Struktur-Funktionsanalyse der Gβγ-vermittelten Aktivierung der Phosphoinositid 3-Kinase γ

(Structural and functional analysis of Gβγ-induced stimulation of phosphoinositide 3-kinase γ)

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ABBREVIATIONS

β-AR	β-adrenergic receptor
AC	adenylyl cyclase
AMF	chemical combination added to buffer: 40 µM aluminium chloride, 6 mM magnesium chloride and 10 mM sodium fluoride
APS	ammonium persulfate
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
cGMP	cyclic guanosine-3',5'-monophosphate
CHAPS	3 [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Ci	Curie
cpm	counts per minute
CTX	cholera toxin secreted by Vibrio cholerae
$C_{12}E_{10}$	polyoxyethylene-(10)-lauryl ether
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC_{50}	the molar concentration of a substance, which produces 50 % of the maximum possible response for that substance.
EDTA	ethylenediamine-N,N,N'N'-tetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
Fig.	figure
(x) g	centrifugal force (9,81 m/s^2)
GABA	γ-aminobutyric acid
GDP	guanosine-5'-diphosphate
G-protein	heterotrimeric guanine nucleotide binding protein
GST	glutathione-S-transferase
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-[γ-thio]- triphosphate
h	hours
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
His-tag	hexahistidine-tag
HPLC	high pressure (performance) liquid chromatography
IP_3	inositol-1,4,5-trisphosphate
kDa	kilodalton (a unit of mass, being 1000 daltons)
Lubrol PX	polyoxyethylene-(9)-lauryl ether
M	mol per liter
MALDI	matrix assisted laser desorption ionisation
min	minutes
MOI	multiplicity of infection
NAD	reduced form of nicotinamide adenine dinucleotide
Ni^{2+}-NTA	nickel nitrilotriacetic acid

p84	a novel regulatory subunit of class I_B PI3Kγ (also known as $p87^{PIKAP}$)
p85	non-catalytic subunit of class I_A PI3Ks α, β and δ
$p87^{PIKAP}$	a novel regulatory subunit of class I_B PI3Kγ (also known as p84)
p101	non-catalytic subunit of class I_B PI3Kγ
p110	catalytic subunit of class I PI3Ks
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pfu	plaque-forming unit: a virus or group of viruses which cause a plaque
pH	logarithmic measure of hydrogen ion concentration (a measure of the acidity or alkalinity of a solution)
PI	phosphatidylinositol
PI-4,5-P_2	phosphatidylinositol-4,5-bisphosphate (PIP_2)
PI-3,4,5-P_3	phosphatidylinositol-3,4,5-triphosphate (PIP_3)
PI3K	phosphatidylinositol-3-kinase
PLC	phospholipase C
PTX	pertussis toxin secreted by Bordetella pertussis
PVDF	polyvinylidene fluoride
rpm	revolutions per minute
s	seconds
S.D.	standard deviation
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
Sf9 cells	cells derived from the pupal ovary of Spodoptera frugiperda
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Tween 20	polyoxyethylene-(20)-monolaurate
UV	ultraviolet
% (v/v)	volume/volume percent
% (w/v)	weight/volume percent

Amino acids

A[1]	**Ala**[2]	Alanine[3]	**C**	**Cys**	Cysteine	**D**	**Asp**	Aspartic acid
E	**Glu**	Glutamic acid	**F**	**Phe**	Phenylalanine	**G**	**Gly**	Glycine
H	**His**	Histidine	**I**	**Ile**	Isoleucine	**K**	**Lys**	Lysine
L	**Leu**	Leucine	**M**	**Met**	Methionine	**N**	**Asn**	Asparagine
P	**Pro**	Proline	**Q**	**Gln**	Glutamine	**R**	**Arg**	Arginine
S	**Ser**	Serine	**T**	**Thr**	Threonine	**V**	**Val**	Valine
W	**Trp**	Tryptophan	**Y**	**Tyr**	Tyrosine			

[1] One-letter symbol
[2] Three-letter abbreviation
[3] Name of amino acid

1 INTRODUCTION

1.1 General principles of transmembrane signal transduction

Signal transduction is a process by which a cell converts one kind of signal or stimulus into another. The communication between cells in higher animals is realized by hundreds of signaling molecules such as proteins, small peptides, amino acids, nucleotides, steroids, retinoids, fatty acid derivatives. Dissolved gases such as nitric oxide and carbon monoxide also participate in signal transduction process (Kroeze et al., 2003). Most of these molecules are secreted from the signaling cell by exocytosis. Others are released by diffusion through the plasma membrane, while some remain tightly bound to the cell surface and influence only cells that contact the signaling cell (Snyder, 1985). Many extracellular stimuli (ligands) are hydrophilic and are unable to go through the plasma membrane. Regardless of the nature of the signal, the target cell responds to a stimulus through interaction with a specific protein faced outwards from the membrane, called a receptor (Landry et al., 2006). There are also intracellular DNA-binding receptors such as steroid hormone and thyroid hormone receptors (Evans, 1988, Parker, 1993). They bind lipophilic ligands that pass through the membrane and directly address genes.

Most cell-surface receptors belong to one of three classes (Fig. 1.1), which are defined by the transduction mechanism used. Ion-channel-coupled receptors, also known as ligand-gated ion channels, are prototypically involved in rapid synaptic signaling between electrically excitable cells. This type of signaling is mediated by a number of neurotransmitters that transiently open or close the ion channel formed by the protein to which they bind. The binding of the neurotransmitters briefly changes the ion permeability of the plasma membrane, and thereby the excitability of the postsynaptic cell (Fig. 1.1A). Nicotinic receptors, 5-HT_3 receptors, $GABA_A$ and $GABA_C$ receptors, glycine receptors, and some glutamate, histamine and serotonin activated anionic channels are examples of ion-channel-coupled receptors (Kotzyba-Hibert et al., 1999, MacDermott et al., 1999, Whiting et al., 1999).

Enzyme-coupled receptors are transmembrane proteins with their ligand-binding domain on the outer surface of the plasma membrane. The cytosolic domains have either an intrinsic enzyme activity or are associated directly with an enzyme (Fig. 1.1B). There are five known classes of enzyme-coupled receptors: (1) receptor guanylyl cyclases, which catalyze the production of cyclic GMP in cytosol (Pyriochou and Papapetropoulos, 2005); (2) receptor tyrosine kinases, which phosphorylate specific tyrosine residues on a small set of intracellular signaling proteins (Fantl et al., 1993); (3) tyrosine-kinase-associated receptors, which associate with proteins that have tyrosine kinase activity (Giliani et al., 2005); (4) receptor

tyrosine phosphatases, which remove phosphate groups from tyrosine residues of specific intracellular signaling proteins (Irie-Sasaki et al., 2003, Huntington and Tarlinton, 2004), and (5) receptor serine/threonine kinases, which phosphorylate specific serine or threonine residues on some intracellular proteins (ten Dijke and Hill, 2004, Feng and Derynck, 2005).

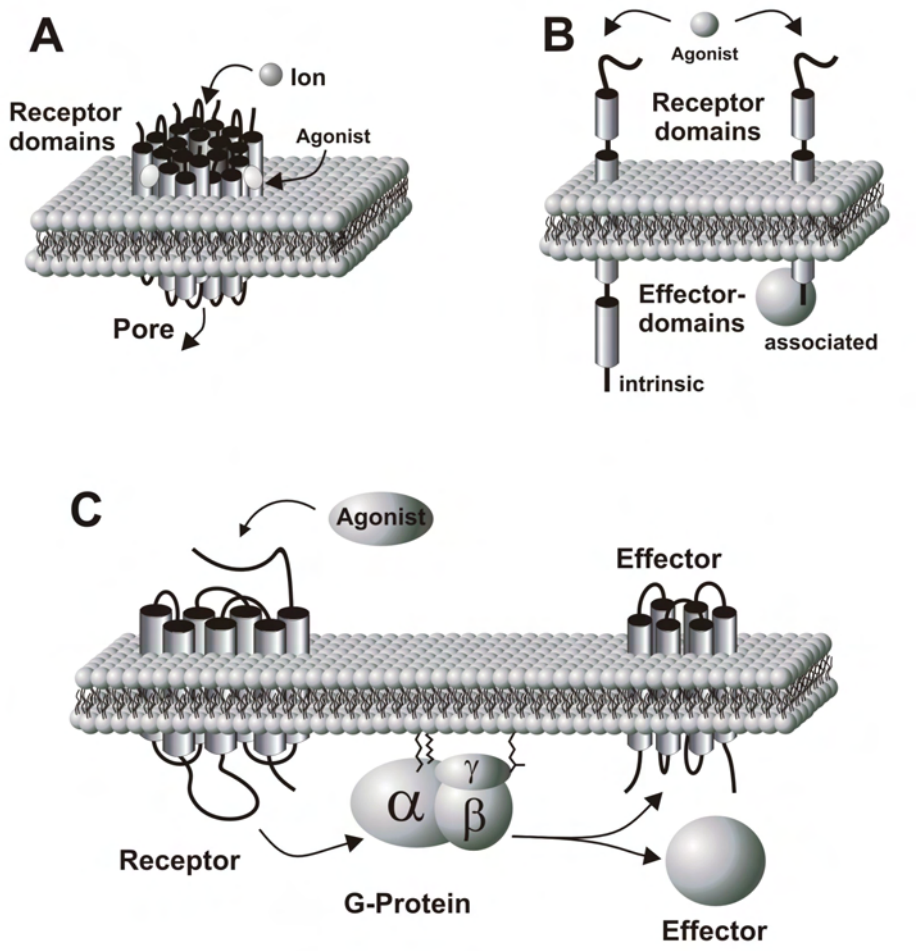


Fig. 1.1: Cell-surface receptors

Ion-channel-coupled receptor (A) contains an extracellular receptor domain and forms a transmembrane pore. Various ligands “close” or “open” the channel. **Enzyme-coupled receptor (B)** may have intrinsic enzyme activity or rely on associated enzymes. **G-protein-coupled receptor (C)** transduces extrtacellular signals to membrane or cytosolic effectors via heterotrimeric G-protein.

G-protein-coupled receptors (GPCR) represent the largest group of cell surface receptors. The investigation of human genome has identified more than 800 different GPCR genes (Wise et al., 2004). GPCRs are integral membrane proteins that possess seven transmembrane domains. These receptors transduce the activation or inactivation of plasma-membrane-bound and cytosolic enzymes or ion channels via heterotrimeric GTP-binding proteins (G-proteins, Fig. 1.1C) (Wymann and Pirola, 1998, Kurachi and Ishii, 2004, Watts and Neve, 2005). Some GPCRs activate or inactivate adenylyl cyclase, thereby changing the intracellular concentration of the intracellular mediator, or second messenger, cyclic AMP (Watts and

Neve, 2005). Others GPCRs may activate class I phosphoinositide 3-kinases (PI3Ks) which phosphorylate the D3 position of the inositol ring of phosphoinositides thus generating intracellular lipid second messengers (Wymann and Pirola, 1998, Maier et al., 1999, Hirsch et al., 2000). These lipid products transmit signals by recruiting intracellular effector molecules to the membrane which contain a pleckstrin homology (PH) domain (Cozier et al., 2004, Halet, 2005, Lemmon, 2005). GPCRs also activate phopholipase Cβ-isoforms which hydrolyze phosphatidylinositol bisphosphate (PIP_2) to generate two second messengers: inositol triphosphate (IP_3) which releases Ca^{2+} from the endoplasmatic reticulum and thereby increases the concentration of Ca^{2+} in the cytosol, and diacylglycerol which remains in the plasma membrane and activates protein kinase C (Ohno-Shosaku et al., 2005, Landry et al., 2006). A rise in cyclic AMP or Ca^{2+} levels affect cells to stimulate cyclic-AMP-dependent protein kinases and Ca^{2+}/calmodulin-dependent protein kinases, respectively. Subsequently, these kinases phosphorylate specific target proteins on serine or threonine residues, and thereby alter the activity of the proteins (Soderling, 1999, Langeberg and Scott, 2005). The responses to extracellular signals can be greatly amplified through the intracellular signaling cascades activated by GPCRs.

1.2 G-protein-dependent signal transduction

1.2.1 Structure and function of G-proteins

Heterotrimeric G-proteins are membrane-bound and closely associated with the intracellular domains of GPCRs (Chen and Manning, 2001). The G-proteins are composed of a Gα, a Gβ and a Gγ subunits. GDP-bound Gα subunits bind tightly to heterodimeric Gβγ. This association allows Gα to localize to the plasma membrane which is essential for coupling to GPCRs (Chen and Manning, 2001, Evanko et al., 2001). In addition, Gβγ binding to GDP-bound Gα slows the spontaneous rate of GDP release, thus acting as a guaninenucleotide dissociation inhibitor (GDI) (Brandt and Ross, 1985, Higashijima et al., 1987). The GDP-bound Gα represents the inactive state of the G-protein. Agonist-bound GPCRs act as guanine nucleotide exchange factors (GEFs), promoting the release of bound GDP from Gα. Nucleotide-free Gα binds GTP which is present at a significant molar excess over GDP in cells. The binding of GTP to Gα results in conformational changes within the three flexible switch regions of Gα designated switch I, II, and III (Sprang 1997, Wall et al., 1998) resulting in the dissociation of Gβγ. Both GTP-bound Gα and free Gβγ interact with downstream effector proteins. The intrinsic guanosine triphosphatase (GTPase) activity of the Gα subunit hydrolyzes bound GTP to GDP which turns Gα to its inactive state. Additionally,

the GTPase activity can be accelerated by a new family of GTPase-accelerating proteins (GAPs) for Gα proteins, which are called “regulators of G-protein signaling” or RGS proteins (Arshavsky and Pugh, Jr., 1998, Snow et al., 1999, Sondek and Siderovski, 2001). Reassociation of Gβγ with Gα-GDP terminates all effector interactions (Ford et al., 1998, Li et al., 1998). Thus, the model of GPCR signaling assumes that the duration of the hydrolysis of GTP to GDP in Gα subunit controls the time of signaling for both Gα-GTP and free Gβγ subunits.

Two bacterial toxins specifically interfere with the G-protein activation-inactivation cycle and are useful tools in studying G-protein-mediated signalling. Pertussis toxin, which is produced by *Bordetella pertussis*, catalyzes the adenosine diphosphate (ADP)-ribosylation of some G-proteins at a cysteine residue near the C-terminus resulting in uncoupling of receptor with the G-protein (Locht and Antoine, 1997; Nürnberg, 1997). Cholera toxin, from *Vibrio cholerae*, ADP-ribosylates the α-subunit of various G-protein subtypes at an internal arginine residue. This ribosylation leads to the constitutive activation of the Gα subunit by blocking the GTPase activity (Zhang et al., 1997).

1.2.1.1 Gα subunits

There are 16 Gα genes in the human genome which encode 23 known Gα proteins. These proteins can be divided into four major families based on sequence similarity: $G\alpha_{(s\ /\ olf)}$, $G\alpha_{(i1\ /\ i2\ /\ i3\ /\ o\ /\ t\text{-}rod\ /\ t\text{-}cone\ /\ gust\ /\ z)}$, $G\alpha_{(q\ /\ 11\ /\ 14\ /\ 16)}$ and $G\alpha_{(12\ /\ 13)}$ (Simon et al., 1991). Gα subunits range in size from 39 to 45 kilodaltons (kDa) (Nürnberg et al., 1995).

G-proteins of the $G\alpha_s$ family (Table 1.1) participate in GPCR-transduced stimulation of adenylyl cyclases. The two main members of this family that are known are $G\alpha_s$ and $G\alpha_{olf}$. The ubiquitously expressed $G\alpha_s$ gene (Gnas) gives rise to several splice variants. Four splice variants, two short forms ($G\alpha_{s(s)}$) and two long forms ($G\alpha_{s(l)}$), are closely related in structure and appear to be functionally indistinguishable. All adenylyl cyclase isoforms are activated by $G\alpha_s$ (Sunahara et al., 1996). Additional extra-long splice forms of $G\alpha_s$, termed xl and xxl, have been described. It is known that they are located at the plasma membrane, bind GTP, and can also regulate adenylyl cyclase activity. Whether extra-long splice forms of $G\alpha_s$ can be activated through GPCRs is not clear (Klemke et al., 2000, Pasolli et al., 2000, Bastepe et al., 2002). Another member of the $G\alpha_s$ family, $G\alpha_{olf}$ is expressed in olfactory sensory neurons and in the central nervous system (Luo et al., 2002).

The most widely expressed $G\alpha_i$ family members are $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, which mediate receptor dependent inhibition of various types of adenylyl cyclases (Offermanns, 2003).

Table 1.1: Classification and functional properties of Gα subunits

Family	Isoform[1]	Expression	Effectors[2]	PTX/CTX sensitivity[2]
G_s	$G\alpha_{s(s),\ s(l)}$	Ubiquitous	AC ↑	CTX
	$G\alpha_{s(xl),\ s(xxl)}$	Neuronal, neuroendocrine	AC ↑	CTX
	$G\alpha_{olf}$	Olfactory epithelium, brain, testes, pancreas	AC ↑	CTX
G_i	$G\alpha_{t(r)}$	Retinal rods, taste cells	cGMP-PDE ↑	CTX, PTX
	$G\alpha_{t(c)}$	Retinal cones	cGMP-PDE ↑	CTX, PTX
	$G\alpha_{gust}$	Taste cells, GI brush cells	PDE ↑?	CTX, PTX
	$G\alpha_{i1}$	Widely, preferentially neuronal	AC I, V, VI ↓, (GIRK ↑, PLCβ ↑?)	PTX, (CTX)
	$G\alpha_{i2}$	Ubiquitous	AC I, V, VI ↓, (GIRK ↑, PLCβ ↑, PI3K ↑)	PTX, (CTX)
	$G\alpha_{i3}$	Widely, preferentially non-neuronal	AC I, V, VI ↓, (GIRK ↑, PLCβ ↑, PI3K ↑?)	PTX, (CTX)
	$G\alpha_{o1,\ o2,\ o3}$	Neuronal, neuroendocrine	AC ↓, VDCC ↓, GIRK ↑, PLCβ ↑?	PTX, (CTX)
	$G\alpha_z$	Neuronal, endocrine, platelets	AC I, V ↓, (GIRK ↑, VDCC ↓)	
G_q	$G\alpha_q$	Ubiquitous	PLCβ ↑, Rho-GEF	
	$G\alpha_{11}$	Widely, not platelets	PLCβ ↑, Rho-GEF	
	$G\alpha_{14}$	Testes, haematopoetic cells and tissues	PLCβ ↑	
	$G\alpha_{15/16}$[3]	Haematopoetic cells and tissues	PLCβ ↑	
G_{12}	$G\alpha_{12}$	Ubiquitous	Rho-GEF ↑, Btk ↑, $Gap1^m$ ↑, cadherin	
	$G\alpha_{13}$	Ubiquitous	Rho-GEF ↑, radixin	

[1] (s) and (l) indicate short and long splice variants of $G\alpha_s$; (xl) and (xxl) indicate additional (extra)-long splice variants of $G\alpha_s$.

[2] ↑ = stimulation; ↓ = inhibition; AC, adenylyl cyclase; Btk, Bruton's tyrosine kinase; cGMP-PDE, cGMP-dependent phosphodiesterase; CTX, cholera-toxin-sensitive; (CTX), CTX-sensitive only in the presence of agonist-activated receptors; $Gap1^m$, Ras GTPase-activating protein; GI, gastrointestinal; GIRK, G-protein-regulated inward rectifier K^+ channel; PI3K, phosphatidylinositol 3-kinase; PLCβ, phospholipase Cβ; PTX, pertussistoxin-sensitive; Rho-GEF, guanine-nucleotide-exchange factor of the Rho GTPase; VDCC, voltage-dependent Ca^{2+} channel.

[3] $G\alpha_{15}$ and $G\alpha_{16}$ are the mouse and human homologous of the GNA15 gene product, respectively.

(from Nürnberg, 2004)

Another member of the $G\alpha_i$ family, $G\alpha_o$, is most abundant in neurons where it constitutes about 0.5 % of membrane protein. It is also present, although at lower levels, in other tissues such as heart, pituitary or pancreas. Despite the high abundance of $G\alpha_o$ in the nervous system, its function is still poorly understood. In contrast to other G-proteins it appears that most of the effects following G_o activation are mediated by the $G\beta\gamma$ heterodimer of this protein whereas the direct regulation of effectors by $G\alpha_o$ subunit is controversial (Anis et al., 1999, Exner et al., 1999, Offermanns, 2003). The pertussis toxin insensitive $G\alpha_z$ is expressed in various tissues such as the nervous system, platelets, or the adrenal gland (Table 1.1). It inhibits adenylyl cyclases types I and V, and it is recognized by several RGS proteins. Its physiological role is not completely clear. However, the data obtained in $G\alpha_z$-deficient mice have demonstrated a possible role of $G\alpha_z$ in platelet activation and in the CNS (Ho and Wong, 2001). The $G\alpha_i$ family also contains some α-subunits such as gustducin ($G\alpha_{gust}$) or transducin ($G\alpha_{t(r)}$, $G\alpha_{t(c)}$), which are involved in the regulation of gustatory and visual systems, respectively (Arshavsky et al., 2002, Margolskee, 2002). In addition, $G\alpha_i$ proteins are also found on endomembranes and hence may function as universal switches of a broad range of cellular processes (Nürnberg and Ahnert-Hilger, 1996, Ahnert-Hilger et al., 1998, Höltje et al., 2000, Gohla et al., 2007).

The $G\alpha_q$ family consists of four members whose α-subunits are encoded by four individual genes (Krumins and Gilman, 2006). They are all responsible for coupling of receptors to PLCβ isoforms in a pertussis toxin insensitive manner (Exton, 1996, Rhee, 2001). Although $G\alpha_q$ and $G\alpha_{11}$ appear to be expressed ubiquitously and widely, respectively, the murine $G\alpha_{15}$ and its human analog $G\alpha_{16}$ are only expressed in hematopoietic cells, and the expression of $G\alpha_{14}$ is limited to certain organs such as kidney, testis, and lung (Amatruda, III et al., 1991, Wilkie et al., 1991, Davignon et al., 2000).

The G-proteins $G\alpha_{12}$ and $G\alpha_{13}$ represent the $G\alpha_{12}$ family, and are expressed ubiquitously (Strathmann and Simon, 1990). It was shown that the guanine nucleotide exchange factors (GEFs) for Rho, p115RhoGEF, PDZ-RhoGEF and LARG are effectors of $G\alpha_{12}$ and $G\alpha_{13}$ (Gohla et al., 1999, Suzuki et al., 2003). In addition, various other proteins like Bruton's tyrosine kinase (Btk), Ras GTPase-activating protein ($Gap1^m$), cadherin or radixin have been shown to interact with $G\alpha_{12}$ and $G\alpha_{13}$ (Gohla et al., 2000, Meigs et al., 2002).

$G\alpha$ subunits are N-terminally modified by the covalent attachment of the fatty acids myristate and/or palmitate (Casey, 1994). All $G\alpha$ subunits except the photoreception-specific $G\alpha_t$, contain a 16-carbon palmitate attached by a reversible thioester bond to a cysteine near the N-terminus (Wedegaertner et al., 1995). Palmitoylation and/or irreversible myristoylation of $G\alpha$ subunits allows targeting to specific cell membrane regions and regulates interactions with other proteins such as adenylyl cyclase, $G\beta\gamma$, and GPCRs (Peitzsch and McLaughlin,

1993, Myung et al., 1999, Moffett et al., 2000). Although myristoylation also contributes to membrane localization, expression of myristoylated but not palmitoylated $G\alpha_{i/o}$ results in the localization of a large part of the $G\alpha$ subunits to the cytosol (Mumby et al., 1994).

1.2.1.2 Structure of Gβγ dimer

The Gβγ dimer is made up of two subunits, Gβ and Gγ. Nevertheless, functionally it acts as a monomer because the two subunits are considered not to dissociate physiologically. There are 5 human genes encoding Gβ isoforms (Clapham and Neer, 1997, Snow et al., 1998) and 12 human genes encoding Gγ isoforms (Table 1.2), which result in a high number of potential combinations of Gβγ dimers (Simon et al., 1991, Clapham and Neer, 1997).

Table 1.2: Diversity and expression of Gβ and Gγ subunits

Gβ subunits		**Gγ subunits**	
Isoform	***Expression***	***Isoform***	***Expression***
$G\beta_1$	Ubiquitous	$G\gamma_1$[1] ($G\gamma_{rod}$)	Retinal rods, neuronal
$G\beta_2$[1]	Ubiquitous	$G\gamma_2$ ($G\gamma_6$)	Widely, neuronal
$G\beta_3$	Widely, cone cells, taste cells	$G\gamma_3$	Widely, neuronal, haematopoietic cells
$G\beta_4$	Widely, brain, lung, placenta	$G\gamma_4$	Widely
$G\beta_5$[2]	Neuronal, lung, germ cells, lymph, ovary	$G\gamma_5$	Widely, placenta, liver
$G\beta_{5(l)}$[3]	Retina	$G\gamma_7$	Widely, brain, thymus, eye
		$G\gamma_8$ ($G\gamma_{olf}$)	Neuronal, olfactory epithelia
		$G\gamma_9$ ($G\gamma_{cone}$, $G\gamma_{14}$)	Retinal cones, neuronal
		$G\gamma_{10}$	Widely, brain, placenta
		$G\gamma_{11}$	Widely, non-neuronal
		$G\gamma_{12}$	Ubiquitous
		$G\gamma_{13}$	Widely, lingual and olfactory epithelium, neuronal

[1] $G\beta_2$ cannot form dimers with $G\gamma_1$.
[2] $G\beta_5$ forms dimers with certain regulators of G-protein signalling (RGS6, 7, 9, 11); dimerization of $G\beta_5$ with Gγ has been observed following recombinant expression of $G\beta_5$ and Gγ.
[3] "long" splice variant of $G\beta_5$.
(from Nürnberg, 2004)

The Gβ subunits are highly homologous, with $G\beta_1$ through $G\beta_4$ sharing 78-88 % identity of amino acids (Schwindinger and Robishaw, 2001). The $G\beta_5$ subunit differs from the other Gβ subunits in gene structure and shares only 51-53 % amino acid identity (Snow et al., 1998, Maier et al., 2000).

The Gγ subunits are more diverse than Gβ subunits. They share only 27-76 % amino acid identity (Huang et al., 1999, Schwindinger and Robishaw, 2001). All Gγ subunits are prenylated post-translationally at the C-terminus: $G\gamma_1$, $G\gamma_8$ and $G\gamma_{11}$ contain a 15-carbon farnesyl group, whereas all others harbor a 20-carbon geranylgeranyl group (Casey, 1994, Wedegaertner et al., 1995). This lipid modification of the Gγ polypeptide is important for the membrane localization of the Gβγ dimer.

The Gβγ heterodimer forms a stable structural unit (Fig. 1.2). All Gβ subunits contain seven WD-40 repeats, a tryptophan-aspartic acid sequence that repeats approximately every 40 amino acids and forms small antiparallel β strands (Neer et al., 1994).

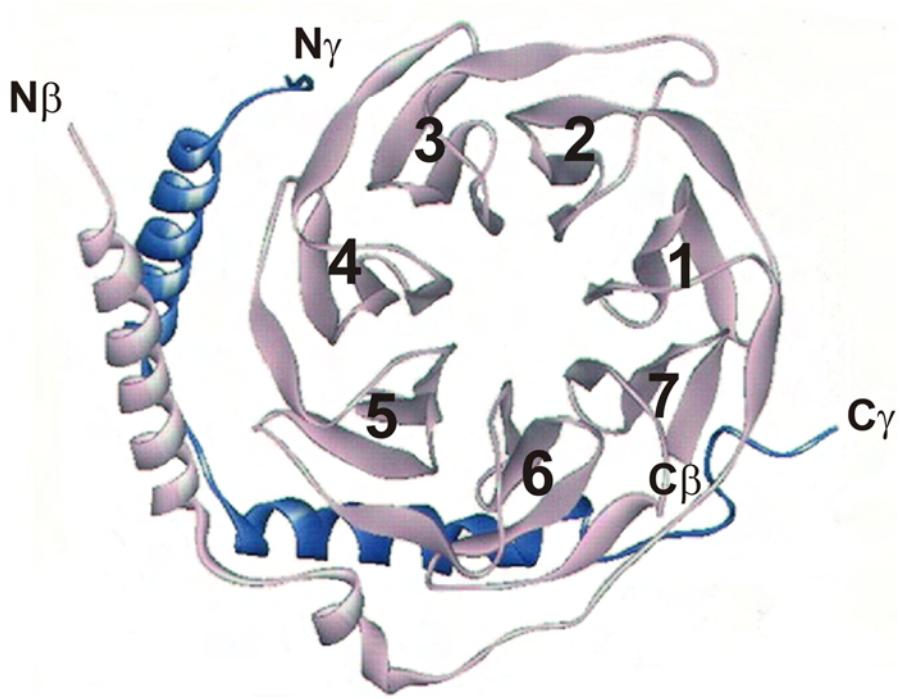


Fig. 1.2: The $G\beta_1\gamma_1$ heterodimer seen from the surface that faces Gα

The structure of the $G\beta_1\gamma_1$ dimer shows that Gβ (purple) forms a seven-blade propeller consisting of seven WD-40 repeats. The blades of $G\beta_1$ are numbered so that the first WD repeat occurs in blade 1. $G\gamma_1$ (blue) forms two α-helices that bind to the single α-helix of Gβ and to several of the WD-40 blades (Wall et al., 1995, Lambright et al., 1996).

Crystal structures of the Gβγ dimer and Gαβγ trimer revealed that the seven WD-40 repeats of the Gβ subunit fold into a seven-bladed β-propeller or torus-like structure. The N-terminus of Gβγ forms an α-helix (Gautam et al., 1998, Wall et al., 1998). Gγ subunit has two α-helices; the N-terminal helix forms a coiled-coil with the α-helix of Gβ, whereas the

C-terminal helix makes contacts with the base of the Gβ torus (Lambright et al., 1996, Sondek et al., 1996). In contrast to Gα subunit, the Gβγ dimer appears not to change conformation during the G-protein dissociation. (Sondek et al., 1996).

There is evidence suggesting the role of specific Gβγ combinations in receptor and effector coupling (Jones et al., 2004, Dingus et al., 2005). For example, $G\beta_1\gamma_1$ interacts more strongly with rhodopsin and phosducin than other Gβγ combinations (Müller et al., 1996, Hamm, 2001). $G\beta_2\gamma_2$ selectively mediates inhibition of α1H low-voltage-activated T-type (Cav3.2) calcium channels (Wolfe et al., 2003). $G\gamma_3$ subunit is important for coupling the somatostatin receptor to voltage-sensitive L-type calcium channels, whereas $G\gamma_4$ is required for coupling the muscarinic receptor to the same channels (Kleuss et al., 1993). The role of $G\gamma_7$ in β-adrenergic receptor signaling have been shown (Schwindinger et al., 2003).

1.2.1.3 Gβγ effectors

Previously, it was assumed that the Gβγ may solely act as a Gα inhibitor by facilitating Gαβγ reassociation. However, it is now appreciated that following dissociation of Gα-GTP, Gβγ is able to activate a large number of its own effectors (Clapham and Neer, 1997, Jones et al., 2004). The G-protein-regulated inward rectifier K^+ channels (GIRK or Kir3 channels) were the first identified Gβγ effectors (Logothetis et al., 1987). Since then, Gβγ has been found to regulate many different effectors (Table 1.3). In general, the mechanism of Gβγ interaction with its effectors is not entirely clear. Many Gβγ effectors contain pleckstrin homology (PH) domains; however, not all PH domain-containing proteins interact with Gβγ (McCudden et al., 2005).

1.2.2 Molecular interaction of Gβγ dimer with Gα subunit and effectors

The crystal structures of the Gαβγ heterotrimer revealed two regions on Gβγ that interact with Gα subunit (Wall et al., 1995, Lambright et al., 1996). The first Gα/Gβ interface is between the N-terminal helix of Gα and the first blade of the Gβ propeller (Gβ residues Leu-55, Lys-78, Ile-80, and Lys-89). The second interface between Gα and Gβγ is made up of residues on the top surface of the Gβ torus (Gβ residues Lys-57, Tyr-59, Ser-98, Trp-99, Met-101, Leu-117, Asn-119, Thr-143, Asp-186, Asp-228, and Trp-332) (Ford et al., 1998, Li et al., 1998). Previously, it was found that Gα subunits turn off the Gβγ-dependent activation of effectors (Clapham and Neer, 1997, Ford et al., 1998).

Table 1.3: Gβγ-dependent effectors

Effector	Response to Gβγ	Reference
ACI	↓	(Sunahara et al., 1996)
ACII, IV[(1)], VII[(1)]	↑	(Sunahara et al., 1996)
GIRK1-4	↑	(Coetzee et al., 1999)
N-, P/Q-, R-, T-type VDCC	↓	(Vignali et al., 2006)
$PLC\beta_{1-3}$	↑	(Exton, 1997)
PLA_2	↑	(Jelsema and Axelrod, 1987)
PI3K-β[(2)],-γ	↑	(Maier et al., 1999)
GRK2,3	↑	(Tesmer et al., 2005)
Raf-1	↑	(Pumiglia et al., 1995)
$p140^{Ras\text{-}GEF}$	↑	(Mattingly and Macara, 1996)
P-Rex1[(3)]	↑	(Mayeenuddin et al., 2006)
Btk	↑	(Tsukada et al., 1994)
Tsk	↑	(Langhans-Rajasekaran et al., 1995)
Phosducin, phosducin-like proteins	(-)[(4)]	(Schulz, 2001)

[1] AC activity is superactivated by Gβγ only if coactivated by $G\alpha_s$.
[2] Stimulation has been demonstrated under in vitro conditions only.
[3] P-Rex1, a guanine-nucleotide-exchange factor of the Rac GTPase, is synergistically activated by phosphatidylinositol-3,4,5-trisphosphate and Gβγ.
[4] Phosducin and phosphoducin-like proteins regulate G-protein-mediated signalling by binding to Gβγ and removing the dimer from cell membranes.
↑ = stimulation; ↓ = inhibition; AC, adenylyl cyclase; Btk, Bruton's tyrosine kinase; GIRK, G-protein-regulated inward rectifier K^+ channel; GRK, G-protein-coupled-receptor-kinase; $p140^{Ras\text{-}GEF}$, guanine-nucleotide exchange factor of the Ras GTPase; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; Raf-1, member of the raf/mil subfamily of serine–threonine protein kinases; Tsk, interleukin-2 (IL-2)-inducible tyrosine kinase (Itk); VDCC, voltage-dependent Ca^{2+} channel
(from Nürnberg, 2004)

This observation suggests that the sites on Gβγ for binding Gα and Gβγ effectors may overlap. The mutagenesis of the amino acids of Gβ subunit which contact Gα has little or no effect on the capability of Gβγ to properly fold and assemble. However, mutations of these residues have differential effects on the ability of Gβγ to activate its effectors. Results suggest that the Gα subunit and Gβγ effectors share at least part of their binding sites on the surface of the Gβγ subunit (Clapham and Neer, 1997, Sprang, 1997, Ford et al., 1998, Li et al., 1998).

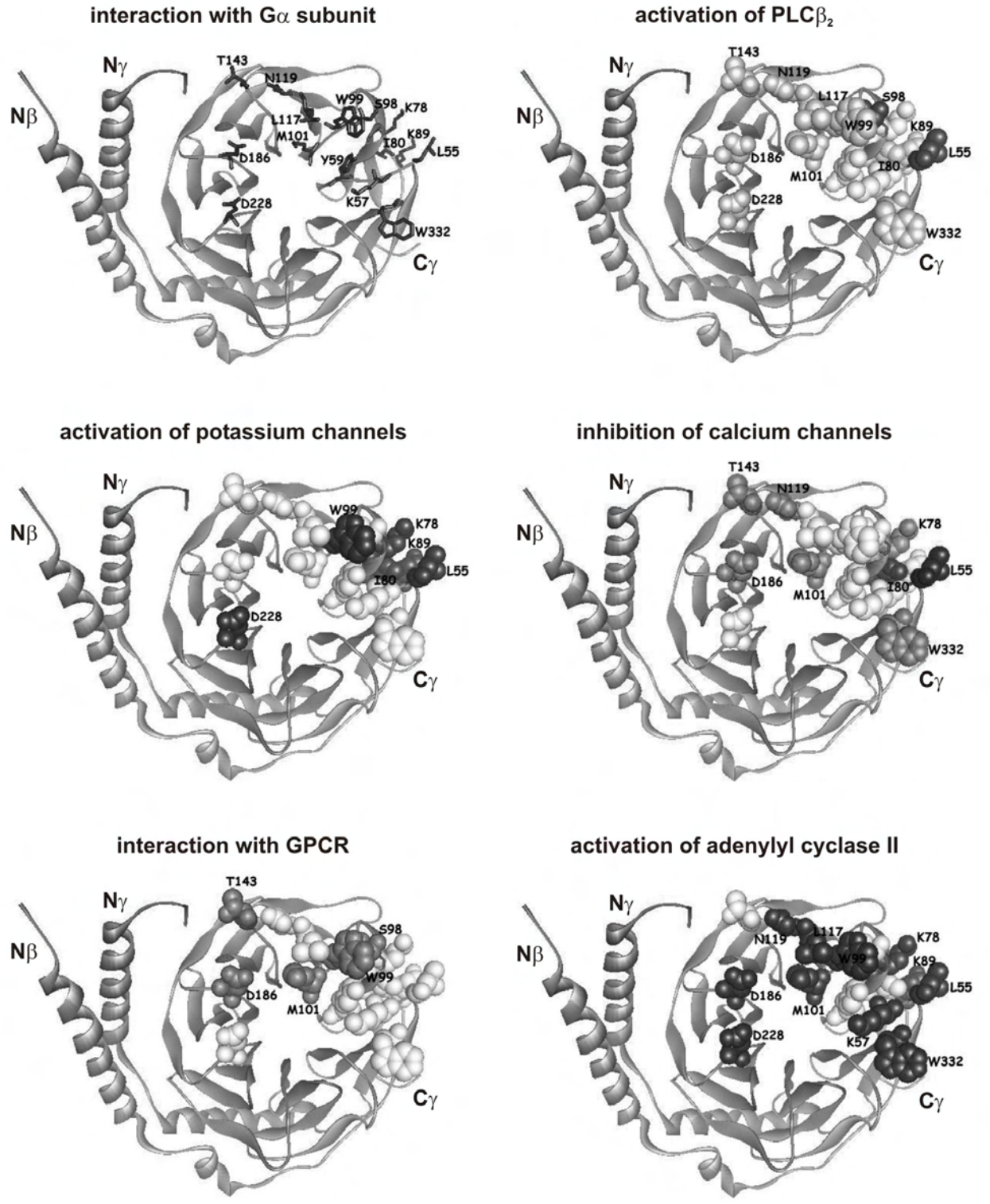


Fig. 1.3: A schematic representation of the regions of $G\beta_1$ involved in interaction with effectors and Gα subunit

$G\beta_1$ residues important for interaction with Gα subunit were substituted with alanine, and each $G\beta_1$ mutant was expressed with either $G\gamma_1$ or $G\gamma_2$. Amino acids of $G\beta_1$ that involved in interaction with effectors are indicated by grey or black colours; those with activity indistinguishable to the wild type are indicated by white colour.
(from Ford et al., 1998).

The crystal structure of $G\beta_1\gamma_2$ bound to a SIGK (SIGKAFKILGYPDYD), a peptide derived from the phage display screen, at a resolution of 2.7 Å showed that the peptide is a structural mimic of the Gβ interacting switch II flexible region of the Gα, and occupies a site on $G\beta_1$ subunit that is used by several Gβγ binding effectors (Davis et al., 2005).

Fig. 1.3 shows that the effector "fingerprint" regions are located on Gβ subunit such that they partially overlap. This allows for one key regulator, namely Gα, to regulate Gβγ signal transduction to different effectors.

1.3 Phosphoinositide 3-kinases (PI3Ks)

1.3.1 The PI3K family

Phosphoinositide (PI) 3-kinase (PI3K) was first observed in 1984 as a minor inositol lipid kinase activity which was associated with immunoprecipitated oncogene products, such as Src, Abl and polyoma mT antigen (Macara et al., 1984, Sugimoto et al., 1984, Whitman et al., 1985). Additionally, it was present in activated growth factor receptor complexes such as the PDGF receptor (Kaplan et al., 1987). Nowadays there are many known PI3Ks which were identified by biochemical approaches and cloning strategies. PI3Ks have been isolated from diverse eukaryotes, including mammals, yeast, flies, slime mold, plants and algae. These enzymes phosphorylate the D3 position of the inositol ring of phosphatidylinositol or phosphoinositides (Fig. 1.4) (Fruman et al., 1998, Vanhaesebroeck et al., 2001). PI3Ks are divided into three classes. This classification is based on the sequence data, type of adaptor proteins and substrate specificity of enzymes (Table 1.4) (Toker and Cantley, 1997, Wymann and Pirola, 1998, Vanhaesebroeck et al., 2001, Foster et al., 2003, Piekorz and Nürnberg, 2004, Nürnberg and Jeanclos, 2007). All PI3K catalytic subunits have a homologous region that consists of a catalytic core domain (HR1; homology region 1) linked to the PIK or PI kinase homology domain (HR2) and a C2 domain (HR3) (Walker et al., 1999, Djordjevic and Driscoll, 2002).

1.3.1.1 Class I PI3Ks

Four distinct protein species of approximately 110 kDa, which include p110α, p110β, p110δ and p110γ, belong to the class I family of PI3K enzymes. All class I enzymes share the majority of their structural organization and a common substrate specificity (Rameh and Cantley, 1999, Fry 2001, Katso et al., 2001). In vitro, all class I PI3Ks are capable of

phosphorylating the D3 position of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2), producing phosphatidylinositol-3-phosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2), and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3), respectively. However, PI-4,5-P_2 is the preferred lipid substrate in vivo (Fig. 1.4) (Stephens et al., 1991, Hawkins et al., 1992, Vanhaesebroeck et al., 2001).

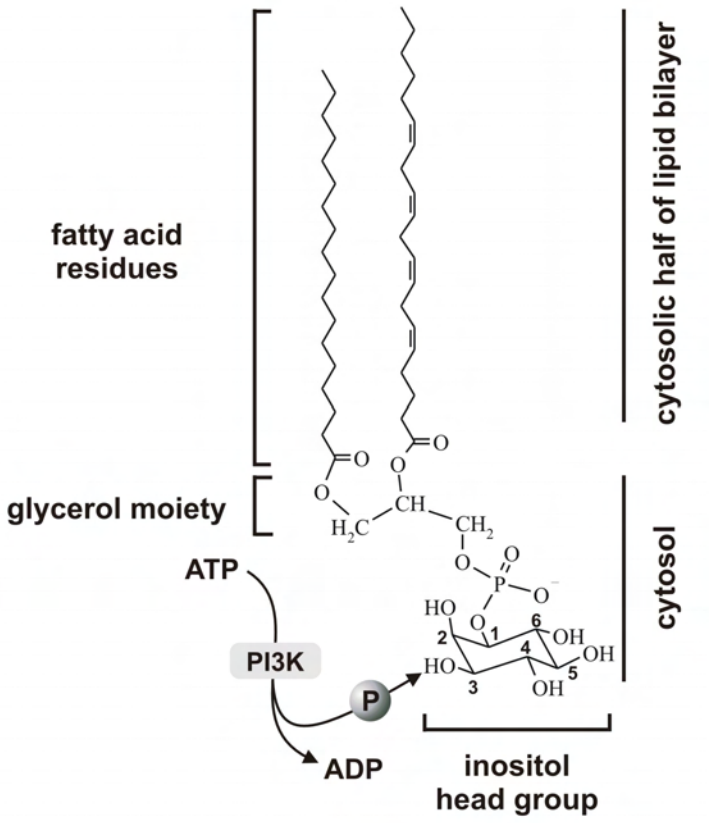


Fig. 1.4: PI3Ks phosphorylate the D3 position of the inositol ring of phosphoinositides

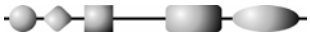
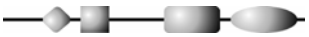
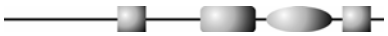
Phosphoinositides belong to the class of glycerophospholipids (or phosphoglycerides). Phosphoinositides consist of sn-glycerol-3-phosphate esterified at its C1 and C2 positions to fatty acids and its phosphoryl group to phosphorylated or non-phosphorylated myo-inositol. Phosphoinositides are therefore amphiphilic molecules with a nonpolar aliphatic "tail" and a polar phosphoryl-inositol "head". The PI3Ks transfer the γ-phosphate from ATP to the D3 position of inositol ring.

Class I PI3Ks are mainly cytosolic in resting cells, but upon stimulation they are recruited to membranes via interactions with receptors or adaptor proteins. Moreover, there are evidences that class I PI3Ks may not only be associated with plasma membranes but also with vesicular and nuclear membranes (Rameh and Cantley, 1999, Vanhaesebroeck et al., 2001). Class I PI3Ks are involved in regulation of different cellular functions such as cell motility, survival, differentiation, and proliferation (Fry, 2001, Katso et al., 2001). The class I PI3Ks are subdivided into two groups on the basis of their regulatory proteins and mechanisms of activation.

1.3.1.1.1 Class I_A PI3Ks

There are three class I_A catalytic subunits: p110α, p110β, and p110δ. All of these PI3Ks interact with a family of Src homology 2 (SH2)-domain-containing regulatory adaptor proteins, i.e. p85 proteins. Three distinct genes encode the p85α, p85β and p55γ adaptors, each with a number of splice variants (Vanhaesebroeck et al., 1997a, Wymann and Pirola, 1998).

Table 1.4: Structural features of PI3K family members

Class	Modular structure of catalytic subunits	Subunits		Lipid substrates	
		catalytic	*non-catalytic*	*in vitro*	*in vivo*
I_A		p110α p110β p110δ	p50α p55α,γ p85α,β	PI PI-4-P PI-4,5-P_2	PI-4,5-P_2
I_B		p110γ	p101 p84 ($p87^{PIKAP}$)		
II		PI3K-IIα PI3K-IIβ PI3K-IIγ PI3K68D	Grb2 Clathrin	PI PI-4-P	?
III		Vps34p	Vps15p p150	PI	PI

p85-binding domain · Ras-binding domain · PIK domain · Kinase domain · C2 domain

(modified from Vanhaesebroeck et al., 2001)

The kinase core domain is found in all PI3Ks. This domain exhibits weak homology to protein kinases. C2 domains in other proteins mediate interactions with lipids or with other proteins. The existance of this domain in the PI3K suggests a role in membrane binding and possibly substrate targeting (Walker et al., 1999). The Ras-binding domain has only been found in class I and II PI3Ks. The p85-binding domain belongs to class I_A PI3K.

The adaptor proteins bind to phosphorylated tyrosine residues that are generated by activated tyrosine kinases of receptors and to various receptor-associated adaptor proteins. The class I_A adaptors prefer to bind to proteins containing a phosphotyrosine within a Y(P)xxM motif, where "x" is any amino acid (Nolte et al., 1996, Breeze et al., 1996). Phosphotyrosine binding

of adaptor protein results in translocation of the cytosolic PI3Ks to the membranes where their lipid substrates reside. Furthermore, class I_A PI3Kβ is also sensitive to Gβγ dimer under in vitro conditions (Kurosu et al., 1997, Maier et al., 1999, Czupalla et al., 2003a). Therefore, PI3Kβ may integrate tyrosine kinase- and G-protein-dependent signals.

Whereas p110α and β are widely distributed in mammalian tissues, p110δ is mainly found in leukocytes (Vanhaesebroeck et al., 1997b). Class I_A PI3K family members have not been found in yeast or plants (Vanhaesebroeck et al., 1997a). The biological roles of individual isoforms of class I_A PI3K are distinct but, possibly, overlapping. They are linked to regulation of cell size in Drosophila and in mammals (Kozma and Thomas, 2002, Caldwell et al., 2005). The p110α isoform regulates the size of the adult heart (Crackower et al., 2002). Murine knockouts of the p110α or p110β genes result in embryonic lethality thereby underlining the necessity of these isoforms (Bi et al., 1999, 2002). Reports suggest that p110α may play a role in cell survival whereas p110β may be more important in promoting cell proliferation (Benistant et al., 2000). The data suggest a role for p110α in various cancers (Fry, 2001). Studies with p110δ knock-in and knock-out mice demonstrate its critical role for full B- and T-cell antigen receptor signalling (Jou et al., 2002, Okkenhaug et al., 2002, Okkenhaug and Vanhaesebroeck, 2003, Bilancio et al., 2006).

1.3.1.1.2 Class I_B PI3Ks

Previously, it was shown that neutrophils exposed to a formylated tripeptide, N-formyl-Met-Leu-Phe (fMLP), rapidly produce PI-3,4,5-P_3 (Traynor-Kaplan et al., 1988, 1989, Stephens et al., 1993). Although fMLP binds to a seven transmembrane helix receptor, it was not clear how this agonist stimulated PI-3,4,5,-P_3 formation. Then, a novel PI3K isoform, PI3Kγ, present in white blood cells was shown to be activated by Gβγ subunits (Thomason et al., 1994, Stoyanov et al., 1995, Stephens et al., 1997, Tang and Downes, 1997). There is only one class I_B catalytic subunit, p110γ, which reveals similarities in homology regions to the class I_A PI3K, but without an N-terminal p85-binding site (Stoyanov et al., 1995, Stephens et al., 1997). Instead, p110γ was found associated with a 101 kDa protein termed p101 (Stephens et al., 1997). Nowadays, the role of the p101 subunit in the activation of PI3Kγ by Gβγ is controversial. p101 has been proposed to be essential in conferring p110γ sensitivity to Gβγ dimers (Stephens et al., 1997). Others have reported that p101 suppresses Gβγ-induced phosphorylation of PI, and concurrently facilitates the Gβγ-induced activation in the presence of PI-4,5,-P_2 (Maier et al., 1999).

Both subunits of PI3Kγ bind Gβγ in vitro, albeit p101 does so with at least a 5-fold higher affinity (Stephens et al., 1997, Maier et al., 1999). Deletion studies have demonstrated

that p110γ harbors at least two Gβγ-binding regions (Leopoldt et al., 1998). One binding region is N-terminally located whereas the other is close to the catalytic core (HR1), indicating that the PI3Kγ represents a horse shoe conformation (Vanhaesebroeck et al., 1996, Leopoldt et al., 1998). Furthermore, an N-terminal domain of p101 play role in heterodimerization with the p110γ subunit, whereas Gβγ dimer binds to a C-terminal domain of this regulatory subunit (Voigt et al., 2005).

Recent publications have described a second regulatory subunit of p110γ termed p84 (Suire et al., 2005) or $p87^{PIKAP}$ (Voigt et al., 2005, 2006). This regulatory subunit exhibits only 30 % homology with p101 and is mainly expressed in cells of haematopoietic origin. p84 ($p87^{PIKAP}$) can bind to p110γ in vitro and in vivo, and by doing so it significantly increases the activation of p110γ by Gβγ. Nevertheless, the Gβγ-dependent stimulation of p110γ/p101 is more potent than of p110γ/$p87^{PIKAP}$.

Class I_B PI3K was found only in mammals, where it shows a limited tissue distribution, being abundant only in white blood cells. This may explain why GPCRs do not induce the production of PI-3,4,5,-P_3 in all cell types (Vanhaesebroeck et al., 2001).

1.3.1.2 Class II PI3Ks

Class II PI3Ks were identified by using PCR cloning approaches. Therefore, they are the least well understood class of PI3K (Fry, 2001). The Drosophila PI3K68D was the first member of the class II PI3Ks (MacDougall et al., 1995, 2004). Members of this class are 170-210 kDa in size and exclusively phosphorylate PI and PI-4-P in vitro (Domin et al., 1997, 2005). Class II PI3Ks display homologies with class I enzymes in HR1 to HR4 regions, but have different N-terminus (Wheeler and Domin, 2006).

Following the identification of PI3K68D in Drosophila, numerous mammalian counterparts were cloned. Mammalian class II PI3Ks are subdivided in three subclasses: PI3K-IIα, PI3K-IIβ, and PI3K-IIγ (Table 1.4). In contrast to class I PI3Ks, the class II PI3Ks are constitutively associated with membrane structures by adapter proteins, such as Grb2 and clathrin (Wheeler and Domin 2001, 2006). Accordingly, the class II PI3Ks are associated with plasma membranes, intracellular membranes and nuclei (Didichenko and Thelen, 2001, Fry, 2001). The class II PI3Ks are activated by several known extracellular signals such as growth factors and chemokines (Brown and Shepherd, 2001). The mechanism of activation and the cellular role of these enzymes are currently less clear.

1.3.1.3 Class III PI3Ks

Class III PI3Ks are represented by the Saccharomyces cerivisiae PI3K, Vps34 (Fry, 1994, Wurmser et al., 1999, Odorizzi et al., 2000). This enzyme phosphorylates only PI, and likely is responsible for the production of most of the PI-3-P in cells (Table 1.4) (Vanhaesebroeck et al., 2001). The class III PI3K catalytic subunit has been identified in all eukaryotic species. In yeast and mammals, this catalytic subunit exists in a complex with a Ser/Thr protein kinase, i.e. Vps15p in yeast and p150 in mammals, respectively (Panaretou et al., 1997, Foster et al., 2003). Class III PI3Ks are located predominantly on intracellular membranes. This class of PI3Ks play roles in the fusion of endosomes (Wurmser et al., 1999), autophagy (Kihara et al., 2001), formation of phagosomes (Vieira et al., 2001), transport at the nuclear membrane (Roggo et al., 2002).

1.3.2 Regulation of PI3Kγ activity

1.3.2.1 Gβγ-dependent stimulation of lipid and protein kinase activity of PI3Kγ

The G-protein-regulated PI3Kγ is assumed to transmit specific extracellular signals through its inherent lipid and protein kinase activities, making PI3Kγ a bifunctional enzyme (Fig. 1.5). Gβγ dimers which dissociate from their Gα subunits by the action of a stimulated GPCR, simultaneously recruit PI3Kγ to the plasma membrane and allosterically stimulate either enzymatic activity. Our group has shown that Gβγ activates membrane associated dimeric and monomeric PI3Kγ, p110γ/p101 and p110γ, respectively, in vitro and in vivo (Leopoldt et al., 1998, Maier et al., 1999, 2000, Brock et al., 2003, Czupalla et al., 2003a). These lipid kinases do not only generate intracellular lipid second messengers (see Fig. 1.5) but also possess an intrinsic protein kinase activity in vitro (Carpenter and Cantley, 1998, Czupalla et al., 2003a, Foukas and Shepherd, 2004). Studies by Bondeva et al. (1998) imply that two different effector pathways are regulated upon activation of PI3K: lipid kinase activity activates protein kinase B (PKB) and protein kinase activity activates the mitogen-activated protein kinase (MAPK) pathway. The role of the protein kinase activity of PI3Kγ is not well understood; however, there is evidence that PI3Kγ also phosphorylates the cytoskeletal non-muscle tropomyosin (Naga Prasad et al., 2005). The authors have proposed that phosphorylation of non-muscle tropomyosin by PI3Kγ is necessary for the proper actin cytoskeletal remodelling that is important for the β_2-adrenergic receptor internalization.

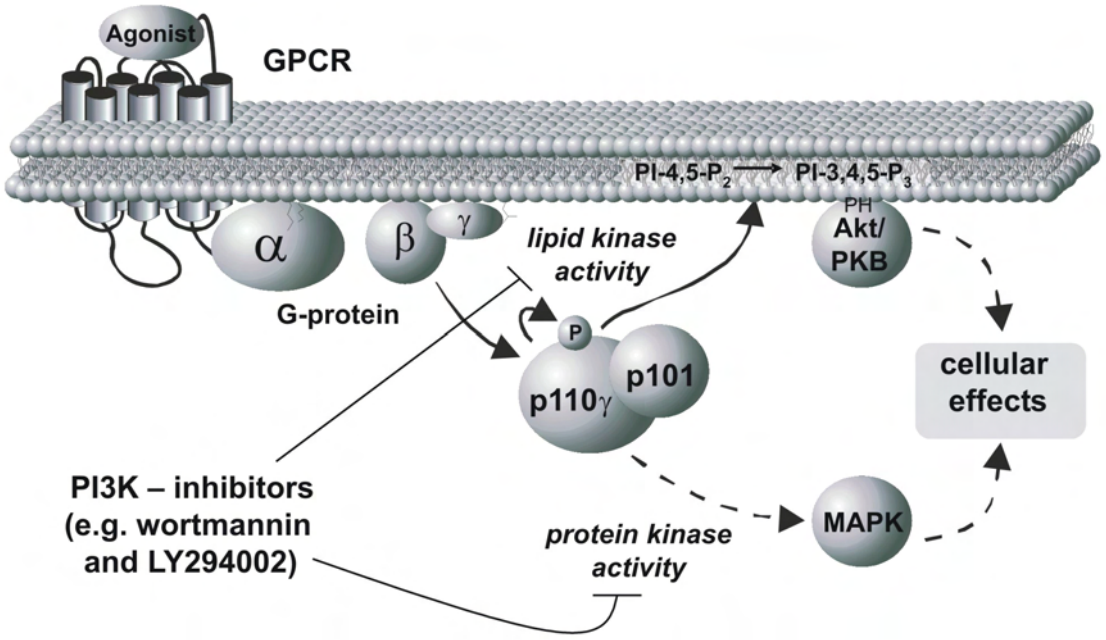


Fig. 1.5: Schematic representation of the signal transduction of PI3Kγ

Upon GPCR stimulation, the G-protein dissociates into free Gα and Gβγ subunits. Gβγ recruits PI3Kγ to the plasma membrane, and allosterically stimulates lipid and intrinsic protein kinase activities of this enzyme. PI3Kγ phosphorylates the phosphoinositides at the D3 position of the inositol ring, thus generating an intracellular phospholipid second messenger. PI3Kγ phospholipid products transmit signals by recruiting intracellular effector molecules to the membrane, which contain PH domain. Wortmannin and LY294002 inhibit lipid and protein kinase activities in vitro.

An additional type of Gβγ-induced protein kinase activity, autophosphorylation, has been found (Stoyanova et al., 1997, Maier et al., 1999). Gβγ induces the autophosphorylation of PI3Kγ catalytic subunit at serine-1101 (Czupalla et al., 2003a, b). It is assumed that autophosphorylation of p110γ has functions distinct from its lipid kinase activity. Czupalla et al. (2003a) proposed the existence of PI3Kγ binding partners that specifically interact with the autophosphorylated form of PI3Kγ. Nevertheless, the functional relevance of PI3Kγ autophosphorylation is not clear.

1.3.2.2 PI3Kγ inhibitors

PI3Kγ seems to be a promising drug target for the treatment of chronic inflammatory disorders. In order to validate the PI3Kγ as a target for human therapy researchers invented a set of tools, including genetically modified mice (Hirsch et al., 2000, Sasaki et al., 2000, Patrucco et al., 2004), neutralizing antibodies (al Aoukaty et al., 1999) and small-molecular inhibitors (Arcaro and Wymann, 1993, Vlahos et al., 1994, Ohashi and Woodgett, 2005). Wortmannin and LY294002 belong to the first generation of PI3Ks inhibitors (Fig. 1.6).

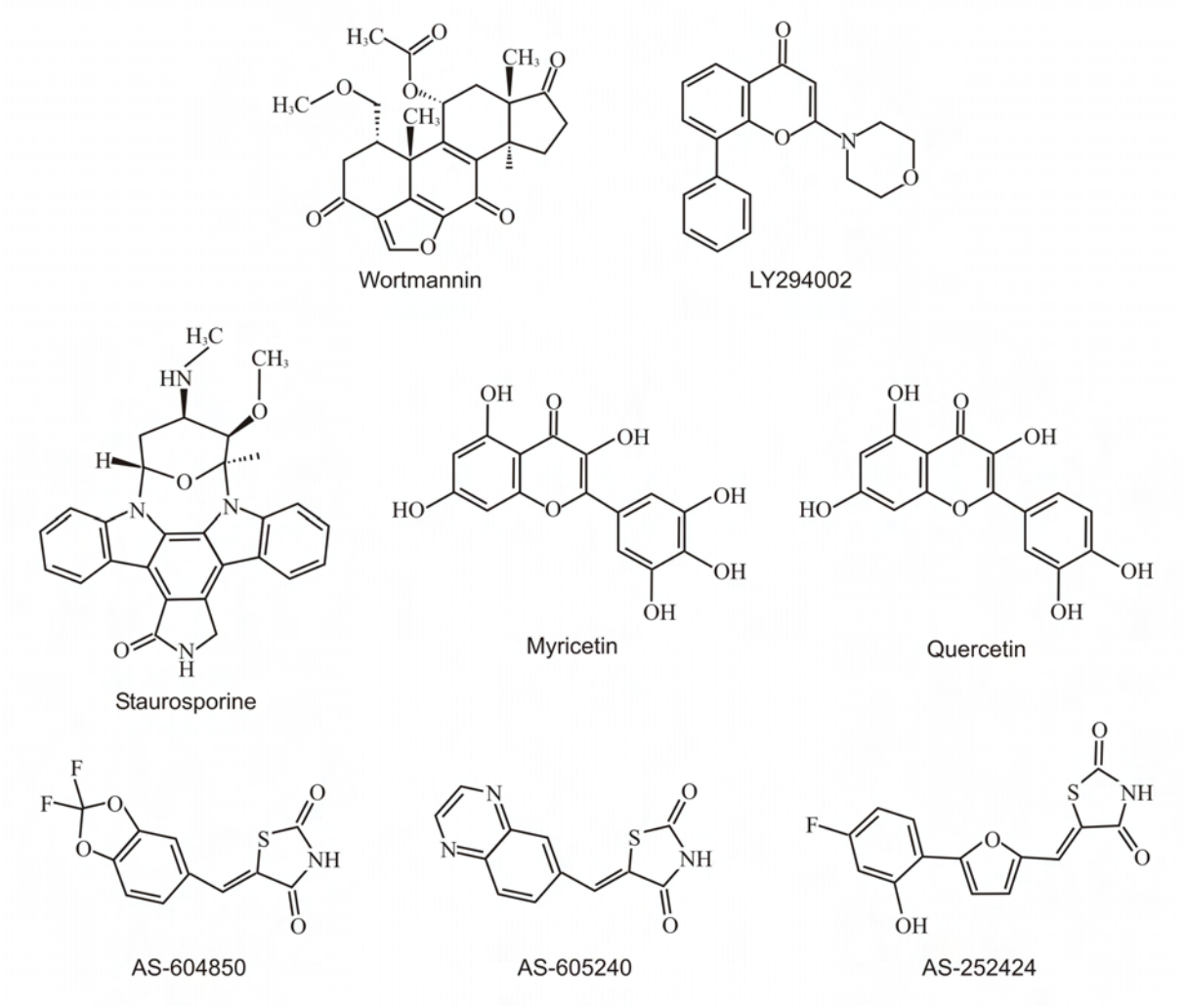


Fig. 1.6: Inhibitors of PI3K

Wortmannin, LY294002, staurosporine, myrecitin, and quercetin are inhibitors which lack the PI3K isoform selectivity (Powis et al., 1994, Walker et al., 2000, Pomel et al., 2006). AS-604850, AS-605240, and AS-252424 are selective inhibitors of PI3Kγ (Camps et al., 2005, Barber et al., 2005, Pomel et al., 2006).

The later are cell-permeable compounds which are widely used for investigation of the cellular functions of PI3Ks (Arcaro and Wymann, 1993, Vlahos et al., 1994, Ui et al., 1995). Wortmannin, a fungal metabolite, is the most potent PI3K inhibitor. In the ATP binding pocket of PI3Kγ, wortmannin is bound more tightly than ATP since it covalently reacts with a Lys-802 of p110α or a Lys-833 of p110γ (Wymann et al., 1996, Walker et al., 2000). Therefore, this inhibitor changes the conformation of the PI3Kγ active site (Walker et al., 2000, Vanhaesebroeck et al., 2001). Wortmannins in vitro 50 % inhibitory concentration (IC_{50}) is approximately 5 nM for PI3Ks (Powis et al., 1994, Maier et al., 1999, Vanhaesebroeck et al., 2001). Wortmannin exhibits a similar potency of inhibition for all PI3Ks with the exception of PI3K-C2α, which is 10-fold less sensitive (Domin et al., 1997, Stein and Waterfield, 2000, Vanhaesebroeck et al., 2001). The disadvantage of wortmannin is that it has relatively short half-life (Jones et al., 1999). Therefore, this substance is not very

suited for prolonged experiments. Moreover, wortmannin also inhibits PI3K-related enzymes such as the mammalian target of rapamycin (mTOR), nuclear protein kinase ataxia telangiectasia mutated (ATM), and DNA-dependent protein kinase (DNA-PK). Nevertheless, the IC_{50} for these enzymes is 20- to 100-fold higher than for PI3K inhibition (Brunn et al., 1996, Banin et al., 1998, Izzard et al., 1999). Wortmannin shows a similar potency of inhibition for lipid and protein kinase activities of PI3K in vitro (Stoyanova et al., 1997). There are another natural PI3K inhibitors, such as quercetin, myricetin, and staurosporin (Walker et al., 2000). However, these compounds are not highly selective to PI3K and may also inhibit a broad range of other protein kinases (Vanhaesebroeck et al., 2001, Pomel et al., 2006).

LY294002, which is illustrated in Fig. 1.6, was the first synthetic flavonoid-based compound (Vlahos et al., 1994). This is the most widely experimentally used PI3K inhibitor because of the short half-life of wortmannin (Jones et al., 1999). The PI3Ks of all classes, with the exception of PI3K-C2α, have a similar in vitro sensitivity to LY294002 with an IC_{50} of approximately 1 μM (Vanhaesebroeck et al., 2001). In contrast to wortmannin, LY294002 does not covalently bind to the PI3Kγ active site (Walker et al., 2000).

The usefulness of wortmannin and LY294002 has been restricted because of their lack of specificity to the different isoforms of PI3K and their high toxicity (Berrie, 2001, Sodhi et al., 2004). Therefore, in recent times, the synthesis of PI3K isoform-selective small-molecule inhibitors have been initiated (Rückle et al., 2006). Camps et al. (2005) identified two inhibitory compounds, AS-604850 and AS-605240, that exhibit high selectivity towards PI3Kγ (Fig. 1.6). The authors have demonstrated that PI3Kγ-deficient mice are protected in mouse models of rheumatoid arthritis. Interestingly, oral administration of AS-605240, which is more potent as compared to AS-604850, exhibited the same effect. This study showed that AS-605240 and, perhaps, AS-604850 are potential molecules for the treatment of chronic inflammatory disorders such as rheumatoid arthritis. Additionally, Barber et al. (2005) examined AS-605240 in MRL-lpr mice which develop symptoms of an autoimmune lupus erythematosus - like disease with early onset. It was shown that treatment of MRL-lpr mice with AS-605240 reduced glomerulonephritis and prolonged lifespan, suggesting that this compound may be a useful tool in the treatment of systemic lupus erythematosus. Pomel et al. (2006) identified another selective small-molecule PI3Kγ inhibitory compound, AS-252424 (Fig. 1.6). Oral administration of AS-252424 in a mouse model of acute peritonitis led to a similar reduction in leukocyte infiltration as was observed in PI3Kγ-deficient mice.

Currently, there are a number of PI3K isoform-selective compounds described in the patent literature (Rückle et al., 2006 and references within). However, no PI3Kγ specific inhibitor has yet entered to the clinical trials.

1.3.2.3 The role of PTEN and SHIP in regulation of PI3Kγ activity

Upon activation of PI3Ks by diverse extracellular stimuli, the concentration of PI-3,4,5-P_3 dramatically increases thereby recruiting pleckstrin homology (PH) domain-containing proteins to the plasma membrane (see section 1.3.3) and thus initiating various signaling pathways. In order to suppress or terminate these pathways, specific phosphatases are involved in the metabolism of phosphoinositides (Leslie et al., 2001, Leslie and Downes, 2004).

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a phosphatase, which dephosphorylates all 3-phosphorylated PIs (Fig. 1.7) and Ins-1,3,4,5-P_4 (Katso et al., 2001, Vanhaesebroeck et al., 2001, Sly et al., 2003). It was shown that PTEN locates at the plasma membrane (Gjorloff-Wingren et al., 2000, Leslie and Downes, 2004), in the cytosol (Li and Sun, 1997), and in the nucleus of some cells (Sly et al., 2003, Wu et al., 2003). PTEN exhibits a tumor suppression activity in cells by reducing level of PI-3,4,5-P_3. Dephosphorylation of this phosphoinositide results in suppression of Akt/PKB. Inhibition of Akt/PKB allows transcription factors to up-regulate the cell cycle inhibitor p27 (Li and Sun, 1997, Sly et al., 2003). Almost 50 % of human cancers bear inactivating mutations of both alleles of PTEN (Cantley and Neel, 1999). The role of PTEN was also linked with reduced ability of cells to form focal adhesions, migrate, and respond to integrin-induced cell distribution (Tamura et al., 1999a, b, Sly et al., 2003).

The SH2-containing inositol 5-phosphatases (SHIP1 and SHIP2) hydrolyze the 5-phosphate from PI-3,4,5-P_3 (Fig. 1.7) (Damen et al., 1996, Blero et al., 2005) and Ins-1,3,4,5-P_4 (Valderrama-Carvajal et al., 2002). SHIP1 is predominantly expressed in cells of hematopoietic origin and plays role in signaling in immune system (Geier et al., 1997, Liu et al., 1998, Vanhaesebroeck et al., 2001). SHIP2 is more widely expressed than SHIP1 (Sly et al., 2003). SHIP2 is a potent negative regulator of insulin signalling and plays role in diabetes (Clement et al., 2001).

1.3.3 The targets and biological significance of 3-phosphoinositides

As discussed above, the interaction of the ligand with its receptor promotes the production of PI-3,4,5-P_3. Subsequently, specific phosphoinositide 5-phosphatases, such as SHIP (see section 1.3.2.3), produce PI-3,4-P_2 by dephosphorylating PI-3,4,5-P_3 (Katso et al., 2001, Sly et al., 2003). PI-3,4-P_2 and PI-3,4,5-P_3 are the two major 3-phosphoinositides which participate in transducing of PI3K signaling. Different intracellular proteins, which act as PI3K-signaling effectors, contain the pleckstrin homology (PH) lipid-binding domain

(Wymann and Pirola, 1998, Rameh and Cantley, 1999). PH domains bind to the phosphoinositides thereby facilitating the recruitment of intracellular effectors with the membrane structures (Fig. 1.7). PI-3,4-P_2- and/or PI-3,4,5-P_3-bound PH domain-containing effectors become activated and regulate important cellular processes, such as survival pathways, the regulation of gene expression, metabolism and cell motility (Rameh and Cantley, 1999, Katso et al., 2001, Vanhaesebroeck et al., 2001, Wymann et al., 2003)

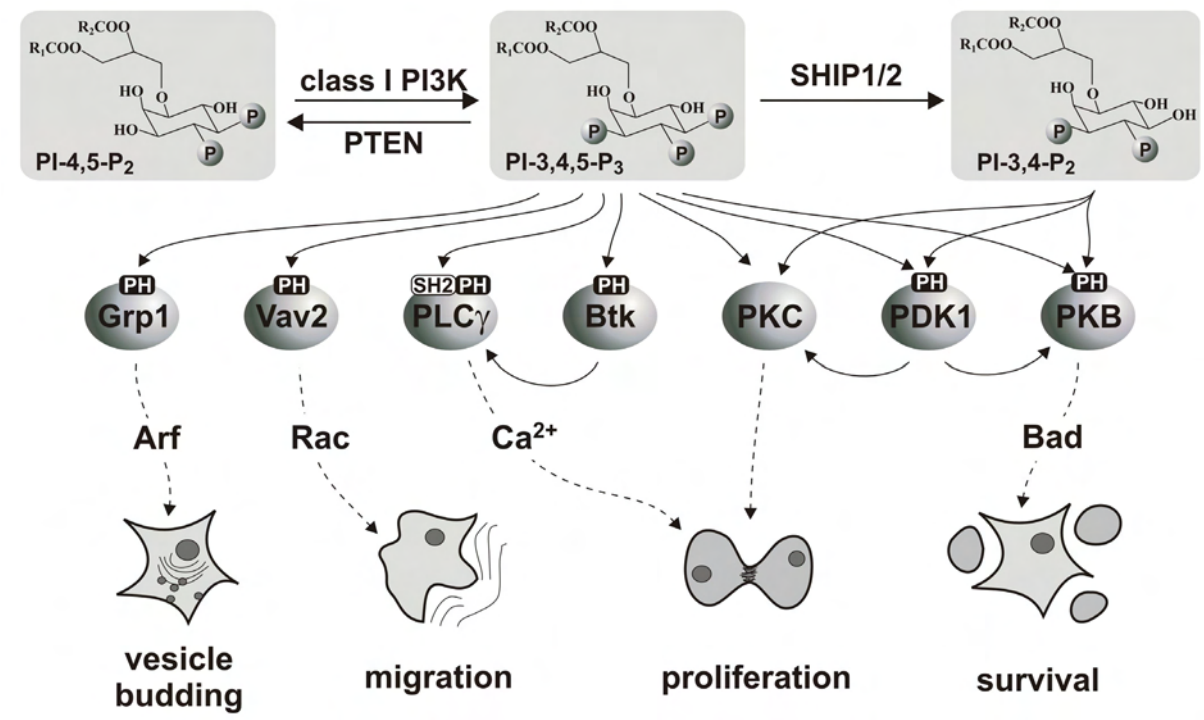


Fig. 1.7: Signaling through PI3K lipid products

ARF, ADP-ribosylation factor (small GTPase); Btk, bruton's tyrosine kinase; Grp, general receptor for phosphoinositides; PDK, phosphoinositide dependent kinase; PH, pleckstrin homology domain (phosphoinositide binding domain); PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rac, small GTPase; SH2, Src homology 2 (phosphoinositide binding domain); SHIP, SH2-containing inositol 5-phosphatase; Vav, nucleotide exchange factor.
(modified from Rameh and Cantley, 1999)

The serine/threonine protein kinase B (PKB), also known as Akt, is the most well characterized target of PI-3,4-P_2 and PI-3,4,5-P_3. Akt/PKB contains a PH domain which promotes translocation to the plasma membrane (Andjelkovic et al., 1997, Franke et al., 1997, Klippel et al., 1997). Thereafter, Akt/PKB becomes activated by phosphorylation at Thr-308 and Ser-473 (Alessi et al., 1996, Andjelkovic et al., 1996). The phosphorylation of Akt/PKB requires phosphoinositide-dependent kinases (PDKs), such as PDK1 (McManus et al., 2004, Bayascas and Alessi, 2005). It was shown that PDK1 also contain a PH domain which allows to interact with PI-3,4,5-P_3 and PI-3,4-P_2 (Alessi et al., 1997a, b, Stokoe et al., 1997).

Furthermore, PDK1 phosphorylates and activates protein kinases C (PKC) ζ and δ isoforms (Le Good et al., 1998).

Activated Akt/PKB phosphorylates and inactivates Bad, a protein involved in promoting cell death, and hence, Akt/PKB mediates the transduction of cell survival signals (Franke and Cantley, 1997, Rameh and Cantley, 1999, Wang et al., 1999). Glycogen synthase kinase 3 and phosphofructokinase are also substrates for Akt/PKB (Cross et al., 1995) Therefore, PI3K lipid products are involved in insulin-dependent regulation of blood sugar.

Recently, an interesting role of PI-3,4-P_2 and its related targets have been shown. The tandem PH domain-containing protein (TAPP1) selectively binds to PI-3,4-P_2 (Dowler et al., 2000, Allam and Marshall, 2005). It was proposed that a pool of protein tyrosine phosphatase (PTPL1) is mainly maintained in the cytoplasm of resting cells through its interaction with TAPP1. Following activation of PI3K and action of phosphoinositide 5-phosphatases, significant amount of PI-3,4-P_2 accumulates at the membrane allowing to bind the PTPL1-TAPP1 tandem. At the plasma membrane PTPL1 can dephosphorylate receptor(s) and/or adaptor protein(s) (see section 1.3.1.1.1), thereby inducing the inactivation of PI3K (Kimber et al., 2003). Thus, PI-3,4-P_2 could initiate a negative feedback loop to deactivate PI3K.

The Bruton's tyrosine kinase (Btk) is an essential protein for development and function of B lymphocytes (Hendriks et al., 1996, Jumaa et al., 2005). It was shown that class I_B PI3Kγ enhances autophosphorylation of Btk and Src family kinase-mediated tyrosine phosphorylation of Btk (Rameh and Cantley, 1999). This phosphorylation was inhibited by wortmannin and depended on the PH domain of Btk (Li et al., 1997). Btk is able to phosphorylate and activate PLCγ (Fluckiger et al., 1998). Phosphorylation of Btk and subsequent activation of PLCγ lead to B cell receptor-mediated, PI-3,4,5-P_3-dependent calcium release (Wymann and Pirola, 1998, Rameh and Cantley, 1999).

In contrast to other PH containing proteins, the protein Grp1 (general receptor for phosphoinositides) is very selective for PI-3,4,5-P_3 (Cronin et al., 2004, Varnai et al., 2005). Grp1 contains the Sec7 homology domain which acts as a guanine nucleotide exchange factor for the small G proteins Arf1 and Arf5. This nucleotide exchange activity of PI-3,4,5-P_3-associated Grp1 towards membrane-bound Arf demonstrates the possible role of PI-3,4,5-P_3 in regulation of budding of intracellular vesicles (Klarlund et al., 1998, Rameh and Cantley, 1999). Previously, it was shown that that PI-3,4,5-P_3 binds to the PH domain of nucleotide exchange factors, such as P-Rex (Welch et al., 2002, Hill et al., 2005, Mayeenuddin et al., 2006) and Vav (Han et al., 1998), and allosterically activates their exchange activity toward Rac. Subsequently, activated Rac is involved in cytoskeleton rearrangements that lead to cell migration (Tapon and Hall, 1997, Rameh and Cantley, 1999, Bagchi et al., 2005).

2 AIMS OF THE STUDY

Receptor-released Gβγ dimers recruit bifunctional PI3Kγ to the plasma membrane and stimulate simultaneously the lipid and the protein kinase activity of this enzyme. Previously, it was shown that GDP-bound Gα subunits can deactivate the Gβγ-dependent signaling. This suggests that the sites of Gβ subunit implicated in interaction between Gα and Gβγ overlaps with the sites of interaction between Gβγ and effectors. The binding surface between Gα and Gβγ is defined. Moreover, molecular determinants indispensable for interaction of Gβγ with several effectors like adenylyl cyclase II, PLCβ_2 or ionic channels have been resolved. Nevertheless, very little is understood about the interaction of Gβγ dimers with PI3Kγ at a molecular lever. Furthermore, no pharmacological modulators are known which selectively discriminate between Gβγ-induced stimulation of different forms and enzymatic activities of PI3Kγ.

Additionally, the role of non-catalytic subunit (p101) of PI3Kγ is controversial. p101 has been proposed to act as an adaptor by conferring p110γ sensitivity to Gβγ dimers. Furthermore, it is believed that p101 differently modulates the protein and lipid kinase activity of PI3Kγ. On the other hand, there are data reporting that p101 discriminates between Gβγ-induced phosphorylation of different phosphoinositides.

Therefore, we propose:

- to investigate the molecular basis of Gβγ interaction and activation of PI3Kγ. For this purpose, we have examined a series of effector-relevant Gβ_1 mutants for their capability to bind and activate lipid and protein kinase activities of monomeric (p110γ) and dimeric (p110γ/p101) PI3Kγ.

- to determine whether the stimulation of different forms and catalytic activities of PI3Kγ is dissectible.

- to elucidate the role of non-catalytic p101 subunit of PI3Kγ.

3 MATERIALS

3.1 List of manufacturers and distributors

(1) Amersham Biosciences, Freiburg
(2) Beckman, Munich
(3) Biochrom, Berlin
(4) Bio-Rad, Munich
(5) Dianova, Hamburg
(6) Gibco BRL, Eggenstein
(7) Hartmann Analytic, Braunschweig
(8) Invitrogen via ITC Biotechnology, Heidelberg
(9) Kodak, Rochester, USA
(10) List Biological Laboratories, Campbell, USA
(11) Merck, Darmstadt
(12) Millipore, Eschborn
(13) Pierce, Rockford, USA
(14) Polaroid, Berlin
(15) Promega via Serva, Heidelberg
(16) Qiagen, Hilden
(17) Raytest, Straubenhardt
(18) Roche Diagnostic, Mannheim
(19) Roth, Karlsruhe
(20) Santa Cruz, Heidelberg
(21) Schering, Berlin
(22) Schleicher & Schuell, Dassel
(23) Serva, Heidelberg
(24) Sigma, Deisenhofen
(25) Whatman, Maidstone, UK

3.2 Chemicals

Acetic acid	(11)
Acrylamide	(19)
Agarose, “low-melting”	(1)
Aluminium chloride	(24)
Ammonium persulfate	(4)

Bovine serum albumin (BSA)	(24)
Bromphenol blue	(23)
CHAPS	(24)
Coomassie Brilliant Blue G-250	(15)
Dimethyl sulfoxide (DMSO)	(11)
Disodium hydrogen phosphate	(11)
Dithiothreitol (DTT)	(24)
ECL Western Blotting Detection System	(1)
EDTA	(23)
EGTA	(23)
Folin-Ciocalteau phenol reagent	(11)
Formaldehyde (40 %)	(24)
Glutathione	(24)
Glycerine	(11)
β-Glycerophosphate	(24)
Glycine	(23)
HEPES	(11), (19)
Imidazole	(24)
Magnesium chloride	(24)
Manganese chloride	(24)
β-Mercaptoethanol	(24)
N, N'- Methylenebisacrylamide (bisacrylamide)	(19), (23)
Octyl glucoside	(18)
Octyl thioglucoside	(18)
Pefabloc SC	(18)
L-α-Phosphatidylcholine	(24)
L-α-Phosphatidylethanolamine	(24)
Phosphatidylinositol	(24)
Phosphatidylinositol-4-phosphate	(24)
Phosphatidylinositol-4,5-bisphosphate	(24)
L-α-Phosphatidylserine	(24)
Pierce BCA Protein Assay Kit	(13)
Polyoxyethylene-10-dodecyl ether ($C_{12}E_{10}$)	(24)
Ponceau S	(11)
Potassium dihydrogen phosphate	(11)
n-Propanol	(19)
Roti-Block	(19)

Saccharose monolaurate	(18)
Scintillation liquid	(2)
Silver nitrate	(11)
Sodium cholate	(24)
Sodium dodecyl sulfate (SDS)	(24)
Sodium fluoride	(24)
Sodium thiosulfate pentahydrate	(24)
Sphingomyelin	(24)
N,N,N',N'- Tetramethylethylenediamine (TEMED)	(4)
N-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK)	(24)
Tris	(11), (23)
Triton X-100	(23)
Trypsin (TPCK-treated)	(24)
Tween 20	(24)
Urea	(24)

3.3 Enzymes, proteins, peptides and other biological active substances

Anti-GST antibody	(20)
Aprotinin	(24)
Benzamidine	(24)
DNAase	(18)
Horseradish peroxidase (HRP) anti-goat IgG	(24)
Horseradish peroxidase (HRP) anti-mouse IgG	(24)
Horseradish peroxidase (HRP) anti-rabbit IgG	(24)
Leupeptin	(24)
Trypsin inhibitor	(18)
Wortmannin	(21)

3.4 Non-radioactively labeled nucleotides

Adenosine-5'-triphosphate (ATP)	(18)
Guanosine-5'-diphosphate (GDP)	(18)
Guanosine-5'-triphosphate (GTP)	(18)

3.5 Radioactively labeled nucleotides

[γ-^{32}P]ATP	(7)
[γ-^{32}P]GTP	(7)

3.6 Cell cultures, cell culture mediums, and supplements

Foetal calf serum (FCS)	(3), (6)
Glutamine	(3)
Lipid Medium Supplement	(3)
Penicillin/Streptomycin (10,000 U/ 10,000 µg/ml)	(3)
Sf9 cells (ovarian cells from Spodoptera frugiperda)	(6)
TC-100 Medium	(3)
TNM-FH Medium	(24)

3.7 Protein standards

High Molecular Weight (HMW) Electrophoresis Calibration Kit	(1)
Low Molecular Weight (LMW) Electrophoresis Calibration Kit	(1)
HMW Gel Filtration Calibration Kit	(1)
LMW Gel Filtration Calibration Kit	(1)

3.8 Chromatography and separation materials

Glutathione Sepharose 4B	(1)
Ni^{2+}-NTA-Superflow	(16)
Protein A Sepharose CL-4B	(1)
Resource 15Q	(1)
Resource 15S	(1)
Resource Q HR 5/5 (1 ml)	(1)
Resource S HR 5/5 (1 ml)	(1)
Thin layer chromatography (TLC) plates with silica gel 60 (20 x 20 cm) (without fluorescence indicator)	(25)
Tris-HCl Ready Gel	(4)

3.9 Membranes and filters

Centricon concentrators 10, 30	(12)
Hybond-C-Extra (membrane optimized for protein transfer)	(1)
Immobilon P (PVDF-Membrane)	(12)
Membrane filters NC 45	(22)
Whatman GF/B	(25)

3.10 Autoradiography films, screens, and accessories

Imaging plates	(17)
X-ray films (CRT7, R, X-OMAT)	(9)
X-ray film cassette (Number 553)	(14)

All other substances used in this work were purchased from Roth (Karlsruhe), Merck (Darmstadt) or Sigma (Deisenhofen) with “pro analysi (pA)” grade.

The baculoviruses used in this study were obtained from Prof. Dr. H. E. Hamm (Nashville, TN, USA), Prof. Dr. R. Wetzker (Jena), Prof. Dr. A.G. Gilman (Dallas, TX, USA), Dr. T. Hanck (Magdeburg), Dr. C. Harteneck (Berlin), Dr. C. Kleuss (Berlin), Prof. Dr. M. Lohse (Würzburg) and Prof. Dr. M. D. Waterfield (London, UK).

Antibodies against Gα and Gβ subunits were produced by Dr. K. Spicher and Prof. Dr. Dr. B. Nürnberg at the Institute of Pharmacology (Free University of Berlin). Monoclonal anti-PI3Kγ antibodies, i.e. mAb and mAb 641, directed against intact p110γ were obtained from Prof. Dr. R. Wetzker (Jena).

Wortmannin was a gift from Dr. A. Steinmeyer (Schering AG, Berlin).

4 EXPERIMENTAL PROCEDURES

4.1 Standard biochemical methods

4.1.1 Measurement of protein concentration

Protein concentrations were measured using different methods. All of these methods are based on specific protein-dependent colour-complex formation.

The BCA™ Protein Assay (Pierce) allows sensitive and selective colorimetric detection and quantitation of total protein content. The assay includes two steps: 1) reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction); 2) the colorimetric detection of the Cu^{+1} by a reagent containing bicinchoninic acid (Smith et al., 1985). Chelation of two molecules of BCA with one cuprous ion leads to formation water-soluble purple-coloured product with maximal absorbance peak at 562 nm. The BCA™ Protein Assay is detergent-compatible and has a broad working protein concentrations range (0.02 - 2 mg/ml).

For protein preparations containing high concentrations of detergent, we used the method by Lowry (Lowry et al., 1951) modified by Peterson (Peterson, 1983). The Lowry method is based on the reactivity of the peptide nitrogen[s] with the Cu^{+2} under alkaline conditions and reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive for the protein concentrations ranging from 0.005 - 0.1 mg/ml.

Another method is detection of proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following staining with Coomassie Brilliant Blue (see sections 4.1.2 and 4.1.3). Gels were digitalized using a transmitted-light scanner (Diana-III, Raytest) and densitometrically evaluated using “Aida” software (Raytest). The working detection range for this method is 50 - 1000 ng/lane. For the methods described above bovine serum albumin was used as the standard.

4.1.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is the common method for separating proteins according their molecular masses using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. (Laemmli, 1970). SDS-PAGE is used for the estimation of the relative molecular weight of proteins. Furthermore, SDS-PAGE is useful to follow the progress of a purification procedure and to determine the purity of proteins in the fractions

from the purification. This method is also suitable for the quantification of protein concentration (see section 4.1.1).

The protein samples were mixed with Laemmli buffer (62.5 mM Tris/HCl, 10 % (v/v) glycerine, 5 % (v/v) β-mercaptoethanol (β-ME), 2 % (w/v) SDS, and 0.02 % (w/v) bromphenolblue). SDS is an anionic detergent which denatures proteins and confers a negative charge on the polypeptide in proportion to its length. β-mercaptoethanol is a reducing agent which prevents formation of disulfide bonds. Disulfide bonding is covalent and is not disrupted by SDS. The electrophoresis is curried out in running buffer (25 mM Tris (pH 8.3 - 8.7), 192 mM glycine, and 0.1 % (w/v) SDS). Proteins are run at 100 V in the stacking gel to concentrate them, and then they are separated in the running gel at 150 V. The "block" gel prevents the proteins from running off the bottom of the gel.

	STACKING GEL	RUNNING GEL	"BLOCK" GEL
Acrylamide/Bisacrylamide (30 % / 0.8 %)	4.5 % (w/v)	10 % (w/v)*	30 % (w/v)
SDS	0.1 % (w/v)	0.1 % (w/v)	
APS	0.1 % (w/v)	0.1 % (w/v)	1.5 % (w/v)
TEMED	0.1 % (w/v)	0.1 % (w/v)	1.5 % (w/v)
Tris/HCl	125 mM, pH 6.8	375 mM, pH 8.8	

* For better resolution between 7 - 30 kDa a 15 % running gel was used.

4.1.3 Coomassie staining of SDS-polyacrylamide gels

Coomassie Blue staining is based on the nonspecific binding of the dye Coomassie Brilliant Blue R250 to almost all proteins. The gel is soaked in a solution of the dye (1 % (w/v) Coomassie Brilliant Blue in 45 % (v/v) methanol and 10 % (v/v) acetic acid) for at least 30 min. The gel is then destained using a mixture of 5 % methanol and 10 % acetic acid to remove the background colour prior to drying and analysis of the gel.

4.1.4 Immunoblotting

After separation of the protein samples by SDS-PAGE, proteins are transferred to a nitrocellulose or PVDF membranes (Towbin et al., 1979). The protein transfer to the nitrocellulose membrane is carried out for 90 min at 250 mA in transfer buffer (25 mM Tris, 192 mM glycine, 0.02 % (w/v) SDS in 20 % (v/v) methanol). In order to visualize the transferred proteins, they are stained with a Ponceau S solution for 5 min. The stain is

removed by washing the membrane with demineralised H_2O. After the temporary detection of the proteins by Ponceau S, the membranes were blocked with a 10 % solution of Roti-Block (Roth) for 1 hour or overnight to avoid non-specific binding of the antibodies or detection reagents in subsequent steps. Blocking was repeated after each successive binding step, because it is essential to minimize any nonspecific binding that would increase the background signal. Non-specifically bound reagents are removed after each of the binding steps in the procedure by washing the membranes in Tris-buffered-saline-Tween buffer (TBST-buffer: 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05 % (v/v) Tween).

After blocking and washing, the nitrocellulose membranes were incubated with primary antibodies (dilution 1:150 - 1:2000) which recognize and bind to the target antigen bound to the membrane (Leopoldt et al., 1997, Exner and Nürnberg, 1999). Membranes were incubated with the primary antibodies for 1 hour or overnight. Antibodies used in this work and peptides utilized for antibody production are shown in Table 4.1.

Table 4.1: Antibodies used for immunological detection of different proteins

Antibody	Peptide sequence	Reference
anti-α_{common} (AS 8)	(*c*)GAGESGKSTIVKQMK	Nürnberg et al., 1994
anti-β_{common} (AS 398)	(*c*)TDDGMAVATGSWDDSFLKIWN	Leopoldt et al., 1997
anti-β_1 (AS 28)	(*c*)CTTTFTGHTG	Hinsch et al., 1989
anti-$\gamma_{2/3}$ (AS 292)	(*c*)SENPFREKKFFC	Dietrich et al., 1992
anti-p101	(*c*)YERPRRPGGHERRG	Viard et al., 1999
anti-p110γ (mAb)	p110γ full length purified from Sf9 cells	Leopoldt et al., 1998
anti-p110γ (mAb 641)	p110γ full length purified from Sf9 cells	Czupalla, 2002

(*c*) - cysteine, which was introduced for the coupling of peptides with KLH (**k**eyhole **l**impet **h**emocyanin).

Commercially available horseradish peroxidase (HRP) - conjugated secondary antibodies used are specific to the species in which the primary antibody was raised. After 1 hour incubation with secondary antibody (dilution 1:1000 - 1:5000) membranes were several times washed with TBST-buffer following luminol-based detection of chemiluminescence (ECL-kit, Amersham).

4.1.5 Stripping and reprobing of membranes

Nitrocellulose membranes can be reprobed by completely stripping bound primary and secondary antibodies and incubating with different antibodies. The nitrocellulose membrane is submerged in stripping buffer (62.5 mM Tris/HCl (pH 6.7), 100 mM β-ME, 2 % SDS) and incubated at 50 °C for 30 minutes with occasional agitation. Then, the membrane is washed in large volumes of TBST-buffer 3 times for 10 minutes each. Following the wash steps the membrane is immersed in a 10 % solution of Roti-Block for 1 hour, and the nitrocellulose membrane can be reprobed for immunodetection (see section 4.1.4).

4.1.6 Immunoprecipitation of recombinant monomeric and dimeric PI3Kγ

Different amounts of recombinant monomeric or dimeric PI3Kγ purified from Sf9 cells (see section 4.3.2) were mixed with 3 µl of anti-p110γ specific monoclonal antibodies (mAb or mAb 641) in precipitation buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2 mM β-ME). Immunoprecipitation was conducted in a final volume of 500 µl. After an incubation period of 1 hour at 4 °C, 10 µl of Protein A Sepharose CL-4B beads (Amersham) preincubated in blocking buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 % BSA) for 30 min were added. The mixture was gently shaken overnight at 4 °C. Thereafter, Protein A Sepharose CL-4B beads were pelleted by centrifugation (2 min at 2,000 g, 4 °C) and washed twice with precipitation buffer. Bound proteins were eluted by adding 1x Laemmli sample buffer (Laemmli, 1970). Immunoprecipitates were separated by SDS-PAGE (see section 4.1.2) and analyzed by immunoblotting (see section 4.1.4) with anti-p110γ monoclonal antibodies (mAb and mAb 641).

4.1.7 Determination of nucleotide concentrations

Nucleotides were quantified by measuring the UV absorbance at 260 nm according to the Beer-Lambert law:

$$\mathbf{A = \varepsilon c L}$$

where A – absorbance (optical density units); ε – molar extinction coefficient (M^{-1} cm^{-1}); c – concentration (M); L – light path length (cm).

One optical density unit is defined as the amount of oligonucleotide, dissolved in 1 ml of water, which results an absorbance of 1 when measured at 260 nm in a 1 cm path-length quartz cuvette (Table 4.2).

Table 4.2: Molar extinction coefficient of some nucleotides (Dawson et al., 1986)

Nucleotide	pH	ε_{260}
Adenosine triphosphate	2	14300
	7	15400
	11	15400
Adenosine diphosphate	2	14500
	7	15400
	11	15400
Guanosine triphosphate	1	11800
	7	11700
	11	11800
Guanosine diphosphate	1	11800
	7	11800
	11	11700

4.2 Expression of recombinant proteins in insect cells

Sf9 cells are a clonal isolate of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE. This cell line, established from ovarian tissue of the fall armyworm, is widely used for expression of recombinant proteins. Recombinant proteins can be produced at levels ranging between 0.1 % and 50 % of the total insect cell protein. For expression of recombinant proteins Sf9 cells were infected with lytic baculoviruses.

Baculoviruses (family *Baculoviridae*) belong to a diverse group of large doublestranded DNA viruses that infect many different species of insects. The replication of DNA starts at approximately 6 h post-infection. In both in vivo and in vitro conditions, the baculovirus infection cycle can be divided into two different phases, early and late. During the early phase, the infected insect cell releases extracellular virus particles (ECV) which may infect further cells. During the late phase of the infection cycle, occluded virus particles (OV) are assembled inside the nucleus. The OV are surrounded by a homogenous matrix made predominantly of a single protein, the polyhedrin protein. Although the polyhedrin is one of the most abundant proteins in infected insect cells, it is not essential for the baculovirus life cycle in tissue culture. Therefore, the polyhedrin gene may be replaced by genes of interest. Since the baculovirus genome is generally too large to easily insert foreign genes, heterologous genes are cloned into transfer vectors. Co-transfection of the transfer vector and baculovirus DNA into Sf9 cells allows recombination between homologous sites, transferring the heterologous gene from the vector to the baculovirus DNA. Baculovirus infection of Sf9 cells results in the shut-off of host gene expression allowing a high rate of recombinant mRNA and protein production.

For more information about baculovirus biology, refer to published references (Luckow, 1991, King and Posee, 1992, O'Reilly et al., 1992). For additional information about insect cell culture, refer to the "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques" available from Invitrogen at their Web site (www.invitrogen.com).

4.2.1 Culture of Sf9 cells

In culture, healthy Sf9 cells attach to the bottom of the plate forming a monolayer. In contrast to healthy cells, infected Sf9 cells become uniformly round, enlarged, develop enlarged nuclei, do not form a monolayer as well, and stop dividing. Healthy Sf9 cells generally double every 18 - 24 h when grown in TNM-FH media supplemented with 10 % fetal calf serum and 1 % penicillin/streptomycin. Sf9 cells may also be grown in suspension. The cells in suspension are cultivated in the same medium as those in a monolayer with the addition of 1 % lipid medium supplement.

Sf9 cells grow at temperatures between 26 - 28 °C. However, after infection it is important to keep the temperature at 27 °C±0.5 °C, otherwise recombinant protein production may be poor even though cells are infected. Cell viability is controlled by trypan blue. To 1 ml of cells add 0.1 ml of a 0.4 % stock solution of trypan blue in phosphate-buffered saline (PBS). Healthy, log-phase cultures should contain more than 97 % unstained, viable cells.

4.2.2 Recombinant virus amplification

Large stocks of virus were prepared by infecting insect cells at a concentration of 1.0×10^6 cells/ml with a low multiplicity of infection (MOI) equal to 0.1. The virus was harvested after 5 days. It is critical to use a low MOI because passaging the virus at high MOI increases the number of viruses with mutations in their genome. After virus amplification, cells and cell fragments were pelleted by centrifugation at 7,500 x g at 4 °C for 20 min. The supernatant containing the baculovirus can be used for the infection of Sf9 cells and the expression of recombinant protein after the virus titer is determined (Luckow, 1991, King and Posee, 1992, O'Reilly et al., 1992).

4.2.3 Estimation of the virus titer (Plaque Assay)

The aim of the plaque assay is to purify virus and/or to determine the viral titer in plaque-forming units per ml (pfu/ml) which allows to infect insect cells with the known amounts of virus. The Sf9 cells (1.0×10^6 cells) were transferred in 10 cm^2 culture dish and incubated for 1 hour to let them attach the bottom. Meanwhile, the baculovirus (see section 4.2.2) was prepared at dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. After the cells have attached to the culture dish, the media and non-attached cells were removed by aspiration. One ml of diluted virus was added to each plate and incubated for 1 hour at 27 °C. The solution of "low-melting" agarose in water (2.5 % (w/v)) was cooled to 37 °C and mixed with TNM-FH media in 1:2 ratios. The diluted viruses were then aspirated and the plates were filled with 2 ml of agarose/media solution. In order to prevent the desiccation of the culture dishes during the 7 days of incubation at 27 °C, the agarose was covered with 2 ml of TNM-FH media. The agarose keeps the cells stable and limits the spread of virus. The infected Sf9 cell lyses and releases the virus which, subsequently, can infect only the neighbouring cells. The group of infected cells form a plaque which can be visualized either by the naked eye or by microscopy in diffused sidelight. Each plaque represents a single virus. Therefore, it is possible to purify a certain virus by isolating individual plaques. Additionally, individual plaques obtained from varying dilutions of a viral stock can be counted to determine the viral titer (pfu/ml) (Luckow, 1991, King and Posee, 1992, O'Reilly et al., 1992), which is calculated using the following equation,

$$\text{pfu/ml} = \frac{\text{number of plaques x dilution factor}}{\text{volume of diluted virus (ml)}}$$

After determination of the virus titer the cells are infected with a certain MOI. MOI is defined as the number of virus particles per cell. In the current work, cells were infected at a MOI = 1.

4.2.4 Recombinant protein expression

For heterologous protein expression, Sf9 cells (1.5×10^6 cells/ml) were incubated with a MOI of 1 virus per cell. Subunits of PI3K and G-proteins were coexpressed at equal MOI numbers and used for functional studies. After 48 - 60 hours of infection, cells were pelleted by centrifugation at 1,000 x g for 5 min and washed twice with phosphate-buffered saline (PBS: 12 mM Na_2HPO_4, 4 mM KH_2PO_4, 120 mM NaCl, pH 7.4). Pellets were either immediately used for the work or after shock-freezing in liquid nitrogen stored at -70 °C for a longer period of time.

4.3 Protein purification

4.3.1 Purification of recombinant membrane-attached His-tagged $G\beta_1\gamma_2$ dimers and their mutants from Sf9-cells

4.3.1.1 Preparation of plasma membrane extracts

Construction and characterization of recombinant baculoviruses for expression of $G\beta_1$ variants, $G\gamma_2$, and $G\gamma_{2\text{-His}}$ were described previously (Ford et al., 1998, Maier et al., 1999, 2000). PBS-washed cells containing recombinantly expressed $G\beta_1\gamma_2$ variants (see section 4.2.4) were resuspended in ice-cold buffer A (50 mM HEPES (pH 8.0), 110 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM β-ME, 10 µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, and 0.2 mM Pefabloc® SC). Cells were disrupted by N_2 cavitation for 30 min at 4 °C, 30 bar, or by forcing the cell suspension through a 22-gauge needle five times and subsequently through a 26-gauge needle 10 times. The lysate was centrifuged at 800 x g for 2 min to remove nuclei and intact cells. Membranes were recovered by centrifugation at 80,000 x g for 30 min and subsequently extracted by incubating membranes with 1 % sodium cholate for 1 h (Maier et al., 1999, 2000).

4.3.1.2 Affinity-chromatography

The membrane extracts (see section 4.3.1.1) were clarified by ultracentrifugation (100,000 x g, 1 h) and diluted five times with buffer B (20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 % polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$), 10 mM β-ME, and 25 mM imidazole). The cleared extracts were incubated for 1 hour with Ni^{2+}-NTA-Superflow resin (1 ml of slurry/7.5 x 10^8 infected cells) that was prewashed with buffer B. The mixture was loaded onto a column cartridge and extensively washed with buffer B containing 20 mM imidazole. Thereafter, bound insect Gα subunits were removed by elution with buffer C (20 mM HEPES (pH 8.0), 100 mM NaCl, 50 mM $MgCl_2$, 0.9 % sodium cholate, 10 mM β-ME, 10 mM GDP, 30 mM $AlCl_3$, and 10 mM NaF). Subsequently, His-tagged Gβγ dimers were eluted from the Ni^{2+}-NTA matrix using buffer D (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 % $C_{12}E_{10}$, 200 mM imidazole, and 10 mM β-ME).

4.3.1.3 Anion exchange chromatography

$G\beta_1\gamma_{2\text{-His}}$ eluted from the Ni^{2+}-NTA-Superflow was loaded onto 1 ml Resource 15Q anion exchange column equilibrated with buffer E (20 mM Tris-HCl (pH 8.0), 0.1 % $C_{12}E_{10}$, and 2 mM DTT). Bound proteins were fractionated, using continuous NaCl gradient elution (0 - 800 mM NaCl in buffer E within 10 column volumes). Purified proteins were quantified by Coomassie Blue staining following SDS-PAGE with bovine serum albumin as a standard. Proteins were stored at -70 °C.

4.3.1.4 Gel filtration (Size exclusion chromatography)

Gel filtration allows the separation of proteins according to their size. This technique is used as a fast method to exchange buffers. Moreover, gel filtration is also used to separate monomers from aggregates, to determine the relative molecular weight of proteins, to facilitate the refolding of denatured proteins by changing buffer conditions (Amersham Pharmacia Biotech, 2001a, b, Amersham Biosciences, 2002).

In this study, the gel filtration was applied as an additional purification step in the $G\beta_1\gamma_{2\text{-His}}$ purification procedure (see section 5.1). $G\beta_1\gamma_{2\text{-His}}$ dimers eluted from the Resource 15Q column were loaded onto Superdex 200 HR 10/30 gel filtration column equilibrated with buffer F (20 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM β-ME) containing 0.1 % $C_{12}E_{10}$ or 0.9 % sodium cholate. Thereafter, proteins were fractionated by elution in the same buffer.

Additionally, we used the gel filtration technique to purify anti-p110γ monoclonal antibody (mAb 641) (see section 5.4.3). mAb 641 obtained from mouse hybridoma cells supernatant was concentrated using Centricon-30 concentrators. The concentrated antibody (0.5 - 1 ml) was applied to a Superdex 200 HR 10/30 and eluted with buffer F.

In order to determine the relative molecular weight of eluted proteins, the gel filtration column Superdex 200 HR 10/30 was calibrated with standard proteins and molecular weight calibration curve was plotted (Fig. 4.1A and B). After determination of elution volumes (Ve), the relative molecular masses of eluted proteins were estimated using equation shown in Fig. 4.1B.

A

Standard protein	MW [kDa]	Ve [ml]
Ribonuclease A	13.7	16.95
Chymotrypsinogen A	25	16.35
Ovalbumin	43	14.54
Albumin	67	13.57
Aldolase	158	12.46
Catalase	232	12.21
Ferritin	440	10.58
Thyroglobulin	669	9.03

B

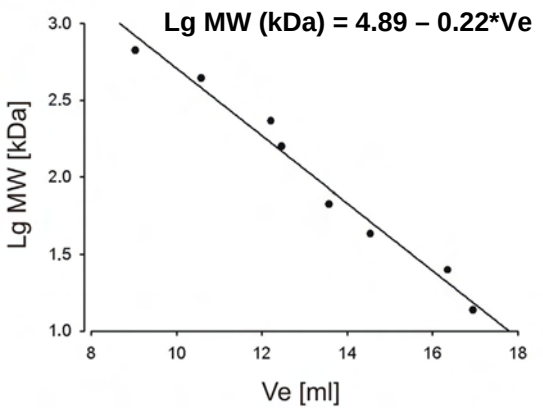


Fig. 4.1: Calibration of Superdex 200 HR 10/30

(A) 10-20 µg of each standard protein were dissolved in 100 µl of buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM β-ME) and applied to the column following isocratic elution using dissolving buffer. The elution volume (Ve) of each standard protein was measured. **(B)** A molecular weight calibration curve of the gel filtration column. The elution volume of a set of standard proteins versus base-10 logarithm of molecular weight was plotted.

4.3.2 Purification of cytosolic His- and GST-tagged PI3Kγ

Construction and characterization of recombinant baculoviruses for expression of non-tagged p110γ and p101, N-terminally tagged $p110\gamma_{\text{-His}}$, $p110\gamma_{\text{-His}}$ (△1-34), $p110\gamma_{\text{-GST}}$ (△1-34), $p110\gamma_{\text{-His}}$ (S1101A), $p110\gamma_{\text{-His}}$ (S1101D), $p110\gamma_{\text{-His}}$ (S1101E), $p101_{\text{-His}}$, and $p101_{\text{-GST}}$ were described previously (Stoyanov et al., 1995, Stephens et al., 1997, Leopoldt et al., 1998, Maier et al., 1999, Czupalla et al., 2003a). Subunits of heterodimeric PI3K were coexpressed at equal MOI numbers in Sf9 cells (see section 4.2.4). After 48 h of infection, cells were pelleted by centrifugation (1,000 x g) and washed two times with PBS. For purification of GST fusion proteins, cells were resuspended in ice-cold buffer A containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 2 mM DTT, 10 mg/ml each of L-1-tosylamido-2-phenylethyl chloromethyl ketone, benzamidine, leupeptin, and 0.2 mM Pefabloc® SC. They were disrupted by N_2 cavitation for 30 min at 4 °C, 30 bar, or by forcing the cell suspension through a 22-gauge needle 5 times and then through a 26-gauge needle 10 times. Nuclei and debris were discarded. The cytosolic fraction was separated from the particulate by centrifugation at 100,000 x g for 50 min. Cytosol was incubated for 3 - 4 h with glutathione-Sepharose 4B beads (Amersham) that were prewashed with buffer A. GST fusion proteins were eluted with buffer B consisting of buffer A with 10 mM glutathione for 1 h at 4 °C (Maier et al., 1999).

For purification of His-tagged PI3Kγ, cells were disrupted using the same procedure as described above in buffer C (20 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM β-ME) containing 10 mg/ml each of L-1-tosylamido-2-phenylethyl chloromethyl ketone, benzamidine, leupeptin, and 0.2 mM Pefabloc® SC. The cytosolic fraction was incubated for 1 - 2 h with Ni^{2+}-NTA-Superflow (Qiagen) that was prewashed with buffer C containing 20 mM imidazole. After extensive washing with buffer C proteins were eluted with buffer C containing 200 mM imidazole (Maier et al., 1999).

Solutions containing His- and GST-tagged PI3Kγ were then applied to a Resource Q HR 5/5 anion exchange column mounted on a "Äkta Purifier 10" FPLC equipment (Amersham), which was equilibrated with buffer D (20 mM Tris-HCl (pH 8.0), 0.033 % $C_{12}E_{10}$, 2 mM DTT). Proteins were eluted with a linear gradient of 0 - 500 mM NaCl in buffer D. Purified proteins were quantified by Coomassie Blue staining following SDS-PAGE with bovine serum albumin as the standard and immunoblotting.

4.3.3 Copurification of $G\beta_1\gamma_2$ with PI3Kγ subunits

For copurification of $G\beta_1\gamma_2$ with His-tagged recombinant subunits of PI3Kγ, such as p101 and p110γ, equal MOI numbers for all recombinant baculoviruses were used. Sf9 cell lysates were obtained by forcing the cell suspension through a 22-gauge needle 5 times and subsequently through a 26-gauge needle 10 times. The suspension was then incubated for 30 min with a buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM β-ME, 0.5 % $C_{12}E_{10}$. After a 2 hour incubation with Ni^{2+}-NTA-Superflow resin proteins were eluted and analyzed for bound $G\beta_1\gamma_2$.

4.4 Limited trypsin proteolysis of $G\beta_1\gamma_2$

The tryptic protection assay (Fung and Nash, 1983, Winslow et al., 1986, Thomas et al., 1993) with some modifications was performed to ascertain properly formed Gβ mutants. Although the Gβ subunit contains 32 potential tryptic sites, the Gβγ dimer is highly resistant to proteolysis. Trypsin only cleaves the Gβ at position Arg-129 producing two proteolytic fragments of 26 and 14 kDa (Winslow et al., 1986, Thomas et al., 1993, Ford et al., 1998). Proteins were cleaved with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin. The reaction volume was 30 µl, and the protein concentration was 167 µg/ml. The purified Gβγ was in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM DTT and 0.033 % $C_{12}E_{10}$. TPCK-treated trypsin was diluted in the same buffer and added to the sample at a ratio of

1:25, trypsin to substrate. The samples were incubated for 40 min at 30 °C. Proteolysis was terminated by the addition of Laemmli sample buffer (Laemmli, 1970) and boiled for 1 min. The reactions were analyzed by SDS-PAGE.

4.5 Determination of PI3Kγ activity

The G-protein-regulated PI3Kγ transmits specific signals through its inherent lipid and protein kinase activities. The lipid kinase activity of PI3Kγ results in the phosphorylation of the D3 position of the inositol ring of phosphoinositides, thus generating intracellular lipid second messengers. In addition to its lipid kinase activity, PI3Kγ possesses an intrinsic protein kinase activity in vitro. $G\beta_1\gamma_2$-dependent autophosphorylation, an additional type of protein kinase activity, occurs at the C-terminal serine-1101 of p110γ (Czupalla et al., 2003).

4.5.1 Measurement of lipid kinase activity

The assays were conducted in a final volume of 50 µl containing 40 mM HEPES (pH 7.4), 0.1 % bovine serum albumin, 1 mM EGTA, 7 mM $MgCl_2$, 120 mM NaCl, 1 mM DTT, 1 mM β-glycerophosphate (buffer A) as previously described (Leopoldt et al., 1998, Maier et al., 1999) with some modifications. A 30 µl lipid vesicle mixture, containing 320 µM phosphatidylethanolamine, 300 µM phosphatidylserine, 140 µM phosphatidylcholine, 30 µM sphingomyelin supplemented with either 300 µM PI or 40 µM PI-4,5-P_2 in buffer A, was dried down and sonicated into buffer A. Subsequently, the lipid vesicles were mixed with Gβγ, and incubated on ice for 10 min. It was ensured that Gβγ-containing phospholipid vesicles did not suppress activity of PI3Kγ. In addition, samples containing different amounts of Gβγ were adjusted to identical detergent concentrations, such as 0.002 % of $C_{12}E_{10}$. For inhibition assays, kinase preparations were preincubated with wortmannin for 10 min at 30 °C. Thereafter, the enzyme fraction (10 ng) was added, and the mixture was incubated for a further 10 min at 4 °C in a final volume of 40 µl. Then, the assay was started by adding 40 µM ATP (1 µCi of [γ-^{32}P]ATP, Hartmann Analytic) in 10 µl of the above assay buffer at 30 °C. After 15 min, the reaction was stopped with 150 µl of ice-cold 1 N HCl and the tubes were placed on ice. The lipids were extracted by vortexing the samples with 500 µl of a 1:1 chloroform : methanol solution. After centrifugation, the organic phase was washed twice with 200 µl of 1 N HCl. Subsequently, 70 µl of the organic phase was resolved on a potassium oxalate-pretreated TLC plate (Whatman) with 35 ml of 2 N acetic acid and 65 ml of *n*-propyl alcohol as the mobile phase. Dried TLC plates were exposed to Fuji imaging

plates, and autoradiographic signals were quantitated with a Fujifilm FLA-5000 imaging system (Raytest).

4.5.2 Measurement of protein kinase activity

The in vitro protein kinase activity was determined as described for the lipid kinase activity (see section 4.5.1) with some modifications. The total assay volume was 25 µl (2 µCi of [γ-^{32}P]ATP/tube), the vesicle buffer contained 10 mM $MgCl_2$, and the lipid vesicles lacked PI-4,5-P_2 (unless otherwise stated). After an incubation period of 30 min at 30 °C the reaction was stopped by adding 10 µl of 4x Laemmli sample buffer (Laemmli, 1970). Following separation on SDS-PAGE, proteins were transferred to nitrocellulose membranes. Dried membranes were exposed to Fuji imaging plates, and autoradiographic signals were quantitated.

4.6 Lipid vesicle pull-down assay

Experimental conditions to determine the Gβγ-dependent association of PI3Kγ with lipid vesicles were similar to measurement of the enzymatic activity of PI3K with some modifications. Usually, the assay did not contain radioactively labelled ATP (unless otherwise stated) and had higher amount of PI3Kγ (200 - 400 ng). After an incubation period of 15 min at 30 °C, the mixture was put on ice and centrifuged for 2 min at 12,000 x g at 4 °C. The supernatant and pellet were separated. The supernatant was supplemented with 4x Laemmli sample buffer. The pellet was resuspended and washed twice with buffer A (see section 4.5.1). Subsequently, the pellet was resolved in 1x Laemmli sample buffer. The samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Semiquantitative analysis of immunoblots was done using specific antisera against p110γ, p101 and $Gβ_1$ subunits. As a control, PI3Kγ enzymatic activities were measured in parallel experiments.

4.7 Generation and presentation of the dose-response curves of PI3Kγ stimulation induced by $Gβ_1γ_{2\text{-}His}$ mutants

Stimulation of lipid and protein kinase activities of PI3Kγ by various $Gβ_1γ_{2\text{-}His}$ variants was analyzed in assays containing phospholipid vesicles (see section 4.5.1). It was noticed that Gβγ-induced stimulation of PI3Kγ enzymatic activities, during the course of this study,

revealed a significant deviation of kinetic parameters (Fig. 4.2). There are some possible reasons of such effect: different lots of phospholipids and other commercial reagents, efficiency of nitrogen drying of phospholipids and sonication into buffer, maintaining of exact temperature, different batches of purified recombinant proteins, etc.

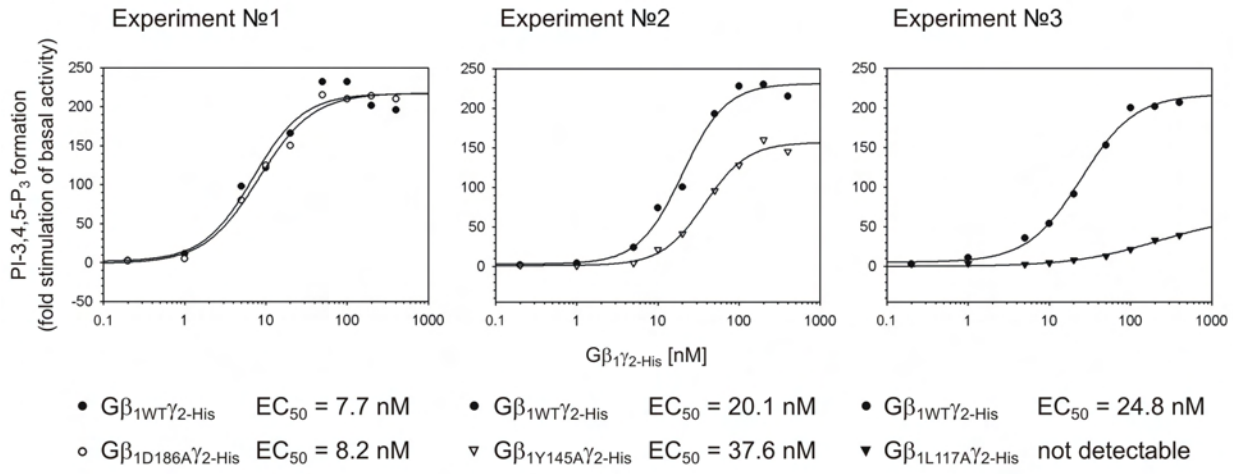


Fig. 4.2: Deviation of kinetic parameters of the $G\beta_1\gamma_{2\text{-His}}$-induced stimulation of lipid kinase activity of dimeric PI3Kγ

Three experiments representing the stimulation of lipid kinase activity of $p110\gamma_{\text{-His}}$/p101 in response to increasing concentrations of $G\beta_{1WT}\gamma_{2\text{-His}}$ and its alanine mutants are shown. Assays were performed as detailed under section 4.5.1. Gβγ-induced activation of dimeric PI3Kγ is illustrated as fold-stimulation of basal activity.

For that reason, ability of $G\beta_1\gamma_{2\text{-His}}$ alanine mutants to simulate PI3Kγ was studied with simultaneous parallel stimulation of PI3Kγ by wild-type $G\beta_1\gamma_{2\text{-His}}$, which was considered as "real-time" control. Fig. 4.2 is example of three independent experiments were EC_{50} values for $G\beta_{1WT}\gamma_{2\text{-His}}$-dependent stimulation of $p110\gamma_{\text{-His}}$/p101 considerably vary. In order to show $G\beta_1\gamma_{2\text{-His}}$ mutants belonging to two distinct groups only in two figures, we proposed to combine the data obtained with different kinetic parameters. For this, recalculations of every data which were used for fitting of dose-response curve were performed as demonstrated in Table 4.3.

Fig. 4.3 demonstrates an example of the final presentation of the data. Therefore, in this case, the kinetic parameters (f. ex. EC_{50} value for $G\beta_{1Y145A}\gamma_{2\text{-His}}$) are not absolute but relative to a "fixed" wild-type control.

Table 4.3: Example of calculation of relative data for the presentation of $G\beta_{1Y145A}\gamma_{2\text{-}His}$– and $G\beta_{1L117A}\gamma_{2\text{-}His}$–induced stimulation of dimeric PI3Kγ

Type of $G\beta_1\gamma_{2\text{-}His}$ mutant	$G\beta_1\gamma_{2\text{-}His}$ [nM]								
	0.2	**1**	**5**	**10**	**20**	**50**	**100**	**200**	**400**
***Data of experiment №1*[1]**									
WT_1[2]	2.5[3]	11.4	98.1	121.8	166.2	232.3	232.1	201.4	196.8
D186A	2	5.3	80.2	124.7	149.8	215.3	209.7	214.2	209.6
Data of experiment №2									
WT_2	2.1	3.9	24.2	73.8	100.4	193.2	227.8	230.1	215.3
Y145A	1.6	0.7	4.4	21.4	41.2	96.1	127.8	160.3	145.3
WT_2/Y145A	1.3	5.6	5.5	3.4	2.4	2.0	1.8	1.4	1.5
Data of experiment №3									
WT_3	3.1	10.9	35.7	54.3	91.5	153.2	197.2	202.4	207.6
L117A	3.0	3.9	2.6	4.5	8.3	13.2	21.6	33.1	38.8
WT_3/L117A	1.03	2.8	13.7	12.1	11.0	11.6	9.1	6.1	5.4
Resulting data for presentation									
WT_1	2.5	11.4	98.1	121.8	166.2	232.3	232.1	201.4	196.8
D186A	2	5.3	80.2	124.7	149.8	215.3	209.7	214.2	209.6
$Y145A_{(added)}$ = WT_1/(WT_2/Y145A)	1.9	2.0	17.8	35.8	69.3	116.2	128.9	143.9	131.2
$L117A_{(added)}$ = WT_1/(WT_3/L117A)	2.4	4.1	7.2	10.1	15.1	20.0	25.5	33.0	36.5

[1] data of experiments shown in Fig. 4.2.
[2] $WT_{1\text{-}3}$: data of wild-type $G\beta_1\gamma_{2\text{-}His}$-induced stimulation of $p110\gamma_{\text{-}His}$/p101 obtained in three independent experiments.
[3] fold stimulation of basal activity

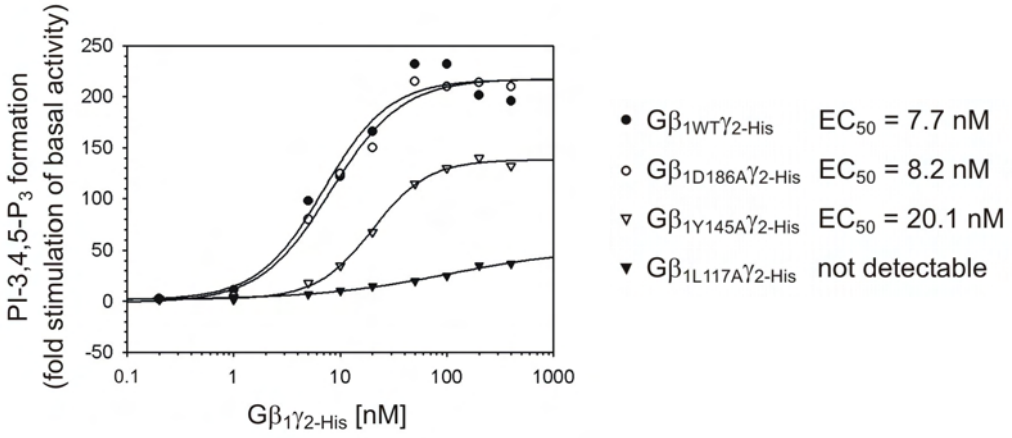


Fig. 4.3: Final combination of the sigmoidal dose-response curves obtained in three independent experiments

Sigmoidal dose-response curves obtained from the data of Experiments №2 and №3 (see Fig. 4.2) were recalculated as demonstrated in Table 4.3. Thereafter, the relative sigmoidal plots for $G\beta_{1Y145A}\gamma_{2\text{-}His}$– and $G\beta_{1L117A}\gamma_{2\text{-}His}$–induced stimulation of the dimeric PI3Kγ were fitted and added in figure obtained from the data of Experiment №1 (see Fig. 4.2).

5 RESULTS

5.1 Purification of recombinant $G\beta_1\gamma_{2\text{-His}}$ from Sf9 cells

According to previous reports (Maier et al., 1999, 2000, Czupalla et al., 2003a), $G\beta_1\gamma_{2\text{-His}}$ dimers were isolated from the membrane fraction of Sf9 cells infected with baculoviruses encoding $G\beta_1$ and $G\gamma_{2\text{-His}}$ using two column chromatography (Ni^{2+}-NTA and Resource Q, see section 4.3.1). $G\beta_1\gamma_{2\text{-His}}$ dimers were purified with a high degree of purity (> 90 %) as illustrated by Coomassie staining of SDS-polyacrylamide gel (Fig. 5.1A).

In order to verify that the purified $G\beta_1\gamma_{2\text{-His}}$ contains no enzymatic contaminants, the complex was tested in lipid kinase assay (see section 4.5.1) for its ability to accumulate phospholipids in the absence of PI3Kγ. Although no evident contaminant protein appeared in stained SDS-polyacrylamide gel, our preparation of $G\beta_1\gamma_{2\text{-His}}$ exhibited lipid kinase activity (Fig. 5.1B). Additionally, this activity increased dose-dependently with the concentration of $G\beta_1\gamma_{2\text{-His}}$ up to 1500 nM. At this concentration the production reaches approximately 80 fmol of ^{32}P-labeled phospholipids.

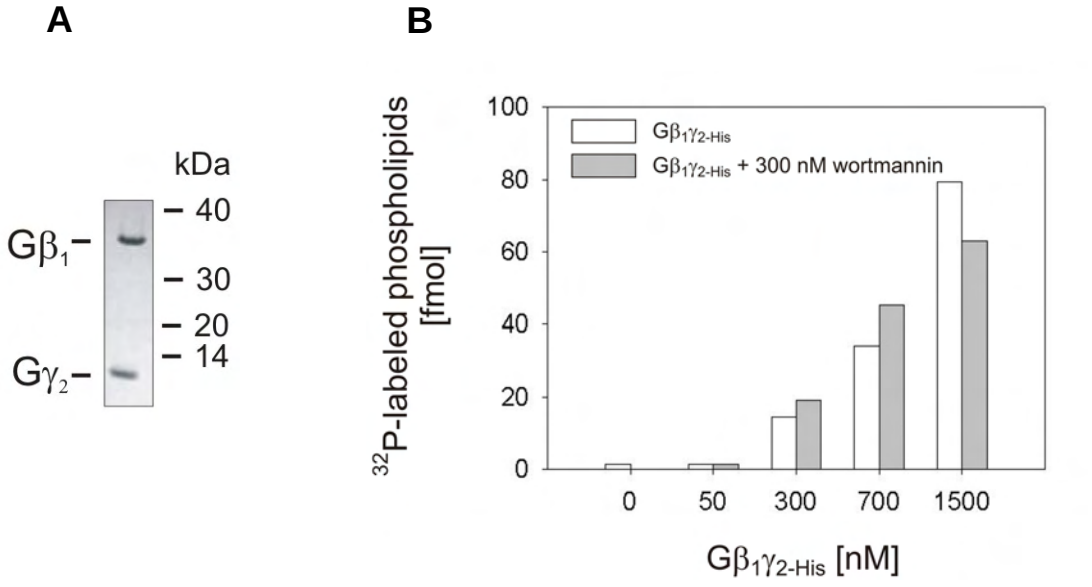


Fig. 5.1: $G\beta_1\gamma_{2\text{-His}}$ copurified with a wortmannin-insensitive lipid kinase specific activity

(A) Recombinant $G\beta_1\gamma_{2\text{-His}}$ was purified from Sf9 cells as detailed under section 4.3.1. Proteins were subjected to SDS-PAGE (10 % acrylamide) and analyzed by Coomassie staining. Apparent molecular masses of marker proteins are indicated. **(B)** Increasing concentrations of purified recombinant $G\beta_1\gamma_{2\text{-His}}$ were preincubated in the absence (clear bars) or in the presence (grey bars) of 300 nM wortmannin and subsequently tested in lipid kinase assay (see section 4.5.1). These experiments were performed in the absence of any PI3Kγ using PI-4,5-P_2 as a substrate. Shown is one representative experiment.

Interestingly, wortmannin, a selective inhibitor of PI3Ks (see section 1.3.2.2), was unable to inhibit the accumulation of ^{32}P-labeled phospholipids even at a 60 fold higher concentration than its IC_{50} (Fig. 5.1B). This activity most likely resulted from a copurified wortmannin-insensitive insect lipid kinase. Correspondingly, by immunoblotting of purified $G\beta_1\gamma_{2\text{-His}}$ preparations we excluded the presence of p110γ using a monoclonal mouse antibody against recombinant human p110γ or a polyclonal antiserum SC-7177 (H199) raised against N-terminus of p110γ [aa 331 - 530] (data not shown). In order to separate the unspecific lipid kinase activity from $G\beta_1\gamma_{2\text{-His}}$, we performed an additional purification step, i.e. gel filtration (see section 4.3.1.4). To establish size exclusion chromatography, we tested two different detergents: the non-ionic polyoxyethylene 10-laurylether ($C_{12}E_{10}$) at a concentration of 1.6 mM (0.1 %) and the ionic sodium cholate at a concentration of 20 mM (0.9 %).

The fractionation in the presence of $C_{12}E_{10}$ revealed two major peaks corresponding to 138-253 kDa and 30-50 kDa (Fig. 5.2A, upper panel).

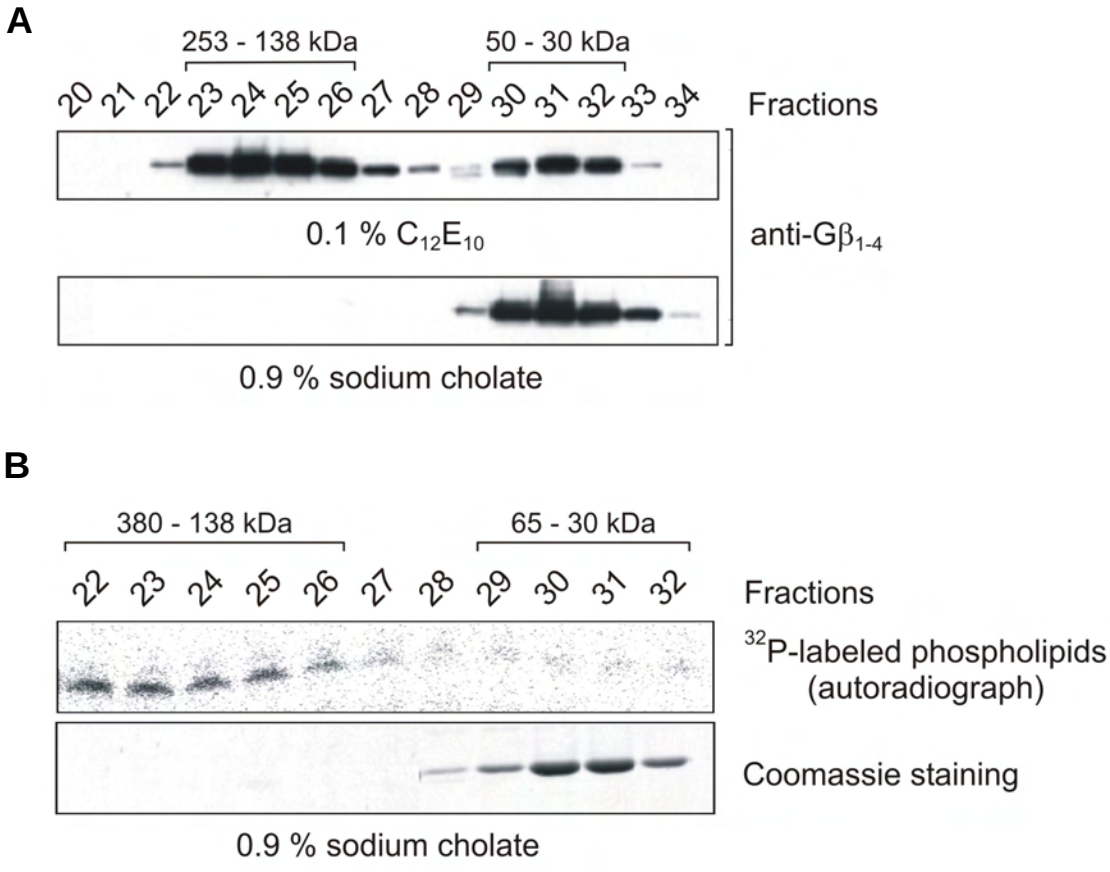


Fig. 5.2: Gel filtration of recombinant $G\beta_1\gamma_{2\text{-His}}$ on Superdex 200 HR 10/30

(A) $G\beta_1\gamma_{2\text{-His}}$ was purified as described (see section 4.3.1). Aliquots of the recombinant protein were applied to a Superdex 200 HR 10/30 and eluted with buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM β-ME) containing 0.1 % $C_{12}E_{10}$ (upper panel) or 0.9 % sodium cholate (lower panel). Fractions were analyzed by immunoblotting using a $G\beta_{1\text{-}4}$–specific antisera. **(B)** Separation of $G\beta_1\gamma_{2\text{-His}}$ from copurified lipid kinase activity. Two microliters of each fraction were analyzed by a lipid kinase assay with PI-4,5-P_2 as the substrate to identify fractions which lead to production of ^{32}P-labeled phospholipids (upper panel). Aliquots of eluted fractions were subjected to SDS-PAGE and analyzed by Coomassie staining (lower panel).

In contrast, only one peak corresponding to 30-50 kDa was observed in the presence of sodium cholate (Fig. 5.2A, lower panel). This difference may be explained by different critical micellar concentrations (CMC) of the different detergents used in this study (Dawson et al., 1986). Indeed, in our experiments $C_{12}E_{10}$ was used at a concentration more than 40 fold higher than its CMC (40 µM) whereas the concentration of sodium cholate was about its CMC (14 mM). The peak corresponding to $G\beta_1\gamma_{2\text{-His}}$ dimers according to their respective molecular masses of 37.4 kDa for $G\beta_1$ and 7.9 kDa for $G\gamma_2$ was expected to be around 45 kDa. The peak of higher molecular mass was most likely due to micelles formed by $C_{12}E_{10}$. However, we can not exclude that non-ionic $C_{12}E_{10}$ and ionic sodium cholate may produce lipid micelles which have different micellar size (Strop and Brunger, 2005).

Fig. 5.2B shows that the presence of 0.9 % sodium cholate allowed separation of $G\beta_1\gamma_{2\text{-His}}$ from unspecific kinase activity during gel filtration. The contaminant peak eluted in early fractions corresponding to 140-380 kDa (Fig. 5.2B, upper panel), whereas $G\beta_1\gamma_{2\text{-His}}$ appeared in fractions with an appropriate mass of 30-60 kDa (Fig. 5.2B, lower panel). Nevertheless, for further use the exchange of sodium cholate for the non-ionic detergent $C_{12}E_{10}$ which is compatible with lipid and protein kinase assays was mandatory. The fractions number 29 - 32 (Fig. 5.2B) were pooled and subjected onto Ni^{2+}-NTA Superflow. Subsequently, $G\beta_1\gamma_{2\text{-His}}$ was eluted in a buffer supplemented with $C_{12}E_{10}$. In order to remove imidazole and to concentrate the protein, the protein peak eluted from the Ni^{2+}-NTA Superflow was once again passed through anion-exchange Resource 15Q column (1 ml).

The addition of the gel filtration step in the purification of $G\beta_1\gamma_{2\text{-His}}$ eliminated the contaminating lipid kinase activity as illustrated in Fig. 5.3A. After this procedure, $G\beta_1\gamma_{2\text{-His}}$ stimulated the dimeric PI3Kγ and the production of specific ^{32}P-labeled phospholipids in a wortmannin-sensitive fashion (Fig. 5.3B).

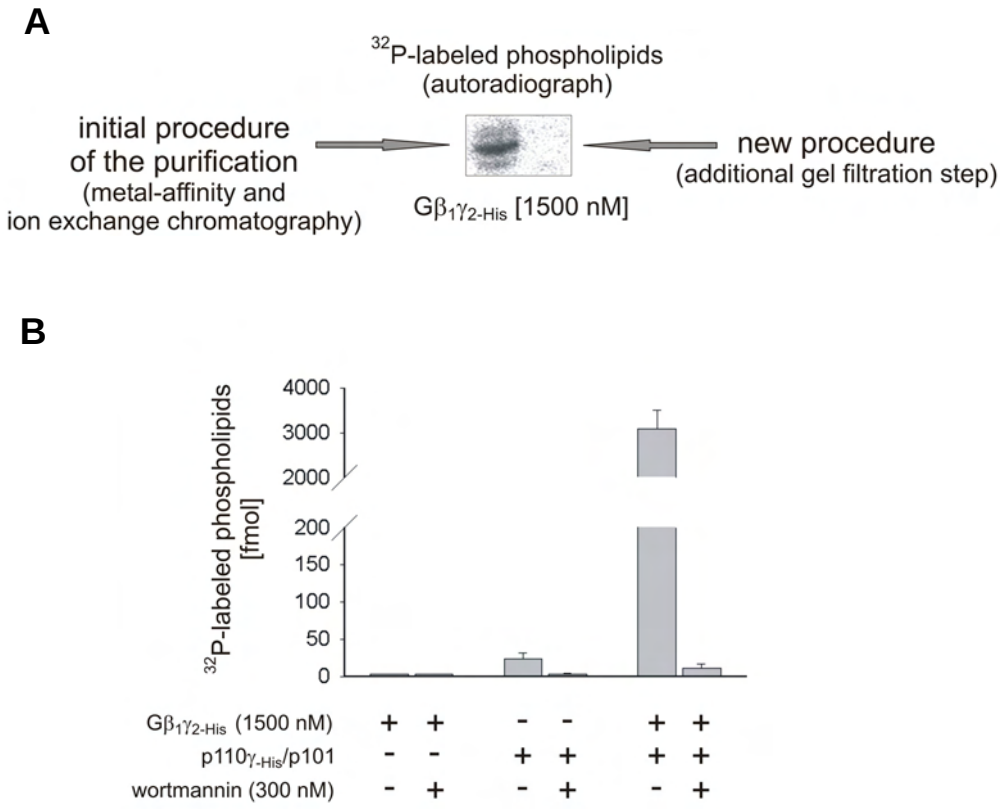


Fig. 5.3: Functional characterization of the purified recombinant $G\beta_1\gamma_{2\text{-His}}$ after gel filtration

(A) Elimination of a lipid kinase specific activity after additional gel filtration step. $G\beta_1\gamma_{2\text{-His}}$ purified by two different protocols was tested in lipid kinase assay (see section 4.5.1) in the absence of PI3Kγ for its ability to produce ^{32}P-labeled phospholipids. PI-4,5-P_2 was used as a substrate. **(B)** Influence of wortmannin on $G\beta_1\gamma_{2\text{-His}}$–dependent stimulation of lipid kinase activity of $p110\gamma_{\text{-His}}$/p101. Indicated are mean values ± S.D. of three independent experiments.

5.2 Characterization of purified recombinant $G\beta_1\gamma_2$ and PI3Kγ

5.2.1 Purity and trypsin sensitivity of $G\beta_1\gamma_{2\text{-His}}$ variants

Classical protocols of purification of recombinant $G\beta_1\gamma_{2\text{-His}}$ describe infection of Sf9 cells with baculoviruses encoding $G\beta_1$, $G\gamma_2$ and $G\alpha_{x\text{-His}}$ (e.g. $G\alpha_{i1\text{-His}}$). $G\alpha_{i1\text{-His}}\beta_1\gamma_2$ complexes were bound to Ni^{2+}-NTA column and subsequently $G\beta_1\gamma_2$ dimers were purified by activation of the heterotrimer and elution with aluminium fluoride (Kozasa and Gilman, 1995). However, in this study, we used $G\beta_1$ mutants where critical amino acids were substituted for alanine. In particular, those amino acids of $G\beta_1$ were exchanged for alanine which were proposed to be important for association of Gβγ with Gα (Ford et al., 1998). For that reason, recombinant $G\beta_1\gamma_{2\text{-His}}$ and its mutants were purified as dimers. Nevertheless, only those mutants, which were properly formed as dimers, were used in the current study. By applying the purification protocol described above (see section 5.1), we purified the mutants to apparent homogeneity.

Fig. 5.4 demonstrates the quality of the purified recombinant $G\beta_{1WT}\gamma_{2\text{-His}}$ and its alanine mutants as well as cytosolic recombinant monomeric and dimeric PI3Kγ (Fig. 5.4B). The purity of proteins was more than 90 %.

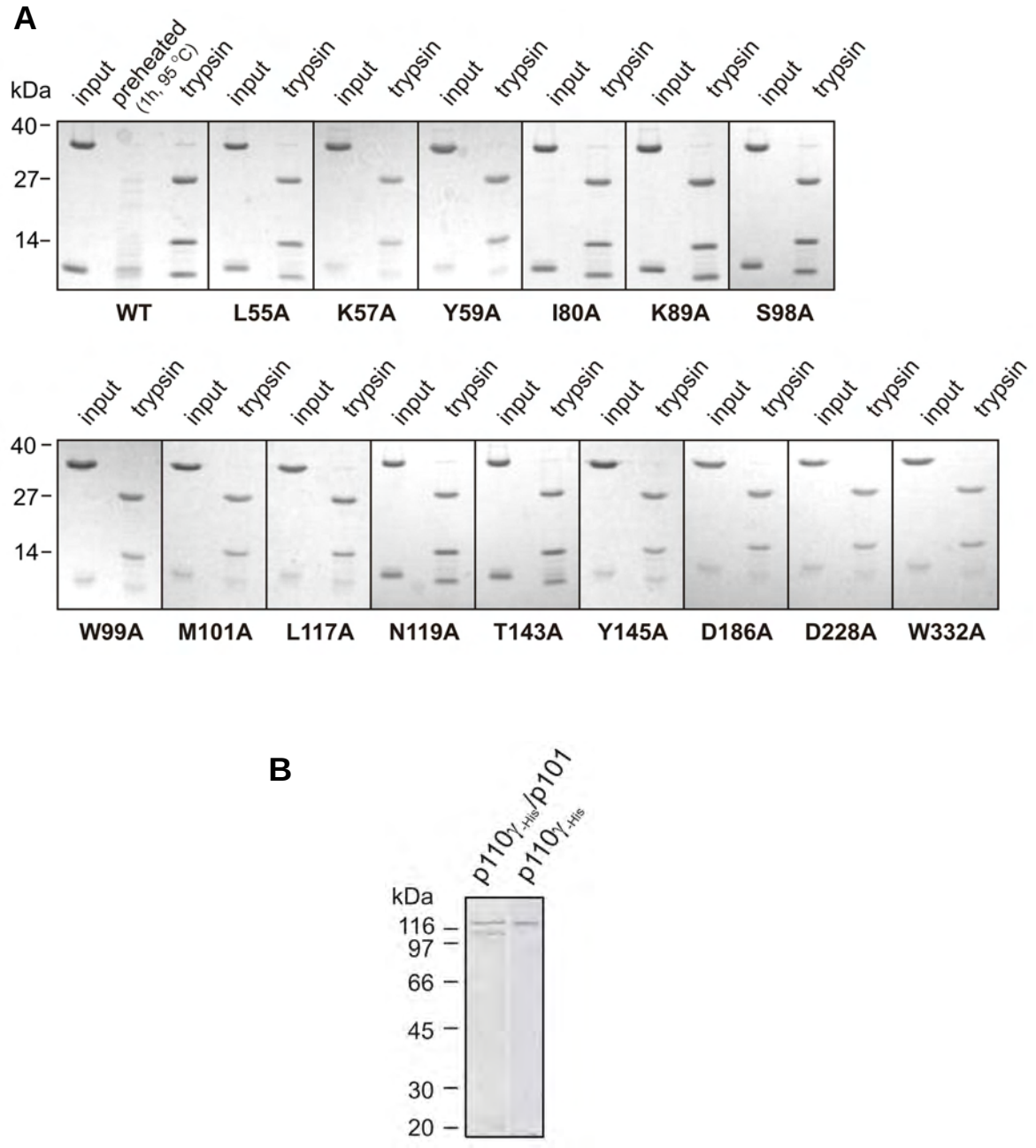


Fig. 5.4: Purity of recombinant proteins and sensitivity of $G\beta_1\gamma_{2\text{-His}}$ variants to trypsin digestion

(A) The tryptic protection assay was performed as described under section 4.4. Five microgramm of each protein were incubated with 0.2 µg of TPCK-treated trypsin for 40 min at 30 °C in total volume of 30 µl. Proteolysis was terminated by adding 10 µl of 4x sample buffer according to Laemmli (Laemmli, 1970). Proteins were subjected to SDS-PAGE and analyzed by Coomassie staining. $G\beta_{1WT}\gamma_{2\text{-His}}$ boiled for 1 hour at 95 °C followed by the tryptic protection assay was considered as a negative control. **(B)** SDS-PAGE of monomeric ($p110\gamma_{\text{-His}}$) and dimeric ($p110\gamma_{\text{-His}}$/p101) PI3Kγ. Apparent molecular masses are indicated.

The correct assembly of $G\beta_1\gamma_{2\text{-His}}$ was tested using the limited trypsin digestion assay (Winslow et al., 1986, Thomas et al., 1993, Ford et al., 1998). The assay is based on the fact that despite the presence of 32 potential tryptic sites on Gβ, intact Gβγ dimers are highly resistant to proteolysis. Indeed, trypsin only cleaves correctly folded Gβ at position Arg-129 leading to two proteolytic fragments of 26 and 14 kDa. These fragments are quite resistant to further degradation by trypsin. The Gγ subunit seems not to be cleaved by trypsin (Fung and Nash, 1983).

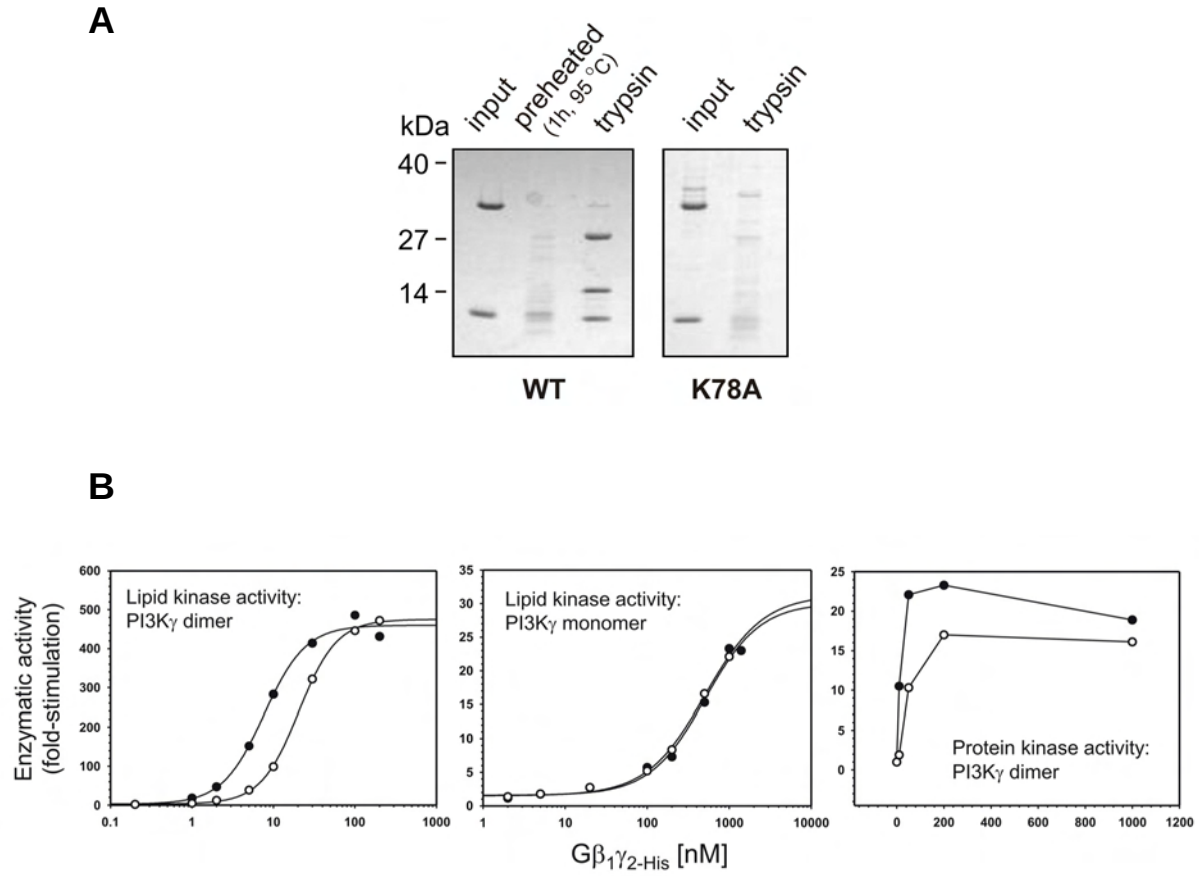


Fig. 5.5: Sensitivity of $G\beta_{1K78A}\gamma_{2\text{-His}}$ to trypsin digestion and its ability to stimulate the PI3Kγ enzymatic activities

(A) Incubation of $G\beta_{1K78A}\gamma_{2\text{-His}}$ with TPCK-treated trypsin produces more than two proteolytic fragments. $G\beta_{1WT}\gamma_{2\text{-His}}$ boiled for 1 hour at 95 °C followed by the tryptic protection assay was considered as a negative control. The tryptic protection assay was done as described in section 4.4. Five microgramm of each protein were incubated with 0.2 µg of TPCK-treated trypsin for 40 min at 30 °C in total volume of 30 µl. Proteolysis was terminated by adding 10 µl of 4x sample buffer according to Laemmli (Laemmli, 1970). Proteins were subjected to SDS-PAGE and analyzed by Coomassie staining. **(B)** Recombinant $G\beta_{1K78A}\gamma_{2\text{-His}}$ mutant (○) shows the same ability to stimulate the PI3Kγ activity as wild-type $G\beta_1\gamma_{2\text{-His}}$ (●). Lipid kinase activities of $p110\gamma_{\text{-His}}$/p101 and $p110\gamma_{\text{-His}}$ and protein kinase activity of $p110\gamma_{\text{-His}}$/p101 were tested (see section 4.5). Gβγ-induced activation of different forms of PI3Kγ is illustrated as fold-stimulation of basal activity.

The tryptic sensitivity assay applied to all $G\beta_1$ alanine mutants in the $G\beta_1\gamma_{2\text{-His}}$ complexes yielded two proteolytic fragments of 26 and 14 kDa indicating that $G\beta_1$ assembled correctly with $G\gamma_2$ (Fig. 5.4A). An incorrect folding of $G\beta_1\gamma_{2\text{-His}}$ is illustrated in Fig. 5.4A (upper panel), following denaturation of $G\beta_{1WT}\gamma_{2\text{-His}}$ by heating for 1 hour at 95 ºC.

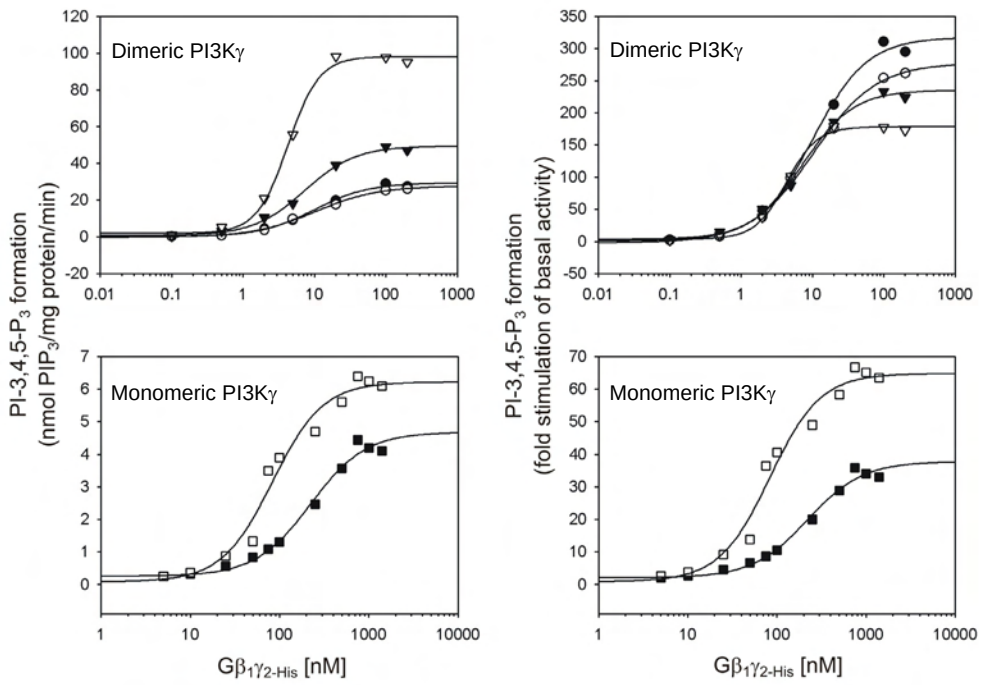
Among the mutants tested, only the mutation of $G\beta_1$ at Lys-78 led to trypsin-sensitive state of $G\beta_1\gamma_{2\text{-His}}$. Indeed, the tryptic protection assay revealed more than two proteolytic fragments (Fig. 5.5A, right panel). This result suggests that alanine mutation of Lys-78 leads to incorrect folding of $G\beta_1$ thereby misassembling with $G\gamma_2$. Nonetheless, this protein was purified and subjected to lipid and protein kinase assays. Surprisingly, in initial studies we could not find any impairment of its ability to stimulate different enzymatic forms (monomer and dimer) and enzymatic qualities (protein and lipid kinase activity) in comparison to $G\beta_{1WT}\gamma_{2\text{-His}}$ (Fig. 5.5B). Nevertheless, this mutant was not considered for further analysis.

5.2.2 Influence of N-terminal His- and GST-tags and N-terminal fragment (amino acids 1 - 34) of p110γ on the lipid kinase activity of PI3Kγ

To facilitate purification, target proteins were fused to affinity tags. The hexahistidine (His)-tag binds to nickel or cobalt ions immobilised on a support (Agarose or “Superflow”). GST-fusion (glutathion-S-transferase) proteins were purified by glutathione affinity chromatography. GST is a tag of higher molecular mass (26 kDa) compared with the His-tag (approximately 0.7 kDa). These two affinity tags are commonly used for the purification of recombinant proteins (Leopoldt et al., 1998, Maier et al., 1999, 2000, Czupalla et al., 2003a, Scheich et al., 2003).

In initial experiments, we have examined the enzymatic activity of His- and GST-fused PI3Kγ. The activities of the dimeric PI3Kγ showed no difference whether the His-tag was bound to the N-terminus of p110γ or p101 (Fig. 5.6A, closed and open circles). The EC_{50} (10 nM) and the V_{max} (~30 nmol PIP_3/mg of protein/min) of the stimulation of the lipid kinase activity by $G\beta_1\gamma_{2\text{-His}}$ are similar in the dimeric $p110\gamma_{\text{-His}}$/p101 and $p110\gamma/p101_{\text{-His}}$ (Fig. 5.6A, table). The fusion of GST-tag to the N-terminus of p101 but not p110γ significantly increased the basal activity as well as the V_{max} of the dimeric PI3Kγ compared to His-tagged versions (Fig. 5.6A, table, triangles versus circles). However, the evaluation of these data as fold stimulation over the basal activity demonstrated that the potency of stimulation of $p110\gamma/p101_{\text{-GST}}$ was significantly reduced compared to $p110\gamma_{\text{-His}}$/p101 (Fig. 5.6A, upper panel, right, open triangles versus closed circles, and table). The differences between other combinations of His- and GST-fused dimeric PI3Kγ were insignificant.

A



PI3Kγ			EC_{50} [nM]	PI-3,4,5-P_3 formation (nmol PIP_3 / mg protein / min)		Stimulation of basal activity (folds)
				basal activity	V_{max}	
●	**p110γ$_{\text{-His}}$/p101**	1	10.1 ± 5.8	0.093 ± 0.07	29.5 ± 4.9	317.2 ± 52.7
○	**p110γ/p101$_{\text{-His}}$**	2	10.5 ± 4.3	0.11 ± 0.05	27.7 ± 6.7	251.8 ± 60.9
▼	**p110γ$_{\text{-GST}}$(Δ1-34)/p101**	3	7.3 ± 3.7	0.21 ± 0.09	49.5 ± 13.4	235.7 ± 63.8
▽	**p110γ/p101$_{\text{-GST}}$**	4	4.3 ± 2.2	0.55 ± 0.14	98.1 ± 25.8	178.4 ± 46.9
■	**p110γ$_{\text{-His}}$**	5	222.6 ± 25.7	0.12 ± 0.06	4.7 ± 0.7	39.2 ± 5.8
□	**p110γ$_{\text{-GST}}$(Δ1-34)**	6	83.2 ± 18.7	0.10 ± 0.04	6.2 ± 0.5	62.0 ± 5.1

B

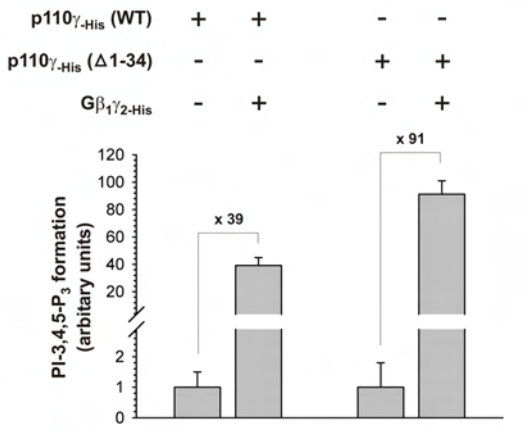


Fig. 5.6: Influence of the N-terminal tags and N-terminal truncation of p110γ on the lipid kinase activity of the recombinant PI3Kγ

(A) Purified recombinant monomeric and heterodimeric PI3Kγ were stimulated with increasing concentrations of recombinant $G\beta_1\gamma_{2\text{-His}}$ using PI-4,5-P_2 as a substrate. ^{32}P-labeled lipid products were isolated and quantified as described under section 4.5.1. After termination of reaction, probes were subjected to thin layer chromatography (TLC) plates. Dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system. Shown is one typical experiment out of three. The kinetic parameters are shown in table. The baculoviruses, which were used for expression of p110$\gamma_{\text{-GST}}$ alone or in combination with p101, encoded N-terminally (amino acids 1 - 34) truncated p110$\gamma_{\text{-GST}}$ (Stoyanov et al., 1995, Stephens et al.,

1997). Data are means ± S.D. of duplicate determinations in three independent experiments. Statistical analysis for basal activity: 2 versus 1, P > 0,05; 3 versus 1, P > 0,05; 4 versus 3, P < 0,05; 4 versus 2, P < 0,01; 4 versus 1, P < 0,01; 6 versus 5, P > 0,05. Statistical analysis for V_{max}: 2 versus 1, P > 0,05; 3 versus 1, P > 0,05; 4 versus 3, P < 0,05; 4 versus 2, P = 0,01; 4 versus 1, P = 0,01; 6 versus 5, P < 0,05. Statistical analysis for fold stimulation of basal activity: 2 versus 1, P > 0,05; 3 versus 1, P > 0,05; 4 versus 3, P < 0,05; 4 versus 2, P > 0,05; 4 versus 1, P < 0,05; 6 versus 5, P < 0,01. **(B)** Lipid kinase activities of full-length wild-type (WT) and N-terminally (△1-34) truncated $p110\gamma_{\text{-His}}$ were tested in the absence (-) and in the presence (+) of 600 nM $G\beta_1\gamma_{2\text{-His}}$. Assays were performed as described under section 4.5. Activities are shown as arbitary units. Basal lipid kinase activity of monomeric PI3Kγ was considered as "1". One arbitary unit of PI-3,4,5-P_3 formation corresponds to basal lipid kinase activity of monomeric PI3Kγ which was 0.12 ± 0.06 nmol PIP_3/mg protein/min (n = 3). Fold-stimulation of basal activity is shown over the bars. Shown are mean values ± S.D. of three independent experiments. The difference between $G\beta_1\gamma_{2\text{-His}}$-stimulated lipid kinase activities of $p110\gamma_{\text{-His}}$ (WT) and $p110\gamma_{\text{-His}}$ (△1-34) is significant (P < 0.05).

The lipid kinase activity of monomeric PI3Kγ also clearly depended on the type of tag used. Fig. 5.6A demonstrates that GST-tagged p110γ (open squares) exhibited a specific activity approximately 1.5-fold higher than the one of the His-tagged kinase (closed squares), whereas the basal activities of both tagged enzymes were unaltered (table). There are evidences that GST stabilizes recombinant proteins (Slater et al., 1998, Houten et al., 1999, Amersham Pharmacia Biotech, 2001b). According to these reports, we assume that the increase of specific activity of GST-fused PI3Kγ was a result of either an improved folding of recombinant PI3Kγ during the expression in insect cells and/or stabilization of already isolated kinase. It was also reported that the GST fusion leads to homodimerization of p110γ (Krugmann et al., 1999). Moreover, Yamada et al. (2005) stated that GST-fused actin binding proteins, α-catenin and β-catenin, interact through homodimerization of GST-tags rather than through a direct α-catenin-β-catenin interaction. Therefore, in contrast to assumption described above, the increase of specific activities of GST-fused monomeric and dimeric PI3Kγ may also be explained by assembling of PI3Kγ proteins due to dimerization of GST-tags.

Since in this study we used the baculoviruses encoding N-terminally (amino acids 1 - 34) truncated $p110\gamma_{\text{-GST}}$ (Stoyanov et al., 1995, Stephens et al., 1997), we can not exclude that the absence of this N-terminal fragment may facilitate lipid kinase activities of $p110\gamma_{\text{-GST}}$ and $p110\gamma_{\text{-GST}}$/p101. Therefore, we purified and tested lipid kinase activity of His-tagged p110γ which has the same truncation as $p110\gamma_{\text{-GST}}$. Fig. 5.6B demonstrates that the N-terminally truncated version of $p110\gamma_{\text{-His}}$ exhibited 2.5-times higher lipid kinase activity in the presence of 600 nM $G\beta_1\gamma_{2\text{-His}}$ than the full-length enzyme, whereas basal lipid kinase activities of both enzymes were similar. These data indicate that the increase of $G\beta_1\gamma_{2\text{-His}}$-stimulated lipid kinase activities of $p110\gamma_{\text{-GST}}$ and $p110\gamma_{\text{-GST}}$/p101 may also be due to the absence of the first N-terminal amino acids (1 - 34). However, this assumption does not explain the increase of lipid kinase activity of $p110\gamma/p101_{\text{-GST}}$ compared to $p110\gamma/p101_{\text{-His}}$ (Fig. 5.6A, open triangles versus open circles), since these enzymes contained full-length p110γ. According to obtained data, it seems that both N-terminal GST-tag and N-terminal

amino acids may influence lipid kinase activity of monomeric and dimeric PI3Kγ stimulated by Gβγ. Thus, we preferably used recombinant p110$\gamma_{\text{-His}}$/p101 and p110$\gamma_{\text{-His}}$ preparations throughout the study.

5.2.3 *The non-ionic detergent $C_{12}E_{10}$ affects the lipid kinase activity of PI3Kγ*

The non-ionic detergent $C_{12}E_{10}$ (polyoxyethylene 10-laurylether) contains uncharged, hydrophilic head group that contains a polyoxyethylene moiety. The non-ionic detergents are considered to be non-denaturant and thus being widely used to isolate membrane proteins in their biologically active form (Maier et al., 1999, 2000, Tan and Ting, 2000). In this study, $C_{12}E_{10}$ was used to purify membrane associated G$\beta_1\gamma_{2\text{-His}}$. Additionally, this detergent was also used for the isolation of the cytosolic PI3Kγ in order to prevent non-specific binding of PI3Kγ to the gel filtration and the affinity chromatography supports.

The PI3Kγ activity was measured in solutions containing not only proteins but also lipid vesicles composed of phospholipids (see section 4.5.1). However, detergents were required to prevent the aggregation of membrane proteins, although they can destroy lipid vesicles. Therefore, it was crucial to find the appropriate detergent concentrations suitable for proteins and lipid vesicles integrity. Fig. 5.7A shows that $C_{12}E_{10}$ dramatically decreases the lipid kinase activity of the dimeric PI3Kγ. Increasing the concentration of detergent from 0.0004 % (○) to 0.006% (▽) led to a decline of maximal G$\beta_1\gamma_2$-induced PI-3,4,5-P_2 formation around 7 times (Fig. 5.7A, left panel, Supplementary Table 12.1A in section 12). The detergent $C_{12}E_{10}$ at the concentration of 0.01 % (△) almost completely suppressed the ability of G$\beta_1\gamma_{2\text{-His}}$ to stimulate p110$\gamma_{\text{-His}}$/p101 (Fig. 5.7A, left panel, Supplementary Table 12.1A). The $C_{12}E_{10}$ also affected the activity of the monomeric kinase. The lipid kinase activity of p110$\gamma_{\text{-His}}$ was significantly reduced by rising the concentration of $C_{12}E_{10}$ from 0.002 % to 0.004 % (Fig. 5.7B, left panel, Supplementary Table 12.1B in section 12). However, the evaluation of these data as fold-stimulation of the basal enzymatic activity demonstrated different results. Despite the strong reduction of the lipid kinase specific activity of p110$\gamma_{\text{-His}}$/p101 when rising the $C_{12}E_{10}$ concentration from 0.0004 % to 0.006 % (Fig. 5.7A, ○ versus ▽, left panel), the dynamic of the activation process and fold-stimulation of basal activity were unaltered (Fig. 5.7A, ○ versus ▽, right panel). This effect is caused by proportional $C_{12}E_{10}$-induced reduction of both basal and maximal G$\beta_1\gamma_2$-dependent PI-3,4,5-P_3 formation, which was approximately 7 times (see Supplementary Table 12.1A in section 12).

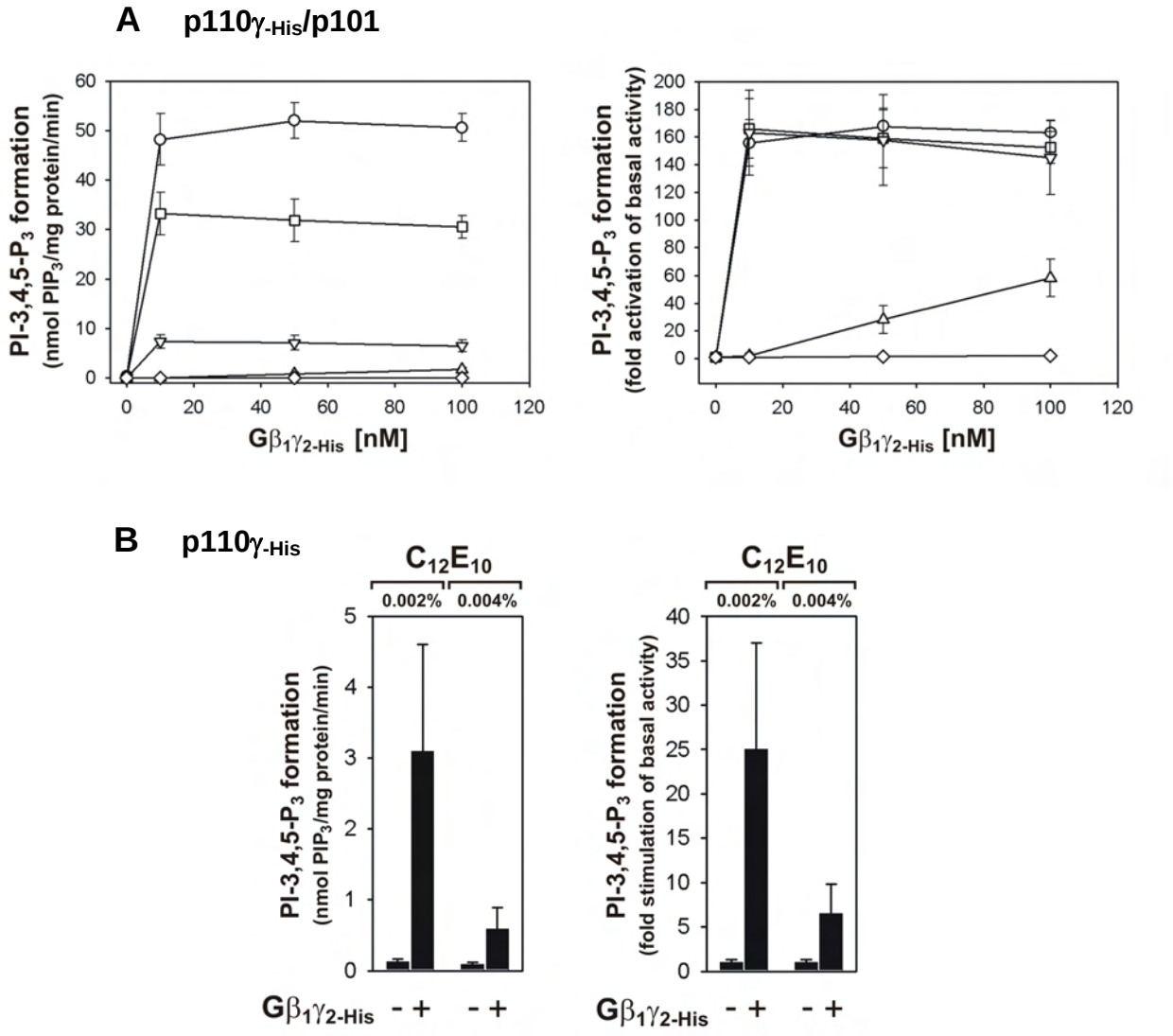


Fig. 5.7: Sensitivity of lipid kinase activity of PI3Kγ to the non-ionic detergent $C_{12}E_{10}$

(A) Purified recombinant p110$\gamma_{\text{-His}}$/p101 was stimulated with increasing concentrations of recombinant G$\beta_1\gamma_{2\text{-His}}$ in a buffer containing 0.0004 % (○), 0.002 % (□), 0.006 % (▽), 0.01 % (△), and 0.05 % (◇) of $C_{12}E_{10}$. **(B)** Lipid kinase activity of p110$\gamma_{\text{-His}}$ in assays including 0.002 % and 0.004 % of $C_{12}E_{10}$.
^{32}P-labeled PI-3,4,5-P_3 were isolated and quantified as detailed under section 4.5.1. After termination of reaction, probes were subjected to thin layer chromatography (TLC) plates. Dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system. Shown are mean values (± S.D.) of duplicate determinations in two independent experiments.

In contrast, for the activity of monomeric PI3Kγ, the increase of $C_{12}E_{10}$ concentration from 0.002 % to 0.004 % showed not only the decline of the specific PI-3,4,5-P_3 formation but also almost a complete abolition of the p110$\gamma_{\text{-His}}$ activity expressed as a fold-stimulation over the basal lipid kinase activity (Fig. 5.7B, right panel, Supplementary Table 12.1B in section 12).

Based on these data, we have decided that the appropriate concentration of $C_{12}E_{10}$ to use for measure of activation of PI3Kγ by G$\beta_1\gamma_{2\text{-His}}$ would be 0.002 % or less. For convenience, the $C_{12}E_{10}$ final concentrations in reactions were adjusted to 0.002 % throughout the study (unless another concentration of $C_{12}E_{10}$ is indicated in figure legends).

5.3 $G\beta_1\gamma_{2\text{-}His}$–induced autophosphorylation of PI3Kγ

In addition to the lipid kinase activity, PI3Kγ possesses an intrinsic protein kinase activity in vitro (Stoyanova et al., 1997), which is sensitive to stimulation by $G\beta_1\gamma_2$ dimers (Maier et al., 1999). This enzymatic quality was characterized as an autophosphorylation of the catalytic subunit of PI3Kγ, which occurs at serine-1101 (Czupalla et al., 2003a, b). In the present study, the stimulation of protein kinase activity by purified recombinant $G\beta_1\gamma_{2\text{-}His}$ and its mutants was considered as one of the screening parameters.

5.3.1 Autophosphorylation of PI3Kγ catalytic subunit occurs at Ser-1101

We examined the $G\beta_1\gamma_{2\text{-}His}$-induced protein kinase activity of heterodimeric PI3Kγ variants containing either wild-type p110γ or a mutated p110γ in which Ser-1101 was replaced by either alanine or the negatively charged aspartic and glutamic acid (Fig. 5.8A). $G\beta_1\gamma_{2\text{-}His}$ significantly induces an incorporation of radioactively labelled phosphate into the catalytic subunit of $p110\gamma_{WT\text{-}His}$/p101. The mutation of Ser-1101 for alanine, aspartic or glutamic acids prevents the incorporation of radioactive phosphate demonstrating that Ser-1101 is the phosphorylation site on p110γ. These findings correlate with previously published data (Czupalla et al., 2003a, Czupalla et al., 2003b).

In contrast to their protein kinase activity, all these PI3Kγ variants were able to produce PI-3,4,5-P_3 in the presence of increasing concentrations of recombinant $G\beta_1\gamma_{2\text{-}His}$ (Fig. 5.8B). No differences were observed between wild-type and alanine substituted kinases, whereas PI3Kγ proteins containing either aspartate or glutamate were significantly more active. The higher activity of PI3Kγ containing aspartate or glutamate at position of 1101 in the presence of $G\beta_1\gamma_{2\text{-}His}$ was unexpected and in disagreement with earlier data published by Czupalla et al. (2003a). The work of this group demonstrated that $G\beta_1\gamma_{2\text{-}His}$-stimulated lipid kinase activity of S1101E and S1101D mutants of PI3Kγ did not differ from those of wild-type PI3Kγ. According the fact that wild-type PI3Kγ is able to autophosphorylate at serine-1101, it is rather surprising that PI3Kγ with substitution of this serine for negatively charged aspartic or glutamic acids, which are assumed to mimic incorporated phosphate, displayed higher activity compared to the wild-type. Additional experiments will be needed to explain the impact of aspartic and glutamic acids at position 1101 of PI3Kγ. At this point we can only speculate that substitution of Ser-1101 for negatively charged residues facilitates the $G\beta_1\gamma_{2\text{-}His}$-induced PI3Kγ by a different manner than just mimicking the phosphate.

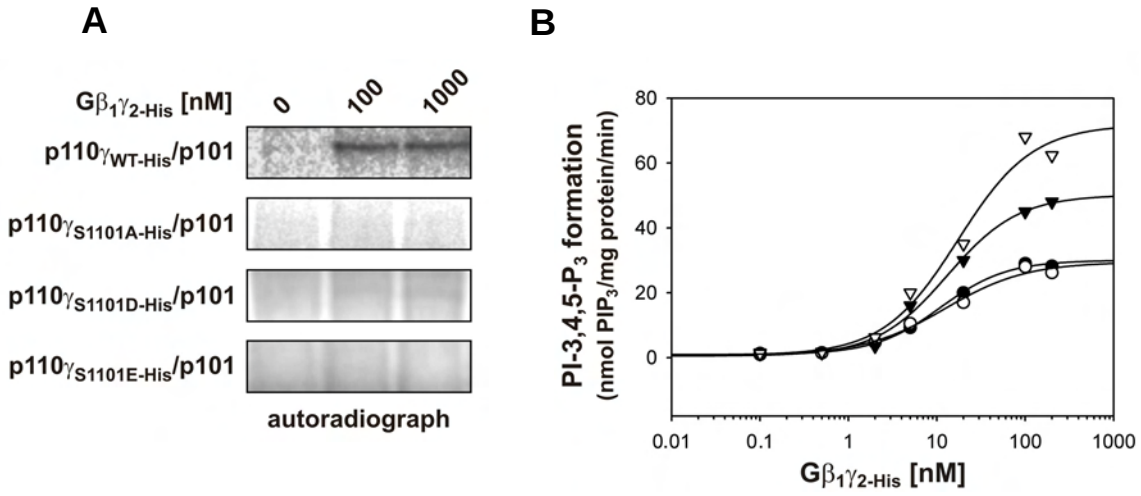


Fig. 5.8: Protein and lipid kinase activities of PI3Kγ variants

(A) Equal amounts (40 ng) of heterodimeric purified PI3Kγ containing either wild-type $p110\gamma_{\text{-His}}$ or its mutants (S1101A, S1101D, S1101E) were tested in a protein kinase assay (see section 4.5.2) in the absence or presence of 100 nM or 1000 nM $G\beta_1\gamma_{2\text{-His}}$. Representative autoradiographs are shown. **(B)** Lipid kinase activity of purified $p110\gamma_{\text{WT-His}}$/p101 (●), $p110\gamma_{\text{S1101A-His}}$/p101 (○), $p110\gamma_{\text{S1101D-His}}$/p101 (▼), and $p110\gamma_{\text{S1101E-His}}$/p101 (▽) was assayed in the presence of increasing concentrations of recombinant $G\beta_1\gamma_{2\text{-His}}$. Assays were performed in the presence of 10 ng of PI3Kγ variants. ^{32}P-labeled PI-3,4,5-P_3 were isolated and quantified as detailed under section 4.5.1. Shown is one representative experiment of three.

In this context previous reports are of interest which provided evidences indicating that aspartic or glutamic acid substitutions are not always proper mimics of phosphorylated amino acids (Ku et al., 1998, Davy et al., 2002). Nevertheless, the data obtained in our study underline that mutation of Ser-1101 for alanine, aspartic or glutamic acids does not hamper the lipid kinase activity of PI3Kγ, whereas autophosphorylation of the enzyme is abolished. In view of that, our results are in accordance with the previously published data (Czupalla et al., 2003a) suggesting that autophosphorylation of p110γ does not inhibit PI3Kγ lipid kinase activity.

5.3.2 Phospholipid vesicles are indispensable for Gβγ-induced stimulation of PI3Kγ autophosphorylation

We have examined whether phospholipid vesicles are required for PI3Kγ autophosphorylation. Fig. 5.9A shows the $G\beta_1\gamma_{2\text{-His}}$-dependent stimulation of heterodimeric PI3Kγ autophosphorylation in phospholipid vesicles and in supernatant. Surprisingly, radioactively labeled kinase was found in both the lipid and the aqueous phase. Moreover, the supernatant appeared to contain the majority of radioactive PI3Kγ (Fig. 5.9A). This observation raises questions about the role of phospholipid vesicles for autophosphorylation of PI3Kγ. Therefore, we studied protein kinase activity of heterodimeric PI3Kγ in the absence

and presence of lipid vesicles. In the absence of phospholipid vesicles, $G\beta_1\gamma_{2\text{-His}}$ did not increase autophosphorylation of $p110\gamma_{\text{-His}}$/p101 (Fig. 5.9B, closed triangles). In contrast, the addition of phospholipid vesicles led to a significant $G\beta_1\gamma_{2\text{-His}}$-dependent stimulation of PI3Kγ autophosphorylation (Fig. 5.9B, circles). Interestingly, the protein kinase activity of PI3Kγ was independent of the presence of phosphoinositides, i.e. PI-4,5-P_2 (Fig. 5.9B, closed and open circles). Although the lipid kinase activity of the enzyme is in a "switched on state" in the presence of PI-4,5-P_2, it does not affect autophosphorylation. On the other hand, autophosphorylation does not affect lipid kinase activity (Fig. 5.8B, open and closed circles, and Czupalla et al., 2003a). The data described above and shown in Fig. 5.9B may indicate that there is no cross-reactivity between these two activities; lipid and protein kinase activities are autonomous and do not depend on each other.

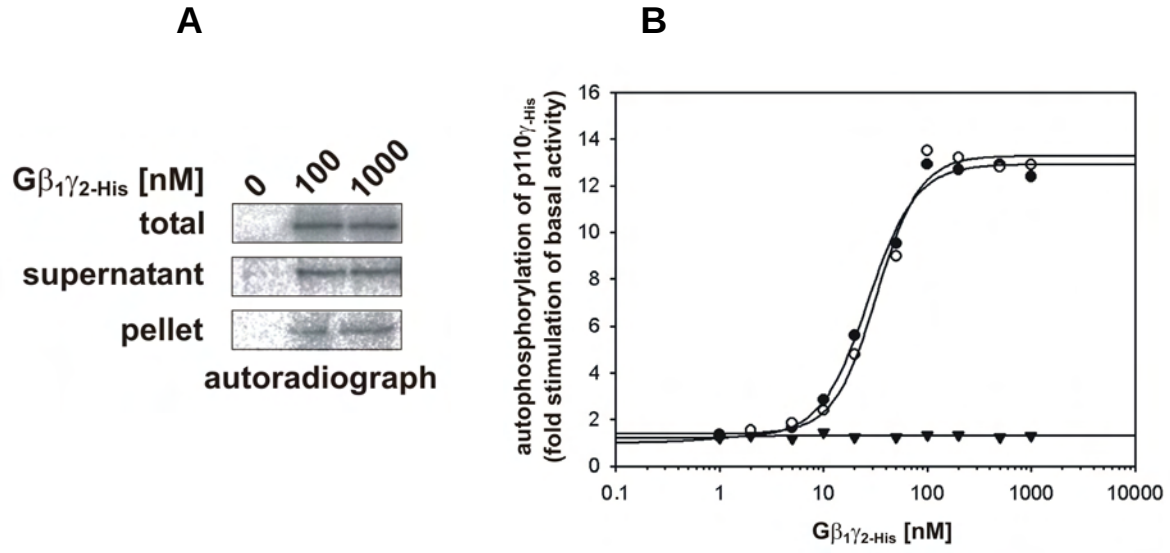


Fig. 5.9: $G\beta_1\gamma_{2\text{-His}}$-induced stimulation of PI3Kγ autophosphorylation requires phospholipid vesicles but not PI-4,5-P_2

(A) Protein kinase activity of recombinant $p110\gamma_{\text{-His}}$/p101 was measured in response to increasing concentrations of $G\beta_1\gamma_{2\text{-His}}$ in the presence of [γ-^{32}P]ATP as detailed under section 4.5.2. After termination of the reaction, supernatant and lipid vesicles were separated by centrifugation as described in section 4.6. One representative autoradiograph of two independent experiments is shown. **(B)** Phospholipid vesicles are necessary for Gβγ stimulation of PI3Kγ autophosphorylation. $G\beta_1\gamma_{2\text{-His}}$–induced autophosphorylation of $p110\gamma_{\text{-His}}$/p101 was tested in the absence (▼) and presence of phospholipid vesicles containing (●) or lacking (○) PI-4,5-P_2. Shown is one representative experiment of three.

5.3.3 Protein kinase activity of the monomeric PI3Kγ

In contrast to $p110\gamma_{\text{-His}}$/p101, monomeric PI3Kγ ($p110\gamma_{\text{-His}}$) exhibited a very high basal protein kinase activity (Fig. 5.10A). Additional stimulation with recombinant $G\beta_1\gamma_{2\text{-His}}$ led to only 1.6-fold stimulation over basal activity whereas $G\beta_1\gamma_{2\text{-His}}$-induced stimulation of dimeric PI3Kγ autophosphorylation was 12 fold (Fig. 5.10A and B).

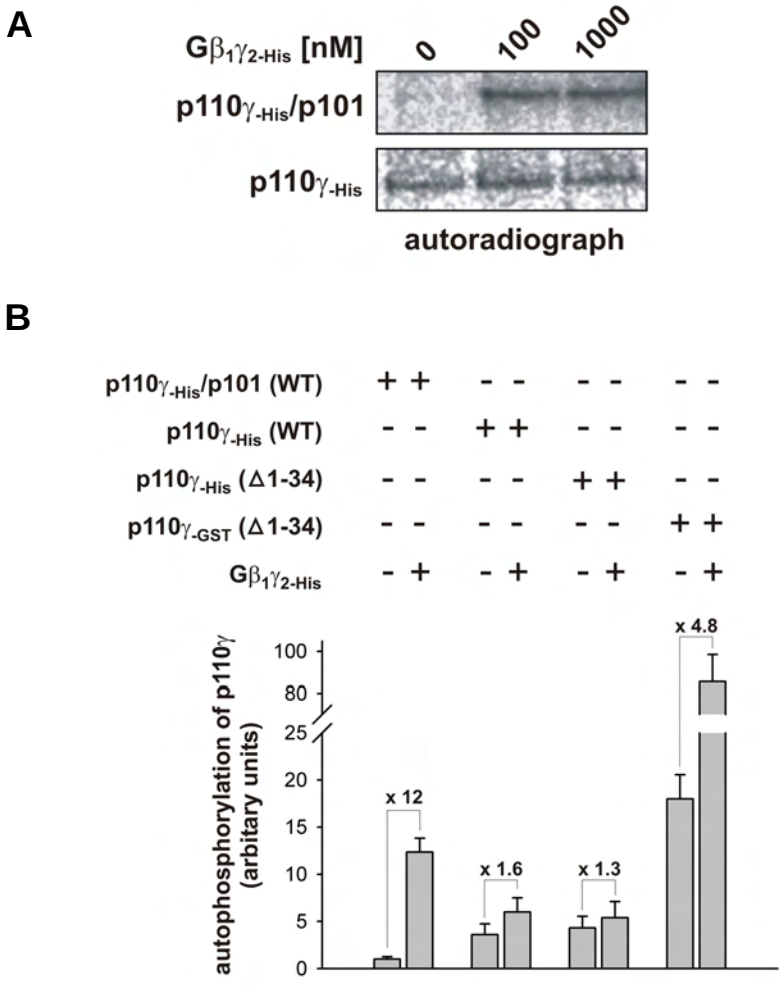


Fig. 5.10: Influence of $G\beta_1\gamma_{2\text{-His}}$ dimers on protein kinase activity of N-terminally GST- and His-tagged monomeric and dimeric PI3Kγ

(A) Protein kinase activity of purified full-length $p110\gamma_{\text{-His}}$/p101 and $p110\gamma_{\text{-His}}$ was tested in response to increasing concentrations of purified recombinant $G\beta_1\gamma_{2\text{-His}}$ as detailed under section 4.5.2. Assays were performed in the presence of 40 ng of dimeric or monomeric PI3Kγ. One representative autoradiograph is shown. **(B)** Protein kinase activity of full-length wild-type $p110\gamma_{\text{-His}}$/p101 and $p110\gamma_{\text{-His}}$ and N-terminally (amino acids 1 - 34) truncated $p110\gamma_{\text{-His}}$ and $p110\gamma_{\text{-GST}}$ was tested in the absence (-) or in the presence (+) of 600 nM $G\beta_1\gamma_{2\text{-His}}$. Assays were performed in the presence of 40 ng of each PI3Kγ variants as described under section 4.5.2. Following separation on SDS-PAGE, proteins were transferred to nitrocellulose membranes. Dried membranes were exposed to Fuji imaging plates and subsequently scanned using Fujifilm FLA-5000 imaging system. Autoradiographic signals were quantitated in photostimulated luminescence (PSL) units, which are proportional to radioactivity, using densitometry software (Aida Image Analyzer 3.45). Protein kinase activity is shown as arbitary units. Basal protein kinase activity of dimeric PI3Kγ was considered as "1". One arbitary unit corresponds to basal lipid kinase activity of dimeric PI3Kγ which was 133.5 ± 32.7 PSL units (n = 3). Fold-stimulation of basal activity in each reaction group is shown over the bars. Shown are mean values ± S.D. of three independent experiments.

Gβγ-induced autophosphorylation of $p110\gamma_{\text{-His}}$/p101 observed in our work correlates with published data from our group (Maier et al., 1999). Although these authors used dimeric PI3Kγ where p110γ was GST-fused and N-terminally truncated (Stoyanov et al., 1995, Stephens et al., 1997), Gβγ purified from bovine brain led to roughly 17-fold stimulation over basal activity. However, regarding to $p110\gamma_{\text{-His}}$ autophosphorylation, our results are in apparent contrast to data published by Maier et al. (1999). This former work described an

approximately 5-fold stimulation of basal activity of GST-fused N-terminally (amino acids 1 - 34) truncated monomeric PI3Kγ in response to increasing concentrations of Gβγ. Therefore, we asked whether N-terminal fragment of p110γ or N-terminal GST-tag may affect protein kinase activity of monomeric PI3Kγ. In order to answer this question, we i) purified His-tagged p110γ, which had the same N-terminal truncation as $p110\gamma_{\text{-GST}}$, and tested its protein kinase activity, and ii) re-examined Gβγ-induced autophosphorylation of $p110\gamma_{\text{-GST}}$ (△1 - 34).

No differences were observed between Gβγ-induced autophosphorylation of full-length and N-terminally truncated $p110\gamma_{\text{-His}}$ (Fig. 5.10B). Interestingly, in contrast to autophosphorylation, we found that the shorter version of $p110\gamma_{\text{-His}}$ exhibited 2.3-times higher lipid kinase activity than the full-length enzyme (Fig. 5.6B in section 5.2.2). According to data obtained, we propose that the first N-terminal amino acids of p110γ may affect lipid but not protein kinase activity of p110γ.

Next, we re-examined autophosphorylation of GST-fused monomeric PI3Kγ (△1 - 34). This enzyme displayed considerably high basal activity of autophosphorylation (Fig. 5.10B). Notably, this activity was roughly the same as maximal $G\beta_1\gamma_{2\text{-His}}$-induced stimulation of $p110\gamma_{\text{-His}}$/p101 autophosphorylation. Additional stimulation of $p110\gamma_{\text{-GST}}$ (△1 - 34) with 600 nM $G\beta_1\gamma_{2\text{-His}}$ led to about 5-fold stimulation over the basal level, as shown before (Maier et al., 1999). Since we excluded that N-terminal amino acids (1 - 34) may have an influence on the autophosphorylation process of monomeric PI3Kγ, we propose that increase of both basal and Gβγ-induced stimulation of $p110\gamma_{\text{-GST}}$ (△1 - 34) was due to the N-terminal GST-tag.

Based on the results described above, the lipid kinase activity of monomeric (p110γ) and dimeric (p110γ/p101) PI3Kγ and the protein kinase activity of dimeric PI3Kγ were selected as screening parameters for the $G\beta_1\gamma_2$ mutants. All enzymatic forms of PI3Kγ contained full-length p110γ subunit fused with His-tag at the N-terminus. Because of the weak $G\beta_1\gamma_{2\text{-His}}$-dependent stimulation of protein kinase activity of monomeric PI3Kγ, this enzymatic form was not considered for further analysis of protein kinase activity.

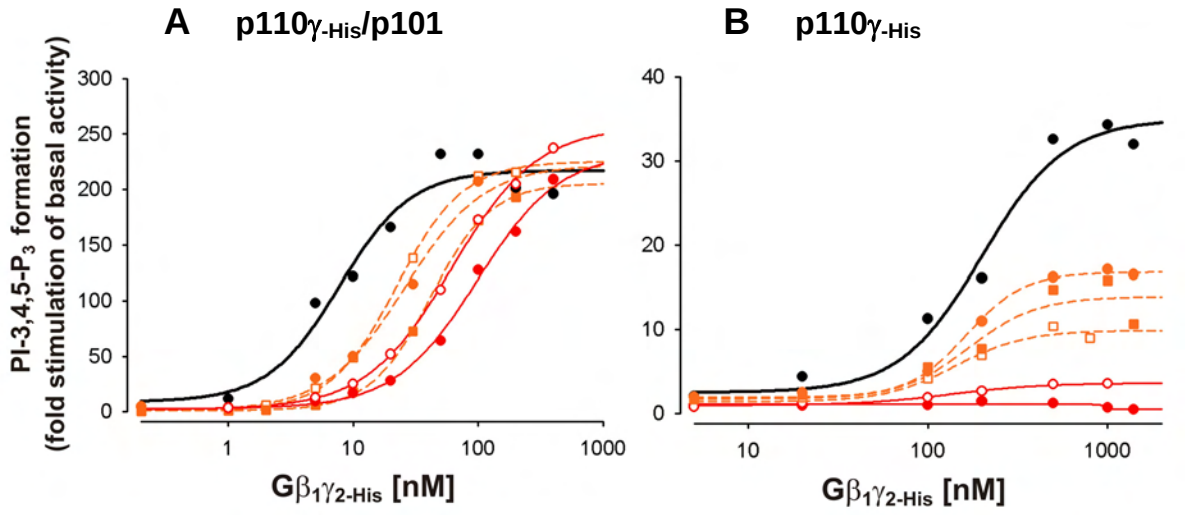
5.4 $G\beta_1$ residues relevant for interaction and stimulation of PI3Kγ enzymatic activities

5.4.1 $G\beta_1$ amino acids essential for stimulation of monomeric and dimeric PI3Kγ enzymatic activities

Specific amino acid residues of $G\beta_1$, i.e. Leu-55, Lys-57, Tyr-59, Lys-78, Ile-80, Lys-89, Ser-98, Trp-99, Met-101, Leu-117, Asn-119, Thr-143, Tyr-145, Asp-186, Asp-228, and Trp-332, were previously shown to be important for the interaction of $G\beta_1$ with $G\alpha$ (see section 1.2.2 of this work and Wall et al., 1995, Lambright et al., 1996, Ford et al., 1998, Panchenko et al., 1998, Davis et al., 2005). We have examined the effect of the exchange of these residues for alanine on the ability of $G\beta_1\gamma_{2\text{-His}}$ to recruit and activate monomeric ($p110\gamma_{\text{-His}}$) and dimeric ($p110\gamma_{\text{-His}}$/p101) PI3Kγ.

$G\beta_1\gamma_{2\text{-His}}$ significantly stimulated lipid kinase activity of the dimeric (Fig. 5.11A, black closed circles) and monomeric (Fig. 5.11B, black closed circles) PI3Kγ in a dose-dependent manner. Substitutions of amino acids of $G\beta_1$ for alanine pertaining Leu-55, Lys-57, Lys-78, Ile-80, Lys-89, Ser-98, Asn-119, or Thr-143 led to similar stimulation of lipid and protein kinase activities of PI3Kγ compared to the wild-type sequence of $G\beta_1\gamma_{2\text{-His}}$ (see Supplementary Fig. 12 in section 12). Moreover, these mutants had similar ability as the wild-type $G\beta_1\gamma_{2\text{-His}}$ to recruit PI3Kγ to phospholipid vesicles (data not shown). Hence, these residues appear to be not essential for binding and stimulation of PI3Kγ.

Interestingly, we identified several $G\beta_1$ mutants (alanine substitutions in positions Tyr-59, Trp-99, Met-101, Asp-228, or Trp-332) which substantially stimulated lipid kinase activity of $p110\gamma_{\text{-His}}$/p101, albeit with reduced potency than the wild-type $G\beta_1\gamma_{2\text{-His}}$ (Fig. 5.11A). $G\beta_{1W99A}\gamma_{2\text{-His}}$ (Fig. 5.11A, red closed circles) and $G\beta_{1M101A}\gamma_{2\text{-His}}$ (Fig. 5.11A, red open circles) affected the ability of $G\beta_1\gamma_{2\text{-His}}$ most severely to stimulate PI3Kγ. Indeed, exchange of these amino acids for alanine led to an approximately 10-fold increased EC_{50} value, whereas the maximal efficiency (V_{max}) was unaltered to wild-type $G\beta_1\gamma_{2\text{-His}}$. In contrast to the dimer, the stimulation of the monomeric PI3Kγ by $G\beta_{1W99A}\gamma_{2\text{-His}}$ was completely lost at any concentration tested. Other mutants of this group, i.e. $G\beta_{1Y59A}\gamma_{2\text{-His}}$, $G\beta_{1M101A}\gamma_{2\text{-His}}$, $G\beta_{1D228A}\gamma_{2\text{-His}}$, and $G\beta_{1W332A}\gamma_{2\text{-His}}$, were able to stimulate the lipid kinase activity of $p110\gamma_{\text{-His}}$, however, with a reduced efficiency (Fig. 5.11B). Surprisingly, mutations of the indicated $G\beta_1$ amino acids affected only the maximal stimulation of $p110\gamma_{\text{-His}}$ lipid kinase activity, while EC_{50} values were similar with wild-type $G\beta_1\gamma_{2\text{-His}}$. Regarding the ability of $G\beta_{1WT}\gamma_{2\text{-His}}$ to stimulate the protein kinase activity of dimeric PI3Kγ, the mutants $G\beta_{1W99A}\gamma_{2\text{-His}}$ and $G\beta_{1M101A}\gamma_{2\text{-His}}$ were at least less potent than the wild-type (Fig. 5.11C).



Gβ1γ2-His variants	p110γ-His/p101		p110γ-His	
	EC50 [nM]	maximal stimulation (fold)	EC50 [nM]	maximal stimulation (fold)
● Gβ1WTγ2-His	7.7	217.3	200.7	34.9
● Gβ1Y59Aγ2-His	25.7	222.2	164.2	16.8
● Gβ1W99Aγ2-His	98.6	220.4	not detectable	1.1
○ Gβ1M101Aγ2-His	61.7	246.3	134.9	3.7
□ Gβ1D228Aγ2-His	22.3	228.3	142.0	9.9
■ Gβ1W332Aγ2-His	41.7	205.6	168.9	13.9

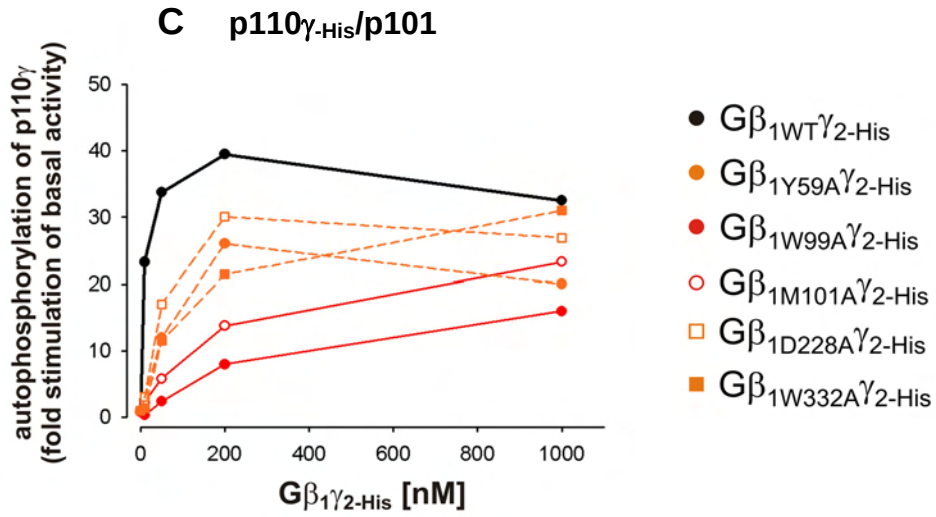


Fig. 5.11: Amino acids of Gβ$_1$ crucial for all forms of PI3Kγ enzymatic activities

Stimulation of lipid kinase activities of p110γ-His/p101 **(A)**, p110γ-His **(B)**, and protein kinase activity of p110γ-His/p101 **(C)** in response to increasing concentrations of Gβ1WTγ2-His and its alanine mutants. Assays were performed as detailed under section 4.5. The kinetic parameters for **(A)** and **(B)** are shown in table. Gβγ-induced activation of different forms of PI3Kγ is shown as fold-stimulation of basal activity. Generation and presentation of the dose-response curves is detailed in section 4.7 (n = 3).

The previous experiments have shown that $G\beta_1\gamma_{2\text{-His}}$ stimulated the dimeric PI3Kγ more effectively than the monomeric (about 200- vs. 30-fold stimulation of basal activity). In order to elucidate this phenomenon, we analyzed the capability of $G\beta_{1WT}\gamma_{2\text{-His}}$ and its mutants to recruit the different forms of PI3Kγ to phospholipid vesicles. Initial experiments revealed that $p110\gamma_{\text{-His}}$ and $p110\gamma_{\text{-His}}$/p101 were associated with phospholipid vesicles in the absence of Gβγ (Fig. 5.12, right panels, Maier et al., 2000). However, increasing concentrations of $G\beta_1\gamma_{2\text{-His}}$ significantly enhanced the association of the dimeric (Fig. 5.12, upper panel, right) but not the monomeric (Fig. 5.12, lower panel, right) PI3Kγ to the lipid vesicles. The increase of amount of the dimeric kinase in the pellet (lipid compartment) correlated with a decreased amount in supernatant (Fig. 5.12, upper panel, left). All mutants of the tested group, except $G\beta_{1W99A}\gamma_{2\text{-His}}$, demonstrated a comparable ability to recruit the various forms of PI3Kγ to lipid vesicles (data not shown). Conversely, it should be noted that monomeric and dimeric PI3Kγ did not affect the recruitment of $G\beta_{1WT}\gamma_{2\text{-His}}$ to phospholipid vesicles (Table 5.1).

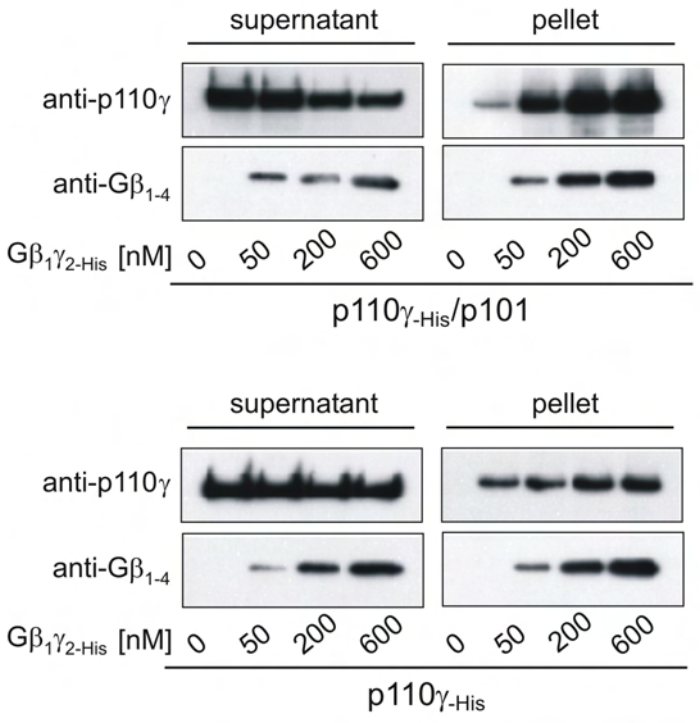


Fig. 5.12: Recombinant $G\beta_1\gamma_{2\text{-His}}$ significantly enhances association of dimeric but not monomeric PI3Kγ to the lipid vesicles

$G\beta_1\gamma_{2\text{-His}}$ was tested for its ability to recruit $p110\gamma_{\text{-His}}$/p101 and $p110\gamma_{\text{-His}}$ to the lipid vesicles as detailed in section 4.6. Assays were performed in the presence of 400 ng of dimeric or monomeric PI3Kγ. Aliquots of sedimented vesicles and supernatant were subjected to SDS-PAGE followed by immunoblotting. The proteins were visualized using specific antisera.

Table 5.1: PI3Kγ does not influence association of $G\beta_1\gamma_{2\text{-His}}$ with lipid vesicles

Coincubation of $G\beta_1\gamma_{2\text{-His}}$ with	Recruitment of $G\beta_1\gamma_{2\text{-His}}$ to lipid vesicles (%)
——	33.2 ± 7.1
$p110\gamma_{\text{-His}}$	42.3 ± 15.9
$p110\gamma_{\text{-His}}$/p101	41.0 ± 8.8

Phospholipid association of recombinant purified wild-type $G\beta_1\gamma_{2\text{-His}}$ (600 nM) alone or coincubated with 200 ng of monomeric or dimeric PI3Kγ was tested as detailed in section 4.6. Aliquots of sedimented vesicles and supernatant were subjected to SDS-PAGE followed by immunoblotting. Percentage of phospholipid vesicle-recruited $G\beta_1\gamma_{2\text{-His}}$ was estimated by immunoblot using anti-$G\beta_{1\text{-}4}$ specific antiserum. Total amount of the $G\beta_1\gamma_{2\text{-His}}$ in assays was considered as 100 %. Shown are mean values (±S.D.) of three separate experiments. The differences between recruitment of $G\beta_1\gamma_{2\text{-His}}$ in the absence of PI3Kγ and in the presence of monomeric or dimeric PI3Kγ are insignificant ($P > 0.05$, $n = 3$).

Next, we examined whether the reduced potency of $G\beta_{1W99A}\gamma_{2\text{-His}}$ to stimulate lipid kinase activity of dimeric PI3Kγ was due to an impaired recruitment of Gβγ to the vesicles or an impaired binding of Gβγ to PI3Kγ. Fig. 5.13A shows that in these experiments $G\beta_{1WT}\gamma_{2\text{-His}}$ and $G\beta_{1W99A}\gamma_{2\text{-His}}$ were equally associated with the lipid vesicles. According to this findings and data from Table 5.1 demonstrating that recruitment of $G\beta_1\gamma_{2\text{-His}}$ to phospholipid vesicles is PI3Kγ-independent process, we conclude that the alanine mutation of $G\beta_1$ in position 99 does not disturb the association of Gβγ with the lipid compartment. However, the alanine mutation of the tryptophan-99 led to a reduction of association of dimeric PI3Kγ with vesicles compared to wild-type Gβγ (Fig. 5.13A).

We next tested the ability of $G\beta_{1WT}\gamma_2$ and $G\beta_{1W99A}\gamma_2$ to bind PI3Kγ subunits. The wild-type Gβγ strongly associated with the regulatory subunit of PI3Kγ ($p101_{\text{-His}}$), whereas the interaction with the catalytic subunit ($p110\gamma_{\text{-His}}$) was much weaker (Fig. 5.13B, left). These results are in accordance with previously published data (Stephens et al., 1997, Maier et al., 1999). In contrast, $G\beta_{1W99A}\gamma_2$ did not efficiently copurified with $p101_{\text{-His}}$ as compared to $G\beta_{1WT}\gamma_2$ (Fig. 5.13B, right). However, the copurification of both Gβγ dimers with catalytic subunit of PI3Kγ was comparable. The data shown in Fig. 5.13B clearly demonstrate that $G\beta_{1W99A}\gamma_2$ had a significantly reduced ability to interact with regulatory subunit of PI3Kγ. These findings are in agreement with the reduced recruitment of dimeric PI3Kγ to phospholipid vesicles by $G\beta_1\gamma_{2\text{-His}}$ when tryptophan-99 is mutated to alanine in $G\beta_1$ (Fig. 5.13A).

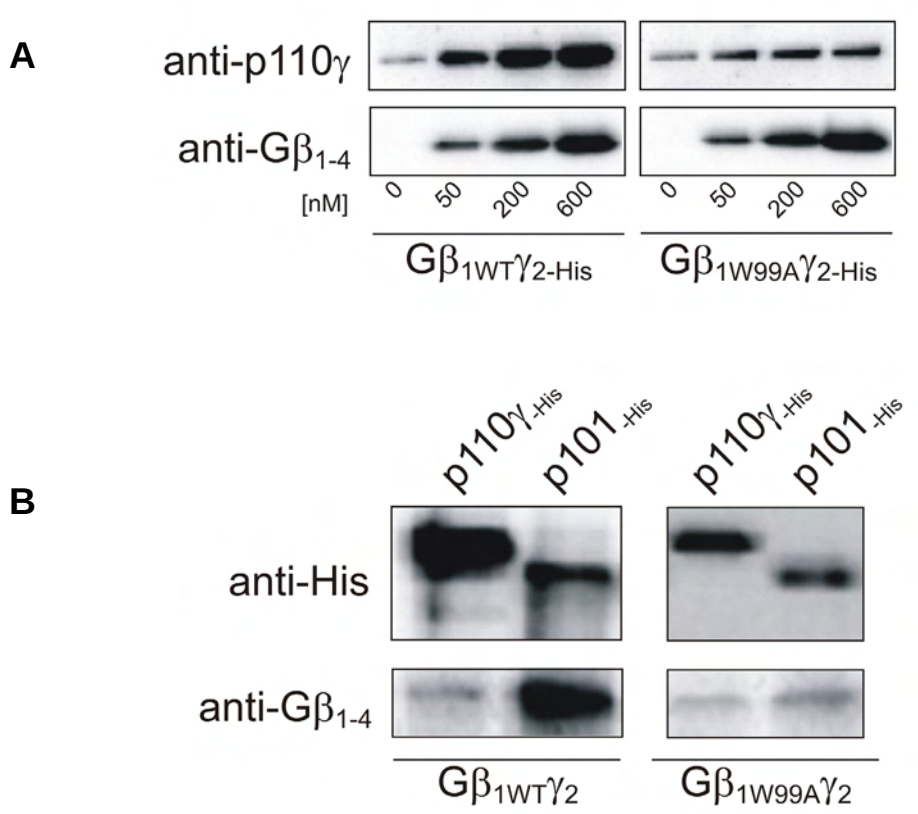


Fig. 5.13: Exchange of Trp-99 to Ala in $G\beta_1$ disturbs the association of $G\beta_1\gamma_{2\text{-His}}$ with PI3Kγ

(A) $G\beta_{1W99A}\gamma_{2\text{-His}}$ reduces vesicular association of $p110\gamma_{\text{-His}}$/p101. The $G\beta_1\gamma_{2\text{-His}}$ mutant was tested for its ability to recruit the dimeric PI3Kγ (400 ng) to lipid vesicles as detailed in section 4.6. Aliquots of pelleted vesicles were subjected to SDS-PAGE followed by immunoblotting. Proteins were visualized using specific antisera. **(B)** Binding of $G\beta_{1WT}\gamma_2$ and $G\beta_{1W99A}\gamma_2$ to PI3Kγ subunits. Recombinant $G\beta_1\gamma_2$ was coexpressed with baculoviruses encoding $p110\gamma_{\text{-His}}$ or $p101_{\text{-His}}$ in Sf9 cells and purified as described in section 4.3.3. Following purification on Ni^{2+}-NTA Superflow resin, bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-hexahistidine and anti-$G\beta_{1\text{-}4}$ specific antisera.

Interestingly, based on the data obtained from the study of the $G\beta_1\gamma_{2\text{-His}}$ mutants, we have found a positive correlation between lipid and protein kinase activities. The potency of $G\beta_{1Y59A}\gamma_{2\text{-His}}$, $G\beta_{1W99A}\gamma_{2\text{-His}}$, $G\beta_{1M101A}\gamma_{2\text{-His}}$, $G\beta_{1D228A}\gamma_{2\text{-His}}$, and $G\beta_{1W332A}\gamma_{2\text{-His}}$ to stimulate lipid kinase activity of the dimeric PI3Kγ is positively correlated with their efficiency to stimulate protein kinase activity of the dimeric PI3Kγ (Fig. 5.14, upper panel) and with the efficiency to stimulate lipid kinase activity of the monomeric PI3Kγ (Fig. 5.14, lower panel). We speculate that $G\beta_{1W99A}\gamma_{2\text{-His}}$ and $G\beta_{1M101A}\gamma_{2\text{-His}}$ form a core region that is indispensable for stimulation of all enzymatic qualities of PI3Kγ.

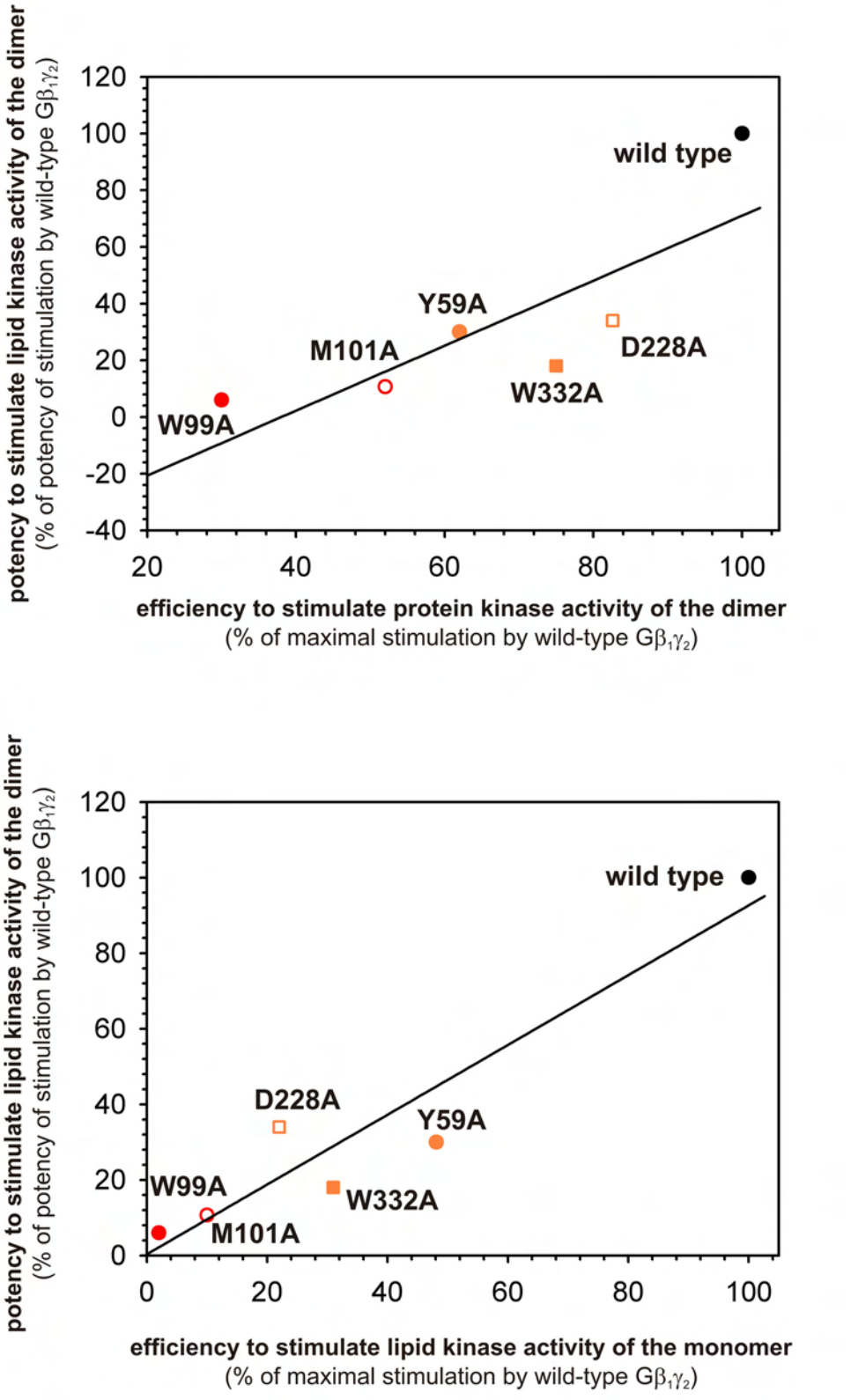


Fig. 5.14: Correlation between PI3Kγ activities stimulated by $G\beta_1\gamma_{2\text{-His}}$ variants

The potency of $G\beta_1\gamma_{2\text{-His}}$ variants to stimulate lipid kinase activity of $p110\gamma_{\text{-His}}$/p101 positively correlates with the efficiency to stimulate protein kinase activity of $p110\gamma_{\text{-His}}$/p101 and with the efficiency to stimulate lipid kinase activity of $p110\gamma_{\text{-His}}$. Positions of $G\beta_1\gamma_2$ residues exchanged for alanine are shown. Potency (EC_{50}) and efficiency (V_{max}) of stimulation of all PI3Kγ activities by wild-type $G\beta_1\gamma_{2\text{-His}}$ were defined as 100 %.

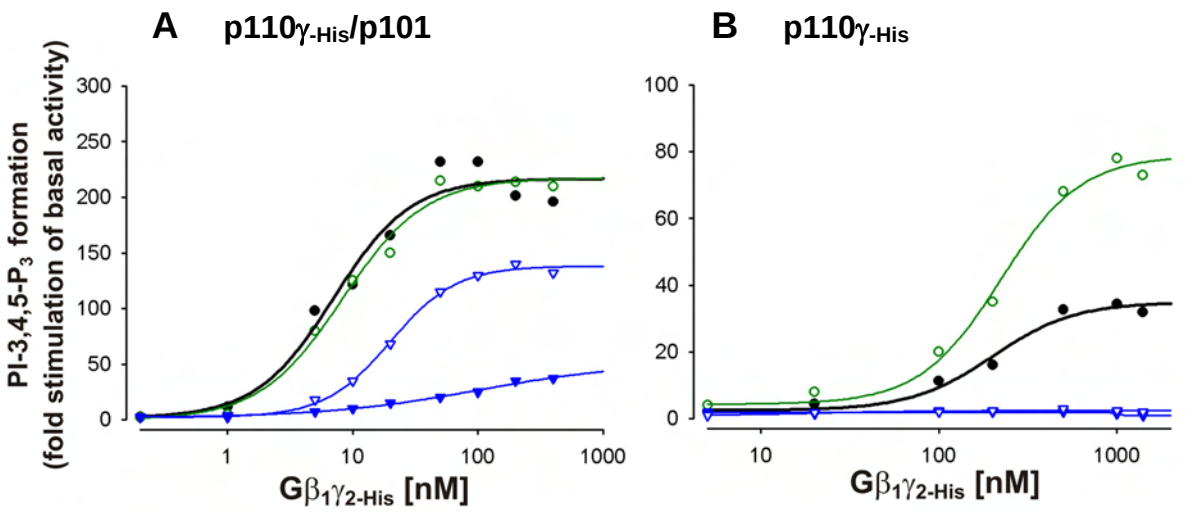
Potency of stimulation = 100 % * (EC_{50} of wild-type $G\beta_1\gamma_2$ / EC_{50} of mutated $G\beta_1\gamma_2$)

Efficiency of stimulation = 100 % * (V_{max} by mutated $G\beta_1\gamma_2$ / V_{max} by wild-type $G\beta_1\gamma_2$)

5.4.2 Gβ$_1$ alanine mutants relevant for discrimination between different enzymatic qualities of PI3Kγ

Furthermore, we identified three additional $G\beta_1\gamma_{2\text{-His}}$ mutants, which show unique stimulating qualities on PI3Kγ (Fig. 5.15). For instance, $G\beta_{1L117A}\gamma_{2\text{-His}}$ only slightly stimulated lipid kinase activity of the dimeric PI3Kγ (Fig. 5.15A, blue closed triangles), whereas lipid kinase activity of the monomer was completely lost at any concentration tested (Fig. 5.15B, blue closed triangles). $G\beta_{1Y145A}\gamma_{2\text{-His}}$ showed not only a reduced potency but also a decreased efficiency to stimulate lipid kinase activity of the dimeric PI3Kγ, whereas lipid kinase activity of the monomeric kinase could not be stimulated (Fig. 5.15A and B, blue open triangles). Surprisingly, the exchange of aspartate-186 for alanine resulted in 2-fold increased activation of the monomeric PI3Kγ (Fig. 5.15B, green open circles), whereas sensitivity of the dimeric kinase towards this Gβγ mutant was unaltered (Fig. 5.15A, green open circles). In contrast to the lipid kinase activity, alanine mutations at positions 117, 145 and 186 seemed not to be crucial for the stimulation of the protein kinase activity of the $p110\gamma_{\text{-His}}$/p101, although a reduced potency was observed (Fig. 5.15C). Most surprisingly, even $G\beta_{1L117A}\gamma_{2\text{-His}}$ stimulated protein kinase activity of PI3Kγ, however with reduced potency.

Among these mutants, only $G\beta_{1L117A}\gamma_{2\text{-His}}$ was unable to recruit dimeric PI3Kγ in a concentration-dependent manner to phospholipid vesicles (Fig. 5.16A). This finding may explain the impaired capability of this mutant to stimulate lipid kinase activity of monomeric and dimeric PI3Kγ. The other $G\beta_1$ mutants of this group, i.e. Tyr-145 and Asp-186, significantly enhanced association of dimeric PI3Kγ to the pellet. Recruitment of the dimeric kinase by $G\beta_{1Y145A}\gamma_{2\text{-His}}$ and $G\beta_{1D186A}\gamma_{2\text{-His}}$ was comparable with wild-type $G\beta_1\gamma_{2\text{-His}}$ (Fig. 5.16A). In contrast to dimeric PI3Kγ, the recruitment of monomeric kinase by $G\beta_1\gamma_{2\text{-His}}$ and its alanine mutants was not significant (Fig. 5.16B).



Gβ1γ2-His variants	p110γ-His/p101		p110γ-His	
	EC50 [nM]	maximal stimulation (fold)	EC50 [nM]	maximal stimulation (fold)
● Gβ1WTγ2-His	7.7	217.3	200.7	34.9
▼ Gβ1L117Aγ2-His	not detectable	43.2	not detectable	1.6
▽ Gβ1Y145Aγ2-His	20.1	138.3	not detectable	2.4
○ Gβ1D186Aγ2-His	8.2	217.4	221.9	78.9

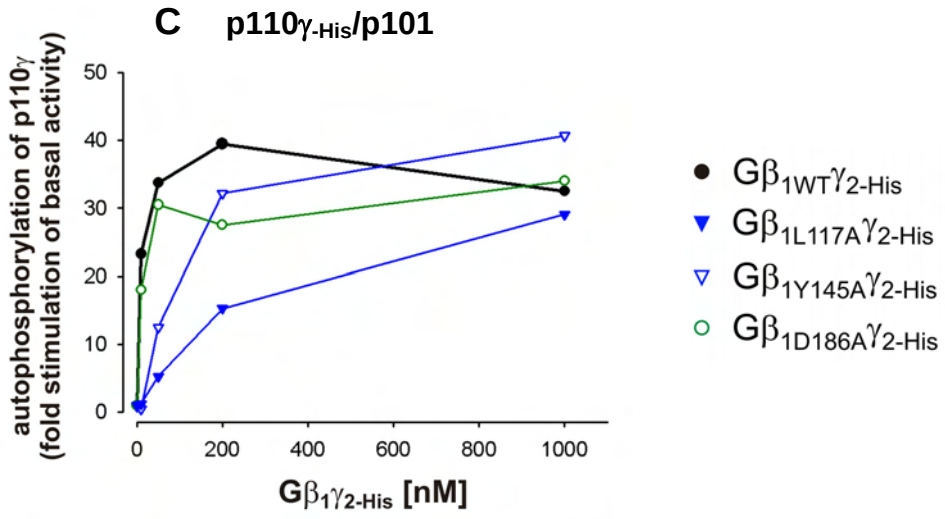


Fig. 5.15: Discrimination between different enzymatic forms of PI3Kγ

Stimulation of lipid kinase activities of p110γ-His/p101 **(A)**, p110γ-His **(B)**, and protein kinase activity of p110γ-His/p101 **(C)** in response to increasing concentrations of Gβ1WTγ2-His and its alanine mutants. Assays were performed as detailed under section 4.5. The kinetic parameters for **(A)** and **(B)** are shown in table. Gβγ-induced activation of different forms of PI3Kγ is shown as fold-stimulation of basal activity. Generation and presentation of the dose-response curves is detailed in section 4.7 (n = 3).

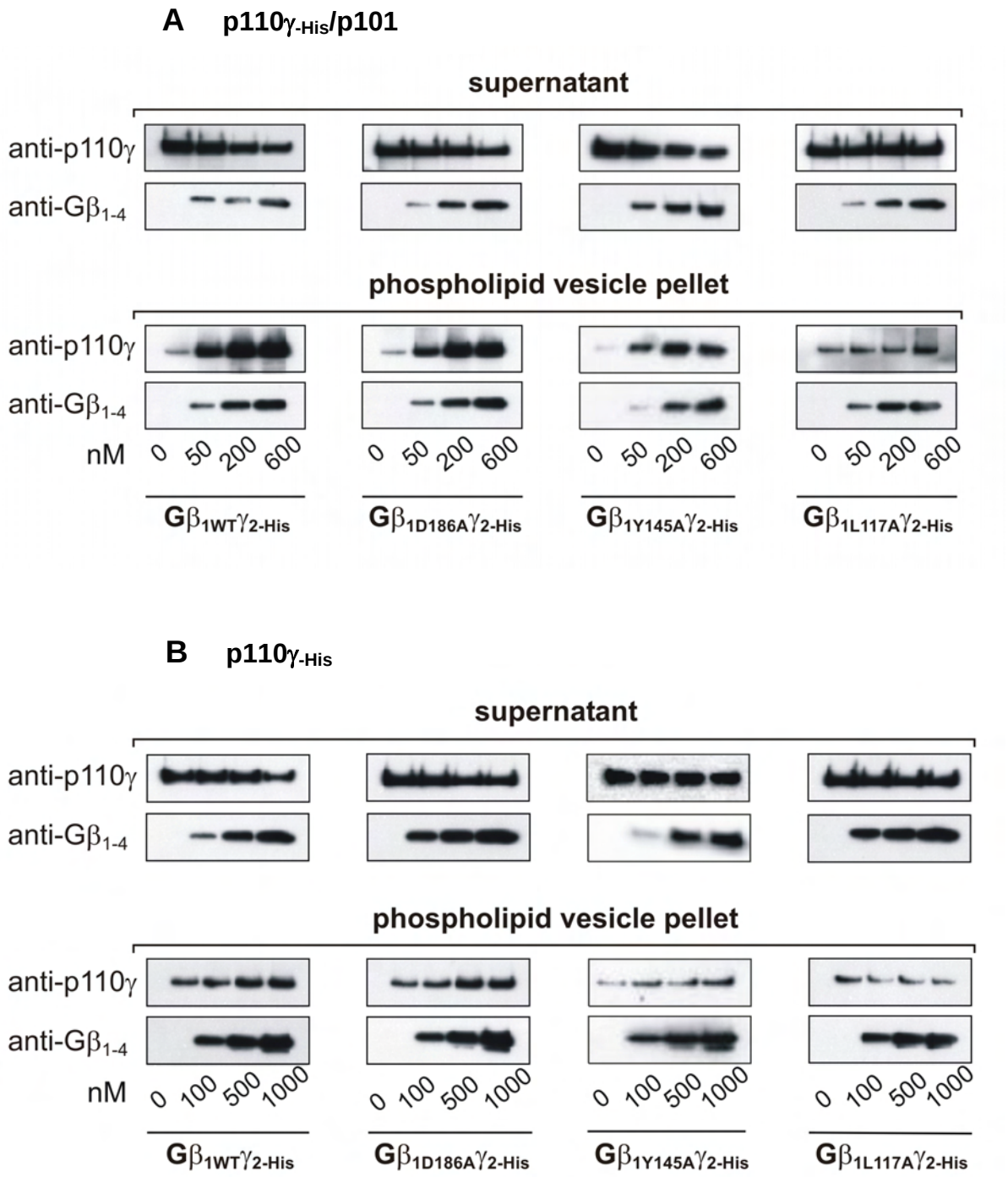


Fig. 5.16: Lipid vesicle association of PI3Kγ induced by $G\beta_1\gamma_{2\text{-His}}$ variants

Purified recombinant $G\beta_1\gamma_{2\text{-His}}$ mutants were tested for their ability to recruit dimeric **(A)** and monomeric **(B)** PI3Kγ to lipid vesicles as described under section 4.6. Assays were performed in the presence of 400 ng of dimeric or monomeric PI3Kγ. Aliquots of sedimented vesicles and supernatant were subjected to SDS-PAGE followed by immunoblotting. The proteins were visualized using specific antisera.

5.4.3 *Monoclonal anti-p110γ antibody (mAb 641) discriminates between Gβ$_1$γ$_2$-induced stimulation of monomeric and dimeric PI3Kγ activities*

In order to confirm the data presented in section 5.4.2 which suggest the possibility to discriminate between Gβ$_1$γ$_2$-stimulated PI3Kγ activities, we looked for specific tools. Recently, the difference in ability to recognize PI3Kγ between two anti-p110γ specific monoclonal antibodies (mAb and mAb 641) which were raised against the entire intact p110γ protein was shown (Czupalla, 2002). It was found that in contrast to mAb, mAb 641 was unable to recognize denatured dimeric PI3Kγ in SDS-PAGE. Nevertheless, mAb 641 could be used for immunoprecipitation of native PI3Kγ from HEK293 cells thereby demonstrating that this antibody specifically recognises intact PI3Kγ.

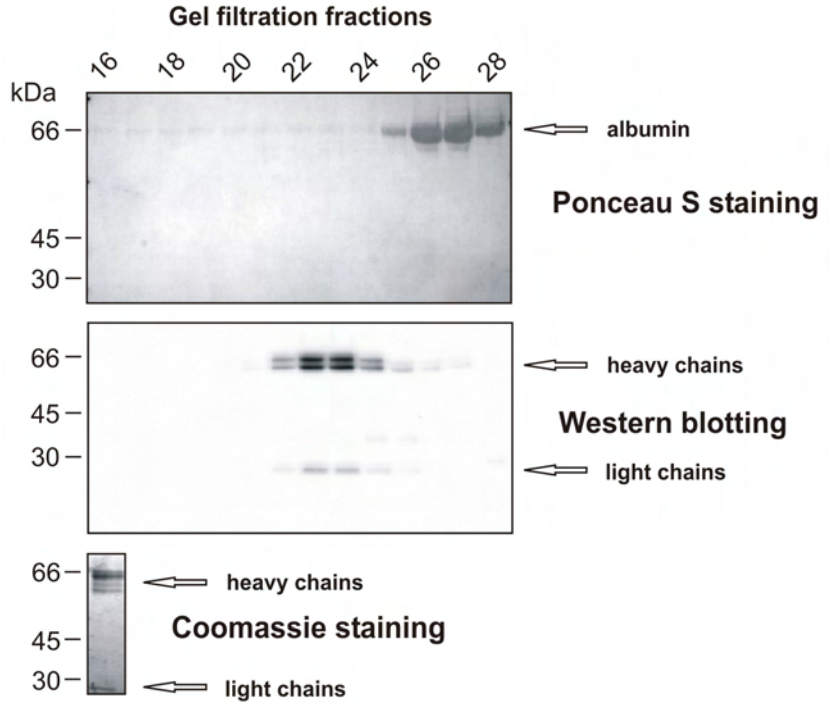


Fig. 5.17: Gel filtration of a monoclonal anti-p110γ antibody (mAb 641)

The hybridoma supernatant containing mAb 641 (10 ml) was concentrated using Centricon-30 concentrators. The concentrated antibody (0.5 - 1 ml) was applied to a Superdex 200 HR 10/30 and eluted with buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM β-ME). Fractions were subjected to SDS-PAGE followed by Western blotting. The nitrocellulose membrane was stained with Ponceau S (upper panel) and, subsequently, analyzed by immunoblotting using anti-mouse-HRP secondary antibody (middle panel). The albumin-free fractions were pooled and concentrated using Centricon-30 concentrator. mAb 641 was subjected to SDS-PAGE and analyzed by Coomassie staining (lower panel).

Since the mAb 641 binding sites are unknown, we analyzed the effect of mAb 641 on lipid and protein kinase activities of the monomeric and dimeric PI3Kγ. mAb 641 was produced using the mouse hybridoma technology. Generally, all supernatants of mouse hybridoma cells

contain low concentration of antibody, whereas the concentrations of other components (e.g. albumin) are usually very high. In order to prevent the cross-reactivity between analyzed PI3Kγ and proteins of mouse hybridoma cells supernatant, we purified and concentrated mAb 641. The simplest way to separate IgG from albumin is gel filtration (Fig. 5.17). Peak fractions containing mAb 641 were identified in fractions 22-23, whereas more than 90 % of albumin was eluted in fractions 25-28 (Fig. 5.17, upper and middle panels). The albumin-free fractions of mAb 641 (fractions 22 and 23) were pooled and concentrated. The antibody was visualized by Coomassie staining (Fig. 5.17, lower panel) and, subsequently, used for experiments.

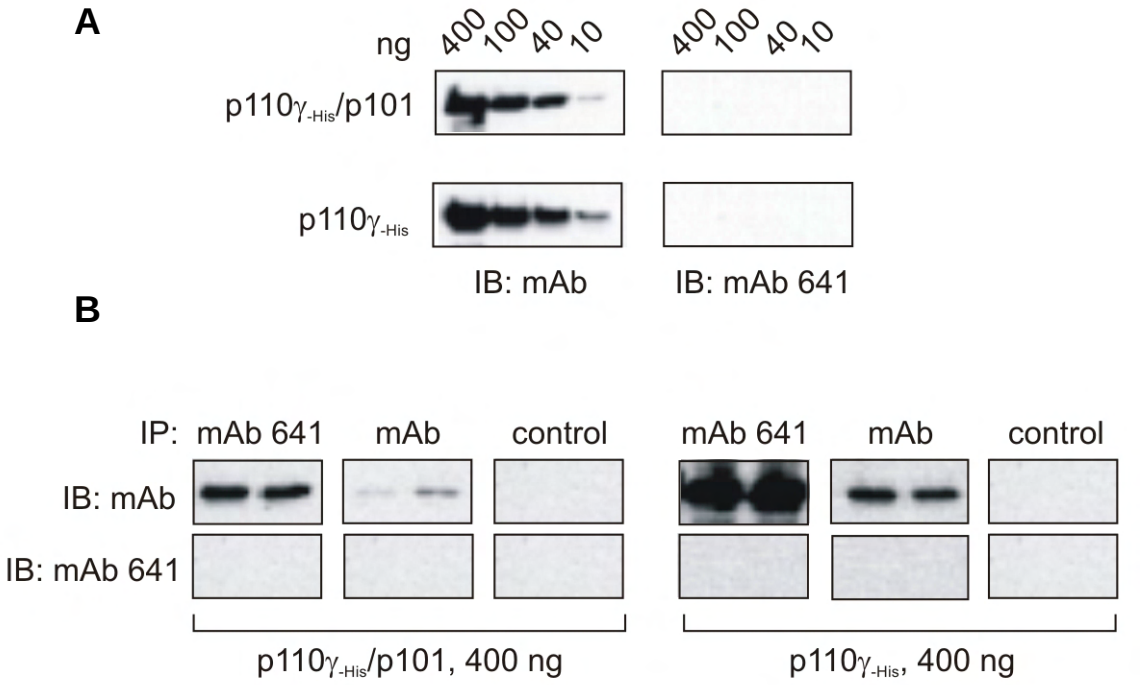


Fig. 5.18: Characterization of monoclonal anti-p110γ antibodies (mAb and mAb 641)

(A) p110γ-His and p110γ-His/p101 were purified from Sf9 cells as detailed under section 4.3.2. Different amount of the protein was subjected to SDS-PAGE followed by immunoblotting (IB). Proteins were visualized using monoclonal anti-p110γ antibodies (mAb and mAb 641). **(B)** Heterodimeric (left) and monomeric (right) PI3Kγ purified from Sf9 cells were immunoprecipitated (IP) using 3 µl either mAb 641 or mAb and 10 µl of Protein A Sepharose CL-4B as detailed under section 4.1.6. For control, p110γ unspecific antibody was used. Each immunoprecipitation was done in duplicates. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting (IB) with anti-p110γ monoclonal antibodies (mAb and mAb 641). Note that for immunoprecipitation, mAb 641 was purified from the mouse hybridoma cells supernatant and, subsequently, concentrated (see Fig. 5.17) whereas mAb from the supernatant was used without additional purification steps.

Next, we ensured that purification and concentration of mAb 641 did not affect its properties described above. The immunoblotting analysis of mAb demonstrated a high sensitivity towards the purified recombinant PI3Kγ (Fig. 5.18A, left panels). In contrast, mAb 641 was unable to recognize denatured dimeric and monomeric PI3Kγ (Fig. 5.18A, right panels).

However, mAb 641 specifically interacts with both forms of intact PI3Kγ since immunoblotting with mAb clearly revealed monomeric and dimeric PI3Kγ in mAb 641 immunoprecipitates (Fig. 5.18B). These data are in accordance with findings of Cornelia Czupalla (2002).

We tested mAb 641 for its ability to affect PI3Kγ activities stimulated by $G\beta_1\gamma_{2\text{-His}}$. $G\beta_1\gamma_{2\text{-His}}$ at a concentration of 600 nM led to a maximal stimulation of lipid and protein kinase activities of monomeric and dimeric PI3Kγ (see Fig. 5.11A, B, and C, black closed circles). Increasing concentrations of mAb 641 diminished the $p110\gamma_{\text{-His}}$ lipid kinase activity stimulated by 600 nM $G\beta_1\gamma_{2\text{-His}}$ (Fig. 5.19, red) while the $G\beta_1\gamma_{2\text{-His}}$-stimulated $p110\gamma_{\text{-His}}$/p101 activity was unchanged (Fig. 5.19, black). Moreover, mAb 641 affected the $G\beta_1\gamma_{2\text{-His}}$-induced intrinsic protein kinase activity (approximately 40 % reduction) of $p110\gamma_{\text{-His}}$/p101 (Fig. 5.19, blue).

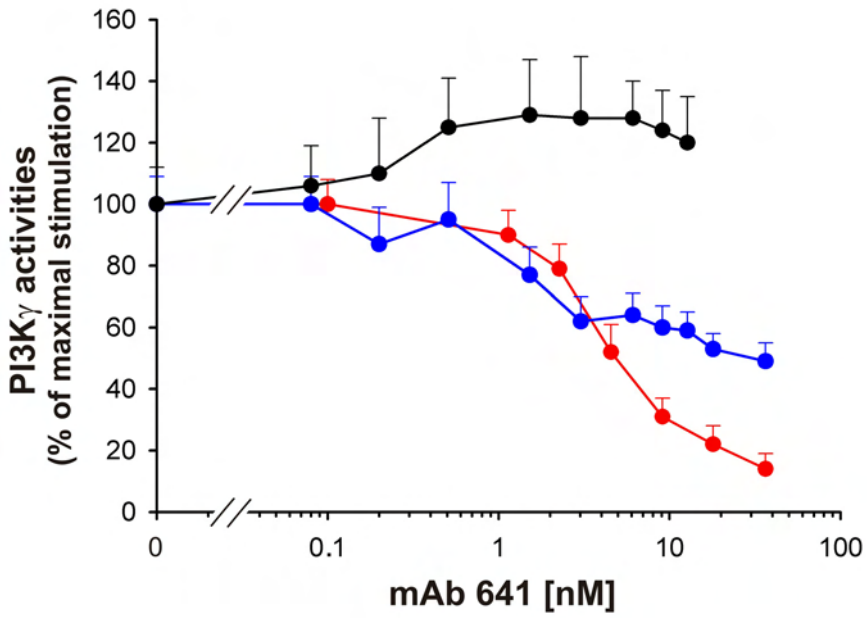


Fig. 5.19: mAb 641 differentially affects lipid and protein kinase activities of monomeric and dimeric PI3Kγ maximally stimulated by $G\beta_1\gamma_{2\text{-His}}$

Lipid kinase activity of $p110\gamma_{\text{-His}}$/p101 (black) or $p110\gamma_{\text{-His}}$ (red), and protein kinase activity of $p110\gamma_{\text{-His}}$/p101 (blue) were stimulated by 600 nM $G\beta_1\gamma_{2\text{-His}}$ in the absence or in the presence of increasing concentrations of mAb 641. Stimulation of PI3Kγ by 600 nM $G\beta_1\gamma_{2\text{-His}}$ led to a maximal activation of all PI3Kγ enzymatic qualities. $G\beta_1\gamma_{2\text{-His}}$-induced maximal activities of each PI3Kγ enzymatic quality were considered as 100 %. Assays were performed as detailed under section 4.5. The lipid kinase assays were performed in the presence of 10 ng of dimeric or monomeric PI3Kγ, whereas the protein kinase assays were done in the presence of 40 ng of dimeric PI3Kγ. Shown are mean values (± S.D.) of duplicate determinations in three independent experiments.

These data suggest that $G\beta_1\gamma_2$-induced lipid and protein kinase activities of the dimeric PI3Kγ may be distinguished since the antibody blocked protein kinase activity but not lipid kinase activity (Fig. 5.19, blue versus black). Compared to lipid kinase activity of monomeric PI3Kγ

(Fig. 5.19, red), lipid kinase activity of dimeric PI3Kγ (Fig. 5.19, black) was resistant to antibody inhibition.

However, it is possible that mAb 641 may interfere with PI3Kγ enzymatic activities which are maximally stimulated with $G\beta_1\gamma_{2\text{-His}}$. In fact, $G\beta_1\gamma_{2\text{-His}}$ at a concentration of 600 nM was in high excess for the maximal stimulation of lipid and protein kinase activity of dimeric PI3Kγ, whereas for lipid kinase activity of monomeric PI3Kγ this concentration of $G\beta_1\gamma_{2\text{-His}}$ was a "starting point" leading to maximal stimulation (see Fig. 5.11A, B, and C, black closed circles). Therefore, we tested the effect of increasing concentrations of mAb 641 on lipid kinase activities of monomeric and dimeric PI3Kγ which were half-maximally stimulated by $G\beta_1\gamma_{2\text{-His}}$. In contrast to data demonstrated in Fig. 5.19, we found that mAb 641 significantly inhibited lipid kinase activity of both monomeric and dimeric PI3Kγ stimulated by $G\beta_1\gamma_{2\text{-His}}$ at EC_{50} (Fig. 5.20).

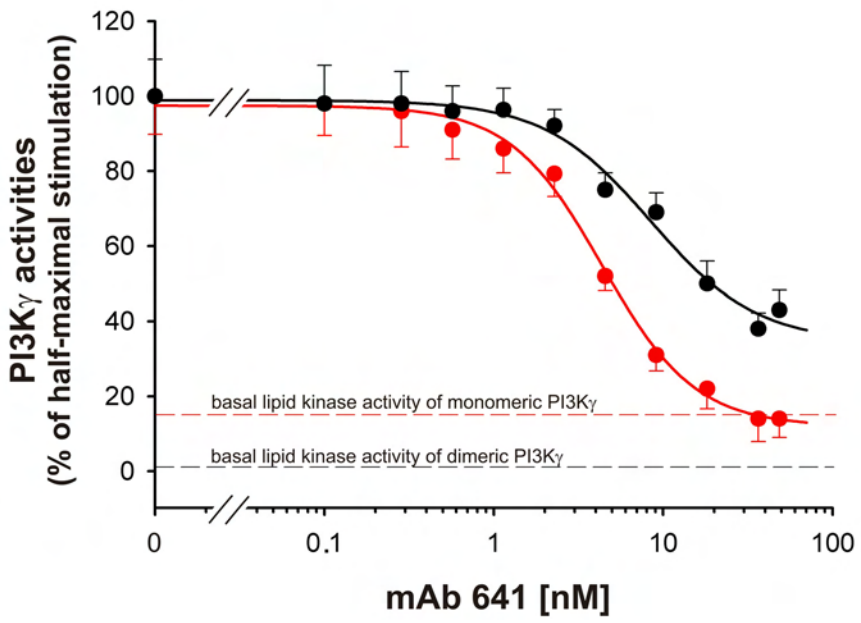


Fig. 5.20: mAb 641 differentially affects lipid kinase activities of monomeric and dimeric PI3Kγ half-maximally stimulated by $G\beta_1\gamma_{2\text{-His}}$

Lipid kinase activity of $p110\gamma_{\text{-His}}$/p101 (black) and $p110\gamma_{\text{-His}}$ (red) were stimulated by half-effective $G\beta_1\gamma_{2\text{-His}}$ concentrations, i.e. 20 nM and 200 nM, respectively, in the absence or in the presence of increasing concentrations of mAb 641. $G\beta_1\gamma_{2\text{-His}}$-induced half-maximal stimulation of lipid kinase activities of monomeric and dimeric PI3Kγ were considered as 100 %. Basal lipid kinase activities, i.e. in the absence of $G\beta_1\gamma_{2\text{-His}}$ and mAb 641, of monomeric and dimeric PI3Kγ were estimated in percents regarding to corresponding half-maximal stimulation and were 14.8 ± 3.7 and 1.3 ± 0.6 %, respectively (n = 3, see Table 12.2 in section 12). Assays were performed in the presence of 10 ng of monomeric or dimeric PI3Kγ as detailed under section 4.5.1. Shown are mean values (± S.D.) of duplicate determinations in three independent experiments. Note that EC_{50} value for the stimulation of the dimeric PI3Kγ during the completing these experiments was 20 nM of $G\beta_1\gamma_{2\text{-His}}$ instead of 7.7 nM indicated above in this study (for explanations see section 4.7).

Nevertheless, mAb 641 at a concentration of 40 nM completely blocked $G\beta_1\gamma_2$-stimulated lipid kinase activity of monomeric PI3Kγ, whereas $G\beta_1\gamma_2$-stimulated lipid kinase activity of the dimer was still 30 times higher compared to corresponding basal lipid kinase activity (Fig. 5.20 and Supplementary Table 12.2 in section 12). According to obtained data, we conclude that mAb 641 may distinguish between different forms and enzymatic activities of PI3Kγ which are stimulated by $G\beta_1\gamma_2$.

5.5 Role of Gβγ dimer and non-catalytic subunit (p101) in activation of PI3Kγ

We and other groups (Pacold et al., 2000, Brock et al., 2003, Czupalla et al., 2003a) have demonstrated that Gβγ dimers recruit PI3Kγ to the plasma membrane thereby stimulating its enzymatic activity. However, the precise molecular mechanism of this G-protein-dependent activation of PI3Kγ is currently unknown. Previous in vivo data imply that $G\beta_1\gamma_2$ recruits PI3Kγ to the membrane through interaction with p101 (Brock et al., 2003). Therefore, p101 may be considered to function as an adaptor. It is unknown whether p101 has additional functions. In order to understand the mechanism of activation following association to the membrane compartment, we examined the activity of PI3Kγ bound to phospholipid vesicles. Earlier, it was shown that anionic phospholipids such as phosphatidic acid (PA) and phosphatidylserine (PS) bind PTEN and PI3Kγ thereby modulating their activity (Kirsch et al., 2001, Walker et al., 2004). In this section we studied the role of Gβγ dimer and p101 in stimulation of PS-recruited p110γ.

5.5.1 Effect of phosphatidylserine on association of $G\beta_1\gamma_{2\text{-}His}$ to the lipid compartment

We have prepared vesicles (see section 4.5.1) with different concentrations of phosphatidylserine (PS). Subsequently, the association of recombinant $G\beta_1\gamma_{2\text{-}His}$ with lipid compartment by lipid vesicle pull-down assay was tested.

Interestingly, increasing concentrations of PS do not significantly affect recruitment of $G\beta_1\gamma_{2\text{-}His}$, regardless whether the G-protein was alone in a reaction mixture or coincubated together with monomeric or dimeric PI3Kγ (Table 5.2). Even the vesicles lacking PS were capable to recruit $G\beta_1\gamma_{2\text{-}His}$, therefore demonstrating that this phospholipid is not essential for the binding of Gβγ with the membrane.

Table 5.2: Recruitment of recombinant $G\beta_1\gamma_{2\text{-His}}$ to phospholipid vesicles

Coincubation of $G\beta_1\gamma_{2\text{-His}}$ with	Phosphatidylserine [mM]				
	0	0.3	0.6	0.9	1.2
—	39.3 ± 7.8 %	33.2 ± 7.1 %	37.5 ± 3.5 %	46.0 ± 17.2 %	33.3 ± 7.8 %
$p110\gamma_{\text{-His}}$	37.0 ± 5.6 %	42.3 ± 15.9 %	34.5 ± 3.0 %	47.0 ± 9.7 %	41.1 ± 12.8 %
$p110\gamma_{\text{-His}}$/p101	34.0 ± 10.0 %	41.0 ± 8.8 %	40.0 ± 4.0 %	47.8 ± 12.0 %	44.5 ± 5.2 %

Phospholipid association of recombinant purified $G\beta_1\gamma_{2\text{-His}}$ (600 nM) alone or coincubated with 200 ng of monomeric or dimeric PI3Kγ was tested at increasing concentrations of phosphatidylserine. For assay conditions see section 4.6. Percentage of phospholipid vesicle-recruited $G\beta_1\gamma_{2\text{-His}}$ was estimated by immunoblot. Total amount of the $G\beta_1\gamma_{2\text{-His}}$ in assay was considered as 100 %. Shown are mean values (±S.D.) of at least two separate experiments. The differences between recruitment of $G\beta_1\gamma_{2\text{-His}}$ in the absence of PI3Kγ and in the presence of monomeric or dimeric PI3Kγ are insignificant ($P > 0.05$).

5.5.2 Effect of $G\beta_1\gamma_{2\text{-His}}$ on stimulation of lipid vesicle-bound PI3Kγ

According to previous reports (Kirsch et al., 2001, Walker et al., 2004), we have found a correlation between the concentration of PS in phospholipid vesicles and the amount of vesicle-recruited monomeric and dimeric PI3Kγ. Indeed, the increase of PS concentration enhanced recruitment of both enzymatic PI3Kγ forms (Fig. 5.21A and Supplementary Table 12.3 in section 12). Therefore, the increase of basal lipid kinase activity of monomeric and dimeric PI3Kγ observed in Fig. 5.21B was due to enhanced recruitment of PI3Kγ by PS rather than direct stimulation by this phospholipid.

The coincubation of $p110\gamma_{\text{-His}}$ together with 600 nM $G\beta_1\gamma_{2\text{-His}}$ did not increase recruitment of p110γ to lipid vesicles by PS. This observation confirms the data described in section 5.4.1 where $G\beta_1\gamma_{2\text{-His}}$ was not able to significantly recruit monomeric PI3Kγ. In contrast, $G\beta_1\gamma_{2\text{-His}}$ considerably recruited $p110\gamma_{\text{-His}}$/p101 to the lipid compartment (Fig. 5.21A and Supplementary Table 12.3 in section 12).

Although $G\beta_1\gamma_{2\text{-His}}$ did not recruit monomeric PI3Kγ, it significantly stimulated lipid kinase activity of $p110\gamma_{\text{-His}}$. This was observed at all PS conditions tested (Fig. 5.21A and B, Supplementary Tables 12.3 and 12.4 in section 12) and suggests that G$\beta\gamma$ dimers stimulate phospholipid vesicles-associated monomeric PI3Kγ.

In order to examine whether additional $G\beta_1\gamma_{2\text{-His}}$-induced stimulation is required for phospholipid vesicles-associated dimeric PI3Kγ or recruitment itself is sufficient for full activation of the enzyme, we designed lipid vesicles containing equal amount of kinase in the absence and presence of $G\beta_1\gamma_{2\text{-His}}$.

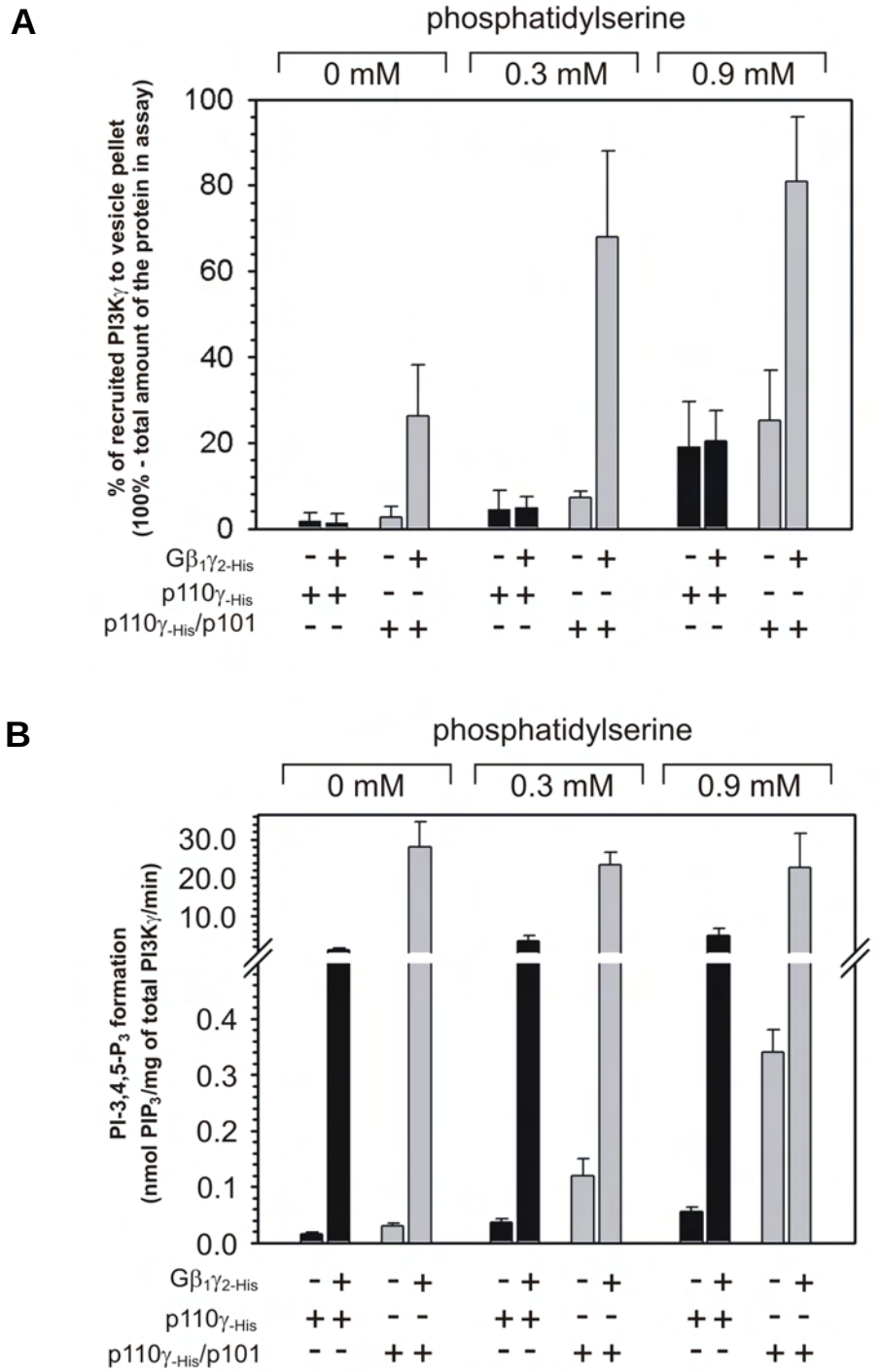


Fig. 5.21: Influence of phosphatidylserine on the recruitment of monomeric and dimeric PI3Kγ to the phospholipid compartment and their lipid kinase activity

(A) Phosphatidylserine was tested for its ability to recruit 200 ng of monomeric (black bars) or dimeric (grey bars) PI3Kγ in the absence (-) or presence (+) of 600 nM $G\beta_1\gamma_{2\text{-His}}$. Assays were done as detailed under section 4.6. Aliquots of pelleted phospholipid vesicles and the supernatants were subjected to SDS-PAGE followed by Western blotting. Proteins were visualized using specific antiserum (see section 3 and 4.1.4). Chemiluminescence signals were estimated by CCD camera. Total amount of monomeric or dimeric PI3Kγ in assay was considered as 100 %. Data are given as means ± S.D. of duplicate determinations in three independent experiments. **(B)** Lipid kinase activity of PI3Kγ under the same conditions as recruitment. Assays were done as detailed under section 4.5.1. After termination of reaction, probes were subjected to thin layer chromatography (TLC) plates. Dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system. Shown are mean values (± S.D.) of duplicate determinations in three independent experiments.

For instance, the coincubation of $G\beta_1\gamma_{2\text{-His}}$ and $p110\gamma_{\text{-His}}$/p101 with vesicles lacking PS exhibits an amount of phospholipid-associated kinase (26.2 %) comparable with association of dimeric PI3Kγ coincubated with vesicles containing 0.9 mM PS in the absence of $G\beta_1\gamma_{2\text{-His}}$ (25.2 %) (Fig. 5.21A and Supplementary Table 12.3 in section 12). Nonetheless, lipid kinase activities were significant different (0.34 nmol/mg of total PI3Kγ/min in the absence of $G\beta_1\gamma_{2\text{-His}}$ (0.9 mM PS) versus 28.1 nmol/mg of total PI3Kγ/min in the presence of $G\beta_1\gamma_{2\text{-His}}$ (0 mM PS)) (Fig. 5.21B and Supplementary Table 12.4 in section 12). Despite of same efficiency of recruitment, the lipid kinase activity of the dimeric PI3Kγ is approximately 80 times higher in the presence of 600 nM $G\beta_1\gamma_{2\text{-His}}$ (Fig. 5.21A and B, Supplementary Tables 12.3 and 12.4 in section 12). Interestingly, we found that in the presence of 0.9 mM PS in phospholipid vesicles, i.e. 3 times more than in normal conditions, the fold stimulation of basal activity by $G\beta_1\gamma_{2\text{-His}}$ was the same for monomeric and dimeric PI3Kγ. Taken together, the presented data demonstrate that $G\beta_1\gamma_{2\text{-His}}$ dimers coordinately control both recruitment and activation of PI3Kγ.

5.5.3 p101 serves as a potential activator of p110γ

The conditions under which similar amounts of monomeric and dimeric PI3Kγ were associated with Gβγ-free phospholipid vesicles were established. Fig. 5.21A shows that 0.9 mM of PS in lipid vesicles allowed recruitment of roughly the same amount of both monomeric and dimeric PI3Kγ. In contrast, the basal lipid kinase activity of associated dimeric PI3Kγ was significantly, e.g. 6 times in the presence of 0.9 mM PS, higher than the basal lipid kinase activity of monomeric PI3Kγ (Fig. 5.21B and Supplementary Table 12.4 in section 12). Thus, we assume, that p101 may directly affect the enzymatic activity of p110γ. Nevertheless, we can not exclude the possibility that monomeric PI3Kγ partially lost its intrinsic lipid kinase activity during the purification procedure. In order to clarify the role of the non-catalytic subunit, we purified p101 alone and performed a set of reconstitution experiments with p110γ.

p101 was expressed in Sf9 cells and purified using standard purification procedure for PI3Kγ (Fig. 5.22A) as described under section 4.3.2. This purification procedure typically yielded 20 to 50 µg of $p101_{\text{-His}}$ per 1.5 x 10^9 Sf9 cells of an estimated purity ranging from 30 to 50 %, whereas yield and purity of $p110\gamma_{\text{-His}}$ was much higher, i.e. from 600 to 1000 µg per 1.5 x 10^9 Sf9 cells of an estimated purity more than 90 %.

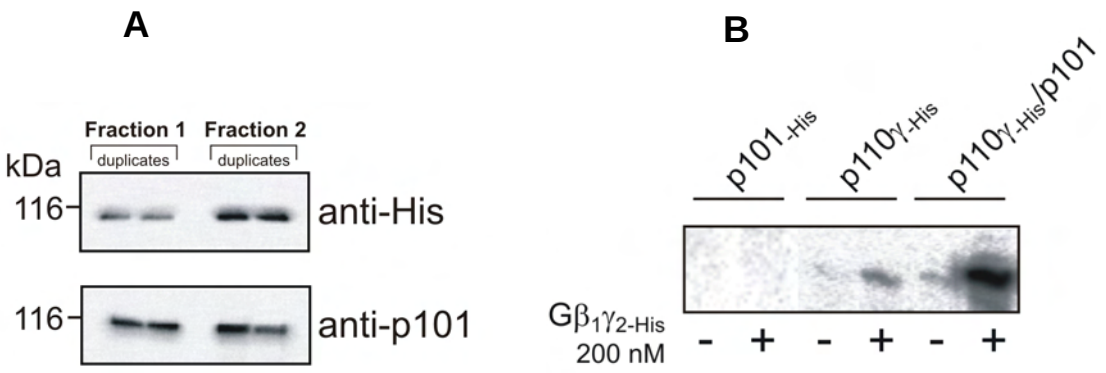


Fig. 5.22: Purification and characterization of recombinant p101$_{\text{-His}}$

(A) p101$_{\text{-His}}$ was purified using regular purification protocol for His-tagged PI3Kγ described under section 4.3.2. Two peak fractions eluted from Resource 15Q (1 ml) in duplicates were subjected to SDS-PAGE, followed by Western blotting. Specific antibodies against His-tag and p101 have been used. Apparent molecular mass of marker protein is indicated. **(B)** Lipid kinase assay of recombinant purified p101$_{\text{-His}}$ (50 ng), p110γ$_{\text{-His}}$ (10 ng), and p110γ$_{\text{-His}}$/p101 (10 ng). Fractions containing p101$_{\text{-His}}$ were tested for endogenous Gβ$_1$γ$_2$–sensitive lipid kinase activity as detailed under section 4.5.1. After termination of reactions, the probes were subjected to TLC plates. Subsequently, dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system.

Purified p101$_{\text{-His}}$ was identified by immunoblotting using specific anti-hexahistidine and anti-p101 antibodies. The fractions containing p101$_{\text{-His}}$ did not exhibit Gβ$_1$γ$_2$-sensitive lipid kinase activity thereby clearly demonstrating that p101$_{\text{-His}}$ was not copurified with endogenous p110γ (Fig. 5.22B). Additionally, we found that independently expressed and purified p101 and p110γ subunits seem to form a complex (Fig. 12.2 in supplementary section 12.1).

These data allowed us to ask whether purified recombinant p101$_{\text{-His}}$ may reconstitute functionally active dimeric PI3Kγ from purified monomeric p110γ$_{\text{-His}}$. Therefore, lipid and protein kinase activities as well as recruitment of PI3Kγ to phospholipid vesicles were studied. Coincubation of individually expressed and purified p101$_{\text{-His}}$ and p110γ$_{\text{-His}}$ led to a reconstitution of lipid kinase activity similar to a coexpressed p110γ$_{\text{-His}}$/p101 dimer (Fig. 5.23A). Furthermore, the reassociation of p110γ$_{\text{-His}}$ with p101$_{\text{-His}}$ demonstrated a Gβ$_1$γ$_{2\text{-His}}$-induced autophosphorylation of catalytic subunit comparable with regular dimeric kinase (Fig. 5.23B). It should be kept in mind that autophosphorylation of monomeric PI3Kγ, i.e. p110γ, is insensitive to Gβ$_1$γ$_{2\text{-His}}$ stimulation (see section 5.3.3 and Fig. 5.23B, top). As detailed before (section 5.3.3), monomeric PI3Kγ (p110γ$_{\text{-His}}$) exhibited a high intrinsic protein kinase activity. Surprisingly, addition of p101$_{\text{-His}}$ suppressed this autophosphorylating activity (Fig. 5.23B, top). Stimulation of the autophosphorylation of the reconstituted dimeric enzyme by Gβ$_1$γ$_{2\text{-His}}$ was similar as the intact dimeric enzyme. The presence of p101$_{\text{-His}}$ also promoted the Gβ$_1$γ$_{2\text{-His}}$-dependent recruitment of p110γ$_{\text{-His}}$ to phospholipid vesicles. Vesicle binding of reconstructed dimer and dimeric PI3Kγ shows no difference (Fig. 5.23C).

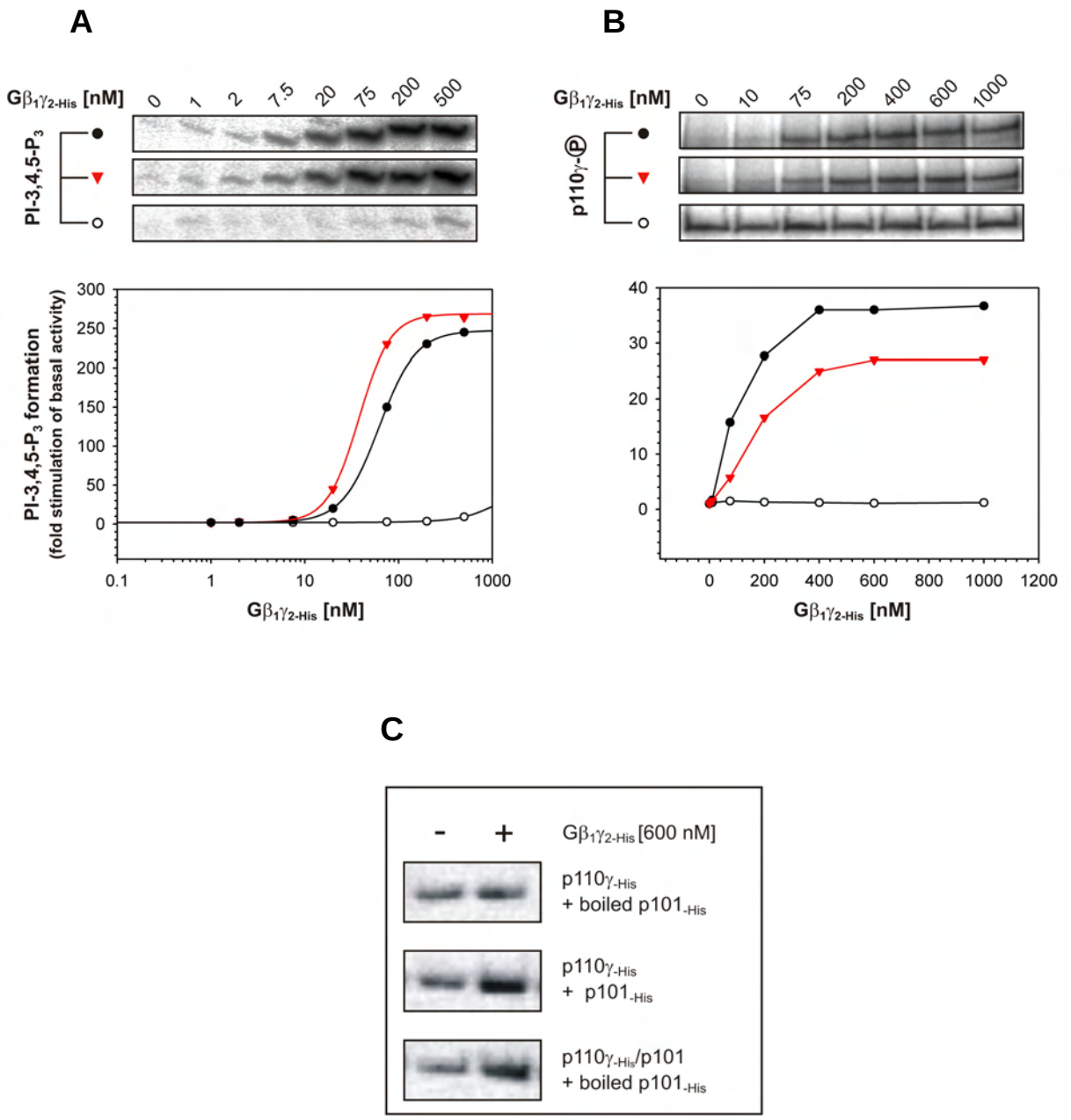


Fig. 5.23: Recombinant p101$_{\text{-His}}$ forms functionally active dimeric PI3Kγ from monomeric p110γ$_{\text{-His}}$

Stimulation of lipid **(A)** and protein **(B)** kinase activities of p110γ$_{\text{-His}}$/p101 (black closed circles), p110γ$_{\text{-His}}$ (black open circles), and p110γ$_{\text{-His}}$ preincubated with p101$_{\text{-His}}$ (red triangles) in response to increasing concentrations of Gβ$_1$γ$_{2\text{-His}}$. Assays were done as detailed under section 4.5. The lipid kinase assays were performed in the presence of 10 ng of dimeric or monomeric PI3Kγ and 50 ng of p101$_{\text{-His}}$, whereas the protein kinase assays were done in the presence of 40 ng of dimeric or monomeric PI3Kγ and 50 ng of p101$_{\text{-His}}$. Autoradiographs of one representative experiment are shown (top). Gβγ-induced activation of PI3Kγ enzymatic activities is illustrated as fold-stimulation of basal activity from one representative experiment out of three (bottom). **(C)** Recruitment of p110γ$_{\text{-His}}$/p101, p110γ$_{\text{-His}}$, and p110γ$_{\text{-His}}$ coincubated with p101$_{\text{-His}}$ to lipid vesicles (see section 4.6). The lipid pull-down assays were performed in the presence of 200 ng of dimeric or monomeric PI3Kγ and 200 ng of p101$_{\text{-His}}$. Sedimented phospholipid vesicles were subjected to SDS-PAGE followed by immunoblotting. Proteins were visualized using specific anti-p110γ antiserum.
In order to achieve the same buffer content, monomeric and dimeric PI3Kγ were preincubated with 50 ng (figure **A** and **B**) or 200 ng (figure **C**) of p101$_{\text{-His}}$ which was boiled for 45 min at 95 °C.

Taken together, on the one hand, our data exclude the possibility that monomeric PI3Kγ partially lost its lipid kinase activity during the purification procedure. Coincubation of p110γ with individually purified p101 brings monomeric catalytic subunit to an enzymatic state identical to dimeric kinase. On the other hand, we provide direct evidence that p101 regulate lipid and protein kinase activity of p110γ in opposite ways, i.e. stimulation of basal lipid kinase activity and suppression of basal autophosphorylation. In consequence, the results obtained in reconstitution analysis support our previous finding described in this study that p101 serves not only as an adaptor for $G\beta_1\gamma_2$ but also is involved in the regulation of the enzymatic activities of p110γ.

6 DISCUSSION

Class I_B phosphoinositide 3-kinase γ (PI3Kγ), which regulates various cellular processes such as mitogenesis, apoptosis and cytoskeletal functions, is an important Gβγ effector (Katso et al., 2001, Vanhaesebroeck et al., 2001). PI3Kγ signals through its inherent lipid and protein kinase activity (Bondeva et al., 1998, Leopoldt et al., 1998, Maier et al., 1999, 2000, Brock et al., 2003, Czupalla et al., 2003a). Receptor-released Gβγ dimers recruit PI3Kγ to the membrane and stimulate these enzymatic activities. However, the molecular basis of this interaction is still unclear. Therefore, it was the aims of this study i) to map the sites of $G\beta_1$ important for binding and activation of different forms and enzymatic activities of PI3Kγ, and ii) to elucidate the role of non-catalytic p101 subunit in activation of PI3Kγ.

6.1 Purification and analysis of recombinant $G\beta_1\gamma_{2\text{-}His}$ variants from Sf9 cells

It was shown that the Gα subunit switch off the ability of the Gβγ dimer to activate its effectors suggesting that Gα- and Gβγ-dependent effectors overlap in binding sites on the surface of Gβγ (Clapham and Neer, 1997, Ford et al., 1998, Li et al., 1998, Panchenko et al., 1998, Scott et al., 2001, Goubaeva et al., 2003). Two regions on Gβγ that interact with Gα have been defined by the X-ray crystallography of heterotrimeric Gαβγ (Wall et al., 1995, Lambright et al., 1996, Sondek et al., 1996). G-protein β subunit residues Leu-55, Lys-78, Ile-80, and Lys-89 form the first Gα/Gβ interface, whereas amino acids Lys-57, Tyr-59, Ser-98, Trp-99, Met-101, Leu-117, Asn-119, Thr-143, Asp-186, Asp-228, and Trp-332 belong to the second interface between Gα and Gβ subunits (Ford et al., 1998). The crystal structure of $G\beta_1\gamma_2$ bound to a synthetic peptide SIGK (SIGKAFKILGYPDYD) at a resolution of 2.7 Å revealed that Gβ residue Tyr-145 is also essential for binding to Gα (Davis et al., 2005). Molecular determinants indispensable for interaction of Gβγ with such effectors as type I (AC I) and type II (AC II) adenylyl cyclase, phospholipase $C\beta_2$ ($PLC\beta_2$), β-adrenergic receptor kinase (βARK), muscarinic potassium channel (GIRK1/GIRK4), calcium channel α1B subunit (CCα1B) have been resolved (Ford et al., 1998, Li et al., 1998). However, the exact nature of the interaction between Gβγ variants and PI3Kγ was unclear. Accordingly, 16 different residues substituted for alanine were chosen in this study for mapping of $G\beta_1$ regions, which are involved in PI3Kγ binding and activation.

To investigate the properties and functions of G-proteins, Gβγ heterodimers were previously purified from the natural tissues (e.g. bovine brain) (Neer et al., 1984, Thomas et al., 1993, Leopoldt et al., 1998, Exner et al., 1999). However, in this case, the Gβγ purified

pool represented a mixture of various Gβ and Gγ isoforms. There are also different heterologous expression systems (e.g Sf9 cells) where it is possible to express defined Gβγ subsets (Iniguez-Lluhi et al., 1992, Hepler et al., 1993, Leopoldt et al., 1997). In order to purify recombinant Gβγ, Sf9 cells should be infected with baculoviruses encoding Gβ, Gγ and Gα. It was believed that only such expression and purification procedures of G-protein subunits lead to proper formation of heterotrimer and subsequent isolation of the Gβγ dimer. However, such a standard procedure of G-proteins expression and purification could not be used for $G\beta_1\gamma_2$ purification where $G\beta_1$ residues important for the assembling into heterotrimers with Gα were substituted for alanine. A recent work demonstrated the possibility of purification of Gβγ dimers, i.e. $G\beta_1\gamma_{2\text{-His}}$, in the absence of Gα in Sf9 cells (Maier et al., 2000). These dimers were as active as native Gβγ proteins in their ability to activate lipid kinase activity of recombinant PI3Kγ.

Therefore, we isolated all $G\beta_1\gamma_{2\text{-His}}$ variants using two column chromatography (Ni^{2+}-NTA-Superflow and Resource 15Q, see section 4.3.1) with an estimated purity of more than 90 %. Ford et al. (1998), who originally created the recombinant baculoviruses of $G\beta_1$ variants, investigated in their study $G\beta_1\gamma_{2\text{-His}}$ protein preparations, which were isolated from membrane fraction of Sf9 cells using one purification step, i.e. Ni^{2+}-NTA resin. The purity of these $G\beta_1\gamma_{2\text{-His}}$ mutants ranged from 30 % to 50 % (Ford et al., 1998). Although the purity of our $G\beta_1\gamma_{2\text{-His}}$ variants was more than 90 %, we observed that these proteins contain a wortmannin-insensitive lipid kinase activity (data shown only for wild-type $G\beta_1\gamma_{2\text{-His}}$, Fig. 5.1). We assume that this unspecific activity is due to a copurified endogenous phospholipid kinase. To prevent the interference between the intrinsic lipid kinase activity of tested PI3Kγ and the Gβγ-copurified unknown phospholipid kinase in our assays, we further improved the purification procedure of recombinant Gβγ by an additional gel filtration step (data shown only for wild-type $G\beta_1\gamma_{2\text{-His}}$, Fig. 5.2 and 5.3). This allowed separation of the phospholipid kinase from Gβγ suggesting that this insect enzyme is not a tightly bound Gβγ interacting protein.

Substitution of amino acids may cause an incorrect folding of the protein and wrong assembly with its co-partners. Previously, it was shown that limited proteolysis can be a useful tool for the examination of the conformational states of G-proteins (Fung and Nash, 1983, Winslow et al., 1986, Thomas et al., 1993). Based on this experimental approach, the data of section 5.2.1 demonstrate that fifteen $G\beta_1$ variants assemble correctly with $G\gamma_2$. Only Lys-78 mutation was sensitive to trypsin digestion. Although Ford et al. (1998) have reported that $G\beta_{1K78A}\gamma_{2\text{-His}}$ and other $G\beta_1\gamma_{2\text{-His}}$ variants isolated from Sf9 cells were stable against trypsin digestion, they did not show the data of proteolysis assay. We found that the ability of $G\beta_{1K78A}\gamma_{2\text{-His}}$ to stimulate lipid and protein kinase activities of PI3Kγ was comparable to $G\beta_{1WT}\gamma_{2\text{-His}}$ (Fig. 5.5). Our possible explanation of this is that alanine mutation of $G\beta_1$ in

position Lys-78 leads to conformational changes, which do not hamper the interaction and stimulation of PI3Kγ but expose one or more of 32 potential tryptic sites. Trypsin cleavage of this site promotes unfolding and, consequently, full digestion of $G\beta_1$. Alternatively, $G\beta_{1K78A}\gamma_{2\text{-His}}$ may gain a correct conformation upon interaction with the effector, i.e. PI3Kγ.

6.2 Autophosphorylation of class I_B PI3K

All members of the PI3K family are bifunctional enzymes characterized by the ability to catalyze both lipid and protein phosphorylation (Carpenter et al., 1993, Dhand et al., 1994). Whereas the role of 3-phosphorylated phosphoinositides generated by the lipid kinase activity of PI3K in signal transduction is well documented, very little is known about the role of the protein kinase activity. Previously, it has been shown that protein kinase activity of PI3Kγ activates the MAPK pathway and phosphorylates cytoskeletal non-muscle tropomyosin (Bondeva et al., 1998, Naga Prasad et al., 2005). Phosphorylation of cytoskeletal non-muscle tropomyosin is essential for the proper actin cytoskeletal remodelling and β_2-adrenergic receptors internalization. An additional type of PI3K intrinsic protein kinase activity, so called autophosphorylation, has been found (Bondeva et al., 1998, Czupalla et al., 2003a). The PI3K has a conserved protein kinase domain that shares similarity to the Src family of tyrosine protein kinases (Walker et al., 1999). However, PI3K catalyzes serine rather than tyrosine phosphorylation (Dhand et al., 1994). Autophosphorylation sites of both PI3K β and γ isoforms were mapped to C-terminal serine residues of the catalytic p110 subunit (i.e. serine-1070 of p110β and serine-1101 of p110γ) (Czupalla et al., 2003a). Moreover, the C-terminal serine-1039 was detected as the site of p110δ autophosphorylation (Vanhaesebroeck et al., 1999).

We have shown that $G\beta_1\gamma_{2\text{-His}}$ significantly induces autophosphorylation of the catalytic subunit of $p110\gamma_{WT\text{-His}}$/p101. Exchange of serine in position 1101 of the catalytic subunit for alanine, aspartic or glutamic acids abolished the autophosphorylation of dimeric PI3Kγ supporting previous data which demonstrated that Ser-1101 is the dominant autophosphorylation site of p110γ subunit (Fig. 5.8A, and Czupalla et al., 2003a, b). In contrast to their protein kinase activity, all these PI3Kγ variants were able to produce PI-3,4,5-P_3 in the presence of increasing concentrations of recombinant $G\beta_1\gamma_{2\text{-His}}$. No differences in lipid kinase activity were observed between wild-type and alanine substituted PI3Kγ, which is in agreement with previous report (Czupalla et al., 2003a). It was assumed that substitution of Ser-1101 for negatively charged aspartic or glutamic acids transfers the PI3Kγ in to the “constitutively phosphorylated” state by mimicking an incorporated phosphate. Accordingly, we expected to observe similar lipid kinase activity exhibited by

these mutants as compared to the wild-type PI3Kγ. Surprisingly, we obtained a 2 - 3 fold higher activity with S1101D and S1101E mutants of PI3Kγ (Fig. 5.8B). Additional experiments will be required to understand the impact of aspartic and glutamic acids at position 1101 on the structure-function relationship of PI3Kγ.

Earlier studies have shown that autophosphorylation of the p110 α, β and δ isoforms led to a reduction of their lipid kinase activity (Carpenter et al., 1993, Dhand et al., 1994, Vanhaesebroeck et al., 1999, Czupalla et al., 2003a). We in this study and Czupalla et al. (2003a) clearly demonstrated that neither autophosphorylation of wild-type PI3Kγ nor S1101A, S1101D or S1101E mutants inhibited their lipid kinase activity.

Yart et al. (2002) have shown that a "protein kinase-only" mutant of p110β, which was obtained by deleting amino acids 946 to 955, exhibited a higher protein kinase activity than the wild-type enzyme. We did not have a "protein kinase-only" mutant of p110γ. Nevertheless, the lipid kinase activity of the PI3Kγ may occur only in the presence of phosphoinositides with non-phosphorylated D3 position of the inositol ring. Therefore, the absence of such phosphoinositides in the phospholipid vesicles gave us the opportunity to investigate wild-type p110γ which exert only protein kinase activity. We did not find any differences in the autophosphorylation activity of p110γ, regardless whether phospholipid substrates, such as PI-4,5-P_2, were present (Fig. 5.9B). According to data described above, we assume that lipid and protein kinase activities are self-directed and do not depend from each other during the Gβγ-induced stimulation. In order to prove this speculation, further investigations will be needed. It was shown that the catalytic domain of p110γ contains an ATP-binding site and a binding site for phospholipid substrates (Walker et al., 1999). The consensus sequence region of p110γ ($_{-943}$GIGDRHNDN$_{951-}$) represents a catalytic loop of catalytic domain which is responsible for deprotonating the substrate, thereby producing the nucleophile that attacks the γ-phosphate of ATP (Walker et al., 1999, Vanhaesebroeck et al., 2001). Therefore, mutations in catalytic loop of p110γ or use of PI-4,5-P_2 analogue lacking hydroxyl (–OH) group in the D3 position of the inositol ring would help to elucidate the correlation between functions of lipid and protein kinase activities of PI3Kγ.

Bondev et al. (1999) observed autophosphorylation of PI3Kγ in assays lacking phospholipids. Fig. 5.9B of this study and others results (Maier et al., 1999, 2000, Czupalla et al., 2003a) demonstrated the necessity of lipid vesicles for Gβγ-induced autophosphorylation. Notably, we found phosphorylated enzyme in both lipid and aqueous phases of reaction mixture (Fig. 5.9A). The reason remains unclear. However, we can speculate that phosphorylation of p110γ could interfere with Gβγ binding, p101 binding or membrane binding. Based on our data presented in this study which show that p110$\gamma_{WT\text{-}His}$/p101 and p110$\gamma_{S1101A\text{-}His}$/p101 exhibit similar Gβγ-mediated lipid kinase activity (Fig. 5.8), we exclude the first two possibilities. There are some evidences demonstrating that protein

phosphorylation is accompanied with their dissociation from the plasma membrane. For instance, Bivona et al. (2006) reported that phosphorylation of K-Ras by protein kinase C (PKC) promotes its displacement from the plasma membrane and association with intracellular membranes (e.g. outer membrane of mitochondria). Furthermore, PKC phosphorylates the myristoylated alanine-rich C kinase substrate (MARCKS), thereby displacing MARCKS from the plasma membrane (Thelen et al., 1991). Subsequent dephosphorylation of MARCKS leads to its reassociation with the membrane. According to these reports and data obtained in this study (Fig. 5.9A), we propose that phosphorylation of dimeric PI3Kγ may lead to detachment of enzyme from the lipid compartment. In this scenario, increase of PI-3,4,5-P_3 concentration upon Gβγ-dependent stimulation of lipid kinase activity of PI3Kγ may incrementally increase the total negative charge on lipid vesicles thereby promoting the displacement of phosphorylated PI3Kγ (Fig. 6.1).

In contrast to p110$\gamma_{\text{-His}}$/p101, monomeric PI3Kγ (p110$\gamma_{\text{-His}}$) did not exhibit considerable Gβγ-induced autophosphorylation. Interestingly, p110$\gamma_{\text{-His}}$ showed high basal level of autophosphorylation. Coincubation of p110$\gamma_{\text{-His}}$ with 600 nM G$\beta_1\gamma_{2\text{-His}}$ showed only 1.6-fold stimulation over the basal activity (Fig. 5.10). Contrary to N-terminally His-tagged monomeric PI3Kγ, we in this study and Maier et al. (1999) demonstrated about 5-fold activation of autophosphorylation of GST-fused N-terminally truncated (amino acids 1 - 34) monomeric PI3Kγ in response to increasing concentrations of Gβγ. Based on these data, we assumed that N-terminal fragment of p110γ may interfere with the autophosphorylation process. Therefore, we purified His-tagged p110γ, which had the same N-terminal truncation as p110$\gamma_{\text{-GST}}$, and tested its protein kinase activity. We found that G$\beta_1\gamma_2$-induced autophosphorylation of p110$\gamma_{\text{-His}}$ full-length and p110$\gamma_{\text{-His}}$ (△1 - 34) was similar (Fig. 5.10B). This finding excluded the possibility that N-terminal fragment of p110γ may affect its protein kinase activity. Most probably, the higher autophosphorylation ability of p110$\gamma_{\text{-GST}}$ (△1 - 34) was due to the N-terminal GST-tag. What effect of the GST-tag on the p110$\gamma_{\text{-GST}}$ (△1 - 34) is, is difficult to judge at this stage. However, there are evidences that GST fusion may promote dimerization (Krugmann et al., 1999, Yamada et al., 2005) and stabilization (Slater et al., 1998, Houten et al., 1999, Amersham Pharmacia Biotech, 2001b) of recombinant proteins. Therefore, we assume that structural stabilization of recombinant PI3Kγ by bulky GST-tag may probably facilitate phosphorylation of p110γ. On the other hand, the strategy of crystallization of p110γ which requires stable protein is in apparent contrast to our assumption. In order to crystallize p110γ and determine its structure, Walker et al. (1999) and Pacold et al. (2000) purified His-tagged p110γ from Sf9 cells.

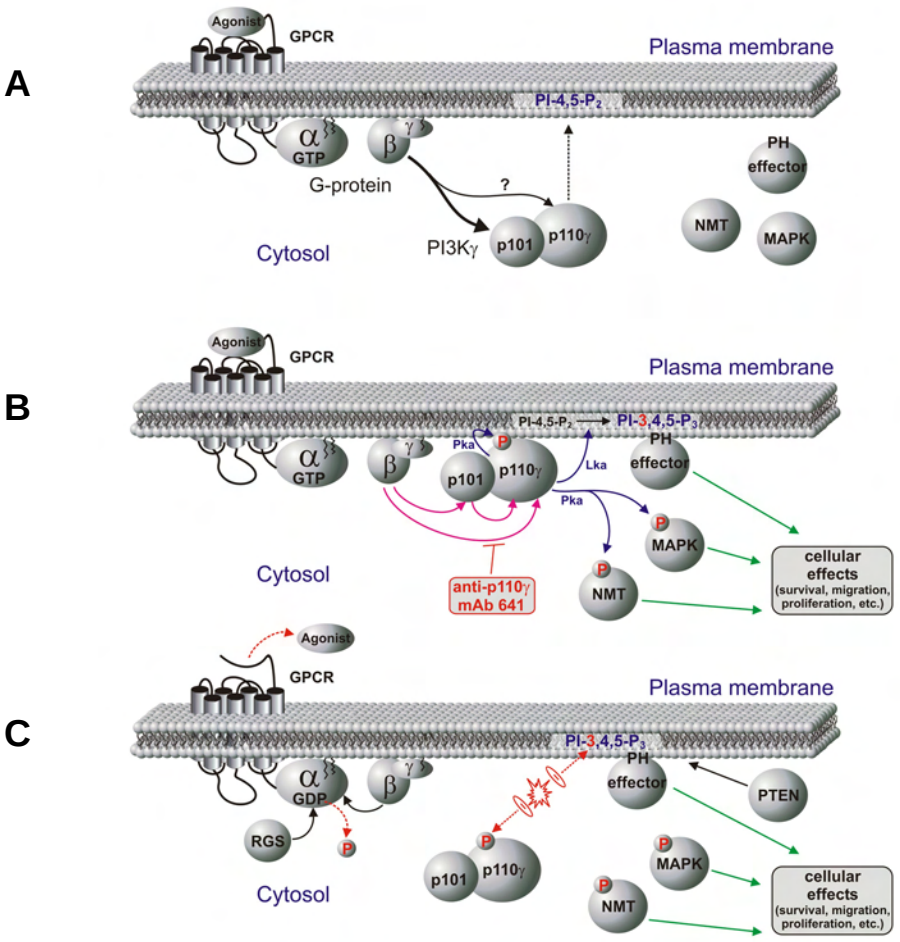


Fig. 6.1: Hypothetical model for activation of the PI3Kγ pathway

(A) Switch-on PI3Kγ signaling. Upon GPCR stimulation by an agonist, G-protein dissociate into both free Gα subunit and Gβγ dimer. Gβγ dimer recruits PI3Kγ to the plasma membrane (dotted arrow) by binding to p101 and, probably, to p110γ (solid arrows). **(B)** Activation of PI3Kγ and its pathways. Gβγ dimer activates PI3Kγ by direct interaction with p101 and p110γ. Additionally, p101 facilitates activity of p110γ. Activation of PI3Kγ is shown by magenta arrows. Activated PI3Kγ exhibits its inherent lipid and protein kinase activities (blue arrows). The lipid kinase activity of PI3Kγ produces PI-3,4,5-P_3 by phosphorylating PI-4,5-P_2. Subsequently, PI-3,4,5-P_3 recruits PH domain-containing effectors to the plasma membrane where they become activated and initiate downstream effects. The protein kinase activity of PI3Kγ leads to phosphorylation of MAPK and NMT (see abbreviations below) thereby regulating their pathways. Moreover, upon Gβγ stimulation, dimeric PI3Kγ phosphorylates its catalytic subunit at Ser-1101 (autophosphorylation). Under experimental conditions, anti-p110γ monoclonal antibody (mAb 641) blocks the interaction between Gβγ dimer and p110γ. **(C)** Switch-off PI3Kγ signaling. Elevation of PI-3,4,5-P_3 concentration increase the total negative charge on plasma membrane. This event may promote the displacement of phosphorylated PI3Kγ from the plasma membrane and translocation to cytosol. RGS proteins facilitate GTPase activity of the Gα which hydrolyzes bound GTP to GDP. Gβγ dimers become higher affinity to Gα than to PI3Kγ and, subsequently, completely reassociate with Gα subunits. GPCR releases an agonist. PTEN dephosphorylates the D3 position of the inositol ring of PI-3,4,5-P_3 thereby generating PI-4,5-P_2. This leads to membrane detachment of PI-3,4,5-P_3-dependent PH domain-containing effectors and deactivation of their pathways.

GPCR, a heptahelical G-protein-coupled receptor; G-protein, heterotrimeric GTP-binding proteins composed of a Gα, a Gβ and a Gγ subunits; Lka, lipid kinase activity of PI3Kγ; NMT, cytoskeletal non-muscle tropomyosin; PH-effectors, pleckstrin homology domain-containing effectors such as GRP1 or Akt/PKB; PI3Kγ, class I_B PI3K consists of a catalytic p110γ subunit associated with a noncatalytic p101 subunit; PI-3,4,5-P_3, phosphatidylinositol-3,4,5-triphosphate; PI-4,5-P_2, phosphatidylinositol-4,5-bisphosphate; Pka, protein kinase activity of PI3Kγ; PTEN, phosphatase dephosphorylating all 3-phosphorylated phosphoinositides; RGS, regulator of G-protein signaling stimulating the GTPase reaction of the Gα subunit; mAb 641, anti-p110γ specific monoclonal antibody raised against entire p110γ molecule in mouse hybridoma cells; MAPK, mitogen-activated protein kinase.

We have shown that coincubation of $p110\gamma_{\text{-His}}$ with individually purified $p101_{\text{-His}}$ transfers monomeric catalytic subunit to an enzymatic state identical to dimeric PI3Kγ (Fig. 5.23). Although the maximal Gβγ-induced stimulation of $p110\gamma_{\text{-His}}$ autophosphorylation after preincubation of $p110\gamma_{\text{-His}}$ together with $p101_{\text{-His}}$ was unaltered compared to autophosphorylation of $p110\gamma_{\text{-His}}$ alone, basal activity of this reconstituted complex was considerably reduced (Fig. 5.23B, top). The consequence of this event is an increase of folds of Gβγ-induced stimulation of autophosphorylation of reconstituted complex over its basal activity. Based on this data, we conclude that the binding of p101 to p110γ slows down the phosphorylation of Ser-1101 whereas addition of $G\beta_1\gamma_{2\text{-His}}$ to this reconstituted dimeric PI3Kγ accelerates phosphate incorporation. This and other studies have demonstrated that Gβγ dimers recruit dimeric PI3Kγ to the phospholipid vesicles or plasma membrane via interaction with p101 (Fig. 6.1, Stephens et al. 1997, Maier et al. 1999, Brock et al., 2003). Accordingly, we propose that monomeric PI3Kγ is highly phosphorylated at Ser-1101 in a Gβγ-independent manner. There are two alternative mechanisms how Gβγ dimer and p101 might control p110γ autophosphorylation. Binding of p101 to catalytic subunit either partially masks Ser-1101 or alters the conformation of the active site towards switch-off state. Interaction of Gβγ dimers with dimeric PI3Kγ induces conformational changes with subsequent opening and exposition of Ser-1101 or activation of the catalytic center.

6.3 Role of p101 in the activation of PI3Kγ

Class I PI3Ks are subdivided into class I_A p85- or class I_B p101-associated heterodimers. The role of p85 adaptor protein is well investigated. The p85 binds to class I_A PI3K thereby stabilizing p110 catalytic subunit and holding it in a low activity state (Yu et al., 1998). The p85 adaptor protein mediates the activation of class I_A PI3K by binding to growth factor receptors (mainly of the protein-tyrosine kinase family) (Songyang et al., 1993, Luo et al., 2005). Binding of p85 to the receptor reduces its inhibitory action on the catalytic subunit and allows recruitment of cytosolic PI3K to the plasma membrane (Rordorf-Nikolic et al., 1995). In contrast to class I_A PI3K, the only class I_B member, p110γ, associates with p101 subunit (Stephens et al., 1997) or with recently discovered second regulatory subunit termed p84 (Suire et al., 2005) or $p87^{PIKAP}$ (Voigt et al., 2005, 2006). Class I_B PI3K is activated by Gβγ proteins (Fig 6.1) (Stoyanov et al., 1995, Stephens et al., 1997, Leopoldt et al., 1998, Brock et al., 2003). Stephens et al. (1997) proposed that p101 confers the p110γ sensitivity to Gβγ dimer. Moreover, it was assumed that p101 may differently modulate protein and lipid kinase activities of PI3Kγ (Lopez-Ilasaca et al., 1998, Murga et al., 1998, Maier et al., 1999). Maier et al. (1999) have demonstrated that p101 may differently contribute to Gβγ-induced

phosphorylation of different phosphoinositides. However, the functional roles of the p101 subunit in the activation of PI3Kγ by Gβγ remain unclear.

We asked whether p101 functions only as an adaptor linking Gβγ with p110γ or it also modulates enzymatic activity of p110γ. To address these questions, we looked for specific tools which allow recruit similar amount of monomeric or dimeric PI3Kγ to phospholipid vesicles. Kirsch et al. (2001) have shown that PI3Kγ binds to anionic phospholipids, such as phosphatidylserine (PS) and phosphatidic acid (PA). They proposed that the PI3Kγ associates to the phospholipid surface with its N-terminal binding domain for anionic phospholipids allowing C-terminal catalytic domain to phosphorylate the membrane localized substrates. Moreover, previous work of Walker et al. (2004) studying catalysis by PTEN showed that anionic lipids greatly modulate PTEN activity in vitro by recruitment to substrate-containing vesicles or micelles. Based on these findings, we performed assays with phospholipid vesicles lacking PS and vesicles containing different concentrations of this phospholipid.

We have found that PS does not affect binding of $G\beta_1\gamma_{2\text{-His}}$ to the membrane, regardless whether this G-protein was alone in a reaction mixture or coincubated together with both enzymatic forms of PI3Kγ (Table 5.2). However, increasing of PS concentrations led to recruitment of monomeric and dimeric PI3Kγ (Fig. 5.21) supporting previously published data (Kirsch et al., 2001, McConnachie et al., 2003, Walker et al., 2004). We observed that only membrane recruitment of PI3Kγ was not sufficient for complete enzyme activation. For instance, monomeric and dimeric PI3Kγ proteins were already associated with Gβγ-free phospholipid vesicles. $G\beta_1\gamma_2$-containing vesicles demonstrated considerably higher recruitment of dimeric PI3Kγ whereas recruitment of monomeric kinase was drastically weakened (Fig. 5.12) or almost abolished (Fig. 5.21). Nevertheless, phospholipid vesicles-associated $G\beta_1\gamma_{2\text{-His}}$ significantly stimulated lipid kinase activity of $p110\gamma_{\text{-His}}$ (Fig. 5.21). These findings are in accordance with earlier data (Brock et al. 2003). These authors have generated p110γ mutants containing a CAAX-box motif, which constitutively localizes p110γ to the plasma membrane. Coexpression of Gβγ with YFP-p110γ-CAAX in HEK cells demonstrated that membrane-bound PI3Kγ is not fully active, but can be further activated by Gβγ. Concerning the dimeric PI3Kγ, which was significantly recruited by $G\beta_1\gamma_{2\text{-His}}$ to phospholipid vesicles, we designed conditions where phospholipid vesicles contained the same amount of $p110\gamma_{\text{-His}}$/p101, regardless whether or not $G\beta_1\gamma_{2\text{-His}}$ dimer was present in assay (Fig. 5.21). Addition of $G\beta_1\gamma_{2\text{-His}}$ enhanced lipid kinase activity of dimeric PI3Kγ over its basal activity. These observations suggest that $G\beta_1\gamma_{2\text{-His}}$ dimers coordinately control both recruitment and activation of PI3Kγ (Fig. 6.1).

By producing Gβγ-free phospholipid vesicles which contained similar amounts of monomeric or dimeric PI3Kγ, we observed that the basal lipid kinase activity of associated dimeric PI3Kγ was considerably higher than in the case of the monomeric kinase (Fig. 5.21).

In these experiments, p101 itself was only difference between tested PI3Kγ proteins. We propose that p101 transfers the catalytic subunit of PI3Kγ in a high activity state. Thus, p101 functions not only as an adaptor for Gβγ but also as a stimulator of lipid kinase activity of p110γ (Fig. 6.1). In contrast to our observation, Maier et al. (1999) have found that basal lipid kinase activity of p110γ was hardly affected by p101. However, these authors did not show how different forms of PI3Kγ were recruited to phospholipid vesicles. They probably measured accumulation of ^{32}P-labeled phospholipids produced by different amounts of phospholipid vesicles-bound enzymes.

Previously, Yu et al. (1998) have shown that the p85 stabilized the conformation of the p110α. If the role of p101 is also to stabilize the conformation of p110γ, we can not exclude that monomeric PI3Kγ partially lost its intrinsic lipid kinase activity already during the purification procedure and further handling. It was shown that p110γ is stable as a monomer in transfected HEK cells whereas p101 is unstable in the absence of p110γ (Brock et al., 2003). However, structural stability of isolated p110γ in the presence and in the absence of p101 was not studied. We also observed that after expression in Sf9 cells and purification of $p101_{\text{-His}}$, the yield of this protein was about 25 times less compared to $p110\gamma_{\text{-His}}$ which was obtained by using the same purification scheme (data not shown). In order to examine whether $p110\gamma_{\text{-His}}$ exhibits full intrinsic catalytic activity, we performed a set of reconstitution assays with purified recombinant $p101_{\text{-His}}$. If the catalytic activity of $p110\gamma_{\text{-His}}$ would be partially lost during the purification, we would expect reconstituted dimeric PI3Kγ less potent compared to intact dimeric PI3Kγ. We examined lipid and protein kinase activities as well as recruitment to the lipid compartment of reconstituted dimeric PI3Kγ (Fig. 5.23). Interestingly, all parameters tested were almost identical with PI3Kγ originally expressed and purified as dimer. Hence, coincubation of $p110\gamma_{\text{-His}}$ with independently purified $p101_{\text{-His}}$ converts monomeric catalytic subunit to dimeric state. These data exclude the assumption that, due to the handling, $p110\gamma_{\text{-His}}$ partially lost catalytic activity. Hence, purified recombinant monomeric PI3Kγ used in this study was fully active.

Additionally, we provide direct evidence that whereas p101 enhances basal lipid kinase activity, it simultaneously suppresses basal autophosphorylation of PI3Kγ (see Fig. 5.23B and section 6.2). In this context previous reports are of interest which suggested that the p101 subunit differently modulate protein and lipid kinase activities of PI3Kγ (Lopez-Ilasaca et al., 1998, Murga et al., 1998). These authors have shown that p101 subunit was crucial for PI3Kγ-induced activation of protein kinase B (Akt/PKB) and Jun kinase (JNK) but had weak effect on mitogen-activated protein kinase (MAPK) activation.

Interestingly, whereas we propose that the role of p101 is to maintain the lipid kinase activity of the class I_B catalytic subunit in a high activity state, previous data have demonstrated that p85 holds class I_A PI3K in a low activity state (Yu et al., 1998). Such

different roles of both non-catalytic subunits might be important in the separation of pathways induced by class I_A and class I_B PI3Ks. One possibility may be that due to the higher basal lipid kinase activity induced by p101, PI3Kγ maintains low level of activation of specific PI3Kγ-dependent PH domain-containing downstream effectors, even in the absence of extracellular stimuli activating GPCR. In contrast, stimulation of receptor tyrosine kinases and/or GPCR by agonists is a prerequisite for any function of class I_A PI3Ks.

6.4 Gβ$_1$ residues implicated in interaction with class I$_B$ PI3K

Next, we examined the molecular basis of Gβγ interaction (Fig. 6.1A) and activation (Fig. 6.1B) of PI3Kγ using purified recombinant $G\beta_1\gamma_{2\text{-His}}$ variants described in section 6.1. Eight of sixteen Gα-interacting residues of $G\beta_1$ investigated in this study appeared irrelevant in $G\beta_1\gamma_{2\text{-His}}$-dependent stimulation of PI3Kγ. Indeed, stimulation of lipid and protein kinase activities of PI3Kγ by $G\beta_{1L55A}\gamma_{2\text{-His}}$, $G\beta_{1K57A}\gamma_{2\text{-His}}$, $G\beta_{1K78A}\gamma_{2\text{-His}}$, $G\beta_{1I80A}\gamma_{2\text{-His}}$, $G\beta_{1K89A}\gamma_{2\text{-His}}$, $G\beta_{1S98A}\gamma_{2\text{-His}}$, $G\beta_{1N119A}\gamma_{2\text{-His}}$, and $G\beta_{1T143A}\gamma_{2\text{-His}}$ was similar compared to the wild-type $G\beta_1\gamma_{2\text{-His}}$ (Table 6.1, Supplementary Fig. 12.1 in section 12). Moreover, these mutants were not involved in binding and recruitment of PI3Kγ to phospholipid vesicles (Table 6.1). Since the $G\beta_1$ residues 55, 78, 80, and 89 cluster within the first Gα/Gβ interface, we conclude that in contrast to Gα subunit, this region is not involved in interaction with PI3Kγ. The only amino acids of $G\beta_1$ that belong to the second Gα/Gβ interface form a patch which contacts PI3Kγ. In contrast, all $G\beta_1$ residues which cluster the first Gα/Gβ interface are important for the modulation of activity of ion channels, i.e. GIRK and CCα1B, whereas the second region is not significant (Table 6.1). These observations immediately raise an intriguing question whether one Gβγ dimer can simultaneously modulate activity of different effectors. In order to answer this question, further investigations (e.g. coimmunoprecipitation studies, pull-down assays, analysis of activity of different effectors simultaneously stimulated by Gβγ) will be needed.

We identified two groups of $G\beta_1$ mutants clustering within the second Gα/Gβγ interface which have different effects on PI3Kγ functions (Table 6.1). $G\beta_1\gamma_2$ mutants, where amino acids Tyr-59, Trp-99, Met-101, Asp-228, or Trp-332 in $G\beta_1$ are substituted by alanine, were still able to stimulate $p110\gamma_{\text{-His}}$/p101 lipid kinase activity (Fig. 5.11). However, this stimulation had reduced potency (EC_{50}) compared to the wild-type $G\beta_1\gamma_{2\text{-His}}$. Interestingly, the potency to stimulate this catalytic activity of dimeric PI3Kγ positively correlated with the efficiency (V_{max}) to stimulate lipid kinase activity of monomeric PI3Kγ and with the efficiency to induce autophosphorylation of dimeric PI3Kγ (Fig. 5.14).

Table 6.1: Properties of $G\beta_1$ variants tested in this as well as in previous studies

Mutation	Recruitment of p110γ/p101 to phospholipid vesicles[1]	Lka of p110γ/p101[1]	Lka of p110γ[1]	Autophosphorylation of p110γ/p101[1]	Activation of GIRK[2]	Inhibition of CCα1B[2]	Interaction with βARK[2]	Activation of PLCβ$_2$[2]	Inhibition of AC I[3]	Activation of AC II[2]	Assembling into heterotrimer with Gα$_t$[2]	Gαt binding and interaction with rhodopsin[2]
The first Gα/Gβ interface												
Leu-55	=	=	=	=	↓	↑	=	↑	NT	↓↓↓	=	=
Lys-78	=	=	=	=	↓	=	=	NT	NT	↓↓↓	NT	NT
Ile-80	=	=	=	=	↓	↑	=	↓	NT	=	↓	↓
Lys-89	=	=	=	=	↓	=	↑	↓, (=)[3]	↓	↓↓↓, (=)[3]	↓	↓
The second Gα/Gβ interface												
Lys-57	=	=	=	=	=	=	↑	=	NT	↓↓↓	=	=
Tyr-59	=	↓[4]	↓	↓	=	=	↑	=	NT	NT	=	=
Ser-98	=	=	=	=	=	=	NT	↑	NT	NT	=	↓
Trp-99	↓	↓[4]	0	↓↓↓	↓	=	=	↓	NT	↓↓↓	=	↓
Met-101	=	↓[4]	↓↓↓	↓	=	=	=	↓	NT	↓	=	↓
Leu-117	↓↓↓	↓↓↓	0	↓[4]	=	=	↓	↓, (=)[3]	=	↓	↓	↓
Asn-119	=	=	=	=	=	=	=	↓	NT	↓↓↓	NT	↓
Thr-143	=	=	=	=	=	=	↓	↓	NT	=	=	↓
Tyr-145	=	↓	0	=	NT	NT	NT	NT	NT	NT	NT	NT
Asp-186	=	=	↑	=	=	=	↑	↓	NT	↓↓↓	=	↓
Asp-228	=	↓[4]	↓↓↓	↓	↓	=	=	↓	↓	↓↓↓	NT	↓
Trp-332	=	↓[4]	↓	↓	=	=	↑	↓	↓	↓↓↓	↓	↓

[1] data obtained in this study. No recruitment of monomeric p110$\gamma_{\text{-His}}$ to the phospholipid vesicles induced by Gβγ was observed.
[2] data from Ford et al., 1998.
[3] data from Li et al., 1998.
[4] the potency of $G\beta_1\gamma_{2\text{-His}}$ mutant to stimulate enzymatic activity of PI3Kγ was significantly reduced, whereas the efficiency of stimulation was comparable with wild-type $G\beta_1\gamma_{2\text{-His}}$.

↑ - increasing effect compare with wild-type; ↓ - decreasing effect compare with wild-type; ↓↓↓ - very strong decreasing effect compare with wild-type; = - similar effect as the wild-type $G\beta_1$; NT - not tested; 0 - no effect; AC - adenylyl cyclase; βARK - β-adrenergic receptor kinase; CCα1B - calcium channel α1B subunit; GIRK - muscarinic potassium channel; Lka - lipid kinase activity of PI3Kγ; PLCβ$_2$ - phospholipase Cβ$_2$; p110γ - monomeric form of PI3Kγ; p110γ/p101 - heterodimeric form of PI3Kγ.

Among this first group of mutants, Trp-99 and Met-101 seems essential for all types of activation. For instance, although $G\beta_{1W99A}\gamma_{2\text{-His}}$ and $G\beta_{1M101A}\gamma_{2\text{-His}}$ were able to significantly stimulate lipid kinase activity of dimeric PI3Kγ, the potency of such stimulation was about 12 and 8 times less, respectively. The $G\beta_{1W99A}\gamma_{2\text{-His}}$- and $G\beta_{1M101A}\gamma_{2\text{-His}}$-induced autophosphorylation of dimeric PI3Kγ was also impaired. Additionally, stimulation of lipid kinase activity of monomeric PI3Kγ by $G\beta_{1M101A}\gamma_{2\text{-His}}$ was drastically decreased as compared to the wild-type, and the stimulation by $G\beta_{1W99A}\gamma_{2\text{-His}}$ was lost at any concentration tested (Fig. 5.11). All mutants of this group, except $G\beta_{1W99A}\gamma_{2\text{-His}}$, showed a similar ability to recruit dimeric PI3Kγ to phospholipid vesicles (Table 6.1). Previously, it was shown that the largely hydrophobic interaction of $G\beta_1$ with Gα is centered around $G\beta_1$ residue Trp-99 (Sprang, 1997). A mutation of Trp-99 or corresponding Trp-136 in the Gβ subunit of Saccharomyces cerevisiae resulted in disrupted interaction between Gα and Gβγ (Whiteway et al., 1994, Sprang, 1997). The fact that Gα and effectors share a common binding surface on Gβ raises the possibility that mutation of Trp-99 will affect the interaction between Gβγ dimer and its effectors. Indeed, previous data have demonstrated impaired capability of this mutant to activate PLCβ$_2$, AC II and GIRK1/GIRK4 (Table 6.1). We have found that $G\beta_{1W99A}\gamma_{2\text{-His}}$ exhibited diminished ability to recruit dimeric PI3Kγ to phospholipid vesicles. This and other studies (Maier et al., 1999) have demonstrated that Gβγ interacts with p101 and p110γ subunits of PI3Kγ. Therefore, the binding of $G\beta_{1WT}\gamma_2$ and $G\beta_{1W99A}\gamma_2$ to both the catalytic and the regulatory subunit of dimeric PI3Kγ was tested. Our data show that copurification of $G\beta_{1WT}\gamma_2$ and $G\beta_{1W99A}\gamma_2$ with $p110\gamma_{\text{-His}}$ was similar. In contrast, substitution of Trp-99 for Ala hampered interaction of $G\beta_1\gamma_2$ with p101 (Fig. 5.13). Although copurification of $G\beta_{1WT}\gamma_2$ or $G\beta_{1W99A}\gamma_2$ with $p110\gamma_{\text{-His}}$ shows no differences, the stimulation of lipid kinase activity of monomeric PI3Kγ by $G\beta_{1W99A}\gamma_{2\text{-His}}$ was abolished. This finding clearly demonstrates that Trp-99 of $G\beta_1$ is required not only for binding of $G\beta_1\gamma_{2\text{-His}}$ to PI3Kγ but also for activation of the enzyme.

Furthermore, we identified another group of $G\beta_1\gamma_2$ mutants which differently affected the various forms and enzymatic activities of PI3Kγ (Fig. 5.15). Exchange of Leu-117 in $G\beta_1$ for Ala led to a very weak stimulation of lipid kinase activity (~20 % of normal) of the dimeric PI3Kγ as compared to the wild-type $G\beta_1\gamma_{2\text{-His}}$. Stimulation of lipid kinase activity of monomeric PI3Kγ by $G\beta_{1L117A}\gamma_{2\text{-His}}$ was lost at any concentration tested. Previous data have demonstrated that Leu-117 in Gβ was critical for interaction with almost all effectors tested except ion channels (Table 6.1). For instance, mutation of Leu-117 to Ala led to impaired capability of Gβγ to bind $G\alpha_t$ and interact with rhodopsin. Moreover, activation of PLCβ$_2$ and AC II by Gβγ dimer containing $G\beta_{1L117A}$ as well as interaction with βARK was significantly reduced compared to the wild-type. However, our data demonstrate that this mutant in higher concentrations considerably induced autophosphorylation of dimeric PI3Kγ, albeit with

reduced potency. The same is true for $G\beta_{1Y145A}\gamma_{2\text{-His}}$. Mutation of $G\beta_1$ in position 145 diminished ability of $G\beta_1\gamma_2$ to stimulate lipid kinase activity of dimeric PI3Kγ and completely impaired stimulation of lipid kinase activity of the monomer, whereas Gβγ-induced stimulation of PI3Kγ autophosphorylation was not affected. However, in contrast to $G\beta_{1L117A}\gamma_{2\text{-His}}$ mutant which was unable to recruit dimeric PI3Kγ in a concentration-dependent manner to phospholipid vesicles, $G\beta_{1Y145A}\gamma_{2\text{-His}}$-dependent recruitment of the enzyme was unaltered compared to $G\beta_{1WT}\gamma_{2\text{-His}}$. Exchange of Asp-186 for Ala resulted in 2 fold increased activation of monomeric PI3Kγ, whereas the sensitivity of neither lipid nor protein kinase activity of dimeric kinase was altered. In contrast to lipid kinase activity, $G\beta_1$ residue Asp-186 was not important for stimulation of PI3Kγ autophosphorylation. Although we did not find any difference between $G\beta_{1D186A}\gamma_{2\text{-His}}$ and $G\beta_{1WT}\gamma_{2\text{-His}}$ in their ability to recruit monomeric or dimeric PI3Kγ (Fig. 5.16), we observed that Gβγ dimers further activated already phospholipid vesicles-associated PI3Kγ (Fig. 5.21). Our data indicate that $G\beta_1$ residue Asp-186 is involved in direct stimulation of PI3Kγ activity rather than in $G\beta_1\gamma_2$-dependent recruitment of PI3Kγ. Ford et al. (1998) have found that alanine mutation of Asp-186 resulted in increased interaction of $G\beta_1\gamma_2$ with β-adrenergic receptor kinase (βARK). These authors suggested that the reason of this event is that some amino acids of $G\beta_1\gamma_2$ have higher affinity of interaction with Gα subunit than with effectors. This allows inactive Gα subunit to switch off the ability of Gβγ to activate or inhibit its effectors and reassemble into heterotrimeric Gαβγ. We assume that exchange of hydrophilic Asp for hydrophobic Ala might have increased hydrophobicity of the $G\beta_1$ region that is required for modulation of p110γ activity. Consequently, increased hydrophobicity facilitated the affinity of $G\beta_1\gamma_2$ to p110γ thereby increasing activity of the enzyme.

According to our data, we conclude that on the surface of $G\beta_1$ some amino acids (Trp-99, Met-101 and, in less extent, Tyr-59, Asp-228, and Trp-332) form the core region which is indispensable for stimulation of all types of PI3Kγ enzymatic activities (Fig. 6.2). In the surrounding of this core region there are some amino acids (e.g. Leu-117 and Tyr-145) which are differently involved in regulation of lipid and protein kinase activity of PI3Kγ. We have found one $G\beta_1$ residue (e.g. Asp-186) which is differently involved in stimulation of monomeric and dimeric PI3Kγ. Among these $G\beta_1$ residues, Trp-99 and Leu-117 form a patch which is crucial for binding and recruitment of PI3Kγ. Based on the crystal structure of $G\beta_1\gamma_2$ (Wall et al., 1995, Lambright et al., 1996), we propose that the first (Tyr-59), the second (residues Trp-99, Met-101, Leu-117), the third (Tyr-145), the fourth (Asp-186), the fifth (Asp-228) and the seventh (Trp-332) blades of seven-bladed β-propeller contributed to regulation of PI3Kγ (Fig. 6.2). Although these six of seven blades of $G\beta_1$ also contribute to interaction with Gα subunit (Wall et al., 1995, Lambright et al., 1996, Ford et al., 1998), PI3Kγ has its individual “fingerprint” on $G\beta_1$. Our data show that the PI3Kγ interaction sites

are clustered on the surface of $G\beta_1$ such that they partially overlap with the $G\alpha$ subunit interaction interface. Such mode of clustering allows for $G\alpha$ subunit to regulate signal transduction from $G\beta\gamma$ dimers to PI3Kγ.

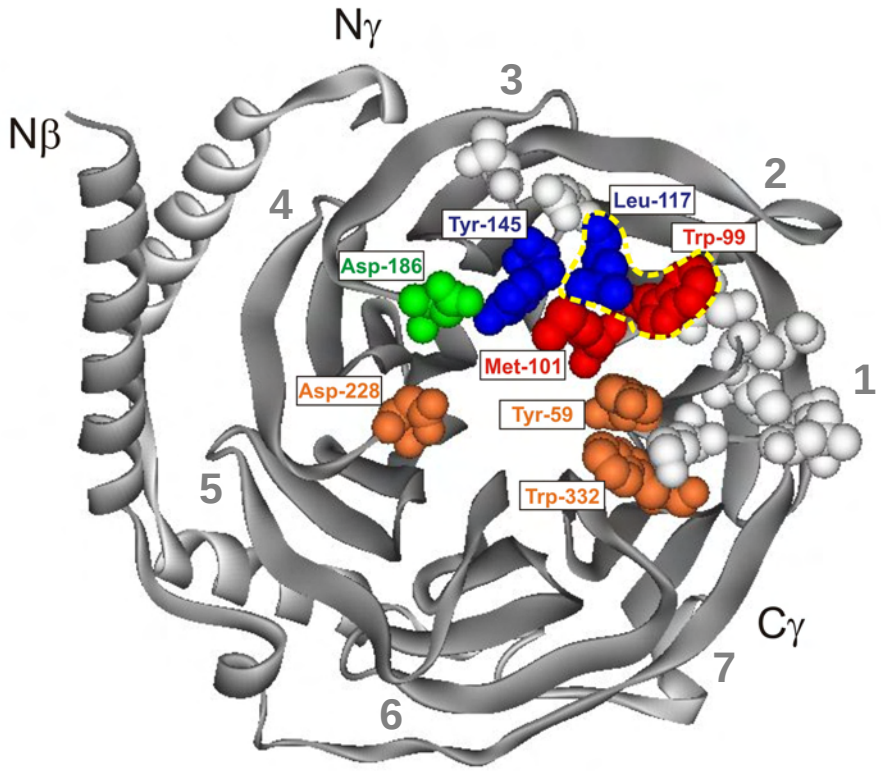


Fig. 6.2: Schematic representation of $G\beta_1$ residues involved in regulation of PI3Kγ enzymatic activities

The structure of the $G\beta_1\gamma_1$ dimer shows that $G\beta$ forms a seven-blade propeller consisting of seven WD-40 repeats (Wall et al., 1995, 1998). The blade 1 includes the first WD repeat. Amino acids which have different effects on PI3Kγ functions are indicated by different colours: **red**, amino acids that form the core region indispensable for stimulation of all types of PI3Kγ enzymatic activities; **orange**, amino acids which exhibit similarly reduced but more effective ability to stimulate PI3Kγ than those coloured by red; **blue**, amino acids which are differently involved in regulation of lipid and protein kinase activity of PI3Kγ; **green**, amino acid which is differently involved in stimulation of monomeric and dimeric PI3Kγ; **white**, amino acids with activity indistinguishable to the wild-type. The region of $G\beta_1$ which is important for binding and recruitment of PI3Kγ is circled in **yellow**.

Although there are five known $G\beta$ isoforms, we used the ubiquitously distributed and well characterized bovine $G\beta_1$. Previously, it was shown that purified recombinant $G\beta_2\gamma_2$ and $G\beta_3\gamma_2$ were also able to stimulate the lipid kinase activity of PI3Kγ (Maier et al., 2000). These preparations exhibited similar potencies of stimulation compared to recombinant $G\beta_1\gamma_2$. Additionally, Kerchner et al. (2004) have demonstrated the ability of recombinant $G\beta_4\gamma_2$ to activate the PI3Kγ which was almost indistinguishable from $G\beta_1\gamma_2$. Furthermore, the $G\beta_{1/2/3/4}$ subunits are highly homologous showing amino acid sequence identity from 78 to 90 % (Wall et al., 1998, Maier et al., 2000, Schwindinger and Robishaw, 2001). Amino acid sequence

alignment between different Gβ isoforms revealed that amino acids of $G\beta_1$ involved in interaction with Gα subunit are identical with those of $G\beta_2$, $G\beta_3$, and $G\beta_4$ (Fig. 6.3). We, therefore, speculate that the residues shown in Fig. 6.2 are also crucial for the stimulation of PI3Kγ by $G\beta_2$, $G\beta_3$, and $G\beta_4$ subunits.

Previous reports have demonstrated that the recombinant $G\beta_5\gamma_2$ dimers were not able to stimulate PI3Kγ (Maier et al., 2000, Kerchner et al., 2004). Such an atypical signaling phenotype of $G\beta_5$, compared to other Gβ subunits, was also shown in experiments with other Gβγ effectors. For example, $G\beta_5\gamma_2$ activated $PLC\beta_1$ or $PLC\beta_2$, but not $PLC\beta_3$, whereas $G\beta_1\gamma_2$ was effective for all PLCβ isoforms tested (Maier et al., 2000). Furthermore, the $G\beta_5\gamma_2$ dimers inhibited AC II, whereas the $G\beta_1\gamma_2$ stimulated AC II (Bayewitch et al., 1998). Sequence alignment between $G\beta_1$ and $G\beta_5$ subunits revealed that of eight amino acids of $G\beta_1$ involved in interaction with PI3Kγ, only one is different in the $G\beta_5$ subunit, i.e. Tyr-59 ($G\beta_1$) → Leu-67 ($G\beta_5$) (Fig. 6.3). However, we have shown that mutation of Trp-99, Met-101, Leu-117 and Tyr-145 drastically reduced the ability of $G\beta_1\gamma_2$ to stimulate PI3Kγ. Fig. 6.3 demonstrates that the $G\beta_5$ subunit contains these corresponding amino acids. Most probably, the reason for such a difference between $G\beta_5\gamma_2$ dimers and $G\beta_{1/2/3/4}/\gamma_2$ pairs in their regulating effects is that $G\beta_5$ subunit has low structural homology (~50 % amino acid identity) compared to other Gβ subunits (Watson et al., 1994, Downes and Gautam, 1999, Albert and Robillard, 2002). Alternatively, as previously proposed (Zhang et al., 1996), $G\beta_1$ and $G\beta_5$ may differ in their capacity to interact with Gγ subunits, thus creating functionally different Gβγ pairs.

6.5 Monoclonal anti-p110γ antibody (mAb 641) differentially affects PI3Kγ enzymatic activities

In order to dissect between different forms and enzymatic activities of PI3Kγ, we employed another approach by using anti-p110γ specific monoclonal antibody (mAb 641). This antibody was produced in mouse hybridoma cells and was raised against p110γ full length purified from Sf9 cells. mAb 641 is able to bind to intact but not to denatured monomeric and dimeric PI3Kγ (see section 5.4.3). Coincubation of maximally $G\beta_1\gamma_{2\text{-His}}$-stimulated PI3Kγ with increasing concentrations of mAb 641 exhibited significant decrease of the $p110\gamma_{\text{-His}}$ lipid kinase activity, whereas lipid kinase activity of dimeric PI3Kγ was not altered. In contrast to lipid kinase activity of $p110\gamma_{\text{-His}}$/p101, the $G\beta_1\gamma_{2\text{-His}}$-induced autophosphorylation of this enzyme was antibody concentration-dependently reduced (Fig. 5.19).

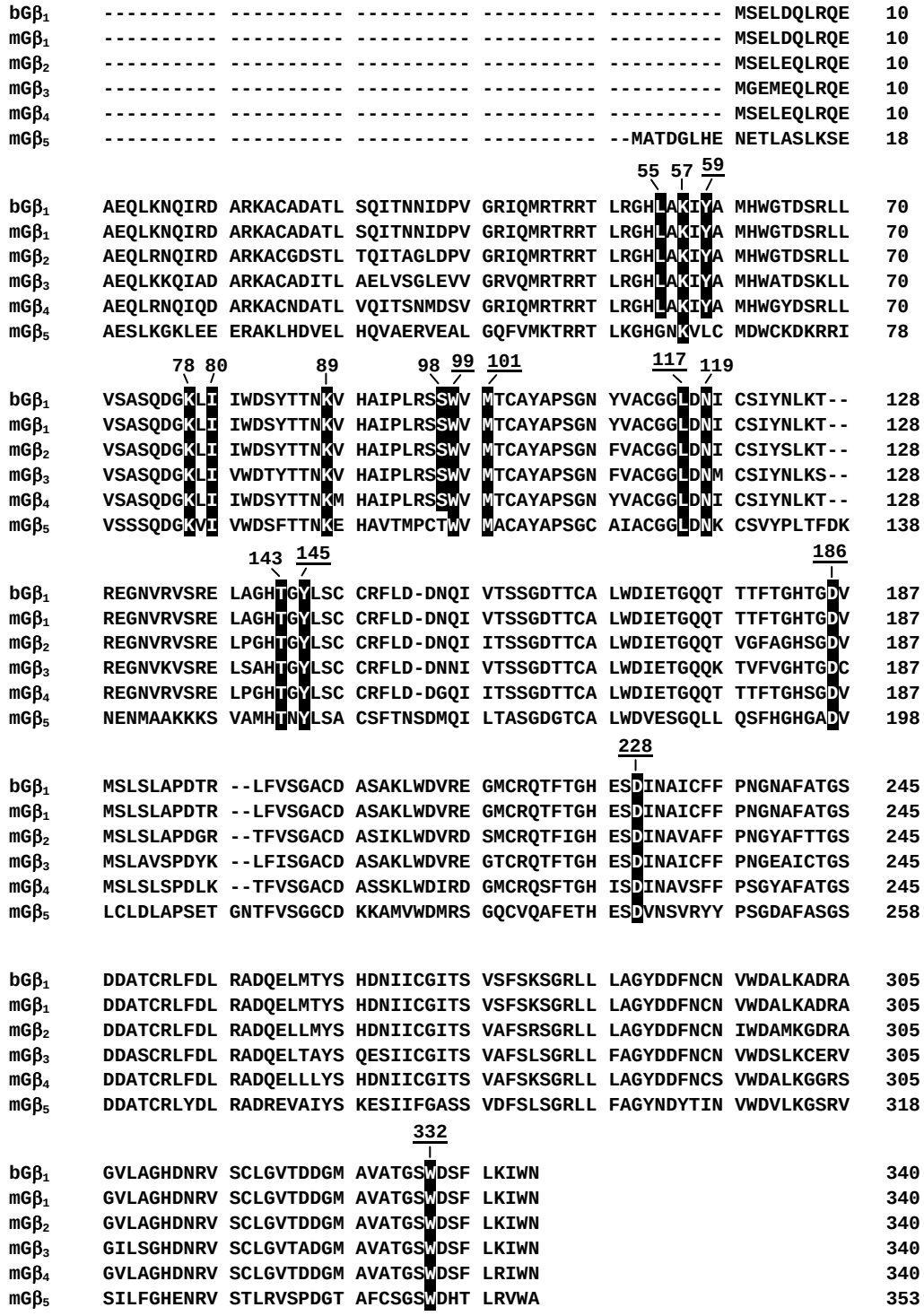


Fig. 6.3: Sequence alignment between Gβ subunits

Amino acid sequence alignment is shown for bovine (b) $G\beta_1$ subunit and all five mouse (m) Gβ isoforms. Positions of $G\beta_1$ residues substituted for alanine are shown. Black boxes illustrate the conserved amino acids among Gβ proteins. $G\beta_1$ residues involved in interaction and activation of PI3Kγ are underlined. GenBank accession numbers are: $bG\beta_1$, NP_86971; $mG\beta_1$, NP_032168; $mG\beta_2$, NP_034442; $mG\beta_3$, NP_038558; $mG\beta_4$, NP_038559; $mG\beta_5$ isoform 2, NP_619733.

In order to exclude interference between mAb 641 and PI3Kγ enzymatic activities which are maximally stimulated with $G\beta_1\gamma_{2\text{-His}}$, we also tested the effect of increasing concentrations of mAb 641 on lipid kinase activities of monomeric and dimeric PI3Kγ stimulated by half-effective $G\beta_1\gamma_{2\text{-His}}$ concentrations. Although mAb 641 inhibited lipid kinase activities of both forms of PI3Kγ, we observed a considerable difference between these effects. Whereas mAb 641 at a concentration of 40 nM totally abolished $G\beta_1\gamma_2$-stimulated lipid kinase activity of monomeric PI3Kγ, the $G\beta_1\gamma_2$-stimulated lipid kinase activity of the dimer was at least 30 times higher compared to its basal lipid kinase activity (Fig. 5.20). Thus, interaction of the antibody with PI3Kγ discriminated among the enzymatic activities of different PI3Kγ forms which confirms the results obtained with $G\beta_1$ mutants. Additionally, the fact that mAb 641 leads to inhibition of autophosphorylation but not the lipid kinase activity of the dimeric PI3Kγ maximally stimulated with $G\beta_1\gamma_{2\text{-His}}$ confirms also our data (section 6.2) showing that lipid kinase activity and autophosphorylation of dimeric PI3Kγ are two dissectible processes. Since mAb 641 drastically inhibited monomeric but not dimeric PI3Kγ, we propose that this antibody prevents the interaction between $G\beta_1\gamma_2$ and p110γ but not between $G\beta_1\gamma_2$ and p101 (Fig. 6.1). According to our data, mAb 641 may be a useful tool for the studies on the involvement of monomeric PI3Kγ and autophosphorylation process of PI3Kγ in signal transduction pathways.

7 SUMMARY

Class I_B phosphoinositide 3-kinase γ (PI3Kγ) is one of the Gβγ effectors, which regulates important cellular processes, such as mitogenesis, apoptosis, and cytoskeletal functions. PI3Kγ signals through its inherent lipid and protein kinase activity. Mechanistically, receptor-released Gβγ dimers recruit PI3Kγ to the membrane and stimulate either enzymatic activity. The first aspect of our study was to map the sites of $G\beta_1$ important for binding and activation of different forms and enzymatic activities of PI3Kγ. In order to do so, we have purified and examined effector-relevant $G\beta_1$ mutants for their capability to recruit and activate monomeric (p110γ) and dimeric (p110γ/p101) PI3Kγ.

The second aspect of our study was to elucidate the role of non-catalytic p101 subunit in activation of PI3Kγ. For this, we designed experimental conditions where Gβγ-free phospholipid vesicles contained similar amounts of monomeric and dimeric PI3Kγ.

We have shown that:

- $G\beta_{1WT}\gamma_{2\text{-His}}$ in a concentration-dependent manner enhanced association of dimeric but not monomeric PI3Kγ with phospholipid vesicles and stimulated lipid and protein kinase activities of the dimer and lipid kinase activity of the monomer. Moreover, already phospholipid vesicles-associated monomeric and dimeric PI3Kγ can be further activated by $G\beta_1\gamma_{2\text{-His}}$.
- basal lipid kinase activity of phospholipid vesicles-associated dimeric PI3Kγ was considerably higher as compared to the monomeric kinase. Basal autophosphorylation of monomeric PI3Kγ was drastically reduced by p101.
- $G\beta_{1W99A}\gamma_{2\text{-His}}$ and $G\beta_{1M101A}\gamma_{2\text{-His}}$ were drastically impaired in regulation of all PI3Kγ activities tested. $G\beta_{1Y59A}\gamma_{2\text{-His}}$, $G\beta_{1D228A}\gamma_{2\text{-His}}$, and $G\beta_{1W332A}\gamma_{2\text{-His}}$ exhibited similarly reduced but more effective ability to stimulate PI3Kγ compared to both $G\beta_{1W99A}\gamma_{2\text{-His}}$ and $G\beta_{1M101A}\gamma_{2\text{-His}}$.
- $G\beta_{1D186A}\gamma_{2\text{-His}}$ resulted in a 2 fold increased activation of monomeric but not dimeric PI3Kγ.
- $G\beta_{1L117A}\gamma_{2\text{-His}}$ stimulated lipid kinase activity of dimeric PI3Kγ very weakly. Stimulation of lipid kinase activity of monomeric PI3Kγ by this mutant was lost at any concentration tested. In contrast to lipid kinase activity, $G\beta_{1L117A}\gamma_{2\text{-His}}$ stimulated protein kinase activity of PI3Kγ, however, with reduced potency. Similarly, ability of $G\beta_{1Y145A}\gamma_{2\text{-His}}$ to stimulate lipid kinase activity of monomeric and dimeric PI3Kγ was diminished, whereas $G\beta_{1Y145A}\gamma_2$-induced activation of protein kinase activity of dimeric PI3Kγ was unaltered.

- $G\beta_{1L117A}\gamma_{2\text{-His}}$ is the only mutant which was unable to recruit dimeric PI3Kγ to phospholipid vesicles. Recruitment of the kinase by $G\beta_{1W99A}\gamma_{2\text{-His}}$ was drastically impaired. All other $G\beta_1\gamma_{2\text{-His}}$ mutants recruited PI3Kγ similarly compared to the wild-type.
- monoclonal anti-p110γ antibody (mAb 641) completely blocked Gβγ-stimulated lipid kinase activity of monomeric but not dimeric PI3Kγ. Additionally, Gβγ-induced autophosphorylation of dimeric PI3Kγ was strongly reduced.

Our data imply that:

- $G\beta_1\gamma_{2\text{-His}}$ dimers coordinately control both recruitment and activation of PI3Kγ.
- p101 maintains the lipid kinase activity of class I_B catalytic p110γ subunit in high activity state and the protein kinase activity in low activity state. Therefore, p101 functions not only as an adaptor for Gβγ but also is involved in regulation of enzymatic activities of p110γ.
- the first (Tyr-59), the second (Trp-99, Met-101, Leu-117), the third (Tyr-145), the fourth (Asp-186), the fifth (Asp-228) and the seventh (Trp-332) blades of β-propeller of the $G\beta_1$ subunits are involved in regulation of PI3Kγ activity.
- Trp-99 and Met-101 represent a part of the core region of $G\beta_1$ that is indispensable for stimulation of all types of PI3Kγ enzymatic activities.
- amino acid residues adjacent to the core region are differently involved in stimulation of monomeric and dimeric PI3Kγ (e.g. Asp-186).
- Gβγ-induced stimulation of lipid and protein kinase activity of PI3Kγ can be dissected apart by distinct $G\beta_1$ mutants (e.g. $G\beta_{1L117A}\gamma_{2\text{-His}}$ and $G\beta_{1Y145A}\gamma_{2\text{-His}}$).
- amino acid residues on the surface of $G\beta_1$ form both PI3Kγ activating (e.g. Tyr-59, Trp-99, Met-101, Leu-117, Tyr-145, Asp-186, Asp-228, and Trp-332) and PI3Kγ binding (e.g. Trp-99 and Leu-117) regions which overlap one another.

8 ZUSAMMENFASSUNG

Die Klasse I_B der Phosphatidylinositol-3-Kinase γ (PI3Kγ) stellt einen wichtigen Effektor von Gβγ-Proteinen dar und reguliert fundamentale zelluläre Prozesse wie Proliferation, Apoptose und die Organisation des Zytoskeletts. PI3Kγ ist eine dual-spezifische Kinase mit Lipidkinase- und Proteinkinase-Aktivität. Beide Eigenschaften sind für die zelluläre Signaltransduktion grundlegend. Nach Stimulation G-Protein gekoppelter Rezeptoren kommt es zur Freisetzung des Gβγ Dimers. Durch Assoziation mit Membran-gebundenem Gβγ erfolgt die Rekrutierung von PI3Kγ an spezielle Membrankompartimente. Gleichzeitig werden dabei beide enzymatischen Aktivitäten stimuliert. Der erste Teilaspekt dieser Arbeit befasste sich mit der funktionellen Charakterisierung von $Gβ_1$ Bindungsstellen welche i) die Interaktion mit verschiedenen PI3Kγ Subtypen vermitteln und ii) eine Stimulation der beiden enzymatischen Aktivitäten bewirken. Insbesondere sollte dabei untersucht werden, ob die Lipidkinase- und Proteinkinase-Aktivität unabhängig voneinander durch verschiedene $Gβ_1$ Peptidsequenzen aktiviert werden können. Zur Beantwortung dieser Fragestellung wurden verschiedene Effektor-relevante $Gβ_1$ Mutanten untersucht, ob diese *in-vitro* die monomere PI3Kγ (p110γ) oder das PI3Kγ Dimer (p110γ/p101) an Membranstrukturen rekrutieren und aktivieren können.

In einem weiteren Projektteil der vorgelegten Arbeit sollte die Rolle der nicht-katalytischen p101 Untereinheit bei der Aktivierung von PI3Kγ untersucht werden. Dazu wurde ein experimentelles Untersuchungssystem etabliert, welches die Untersuchung monomerer und dimerer PI3Kγ Formen an Gβγ-freien Phospholipidvesikeln erlaubte.

Es ergaben sich folgende Resultate:

- Durch $Gβ_{1WT}γ_{2\text{-}His}$ konnte eine konzentrationsabhängige Assoziation von p110γ/p101 mit Phospholipidvesikeln induziert werden. Im Gegensatz dazu fand unter gleichen Bedingungen keine Assoziation der monomeren p110γ statt. Auf enzymatischer Ebene stimulierte $Gβ_{1WT}γ_{2\text{-}His}$ sowohl die Lipid- als auch die Proteinkinase Aktivität des PI3Kγ Dimers, wohingegen das Monomer nur eine erhöhte Lipidkinase-Aktivität aufwies. Zusätzlich konnten die enzymatischen Aktivitäten beider Formen durch $Gβ_{1WT}γ_{2\text{-}His}$ gesteigert werden, wenn die Kinasen zuvor an Phospholipidvesikel gebunden wurden.
- Im Falle einer Assoziation mit Phospholipidvesikeln war die basale Lipidkinase-Aktivität der dimeren PI3Kγ bedeutend höher als die des p110γ Monomers. Die basale Autophosphorylierung des PI3Kγ Monomers durch p101 war drastisch reduziert.

- $G\beta_{1W99A}\gamma_{2\text{-His}}$ und $G\beta_{1M101A}\gamma_{2\text{-His}}$ hatten nur marginalen Einfluss auf die enzymatischen Aktivitäten von PI3Kγ. Im Gegensatz dazu war die durch $G\beta_{1Y59A}\gamma_{2\text{-His}}$-, $G\beta_{1D228A}\gamma_{2\text{-His}}$- und $G\beta_{1W332A}\gamma_{2\text{-His}}$-induzierte Stimulation der PI3Kγ Aktivität deutlich erhöht, erreichte jedoch nicht das Niveau von $G\beta_{1WT}\gamma_{2\text{-His}}$.
- Eine D186A Substitution ($G\beta_{1D186A}\gamma_{2\text{-His}}$) führte zu einem 2-fachen Anstieg der Aktivität des Monomers, zeigte jedoch keinen Effekt bei der Aktivierung des PI3Kγ Dimers.
- Eine schwache Stimulation der Lipidkinase-Aktivität der dimeren PI3Kγ konnte durch $G\beta_{1L117A}\gamma_{2\text{-His}}$ erreicht werden. Diese Mutation hatte jedoch (auch bei hohen Konzentrationen) keinen Einfluss auf die Lipidkinase-Aktivität der monomeren p110γ. Im Vergleich zur geringfügigen Steigerung der Lipidkinase-Aktivität, wurde die Proteinkinase-Aktivität von PI3Kγ durch $G\beta_{1L117A}\gamma_{2\text{-His}}$ stärker stimuliert. Das Aktivierungsniveau lag jedoch niedriger als bei $G\beta_{1WT}\gamma_{2\text{-His}}$. In ähnlicher Weise war durch $G\beta_{1Y145A}\gamma_{2\text{-His}}$ eine verminderte Stimulation der Lipidkinase-Aktivität beider PI3Kγ Formen zu beobachten, wohingegen die Stimulation der Proteinkinase-Aktivität der dimeren PI3Kγ durch $G\beta_{1Y145A}\gamma_{2\text{-His}}$ unverändert war.
- $G\beta_{1L117A}\gamma_{2\text{-His}}$ repräsentiert die einzige Mutante, die keine Rekrutierung der dimeren PI3Kγ an Phospholipidvesikel bewirkte. Im Vergleich zur Wildtypsituation wurde durch $G\beta_{1W99A}\gamma_{2\text{-His}}$ bedeutend weniger PI3Kγ an Phospholipid-Vesikel rekrutiert. Alle anderen $G\beta_{1}\gamma_{2\text{-His}}$ Derivate besaßen ein Rekrutierungspotential, welches dem $G\beta_{1WT}\gamma_{2\text{-His}}$ entsprach.
- Durch den Einsatz eines monoklonalen anti-p110γ Antikörpers (mAb 641) konnte die Gβγ-stimulierte Lipidkinase-Aktivität der monomeren PI3Kγ komplett blockiert werden. Eine solche Wirkung traf für das PI3Kγ Dimer nicht zu. Weiterhin war die Gβγ-induzierte Autophosphorylierung der dimeren PI3Kγ nach Vorinkubation mit mAb 641 stark reduziert.

Aufgrund der erhobenen Daten dieser Arbeit lassen sich folgende Schlussfolgerungen ziehen:

- $G\beta_{1}\gamma_{2\text{-His}}$ Dimere kontrollieren sowohl die Rekrutierung als auch die Aktivierung von PI3Kγ.
- p101 hält die katalytischen Aktivitäten von p110γ in unterschiedlicher Art und Weise aufrecht, einen hoch aktiven Zustand für die Lipidkinase-Aktivität und einen niedrig aktiven Zustand für die Proteinkinase-Aktivität. Aus diesem Grund fungiert p101 nicht nur als molekularer Adapter für Gβγ Proteine, sondern ist auch an der Stimulation der enzymatischen Aktivitäten von p110γ beteiligt.

- Das erste (Tyr-59), das zweite (Trp-99, Met-101, Leu-117), das dritte (Tyr-145), das vierte (Asp-186), das fünfte (Asp-228) und das siebte (Trp-332) Faltblatt des β-Propelles der $G\beta_1$ Untereinheit sind an der Regulation der enzymatischen Aktivitäten der PI3Kγ beteiligt.
- Trp-99 und Met-101 bilden einen Teil der Kern-Region von $G\beta_1$, welche für die Stimulation jeglicher enzymatischen Aktivitäten von PI3Kγ unabdingbar ist.
- Aminosäurereste, die benachbart zur Kern-Region liegen, besitzen unterschiedliche Funktionen bei der Stimulation von monomeren und dimeren Formen der PI3Kγ (z.B. Asp-186).
- Lipidkinase- und Proteinkinase-Aktivitäten von PI3Kγ können durch unterschiedliche $G\beta_1$ Mutanten selektiv gesteuert werden (z.B. $G\beta_{1L117A}\gamma_{2\text{-His}}$ und $G\beta_{1Y145A}\gamma_{2\text{-His}}$).
- Aminosäurereste der $G\beta_1$-Oberfläche sind sowohl für die Aktivierung von PI3Kγ (z.B. Tyr-59, Trp-99, Met-101, Leu-117, Tyr-145, Asp-186, Asp-228, Trp-332) als auch für die Interaktion mit PI3Kγ (z.B. Trp-99 und Leu-117) bedeutend. Die beteiligten Peptidregionen können dabei überlappen.

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10 CURRICULUM VITAE

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Papers

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***Shymanets A**, Weinmann S, Wetzker R, and Nürnberg B. (2005) Gβ residues relevant for interaction and stimulation of phosphoinositide 3-kinase γ (PI3Kγ) enzymatic activities. The 60th Harden Conference "Inositol Phosphates and Lipids – Regulation and Function", 13-18 August 2005, St Martin's College, Ambleside, Lake District, UK, 8.

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* - oral presentations

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12 SUPPLEMENTARY MATERIAL

Table 12.1: Sensitivity of lipid kinase activity of PI3Kγ to the non-ionic detergent $C_{12}E_{10}$

A Lipid kinase activity of $p110\gamma_{\text{-His}}$/p101

$C_{12}E_{10}$, [%]	$G\beta_1\gamma_{2\text{-His}}$, [nM]			
	0	**10**	**50**	**100**
0.0004	0.31 ± 0.07	48.2 ± 5.2	52.0 ± 3.6	50.6 ± 2.8
0.002	0.20 ± 0.05	33.2 ± 4.3	31.8 ± 4.3	30.5 ± 2.3
0.006	0.045 ± 0.020	7.4 ± 1.4	7.1 ± 1.5	6.5 ± 1.2
0.01	0.03 ± 0.02	0.07 ± 0.05	0.85 ± 0.32	1.75 ± 0.42
0.05	0.04 ± 0.03	0.04 ± 0.02	0.06 ± 0.04	0.08 ± 0.06

B Lipid kinase activity of $p110\gamma_{\text{-His}}$

$C_{12}E_{10}$, [%]	$G\beta_1\gamma_{2\text{-His}}$, [nM]	
	0	**600**
0.002	0.12 ± 0.04	3.1 ± 1.5
0.004	0.09 ± 0.03	0.59 ± 0.31

(A) Supplementary table related to Fig. 5.7A. Purified recombinant $p110\gamma_{\text{-His}}$/p101 was stimulated with increasing concentrations of recombinant $G\beta_1\gamma_{2\text{-His}}$ in a buffer containing 0.0004 %, 0.002 %, 0.006 %, 0.01 %, or 0.05 % of $C_{12}E_{10}$. **(B)** Supplementary table related to Fig. 5.7B. Lipid kinase activity of $p110\gamma_{\text{-His}}$ in assays including 0.002 % or 0.004 % of $C_{12}E_{10}$. ^{32}P-labeled PI-3,4,5-P_3 were isolated and quantified as detailed under section 4.5.1. After termination of reaction, probes were subjected to thin layer chromatography (TLC) plates. Dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system.

Specific lipid kinase activity of PI3Kγ is demonstrated as nmol PIP_3 / mg of total PI3Kγ / min. Shown are mean values (± S.D.) of duplicate determinations in two independent experiments.

Fig. 12.1A Lipid kinase activity of p110$\gamma_{\text{-His}}$/p101

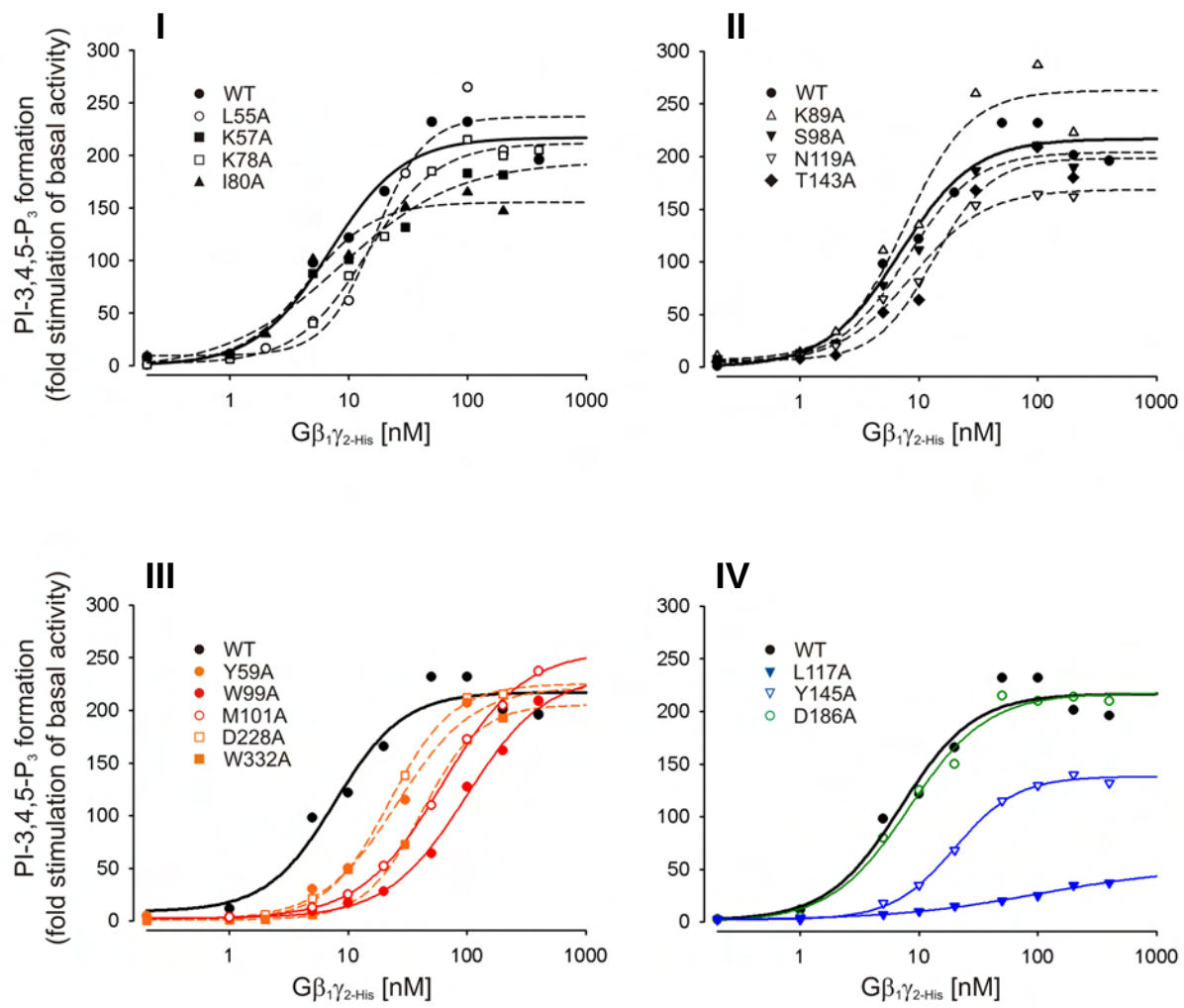
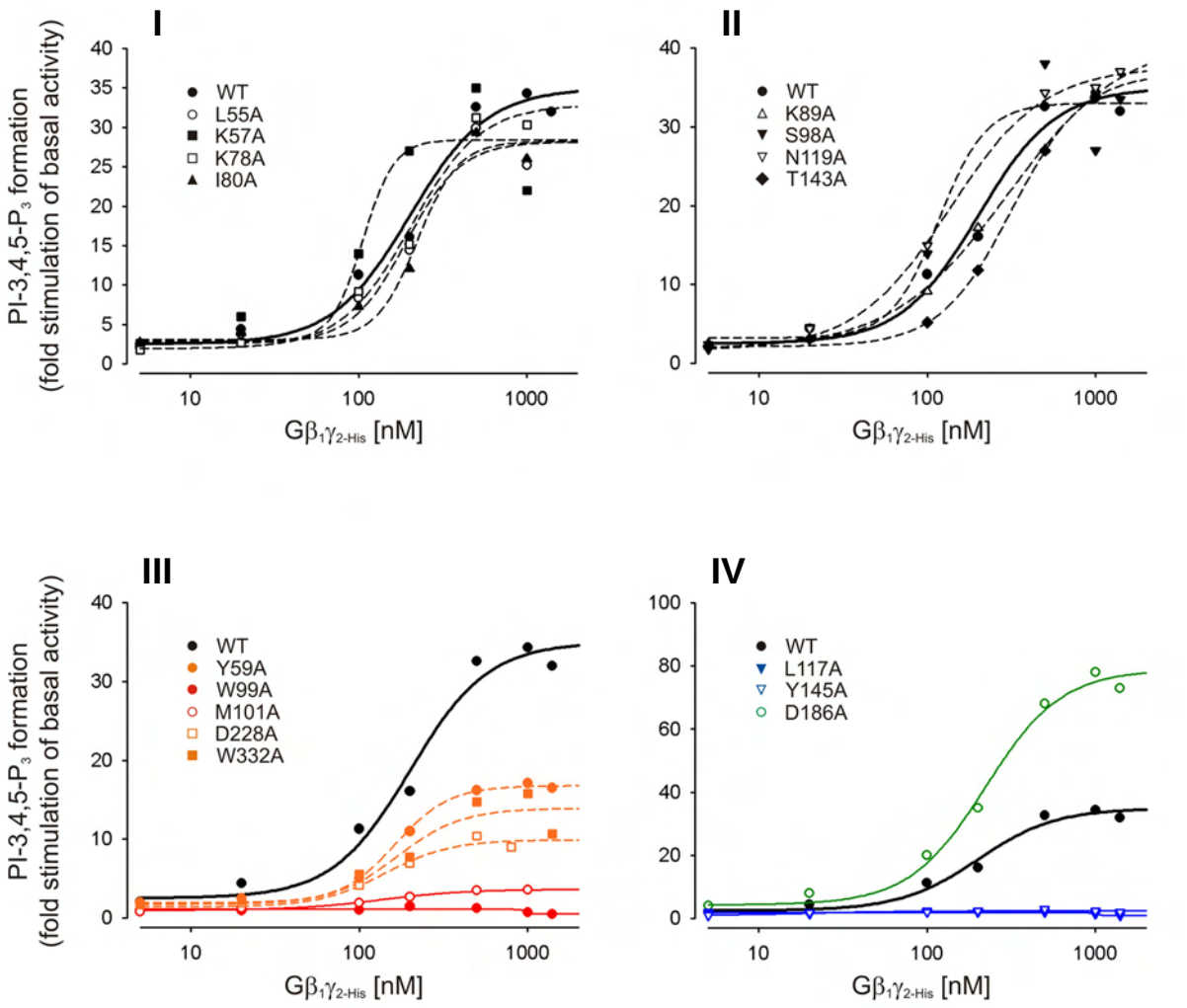


Fig. 12.1B Lipid kinase activity of p110γ-His



Gβ1γ2-His variants	p110γ-His/p101		p110γ-His	
	EC_{50} [nM]	maximal stimulation (fold)	EC_{50} [nM]	maximal stimulation (fold)
WT	7.7	217.3	200.7	34.9
L55A	15.6	236.8	191.1	28.2
K57A	8.9	192.7	107.8	28.4
Y59A	25.7	222.2	164.2	16.8
K78A	14.1	211.6	204.3	32.9
I80A	5.4	155.6	223.6	28.2
K89A	7.7	262.9	303.4	41.2
S98A	8.0	204.3	117.7	33.1
W99A	98.6	220.4	not detectable	1.1
M101A	61.7	246.3	134.9	3.7
L117A	not detectable	43.2	not detectable	1.6
N119A	8.8	168.7	162.2	41.1
T143A	13.3	198.5	299.4	34.4
Y145A	20.1	138.3	not detectable	2.4
D186A	8.2	217.4	221.9	78.9
D228A	22.3	228.3	142.1	9.9
W332A	41.7	205.6	168.9	13.9

Fig. 12.1C Protein kinase activity of p110γ-His/p101

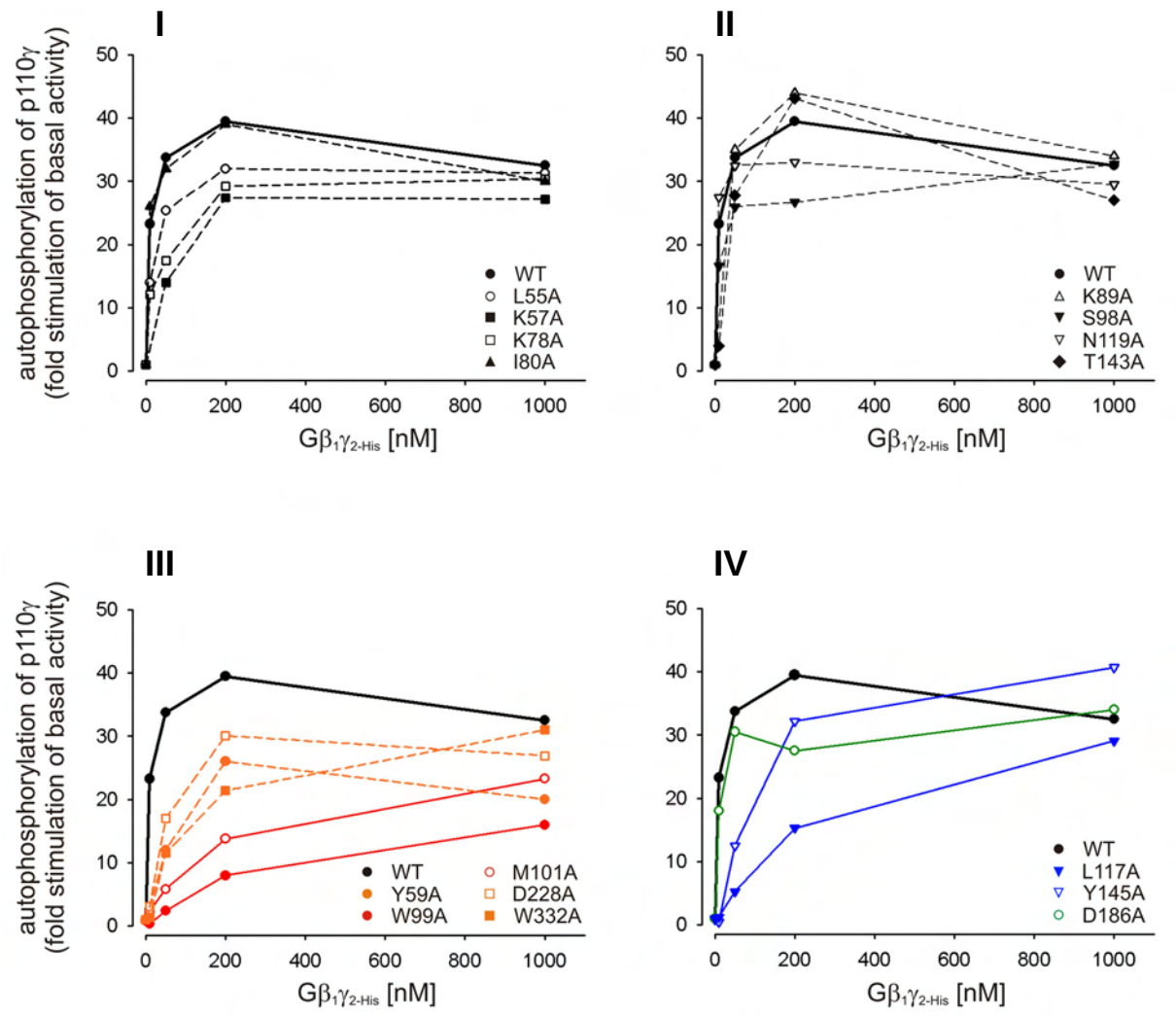


Fig. 12.1: Stimulation of PI3Kγ enzymatic activities by $G\beta_1\gamma_{2\text{-His}}$ variants

Stimulation of lipid kinase activities of $p110\gamma_{\text{-His}}$/p101 **(A)**, $p110\gamma_{\text{-His}}$ **(B)**, and protein kinase activity of $p110\gamma_{\text{-His}}$/p101 **(C)** in response to increasing concentrations of $G\beta_{1WT}\gamma_{2\text{-His}}$ and its alanine mutants. Assays were performed as detailed under section 4.5. Stimulation of PI3Kγ enzymatic activities by $G\beta_{1WT}\gamma_{2\text{-His}}$ is indicated by solid line. Gβγ-induced activation of different forms of PI3Kγ is illustrated as fold-stimulation of basal activity. The stimulation of each PI3Kγ enzymatic activity is demonstrated in four figures (**I**, **II**, **III**, and **IV**). The kinetic parameters for **(A)** and **(B)** are shown in table. Generation and presentation of the dose-response curves is detailed in section 4.7.

Table 12.2: mAb 641 differentially affects lipid kinase activities of monomeric and dimeric PI3Kγ half-maximally stimulated by $G\beta_1\gamma_{2\text{-His}}$ (Supplementary table related to Fig. 5.20)

$G\beta_1\gamma_{2\text{-His}}$	mAb 641, [nM]	PI3Kγ activity (% of maximal stimulation)	
		$p110\gamma_{\text{-His}}$/p101	$p110\gamma_{\text{-His}}$
+	0	100.0 ± 9.8	100.0 ± 10.2
+	0.10	98.1 ± 10.2	97.9 ± 8.5
+	0.29	97.9 ± 8.6	96.2 ± 9.6
+	0.57	96.2 ± 6.7	90.8 ± 7.8
+	1.14	96.3 ± 4.2	85.8 ± 6.5
+	2.28	92.1 ± 5.9	79.3 ± 6.1
+	4.56	75.2 ± 4.2	52.1 ± 3.8
+	9.10	69.1 ± 5.3	31.2 ± 4.1
+	18.20	50.3 ± 6.2	21.9 ± 5.2
+	36.40	38.2 ± 4.4	14.2 ± 4.9
+	48.5	43.3 ± 5.3	14.1 ± 5.2
-	**0**	**1.3 ± 0.6**	**14.8 ± 3.7**

Lipid kinase activity of $p110\gamma_{\text{-His}}$/p101 and $p110\gamma_{\text{-His}}$ were stimulated by half-effective $G\beta_1\gamma_{2\text{-His}}$ concentrations (+), i.e. 20 nM and 200 nM, respectively, in the absence or in the presence of increasing concentrations of mAb 641. $G\beta_1\gamma_{2\text{-His}}$-induced half-maximal stimulation of lipid kinase activities of monomeric and dimeric PI3Kγ were considered as 100 %. Basal lipid kinase activities, i.e. in the absence of $G\beta_1\gamma_{2\text{-His}}$ (-) and mAb 641, of monomeric and dimeric PI3Kγ were estimated in percents regarding to corresponding half-maximal stimulation. Assays were performed as detailed under section 4.5.1. Shown are mean values (± S.D.) of duplicate determinations in three independent experiments. Note that EC_{50} value for the stimulation of the dimeric PI3Kγ during the completing these experiments was 20 nM of $G\beta_1\gamma_{2\text{-His}}$ instead of 7.7 nM indicated above in this study (for explanations see section 4.7).

Table 12.3: Influence of phosphatidylserine on the recruitment of monomeric and dimeric PI3Kγ to the phospholipid vesicles (Supplementary table related to Fig. 5.21A)

Coincubation		Phosphatidylserine [mM]		
		0	**0.3**	**0.9**
$p110\gamma_{\text{-His}}$ (200 ng)	**—**	1.7 ± 2.0 %	4.5 ± 4.3 %	19.1 ± 10.6 %
	600 nM $G\beta_1\gamma_{2\text{-His}}$	1.4 ± 2.0 %	5.0 ± 2.4 %	20.5 ± 7.0 %
$p110\gamma_{\text{-His}}$/p101 (200 ng)	**—**	2.7 ± 2.5 %	7.3 ± 1.4 %	25.2 ± 11.8 %
	600 nM $G\beta_1\gamma_{2\text{-His}}$	26.2 ± 12.0 %	68.0 ± 20.0 %	81.0 ± 15.0 %

Phosphatidylserine was tested for its ability to recruit monomeric or dimeric PI3Kγ (200 ng) in the absence or presence of 600 nM $G\beta_1\gamma_{2\text{-His}}$. Assays were done as detailed under section 4.6. Aliquots of pelleted phospholipid vesicles and the supernatants were subjected to SDS-PAGE followed by immunobloting. Percentage of phospholipid vesicle-recruited monomeric or dimeric PI3Kγ was estimated by immunoblot. Proteins were visualized using specific antiserum (see section 3 and 4.1.4). Chemiluminescence signals were estimated by CCD camera. Total amount of monomeric or dimeric PI3Kγ in assay was considered as 100 %. Data are given as means ± S.D. of duplicate determinations in three independent experiments.

Table 12.4: Influence of phosphatidylserine on lipid kinase activity of monomeric and dimeric PI3Kγ (Supplementary table related to Fig. 5.21B)

Coincubation		Phosphatidylserine [mM]		
		0	**0.3**	**0.9**
$p110\gamma_{\text{-His}}$ (200 ng)	**—**	0.015 ± 0.003	0.036 ± 0.007	0.056 ± 0.007
	600 nM $G\beta_1\gamma_{2\text{-His}}$	1.10 ± 0.48	3.45 ± 1.51	4.78 ± 1.96
$p110\gamma_{\text{-His}}$/p101 (200 ng)	**—**	0.03 ± 0.005	0.12 ± 0.03	0.34 ± 0.04
	600 nM $G\beta_1\gamma_{2\text{-His}}$	28.10 ± 6.72	23.44 ± 3.34	22.8 ± 8.89

Lipid kinase activity of monomeric or dimeric PI3Kγ (200 ng) was tested in the absence or presence of 600 nM $G\beta_1\gamma_{2\text{-His}}$ in a reactions with phospholipid vesicles containing 0 nM, 0.3 nM, or 0.9 nM of phosphatidylserine. Assays were done as detailed under section 4.5.1. After termination of reaction, probes were subjected to thin layer chromatography (TLC) plates. Dried TLC plates were exposed to imaging plates, and autoradiographic signals were quantified with a Fujifilm FLA-5000 imaging system. Specific lipid kinase activity of PI3Kγ is demonstrated as nmol PIP_3 / mg of total PI3Kγ / min. Shown are mean values (± S.D.) of duplicate determinations in three independent experiments.

12.1 Reassociation of recombinant p101 and p110γ subunits

We examined the individually purified $p101_{\text{-His}}$ on its ability to form dimeric complex with p110γ in vitro. For that purpose the gel filtration technique was applied (Fig. 12.2A). Additionally, the reassociation of both preincubated subunits was studied using metal-affinity Ni^{2+}-NTA Superflow column (Fig. 12.2B).

Gel filtrations were performed with $p110\gamma_{\text{-His}}$ only, $p101_{\text{-His}}$ only, and $p110\gamma_{\text{-His}}$ preincubated with $p101_{\text{-His}}$ (Fig. 12.2A). The gel filtration of catalytic subunit of PI3Kγ showed only one main peak corresponding to fraction E1. The gel filtration of $p101_{\text{-His}}$ displayed a broad peak (Fig. 12.2A, upper panel). Coincubation of both subunits led to shift of the main $p110\gamma_{\text{-His}}$ peak from E1 to D12 fraction together with formation of new p101-p110γ containing peak in position of D7-D9 fractions (Fig. 12.2A, lower panel).

Additionally, we found that $p101_{\text{-His}}$ was able to associate with added p110γ as shown by retention of p110γ on the Ni^{2+}-NTA Superflow column as a consequence of binding to $p101_{\text{-His}}$ (Fig. 12.2B). Although p110γ alone, which was expressed without His-tag, had unspecific binding to Ni^{2+}-NTA column, coelution of preincubated $p101_{\text{-His}}$ and p110γ subunits from the column matrix resulted in a higher amount of p110γ compared to elution of p110γ which was not preincubated with $p101_{\text{-His}}$. The data shown in Fig. 12.2A and B demonstrate that both subunits of PI3Kγ, which had been independently expressed and/or purified, may form a complex.

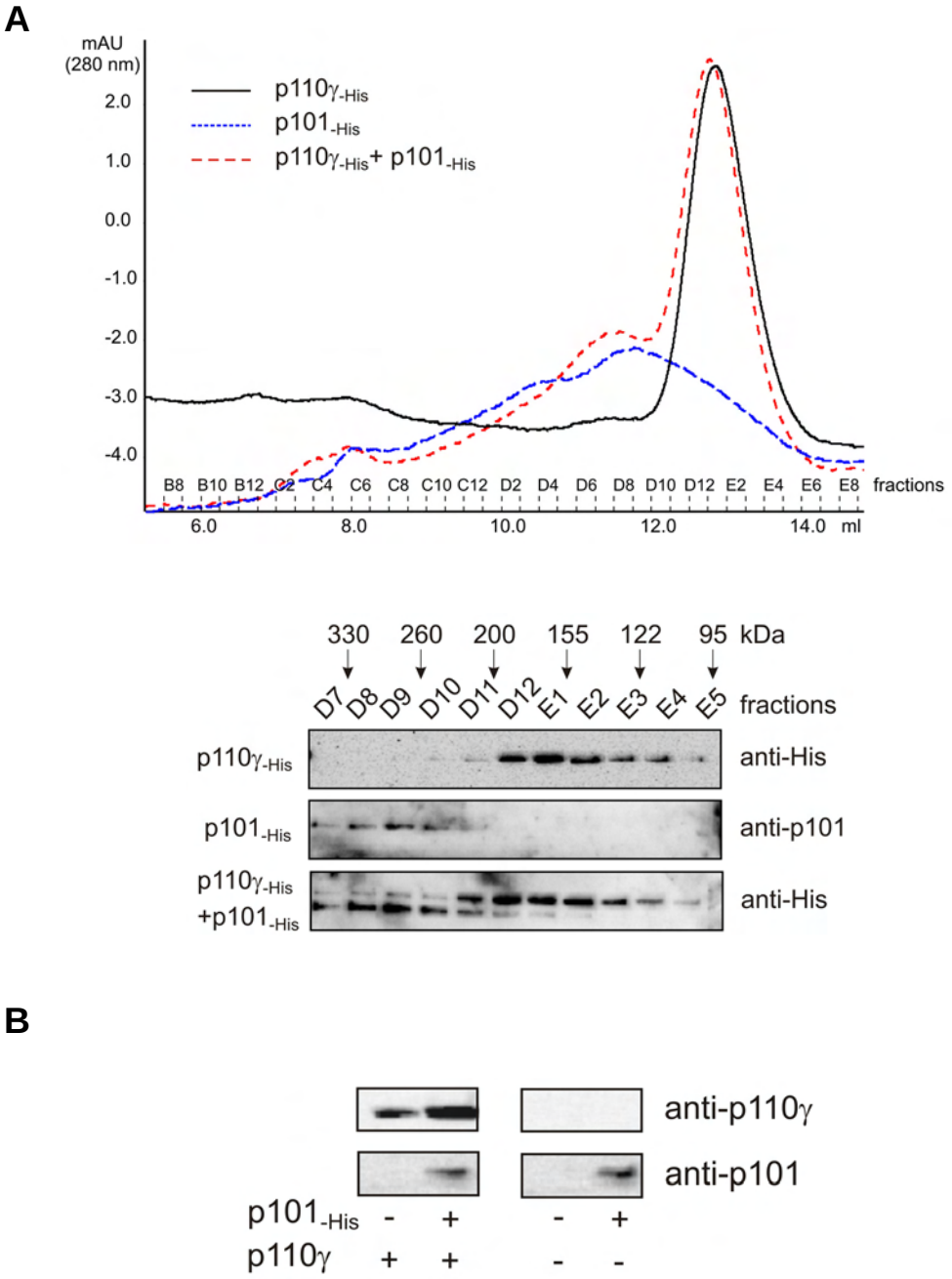


Fig. 12.2: p101 forms a dimeric complex with p110γ

(A) Purified recombinant p101$_{\text{-His}}$ (50 µg), p110γ$_{\text{-His}}$ (40 µg) or p110γ$_{\text{-His}}$ (40 µg) preincubated with p101$_{\text{-His}}$ (50 µg) were loaded onto Superdex 200 HR 10/30 gel filtration column and eluted with buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 % $C_{12}E_{10}$, and 10 mM β-ME (upper panel). Elution of proteins was performed at a flow rate of 0.4 ml/min. The protein elution volumes of each runs were estimated as described under section 4.3.1.4. Fractions (0.5 ml) eluted from Superdex 200 HR 10/30 were analyzed by immunoblotting using specific anti-His and anti-p101 antisera (lower panel). **(B)** Purified recombinant p101$_{\text{-His}}$ (20 µg) was incubated with Sf9 cell cytosol (4 ml) containing (left) or not containing (right) recombinantly expressed p110γ. Subsequently, proteins were subjected to Ni^{2+}-NTA Superflow column (100 µl of beads). Following extensive washing and elution from the Ni^{2+}-NTA column, bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-p110γ and anti-p101 specific antisera.

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Düsseldorf, den 17.04.2007

(Aliaksei Shymanets)