effect in transcription. For DNA repair, sumoylation of TDG, a DNA-repair enzyme that can bind the mismatched DNA and activate the corrupted base, can reduce its binding affinity with DNA and result in the release of the enzyme to the nucleoplasm (Hardeland, Steinacher et al. 2002; Baba, Maita et al. 2005).

In addition to the nuclear targeted effects, sumoylation has recently been found (Harder, Zunino et al. 2004; Rajan, Plant et al. 2005; Dadke, Cotteret et al. 2007)

to occur in many parts of cells including the plasma membrane, the endoplasmic reticulum (ER) and in the cytoplasm. In mitochondria, sumoylation is suggested to be important for maintaining the balance between mitochondrial fission and fusion (Harder, Zunino et al. 2004). At the ER membrane, cell proliferation and growth factor signalling are negatively modulated by dephosphorylation of key receptor tyrosine kinases through ER-associated protein-tyrosine phosphatase-1B (PTP1B). Insulin treatment can cause the sumoylation of PTP1B thereby inhibiting its activity (Dadke, Cotteret et al. 2007). Sumoylation has been observed to have a negative effect on membrane protein K2P1 potassium-leak channel (Rajan, Plant et al. 2005). Other sumoylated membrane related proteins include voltage-gated potassium channel (Benson, Li et al. 2007), metabotropic glutamate receptor-8 (mGluR8) (Tang, El Far et al. 2005) and GluR6 subunit of kainite receptor (Martin, Nishimune et al. 2007).

2 Methods and Materials

2.1 Antiserum and material

Santa Cruz Biotechnology: phospho- β 2-AR T350 (sc-16720), PP2A (sc-14020), β arrestin2 (sc-13140), β 2-AR pSer355/356 (sc-16719-R), Clathrin HC (sc-12734), RACK1 (sc-17754), β 2-AR (sc-569), glutathione S-transferase (GST) (sc-53909).

Cell Signaling Technology: pERK (9106s), MEK1(61B2) (2352), ERK (4695), phospho-MEK1/2 (Ser217/221) (9121s), ERK (4969), pSer412 (2416s), S UMO1 (4930), SUMO2/3 (4871s), MEK2 (4694), Anti-FLAG rabbit (2368), pERK (4377s), MEK1/2 (9128), ERK (9102), MEK2 (9125),.

Millipore: Anti-PP2A C subunit (07-324), Anti-PP2A B subunit (05-529), Active GST-MEK1 (SGT-220), inactive HisMEK1 (14-706)

ABGENT: SUMO4 (AP1263b), SUMO1 N-terminal (AP1222d)

BIOMOL International: SUMO1 (PW9460), SUMO2 (9465)

Sigma Aldrich: Anti-FLAG HRP (A8592-1MG), donkey serum.

Invitrogen: mouse Anti-GMP-1, anti-Src pan (44-655G), anti-His-horseradish peroxidase (P/N56-0707)

Bio-Rad: Bradford reagent

All the antibodies were used as 1:2000 dilution for immuno-blot, and 2 μ l per 50 μ l agarose beads for immunoprecipitation.

PDE4D antibodies are kind gifts from from Dr. S Wolda (ICOS Corp., Seattle, WA). HEKB2 cells were a kind gift from Prof. Graeme Milligan (University of Glasgow). Cell permeable peptides were a kind gift from Prof. Enno Klusmann (Leibniz-Institut für Molekulare Pharmakologie, Berlin)

2.2 Molecular Biology

2.2.1 *Plasmid construction and site-directed mutagenesis*

FLAG-tagged- β arrestin1, which was generated by an insertion of the full ORF of β arrestin1 into the Not1 site of pcDNA3 (Invitrogen), was a kind gift from Dr. Graeme B. Bolger (University of Alabama at Birmingham, USA). Primer 5'-GGGAAAGCGG GCCTTTGTGG CCCACATCGA CCTCGTGGAC C-3 and 5'-GGTCCACGAG GTCGATGTGG GCCACAAAGG CCCGCTTTCC C-3 were used to generate the FLAG-tagged Asp²⁶Ala:Asp²⁹Ala- β arrestin1 mutant with QuikChange site-directed mutagenesis kit (Stratagene). pCHA-MEK1 construct was a kind gift from Prof. Walter Kolch (the Beatson Institute for Cancer Research, Glasgow, UK). It was constructed as described below: Rat MEK1 was tagged at the N terminus with the influenza virus hemagglutinin (HA) epitope. Briefly, adenosine deaminase was excised from pLNCAL7 and replaced with an oligonucleotide specifying a translation initiation codon, HA tag, and unique NotI site, to yield vector pCMV-HA. MEK inserts were generated by PCR amplification of cDNAs and ligated to NotI-digested pCMV-HA, fusing the amino acid sequence MDTKYPYDVHDYAAA in frame with codon 2 of MEK1. Primer 5'-GGAGCTTGAC GAGCAGCAGG CGGCGGCGCT CGAGGCCTTT CTGACG-3 and 5'-CGTCAGAAAG GCCTCGAGCG CCGCCGCTG CTGCTCGTCA AGCTCC-3 were designed to generate Arg⁴⁷Ala:Lys⁴⁸Ala:Arg⁴⁹Ala-MEK1.

2.2.2 *Large scale DNA purification*

DNA was purified using a QIAfilter Plasmid Maxi Kit according to the manufacturers instructions. Briefly, bacterial cultures (3-4 x 10⁹ cells/ml) were harvested by centrifugation (6000 x g for 15 min at 4°C) and the pellet resuspended in 10 ml Buffer P1 containing RNase A. Buffer P2 (10ml) was added and mix thoroughly by inversion 4-6 times, prior to incubation at room temperature for 5 min. Chilled Buffer P3 (10ml) was added to the lysate, and mixed by inversion 4-6 times. The mixture was then decanted to a QIAfilter Cartridge and incubated at room temperature for 10 min. The mixture was then filtered into a QIAGEN-tip 500 pre-equilibrated in Buffer QBT, and allowed to enter by gravity flow. The QIAGEN-tip was washed with 2 x 30 ml Buffer QC and the DNA eluted with 15 ml Buffer QF. The DNA was precipitated by adding 10.5

ml (0.7 volumes) room-temperature isopropanol to the eluted DNA, prior to centrifugation at $\geq 15,000 \times g$ for 30 min at 4°C. The DNA pellet was washed with 5 ml of room-temperature 70% ethanol, and centrifuged at $\geq 15,000 \times g$ for 10 min. The pellet was air-dried for 5-10 min, and the DNA redissolved in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).

2.2.3 Small scale DNA purification

DNA was purified using a QIAprep Spin Miniprep Kit according to the manufacturers instructions. Briefly, bacterial cultures ($3-4 \times 10^9$ cells/ml) were harvested by centrifugation ($6000 \times g$ for 10 min at 4°C) and the pellet resuspended in 250 μ l Buffer P1 containing RNase A. Buffer P2 (250 μ l) was added and mix thoroughly by inversion 4-6 times. Do not allow the lysis reaction to proceed for more than 5 min before add Buffer N3. Chilled Buffer N3 (350 μ l) was added to the lysate, and mixed by inversion 4-6 times. The mixture was then centrifuged at $\geq 17,900$ for 5 min and pipetting the supernatants to the QIAprep spin column. The supernatants were centrifuged for 30-60 s and the flow-through was discarded. Wash the QIAprep spin column by 0.5 ml Buffer PB (centrifuging for 30-60s) and 0.75 Buffer PE (centrifuging for 30-60s) in turn. The flow-through was discarded, and the QIAprep spin column was centrifuged for an additional 1 min to remove residual Buffer PE. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 50 μ l Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column and centrifuging for 1 min.

2.2.4 Site direct mutagenesis (Stratagene QuickChange)

2.2.4.1 Mutant Strand Synthesis Reaction (Thermal Cycling)

1. Prepare the sample reaction(s) as indicated below:

5 μ l of 10 \times reaction buffer

X μ l (5-50 ng) of dsDNA template

X μ l (125 ng) of oligonucleotide primer #1

X μ l (125 ng) of oligonucleotide primer #2

1 μ l of dNTP mix

ddH₂O to a final volume of 50 μ l

Then add

1 μ l of PfuTurbo DNA polymerase (2.5 U/ μ l)

2. If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 μ l of mineral oil.

TABLE I. Cycling Parameters for the QuikChange Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95° C	30 seconds
2	18	95° C	30 seconds
		55° C (or 70° C)	1 minute
		68° C	1minutes/kb of plasmid length

3. Cycle each reaction using the cycling parameters outlined in Table I.

4. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to $\leq 37^{\circ}\text{C}$.

2.2.4.2 *Dpn* I Digestion of the Amplification Products

1~2 l of *Dpn* I restriction enzyme (10 U/ μ l) was added directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip. The reaction mixture was mixed and centrifuged for 1 minute prior to incubation at 37° C for 1 hour to digest the parental (i.e., the non-mutated) supercoiled dsDNA.

2.2.4.3 Transformation of XL1-Blue Supercompetent Cells

1. Gently thaw the XL1-Blue supercompetent cells on ice. For each control and sample reaction to be transformed, aliquot 50 μ l of the supercompetant cells to a pre-chilled 14-ml BD Falcon polypropylene round-bottom tube.

2. Transfer 1–4 μ l of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the supercompetant cells. (Note Carefully remove any residual mineral oil from the pipette tip before transferring the Dpn I-treated DNA to the transformation reaction). As an optional control, verify the transformation efficiency of the XL1-Blue supercompetant cells by adding 1 μ l of the pUC18 control plasmid (0.1 ng/ μ l) to a 50- μ l aliquot of the supercompetant cells. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes.
3. Heat pulse the transformation reactions for 45 seconds at 42°C and then place the reactions on ice for 2 minutes. (Note: This heat pulse has been optimized for transformation in 14-ml BD Falcon polypropylene round-bottom tubes.)
4. Add 0.5 ml of L-Broth preheated to 42°C and incubate the transformation reactions at 37°C for 1 hour with shaking at 225-250 rpm.
5. Plate the appropriate volume (0.25ml) of each transformation reaction, on agar plates containing the appropriate antibiotic for the plasmid vector.
6. Incubate the transformation plates at 37°C for >16 hours.

2.2.4.4 Colony Selection and Storage

About 6 single colonies of each transformation were picked and their DNA were extracted for sequencing. The colonies were grown in 6 ml LB-broth, 0.7ml of which then mixed with 0.3ml 70% (v/v) glycerol and the mixture was stored at -80°C.

2.3 Biochemistry

2.3.1 In Vitro pull down using purified proteins

1nmol of purified GST or GST- β arrestin1 mixed with equal amount of HisMEK1 (Millipore) in 0.5 ml binding buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 2mM MgCl_2 , 1mM DTT, 0.5% Triton X-100). The mixture was incubated for 1 hour at 4°C, then 50 μ l of pre-washed Anti-poly-Histidine-agarose beads (Sigma: A5713)

were added and incubated overnight incubation. Beads were collected by 13,000 xg centrifugation for 1min at 4°C, and washed three times with binding buffer before separation of proteins by SDS-PAGE.

2.3.2 Immuno-precipitation

Wash adherent cells twice in the dish with ice-cold PBS and drain off PBS. Wash non-adherent cells in PBS and centrifuge at 3000xg in a table-top centrifuge for 5 minutes to pellet the cells. Add ice-cold 3T3 lysis buffer or modified RIPA buffer to cells (1 ml per 10⁷ cells/100 mm dish/150 cm² flask; 0.5 ml per 5 x 10⁶ cells/60 mm dish/75 cm² flask). Scrape adherent cells off the dish with a plastic cell scraper that has been chilled in ice-cold PBS. Transfer the cell suspension into a centrifuge tube. Gently rock the tube with suspension on an orbital shaker at 4°C for 20 minutes to obtain cell lysate. Centrifuge the cell lysate at 13,000 x g for 5~10 minutes to separate cell debris, which is pelleted at the bottom of the tube. Immediately transfer the supernatant to a fresh centrifuge tube to obtain clear cell lysate and discard the pellet. Quantify the amount of protein of the clear cell lysate, and dilute it to 1µg/µl or 2µg/µl of total protein with lysis buffer (higher concentration may require when handle low expression proteins).

To prepare protein A or G agarose/sepharose, wash the beads 3 times with lysis buffer and resuspend the beads to a total 500µl volume. Add proper amount of antibody to the beads and rotate for 1 hour at 4°C to allow antibody conjugation to the beads. Aliquot the beads slurry to required number of Eppendorf tubes; spin down the beads at 13000 xg; and carefully discard the supernatant (if using conjugated agarose beads such as FLAG-beads or His-beads, just wash them 3 times and make required aliquot). Cut the tip off of the pipette when manipulating agarose beads to avoid disruption of the beads.

Pre-clear the clear cell lysate by adding 50 µl of either protein A or G agarose/sepharose bead slurry to 500 µl of clear cell lysate and incubating at 4°C for 20 minutes on an orbital shaker. Pre-clearing the lysate will reduce non-specific binding of proteins to the agarose or sepharose. Remove the protein A or G beads by centrifugation at 13,000 x g at 4°C for 1 minute. Transfer the supernatant to prepared antibody conjugated beads.

Gently rock the clear cell lysate/beads mixture for either 2 hours or overnight at 4°C on an orbital shaker. A 2 hour incubation time is recommended for the immunoprecipitation of active enzymes for kinase or phosphatase assays.

Collect the agarose/sepharose beads at 13,000 xg. Discard the supernatant and wash the beads 3 times with 500 µl ice-cold lysis buffer. Occasionally, washing with lysis buffer will strip the immunocomplex off of the agarose/sepharose beads. In these cases, washing with the milder PBS is recommended.

Resuspend the agarose/sepharose beads in 60 µl 2x sample buffer and mix gently. This will allow for sufficient volume to run three lanes.

The agarose/sepharose beads are boiled for 5 minutes to dissociate the immunocomplexes from the beads. The beads are collected by centrifugation and SDS-PAGE is performed with the supernatant. Alternatively, the supernatant can be transferred to a fresh microcentrifuge tube and stored frozen at -20°C for later use. Frozen supernatant should be reboiled for 5 minutes directly prior to loading on a gel.

2.3.3 Cell fractionation

The medium was removed from cells by washing with 5ml ice cold PBS 2 times. The excess PBS was removed by inverting and tapping the dishes on a piece of tissue. 0.5ml complete KHEM was added to 10cm dish (or 0.75ml to 150ml flask). Cells were scrapped and removed into a fresh microcentrifuge tube. Snap freeze and thaw the lysate for 3 times. The cell solution was passaged through needle 10 times by 1ml syringe. Cell solution was homogenized for 10 times. The cell solution was centrifuged at 6000 xg for 2 min and homogenized again for 10 times (Repeat this step if necessary). The cell solution was centrifuged at 6000 xg for 10mins. 0.5ml complete KHEM was added to the pellet to obtain P1 nuclear fraction. Supernatant was transferred to a new microcentrifuge tube. The supernatant was centrifuged at 186000 for 30mins. The supernatant was removed and kept as S fraction which is the cytoplasm. The pellet was washed 1 time with KHEM and was resuspended and centrifuged again. The pellet was resuspended in 100-200 µl(or appropriate) complete KHEM buffer to obtain the membrane fraction.

2.3.4 Protein analysis

2.3.4.1 Whole cell lysate

Cell culture plates were maintained on ice and all buffers used were pre-chilled on ice to minimize protein degradation and denaturing of enzymes. The medium in the culture plates or flask was discarded, washed 3 times with PBS and the plates were tapped on tissues to remove excess buffer. 1ml/10cm of 3T3 lysis buffer (20 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH7.4, 50 mM NaCl, 50 mM NaF, 10 % v/v glycerol, 1 % v/v triton X-100, 10 mM Ethyleneglycol-bis(P-aminoethylether)-N,N,N',N'-Tetra-acetic acid (EGTA), 30 mM sodium pyrophosphate, proteases inhibitor cocktail) was added to the plates. The cells were scraped by a plastic scraper and the cell lysate was transferred to a fresh 1.5ml Eppendorf tube. Snap freeze the lysate by putting the Eppendorf tube into dry ice for several minutes. The lysate was kept at -80°C.

2.3.4.2 Protein quantification (Bradford assay)

The protein assay was carried by using ELISA 96-well plate. A standard protein concentration curve with a range of 1 µg/µl - 5 µg/µl was generated by using 0.5mg/ml BSA (bovine serum albumin) solution. 2µl of each sample lysate was added to the 96-well plate in triplicate. Bradford reagent from Bio-rad was diluted 1:5 with distilled water to make a total volume of 20ml solution per plate. 200 µl of the diluted Bradford reagent was added to each well. The protein concentration was analyzed by the Revelation program and an MRX microtitre plate reader (absorbance read at 590 nm). Protein concentrations were obtained by plotting the standard curve and using the least squared regression analysis to obtain the line of best fit. The equation of the line was used to determine the protein concentration of samples.

2.3.5 Gel electrophoresis and Western blotting

2.3.5.1 Cell lysis samples

Cell lysates were prepared and protein concentration was analyzed as described above. Equal amounts of total protein were mixed with 5x Laemmli sample buffer (260mM TrisCl, pH 6.7, 55.5% v/v glycerol, 8.8% v/w SDS, 0.007% v/w bromophenol blue, 11.1% v/v 2-mercaptoethanol) to obtain 1x loading solution (normally 30µg total protein per well). Loading solution was boiled for 3-5 minutes or 37° C for 1 hour when working with beta2 adrenergic receptors.

2.3.5.2 Nu-Page™ gel system

Pre-cast 4-12%, 10-well, 12-well or 15-well Bis-Tris 1.0mm gels (Invitrogen) were used for protein electrophoresis. The gels were assembled in *Xcell SureLock™* containers (Invitrogen) according to the manufacturer's instructions. Depending on the molecular weight of protein interest, either NuPage™ MOPS SDS running buffer or NuPage™ MES SDS running buffer (Invitrogen) was used. 3-5µl of Bio-Rad Blue prestained broad range precision protein marker was used as standard marker to measure protein weight. An appropriate volume of boiled samples was loaded to each well of the gel. The gels then were run at 160-200V constant (operated by POWER-PAC 300 (Bio-Rad) for about 1 hour or until the lightest protein marker band reaches the bottom of the gel.

2.3.5.3 Protein Transfer

2 pieces of Whatman 3MM paper and 1 piece of nitrocellulose paper (Schleicher& Schuell) were prepared to the gel size. 1 piece of Whatmann 3MM paper was soaked in 1x NuPage Transfer Buffer (Invitrogen). The gel cassette was dis-assembled and the gel was placed on the Whatmann 3MM paper. The pre-soaked nitrocellulose paper and another pre-soaked whatmann 3MM paper were placed onto the gel. A round pen or a glass stick was rolled from one side of the second layer of Whatmann paper to the other side in order to get rid of bubbles between the layers (alternately, assemble all the layers in a tank filled with transfer buffer to avoid bubble formation). The sandwich was moved to the middle of two pieces of foam and this was assembled with the Xcell IITM Blot Module. Make sure the nitrocellulose was prone to the positive electrode. The

tank was filled with 1x transfer buffer and the proteins were transferred at 25V for 1 hour or 10V overnight.

2.3.5.4 Immunoblotting

Once the protein transferred to the nitrocellulose, the membrane was washed with Ponceau S stain (0.1% v/w Ponceau S, 3% v/w Trichloroacetic Acid) for 3-5 minutes, the membrane then was washed with distilled water for 3 times to visualize the proteins. To block all the binding sites on the nitrocellulose, the membrane was incubated with 5% (w/v) skimmed milk in TBST (137mM NaCl, 20mM TrisCl, pH 7.6, 0.1% v/v Tween20) for an hour. After blocking, the nitrocellulose was incubated with primary antibody in 1% milk (w/v, skimmed milk dissolved in TBST) for 1 hour (all the incubations were performed with vigorous shaking). The membrane was washed with TBST for 3 times, each time was 10 minutes. Secondary antibody was an anti-immunoglobulin (IgG) antibody conjugated to horse-radish peroxidase (HRP) that directed against the primary antibody. It was diluted in 1% milk and incubated with the nitrocellulose for 1 hour followed with 3 times TBST washing. To obtain the immunoblot image, the nitrocellulose was incubated with ECL reagents (Amersham) for at least 1 minute according to the manufacturers' instruction. The nitrocellulose (soaked with ECL) was then covered with a piece of x-ray film (Kodak) in a dark room for different time length. It was finally developed by using a Kodak Xomat machine.

2.3.6 Expression and Purification of GST Fusion Proteins

2.3.6.1 GST Fusion Protein Expression

The plasmid DNA of interest was inoculated into 30ml of L-Broth (1% NaCl, 0.5% Bacto Yeast Extract, 1% Bacto-Tryptone pH 7.5) containing 100µg/ml ampicillin, and the culture mixture was incubated at 200 rpm, 37°C overnight. Next morning, the overnight culture was transferred to 500ml of L-Broth (100µg/ml) for further incubation until OD600 reached 0.6-1.0 at 37°C. 1ml of culture mixture was taken as sample 1 (no IPTG sample showing none expressing of interested protein). IPTG was added to the culture at a final concentration of 0.2mM, shaking at 200 rpm at 25-30°C for another 2-5 hours to allow expression of the interested protein. Samples were taken at 2 hours interval in order to monitor the protein expression. 5 hours later, cells were collected at 3000x g at

4°C for 10 minutes and froze under -80°C (in this step, the cell pellet can be kept at -80°C for 1 month).

2.3.6.2 GST Fusion Protein Purification

The frozen cells were thawed on ice, resuspended with 10 ml of ice cold resuspension buffer (50mM TrisCl pH8.0, 10mM sodium chloride, 1mM EDTA, 10mM mercaptoethanol, 1x Roche protease inhibitor tablet). 1/10 of volume of 10mg/ml Lysozyme was added to the resuspended cells and the mixture was kept on ice for 15 minutes. The cell mixture was then sonicated on ice for 5 minutes (5x 30 seconds with 30 seconds rest). 1/500 volume of 10% Triton X-100 was mixed with the sonicated cell mixture and spun down at 12,000x g, 4°C for 15 minutes. 1 ml glutathione beads were washed with wash buffer (50mM TrisCl pH8.0, 10mM sodium chloride, 1mM EDTA, 10mM beta-mercaptoethanol, 1x protease inhibitor tablet, 0.05% NP-40) for 3 times. 10 ml of the supernatant was transferred to a 15ml falcon tube with 1 ml of pre-equilibrated Glutathione Sepharose 4B beads (Amersham Biosciences). The mixture was rotated end to end at 4°C for 1 hour. The beads were collected at 366x g at 4°C for 2 minutes and the supernatant was carefully removed; they were washed by 5 ml wash buffer, collected, and transferred to a 2 ml Eppendorf tube by adding 1 ml fresh wash buffer; the beads were then washed with 1 ml wash buffer for 3 times. The GST fusion protein was eluted from the glutathione beads with elution buffer (10mM glutathione in 50mM TrisCl pH 8.0), rotated at 4°C for 20 minutes, and centrifuged at 13,000 xg for 10 seconds. The supernatant was carefully removed to a fresh microcentrifuge tube. This step was repeated for another 2-3 times and the supernatants were collected. The protein concentration of all of the elutes was quantified and those with a high protein concentration were combined. In order to remove free glutathione, the combined elutes were transferred to Slide-ALyzer® Dialysis Cassette (0.5-3.0ml capacity) (PIERCE) and dialyzed in 700ml chilled dialysis buffer (50mM TrisCl pH8.0, 100mM NaCl, 5% v/v glycerol) overnight. The dialyzed elute was collected for further analysis.

2.3.6.3 Visualization of Proteins

Following gel electrophoresis, the gel was incubated with Coomassie Blue (1.25mg/ml Blue R250, 44.4% methanol, 5.6% acetic acid, 50% distilled water) for 4 hours to stain the protein. The limit of detection of protein by Coomassie

Blue stain is 0.3–1.0 µg. After gel stain, the gel was then destained using destain buffer (44.4% methanol, 5.6% acetic acid, 50% distilled water) until the protein bands could be clearly visualized.

2.3.6.4 SDS Polyacrylamide Gel Dehydration

The SDS gels were dried on a Bio-Rad gel drier. A piece of damp filter paper was placed on the drying surface of the drier, the gel was laid on the filter paper, and covered with cling film and the cover sheet of the drier. A vacuum was used to tight seal all the layers. The gel was dried at 63 °C for 2 hours.

2.3.7 Peptide Array Analysis

Peptide libraries were produced by automatic SPOT synthesis (Kramer and Schneider-Mergener 1998). Peptide libraries were synthesized on continuous cellulose 75 membrane supports on Whatman 50 cellulose membranes according to standard protocols by using Fmoc-chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Koln, Germany). Alanine scan peptide array was undertaken by taking positive interaction spots on peptide array, and substituting each residue of the positive spot to alanine (when alanine is the native residue, then it was substituted with aspartate on the scanning array).

The fresh membrane-immobilised peptide array was immersed in 75% or 100% ethanol to activate the hydrophobic group of the peptide. The membrane was then washed 3 times with TBST (137mM NaCl, 20mM TrisCl, pH 7.6, 0.1% tween20) to exclude ethanol. The peptide array membrane was then incubated with 5% skimmed milk in TBST for 2 hours to block all the non-specific binding sites. The purified protein of interest was diluted in 1% skimmed milk (w/v) to a concentration of 10µg/ml, which was then used to incubate with the blocked peptide array overnight at 4 °C. The presence, on the peptide array, of the bound protein of interest was detected by western blot with corresponding primary and secondary antibody.

2.3.8 Biotin Protection Assay

Cells were grown in 10cm dishes until they reached 80% confluent. The cells were treated with 0.3mg /ml disulfide-cleavable biotin (Pierce) in PBS at 4 °C for

30 mins (5 ml/well). Cells were washed with pre-cooled PBS for 3 times to cease biotinylation. 100% and Strip biotinylation control group were setup and kept under 4 °C. Pre-warmed medium were added (37 °C) and cells were incubated for 15mins. Cells were treated with specific ligands. (a, 507 MEK replace peptide for 2h. b, Iso time course.) At the end of the treatment, the media was removed and the dishes were left on ice immediately to stop the reaction. The dishes were wash 2 times with PBS and the biotinylated receptors was stripped with stripping buffer (0.05M glutathione, 0.3M NaCl, 0.075M NaOH, 1% FBS/NCS in PBS) at 4 °C for 30 mins or 15 mins for 2 times if better stripping was needed. The dishes were wash 2 times with PBS at 4 °C. (Note: if you have a lot of samples to process then it is better to split the dishes into several group and speed up the washing process, but just make sure that the cells always have some media on them so as to prevent cells unexpectedly floating off the plastic surface). Cells were lysed in RIPA (150mM NaCl, 25mM KCl, 10mM Tris-HCl and 0.1% Triton X-100, pH 7.4) and then the cellular debris was removed by centrifugation at 10,000g for 10 mins at 4 °C. The biotinylated receptors was immunoprecipitated with Immobilized NeatrAvidin (Pierce) at 4 °C overnight (50ul beads each). The pellet was washed 3 times with RIPA or PBS. An appropriate amount of 2x loading buffer was added in the pellet and it was incubated at 37 °C for 1 hour or 70 °C for 10 mins. The samples were subjected to Western Blot and blotted with proper antibodies. (*anti-B₂AR*, Santa Cruz).

2.3.9 In Vitro Sumoylation on Peptide Array

A commercial Sumoylation Kit (BIOMOL) was used in these studies.

2.3.9.1 Components of SUMOylation Kit

20x SUMO Activating Enzyme Solution (SUMO E1); 20x SUMO Conjugating Enzyme Solution (SUMO E2); 20x SUMO Enzyme Solutions (SUMO1, SUMO2, SUMO3); 10x SUMOylation Buffer; 20x Mg-ATP Solution.

2.3.9.2 Peptide Array Preparation

Peptide arrays were activated in 100% ethanol for 10 seconds, followed with 3 washes of TBST. The peptide array was then incubated with 5% skimmed milk (in

1x TBST) for 2 hours to block the unoccupied binding sites. The peptide array was washed 5 times, each time 5 minutes to ensure TBST had been removed.

2.3.9.3 In Vitro SUMOylation

All the components (20x SUMO Activating Enzyme Solution (SUMO E1); 20x SUMO Conjugating Enzyme Solution (SUMO E2); 20x SUMO Enzyme Solutions (SUMO1, SUMO2, SUMO3); 10x SUMOylation Buffer; 20x Mg-ATP Solution) were mixed to the volume of 1 ml with distilled water. The mixture was incubated with prepared peptide array at 37°C for 1 hour. The reaction was ceased by washing with TBST for 3 times.

The SUMOylated peptide array was subjected to Western Blot with the kit provided primary antibody. The potential sumoylation site would be identified as a positive dark spot after development.

2.4 Mammalian Cell Culture

2.4.1 HEK293 cell

HEK293 (ATCC CRL 1573) is an epithelial cell line that is derived from Human Embryonic Kidney cells. HEK293 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM glutamine, 10% (v/v) foetal bovine serum (FBS), 100 units/ml penicillin and 100µg/ml streptomycin in an atmosphere of 5% (v/v) CO₂ at 37 °C. Cells were passaged at 80~90% confluence. To passage cells, they were rinsed with pre-warmed PBS. Then 1~2ml 1x trypsin/EDTA solution (Sigma) was added, and incubated at 37°C until cells detached from the flasks. Cells were collected by adding 10ml of cell culture medium and centrifuged at 1000 xg for 3 minutes. The cells were then resuspended by adding fresh media and transferred to new flasks at 10⁵ cells/ml.

2.4.2 HEKB2 cells

HEKB2 cells are HEK293 cells stably expressing GFP/FLAG tagged β_2 adrenergic receptor under the selection pressure of G418 antibiotic. It was developed by Prof. Graeme Milligan (Glasgow). The HEKB2 use a modified culture medium from HEK293 medium. It contains Dulbecco's Modified Eagles Medium (DMEM)

supplemented with 2mM glutamine, 10% newborn calf serum (NCS), 1% penicillin/streptomycin (100units/ml) and 1mg/ml G418 antibiotic. The maintenance and passaging condition are the same as HEK293 that has been describe above.

2.4.3 Making HEK293 cells stably expressing mutated GFP/FLAG tagged β_2 adrenergic receptor

HEK 293 cells were cultured with HEK293 media (Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM glutamine, 10% (v/v) foetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin) to >80% confluence in 10cm Petri-dishes. The HEK293 cells were transfected with GFP/FLAG tagged β_2 AR YY350/354AA mutant by Poyfect (Qiagen) as manufacture's instruction. The next day, the medium was changed to HEKB2 medium (Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM glutamine, 10% newborn calf serum (NCS), 1% penicillin/streptomycin (100units/ml) and 1mg/ml G418 antibiotic) that contained G418 antibiotic. The transfected cells were left in HEKB2 medium and keep topping the vaporized medium for 7 days. Normally, >90% of transfected cells may die under the harsh G418 selection pressure. It was important to avoid vigorous handling of the transfected cells to allow sufficient cell attachment. Discard unattached cells with medium and refill with fresh HEKB2 medium. You may see single cell colonies at this stage. Keep feeding the cells with HEKB2 media for another 2 weeks or wait until cell colony was large enough to transfer to 6-well plate. Individual cell colony was scraped with the large end of 0.2 ml pipette tip (sterile), transferred them to 6-well plates. When cell colony was confluent in 6-well plate, it was passaged to 75 cm² flasks and was analyzed using Confocal Microscopy and Western blot for final characterization. Cells that stable expressing GFP/FLAG tagged β_2 AR YY350/354AA mutant were selected as stable cell line.

2.5 Confocal analyses

HEKB2 cells transfected with FLAG- β arrestin 1 (or interested cells) were seeded on to poly(L-lysine)-treated coverslips at approx 20% confluence. After treatment with indicated ligands, cells were fixed for 10 min in 4%(w/v)

paraformaldehyde followed by three washes with TBS (Tris-buffered saline; 150mM NaCl, 20 mM Tris, pH 7.4). Cells were permeabilised by incubating with 0.2% Triton dissolved in TBS. Cells were blocked for 1 hour with blocking solution (10% donkey serum, 4% BSA in TBS). The primary antibody was diluted to required concentration (normally at 10 fold of the required concentration in Western blotting for the same antibody) using diluent (TBS: Blocking buffer at 1:1 volume). 200µl of diluted primary antibody was added to the coverslip for 1 hour incubation. The coverslips were washed with diluent 3 times before the fluorescent probe was added. The fluorescent probe (Alexa 594-conjugated antibody or Alexa 488-conjugated antibody) was diluted at 1:200 using diluent and added to the coverslip for 1 hour (repeat this procedure if a second fluorescent probe was needed). The coverslips were washed 5 times with TBS before being mounted on microscope slides with Immunomount (sandon). Cells were visualized using the Zeiss Pascal laser-scanning confocal microscope (Zeiss, Oberkochen, Germany). The Alexa 594-conjugated antibody was excited at 543 nm and detected at 590 nm. The Alexa 488-conjugated antibody was excited at 495 nm and detected at 518 nm.

2.6 Buffers

3T3 lysis buffer (20 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH7.4, 50 mM NaCl, 50 mM NaF, 10% (v/v) glycerol, 1% (v/v) triton X-100, 10mM Ethyleneglycol-bis(P-aminoethylether)-N,N,N',N'-Tetra-acetic acid (EGTA), 30 mM sodium pyrophosphate, protease inhibitor cocktail)

5x Laemmli sample buffer (260mM TrisCl, pH 6.7, 55.5% v/v glycerol, 8.8% (v/w) SDS, 0.007% (v/w) bromophenol blue, 11.1% (v/v) 2-mercaptoethanol)

Binding buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 2mM MgCl₂, 1mM DTT, 0.5% (v/v) Triton X-100)

Blocking solution (10% (v/v) donkey serum, 4% (w/v) BSA in TBS)

Coomassie Blue (1.25mg/ml Blue R250, 44.4% (v/v) methanol, 5.6% (v/v) acetic acid)

Destain buffer (44.4% (v/v) methanol, 5.6% (v/v) acetic acid)

EB buffer (10 mM Tris·Cl, pH 8.5)

Elution buffer (10mM glutathione in 50mM TrisCl pH 8.0)

HEKB2 medium (Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM Glutamine, 10% (v/v) newborn calf serum (NCS), 1% penicillin/streptomycin (100units/ml) and 1mg/ml G418 antibiotic)

KHEM (KCl 50mM, EGTA 10 mM, $MgCl_2$ 1.92 mM, dithiothreitol 1 mM, HEPES 50 mM, final pH 7.2,) containing 'complete' protease inhibitors (Boehringer - Mannheim) of final concentrations 40 mg/ml PMSF, 156 mg/ml benzamine, 1 mg/ml aprotonin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A and 1 mg/ml antipain.)

L-Broth or LB medium (1% v/w NaCl, 0.5% (v/w) Bacto Yeast Extract, 1% (v/w) Bacto-Tryptone pH 7.5)

Ponceau S stain (0.1% (v/w) Ponceau S, 3% (v/w) Trichloroacetic Acid)

Resuspension buffer (50mM TrisCl pH8.0, 10mM NaCl, 1mM EDTA, 10mM mercaptoethanol, 1x Roche protease inhibitor tablet)

RIPA (150mM NaCl, 25mM KCl, 10mM Tris-HCl and 0.1% (v/v) Triton X-100, pH 7.4)

Stripping buffer (0.05M glutathione, 0.3M NaCl, 0.075M NaOH, 1% (v/v) FBS/NCS in PBS)

TBS (Tris-buffered saline; 150mM NaCl, 20 mM Tris, pH 7.4)

TBST (137mM NaCl, 20mM TrisCl, pH 7.6, 0.1% v/v Tween20)

TE buffer (pH 8.0, or 10 mM Tris·Cl, pH 8.5)

Wash buffer (50mM TrisCl pH8.0, 10mM sodium chloride, 1mM EDTA, 10mM beta-mercaptoethanol, 1x protease inhibitor tablet, 0.05% NP-40)

3 MEK1 binds β arrestin1 directly

3.1 Introduction

The β arrestins are well studied scaffolding proteins that serve not only as key regulators in heptahelical G protein coupled receptor (GPCR) desensitization, but also determine their recycling and play pivotal roles in many different signalling pathways (DeWire, Ahn et al. 2007; DeFea 2008). The β_2 -adrenergic receptor (β_2 AR) is the most popular receptor used in studies of GPCR and β arrestins. The classic paradigm is that after agonist occupancy with the receptor, GRKs are translocated to the receptor where they phosphorylate it, thereby initiating cytosolic β arrestin recruitment (Ferguson, Downey et al. 1996; Premont and Gainetdinov 2007). The β arrestins then associate with agonist occupied receptors and sterically block their interaction with G proteins. Meanwhile, β arrestin also facilitates GPCR internalization, leading to either recycling or degradation of the target GPCR (Baillie, Sood et al. 2003). In this fast internalization, the interaction between β arrestin and clathrin cages is considered as a key part of the receptor desensitization process (Gurevich and Gurevich 2006). In the case of the β_2 AR, β arrestin can also bring the cAMP degrading PDE4D5 enzyme to the receptor at the site of cAMP synthesis. This controls phosphorylation of the β_2 AR by an AKAP-tethered PKA sub-population, which serves to regulate the switching of β_2 AR coupling between Gs and Gi, thereby providing a further means of regulating receptor desensitization (Baillie, Sood et al. 2003; Lynch, Baillie et al. 2005; Bolger, Baillie et al. 2006).

Without any stimulation, cytosolic β arrestin1 is constitutively phosphorylated by ERK at Ser412, which is located at its distal C-terminus (full length β arrestin1 contains 418 amino acids) (Lin, Miller et al. 1999; Luttrell, Roudabush et al. 2001). After agonist-dependent activation of GPCRs, β arrestin1 is dephosphorylated at Ser412 and recruited to GPCRs. Clathrin recruitment and dynamin phosphorylation are two important features in receptor internalization. The dephosphorylation of Ser412 is considered as a molecular switch to allow the interaction between β arrestin1 and clathrin (Lin, Krueger et al. 1997; Lin, Miller et al. 1999), and also is considered to play a pivotal role in the association of β arrestin1 with c-Src, which leads to the phosphorylation of dynamin (Miller, Maudsley et al. 2000). Thus, the ERK-dependent Ser412 phosphorylation and

dephosphorylation orchestrates the association of β arrestin1 with the endocytic machinery in the internalization of GPCRs (Lin, Krueger et al. 1997).

The c-Raf/MEK/ERK signalling pathway is one of the mitogen-activated protein kinase (MAPK) signalling cascades and contains three kinases where each kinase is phosphorylated and activated by the upstream kinase (Rubinfeld and Seger 2005). The MAPKs play pivotal roles in the regulation of a variety of cellular processes such as cell proliferation, transcription regulation, apoptosis and cell differentiation (Lu and Xu 2006). Due to the great importance of this process in many diseases, ERK is a hot target for drug development. However, there has been little success in using ERK inhibitors therapeutically. Because of the high fidelity of the cascade, more and more attempts have been made to develop a MEK inhibitor to treat cancer and, recently, for chronic inflammatory disorders, such as asthma and rheumatoid arthritis (English and Cobb 2002; Pelaia, Cuda et al. 2005; Sweeney and Firestein 2006).

Luttrell et al suggested that β arrestin may form a complex with ERK and MEK to restrict MAPK signals in defined intracellular compartments (Lefkowitz, Inglesse et al. 1992; Luttrell, Roudabush et al. 2001). A number of studies indicated that MEK recruitment to signalling complexes might be due to its interaction with ERK and c-Raf rather than a direct contact with β arrestin (DeFea, Zalevsky et al. 2000; McDonald and Lefkowitz 2001; Perry and Lefkowitz 2002; Lefkowitz and Whalen 2004; Lefkowitz and Shenoy 2005). However, a very recent report, which came out after the current study was completed, suggested that MEK can bind directly to β arrestin on both its N and C domains (Song, Coffa et al. 2009). However, no functional study of this interaction has been made. Given that β arrestin1 is ERK phosphorylated and it is important in GPCR internalization (Lin, Miller et al. 1999), the nature of the MEK1/ β arrestin1 interaction requires further investigation. Here then, we demonstrate that MEK1 binds directly with β arrestin1, influencing both its phosphorylation and the timing of its isoprenaline-stimulated internalization with the β_2 AR.

3.2 Results

3.2.1 MEK1 binding sites on β arrestin1

MEK1 is a 45kDa MAPK kinase that activates ERK by phosphorylating it at Thr202/Tyr204 of ERK1 or Thr185/Tyr187 Of ERK2. It has been shown to interact with β arrestin by overexpressing multiple key intermediates such as c-Raf, ERK, MEK and β arrestin (Luttrell, Roudabush et al. 2001). However, such studies didn't investigate whether the interaction between β arrestin and MEK depends on a direct contact or depends on a bridging partner, such as ERK, which has been shown to associate with both MEK and β arrestin directly (DeFea, Zalevsky et al. 2000; Tohgo, Pierce et al. 2002; Xu, Baillie et al. 2008). A newly published paper by Song et al., which was published after the current study was completed, proved that MEK can bind to both the N and C domain of β arrestin1 (Song, Coffa et al. 2009). However the nature of this interaction remains in obscure.

Firstly, to investigate whether MEK1 has the ability to interact with β arrestin, a SPOT immobilised peptide array analysis was employed (Bolger, Baillie et al. 2006; Baillie, Adams et al. 2007). To perform the analysis, we generated a library of 25-mer overlapping peptides, each shifted by 5 amino acids, spanning the whole region of β arrestin. These 25-mer peptides were synthesised and immobilized on cellulose membranes. Purified GST and GST-MEK1 were then incubated with the membrane to allow potential association to occur. A further detection by anti-GST antibody identified the 'dark' spots as positive interactions (Fig 3.1a). Spots 3-6 were picked up by the analysis for GST-MEK1 binding but not for GST. Spot 6 that had the strongest interaction was further investigated later by an alanine substitution array procedure as below.

To identify which amino acids may play the pivotal role in this interaction, an alanine substitution array analysis was designed. Spot 6, which gave the strongest signal in the peptide array analysis, represents the amino acids Asp26-Glu50 of β arrestin1. It was used as a template to generate the alanine substitution array by sequentially replacing each amino acid to alanine to provide a family of mutant peptides based upon the parent template. By this method, we observed that the binding between MEK1 and Asp26-Glu50

βarrestin1 was ablated when Asp26 and Asp29 were replaced with alanines (Fig 3.1b). This binding was also ablated when Asp26 to His30 were all substituted to Ala, but not when Tyr46 to Glu50 were replaced with alanines.

Fusion proteins were used in our *in vitro* mix assay in order to prove that MEK1 and βarrestin1 could interact directly *in vitro*. To perform the experiment, His-MEK1 was mixed with either GST-βarrestin1 or GST protein alone in reaction buffer first. Then His-MEK1 was pulled down by His conjugated agarose, and the pull-down pellet was analyzed by Western Blotting (Fig 3.2a). GST-βarrestin1, but not GST, could co-immunoprecipitate with His-MEK1, which confirms our hypothesis that MEK1 can associate with βarrestin1 directly and that this association is not dependent on any other component of the signalling complex.

To evaluate the importance of the DXXD motif in the MEK1 and βarrestin1 interaction, Asp26 and Asp29 were mutated on a FLAG epitope-tagged βarrestin1 to generate a MEK1-binding null mutant. FLAG-βarrestin1 and its D26A/D29A mutant were expressed in HEK293 cells. Cell lysates were collected and immunoprecipitated with FLAG-agarose. Although MEK1 can co-immunoprecipitate with wild type FLAG-βarrestin1, only $8.3 \pm 3.9\%$ (mean; n=3) endogenous MEK1 could be pulled down by the D26A/D29A mutant. However, equal amounts of PDE4D5 were pulled down in both wild type FLAG-βarrestin1 and its mutant (Fig 3.2) due to the fact that PDE4D5 has a different docking site in βarrestin1 (Baillie, Adams et al. 2007).

ERK, being a downstream effector of MEK, was shown to associate with βarrestin1 (Luttrell, Roudabush et al. 2001). Therefore it has been assumed to that ERK might act as adaptor to recruit MEK1 to βarrestin. So is the loss of MEK1 interaction with βarrestin1 due to the loss of ERK? To answer this question, ERK2 was blotted for in FLAG-βarrestin1 immunoprecipitations (Fig 3.2). In our hands it is quite clear that the ERK/βarrestin interaction still occurs in the D26A/D29A mutant as well as with wild type FLAG-βarrestin1 (Fig 3.2).

3.2.2 Beta-arrestin1 interacting region on MEK1

To further investigate how βarrestin1 may bind to MEK1, a full sequence MEK1 peptide array was designed and probed using purified GST-βarrestin1. A potential βarrestin1 binding sequence was identified (spot 10), which consists of

amino acids Gln46-Lys60 (Fig 3.3a). An interesting finding about this peptide is that it includes a cluster of positive charged amino acids, Arg 47, Lys48 and Arg49. An alanine scanning peptide array was designed to test if these positive charged amino acids interact with their negative counterpart DxxD on β arrestin1. β arrestin1 binding was attenuated when Lys48 and Arg49 were replaced with alanine, whereas this binding was abolished when either Arg47 and Lys48 or all these residues were superseded to alanine. However, substitution of three negative charged residues, Asp65, Asp66 and Asp67, had limited effect (Fig 3.3b).

To examine whether this positive charged cluster of MEK1 is involved in the MEK1/ β arrestin1 association, a R47A/K48A/R49A mutant was made using a HA epitope-tagged MEK1 construct. Upon co-transfection of FLAG- β arrestin1 with wild type HA-MEK1 in HEK293 cells, β arrestin1 could be immunoprecipitated with HA-MEK1 (Fig 3.3c). However, R47A/K48A/R49A mutant could not be pulled down with FLAG- β arrestin1 (Fig 3.3c).

3.2.3 Use of a 25-mer short β arrestin peptide to disrupt MEK1/ β arrestin complex in vivo

Two small peptides were generated. One peptide was 6-TRVFKKASPNGKLTVYLGKRDFVD-30 that was derived from β arrestin1 so as to include the DXXD motif, and this was called MEK displacer peptide (T-DFVD); the other peptide was the same core sequence of the MEK peptide but with alanine substitutions at Asp26 and Asp29. This was the control peptide (T-AFVA) TRVFKKASPNGKLTVYLGKRAFVA. Both peptides were modified by N-terminal stearoylation to allow for cell-permeability.

This approach has been used in several studies to disrupt signalling complexes in intact cells (Futaki, Ohashi et al. 2001; Murdoch, Mackie et al. 2007; Smith, Baillie et al. 2007). The peptide was selected based on two rules. First, it contains only one DXXD motif includes the Asp26 and Asp29 which has been shown to play key roles in β arrestin1 association with MEK1. Second, to avoid any steric impact on the disruption of the β arrestin1/MEK1 complex, the stearate group was added away from the DXXD motif at the N-terminal of the peptide.

To inspect the effects of the MEK displacer peptide (T-DFVD) and its control peptide (T-AFVA), wild type HEKb2 cells were harvested after 2 hours treatment with each peptide. In a co-immunoprecipitation experiment, MEK1 could be pulled down by β arrestin1 when treated with control peptide (T-AFVA); and the amount of MEK1 was attenuated when treated with the MEK displacer peptide (Fig.3.4b). However, the endogenous MEK1 pulled down by β arrestin1 was at the edge of our MEK1 detection ability (Fig 3.4b). In order to obtain clear results, we overexpressed FLAG- β arrestin1 in HEKb2 and analyzed effects of the MEK displacer peptide and its control peptide. Similar results were observed in co-immunoprecipitation studies with MEK1 with β arrestin1, where the MEK displacer peptide successfully disrupted the association of MEK1/ β arrestin1, but there was hardly any effect using the control peptide (Fig 3.4a).

3.2.4 MEK1 binding to β arrestin1 regulates its ERK phosphorylation

In the cytosol, β arrestin1 is constitutively phosphorylated by ERK at Ser412 without any G-protein agonist treatment (Lin, Miller et al. 1999). ERK is the downstream effector of MEK, thus either change of MEK activity or its association with ERK would influence ERK activity, leading to potential changes in the phosphorylation status of β arrestin1. Indeed, after 4 hours treatment with the MEK inhibitor UO126 and/or PD98059 we see a clear decrease of β arrestin1 phosphorylation at Ser412 (Fig 3.5a). Furthermore, in contrast to wild-type β arrestin1, the MEK1 binding null mutant D26A/D29A- β arrestin1 has significantly decreased phosphorylation on Ser412 (Fig 3.5a).

When HEK293 were treated with the MEK displacer peptide, a decrease in β arrestin1 Ser412 phosphorylation was seen after 2 hours, while treatment using the control peptide had no such effect (Fig 3.5b). Since a change of two negative charged residues Asp26/Asp29 in the control peptide may potentially affect the peptide's cell-permeability, a fluorescent version of this stearylated peptide was designed to test this notion. After 2 hours treatment at the same condition as Fig.3.5b similar peptide accumulation both at the membrane and in the cytosol was observed using the wild-type and substituted, Asp26/Asp29 peptides by confocal microscopy (Fig.3.6).

We then used EGF under conditions to reach maximum ERK activation so as to explore its influence on phosphor-Ser412 and to further test the efficiency of the peptides. First, we saw that the EGF-treated cells exhibited the same amount of phosphorylated β arrestin1 as untreated cells (5 min of EGF, vehicle control, second lane; normalised 100% ERK phosphorylation of β arrestin1 on Ser412 at basal level) (Fig.3.5c). Second, the MEK displacer peptide retains its efficiency to down-regulate the phosphorylation of β arrestin1 at Ser412, indicating that the displacer peptide has a dominant effect on the disruption of the MEK/ β arrestin1 complex even under increased global ERK activity (Fig.3.5c).

3.2.5 MEK1 binding to β arrestin regulates the association of clathrin and Src to β arrestin

It has been shown that β arrestin1 recruitment to the β_2 AR was necessary for the dephosphorylation at Ser412 of β arrestin1 (Lin, Krueger et al. 1997). Dephosphorylation of β arrestin1 at Ser412 increases its binding affinity with clathrin and Src, leading to clathrin dependent receptor internalization (Lin, Miller et al. 1999; Miller, Maudsley et al. 2000). In HEK293 cells with stable expressed β_2 -adrenergic receptor, upon stimulation of the receptors by isoprenaline, there is enhanced β arrestin1 association with both clathrin and Src. In cells that were pre-treated with MEK displacer peptide, we observed an enhanced association of β arrestin1 with clathrin and Src both under basal and stimulated conditions, yet the control peptide had no such effect (Fig.3.7).

These findings confirm the notion that the MEK displacer peptide (V-DFVD) induces the dephosphorylation of β arrestin1 at Ser412.

3.2.6 MEK displacer peptide accelerate β_2 -adrenergic receptor internalization

Upon stimulation of β_2 AR, β arrestin1 undergoes rapid dephosphorylation at Ser412, leading to enhanced association of with clathrin and Src, and increased β_2 AR internalization. To follow the receptor internalization, we used HEK293 cells stable expressing FLAG and GFP tagged β_2 AR (Li, Huston et al. 2006). A biochemical assay and confocal microscopy were employed to monitor the endocytosis of β_2 -AR. First, we used cleavable biotin to label all β_2 -adrenergic

receptor on the cell surface. After agonist stimulation, internalized receptors were protected from the following biotin cleavage step, which allowed us to quantify the amount of internalized receptor. Here, we see pre-treatment with the MEK displacer peptide facilitated the rate of β_2 AR endocytosis, whilst this was not seen with the control, Asp26Ala:Asp29Ala substituted peptide (Fig.3.8a).

We then set to investigate the receptor internalization by using confocal microscopy. Significant receptor internalization was observed upon MEK displacer peptide treatment (Fig.3.8b,c), but not the control peptide. These findings underpin the notion that disruption of the MEK1/Barrestin1 complex dephosphorylates β arrestin1 at Ser412, enhancing the association of β arrestin1 with clathrin and Src to facilitate β_2 AR internalization.

3.3 Discussion

It has been shown that the phosphorylation of β arrestin1 at Ser412 plays a pivotal role in regulating receptor endocytosis, but has no effect on β_2 -adrenoceptor desensitization (Lin, Miller et al. 1999). β arrestin is located in the cell cytosol in the resting state. Upon receptor stimulation, it translocates to the activated receptor and becomes dephosphorylated at Ser412, which enables the association of β arrestin1 with clathrin and Src after c-Src dependent dynamin phosphorylation that is known to be a key process in GPCR endocytosis. β arrestin2 has also been shown to undergo similar dephosphorylation after challenged with isoproterenol (Lin, Chen et al. 2002). However, the sites at which dephosphorylation occurs are at Ser361 and Thr383, which are regulated by casein kinase 2 (Kim, Barak et al. 2002; Lin, Chen et al. 2002). Insulin treatment can induce the phosphorylation of β arrestin1 at Ser412, and this can block isoprenaline-activated dephosphorylation of Ser412, subsequent β_2 adrenoceptor internalization and subsequent β arrestin-mediated ERK signalling. Previous investigators have reported that either MEK inhibitors or dominant negative MEK (inactive) attenuated Ser412 phosphorylation of β arrestin1 (Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002; Hupfeld, Resnik et al. 2005). Therefore, the phosphorylation of β arrestin1 on Ser412 is dependent on MEK. Indeed, in this study we show that β arrestin1 is phosphorylated by activated ERK, and the activation of ERK is dependent on β arrestin1 interacting MEK.

In this study, we demonstrate that MEK1 binds directly to β arrestin1 and this interaction does not require ERK to orchestrate. Several studies had suggested that c-Raf, MEK and ERK could simultaneously interact with β arrestin (Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002). C-Raf has also been suggested as an adaptor protein involved in MEK/ERK association to β arrestin to facilitate the signalling complex formation (Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002). Nevertheless, a recent study by Song et al. showed that both β arrestin C-domain and N-domain can bind to MEK1 (Song, Coffa et al. 2009). In agreement with Song et al.'s finding, in our experiments we showed that MEK1 binds directly to a DxxD motif on β arrestin's N-domain. Interestingly, another recent study identified an ERK docking site in β arrestin's C-domain (Xu, Baillie et al. 2008), which is distant from the MEK1 docking site on β arrestin's N-domain. Therefore there is a strong possibility that β arrestin works as a scaffold protein to organise complex formation involving ERK, MEK and c-Raf. This report, in agreement with that of Song et al., provides firm evidence that MEK binds directly to β arrestin's N-domain. Whether it is possible to envisage an additional indirect association between β arrestin and MEK via ERK remains to be seen. This would require further investigation.

By using our peptide array and substitution array technology, we identified a cluster of potential MEK1 docking residues on β arrestin1 comprising two amino acids, namely Asp26 and Asp29 (Fig.3.9). Gratifyingly, from the known crystal structure of β arrestin it is evident that Asp26 and Asp29 are partially exposed on the surface of β arrestin in its basal conformation (Protein Data Bank codes 1G4M, 1G4R and 1ZSH (Milano, Kim et al. 2006)). However, they are involved in interactions with neighbouring residues. Thus Asp26 connects to the C-terminal Lys355 and Arg393 by salt bridges, and also interacts with the "phosphate sensor" Arg169. Also, Asp29 forms a salt bridge to Lys170 and contacts the side chain of Gln172 by hydrogen-bonds. These interactions make it unlikely that MEK can bind to these residues of β arrestin1 in such a basal conformation without either competing these interactions out and thereby triggering a conformation change in β arrestin or its interaction is gated by a change that causes disruption of this basal conformation such as could occur through post-translational modification (e.g. phosphorylation) or binding some other partner protein (Fig.3.9). The binding between Asp26 and MEK1 could occur only if the sequestered effects of the β arrestin1 C-terminal sequence on these residues is

removed. Asp26 becomes more accessible when the C-terminal sequence is displaced, but MEK1 binding to Asp26 and Asp29 require further conformational changes. An example of this is that β arrestins undergo a great conformational change upon association with their activated phospho-GPCR partners. This includes GPCR-phosphate engagement with β arrestin phosphate-sensor and repositioning of the C-terminal tail from its location of basal conformation where it is folded across the N-terminal region. β arrestin1 as a scaffold protein has a number of binding partners (Perry and Lefkowitz 2002; Xiao, McClatchy et al. 2007), and it undergoes structural alteration in binding of these proteins. In fact, β arrestin1 could undertake conformational changes upon binding to receptors and post-translational modification (Vishnivetskiy, Hosey et al. 2004). Based on this, it is possible that MEK1 can bind to modified or partner protein sequestered β arrestin1 that enables surface exposure of Asp26 and Asp29. It is also possible that there is equilibrium between β arrestin1's basal conformation and small percentage of "open conformation" that MEK1 and other proteins, such as MEK, may bind. If so, MEK1 binding may stabilize the open conformation of β arrestin1, shifting the equilibrium to generate a sub-population of MEK1 binding β arrestin1. Of interest, the Asp26 to Asp29 sequence is permanently located at the surface of isolated β arrestin1 N-domain. Thus, this sequence must have a similar interaction with MEK1 as Asp26 and Asp29. Another possibility is that if Song suggested MEK1 could associate with both N-domain and C-domain of β arrestin1, the MEK1 binding to the C-domain may act as a trigger of β arrestin1 conformational change, leading to increased accessibility to Asp26 and Asp29 at β arrestin1's N-domain.

Although we found two amino acids, Arg47 and Arg49, on MEK1 that β arrestin1 could bind to, we can't evaluate their accessibility due to lack of this region in a crystal structure of MEK1 (Ohren, Chen et al. 2004). However, considering their position at the upstream of MEK1 kinase domain, it is possible that they are surface exposed for interaction.

We designed and constructed a double alanine substitution β arrestin1 mutant that cannot bind MEK1. We also employed a novel small peptide technology to generate the inhibitory peptide for MEK1 binding to β arrestin1 and, by using this peptide, we demonstrated that it can regulate the phosphorylation of β arrestin1 at Ser412. Disassociation of MEK1 from the β arrestin1/ERK complex decreases

the phosphorylation of β arrestin1 at Ser412, which increases interactions of clathrin and c-Src to β arrestin1. This enlarged association of β arrestin1 with the endocytotic machinery accelerates the internalization of β 2AR, which is known to be induced by dephosphorylation of β arrestin1 at Ser412. In fact, the MEK1 displacer peptide leads to the dephosphorylation of β arrestin1, increases the interaction of β arrestin1 to clathrin and c-Src, and promotes β 2AR internalization indicates that the dephosphorylation of β arrestin1 is a bottleneck for the receptor internalization process.

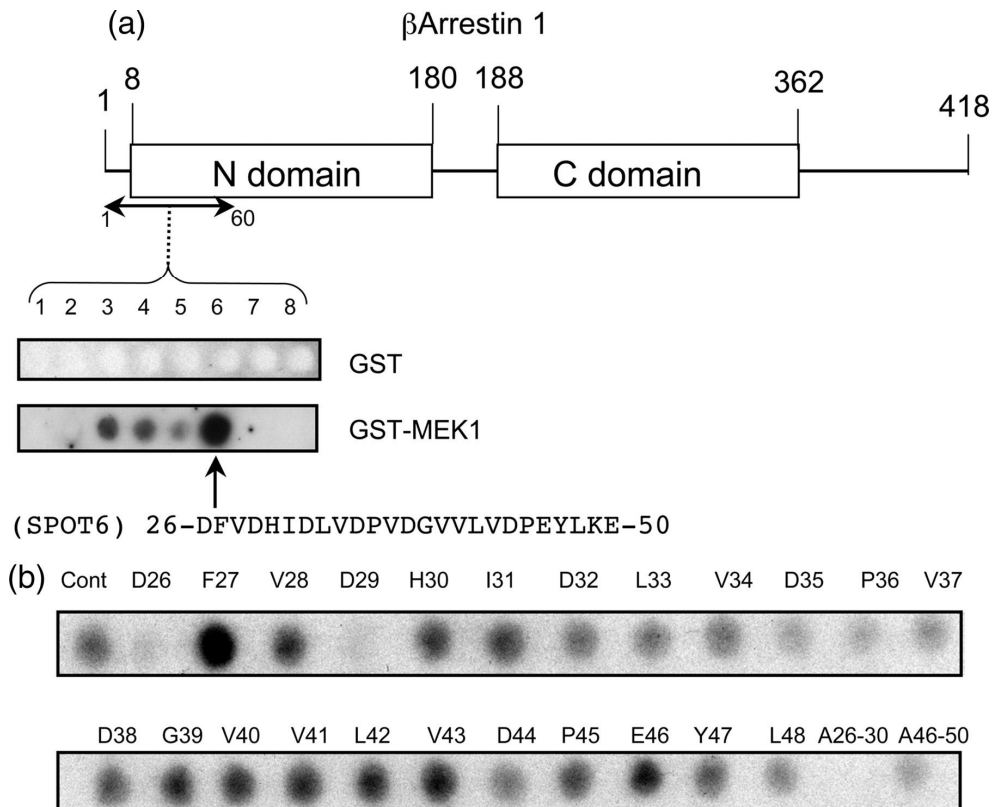


Figure 3.1: Peptide array identified potential MEK1 binding sites on βarrestin1 N-terminal.

(a) Synthesized peptide spots containing 25 -mer peptides were immobilized on nitrocellulose. Each of the peptide is shifted by 5 amino acids, covering the whole length of βarrestin1. The nitrocellulose was activated by a 15 seconds wash in pure ethanol, followed by a 2 hours (minimum) incubation with 5% (w/v) skimmed milk to block the non-specific binding sites. Purified GST-MEK1 or GST proteins were used to probe their interaction sites on the βarrestin1 peptide array. The GST signal was detected by immunoblotting with anti-GST antibody (Santa Cruz). Positive interaction sites are presented in DARK spots. Spot 6 spanning D25-E50 has the strongest interaction with GST-MEK1 and was selected for alanine-scan peptide array analysis. (b) The alanine-scan peptide array for full sequence of D26-E50 (spot6) was designed as below: for the control spot, it represents the identical spot 6 from (a) full length βarrestin1 array; the following spots were also from spot 6 but with its amino acid sequentially substituted with an alanine residue; the A26-30 and A46-50 have 5 amino acids substituted with alanines. The alanine-scan array was prepared and processed as described in (a). Light spots represented attenuated Gst-MEK1 interaction. (Shown are representative immunoblots from three independent experiments. Data provided by George Baillie)

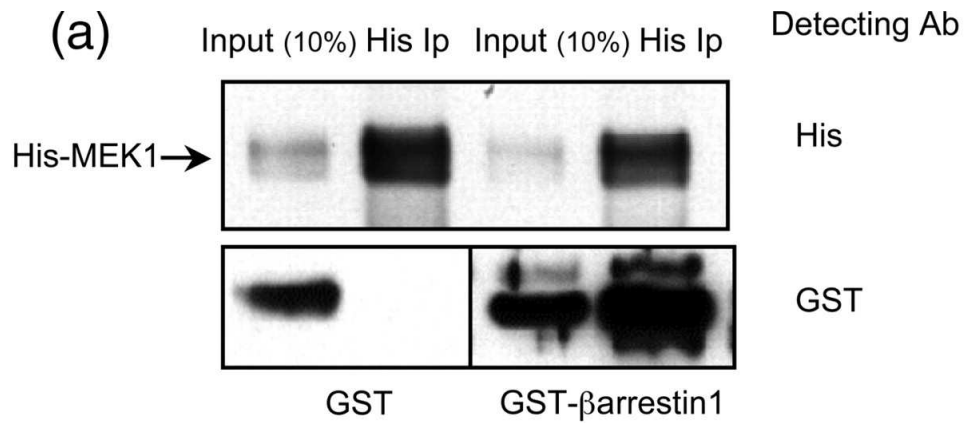


Figure 3.2a: β arrestin1 binds to MEK1 directly *in vitro*.

Purified fusion protein His-MEK1 was mixed with either GST- β arrestin1 or GST protein alone *in vitro* for 1 hour. The His-MEK1 was isolated by immunoprecipitation with anti-His agarose. Pull-down pellets were subjected to Western blot analysis and GST tag was detected by immunoblotting with GST antiserum. (Shown are representative immunoblots from three independent experiments.)

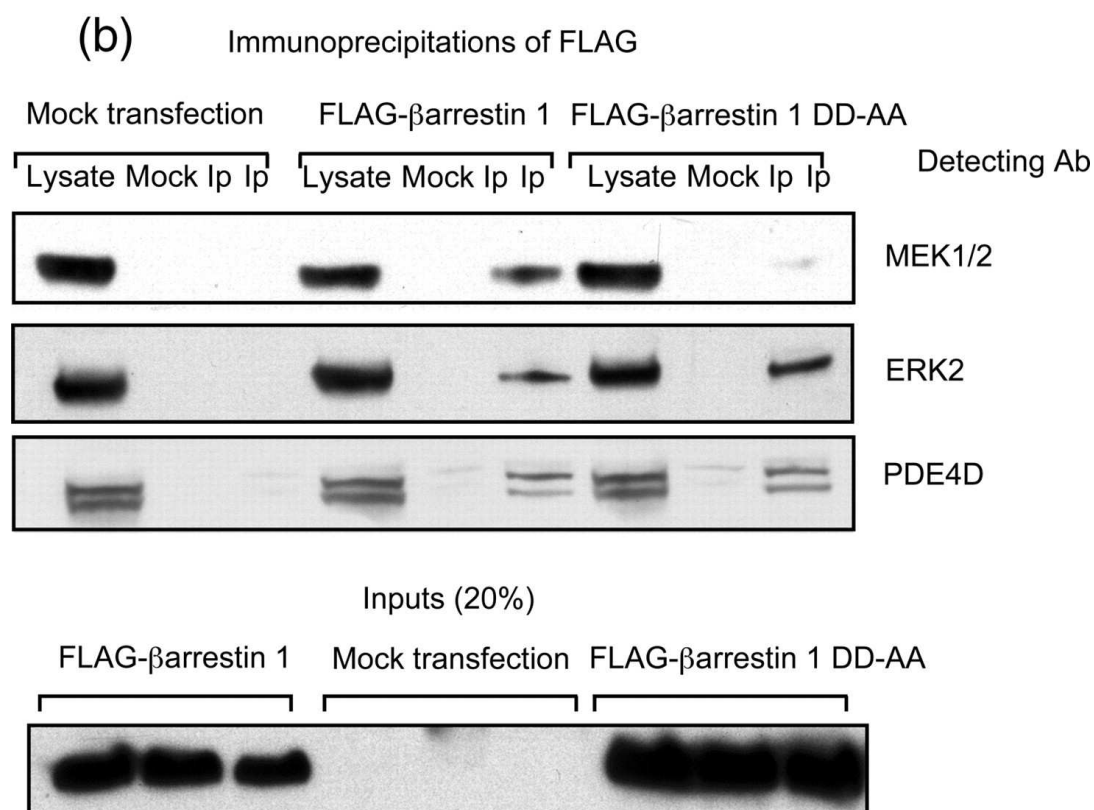


Figure 3.2b: β arrestin1 binds to MEK1 directly *in vivo*.

HEK293 cells were transfected with wild type Flag- β arrestin1 or DD-AA mutant where Asp26 and Asp29 were each mutated to alanine. Cells were lysed in 3T3 lysis buffer and centrifuged at 13,000 for 5 minutes in order to remove the debris. Supernatants were measured for total protein concentration. Equal amounts of supernatants were precleared with Protein G beads before immunoprecipitation by Flag epitope. The immunoprecipitates were then subjected to Western blot and MEK1/2, ERK2, PDE4D were detected with specific antibodies. Lysate, precleared lysate contains 30 μ g of total protein. MOCK IP, protein G beads for MOCK IP. IP, IP with anti-FLAG antibody. Ab, Antibody. (Shown are representative immunoblots from three independent experiments. Data provided by George Baillie)

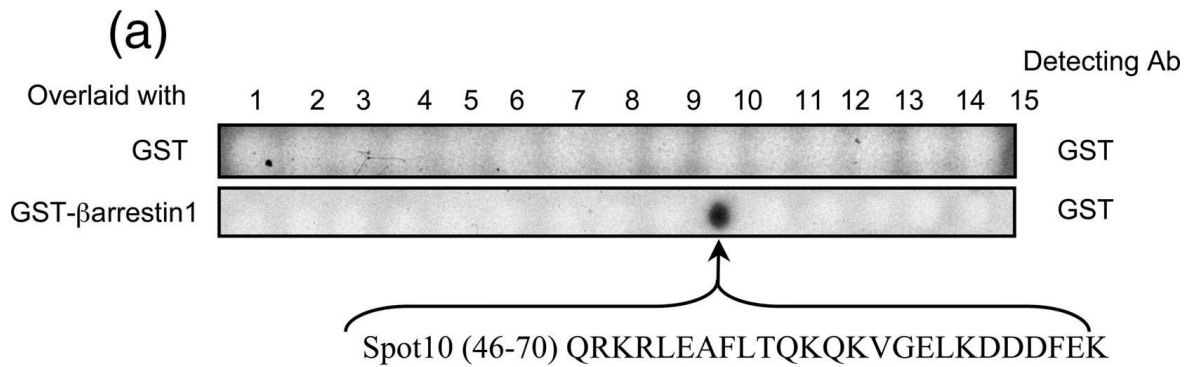


Figure 3.3a: β arrestin1 binds to the N terminal of MEK1 in peptide array.

Synthesized peptide spots containing 25 -mer peptides were immobilized on nitrocellulose. Each of the peptide is shifted by 5 amino acids, covering the entire sequence of MEK1. The nitrocellulose was prepared using the method in Fig 3.1. GST or GST- β arrestin1 interaction with MEK1 peptide array was probed using anti-GST antibody. Spot 10 showed positive interaction with GST- β arrestin1. (Shown are representative immunoblots from two independent experiments. Data provided by Martin Lynch.)



Figure 3.3b: N-terminal residues Arg47/Lys48/Arg49 are identified as β arrestin1 binding sites.

Spot 10 from MEK1 peptide array (Fig 3.3a) was selected for alanine scan analysis. Sequence Q46-K70 and its mutant with alanine substitutions were generated as indicated. (Shown are representative immunoblots from two independent experiments. Data provided by Martin Lynch.)

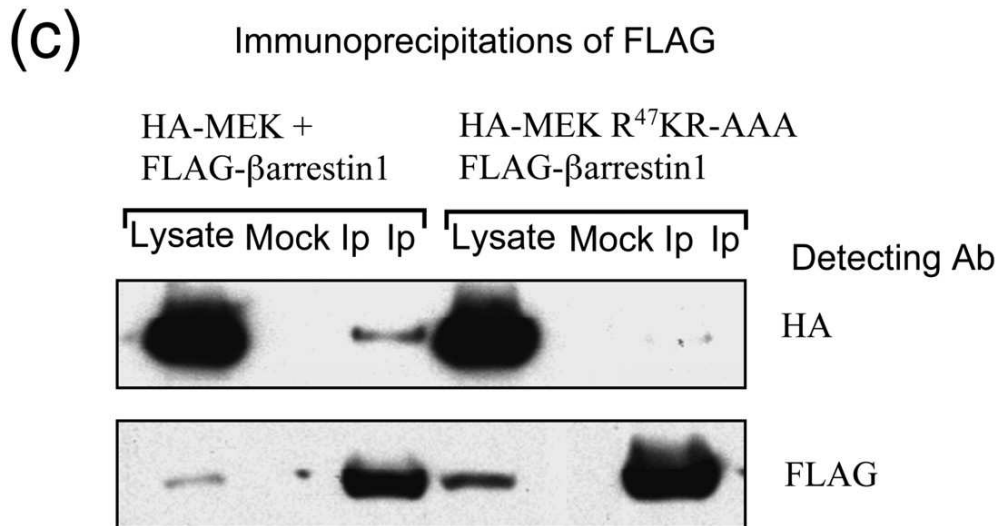


Figure 3.3c: N-terminal residues Arg47/Lys48/Arg49 are identified as β arrestin1 binding sites.

HEK293 cells expressing FLAG- β arrestin1 with either HA-MEK1 or HA-MEK1 R⁴⁷KR-AAA mutants were lysed and prepared as described in Fig 3.1 before being subjected to immunoprecipitation with anti-Flag agarose beads. The pull-down pellets were analysed by Western Blotting. HA-MEK1 and Flag- β arrestin1 were probed with antisera for HA and Flag epitope. Lysate, precleared lysate contains 30 μ g of total protein. MOCK IP, protein G beads for MOCK IP. IP, IP with anti-FLAG antibody. Ab, Antibody. (Shown are representative immunoblots from three independent experiments.)

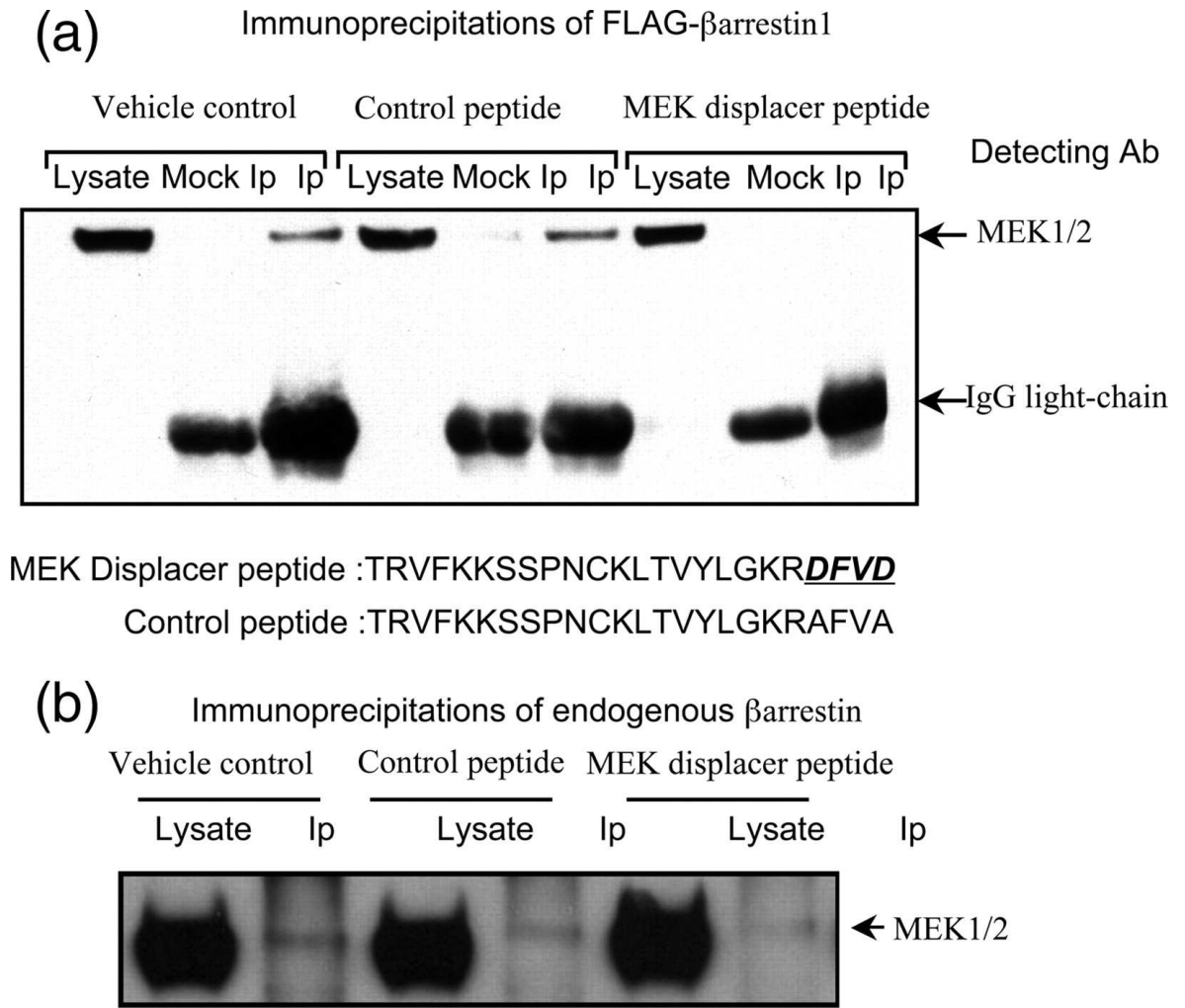


Figure 3.4: Disruption of the MEK1/ β arrestin1 complex via disruptor peptide.

(a) HEK293 cells transfected with FLAG- β arrestin1 were treated with vehicle (DMSO), a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T⁶-DFVD²⁹, MEK displacer peptide, 10 μ m) or a mutant where D26D29 were substituted to alanines (Control peptide, 10 μ m). Cells were lysed and prepared as described in Fig 3.1 before being subjected to immunoprecipitation with anti-Flag agarose beads. The pull-down pellets were analysed by Western Blot. Endogenous MEK1 was probed with anti-MEK1 antiserum. Lysate, precleared lysate contains 30 μ g of total protein. MOCK IP, protein G beads for MOCK IP. IP, IP with anti-FLAG antibody. Ab, Antibody. (b) Wild type HEK293 cells were treated as that in (a), and endogenous MEK1 was probed with the same antibody. (Shown are representative immunoblots from three independent experiments. Thanks George Baillie for his help with the immunoprecipitation.)

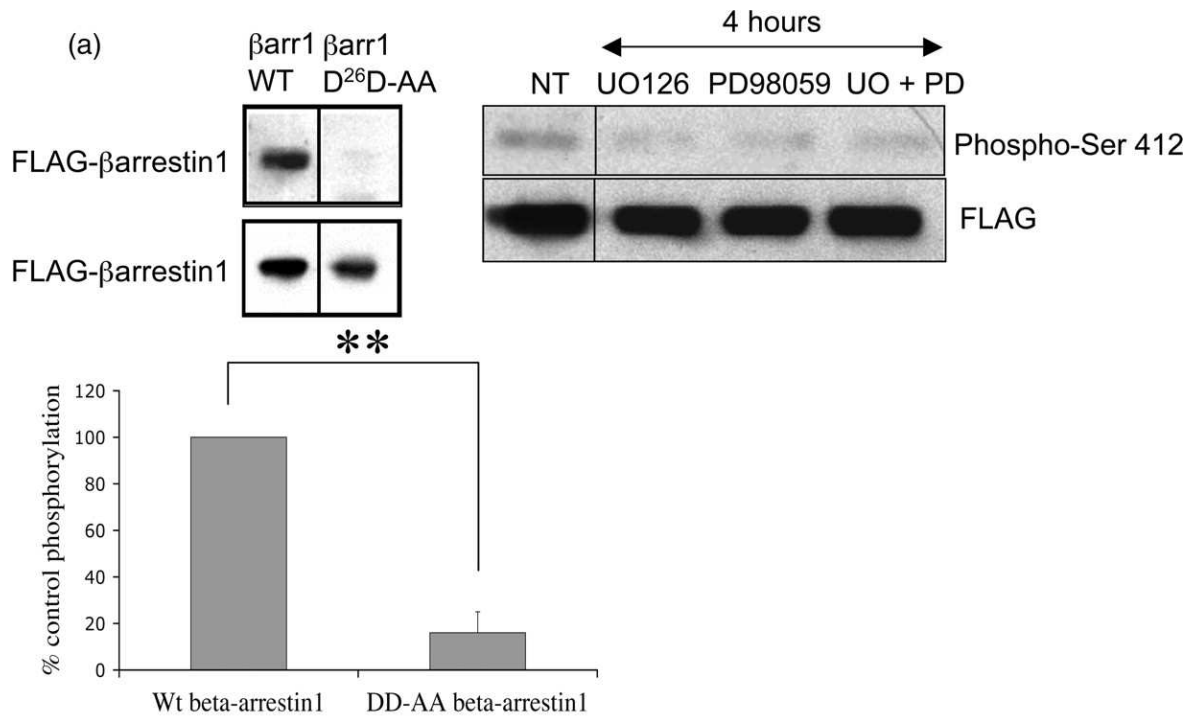


Figure 3.5a: Disruption of the MEK1/Barrestin1 complex by mutagenesis

Right, HEK293 cells transfected with FLAG-βarrestin1 were treated with MEK1 inhibitors UO126 and/or PD98059 (1μM) for 4 hours. Phospho-Ser412 of FLAG-βarrestin1 was detected by an antibody for phospho-Ser412 (Cell Signaling). FLAG epitope was blotted to show equal loading. NT, no treatment. *Left*, HEK293 cells transfected with wild type FLAG-βarrestin1 or its DD-AA mutant for 48 hours. Cells were lysed and prepared as described in Fig 3.1 before being probed with anti-phospho-Ser412 and FLAG antiserums. (Shown are representative immunoblots from three independent experiments.)

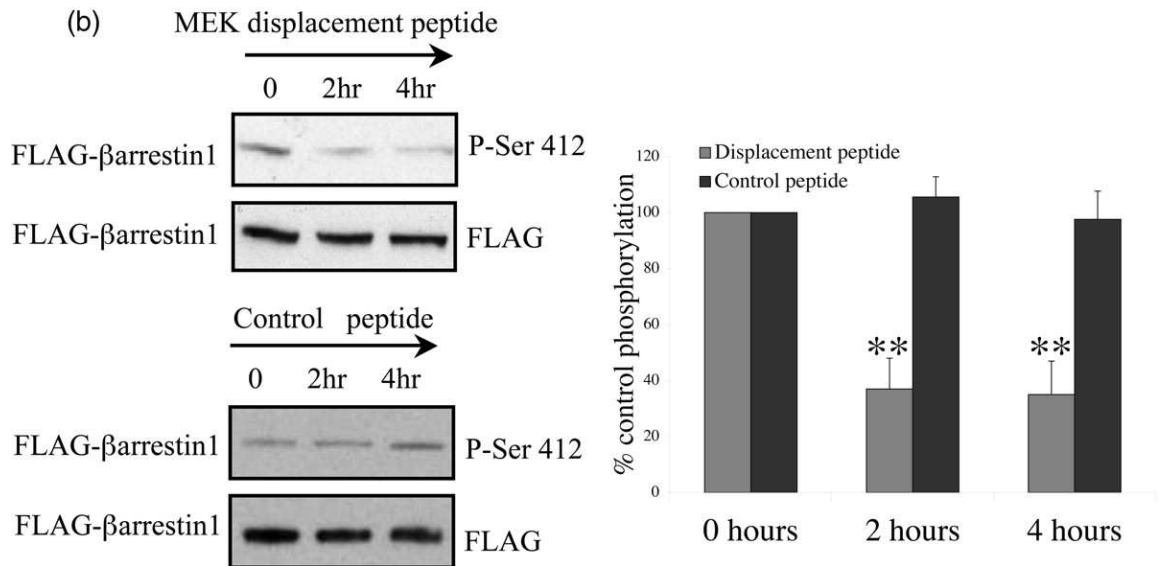


Figure 3.5b: Disruption of the MEK1/ β arrestin1 complex by disruptor peptide attenuates the phosphorylation of β arrestin1.

HEK293 cells transfected with FLAG- β arrestin1 were treated with a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T^6 -DFVD²⁹, MEK displacer peptide, 10 μ m) or a mutant peptide where D26D29 were substituted to alanines (Control peptide, 10 μ m). Cells were lysed and prepared as described in Fig 3.1 before being subjected to Western Blotting. Flag- β arrestin1 was probed with anti-FLAG antiserum. Phospho-Ser412 was detected by a site directed phosphor-antibody. **, statistical significance ($P < 0.01$ using student's t test)

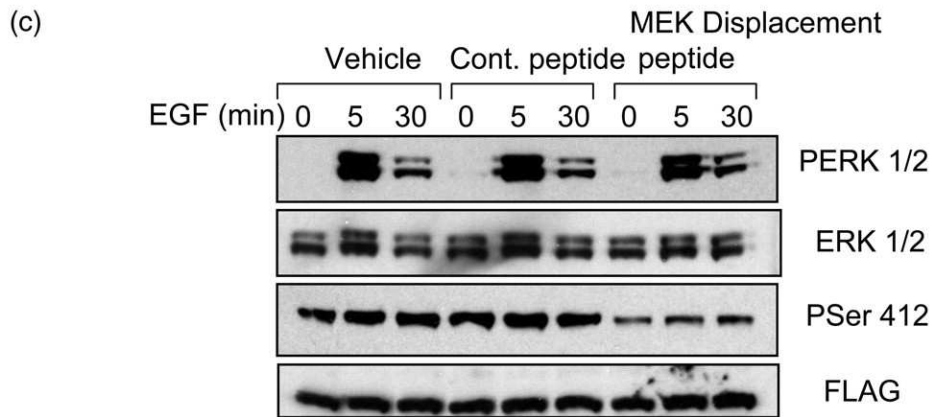


Figure 3.5c: Disruption of the MEK1/Barrestin1 complex by disruptor peptide has no influence on phosphorylation of ERK.

HEK293 cells transfected with FLAG- β arrestin1 were treated with vehicle (DMSO), a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T⁶-DFVD²⁹, MEK displacement peptide, 10 μ m) or a mutant where D26D29 were substituted to alanines (Control peptide, 10 μ m) for 2 hours before treatment of EGF for indicated time. Cells were lysed and prepared as described in Fig 3.1 before being subjected to Western Blot. Cell lysates were immunoblotted for expression using FLAG epitope and phosphorylation at Ser412 of β arrestin1, ERK and phospho-ERK using corresponding antibody. (Shown are representative immunoblots from three independent experiments.)

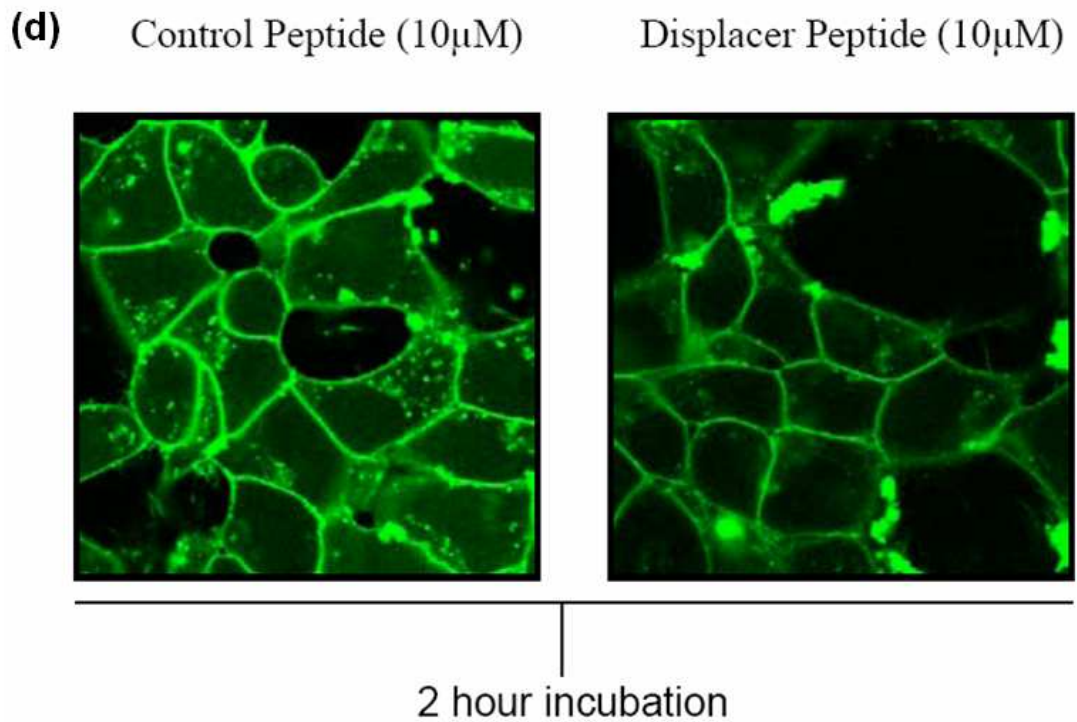


Figure 3.6: Uptake of fluorescently labeled cell-permeable peptides

HEK293 cells transfected with FLAG- β arrestin1 were treated with fluorescently labelled cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T⁶-DFVD²⁹, MEK displacement peptide, 10 μ M) or a mutant where D26:D29 were each substituted to alanine (Control peptide, 10 μ M) for 2 hours. Cells were then fixed and subjected to confocal microscopy in order to visualise the peptides.

(Peptides and data provided by Enno Klusmann, Leibniz-Institut für Molekulare Pharmakologie, Campus Berlin-Buch, Robert-Rössle-Strasse 10, 13125 Berlin, Germany)

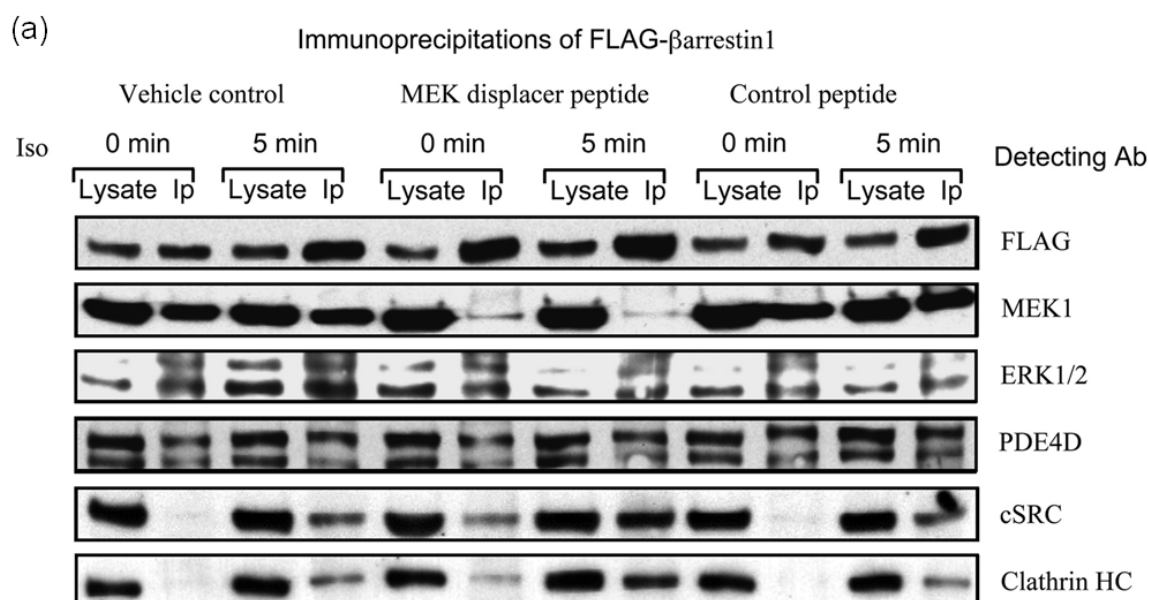


Figure 3.7: Disruption of the MEK1/ β arrestin1 complex by disruptor peptide enhances β arrestin1 association with c-Src and Clathrin.

(a), HEK293 cells transfected with FLAG- β arrestin1 and β_2 AR were treated with vehicle (DMSO), a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T⁶-DFVD²⁹, MEK displacement peptide, 10 μ m) or a mutant where D26:D29 were each substituted with alanine (Control peptide, 10 μ m) for 2 hours before treatment of isoprenaline for 5 minutes. Cells were lysed and prepared as described in Fig 3.1 before immunoprecipitation of Flag- β arrestin1. Cell lysates and pull-down pellets were immunoblotted for expression using FLAG epitope and MEK1, ERK, PDE4D, c-Src, Clathrin (Heavy Chain) using corresponding antisera. (b), Control IP for (a). Same cells as that used in (a) were treated with disruptor peptides and isoprenaline under the same procedure of (a). Cells lysates were immunoprecipitated with anti-VSV agarose. The IP products were blotted for Flag, c-Src, ERK and Clathrin HC (Heavy Chain). (Shown are representative immunoblots from three independent experiments. Thanks George Baillie for his help with the immunoprecipitation.)

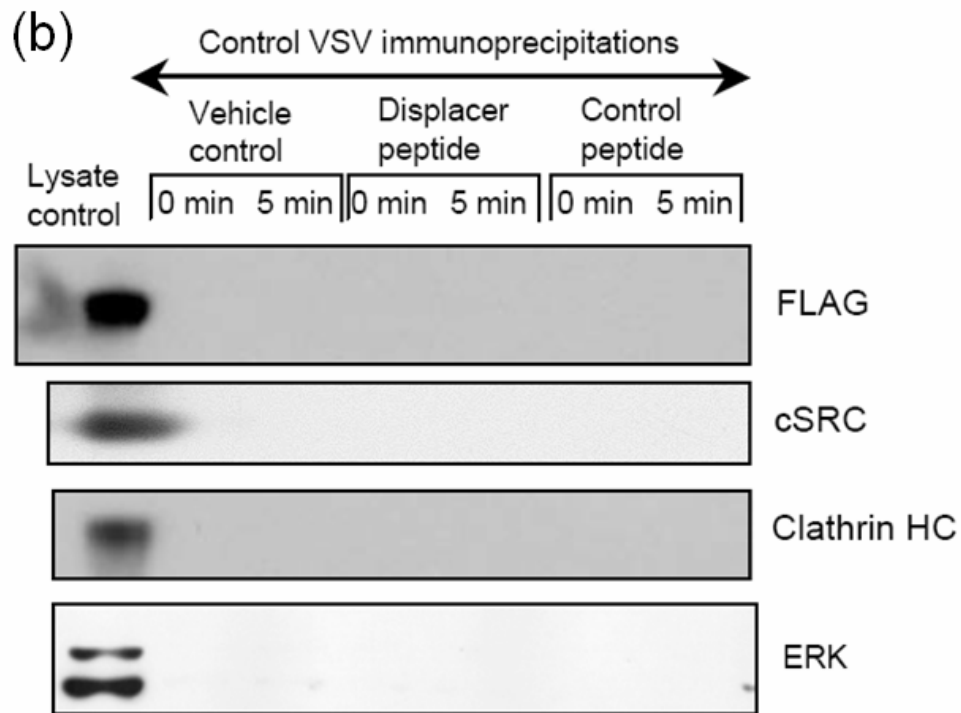


Figure 3.7: Disruption of the MEK1/Barrestin1 complex by disruptor peptide enhances Barrestin1 association with c-Src and Clathrin.

(a), HEK293 cells transfected with FLAG- β arrestin1 and β_2 AR were treated with vehicle (DMSO), a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T⁶-DFVD²⁹, MEK displacement peptide, 10 μ m) or a mutant where D26:D29 were each substituted with alanine (Control peptide, 10 μ m) for 2 hours before treatment of isoprenaline for 5 minutes. Cells were lysed and prepared as described in Fig 3.1 before immunoprecipitation of Flag- β arrestin1. Cell lysates and pull-down pellets were immunoblotted for expression using FLAG epitope and MEK1, ERK, PDE4D, c-Src, Clathrin (Heavy Chain) using corresponding antisera. (b), Control IP for (a). Same cells as that used in (a) were treated with disruptor peptides and isoprenaline under the same procedure of (a). Cells lysates were immunoprecipitated with anti-VSV agarose. The IP products were blotted for Flag, c-Src, ERK and Clathrin HC (Heavy Chain). (Shown are representative immunoblots from three independent experiments. Thanks George Baillie for his help with the immunoprecipitation.)

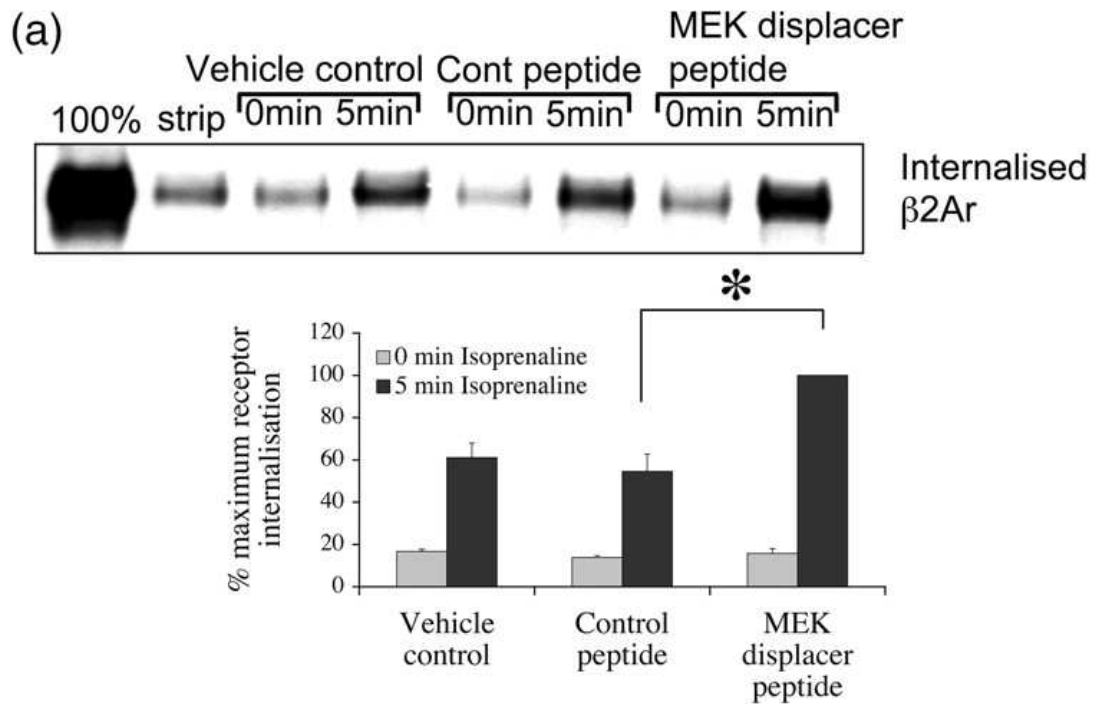


Figure 3.8a: Biotin protection assay shows disruption of the MEK1/Barrestin1 complex by disruptor peptide promotes $\beta_2\text{AR}$ endocytosis upon isoprenaline treatment.

HEKb2 cells were biotinylated and then pretreated with vehicle (DMSO), a cell permeable peptide consisting of amino acids 6-29 of β arrestin1 (T^6 -DFVD²⁹, MEK displacement peptide, 10 μm) or a mutant where D26:D29 were each substituted with alanine (Control peptide, 10 μm) for 2 hours before treatment of isoprenaline for 5 minutes. Biotin-tags on receptors at cell surface were stripped before cells were lysed. The lysates were prepared as described in Figure 3.1 before immunoprecipitation with agarose specific binding to biotin tag. The pull-down products were blotted for $\beta_2\text{AR}$ with a specific antiserum. 100%, full biotinylation of receptors. Strip, biotinylated receptors were stripped before any treatment. *, statistical significance ($P < 0.05$ using student's t test) (Shown are representative immunoblots from three independent experiments.)

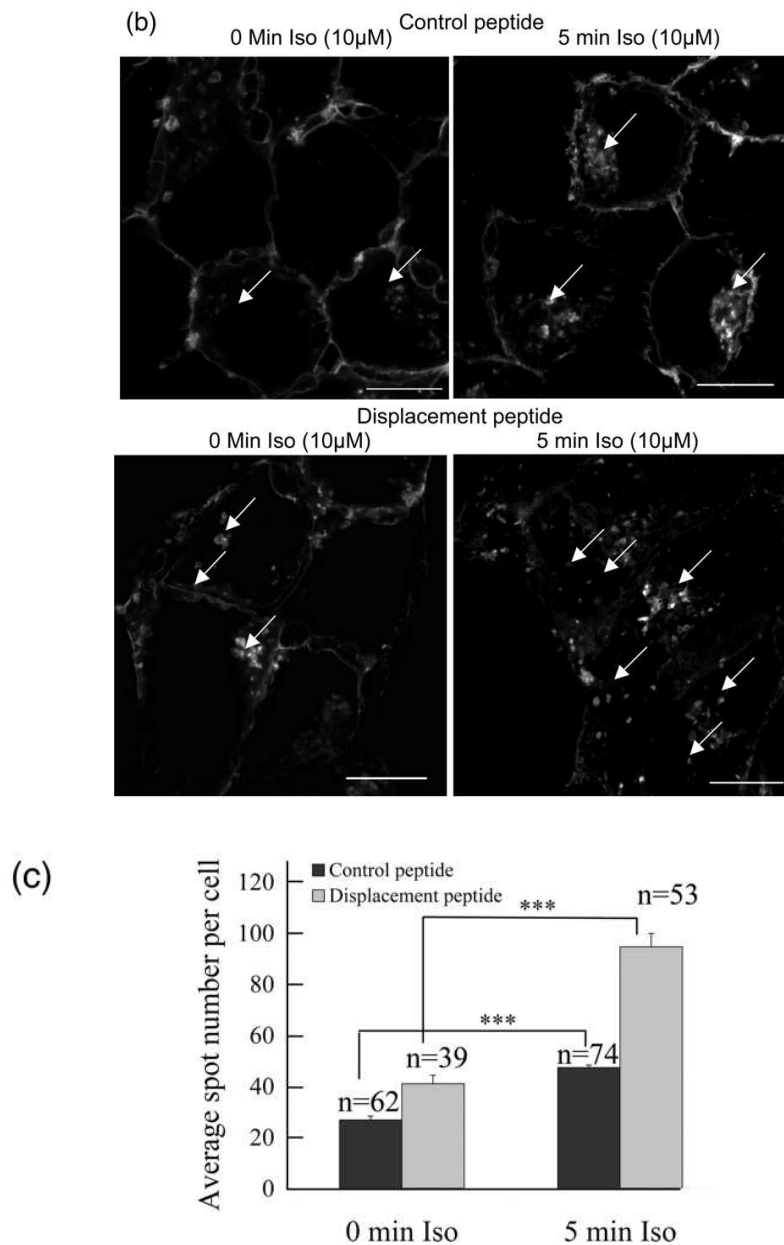


Figure 3.8b,c: Confocal image shows disruption of the MEK1/Barrestin1 complex by disruptor peptide promotes β_2 AR endocytosis upon isoprenaline treatment.

(b), HEKb2 cells were treated with control peptide and disruptor peptide similarly as for Figure 3.7a. Cells were fixed and subjected to confocal microscopy to visualise GFP tag on the β_2 AR. ($P < 0.05$ using student's t test). (c), quantification of internalized β_2 ARs. n= number of cells analysed /treatment. ***, $p < 0.001$ (Thanks Elaine Huston for her help with confocal microscopy.)

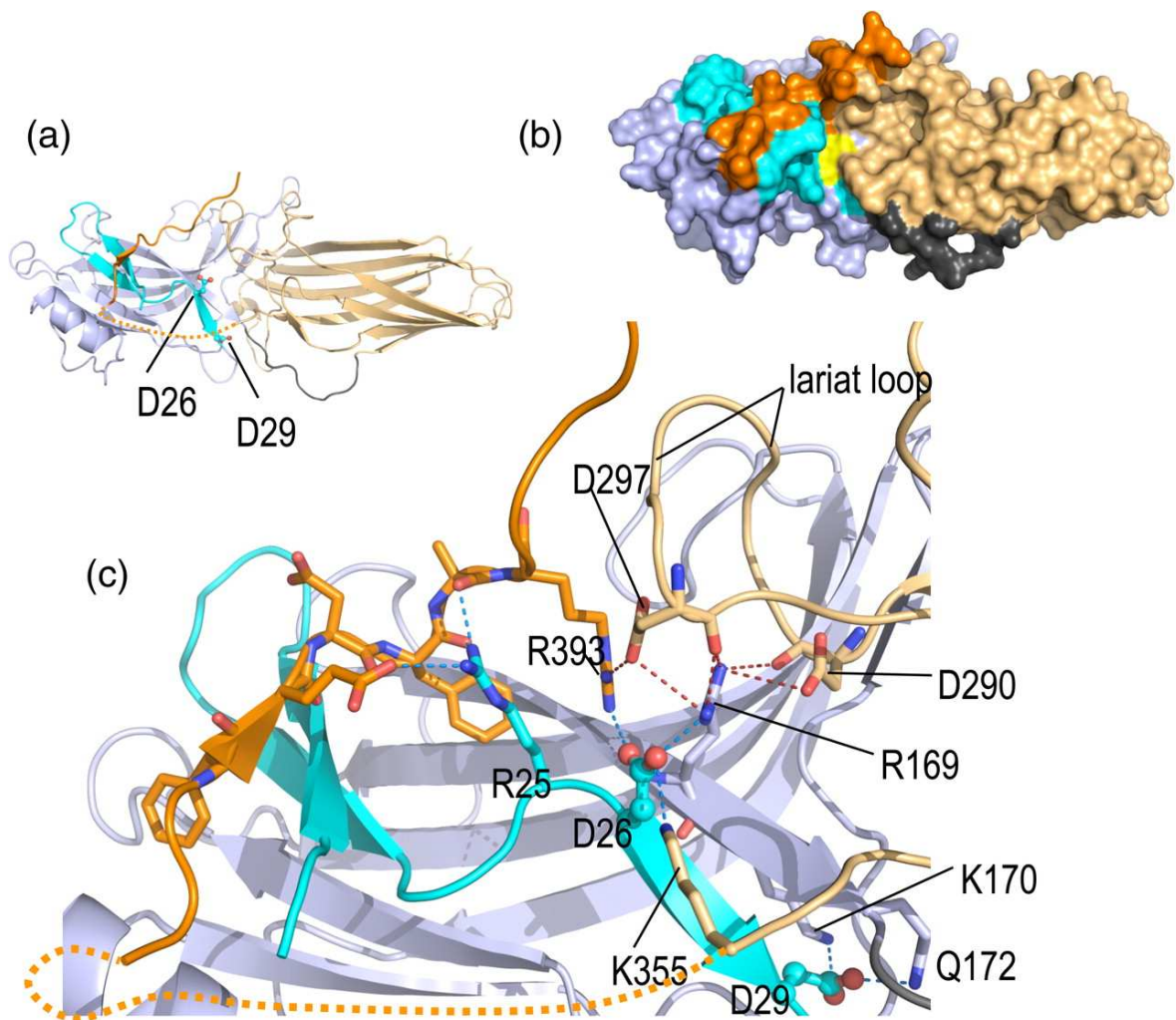


Figure 3.9. Views taken from the crystal structure of bovine _arrestin1 (Protein Data Bank code 1ZSH) to illustrate the environment of Asp26/Asp29 implicated in the binding of MEK1.

(a), ribbon view of basal state arrestin conformation. (b), as (a) but with solvent-accessible surface rendition. c, detail of internal interactions for Asp26 and Asp29 in basal state arrestin conformation. Light blue, N-domain; light orange, C-domain; bright orange, C-terminal peptide latch; gray, interdomain hinge peptide sequence; cyan, 6TRVF~~KK~~ASPN~~GK~~LT~~VY~~LG~~KR~~DFVD29 peptide-6 sequence; yellow, surface exposure of Asp26 in b. The numbering used is taken from human _arrestin1 (P49407); all of the identified residues are conserved between the bovine and human isoforms. Unresolved electron density for the sequence spanning residues 356-384 is indicated by the dotted orange line. (Data provided by Professor David R. Adams, Heriot-Watt University, Edinburgh, UK)

4 PDE4D5 dimerization

4.1 Introduction

cAMP and cGMP are well-studied second messengers that are in charge of transducing extracellular signals such as light, hormones and neurotransmitters into numerous cell functions. Both of them play key roles in lots of physiological progresses that are involved in vision, memory, immunity and cell development (Beavo 1995; Manganiello, Murata et al. 1995; Conti and Jin 1999; Soderling and Beavo 2000 ; Francis, Turko et al. 2001). Therefore, regulation of their intracellular concentration is of high importance. There are two ways to modulate the intracellular cyclic nucleotide synthesis by adenylyl cyclases and degradation by phosphodiesterases. The phosphodiesterases can hydrolyze the phosphodiester bond and produce the corresponding 5'-nucleoside monophosphates.

PDEs consist of 11 subfamilies (PDE1 to PDE11), which are characterized by their sequence and enzymatic properties. They all contain a highly conserved catalytic domain, which is located between the unique N-terminal and C-terminal of each PDE. These N and C termini can undergo post-translational modification such as phosphorylation (by PKA, PKB, ERK1/2, CaMK and PKG) (Chu, Corbin et al. 1997; Grange, Sette et al. 2000) and association with other signalling messengers (cGMP, Ca²⁺-calmodulin and phosphatidic acid) (Sharma and Wang 1986; Thomas, Francis et al. 1990; Sette and Conti 1996; Hoffmann, Baillie et al. 1999; Ahmad, Cong et al. 2000) in order to change PDE activity; or restrict PDE subcellular distribution by interaction with other protein partners and membrane insertion (Yarwood, Steele et al. 1999; Shakur, Takeda et al. 2000; Verde, Pahlke et al. 2001). Thus, the terminal domains work in different combinations to form unique PDEs, and the different PDEs, in turn, form one of the basic regulatory devices of specific cell types.

The cAMP specific PDE4 family is the largest PDE family and consists of over 20 isoforms. They are composed by expression of different variants of 4 genes, namely the PDE4A, PDE4B, PDE4C and PDE4D subfamilies. However, PDE4s can also be divided into long, short and supershort isoforms based on their specific N terminal features. The unique N-terminal distinguishes PDE4 from other PDE

families in that it contains two upstream conserved regions 1 and 2 (UCR1 and UCR2). The long form contains both UCR1 and UCR2, whereas the short form contains only UCR2 or part of UCR2 for supershort PDE4 (Houslay and Adams 2003). The UCR1 and UCR2 domains in the long form PDE4 splice variants have been shown to interact with each other, and this interaction was suggested to be intramolecular (Lim, Pahlke et al. 1999; Beard, Olsen et al. 2000). To form the interaction, two positively charged residues (Arg98 and Arg101 in PDE4D3) of the UCR1 are suggested to contact with a cluster of negatively charged residues (Glu146, Glu147 and Asp149 in PDE4D3) (Beard, Olsen et al. 2000). This interaction can be changed by PKA phosphorylation on a serine residue (Ser54 in PDE4D3). The PKA phosphorylation also activates the long PDE4. Since UCR2 was suggested to have an inhibitory effect on the catalytic region, it is possible that the PKA phosphorylation alters the interaction between UCR1 and UCR2, which in turn remove UCR2 from the centre of catalytic unit to cause enzyme activation (Sette and Conti 1996; Lim, Pahlke et al. 1999; Beard, Olsen et al. 2000). ERK2 phosphorylation of PDE4 long isoforms at C terminus inhibits their activity, whereas phosphorylation of PDE4 short isoform PDE4D1 leads to its activation (Hoffmann, Baillie et al. 1999; MacKenzie, Baillie et al. 2000).

A previous study led to the suggestion that the PDE4 long form PDE4D3, containing both UCR1 and UCR2, existed as a dimer in cells, whereas the short form PDE4D2 behaves as a monomer (Richter and Conti 2002). Truncation of either the N-terminal or C-terminal regions of PDE4D3 ablated dimer formation. However, this interaction can be distinguished from previous reported UCR1/2 intramolecular interaction, since PKA phosphorylation that could ablate the intramolecular interaction has little impact on its dimerization. A following study on PDE4D3 dimerization highlighted the importance of such an effect in determining the regulatory property and inhibitor (rolipram) sensitivity of PDE4D3. From a site-directed mutagenesis study, it was shown that monomeric PDE4D3 cannot be activated by PKA phosphorylation or phosphatidic acid binding.

PDE4D5, a PDE4 long isoform, shares a highly conserved catalytic unit and UCR1/2 with PDE4D3. These isoforms are only different at their extreme N-terminal region. PDE4D3 has a 15 amino acid unrelated N-terminal region, whereas PDE4D5 has a unique region of 88 amino acids (Bolger, Erdogan et al. 1997). However, this small difference is sufficient to bring isoform specificity to

these PDEs (Lynch, Baillie et al. 2005). Indeed, even with high similarity, PDE4D3 and PDE4D5 are not redundant with respect to each other (Lynch, Baillie et al. 2005).

To further understand the isoform specificity of PDE4D5, we investigated the dimerization ability of PDE4D5 in this study.

4.2 Results

4.2.1 *PDE4D5 can interact with itself and other isoforms*

We first set up to test if PDE4D5 can form dimerize with itself (homodimerisation) via yeast two hybrid experiments. All the yeast two hybrid data in this study were obtained from Dr Bolger (the University of Alabama, USA).

Yeast two hybrid assay is a powerful way in screening for interacting protein partners. The molecular basis for this method is that in most eukaryotic transcription factors, their binding and activating domain can function in close proximity without direct binding. In the following experiment, it employs DNA binding domain (BD), activation domain (AD) and the *LacZ* reporter gene to obtain an observable colour change when a protein interaction occurs. The proteins of interest are fused with BD and AD separately and their plasmids are transfected into yeast simultaneously. The protein fused with BD also is referred to as bait, whereas the protein fused with AD is referred to as prey. In yeast, the BD domain can bind to UAS (upstream activating sequence), when there is an interaction between the BD fused protein and AD fused protein, it brings AD domain in close proximity with the BD domain to activate the transcription of the down-stream *LacZ* reporter gene. The product of *LacZ* reporter gene, β -galactosidase, generates a blue colouration through metabolism of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside), therefore giving an positive indication of protein interaction. The yeast two hybrid assay allows for fast screening of interacting partners which is much faster than doing expression studies in mammalian cells, although the key interactions need to be confirmed in mammalian systems. In the yeast two hybrid experiments of this study, either LexA-PDE4D5 or empty vector was used as bait, and different PDE4D constructs were used as prey. It is clear that PDE4D5 can form strong homo-dimerization

with itself. It is also interesting that PDE4D5 can form weaker hetero-dimerization with the PDE4D3 isoform. This finding was a surprise as PDE4D3 and PDE4D5 do not co-immunoprecipitate in mammalian cells (Baillie, MacKenzie et al. 2001; MacKenzie, Baillie et al. 2002; Lynch, Baillie et al. 2005). However, since PDE4D5 and PDE4D3 are long forms that have conserved UCR1 and UCR2 domains, which were shown to play key roles in PDE4D3 dimerization, it is possible that in this model system small numbers of heterodimers can form. In native cells the availability of partner proteins for PDE4D3 and PDE4D5 together with their very low expression cells would be expected to prevent heterodimers from forming.

The EELD motif on UCR2 N-terminal was considered to be important in PDE4 intramolecular interaction but not in dimer formation (Richter and Conti 2004) . Indeed, we see here that mutation of EELD has no detectable effect on PDE4D5 dimerization. Consistent with Conti's finding (Richter and Conti 2004) on PDE4D3, the V172A/F176A/L224A/L227A mutation of PDE4D5 has an attenuated interaction on dimer formation of this mutant with its wild-type isoform. (Figure 4.1)

To show whether PDE4D5 dimerization occurs in mammalian cells, I cotransfected HEK293 cells with differently tagged PDE4D5 forms (vsv tag and FLAG tag), immunoprecipitated FLAG-PDE4D5 with anti-FLAG agarose beads, and immunoblotted for the counter-part vsv-PDE4D5 (Figure 4.2). One problem for this that transfection of equal amount of PDE4D5 constructs normally see different expression levels in the same cell line, which can due to the nature of constructs themselves since some mutations may change their transcription ability, or due to the purity and quality of the DNA since the transfection reagent (Polyfect, QIAGEN) can be affected by impure DNA. To obtain a comparable result, normalization of each PDE4D5 construct used in the co-expression is of high importance. This normalisation includes several aspects: firstly, all constructs under the same procedures; secondly, equal amount of construct is transfected into cells and their individual expression levels are checked. A expression ratio is obtained using mutants to against their native forms such as vsv-QUAD mutant against vsv-4D5. The amounts of construct that will be used in the transfection are adjusted according to the ratio; Thirdly, the vsv tagged 4D5 or its mutant with Flag tagged 4D5 are co-transfected and the expression levels

of each construct are checked again before immunoprecipitation. This process is time consuming and requires a lot of effort to go through all the procedures in normalisation the necessary conditions. However, by doing this I observed clear dimerization of PDE4D5 as seen in the two hybrid experiment.

4.2.2 PDE4D5 binding partners influence its dimerization

PDE4D5 is known to associate with a number of proteins including G-protein coupled receptors, AKAPs, RACK1 and β arrestins (Bolger, McCahill et al. 2002) (McCahill, Warwicker et al. 2002) (Baillie, Sood et al. 2003) (Bolger, Baillie et al. 2006) (Lynch, Baillie et al. 2005) . Many of the interactions are believed to cause structure change of PDE4D5, altering its enzyme activity and influencing its affinity with other proteins. In order to investigate whether these partner proteins can impact on the PDE4D5 dimerization, we requested that Dr Bolger examine the role of RACK1 and β arrestin as these key signalling scaffold proteins interact specifically with PDE4D5 and have functional roles in cells (Bolger, McCahill et al. 2002; McCahill, Warwicker et al. 2002; Kiely, Leahy et al. 2005) .

These scaffolds were co-expressed with bait and prey PDE4D5 in yeast two hybrid experiments. Compared to the control that only has bait and prey PDE4D5, additional RACK1 and β arrestin expression ablated the PDE4D5 dimerization (Figure 4.3). However, when I set out to evaluate this potential disruptive effect in HEK293 cells, I observed that RACK1 and β arrestin failed to intervene in PDE4D5 dimerization (Figure 4.4). This is curious as we have shown (Lynch, Baillie et al. 2005) that PDE4D5 cannot bind both β arrestin and RACK1 at the same time. As these scaffolds bind to overlapping interaction sites on PDE4D5 and so one molecule of PDE4D5 can't bind both β arrestin and RACK1, we surmised from this observation and the 2-hybrid result that these scaffolds disrupted dimers to give monomers complexed with RACK1 or β arrestin. It appears that in mammalian cells there may be some other modification or partner protein that stabilises the dimers even when these scaffold interact. However, if such dimers do occur natively then a PDE4D5 dimers must only be able to bind RACK1 or β arrestin as mixtures are never observed. This also, however, suggests that if β arrestin is bound to one molecule of the dimer its binding must sterically block any binding of RACK1 to the other PDE4D5 molecule in the dimer. However, in yeast two hybrid experiments, we did see that

RACK1 and β arrestin ablated PDE4D5 dimerization (Figure 4.3). This might be due to the nature of yeast-two-hybrid assay, especially if the threshold for the signal to be detected is high such that disruption of only a small number of dimers by overexpression of RACK1 and β arrestin could attenuate the dimerization such that the signal now falls below the minimum detectable concentration in yeast-two-hybrid. Conversely, overexpression of RACK1 and β arrestin may reduce dimer formation by a small amount in mammalian cells but this is not readily detected by the immunoprecipitation assay.

4.2.3 Reversibility of PDE4D5 dimerization

In order to investigate the stability of PDE4D5 dimerization, I co-transfected different tagged versions of PDE4D5, together or separately, into HEK293 cells, cell lysates were subjected to immunoprecipitation. FLAG-PDE4D5 and VSV-PDE4D5 were separately transfected into HEK293 cells; 48 hours later, cells were lysed using 3T3 lysis buffer. Equal amount of cytosolic fractions (total protein) containing either FLAG or VSV tagged PDE4D5 were mixed, and rotated end to end at 4°C for 16 hours. The lysates were then subjected to immunoprecipitation using anti-vsv agarose, and the pull down pellets were analysed for vsv and FLAG tagged PDE4D5 using the corresponding antibody. As can be seen in Figure 4.5 lane 4, co-immunoprecipitation of co-transfected vsv and FLAG-PDE4D5 using anti-vsv agarose can pull-down FLAG-tagged PDE4D5. However, when immunoprecipitating vsv-PDE4D5 from separately expressed lysates mixture, tiny amounts of FLAG-PDE4D5 can be detected (Figure 4.5 lane 1). Furthermore, FLAG-PDE4D5 was also detectable in mixed lysates with separately expressed RACK1 and β arrestin (Figure 4.5 lane 2, 3). These data indicate that PDE4D5 dimerization is quite stable and that a limited percentage of PDE4D5 is subjected to disassociation/reassociation. This *in vitro* disassociation/reassociation is not consistent with Conti's finding (Richter and Conti 2002) that PDE4D3 does not disassociate in vitro. It could be that the disassociation/reassociation of PDE4D5 might be an isoform-specific feature when compared to the closely related PDE4D3, which has no detectable reassociation in vitro (Richter and Conti 2002). In addition, adding RACK1 or β arrestin to the PDE4D5 mixture neither enhanced the disassociation of dimerized PDE4D5 nor prohibited the reassociation of PDE4D5, which suggests

that expression of RACK1 and β arrestin have no significant influence on PDE4D5 dimerization. This may be because PDE4D5 dimerises as it is synthesised in cells.

4.2.4 Mapping the PDE4D5 dimerization domain using peptide array

To narrow down the PDE4D5 dimerization domain, we employed a novel peptide array technology. The full length PDE4D5 peptide library was synthesized on nitrocellulose. Each spot contains a 25 amino acid peptide, and each spot has 20 amino acid overlapping region with the adjacent spot in an order to cover the full length PDE4D5. To prepare the PDE4D5 peptide array, it was first activated in pure ethanol for 10 seconds; after 3 times 10 minutes wash with TBST, it was then incubated with 5% skimmed milk for at least 2 hours to block the non-specific binding sites. Purified GST tagged PDE4D5 was diluted to 1 μ g/ml in 1% milk, and applied to prepared PDE4D5 array for 2 hours. The PDE4D5 peptide array was probed with anti-GST antibody, and analysed with immunoblotting. The putative interaction (dimerisation) sites were recognised as dark spots (Figure 4.6A). From the full length PDE4D5 peptide array, three PDE4D5 dimerisation regions were detected, which includes N-terminal regions of UCR1, UCR2 and part of the catalytic domain.

To further narrow down the dimerization sites, an alanine scan array was performed to locate the putative candidates. Alanine scan array is where the various amino acids of the positively interacting peptides from the primary scan above were each substituted to give a family of peptides where every amino acid in the sequence was sequentially and individually replaced with alanine (note than when the native amino acid was alanine we changed it to aspartic acid). These alanine scanning peptides were probed with GST-PDE4D5 and GST control, with detection using an anti-GST antibody. The amino acids that contribute to potential binding sites were detected as light spots indicating the loss of interaction when substituted to alanine/aspartate. The three putative regions found in PDE4D5 peptide array were subjected to alanine scan array respectively. This yielded data suggesting that key residues forming the first dimerization site are R173/N174/N175 of PDE4D5 UCR1 region (Figure 4.6B). This is consistent with Conti's suggestion (Richter and Conti 2004) that LRTVRNNF is the dimerization site of PDE4D3. The sequence of the putative second dimerization

site is E228/T229/L230 on the UCR2 (Figure 4.6C), which is adjacent to a previously (Richter and Conti 2004) proposed dimerization domain 219E-227L. We also discovered the sequence of the putative two novel PDE4D5 dimerization sites L306/M307/H308 and K323/T324/E325. Both of these are located within the catalytic domain, and the latter one could be a potential sumoylation site based upon it having a sequence of V³²²KTE³²⁵ that is a classic consensus SUMO motif ψ KxE (where ψ is an aliphatic branched amino acid and x could be any amino acid) (Figure 4.6D).

4.2.5 Initial evaluation of RNN, ETL, LMH and KTE mutants

In these experiments I used WT, native PDE4D5; E53 (125), S54(126)A, S54(126)D mutants related to the PKA consensus phosphorylation site at the beginning of UCR1 (Sette and Conti 1996); S579(651)A, S579(651)D, related to the MAPK consensus phosphorylation site (Hoffmann, Baillie et al. 1999) and S239(311)D, related to the oxidative stress kinase site (Hill, Sheppard et al. 2006). (These mutants are numbered as they were in PDE4D3, PDE4D5 numbering in parentheses).

PDE4D5 constructs with RNN, ETL, LMH and KTE mutants were generated with each mutated to AAA. These were co-expressed with wild type PDE4D5 in yeast-two-hybrid experiments. The results showed that the RNN, ETL, LMH and KTE mutants had a minimal effect on the PDE4D5 dimerization (Figure 4.7A). However, this does not mean that these mutated sites do not form the dimerization sites. It is very likely that PDE4D5 dimerization requires more than one dimerizing region, and each of these may contribute partially to overall PDE4D5 dimerization. Therefore, disruption of a single site alone might not be enough to abolish the whole association. Indeed, some of these sites may be regulatory, where changes could enhance or disrupt binding.

To evaluate the effects of combinations of triplet and Quad mutants, we generated constructs expressing PDE4D5 with RNN, ETL, LMH and KTE mutations (to AAA), either in triplet or altogether (the Quad mutant), and tested them in yeast-two-hybrid assays. However, these mutants still had limited effects on PDE4D5 self-dimerization even with the Quad mutant. The only detected effects were increasing PDE4D5 dimerization rather than decreasing it (Figure 4.7B). In

these experiments, all of the mutants were in one of the two dimerized PDE4D5s. Thereby, the mutations might need to be on both dimerizing PDE4D5 to give a detectable result.

Though the main purpose of these yeast-two-hybrid experiments was to test the effects of RNN, ETL, LMH and KTE mutants, we also made several PDE4D5 mutants to test if PKA and ERK phosphorylation had any influence on PDE4D5 self-association. However, most of the mutants had no significant effect on PDE4D5 dimerization except S54A/S579A mutant, which totally abolished interaction with either wild type PDE4D5 or PDE4D3 (Figure 4.7A). This requires further investigation in both 2-hybrid and mammalian cells. However, it is possible that this double mutant triggers some disruptive conformational change. As it does not reflect any modified state, eg the Ser/Asp mutants would reflect a phosphorylated state. We decided it did not have enough priority to evaluate further. .

Apart from PDE4D5 homodimerization, we also analyzed the PDE4D3 heterodimerization with PDE4D5 mutants. Two PDE4D5 mutants S54A, which blocks the enzyme to be phosphorylated by PKA (Sette and Conti 1996), and LMH showed decreased dimerization affinity with PDE4D3 (Figure 4.7A). There are two possibilities for this observation: First, PDE4D3 may indeed dimerize with PDE4D5, but with less affinity than PDE4D5 with itself. Therefore, the mutants indeed caused a reduction that could be detected by yeast-two-hybrid. Second, the PDE4D3 constructs express less well than PDE4D5 constructs. The signal of wild type PDE4D3 with PDE4D5 is lower, as you can see in the wild type positive control, which allows the differences to be more readily apparent for detection. However, further confirmation for these effects is required by immunoprecipitation.

4.2.6 The Quad PDE4D5 can still dimerize in vivo

To assess whether PDE4D5 can dimerize with its Quad mutant, vsv tagged PDE4D5 construct with the Quad mutant was generated. The vsv-Quad mutant and Flag-PDE4D5 were co-expressed in HEK293 cells for 2 days; the cells were then harvested and the lysate was subjected to immunoprecipitation. The Flag-PDE4D5 were detectable in both wild type vsv-PDE4D5 and vsv-Quad mutant pull-

downs indicating that the Quad mutant can still dimerize with wild type PDE4D5 *in vivo* (Figure 4.8), which is consistent with the previous finding in yeast-two-hybrid.

A Korean group has shown that a single ion pair was crucial for dimerization of PDE4B truncated catalytic unit in the PDE4B crystal structure (Lee, Chandani et al. 2002). These paired residues are D322 and R358, and they also suggested that mutation of D322 to arginine disrupted the dimer interface. Therefore, we surmised that it may be possible that the two correspondent residues in PDE4D5, R499 (R358 in PDE4B) and D463 (D322 in PDE4B), could contribute to the dimerization of PDE4D5 (Figure 4.9).

4.2.7 R499-D463 ion pair is crucial for PDE4D5 dimerization

To evaluate (1) the influence of ion pair R499-D463 to PDE4D5 dimerization; (2) the effect of Quad mutation on both part of dimerized PDE4D5, we made PDE4D5 constructs with R499D mutation and/or Quad mutation on both bait and prey in yeast-two-hybrid assay.

Interestingly, in the yeast-two-hybrid assay, the R499D mutant has little effect by itself (Figure 4.10A, third row). This clearly proves that the disruption of the dimer interface in the catalytic unit is not sufficient to stop dimerisation in the full-length molecule. Although we do not have the full length peptide array that covers this site, however, as we see here, disruption of the known catalytic binding site by R/D mutation now allows the Quad mutation to ablate dimerisation (Figure 4.10 fifth row, fifth column; fourth row, sixth column). We see that when PDE4D5 Quad mutant is challenged against itself, there is still strong interaction (Figure 4.10A, fourth row, fifth column). However, when adding R499D to the Quad mutant, it could abolish the dimerization against not only itself (Quad/R499D) but also the Quad mutant itself. This suggests that the PDE4D5 dimerization requires the combination of R499-D463 ion pair and RNN, ETL, LMH, KTE (Quad) sites. Note that shown here PDE4D3 against Quad mutant was a false negative as it was positive on subsequent repeats (data not shown).

Molecular modeling of PDE4D5 3D structure indicated D463 as the counterpart of R499 was also contributing to the charge on the dimerization interface (data not shown). Therefore, mutating D463 to its opposite charged amino acid arginine

was expected to restore interaction lost with the R499D mutant. Indeed, when we tested this hypothesis in yeast-two-hybrid assay, the PDE4D5 D463R mutant alone has no effect on PDE4D5 dimerization when against other mutants (Figure 4.10B third row). And when combining D463R with Quad, it ablates the interaction with Quad and itself (Figure 4.10B fifth column, fourth and fifth row). However, when using D463R Quad mutant against R499D Quad mutant, it rescued the PDE4D5 dimerization by restoring the charge between the ion pair (Figure 4.10B, fifth row, sixth column).

4.2.8 Quad R499D mutant behaves as a monomer in vivo

To confirm that Quad R499D PDE4D5 behaves as a monomer in vivo, we made Flag and vsv tagged PDE4D5 constructs with the mutants that have been tested in the yeast-two-hybrid assay. The mutants were transfected into HEK293 cells for 2 days before lysis with 3T3 lysis buffer. The lysates were then centrifuged to remove the insoluble debris and subjected to Bradford assay in order to quantify the protein concentration. Equal amounts of cell lysates (total protein) were subjected to immunoprecipitation using anti-vsv agarose, and the pull-down pellets were analyzed by Western Blotting.

In the pull-down results, it is clear that Quad (Figure 4.11, lane 4), R499D (Figure 4.11, lane 5, 6) and Quad R499D mutants (Figure 4.11, lane 7) were not enough to disrupt the dimerization with wild type PDE4D5. However, the interaction between Quad R499D and Quad mutants was successfully ablated (Figure 4.11, lane 8). All these results were identical to those we observed in the yeast-two-hybrid assay. Therefore, the Quad R499D mutant is a monomeric PDE4D5. It is noticeable that Flag tagged Quad R499D mutant (Figure 4.11, lane 7, 8) is expressed less well than wild type Flag tagged PDE4D5 and its mutants, therefore, we use Flag-QUAD R499D against vsv-4D5 (Figure 4.11, lane 7) as standard when analyzing the effect of Flag-QUAD R499D against vsv-Quad (Figure 4.11, lane 8). Based on this, we concluded that the Quad R499D mutant forms a monomeric PDE4D5. A possible explanation for the low expression of Quad R499D mutant in HEK293 may be that PDE4D5 dimerization may serve important roles in its synthesis, and disruption of dimerization may lead to decreased PDE4D5 expression. However, we cannot exclude the possibility that the Quad R499D mutant itself is a low expressing construct.

4.2.9 Dissecting out the effects of the various portions of the Quad mutant

As explained above, the Quad mutant contains four triplets, in total 12 amino acids that contributed in PDE4D5 dimerization. To analyze the different contribution of each triplet to dimerization, we made constructs with all the possible combination of the triplets as indicated (Figure 4.12). Then, we compared their effects on PDE4D5 dimerization of Quad R499D and Quad mutants. The RNN triplet was proved to be the key element in the binding to Quad R499D, and no such effect was detected in other triplets (Figure 4.12, bottom row, third column).

4.2.10 Comparing the effect of the RNN-R499D mutant to that of the Quad-R499D mutant

RNN mutant has been shown to represent the Quad effect when against Quad R499D. We therefore tested if RNN may act in the same way as the Quad mutant.

In this yeast-two-hybrid assay, when using Quad R499D as a Gal4 activation domain and RNN as a LexA DNA-binding domain like the Quad mutant in Figure 4.12, the RNN mutant works as well as the Quad mutant (Figure 4.13, third column). However, when using RNN R499D as Gal4 activation domain and comparing it to the full Quad R499D mutant, there is a difference between RNN and Quad when using these as LexA DNA-binding domain. The major difference is the single patch with LexA-Quad-R499D vs. Gal4 AD RNN-R499D: LexA-Quad-R499D does not interact with Gal4 AD Quad-R499D (Figure 4.13, third column, third row), while LexA-Quad-R499D does interact with Gal4 AD RNN-R499D (Figure 4.13, fourth column, third row). This discrepancy may reflect the steric affects of the large fusions on dimerization. The reason for this explanation is that the LexA fusion has to simultaneously bind to DNA as well as its Gal4 AD partner in order to activate beta-galactosidase. Therefore, the LexA fusion may reflect a “global” conformational change in the dimer, which are mutation dependent. This emphasizes that one needs to be very careful when choosing which observation may reflect the nature of the PDE4D5 dimerization. Therefore, expressing these mutants in mammalian cells for further confirming their interaction is absolutely essential.

4.2.11 *Testing the RNN R499D mutant in HEK293 cells*

The RNN R499D PDE4D5 mutant was first considered as a good representative for Quad R499D mutant to be a monomeric PDE4D5 with fewer mutations (Figure 4.12). However, later evidence suggested that it may still dimerize in some way with itself (Figure 4.13 fourth column, bottom). To evaluate its dimerization ability, we created Flag tagged PDE4D5 with RNN mutant and vsv tagged PDE4D5 with RNN R499D mutant, and coexpressed them in HEK293 cells. The cells were collected and analyzed using methods identical to that used in Figure 4.11. Flag tagged RNN-R499D-PDE4D5 may be a better substitution for Flag-RNN mutant, however, due to the low expression of the RNN R499D mutant, it is hard to coexpress both vsv and Flag tagged RNN R499D mutant together in order to get a detectable signal for both of them. Therefore, we chose RNN mutant vs RNN R499D mutant to see if they dimerize in the way that was observed in yeast-two-hybrid assay (Figure 4.13).

The immunoprecipitation result showed that the dimerization between vsv-RNN R499D and Flag-RNN mutants was decreased by around 20% compared to wild vsv and Flag PDE4D5 dimerization (Figure 4.14). This is consistent with results that we found in yeast-two-hybrid, but the association is quite strong compared to that in mammalian cells (Figure 4.11). Therefore, although the RNN R499D mutant has fewer mutations, which means it may have less possible effects on PDE4D5 conformation, compared to Quad R499D, the Quad R499D may be a better choice for studying the nature of monomeric PDE4D5.

4.2.12 *Preliminary data of different agonist effects on PDE4D5 dimerization*

Previous reports indicate disruption of dimerization ablated the activation of PDE4D3 by either protein kinase A phosphorylation or phosphatidic acid binding (Richter, 2004). Additionally, it was claimed that disruption of dimerization reduces the sensitivity of the PDE long isoform to PDE4 inhibitor Rolipram. Therefore, dimerization of PDE4 has been suggested to be an important feature involved in PDE4 regulatory function.

Concerning the importance of PDE long isoform dimerization, an understanding of its regulation may also contribute to our appreciation of the properties of PDE4. For such reasons, we tested a series of agonists which are known to interact with PDE4D5.

We first tested the effects of the PDE4 inhibitors, rolipram and ariflo on the dimerization of PDE4D5. Rolipram and Ariflo (cilomilast, SB 207499) are PDE4 specific inhibitors. According to rolipram binding affinity, there are two conformers of PDE4. One of the conformers termed “high affinity rolipram binding” PDE4 or HARB, can be inhibited by nanomolar concentrations of rolipram. The other conformer termed “low affinity rolipram binding” PDE4 or LARB, requires 10-100 fold higher concentrations of rolipram to inhibit it (Griswold, Webb et al. 1998). HARBs were shown to predominate in the central nervous system (CNS) (Saccomano, Vinick et al. 1991), which is considered to be the main cause for side effects of rolipram, while LARBs usually exist in inflammatory cells (Souness, Griffin et al. 1996). Rolipram has a high binding affinity to HARBs. However, ariflo prefers to interact with LARBs (Christensen, Guider et al. 1998; Griswold, Webb et al. 1998).

HEK293 cells expressing vsv and Flag tagged PDE4D5 were seeded into 10cm culture plate. Rolipram and Ariflo were dissolved in pure DMSO and added to cell culture medium to a final concentration of 10 μ M. The cells were then incubated at 37°C for 10 minutes. After 2 washes with pre-chilled PBS to cease the reaction, the cells were lysed in 3T3 lysis buffer. The lysates were then subjected to immunoprecipitation using anti-vsv agarose. In the IP experiment, there were no detectable differences on PDE4D5 dimerization between rolipram and ariflo treatment (Figure 4.15A). Thus, PDE4D5 dimerization is not affected by PDE4 inhibitors able to distinguish between the HARBs and LARBs conformers (n=2).

We then tested the influence of mitogen-activated protein kinase ERK on PDE4D5 dimerization by activating this pathway using the PKC activator, PMA (Phorbol-12-myristate-13-acetate). PMA was applied to the transfected HEK293 cells at a final concentration of 10 μ M. It is shown that PMA has no effect on PDE4D5 dimerization. This is consistent with our yeast two hybrid assay that a mutation

that mimics erk phosphorylation of PDE4D5, namely the S54D mutation (Figure 4.7A) has little effect on PDE4D5 dimerization (Figure 4.16).

The p38 MAPK activator anisomycin was shown to increase PDE4D5 dimerization by 2 fold (n=2) (Figure 4.15A, B). One explanation is that anisomycin may either triggers the phosphorylation of PDE4D5 by p38MAPK or a downstream kinase or interacts directly with PDE4D5, thereby changing the conformation of PDE4D5 and increasing its dimerization. Further exploration is needed to fully understand this phenomenon.

HEK293 cells expressing Flag and vsv tagged PDE4D5 were also set up to test the influence of PKA phosphorylation on PDE4D5 dimerization. The PDE inhibitor IBMX and adenylyl cyclase activator forskolin were added to prepared cells at a final concentration of 10 μ M to ensure elevated cAMP levels (McCahill, McSorley et al. 2005). Activated PKA leads to the activation of PDE4D5 by phosphorylation at Ser126 (Baillie, MacKenzie et al. 2001). The IBMX + forskolin treatment caused a 60% reduction in PDE4D5 dimerization (Figure.15 A, C). When pretreating the cells with the PKA inhibitor KT5720, it partially rescued this decreased dimerization. This indicated that PKA phosphorylation of PDE4D5 could reduce its dimerization, and inhibiting PKA activity could partially rescue it. Further work is needed to be done to clarify this using other inhibitors and mutants.

4.3 Discussion

Cyclic nucleotide phosphodiesterases provide the only way to degrade the ubiquitous second messenger cAMP/cGMP and have been a popular research subject for decades. Their functions are now far extended from their first identified desensitization ability for G-protein coupled receptors that increase cAMP to playing fundamental roles in cross-talk between different signaling pathways (Hoffmann, Baillie et al. 1999) {O'Connell, 1996 #22}and regulating cAMP compartmentalization (Dodge, Khouangsathiene et al. 2001) (Lynch, Baillie et al. 2007). It has been pointed out that most PDEs, at least members of PDE families 1-6, can exist as dimers (Richter and Conti 2004). For PDE4 subfamilies, the long PDE isoforms have been shown to undergo dimerization via interactions involving their conserved UCR1 and UCR2 domains. One of the PDE4 long

isoforms, PDE4D3 was investigated intensively by Marco Conti's group (Richter and Conti 2002) (Richter and Conti 2004). They suggested that disruption of PDE4D3 dimerization can be achieved through its activation by either protein kinase A phosphorylation or by phosphatidic acid binding and that this led to reduced sensitivity to inhibition by rolipram (Richter and Conti 2004). They also demonstrated that ablating the UCR1 and UCR2 intramolecular interaction did not interfere with dimerization (Richter and Conti 2002).

PDE4D5 is another long PDE4 isoform that shares conserved UCR1&2 and catalytic domains with PDE4D3, but they are not redundant in their functions. PDE4D5 is unique for its function in binding to the scaffold protein, β arrestin which allows it to be recruited to the β 2 adrenoceptor after agonist challenge and generating a compartmentalized cAMP pool adjacent to these receptors (Lynch, Baillie et al. 2005; Lynch, Baillie et al. 2007). Due to the high similarity between PDE4D3 and PDE4D5, it seems likely that PDE4D5 is also dimeric. In order to better understand the function and regulation of PDE4D5, I set out to explore the nature of PDE4D5 dimerization.

Yeast two hybrid and immunoprecipitation results showed that PDE4D5 can dimerize both *in vitro* and *in vivo*. In yeast two hybrid studies, adding PDE4D5 interacting protein RACK1 or β arrestin could successfully disrupt PDE4D5 dimer formation; however, we did not see such an effect in immunoprecipitation studies using HEK293 cells. This is strange as we have shown (Lynch, Baillie et al. 2005) that PDE4D5 cannot bind both β arrestin and RACK1 at the same time. As these scaffolds bind to overlapping interaction sites on PDE4D5 and so one molecule of PDE4D5 can't bind both β arrestin and RACK1, we surmised from this observation and the 2-hybrid result that these scaffolds disrupted dimers to give monomers complexed with rack1 or β arrestin. There are various possible explanations for this observation. One may be that in the yeast two hybrid assay the threshold for a signal to be detected between the prey and bait protein is different from the immunoprecipitation studies such that RACK1 or β arrestin can ablate PDE4D5 dimerization in yeast two hybrid but not in immunoprecipitation. It may also be that in mammalian cells there may be some other modification or partner protein that stabilises the dimers even when these scaffolds interact. However, if such dimers do occur natively then a PDE4D5 dimers must only be able to bind RACK1 or β arrestin as mixtures are never observed. This also,

however, suggests that if β arrestin is bound to one molecule of the dimer its binding must sterically block any binding of RACK1 to the other PDE4D5 molecule in the dimer. I also observed that overexpressing PDE4D5 alone or with RACK1 and β arrestin together significantly increases the amount of PDE4D5. This causes an increased ratio of PDE4D5 against RACK1 and β arrestin, which makes RACK1 and β arrestin less potent to disrupt PDE4D5 dimerization. We also found PDE4D5 dimerization is of high stability, and its disassociation and reassociation is not affected by separately expressed RACK1 and β arrestin. This may mean that PDE4D5 immediately forms dimers upon its synthesis at the plasma membrane.

It has been reported (Richter and Conti 2004) that the dimerization of PDE4D3 could be ablated with mutation of hydrophobic residues V100/F104 on UCR1 (V172/F176 in PDE4D5) and L145/L148/L152/L155 on UCR2 (L217/L220/L224/L227 in PDE4D5) (Richter and Conti 2004). In this study, we employed a novel technology peptide array in order to locate the PDE4D5 dimerization sites. We found four potential dimerization sites, which are R173/N174/N175 in UCR1, E228/T229/L230 in UCR2, L306/M307/H308 and K323/T324/E325 in the catalytic domain. The R173/N174/N175 is adjacent to V172/F176 in UCR1 and E228/T229/L230 is adjacent to L217/L220/L224/L227, which locate within the same region of previously discovered PDE4D3 dimerization sites. However, the constructs with mutated RNN and ETL had little effect on PDE4D5 dimerization, neither did the Quad mutant construct that has all four potential dimerization sites mutated to alanine. As in a previous report indicated that mutation of an ion pair of the catalytic domain can disrupt the oligomerization of the isolated PDE4 catalytic unit, we mutated residues of this ion pair in PDE4D5, in addition to the Quad mutant and found this successfully abolished PDE4D5 dimerization. According to this, we generated the monomeric PDE4D5 Quad R499D mutant. We then investigated their individual contribution to the Quad mutant. The RNN R99D was found to be the replicate for the Quad R499D mutant in yeast two hybrid. However, when we tested the its dimerization in mammalian cells, it worked less well than Quad R499D as a monomeric mutant.

I also obtained some preliminary data testing the ability of PDE4D5 to dimerize in response to several treatments. Rolipram, which binds preferentially to HARB conformers and Ariflo, which binds similarly to HARBS and LARB conformers

exhibited little effect on PDE4D5 dimerization. However, we do not know if the dimerized PDE4D5 exist in their HARBs or LARBs or both, and this may be worth further investigation.

I also showed that anisomycin can increase the PDE4D5 dimerization and this may be due to the activation of its downstream effectors in activating the p38 MAPK pathway. Recent (unpublished) work from our laboratory suggests that this pathway can trigger the phosphorylation of PDE4 isoforms and so making phosphomimetic mutants would be useful to test. Also one could evaluate a selective inhibitor of p38 MAPK to check whether it was exerting its action on PDE4D5 through this pathway or some other.

IBMX/Forskolin treatment leads to a reduction of PDE4D5 dimerization. This might due to PKA phosphorylation on PDE4D5 and further experiments are needed to confirm it. Note in this regard that phosphomimetic mutants for PDE4D5 phosphorylation by PKA appeared to have no effect in 2-hybrid studies (Fig.4.7A). This suggests that if they do occur in mammalian cells they must exert an action through a route that does not just involve PDE4D5 alone. It could be that accessory proteins stabilized PDE4D5 dimers in intact cells, protecting against disruption by Rack1/barrestin. The interaction of these may be disrupted by PKA phosphorylation. It would be interesting then to see if either RACK1/barrestin augmented the disruption of PDE4D5 dimers by Fsk/IBMX.

In our yeast two hybrid experiment, we found that PDE4D5 could form hetero-dimers with PDE4D3. However, its function and regulation remain obscure. We also generated the monomeric PDE4D5, the Quad R499D mutant. Its enzyme activity, compartmental distribution, binding affinity to PDE4D5 interacting proteins and sensitivity to PDE inhibitors are required for further study.

In summary, we find that PDE4D5 can form strong dimerisation with itself in both yeast and mammalian cell lines. The dimerisation requires multiple interaction sites such as R173/N174/N175, E228/T229/L230, L306/M307/H308 and K323/T324/E325 (together named QUAD) in which R173/N174/N175 contributes most in the dimerisation. Association of an ion-pair R499/D463 also proved to be a necessary condition for dimer formation. The PDE4D5 dimerisation can be affected by challenge of the PDE inhibitor anisomycin and cAMP elevating

agonists Fsk/IBMX. However, the mechanism for such phenomena need further investigation.



Figure 4.1: PDE4D5 can interact with itself and with other PDE4 isoforms.

For all the patches, the bait was *LexA*-PDE4D5 or empty vector (*columns*). This was tested against various prey, all expressed as *GAL4* fusions (*rows*). Pink patches are negative and blue patches are positive. (Data provided by Graeme B. Bolger)

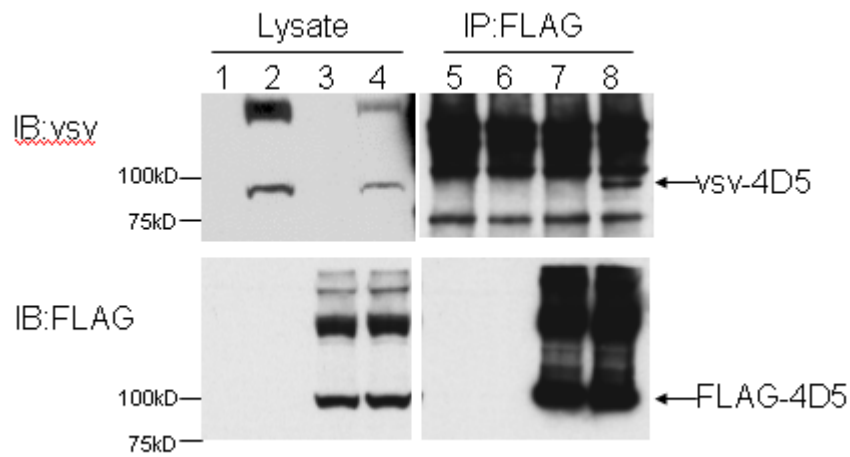


Figure 4.2: Confirmation of PDE4D5 dimerization in vivo by immunoprecipitation.

Vsv-4D5 and FLAG-4D5 were co-expressed in HEK293 cells. Cells were lysed in 3T3 lysis buffer and centrifuged for 5 minutes at 13'000 for preclearing. Equal amount of total proteins were subjected to immunoprecipitation with anti-vsv agarose. Pulled down pellets were then subjected to SDS-PAGE and immunoblotted for vsv and FLAG. 1-4 are precleared cell lysates, 5-8 are IP using anti-FLAG agarose. 1 and 5, HEK cells with polyfect alone as mock control. 2 and 6, transfected with vsv-PDE4D5. 3 and 7, with FLAG-PDE4D5. 4 and 8, with both vsv and FLAG-PDE4D5. (Shown are representative immunoblots from three independent experiments.)

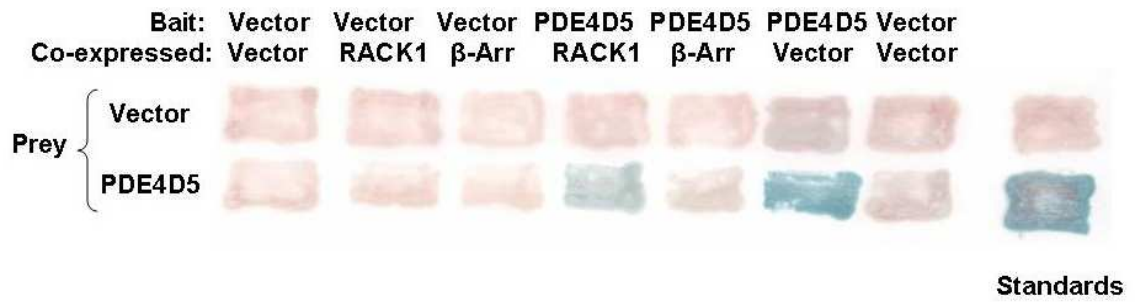


Figure 4.3: Co-expression of either RACK1 or β -arrestin2 significantly attenuates dimerization of PDE4D isoforms.

RACK1 or β -arrestin2 were co-expressed along with the LexA-PDE4D5 and GAL4-PDE4D5 fusions used in Figure 4.1. (Data provided by Graeme B. Bolger)

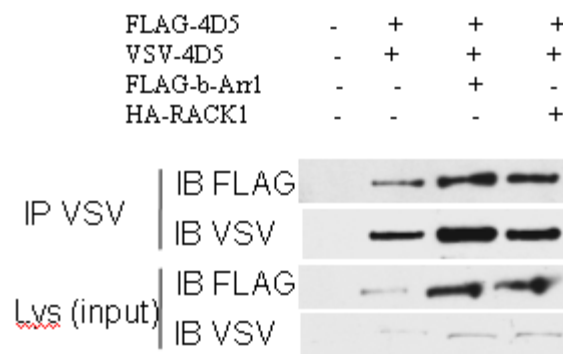


Figure 4.4. PDE4D5 binding partner RACK1& β -arresitn effects on PDE4D5 dimerization *in vivo*.

HEK293 were cotransfected with vsv-4D5, FLAG-4D5 and FLAG- β -arrestin or HA-RACK1. Cell lysates were then centrifuged at 13,000 rpm for 5 minutes to remove debris. Equal amounts of protein were subjected to anti-vsv immunoprecipitation and the differently tagged PDE4D5 were detected by blotting with their corresponding antibody. (Shown are representative immunoblots from two independent experiments.)

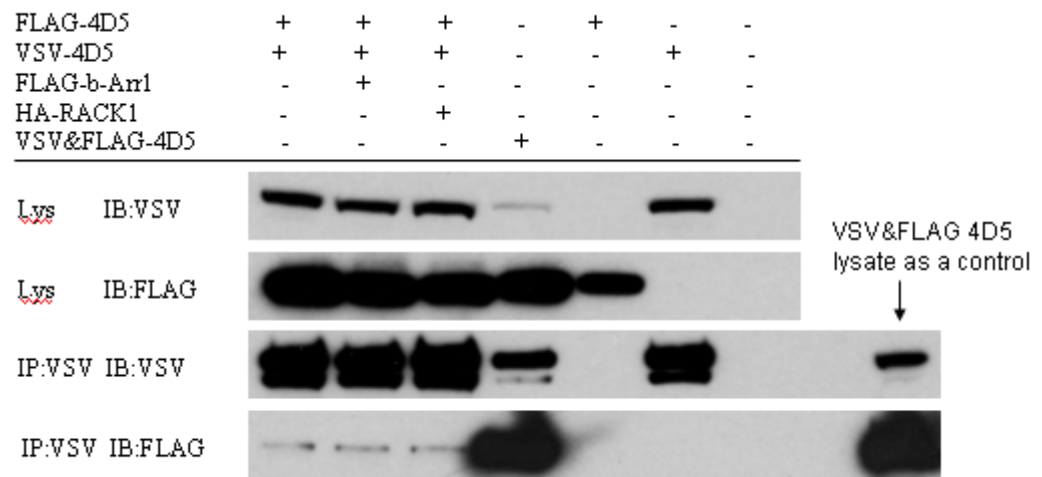


Figure 4.5: PDE4D5 binding partner RACK1& β -arresitn effects on PDE4D5 re-dimerization *in vitro*.

HEK293 cells over-expressing FLAG-4D5, VSV-4D5, FLAG- β -arr1, HA-RACK1 or FLAG&VSV-4D5 were lysed in 3T3 lysis buffer, precleared and measured as described in Figure 4.2. Equal amounts of protein were mixed as indicated, rotated at 4 °C for 20hrs. The lysates were then subjected to immunoprecipitation with anti-vsv and blotted for vsv and FLAG-PDE4D5. (Shown are representative immunoblots from three independent experiments.)

GST-PDE4D5 on PDE4D5 peptide array

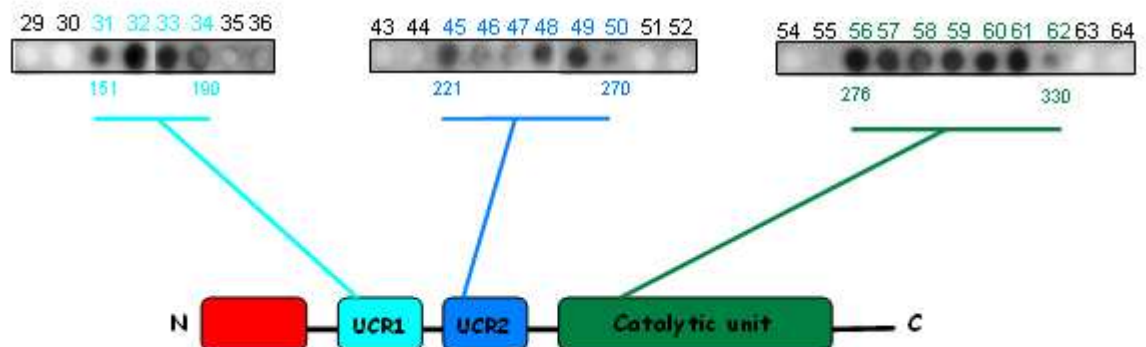


Figure 4.6A: Identification of putative dimerization sites by peptide array.

Synthesized peptide spots containing 25 -mer peptides were immobilized on nitrocellulose. Each of the peptide is shifted by 5 amino acids, covering the entire sequence of PDE4D5. The nitrocellulose was prepared using the method in Fig 3.1. GST-PDE4D5 interaction with PDE4D5 peptide array was probed using anti-GST antibody. Spot 31-34, 45-50 and 56-62 showed positive interactions with GST- β arrestin1. (Shown are representative immunoblots from two independent experiments. Data provided by George Baillie.)

PFSAS1 Alanine Scanning (R173/N174/N175)

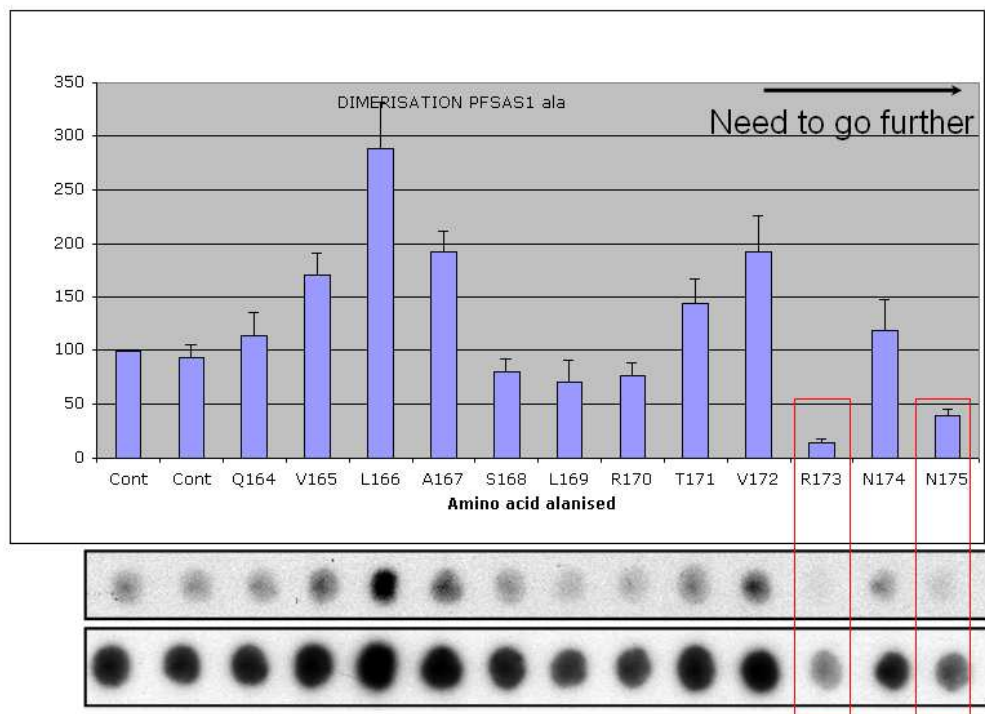


Figure 4.6B: Identification of RNN dimerization site by ALA scan array .

Sequence Q164-N175 of PDE4D5 was subjected to alanine scan analysis. Sequence Q164-N175 and its mutant with alanine substitutions were generated as indicated. GST-PDE4D5 interaction with sequence Q164-N175 was probed using anti-GST antibody. (Shown are representative immunoblots from two independent experiments. Data provided by George Baillie.)

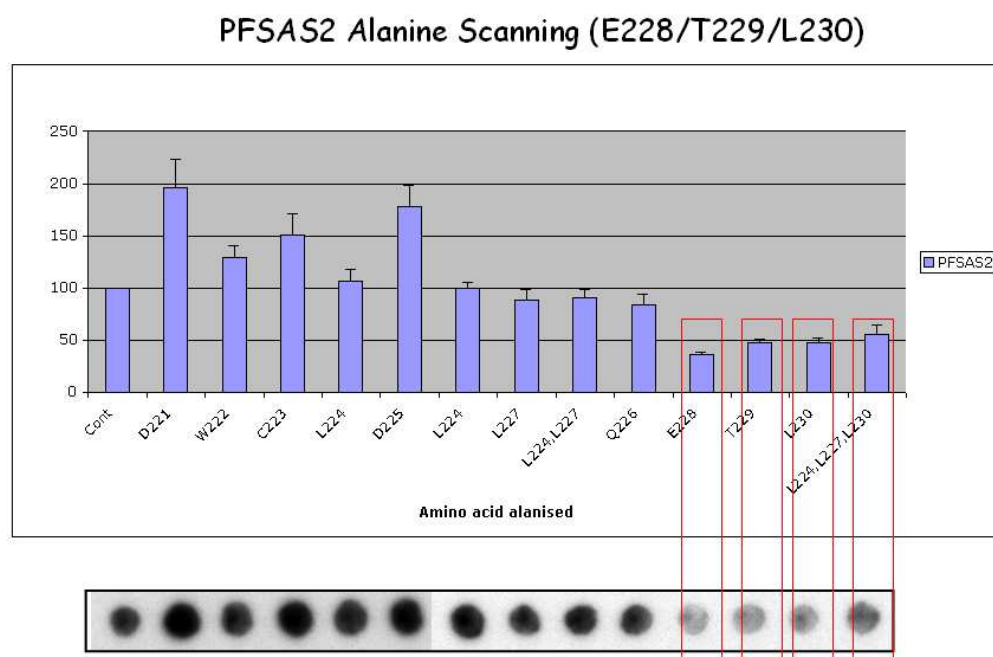


Figure 4.6C: Identification of ETL dimerization site by ALA scan array.

Sequence D221~L230 of PDE4D5 was subjected to alanine scan analysis. Sequence D221~L230 and its mutant with alanine substitutions were generated as indicated. GST-PDE4D5 interaction with sequence D221~L230 was probed using anti-GST antibody. (Shown are representative immunoblots from two independent experiments. Data provided by George Baillie.)

PFSAS3 Alanine Scanning (L306/M307/H308)

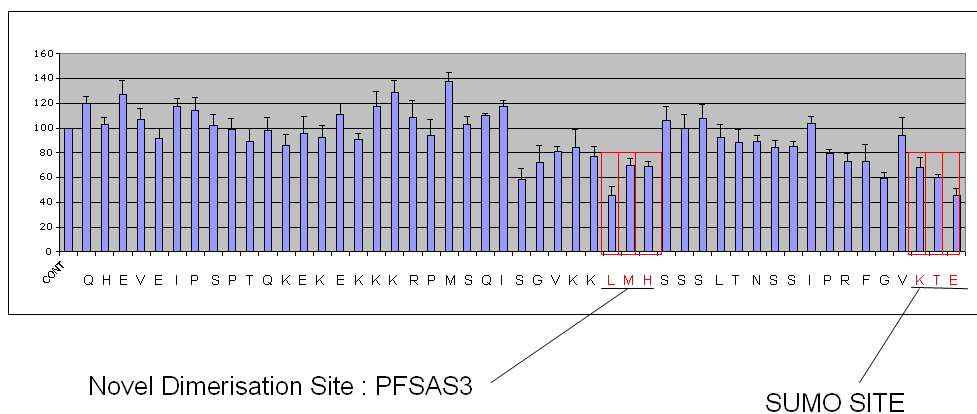


Figure 4.6D: Identification of LMH and KTE dimerization sites by ALA scan array.

Sequence Q277~E325 of PDE4D5 was subjected to alanine scan analysis. Sequence Q277~E325 and its mutant with alanine substitutions were generated as indicated. (Data provided by George Baillie)

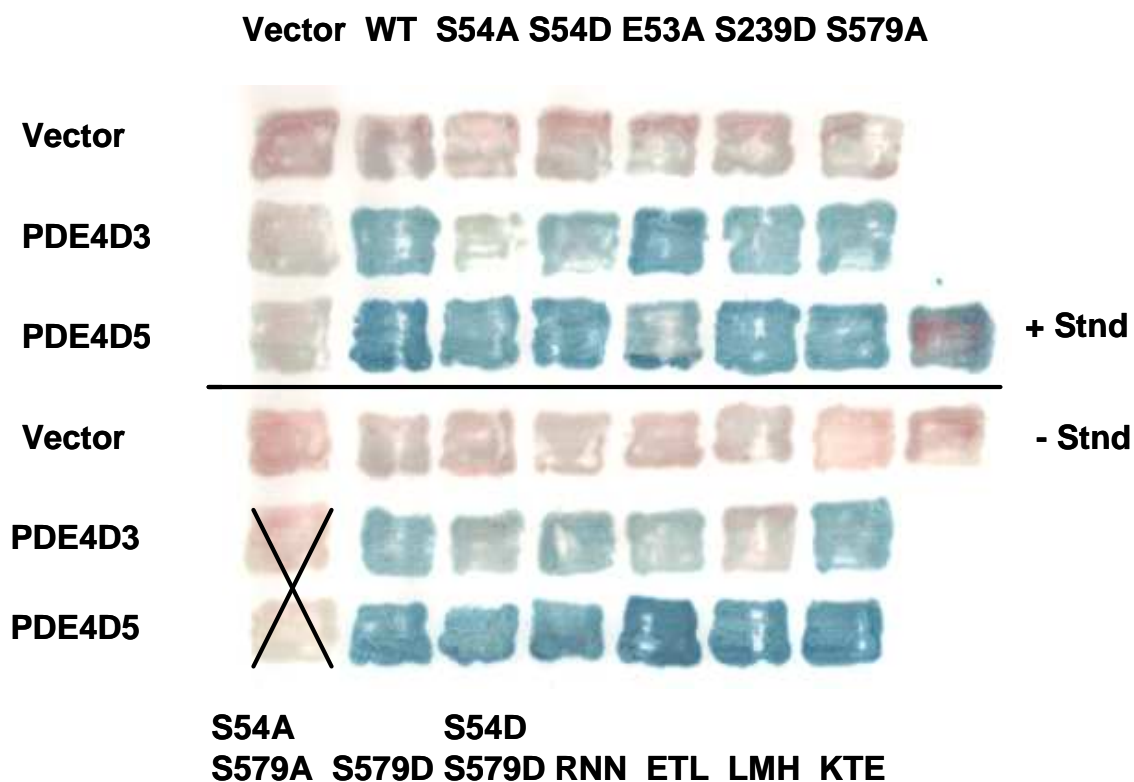


Figure 4.7A: Initial evaluation of the phosphorylation site and RNN, ETL, LMH and KTE mutants

Constructs expressing *LexA*-PDE4D5 with some of the phosphorylation site mutants, as well as RNN, ETL, LMH and KTE (columns) are used as bait. Empty vector, *GAL4*-PDE4D3 and *GAL4*-PDE4D5 (rows) are used as prey. The assay used methods identical to those used in Figure 4.1. (Data provided by Graeme B. Bolger)

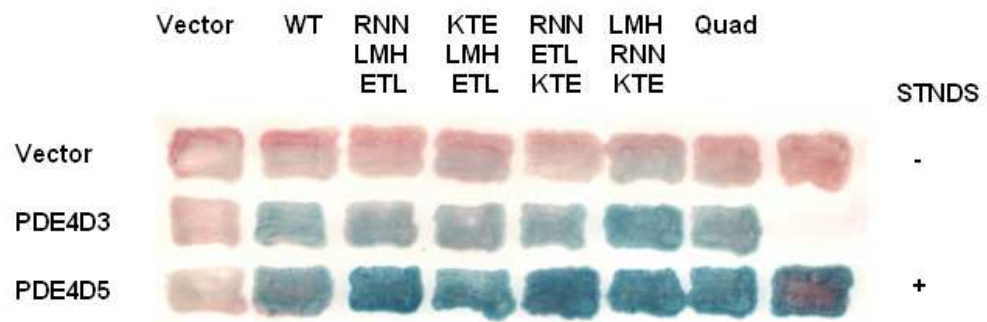


Figure 4.7B: Evaluation of combinations of triplets and the Quad mutant. Constructs expressing *LexA-PDE4D5* with RNN, ETL, LMH and KTE, either in triplets or all together (i.e., the Quad mutant) are used as baits and empty vector, *GAL4-PDE4D3* and *GAL4-PDE4D5* (rows) are used as preys. The assay used methods identical to those used in Figure 4.1. (Date provided by Graeme B. Bolger, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294-3300, U.S.A.)

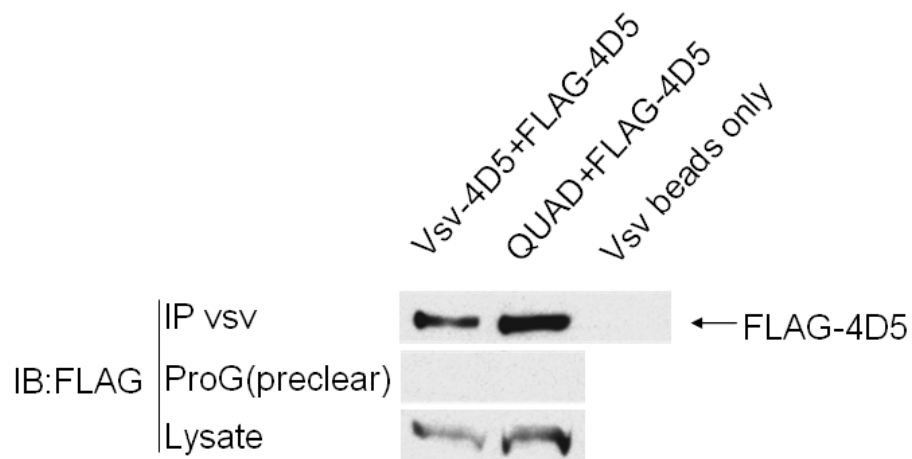


Figure 4.8: Investigate PDE4D5 QUAD mutant and its ability of dimerization.

HEK293 cells were transfected with different constructs as indicated: FLAG-4D5 plus VSV-4D5 (first column), FLAG-4D5 plus vsv tagged Quad mutant (second column). After 48 hours culture at 37°C, cells were lysed in 3T3 lysis buffer, precleared and measured as described in Figure 4.2. Equal amounts of protein were subjected to immunoprecipitation of vsv-PDE4D5 and blotted for vsv and FLAG-PDE4D5 using anti-vsv and anti-Flag antibody. (Shown are representative immunoblots from three independent experiments.)

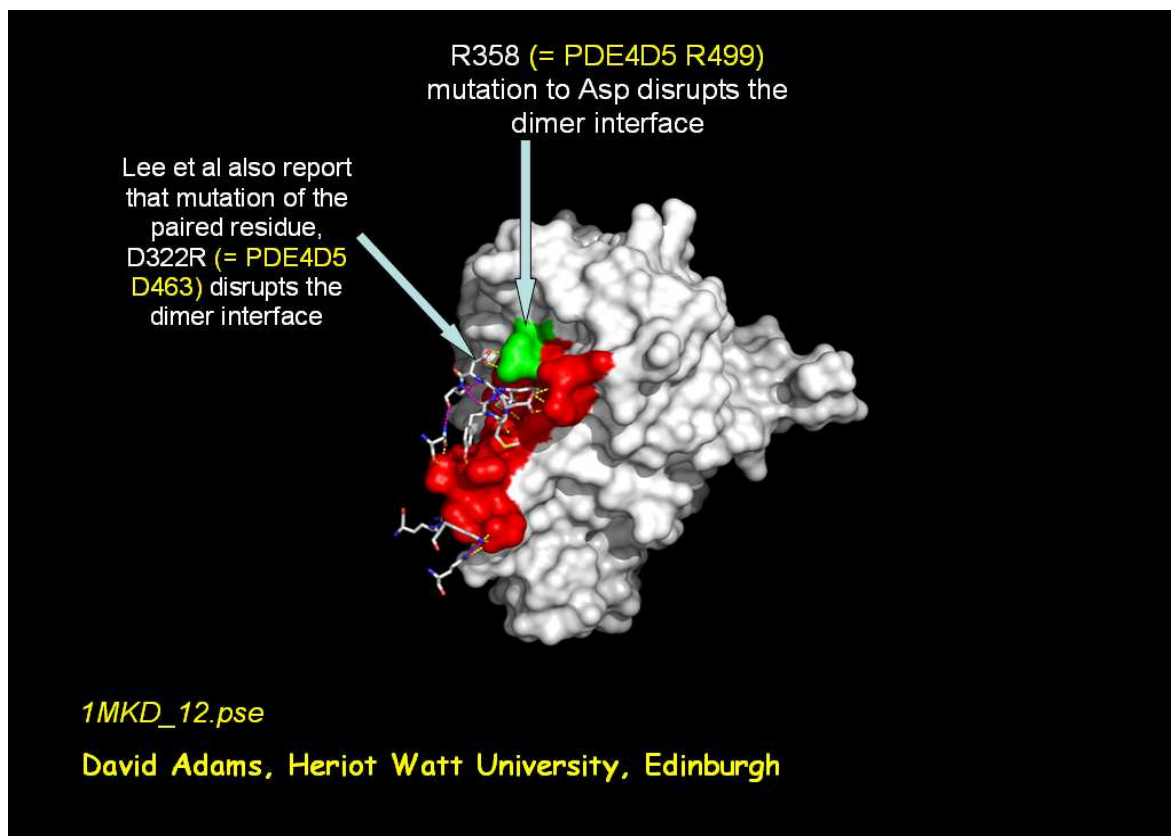


Figure 4.9: PFE4D5 3D structure

This shows a single ion-pair R499 with D463 that may play key roles in PDE4D5 dimerization. (Data provided by David Adams, Heriot Watt University, Edinburgh, U.K.)

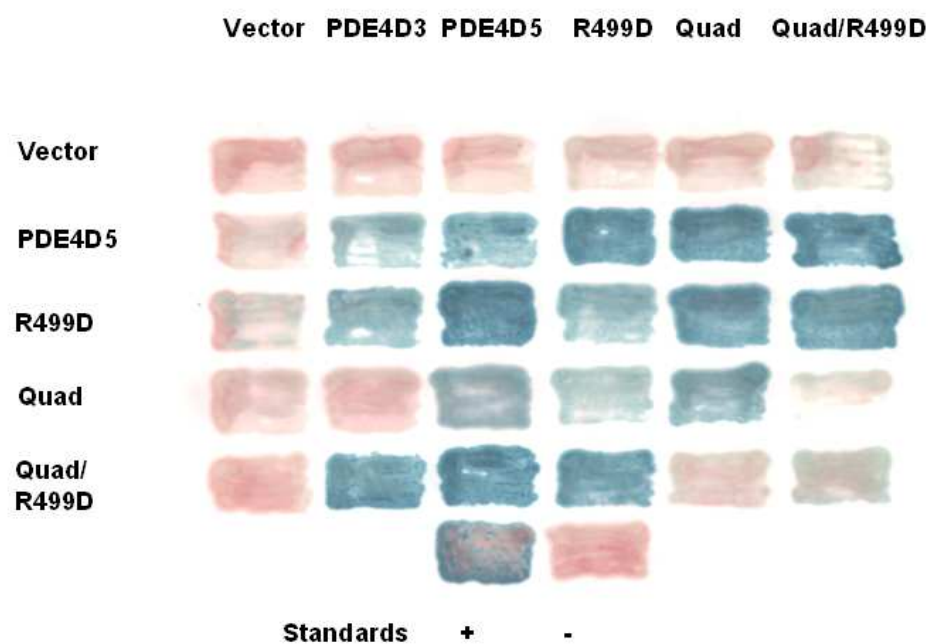


Figure 4.10A: Evaluating the R499D mutant alone, or as part of the Quad set. Constructs expressing PDE4D5 with the Quad mutant plus R499D, or with R499D alone were made, and were tested in two-hybrid assays, using methods identical to those used in Figure 4.1. (Date provided by Graeme B. Bolger)

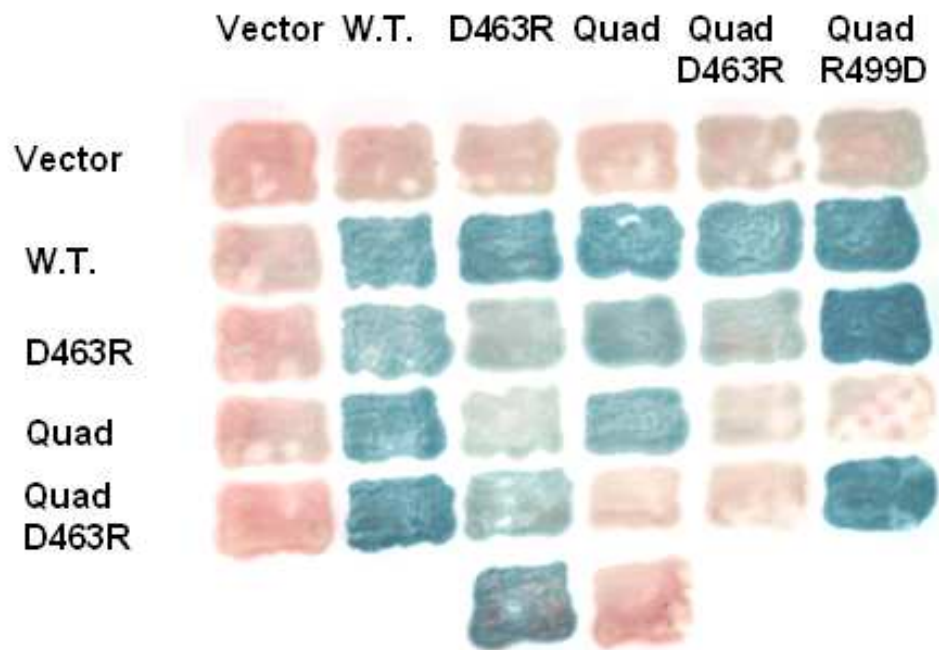


Figure 4.10B: Testing the D463R mutant for its effect on dimerization.

We made constructs expressing PDE4D5 with the D463R mutant, or the Quad mutant plus D463R, and tested them in two-hybrid assays, using methods identical to those used in Figure 4.1. (Date provided by Graeme B. Bolger)

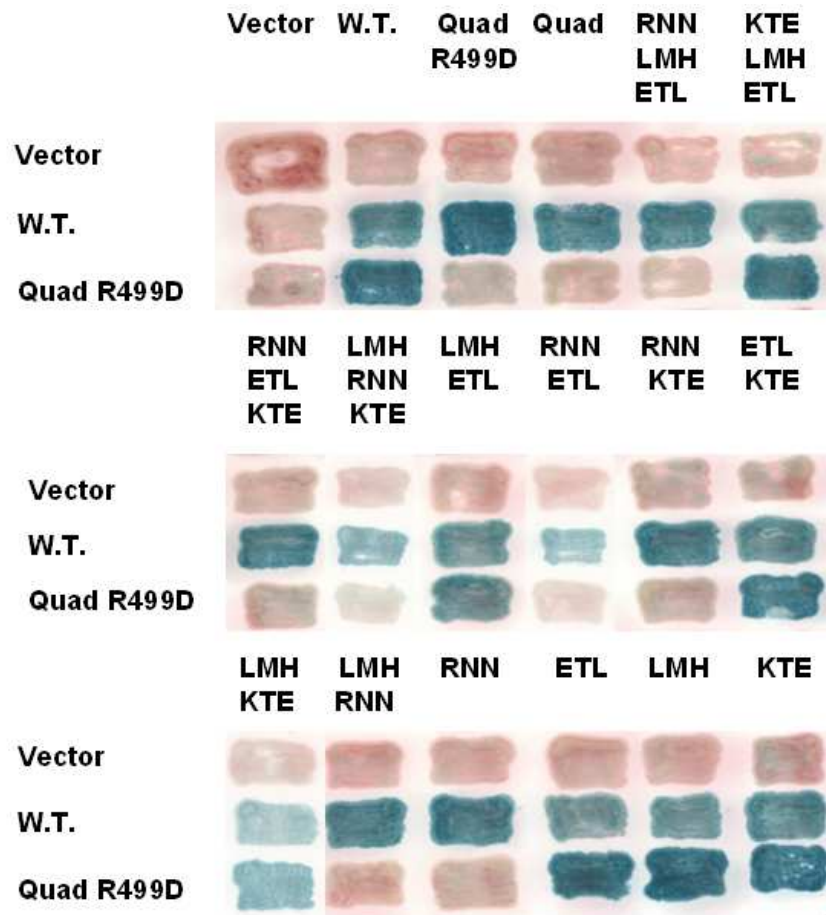


Figure 4.12: Dissecting out the effects of the various portions of the Quad mutant.

The Quad mutant contains four triplets in all. To determine the contribution of each triplet to the overall effect of the Quad mutant, we generated constructs expressing PDE4D5 with each of the triplets, as well as doublets and triplets, and compared their effects to that of the Quad mutant. The mutants were tested in two-hybrid assays, using methods identical to those used in Figure 4.1. GAL4-empty vector, wild type PDE4D5 and Quad R499D mutants are used as prey (rows). Different combinations of the triplets are all LexA fusion and used as bait (columns). (Date provided by Graeme B. Bolger)

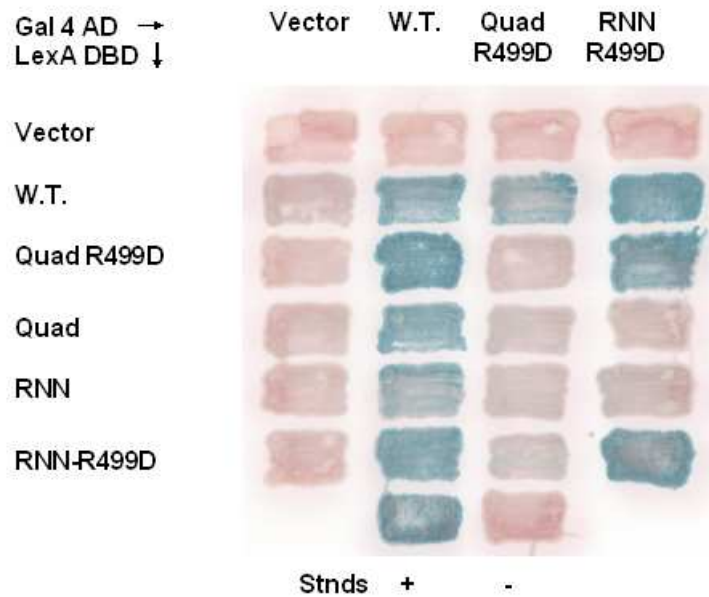


Figure 4.13: Comparing the effect of the RNN-R499D mutant to that of the Quad-R499D mutant.

Since effects of the RNN mutant seem to replicate the effects of the entire Quad set (Figure 4.9, above), we compared the effect of RNN-R499D mutant to that of Quad-R499D. The mutants were tested in two-hybrid assays, using methods identical to those used in Figure 4.1. (Date provided by Graeme B. Bolger)

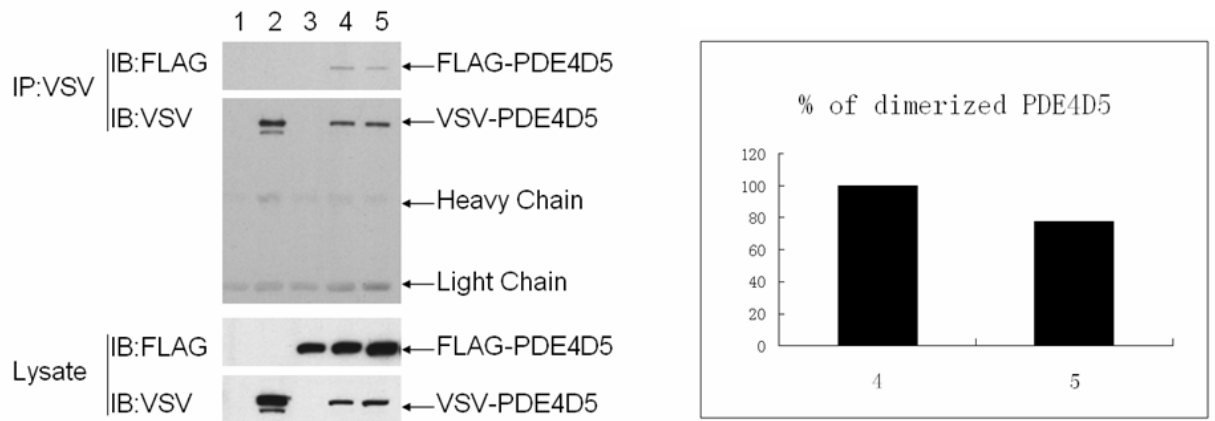


Figure 4.14. Testing the RNN R499D mutant on dimerization *in vivo*.

HKE293 cells expressing different constructs as indicated: (1) Wt HEK293 (2) vsv-4D5 (3) FLAG-4D5 (4) vsv+FLAG-4D5 (5) vsv-RNN R499D+FLAG-RNN were lysed in 3T3 lysis buffer, precleared and measured as described in Figure 4.2. Equal amounts of total protein were subjected to SDS-PAGE and subsequently blotted for each tagged protein. According to this, cell lysates then diluted to contain equal amount of vsv tagged and Flag tagged protein before immunoprecipitate for vsv-PDE4D5. The vsv and Flag tagged PDE4D5 and its mutants are detected by Western blotting using anti-vsv and anti-flag antibody. (This experiment only carried once.)

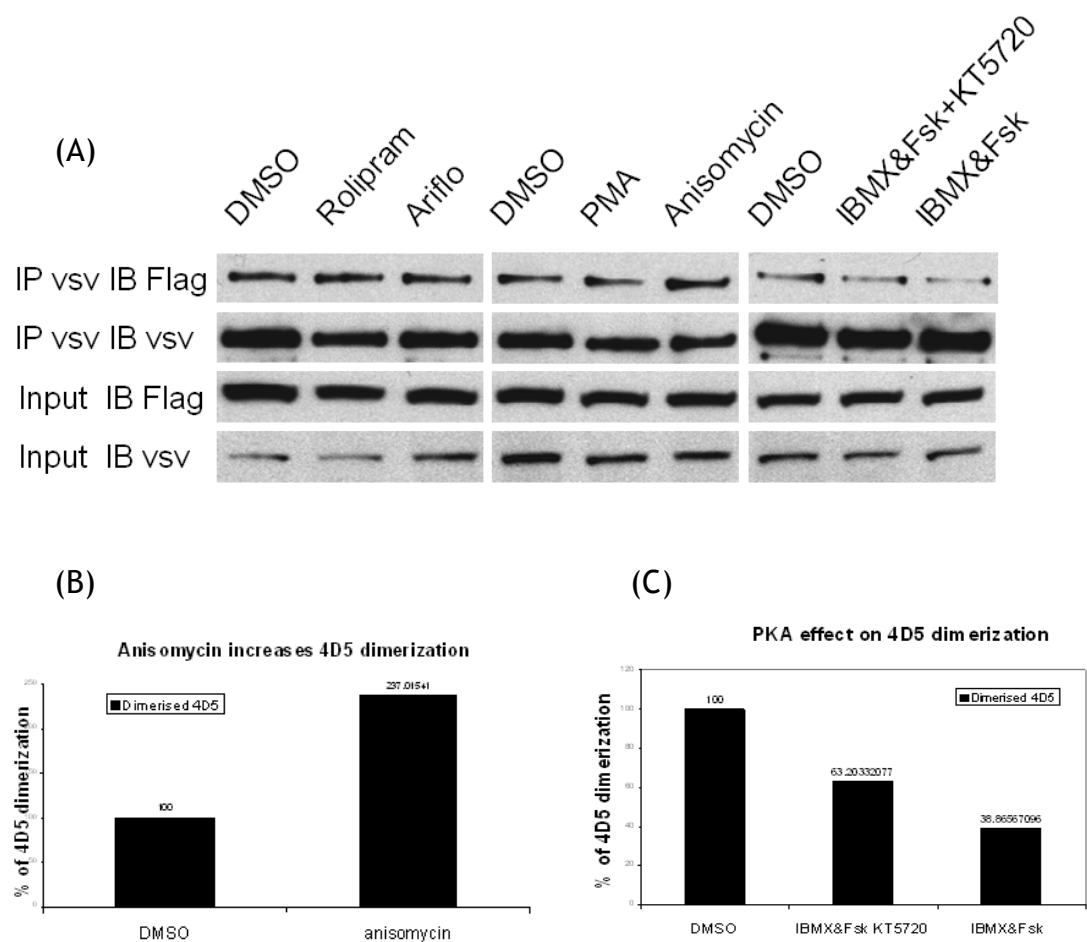


Figure 4.15: Different drug effects on 4D5 dimerization.

HEK293 cells expressing vsv and Flag tagged PDE4D5 were seeded onto 10cm culture plates. All the compounds were dissolved in pure DMSO and added to cell culture medium to a final concentration of 10 μ M. The cells were then incubated at 37°C for 10 minutes. For IBMX/Forskolin and KT5720 treated cells, KT5720 was added to cells for 10 minutes before adding IBMX/Forskolin. After 2 washes with pre-chilled PBS to cease the reaction, the cells were lysed in 3T3 lysis buffer. (A) The lysates were then subjected to immunoprecipitation using anti-vsv agarose and blotted with vsv and flag antibody. Western blot results were scanned in and analyzed with QuantityOne (Biorad). Immunoprecipitated Flag-PDE4D5 (row 1) vs vsv-PDE4D5 (row 2) was plotted in bar chart. The vsv vs Flag ratio of DMSO treated cells was set as 100% dimerization, and comparing this with ratios from other treatments displayed the changes of PDE4D5 dimerization under the corresponding treatments. (B) shows anisomycin effect on PDE4D5 dimerization. (C) shows effect of IBMX/Forskolin (trigger PKA phosphorylation) on dimerization. (Shown are representative immunoblots from two independent experiments.)



Figure 4.16: Co-expression of ERK2 has no major effect on dimerization of PDE4D isoforms.

ERK2 was co-expressed along with the LexA-PDE4D5 and GAL4-PDE4D5 fusions and experiments were carried out identically to those in Figure 4.1. (Date provided by Graeme B. Bolger)

5 Preliminary studies on RACK1

5.1 Introduction

Receptor for activated C-kinase 1 (RACK1) is a 36 -kDa protein consisting of seven internal tryptophan-aspartate 40 (WD40) repeats. The structure of RACK1 is indicated to be a seven-bladed propeller from the WD repeats (Sondek and Siderovski 2001; Steele, McCahill et al. 2001). Each blade of RACK1 is built up by β -sheets as shown in G_{β} crystal structure (Wall, Coleman et al. 1995; Sondek, Bohm et al. 1996). RACK1 and G_{β} subunit share 42% amino acids similarity. RACK1 has also been found to interact with Gbeta-gamma subunit via its WD repeats (Chen, Spiegelberg et al. 2004).

The first function of RACK1 identified was its ability to interact with activated “conventional” protein kinase-C forms (PKC) such as PKC β II (Ron, Luo et al. 1995; Stebbins and Mochly-Rosen 2001) and “novel” PKCs such as PKC ϵ (Besson, Wilson et al. 2002). Conventional PKCs (α , β I, β II, γ) are calcium- and diacylglycerol-dependent protein kinases which are activated upon elevated calcium and diacylglycerol that are generated by the receptor-stimulated hydrolysis of plasma membrane phosphatidylinositol 4, 5-bisphosphate. Novel PKCs are those calcium-independent kinases (δ , ϵ , η , θ , μ) or calcium- and diacylglycerol-independent (atypical) kinases (ζ , λ) (Mellor and Parker 1998).

Another RACK1 interacting protein of particular note is the cAMP-specific phosphodiesterase PDE4D5, which has been shown to contain a RACK1 interacting domain (RAID) in its N-terminal region (Bolger, McCahill et al. 2002). Further investigations revealed that there is another RACK1 interacting site within the PDE4D5 catalytic region (Bolger, Baillie et al. 2006). RACK1 interacts with PDE4D5 via WD repeats 5-7 (Steele, McCahill et al. 2001). The PDE4D5-interacting protein, β -arrestin has overlapping binding sites with RACK1 on PDE4D5.

RACK1 has been reported to mediate and initiate cell migration in many cell types (Buensuceso, Woodside et al. 2001; Cox, Bennin et al. 2003; Kiely, O’Gorman et al. 2006). It works as a scaffolding protein at focal adhesion sites and regulates the formation of focal adhesions (Doan and Huttenlocher 2007;

Onishi, Lin et al. 2007). It has been shown to associate with core kinases of the ERK signalling pathway, Raf, MEK and ERK, and target active ERK to focal adhesions. FAK and paxillin are two focal adhesion proteins whose phosphorylation is modulated by RACK1 (Mamidipudi, Chang et al. 2004; Vomastek, Iwanicki et al. 2007).

Signalling from Insulin-like Growth Factor I (IGF-I) receptor and β 1 integrins are also orchestrated by RACK1 in a way where RACK1 scaffolds the receptors and signalling molecules into a complex (Hermanto, Zong et al. 2002; Lynch, Vodnyanik et al. 2005; Kiely, O'Gorman et al. 2006). The signalling collaboration between IGF-I and β 1 integrin is essential for tumour generation (Goel, Breen et al. 2005).

RACK1 is considered to influence the activity of another member of the MAPK signalling pathway, c-Jun NH₂-terminal kinase (JNK), via its interaction with PKC. JNK activation can be affected by changes in activity or levels of the PKC-RACK1 module. Thereby, JNK activity varies upon the expression level of RACK1 (Lopez-Bergami, Habelhah et al. 2005). Interestingly, enhanced RACK1 expression levels were detected in colon carcinoma (Berns, Humar et al. 2000), melanoma (Lopez-Bergami, Habelhah et al. 2005), non-small-cell lung carcinoma (Berns, Humar et al. 2000), and oral squamous cell carcinoma (Wang, Jiang et al. 2008). The inhibition of RACK1 expression increased the sensitivity of melanoma cells to treatment and reduced their tumorigenicity in a xenograft tumour model. Therefore RACK1 is expected to be important in tumorigenicity (Lopez-Bergami, Habelhah et al. 2005). Understanding RACK1 and its link to JNK and other RACK1 interacting proteins may lead us to new methods in tumour treatment.

Since RACK1 has been shown to relocate the nucleus after ethanol and forskolin treatment (Ron, Vagts et al. 2000; He, Vagts et al. 2002) and SUMOylation may lead to protein nuclear transport, we investigated whether RACK1 is a SUMO substrate and, if so, whether nuclear translocation is related to RACK1 SUMOylation.

Sumoylation is considered to have a nuclear effect as most of SUMO enzymes are enriched in the nucleus, and many researchers focussed on its role in DNA repair, transcription, nuclear bodies and nucleo-cytoplasmic transport (Geiss-Friedlander and Melchior 2007)

Small ubiquitin- related modifier (SUMO) proteins can covalently associate with many proteins as a post-translational modification. Sumo modification participates in various cellular processes, including signal transduction, DNA repair, transcription regulation and nuclear transport (Mahajan, Delphin et al. 1997; Du, Bialkowska et al. 2008). An isopeptide bond between the C-terminal carboxyl group of SUMO and the ϵ -amino group of a lysine residue in the substrate connects the SUMO with its substrate. This SUMO modification requires three different enzymes to attach SUMO to its substrate. SUMOylation starts with an activation procedure by a SUMO-activating enzyme (also named E1), which initiates an ATP-dependent activation of SUMO C-terminus. Activated SUMO is transferred to a SUMO-conjugating enzyme (E2) Ubc9 from E1. In the last step, SUMO is transferred to the specific substrate from Ubc9 with the help of SUMO ligases (E3). The SUMO proteins are attached to the lysine residues within a short consensus sequence Ω KXE, where Ω is a large hydrophobic amino acid such as isoleucine, valine or leucine; K is the lysine residue where the SUMO is attached; X could be any amino acid; and E represents a glutamic acid. The Ubc9 (E2) binds directly to this motif. E2 and E3 control substrate specificity. The SUMO unit can be cleaved off by several cleavage enzymes (Johnson 2004; Hay 2005).

5.2 Results

5.2.1 *RACK1 is a potential SUMOylation substrate*

We first scanned RACK1 full-sequence (NP_006089, NCBI) for classic SUMOylation sites. SUMOplot™ prediction, developed by ABGENT

(www.abgent.com/tools/sumoplot) was used to search for potential target lysine sites in consensus motifs. Two possible SUMOylation motifs were identified in the scan: one is LK²⁷¹QE with a score of 0.91 (red) indicating high possibility, the other one is GK²¹²DG with a score of 0.50 (blue) indicating low possibility. Both motifs are consistent with the SUMO consensus sequence ψ KXE, where ψ is an aliphatic branched amino acid, K is the lysine that SUMO added to and x could be any amino acid (Fig. 5.1).

5.2.2 *In vitro* SUMOylation on RACK1 peptide array

I employed peptide array technology to investigate further whether LK²⁷¹QE and GK²¹²DG are SUMOylation motifs. The full length RACK1 peptide library was synthesized on nitrocellulose and prepared as described in 3.2.1. An *in vitro* SUMOylation kit (BIOMOL) was used according to the instructions: all buffers were mixed and diluted with double distilled water into a total volume of 1 mL before applying to prepared RACK1 peptide array for 1 hour incubation at 37 degree. The reaction was stopped by washing the membrane with TBST 3 times. The SUMOylated RACK1 peptide array was probed with anti-SUMO1 antibody (BIOMOL). As a control, an identical RACK1 peptide array was probed with anti-SUMO1 antibody without *in vitro* SUMOylation. The dark spots were recognised as positive sumoylated peptides.

Two regions were identified as potential SUMO attachment sequences from this *in vitro* SUMOylation experiment. One contains 51-NYGIPQRALR GHSHFVSDVV ISSDGQFALS GSWDG-85 (in green), which does not include any lysine and so was considered false positive. The other is 251-ATGPSIKIWD LEGKIIVDEL KQEVI-275 (in red), which contains the LK²⁷¹QE motif that found in SUMOplot prediction. However, the GK²¹²DG found in SUMOplot prediction was not SUMOylated under the same conditions (Fig. 5.2).

5.2.3 Accessibility of LK²⁷¹QE in the RACK1 crystal structure

From the RACK1 crystal structure, we can see that lysine271 is on the side chain directly opposite to the FAK interaction surface (Fig. 5.3). The lysine271 is accessible and conformational flexibility allows it to poke downwards in the direction of the indicated arrow. However, one thing to be noticed is that among the LK²⁷¹QE the Gln272 is disconnected from Glu273 in this RACK1 crystal structure. A reasonable explanation is that the template proteins used in construction of this homology model have a longer repeat on this repeat of the structure. However, the key information is that the lysine271 is surface exposed and accessible.

5.2.4 *In vitro* sumoylation on alanine/arginine scan array

In full length RACK1 peptide array, I found sequence 251-ATGPSIKIWD LEGKIIVDEL KQEVI-275 containing LK²⁷¹QE can be SUMOylated *in vitro*. The sequence between 251-275 was subjected to alanine scan array and arginine scan array. The alanine/arginine scan array was subjected to *in vitro* SUMOylation using methods identical to those in Fig 5.2. It was then probed with anti-SUMO antisera (Cell signalling and BIOMOL). It is quite striking that mutation of lysine271 to alanine (Fig 5.4a) did not attenuate the SUMOylation of the peptide, which argues against the hypothesis that K271 is the SUMOylation site. Similar results were obtained when lysine271 was mutated to arginine. Note that when immunoblotting the same alanine scan array with anti-SUMO1 from different resources, they gave different results as leucine270 was positive when blotting with anti-SUMO1 from BIOMOL (Fig 5.4a 1), but negative when using anti-serum from Cell Signaling (Fig 5.4a 2). This indicated the non-specific binding of different antisera may give diverse and even opposite results, which highlights the importance in choosing the right antiserum. Another thing to be noticed is that even when using the same antibody, the alanine scan array (Fig 5.4a 1) and arginine scan array (Fig 5.4a) also displayed different results in the *in vitro* SUMOylation analyses. Indeed, amino acid substitution may possibly cause changes in the peptide nature and their subtle structural conformation, which may affect the *in vitro* SUMOylation of the peptide or the antibody specificity to the peptide.

5.2.5 *In vitro* sumoylation on amino acid replacement scan array 251-275

As mentioned above, Leu²⁷¹ substitution with Ala or Arg has different effects on SUMOylation, therefore, in searching for a suitable amino acid replacement to ablate the SUMOylation on Leu²⁷¹, an amino acid replacement scan array was designed and generated. Peptide 251-A T G P S I K I W D L E G K I I V D E L K Q E V I-275 was used as a template in this amino acid replacement scan array. K²⁷¹ of the template was replaced by 19 different amino acids as indicated (Fig 5.5). All the peptides were synthesized and mounted on nitrocellulose, which was SUMOylated and detected using methods identical to that in Fig 5.2. Surprisingly, changing K271 to any other amino acid significantly increased the sumoylation of

this peptide rather than decreasing it. There are two possible conclusions from this. One is that the Leu²⁷¹ is not the substrate for SUMOylation. The other one is that it may be due to the non-specific binding of the antiserum for SUMO that we have observed previously (Fig 5.4).

5.2.6 In vitro sumoylation on purified GST-RACK1 and its K271A mutant

GST fusion RACK1 protein was constructed and its K271A mutant was made using site directed mutagenesis. Equal amounts of purified GST-RACK1 and K271A mutant were subjected to in vitro SUMOylation using a SUMOylation kit (BIOMOL). RanGAP is a known protein that carries a SUMO modification. It was used as a positive control for testing the efficacy of the SUMOylation kit. After in vitro SUMOylation, equal volume of reaction solution were subjected to SDS-PAGE and detected with immunoblotting. When immunoblotting with anti-SUMO1: Lane 1 is SUMOylated RanGAP which showed the same pattern as shown in the kit instructions; Lane 2 is wild type GST-RACK1 with no detectable SUMOylation; Lane 4 is GST-RACK1 K271A mutant with a pattern similar to SUMOylated RanGAP; Lane 3 and 5 are negative controls (Fig 5.6 IB:SUMO1). It seems that RACK1 K271A mutant rather than wild type RACK1 can be SUMOylated (Fig 5.6 IB:SUMO1). SUMO1 is a small protein with a molecular weight of 11 kDa and SUMOylated target protein normally has a 20 kDa size increase in gel electrophoresis. However, when blotting for the GST tag on RACK1, there was no detectable molecular weight migration (Fig 5.6 IB:GST). Thus, GST fusion RACK1 cannot be SUMOylated in vitro. Protein expressed in prokaryotes lacks post translational modification and sometimes can lead to incorrect folding. Therefore, purified GST-RACK1 may have a different structure / conformation than RACK1 generated in eukaryotes, blocking K271 from being SUMOylated.

5.2.7 In vivo sumoylation on RACK1

To test if RACK1 can be SUMOylated in mammalian cells, I made an HA tagged RACK1 construct with K271A mutation. Wild type HA-RACK1 or K271A mutant were transfected with HA-PIASy in HEK293 cells. PIAS is an E3 ligase that contributes to SUMO substrate specificity. To determine whether overexpressing PIAS with the target protein can increase the SUMOylation. PIASy was

overexpressed with RACK1 in HEK293 cells that were lysed in modified RIPA buffer with NEM (de-SUMOylation inhibitor). The lysates were centrifugation at 13,000 for 5 minutes, the supernatant were collected as precleared lysates. The precleared lysates were analyzed for their protein concentration and subjected to immunoprecipitation using anti-HA agarose beads. The pull-down pellets were analyzed by Western Blotting. In this experiment, no SUMOylated RACK1 was detected in the HA IPs (lane 4, Fig 5.7B, D) nor in the lysates (lane 4, Fig 5.7A, C, E). Identical experiments were carried out and immunoblotted for SUMO2/3 and SUMO4 rather than SUMO1. However, no detectable SUMOylation was observed (data not shown). One reason for this is that it might due to the substrate specificity of PIASy. As there are several PIAS subtypes, PIASy may not be the right E3 ligase for RACK1 SUMOylation. Another reason might be that RACK1 SUMOylation is stimuli-dependent. RACK1 cannot be SUMOylated without activation.

5.2.8 PIAS isoforms cannot sumoylate RACK1 in HEK293 cells

Different tagged PIAS1, PIAS3 and PIASy were co-transfected with vsv tagged RACK1 in HEK293 cells. Cells lysates were prepared using methods identical to those used in Fig 5.5. The lysates were then subjected to Western Blot. When immunoblotting for vsv epitope, vsv-RACK1 can be detected but no SUMOylated RACK1 which should have a drift in molecular weight (Fig 5.8). This indicates that co-expressing PIAS isoforms with RACK1 does not cause RACK1 SUMOylation.

5.2.9 Stimuli treatment on RACK1 SUMOylation

Forskolin and Ethanol treatment of G6 cells has been reported to cause RACK1 translocation to the nucleus, and SUMOylation has been shown to cause translocation of proteins to the nucleus (Du, Bialkowska et al. 2008). Therefore, forskolin and ethanol treatment may leads to RACK1 sumoylation.

To test this, HA-RACK1 was transfected in HEK293 cells for 48 hours before adding different agonists. Cells were treated with forskolin, ethanol, insulin and isoprenaline at indicated concentration for 30 minutes (Fig 5.9). Cells were lysed and subjected to Western Blot. There was no detectable shift in migration of vsv-RACK1 to a higher molecular weight species when immunoblotting for vsv-

RACK1 using anti-vsv anti-serum. AS this may due to the cell specificity since forskolin and ethanol can cause RACK1 nuclear translocation in G6 cells, attempts were made to overexpress RACK1 in G6 cells, but without success.

5.3 Discussion

SUMOylation has been a popular research subject since the first sumo substrate Ran GAP was found (Mahajan, Delphin et al. 1997). As a reversible post-transcriptional modification, it is involved in many cellular progresses, such as signal transduction, DNA repair, transcription regulation and nuclear translocation (Hardeland, Steinacher et al. 2002; Baba, Maita et al. 2005; Geiss-Friedlander and Melchior 2007) .

In this study, I found that RACK1 contains a SUMO consensus motif Ω KXE by scanning the full sequence of RACK1 using a sumoylation predication software system (ABGENT). I found that the small peptide A²⁵¹~L K Q E V I²⁷⁵ containing LK²⁷¹QE can be sumoylated *in vitro*, and the LK²⁷¹QE was surface exposed and accessible according to its crystal structure. However, further *in vitro* SUMOylation of Ala/Arg substitution scanning arrays of the corresponding A²⁵¹~L K Q E V I²⁷⁵ sequence showed that mutating the Lysine²⁷¹ to Ala or Arg strangely increased, rather than decreased, the *in vitro* sumoylation of the A²⁵¹~L K Q E V I²⁷⁵ peptide. Purified GST fusion RACK1 cannot be sumoylated *in vitro*, but GST RACK1 K271A mutant displayed a pattern similar to sumoylated RanRAP1. One explanation is that K271A is not the sumoylation site. I thus set out to see if mutation of this site in RACK1 may give any information on whether this is a SUMO site in itself or may lead to a structural conformational change and exposure of another sumoylation site. However, exposing another site is rather unlikely because (1) in A²⁵¹~L K Q E V I²⁷⁵ peptide, there is no other Ω KXE SUMO consensus site; (2) RACK1 K271A mutant has no molecular weight increase that is normally seen in sumoylated proteins. Of course it is possible that the sumoylation signal that was detected in the peptide array might due to the non-specific binding of the antisera. Indeed, anti-sera from different resources did give diverse results (Fig 5.4).

As SUMO E3 ligase PIASy has been shown to assistant sumoylation, I co-transfected PIASy with RACK1 construct into HEK293 cells, but there was no

detectable sumoylation. I then set out to investigate other PIAS substrates PIAS1/3 yet there was still no detectable SUMOylation.

To sum up, RACK1 seems unlikely to be sumoylated (at least under conditions that I have tested). The sumoylation signal detected on peptide array is due to the non-specific binding of anti-sumo anti-sera. However, several reports suggested that there were proteins that could undertake sumoylation without possessing the sumo consensus motif (Hoegel, Pfander et al. 2002), therefore it is possible that RACK1 can be sumoylated in a way that is not related to currently known sumo consensus motifs. Hietakangas, V. also reported several negatively charged residues E/D or phosphorylated residues downstream of the consensus motif Ω KXE may play key roles in regulating substrate sumoylation (Hietakangas, Ankar et al. 2006). This might be an alternative explanation for the observation made here. In *in vitro* SUMOylation of the RACK1 peptide array, the sequence A²⁵¹~L K Q E V I²⁷⁵ containing LK²⁷¹QE was sumoylated (Fig 5.2 and Fig 5.4 control), but not the following sequence containing LK²⁷¹QEVISTSS (Fig 5.2 right to spot B, Fig 5.4, last two spots of each array). This may support the hypothesis that the RACK1 sumoylation (if any) is regulated by the phosphorylation of STSS. Thus, the phosphorylation of STSS may trigger conformational change of RACK1 structure, thereby promote the subsequent sumoylation. If this hypothesis is true, finding the right kinase for this site on RACK1 provides a major challenge.

SUMOplot™ Prediction

Developed by Abgent, copyright 2003-2004

Protein ID:	N/A
Defintion:	N/A
Length:	317 aa

```

1 MTEQMTLRGT LKGHNGWVTQ IATTPQFPDM ILSASRDKI IMWKLTRDET
51 NYGIPQALR GHSHFVSDVY ISSDGQFALS GSWDGLRLW DLTGTITRR
101 FVGHTKDVL VAFSSDNRI VSGSRDKTI LWNTLGVCY TVQDESHSEW
151 VSCVRFSPNS SNPIIVSCGW DKLVKVWNL NCKLKTNHIG HTGYLNTVTV
201 SPDGSLCASG GKDGQAMLWD LNEGKHLTYL DGGDIINALC FSPNRYWLCA
251 ATGPSIKIWD LEGKIIVDEL KQEVISTSSK AEPPQCTSLA WSADGQTLFA
301 GYTDNLVRVW QVTIGTR

```

■ Motifs with high probability

■ Motifs with low probability

■ Overlapping Motifs

No.	Pos.	Group	Score	No.	Pos.	Group	Score
1	K271	IIVDE LKQE VISTS	0.91	2	K212	LCASG GKDG QAMLW	0.50

Figure 5.1: Identification of two SUMO consensus motifs, GK²¹²DG and LK²⁷¹QE, in RACK1.

Full length of RACK1 sequence was scanned by SUMOplot™ prediction developed by ABGENT. Blue indicates motifs with low probability and Red indicates high probability.

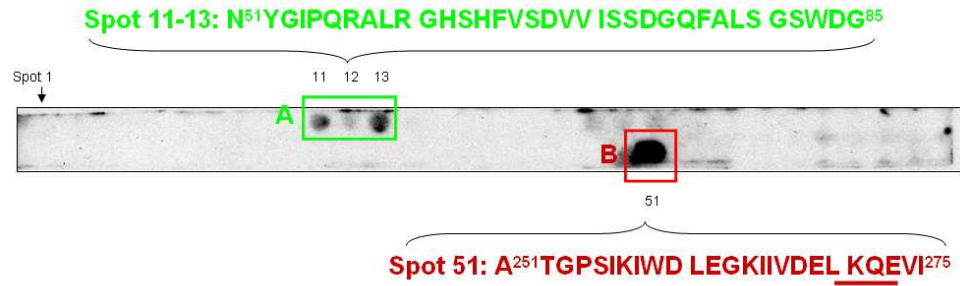


Figure 5.2: *In vitro* SUMOylation of a RACK1 peptide array identified A²⁵¹~I²⁷⁵ containing LK²⁷¹QE which can be SUMOylated.

RACK1 peptide array was *in vitro* SUMOylated as described before. It was then subjected to Western Blot with an antiserum for SUMO1. Dark spots represent the SUMOylation sites. A, sequence N⁵¹YGIPQRALR GHSHFVSDVV ISSDGQFALS GSWDG⁸⁵ (in green). B, sequence A²⁵¹TGPSIKIWD LEGKIIVDEL KQEVI²⁷⁵ (in red). Underlined is LK²⁷¹QE motif. (Shown are representative immunoblots from two independent experiments. Data provided by George Baillie)

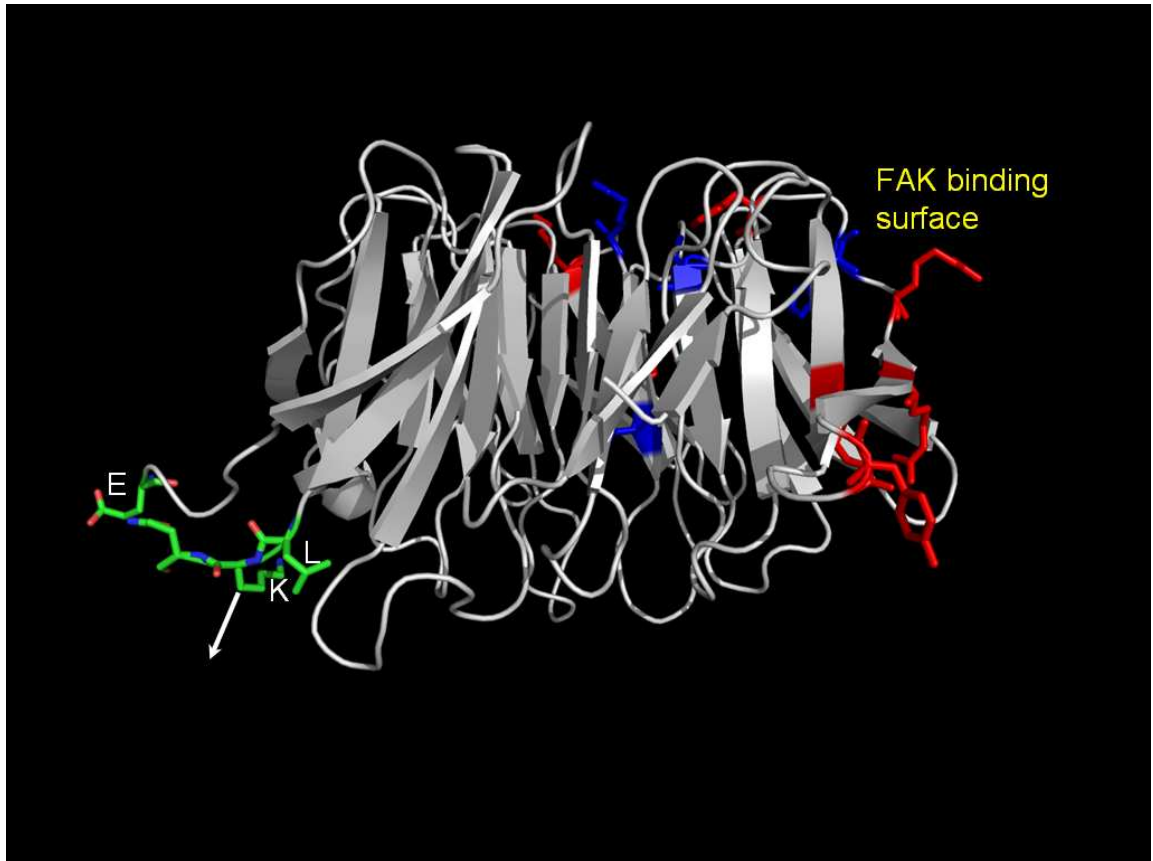


Figure 5.3: Crystal structure of RACK1 showing LK²⁷¹QE motif is surface exposed.

K²⁷¹ is on the side chain directly opposite to the FAK interaction surface as indicated. The green motif is the LK²⁷¹QE. The K²⁷¹ pokes downwards in the direction of the indicated arrow. (Data provided by David R. Adams, Heriot-Watt University, Edinburgh, UK)

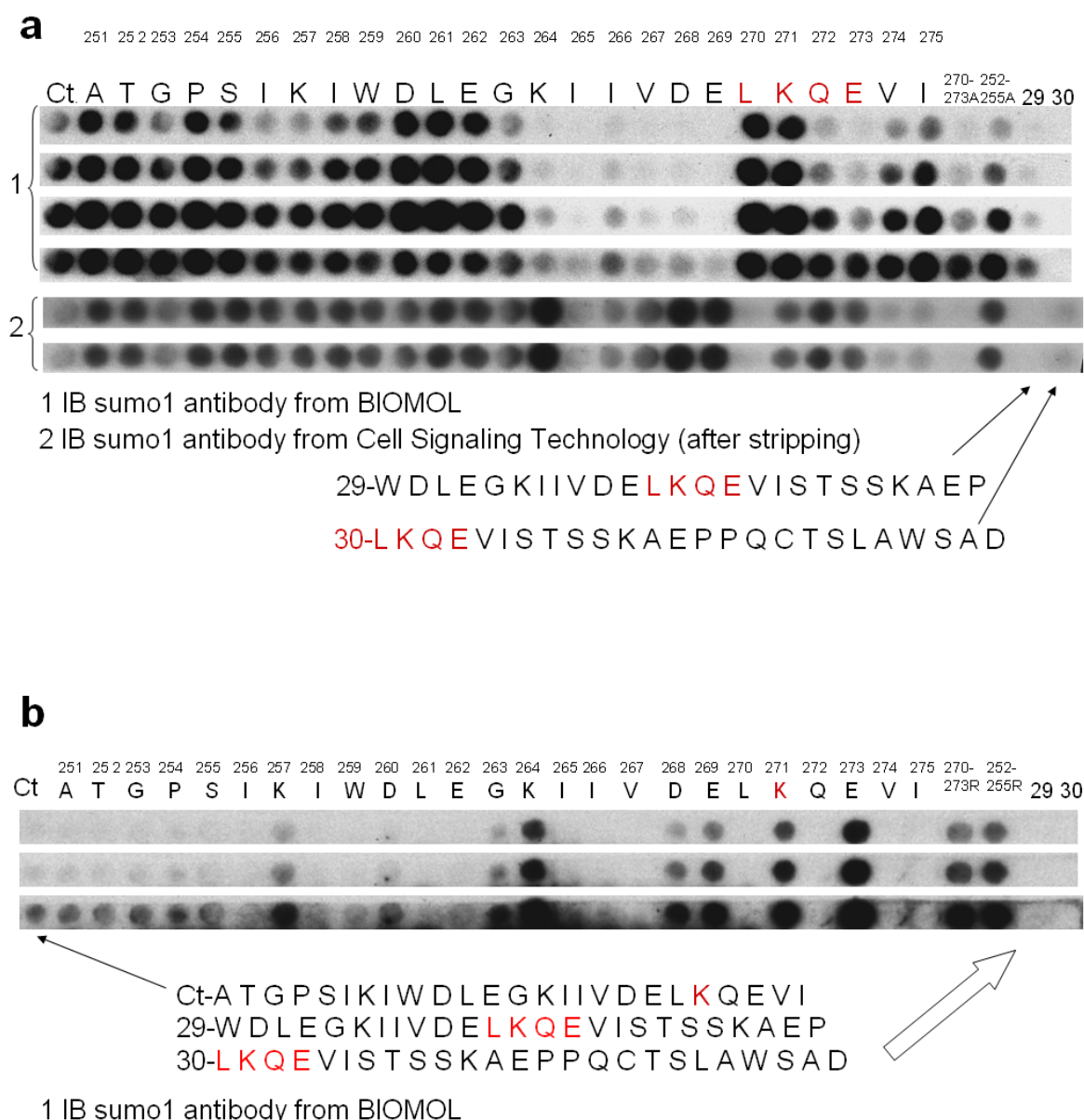


Figure 5.4: *In vitro* SUMOylation of alanine/arginine scan arrays

Sequence 251-A T G P S I K I W D L E G K I I V D E L K Q E V I-275 (Ct) containing LK²⁷¹QE was subjected to alanine scan array (a) and arginine scan array (b). The alanine/arginine scan arrays were subjected to *in vitro* SUMOylation identically to that described in Fig 5.2. They were probed with anti-SUMO antisera: 1 from BIOMOL and 2 from Cell Signaling Technology. 270-273A/R, residues 270-273 are substituted to Ala/Arg. 252-255A/R, residues 252-255 are substituted to Ala/Arg. LK²⁷¹QE is highlighted in RED. (Shown are representative immunoblots from two independent experiments.)

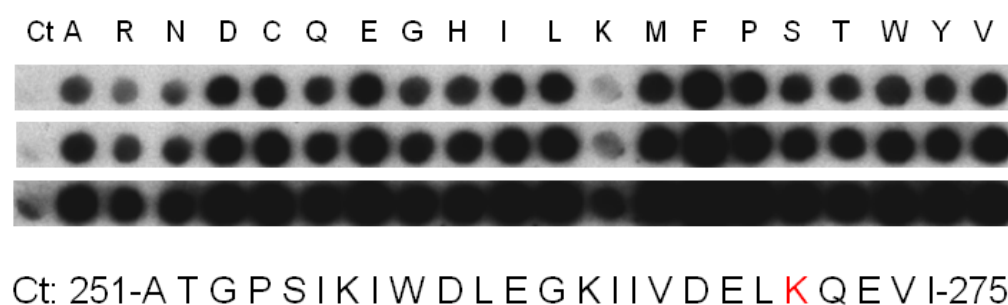


Figure 5.5: *In vitro* SUMOylation of an amino acid replacement scan array 251-275.

Peptide 251-A T G P S I K I W D L E G K I I V D E L K Q E V I-275 was used as a template in an amino acid replacement scan array. K²⁷¹ (RED) of the template was replaced by 19 different amino acids as indicated (Fig 5.5). All the peptides were synthesized and mounted on nitrocellulose, which was SUMOylated and detected using methods identical to that in Fig 5.2. (Shown are representative immunoblots from two independent experiments.)

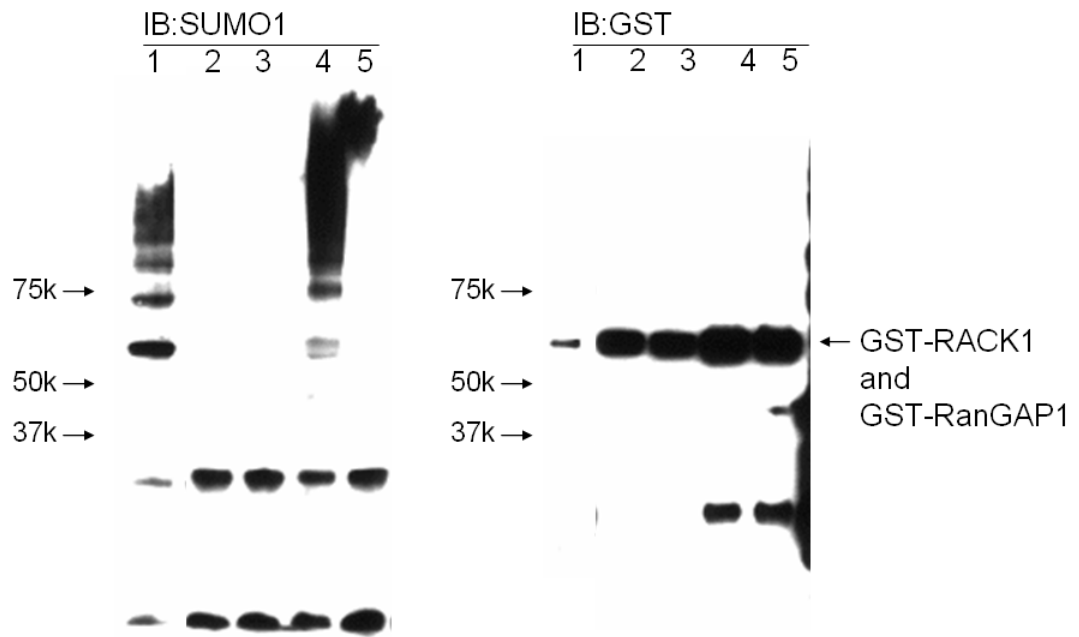


Figure 5.6: *In vitro* SUMOylation of purified GST-RACK1 and its K271A mutant.

In vitro sumoylation kit (BIOMOL) was used in this experiment. + is the positive reaction, - is the negative control lack activation reagent. Purified GST tagged protein and the activation reagent were loaded as described below: (1) GST-RanGAP1 +. (2) GST-RACK1 +. (3) GST-RACK1 -. (4) GST-RACK1 K271A +. (5) GST-RACK1 K271A -. IB: Immunoblot. (Shown are representative immunoblots from two independent experiments.)

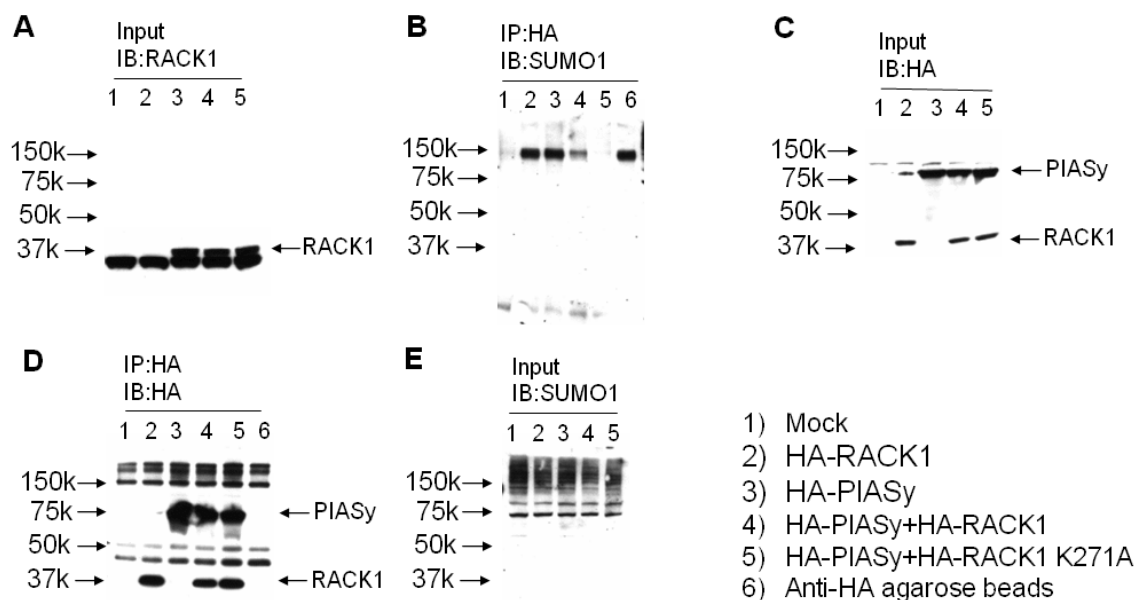


Figure 5.7: *In vivo* SUMOylation of RACK1.

HEK293 cells expressing HA-PIASy and/or HA-RACK1 were lysed and lysates blotted with antibodies to RACK1(A),SUMO1(E) or HA(C). HA-RACK1 was immunoprecipitated using HA-antibodies and blotted with SUMO1(B) or HA(D). (Shown are representative immunoblots from three independent experiments.)

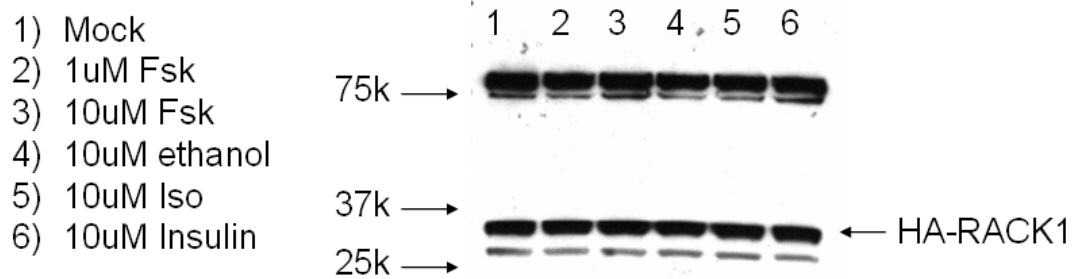


Figure 5.9: Various stimuli have no influence on RACK1 sumoylation.

HEK293 cells overexpressing HA tagged RACK1 were treated with Forskolin (Fsk), isoprenaline (Iso), Ethanol or Insulin for 30 mins at the indicated concentration. Lysates were prepared and immunoblotted with anti-HA antibodies for HA. (Shown are representative immunoblots from two independent experiments.)

6 General discussion and predication

6.1 MEK1 binds directly to β arrestin1

G protein coupled proteins (GPCR) can undertake fast desensitization, receptor internalization and recycling upon receptor stimulation with β arrestins and have been shown to play pivotal roles in regulation of these processes (DeWire, Ahn et al. 2007; DeFea 2008). The β_2 -adrenergic receptor (β_2 AR) has been a topical and important subject relating to investigations of the functioning of GPCRs and β arrestins. The classic signal transduction through GPCR (β_2 AR) is that upon agonist binding to the receptors, they interact with the GTP-binding protein G_s . This releases the GTP-bound form of alpha- G_s which can then activate the downstream effector, adenylyl cyclase, leading to elevated cAMP levels and PKA activation.

However, a process of desensitization is rapidly started to cease the receptor signal transduction: GRKs are translocated to and phosphorylate the receptor, which facilitates cytosolic β arrestin recruitment to the receptor (Ferguson, Barak et al. 1996; Premont and Gainetdinov 2007). The β arrestins then associate with agonist occupied receptors, sterically blocking their interaction with G proteins. The receptor associated β arrestin is crucial for the GPCR internalization, as β arrestins can interact with clathrin cages and bring them to the GPCR to facilitate GPCR internalization (Gurevich and Gurevich 2006). In the case of β_2 AR, β arrestin can sequester the cAMP specific phosphodiesterase, PDE4D5 thereby transferring a cAMP degrading enzyme to the receptor at the plasma membrane close to where cAMP is synthesised. This recruited PDE4D5/ β arrestin complex is able to regulate cAMP levels around the β_2 AR so as to control phosphorylation of the β_2 AR by an AKAP-tethered PKA sub-population. This serves to regulate the switching of β_2 AR coupling between G_s and G_i , thus providing a further means of regulating receptor desensitization (Baillie, Sood et al. 2003; Lynch, Baillie et al. 2005; Bolger, Baillie et al. 2006).

β arrestin1 (418 amino acids), which has an additional 10 amino acids in its C-terminal region, compared to β arrestin2 (409 amino acids), is constitutively phosphorylated by ERK at Ser412 (Lin, Miller et al. 1999; Luttrell, Roudabush et al. 2001). Upon agonist stimulation of GPCRs, β arrestin1 undergoes rapid

dephosphorylation at Ser412 and translocates to the GPCR at the plasma membrane. β arrestin has been considered to regulate clathrin recruitment to the receptors and dynamin phosphorylation, which are two important features in receptor internalization. The dephosphorylation of Ser412 is considered to be a molecular switch that allows interaction between β arrestin1 and clathrin (Lin, Krueger et al. 1997; Lin, Miller et al. 1999), and also is considered to play a pivotal role in the association of β arrestin1 with c-Src, which leads to the phosphorylation of dynamin (Miller, Maudsley et al. 2000). Therefore, the ERK-dependent Ser412 phosphorylation and dephosphorylation of β arrestin1 modulates its interaction with the endocytic machinery (clathrin and dynamin) in the internalization of GPCRs (Lin, Krueger et al. 1997).

The mitogen-activated protein kinase signaling pathway c-Raf/MEK/ERK includes three serine/threonine kinases that are phosphorylated and activated by their upstream kinases (Rubinfeld and Seger 2005). The c-Raf/MEK/ERK complex mediates various cellular processes including cell proliferation, transcription regulation, apoptosis and cell differentiation (Lu and Xu 2006). This signaling system is involved in many diseases, and ERK has become a popular target in searching for a specific inhibitor as a possible therapeutic. However, there has been little success in trying to do this. Considering the high fidelity of this MEK/ERK cascade, a MEK inhibitor would be expected to work equally well in causing ERK inhibition. Therefore, MEK inhibitors have become candidates as potential anti-cancer therapeutics and also for treating chronic inflammatory disorders, such as asthma and rheumatoid arthritis (English and Cobb 2002; Pelaia, Cuda et al. 2005; Sweeney and Firestein 2006).

Previous studies have indicated that either MEK inhibitors or dominant negative MEK (inactive) can attenuate Ser412 phosphorylation of β arrestin1 (Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002; Hupfeld, Resnik et al. 2005). Consistent with this, I have shown that treating cells with MEK inhibitor U0126 or PD98059 does decrease ERK-dependent phosphorylation on Ser412, which confirms that the phosphorylation of β arrestin1 on Ser412 is dependent on MEK.

Previous research suggests that c-Raf, MEK and ERK could form a signaling complex with β arrestin. In this, C-Raf acts as the adaptor protein linking MEK/ERK with β arrestin to facilitate the formation of a signaling complex

(Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002). In addition, a report that came after my research was completed shows that MEK1 can bind to both the C-domain and N-domain of β arrestin (Song, Coffa et al. 2009). In the course of my work, I have shown that MEK1 binds directly to a DxxD motif on β arrestin's N-domain and that this association is not ERK dependent. In another recent study, an ERK docking site has been found to locate in β arrestin's C-domain. Based on these reports, we can conclude that MEK1 binds directly to β arrestin's N-domain and can presume an indirect interaction between β arrestin and MEK via ERK.

To locate the possible interaction motif for β arrestin1 on MEK1 and vice versa, I employed a novel peptide array and substitution array in my study. By this scanning technology, I located the MEK1 docking sites on β arrestin1, namely Asp26 and Asp29, and the corresponding β arrestin1 docking residues on MEK1, namely Arg47 and Arg49. To confirm this finding, I made and tested the MEK1-binding-KO β arrestin1 mutant with the two aspartic acids substituted to alanines and the β arrestin1-binding-KO MEK1 mutant with two Arg residues mutated to Ala. Immunoprecipitation assays showed that these mutations disabled the binding ability to the counterpart. In addition to this, a peptide that inhibited MEK1 binding to β arrestin1 (T-DFVD, with a sequence identical to residue 6-30 of β arrestin1) was designed, generated and evaluated. This small peptide with a N-terminal stearylation can penetrate cell membrane in 2 hours where it regulates the phosphorylation of β arrestin1 at Ser412. Disassociation of MEK1 from the β arrestin1/ERK complex by the inhibitory peptide decreases the phosphorylation of β arrestin1 at Ser412, which increases interactions of clathrin and c-Src to β arrestin1. The dephosphorylation of β arrestin1 at Ser412 thereby increases the association of β arrestin1, which, in turn, facilitates the internalization of β 2AR. All these pieces of evidence indicate that the dephosphorylation of β arrestin1 is a rate-limiting step for the receptor internalization process.

From the known crystal structure of β arrestin it is evident that Asp26 and Asp29 are partially exposed on the surface of β arrestin in its basal conformation (Brookhaven Protein Data Bank codes 1G4M, 1G4R and 1ZSH (Milano, Kim et al. 2006)). However, in the basal / crystalline state they are involved in interactions with neighbouring residues. Thus Asp26 connects to the C-terminal Lys355 and Arg393 by salt bridges, and also interacts with the "phosphate sensor" Arg169.

Also, Asp29 forms a salt bridge to Lys170 and contacts the side chain of Gln172 by hydrogen-bonds. These interactions make it unlikely that MEK can bind to these residues of β arrestin1 in such a basal conformation without either competing these interactions out and thereby triggering a conformation change in β arrestin or its interaction is gated by a conformational change that causes disruption of this basal conformation, such as could occur through post-translational modification (e.g. phosphorylation) or binding some other partner protein. The binding between Asp26 and MEK1 could occur only if the sequestered effect of the β arrestin1 C-terminal sequence on these residues is removed. Asp26 become more accessible when the C-terminal sequence is displaced, but MEK1 binding to Asp26 and Asp29 requires further conformation changes. An example of this is that β arrestins undergo a great conformation change upon association with their activated phospho-GPCR partners. This includes GPCR-phosphate engagement with β arrestin phosphate-sensor and repositioning of the C-terminal tail from its location of basal conformation where it is folded across the N-terminal region. β arrestin1 as a scaffold protein has a number of binding partners (Perry and Lefkowitz 2002; Xiao, McClatchy et al. 2007), and it undergoes structural alteration upon binding of these proteins. Thus the binding of a specific partner protein may also cause a conformational change in this region to allow MEK1 to bind. Indeed, β arrestin has so many possible partners that defined sub-populations might form where the binding of one partner changes the conformation of β arrestin such that only certain other partners can bind. Thus β arrestin1 could undertake conformational changes upon binding to receptors, post-translational modification and binding to specific partners (Vishnivetskiy, Hosey et al. 2004) that then defines which complexes MEK1 can associate with depending upon which modifications and interactions open up the sites for MEK1 interaction. In the future it might be interesting then to harvest β arrestin/MEK1 complexes specifically as see what other proteins are associated with this complex using mass spectrometry/proteomics methods. Based on this, it is possible that MEK1 can bind to modified or partner protein sequestered β arrestin1 that enables surface exposure of Asp26 and Asp29. It is also possible that there is equilibrium between β arrestin1's basal conformation and a small percentage of "open conformation" that MEK1 and other proteins, such as MEK, may bind. If so, MEK1 binding may stabilize the open conformation of β arrestin1, shifting the equilibrium to generate a sub-population of MEK1 binding β arrestin1. To be noted, the Asp26 to Asp29 sequence is permanently

located at the surface of isolated β arrestin1 N-domain. Thus, this sequence must perform similar interaction with MEK1 as Asp26 and Asp29. Another possibility is that if, as Song suggested MEK1 could associate with both N-domain and C-domain of β arrestin1, the MEK1 binding to the C-domain may act as a trigger of β arrestin1 conformational change, leads to increased accessibility to Asp26 and Asp29 at β arrestin1's N-domain. Although we found two amino acids, Arg47 and Arg49, on MEK1 that β arrestin1 could bind to, we can't evaluate their accessibility due to lack of a crystal structure of MEK1 (Ohren, Chen et al. 2004). However, considering their position upstream of MEK1 kinase domain, it is possible that they are surface exposed and thus available for interaction.

6.2 PDE4 dimerization

cAMP is a well studied second messenger that plays key roles in many cellular processes, and its degradation by cyclic phosphodiesterases (PDEs) is the only way to stop signal transduction. PDEs were first identified for their ability to cause desensitization of adenylyl cyclase signalling. The wide range of PDEs places them as important proteins involved in regulation of cAMP compartmentalization (Dodge, Khouangsathiene et al. 2001; Lynch, Baillie et al. 2007) and the cross-talking between different signalling pathways (Hoffmann, Baillie et al, 1999 ; O'Connell, McCallum et al 1996). Dimerization has been noted in many PDE subfamilies including members from PDE1 to PDE6 (Richter and Conti 2004). In the case of the PDE4 subfamily, PDE4D3 has been used in investigating the nature of dimerization. These studies showed that the conserved region UCR1 and UCR2 of PDE4D3 are responsible for its dimerization (Richter and Conti 2002; Richter and Conti 2004).

It has been pointed out that most PDEs, at least members of PDE families 1-6, can exist as dimers (Richter and Conti 2004). In the PDE4 subfamilies, the long PDE isoforms were shown to form dimers via their conserved UCR1 and UCR2 domains. In this, the PDE4D3 isoform was investigated intensively by Macro Conti's group (Richter and Conti 2002) (Richter and Conti 2004). They suggested that disruption of PDE4D3 dimerization abolished its activation by either protein kinase A phosphorylation or phosphatidic acid binding and reduced the sensitivity to inhibition by rolipram (Richter and Conti 2004). They also demonstrated that ablating the UCR1 and UCR2 intramolecular interaction did not interfere with

dimerization (Richter and Conti 2002). PDE4D5, which shares conserved UCR1, UCR2 and catalytic unit with PDE4D3, can be presumed to provide another dimeric PDE4 isoform. Even with high similarity, PDE4D3 and PDE4D5 are not redundant in their functions. PDE4D5 is unique for its β arrestin dependent translocation to the β 2 adrenoceptor after agonist challenge and generating a compartmentalized cAMP pool adjacent to the receptors, whereas PDE4D3 does not (Lynch, Baillie et al. 2005; Lynch, Baillie et al. 2007) but interacts with the ryanodine receptor (Lehnart, Wehrens et al. 2005) and myomegalin (Verde, Pahlke et al. 2001).

In this study, I show that PDE4D5 can form strong homo-dimers. Indeed PDE4D5 can dimerise both *in vivo* and *in vitro* using yeast-two-hybrid assay and immunoprecipitation. Peptide array and scanning substitution array approaches were then used to identify the dimerization sites on PDE4D5, locating four triplets of residues that may be involved in the dimerization process. These are R173/N174/N175 of UCR1, E228/T229/L230 of UCR2, L306/M307/H308 and K323/T324/E325 on the catalytic domain. The R173/N174/N175 and the E228/T229/L230 are adjacent to V172/F176 (V100/F104 in PDE4D3) and L217/L220/L224/L227 (L145/L148/L152/L155 in PDE4D3) respectively, both of which are suggested dimerization sites in PDE4D3 (Richter and Conti 2004), indicating a high possibility that RNN and ETL are involved in PDE4D5 dimerization. However, substitution of each triplet to triple AAA or even mutating all four triplets (also known as Quad) did not ablate the PDE4D5 dimerization. A previous report indicates that mutating an ion pair (D463-R499 in PDE4D5) of the catalytic domain can disrupt the oligomerization of the isolated catalytic units (Lee, Chandani et al. 2002). We then made the Quad mutants with disrupted catalytic unit ion-pairs by mutating either R499 to Asp or D463 to Arg, and showed these Quad R499D and Quad D463R mutants lost their ability to dimerize with Quad mutant, suggesting that the ion-pair is crucial for PDE4D5 dimerization. We then set out to explore the individual contribution of each triplet to PDE4D5 dimerization, and found that R173/N174/N175 alone with R499D mutation could ablate its dimerization with Quad and Quad R499D in yeast hybrid assay, but not the other triplets. However, when we test the monomeric RNN R499D via immunoprecipitation, it ablates the dimerization to a certain percentage rather than the total loss of dimerization that we have seen in Quad R499D.

One thing that should be mentioned is that in the yeast two hybrid and immunoprecipitation studies, the results showed that PDE4D5 can dimerize both *in vitro* and *in vivo*; In yeast two hybrid, adding PDE4D5 interacting protein RACK1 or β arrestin could successfully disrupt PDE4D5 dimer formation; however, we did not see such an effect in immunoprecipitation using HEK293 cells. There are two explanations for this observation. One is due to the nature of yeast two hybrid assay that it has a threshold for the signal to be detected, in another words, the interacted prey and bait protein must reach a certain affinity to initiate the interaction. However, the immunoprecipitation analyses are perhaps more sensitive than yeast two hybrid assay in detecting protein interaction. Therefore, RACK1 or β arrestin can ablate PDE4D5 dimerization in yeast two hybrid assay but not in immunoprecipitation. Another reason is that in mammalian cells, PDE4D5 exists as low transcript number but high activity, RACK1 and β arrestin have more transcripts than PDE4D5. Therefore, RACK1 and β arrestin may sequester PDE4D5 in abundance to intervene in the dimer formation. However, overexpressing PDE4D5 alone or with RACK1 and β arrestin together also significantly increases the amount of PDE4D5, causing an increased ratio of PDE4D5 against RACK1 and β arrestin. This makes RACK1 and β arrestin less potent in disrupting PDE4D5 dimerization. We also found PDE4D5 dimerization is of high stability and its disassociation and reassociation is not affected by separately expressed RACK1 and β arrestin.

PDE4D5 dimerization responses in to several compounds were tested and some preliminary results were obtained. We see rolipram (binds to HARB and LARB conformation of PDE4D5, LARB and HARB are explained in 4.2.12) and ariflo (binds to LARB conformation only of PDE4D5) have little difference in intervening in PDE4D5 dimerization. However, we do not know if the dimerized PDE4D5 exists in the HARBs or LARBs conformation, and this may worth further investigation. Anisomycin can increase the PDE4D5 dimerization and this may due to the activation of its downstream effecters such as P38 and JNK. IBMX/Forskolin treatment leads to a reduction of PDE4D5 dimerization. This might due to PKA phosphorylation on PDE4D5 and further experiments are needed to confirm it.

We found that PDE4D5 could form hetero-dimers with PDE4D3 in yeast two hybrid experiments, but not in cells. Therefore, this hetero-dimer might be an

artefact of the yeast-two-hybrid system. In the case of the monomeric PDE4D5, the Quad R499D mutant, its enzyme activity, compartmental distribution, binding affinity to PDE4D5 interacting proteins and sensitivity to PDE inhibitors are also required for further study.

6.3 RACK1 sumoylation

Sumoylation is a reversible post-translational modification that is involved in DNA repair, transcription, nuclear bodies and nucleo-cytoplasmic transport (Geiss-Friedlander and Melchior 2007). Small ubiquitin-related modifier (SUMO) proteins can be added to substrates via an isopeptide bond between the C-terminal carboxyl group of SUMO and the ϵ -amino group of a lysine residue in the substrate. Three different enzymes E1, E2 and E3 are required in attaching SUMO to its substrate. The only E2 conjugating enzyme, UBC9, can recognise the classic SUMO consensus sequence ψ KXE, where ψ is an aliphatic branched amino acid, K is the lysine that SUMO added to and x could be any amino acid (Johnson 2004; Hay 2005).

RACK1 is a scaffolding protein that can interact with PKC (Ron, Luo et al. 1995; Stebbins and Mochly-Rosen 2001) and PDE4D5 (Bolger, McCahill et al. 2002), mediate and initiate cell migration in many cell types (Buensuceso, Woodside et al. 2001; Cox, Bennin et al. 2003; Kiely, O'Gorman et al. 2006) and affect the activity of MAPK signalling pathway, c-Jun NH2-terminal kinase (JNK), via its interaction with PKC (Lopez-Bergami, Habelhah et al. 2005).

RACK1 can translocate to the nucleus upon challenge with ethanol and forskolin (Ron, Vagts et al. 2000; He, Vagts et al. 2002), and sumoylation can target its substrate to the nucleus. Therefore, we investigated whether RACK1 is a SUMO substrate and obtained some preliminary data.

By doing *in vitro* sumoylation on a RACK1 peptide array and scanning Ala and Arg array, I identified a potential SUMO modification site K271, which lies in the sequence LK²⁷¹QE that is consistent with the SUMO consensus motif ψ KXE. Although the sumoylation is detectable in the *in vitro* sumoylated peptide array, I did not see sumoylation in the *in vitro* sumoylation on purified GST-RACK1. This may be due to the incorrect folding of GST-RACK1 blocking the sumo site LK²⁷¹QE.

As overexpression of the E3 ligase PIASy may facilitate *in vivo* RACK1 sumoylation, I overexpressed PIASy, PIAS1 and PIAS3 respectively with RACK1 in HEK293 cells, however, no sumoylation was detected. Furthermore, challenging cells with forskolin and ethanol has been reported to cause RACK1 translocation to nucleus, and sumoylation was known to cause protein nucleus translocation (Du, Bialkowska et al. 2008). Thereby, forskolin and ethanol may cause RACK1 sumoylation and translocation to nucleus. However, there was no detectable RACK1 sumoylation in forskolin and ethanol treated HEK293 cells. This may due to the low percentage of SUMOylated protein (normally < 5% of target protein) or because I haven't found the correct E3 ligase or the correct cell lines that allow RACK1 to be SUMOylated. Another thing to be considered is that the non-specific binding of different anti-SUMO anti-sera gives diverse results, therefore, choosing a good antiserum is crucial for this study.

For future work, there are several aspects that can be investigated: (1) Using other cell lines such as G6 to see if RACK1 can be sumoylated *in vivo* with or without forskolin and ethanol treatment. (2) Overexpressing the E2 conjugating enzyme UBC9 with PIAS and RACK1 to see if they can facilitate RACK1 sumoylation. (3) Mutating Lys271 of RACK1 to Arg and transfecting the mutant in cells to compare its distribution with native RACK1 under confocal microscopy.

In conclusion, this sumoylation event was only detectable in an *in vitro* sumoylated peptide array. The notion that RACK1 can undertake sumoylation still requires firm evidence and so much more experimental work is required.

Peptide array and the scanning substitution array is very potent technology in searching for the specific interaction sites between proteins, and it has been used through my researches. However, care must be taken when using this technology: (1) Due to the relative large molecular weight and potential non-specific binding to some proteins, GST tagged proteins are not as good as His tagged ones. And the control reaction which uses GST protein to evaluate the non-specific binding to the peptide array is also essential. (2) The small peptides all exist in their own conformations. Deletion or changing even one single residue of the peptide may give diverse results in binding to target proteins. (3) In respect to (1) and (2), the potential interaction sites require further

confirmation using other methods such as site-directed mutagenesis and immunoprecipitation.

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