Ph.D. PROGRAM
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THE PUZZLING UNIQUENESS OF THE HETEROTRIMERIC G15 PROTEIN AND ITS POTENTIAL BEYOND HEMATOPOIESIS

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What is it, Ben?
I'm just...
Worried?
Well...
About what?
I guess about my future.
What about it?
I don't know... I want it to be...
To be what?
... Different.

Mr. Braddock and Benjamin
THE GRADUATE

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Chapter 1

GENERAL INTRODUCTION

G-PROTEIN MEDIATED SIGNALLING

All cells possess trans-membrane signalling systems that allow them to transduce information from extracellular signals, such as hormones, neurotransmitters, or sensory stimuli, into biological responses. This fundamental process enable cells to communicate with each other.

Trans-membrane signalling systems share two basic constituents: (1) a RECEPTOR that is able to recognize a specific extracellular stimulus, and (2) an EFFECTOR whose activity, controlled by receptor, generates an intracellular signal. In some signalling systems (e.g. tyrosine kinase receptors) these two constituents are incorporated in one molecule. In contrast, the G protein-mediated signalling machinery is more complex consisting in a RECEPTOR, a HETEROTRIMERIC G PROTEIN, and an EFFECTOR. This modular design of the G protein-mediated system allows the convergence and divergence of signals at the interfaces between receptor and G protein, as well as between G protein and effector. In addition, each component can be regulated independently by ancillary proteins, soluble mediators, or at transcriptional level.

The complex organization of the G protein-mediated signalling system provides the basis for a huge variety of signalling pathways that are tailored to serve peculiar functions in different cell types. It is probably due to its versatility that such a signalling mode is the mostly employed.

G protein-coupled receptors (GPCRs)

The trans-membrane receptors which mediate their intracellular actions primarily through the activation of heterotrimeric G proteins belong to the G protein-coupled receptors (GPCRs) superfamily.

In the human genome about 1,000 different genes code for GPCRs, among which the majority are taste or olfactory receptors, and ~400-500 recognize non-sensory ligands, such as hormones, neurotransmitters, or autacoids[1-2]. For more than 200 GPCRs the physiological ligands are known (Table 1, Table 2 pag.6), whereas for the remaining part no endogenous ligand has yet been identified, and they are called "orphan" GPCRs [3].

Sensory Stimuli	Receptor	Coupling to G Protein Subclass(es)
Light		
~500 nm (max. absorption)	Rhodopsin (11-*cis*-retinal)	$G_{t\text{-}r}$
~426 nm (max. absorption)	Blue-opsin (11-*cis*-retinal)	$G_{t\text{-}c}$
~530 nm (max. absorption)	Green-opsin (11-*cis*-retinal)	$G_{t\text{-}c}$
~560 nm (max. absorption)	Red-opsin (11-*cis*-retinal)	$G_{t\text{-}c}$
~425–480 nm (max. absorption)	Melanopsin (11-*cis*-retinal)	$G_{q/11}$?
Taste		
Umami	T1R1 + T1R3	G_{gust}?
	mGluR4	$G_{i/o}$
Sweet	T1R2 + T1R3	G_{gust}?
Bitter	T2 receptor group (many; ~25 in human, ~36 in mouse)	G_{gust}?
Odorants	many (~350 in human, ~1,000 in mouse)	G_{olf}
Pheromones	V1 group (few in human, ~150 in mouse)	G_{i2} ?
	V2 group (none in human, ~150 in mouse)	G_{o} ?

Table 1 - Sensory receptors [4]- The first column lists the different sensory stimuli and the third column the corresponding coupled G proteins.

All GPCRs share the same molecular architecture (Figure 1, pag.5), consisting of seven trans-membrane α-helices (7TM), three extracellular loops (EL1, EL2, EL3), three intracellular loops (IL1, IL2, and IL3), an extracellular amino-terminal domain (N-ter) and an intracellular carboxyl terminus (C-ter). This topology is predicted

from the analysis of the hydropathy profiles and from experimental evidences derived from the crystal structure of the visual pigment rhodopsin[5], the GPCR activated by light.

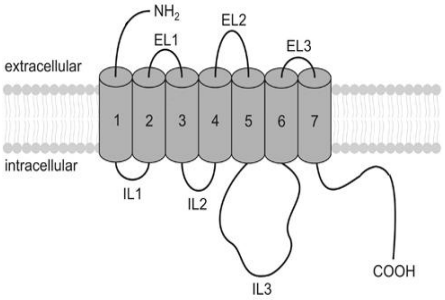

Figure 1 - Schematic representation of the trans-membrane topology of GPCRs.

The majority of GPCRs also share a common signal transduction mechanism that involves the participation of a heterotrimeric G-protein that is interposed between receptor and effector.

Typically, upon activation of a receptor by an endogenous ligand (or an agonist drug), its coupling to the heterotrimeric G protein is facilitated, hence leading to the formation of a high-affinity protein-protein interaction. A number of site-directed mutagenesis experiments and *in silico* modeling studies performed on GPCRs have revealed that some cytoplasmatic domains of the receptors and a cluster of residues in the inner cavity between trans-membrane helices (e.g. TM1, TM2 and TM7), are involved in the interaction between receptors and G proteins[6]. However, despite the determination of the structure of rhodopsin at atomic resolution[5], it is still unclear how the specificity of the receptor-G protein interaction is achieved and how a ligand-induced conformational change in the receptor is transduced to G protein activation [7].

Endogenous Ligand(s)	Receptor	Coupling to G Protein Subclass(es)
Amino acids, dicarboxylic acids		
Glutamate	mGluR1,5	$G_{q/11}$
	mGluR2,3,4,6,7,8	$G_{i/o}$
γ-Aminobutyric acid (GABA)	$GABA_{B1}$ (binding), $GABA_{B2}$ (signaling)	$G_{i/o}$
α-Ketoglutarate	GPR99	$G_{q/11}$
Succinate	GPR91	$G_{q/11}$, $G_{i/o}$
L-Arginine, L-lysine	GPRC6A	$G_{q/11}$?
Biogenic Amines		
Acetylcholine	M_1,M_3,M_5	$G_{q/11}$
	M_2, M_4	$G_{i/o}$
Epinephrine, norepinephrine	α_{1A},α_{1B},α_{1D}	$G_{q/11}$
	α_{2A},α_{2B},α_{2C}	$G_{i/o}$
	β_1,β_2,β_3	G_s
Dopamine	D_1,D_5	G_s
	D_2,D_3,D_4	$G_{i/o}$
Histamine	H_1	$G_{q/11}$
	H_2	G_s
	H_3,H_4	$G_{i/o}$
Melatonin	MT_1,MT_2,MT_3	$G_{i/o}$
Serotonin	$5\text{-}HT_{1A/B/D/E/F}$	$G_{i/o}$
	$5\text{-}HT_{2A/B/C}$	$G_{q/11}$
	$5\text{-}HT_4$,$5\text{-}HT_6$,$5\text{-}HT_7$	G_s
	$5\text{-}HT_{5A/B}$	$G_{i/o}$, G_s
Trace amines	TA1, TA2	G_s
Ions		
Ca^{2+}	CaSR	$G_{q/11}$, $G_{i/o}$
H^+	SPC1, G2A	$G_{q/11}$, $G_{12/13}$
	GPR4, TDAG-8	G_s
Nucleotides/nucleosides		
Adenosine	A_1, A_3	$G_{i/o}$
	A_{2A}, A_{2B}	G_s
ADP	$P2Y_{12}$, $P2Y_{13}$	$G_{i/o}$
ADP/ATP	$P2Y_1$	$G_{q/11}$
ATP	$P2Y_{11}$	$G_{q/11}$, G_s
UDP	$P2Y_6$	$G_{q/11}$
UDP-glucose	$P2Y_{14}$	$G_{i/o}$
UTP/ATP	$P2Y_2$, $P2Y_4$	$G_{q/11}$
Lipids		
Anandamide, 2-arachidonoyl glycerol	CB_1, CB_2	$G_{i/o}$
11-*Cis*-retinal (covalently bound for light-dependent receptor activation; see below)	Rhodopsin	$G_{t\text{-}r}$
	Opsins (green, blue, red)	$G_{t\text{-}c}$
	Melanopsin	$G_{q/11}$?
Fatty acids (C_2–C_5)	GPR41, GPR43	$G_{i/o}$, $G_{q/11}$
(C_{12}–C_{20})	GPR40	$G_{q/11}$
(C_{14}–C_{22})	GPR120	$G_{q/11}$
5-Oxo-ETE	TG1019, GPR170	$G_{i/o}$
Leukotrinene B_4 (LTB_4)	BLT	$G_{i/o}$
LTC_4, LTD_4	CysLT1, CysLT2	$G_{q/11}$
LXA_4	FPRL1 (ALXR)	$G_{i/o}$
Lysophosphatidic acid (LPA)	$LPA_{1/2/3}$ (Edg2/4/7)	G_i, $G_{q/11}$, $G_{12/13}$
Platelet-activating factor (PAF)	PAF	$G_{q/11}$
Prostacyclin (PGI_2)	IP	G_s
Prostaglandin D_2 (PGD_2)	DP	G_s
	$CRTH_2$	G_i
Prostaglandin $F_{2\alpha}$ (PGF)	FP	$G_{q/11}$
Prostaglandin E_2 (PGE_2)	EP_1	$G_{q/11}$
	EP_2, EP_4	G_s
	EP_3	G_s, $G_{q/11}$, G_i
Spingosine-1-phosphate (S1P)	$S1P_{1/2/3/4/5}$ (Edg1/5/3/6/8)	G_i, $G_{q/11}$, $G_{12/13}$
Spingosylphosphorylcholine (SPC)	SPC_1 (OGR1), SPC_2 (GPR4)	G_i
Thromboxane A_2 (TxA_2)	TP	$G_{q/11}$, $G_{12/13}$
Peptides/proteins		
Adrenocorticotrophin (ACTH)	MC_2	G_s
Adrenomedullin	AM_1 (CL+RAMP2), AM_2 (CL+RAMP3)	G_s

Table 2 - GPCRs which respond to non-sensory ligands [4]- The first column lists the ligands and the third column the corresponding coupled G proteins.

Endogenous Ligand(s)	Receptor	Coupling to G Protein Subclass(es)
Amylin	AMY_1 (CT+RAMP1), AMY_2 (CT+RAMP2), AMY_3 (CT+RAMP3)	G_s
Angiotensin II	AT_1	$G_{q/11}$, $G_{12/13}$, $G_{i/o}$
	AT_2	?
Apelin	APJ	$G_{i/o}$
Bradykinin	B_1, B_2	$G_{q/11}$
Calcitonin	CT	G_s, $G_{q/11}$
Calcitonin gene-related peptide (CGRP)	$CGRP_1$ (CL+RAMP1)	G_s, $G_{q/11}$
CC chemokines	CCR1,2,3,4,5,6,7,8,9,10	$G_{i/o}$
CXC chemokines	CXCR1,2,3,4,5,6	$G_{i/o}$
CX_3C chemokines	XCL1, XCL2, CX3L1	$G_{i/o}$
Cholecyctokinin (CCK-8)	CCK_1, CCK_2	$G_{q/11}$, G_s
Complement C3a/C5a	C3a, C5a	$G_{i/o}$
Corticitropin-releasing factor (CRF), urocortin	CRF_1, CRF_2	G_s
Endothelin-1, -2, -3	ET_A (ET-1, ET-2), ET_B (ET-1, -2, -3)	$G_{q/11}$, $G_{12/13}$, G_s
Follicle-stimulating hormone (FSH)	FSH	G_s
Formyl-Met-Leu-Phe (fMLP)	FPR	$G_{i/o}$
Galanin, galanin-like peptide	GAL1, GAL3	$G_{i/o}$
	GAL2	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$
Gastric inhibitory peptide	GIP	G_s
Gastrin	CCK_2	$G_{q/11}$
Gastrin-releasing peptide (GRP), bombesin	BB2	$G_{q/11}$
Ghrelin	GHS-R	$G_{q/11}$
Glucagon	Glucagon	G_s
Glucagon-like peptide	GLP1, GLP2	G_s
Gonadotropin-releasing hormone	GnRH	$G_{q/11}$
Growth hormone-releasing hormone	GHRH	G_s
Kisspeptins, metastin	GPR54	$G_{q/11}$
Luteinizing hormone, choriogonadotropin	LSH	G_s, G_i
Melanin-concentrating hormone	MCH1	$G_{i/o?}$
	MCH2	$G_{q/11}$
Melanocortins	MC_1, MC_3, MC_4, MC_5	G_s
Motilin	GPR38	$G_{q/11}$
Neurokinin-A/-B (NK-A/-B)	NK_2 (NK-A), NK_3 (NK-B)	$G_{q/11}$
Neuromedin-B, bombesin	BB1	$G_{q/11}$
Neuromedin U	NMU1 (FM-3), NMU2 (FM-4)	$G_{q/11}$
Neuropeptide FF & AF	NPFF1, NPFF2	$G_{i/o}$
Neuropeptide W-23, W-30	GRP7, GPR8	$G_{i/o}$
Neuropeptide Y (NPY) etc.	Y_1, Y_2, Y_4, Y_5, Y_6	$G_{i/o}$
Neurotensin	NTS1, NTS2	$G_{q/11}$
Opioids (β-endorphin, Met/Leu-enkephalin, dynorphin A, nociceptin/orphanin FQ)	δ, κ, μ, ORL1	$G_{i/o}$
Orexin A/B	OX1, OX2	G_s, $G_{q/11}$
Oxytocin	OT	$G_{q/11}$, $G_{i/o}$
Parathyroid hormone (related peptide)	PTH/PTHrP	G_s, $G_{q/11}$
Prokineticin-1,2	PK-R1, PK-R2	$G_{q/11}$
Prolactin-releasing peptide	PrRP (GPR10)	$G_{q/11}$
Relaxin, insulin-like 3	LGR7, LGR8	G_s
Secretin	Secretin	G_s
Somatostatin	SST_1, SST_2, SST_3, SST_4, SST_5	$G_{i/o}$
Substance P (SP)	NK_1	$G_{q/11}$
Thyrotropin (TSH)	TSH	G_s, $G_{q/11}$, G_i, $G_{12/13}$
Thyrotropin-releasing hormone (TRH)	TRH-1, TRH-2	$G_{q/11}$
Urotensin II	UT-II (GPR14)	$G_{q/11}$
Vasoactive intestinal polypeptide (VIP), PACAP	$VPAC_1$, $VPAC_2$, PAC_1	G_s
Vasopressin	V_{1a}, V_{1b}	$G_{q/11}$
	V_2	G_s
Proteases (the new NH_2-terminal domain produced by proteolytic cleavage serves as a tethered ligand)		
Thrombin and others	PAR-1, PAR-3, PAR-4	$G_{q/11}$, $G_{12/13}$, $G_{i/o}$
Trypsin and others	PAR-2	$G_{q/11}$

Table 2 continued - GPCRs which respond to non-sensory ligands [4]- The first column lists the ligands and the third column the corresponding coupled G proteins.

Recent studies have suggested that signalling through GPCRs is more diversified and complex than originally thought, first of all because a single GPCR can couple to multiple G-proteins, and secondarily because GPCRs can signal through other scaffold/adaptor proteins independently from coupling to G-proteins.

In particular, GPCRs are able to initiate alternative signalling pathways through different transducers[8] including PDZ-containing proteins (e.g. CN-Ras-GEF[9]), non-PDZ scaffolding proteins (e.g. arrestins[10]), and proteins containing Src homology 2 domain (SH2) (e.g. JAK2[11]), Src homology 3 domain (SH3), and enabled Vasp homology (EVH) domains (e.g. Homer 1[12]).

These scaffolding proteins can also facilitate the receptor-effector interaction by ensuring specificity and efficacy in the activation of downstream signalling cascades, and can promote the appropriate subcellular localization and organization of these signalling complexes [13]. Moreover they can modulate other processes like receptor internalization, phosphorylation, de-phosphorylation, and post-endocytic sorting of receptors to recycling, degradation, or exocytotic pathways [14].

In conclusion, these G-protein-independent transducers make GPCR capable to couple with a different set of effectors thus contributing to the multiplicity of signalling cascades. For this reason some authors prefer to define GPCRs as 7-trans-membrane (7TM), serpentine, or hepta-helical receptors [13] (here the term GPCR is preferred).

Heterotrimeric G protein

Heterotrimeric G proteins are composed of α, β and γ subunits. They dynamically couple activated GPCRs to effectors, and undergo a typical activation-inactivation cycle (Figure 2).

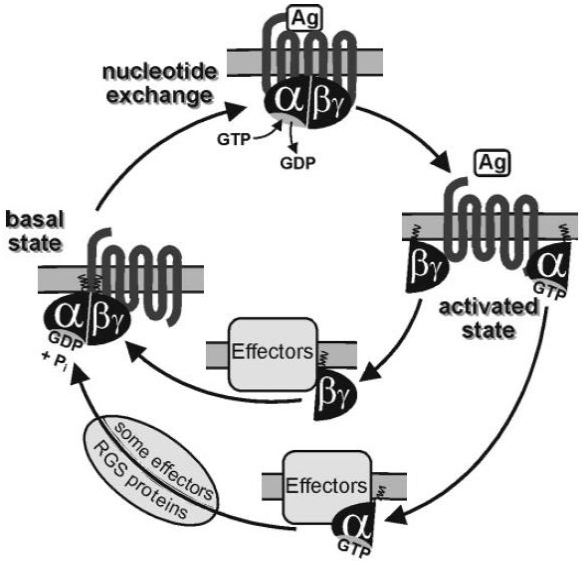

Figure 2 - Functional cycle of G protein activation/inactivation[4] - The binding of an agonist (Ag) to a GPCR promotes the release of GDP from the α-subunit of the heterotrimeric G protein, and results in the formation of GTP-bound Gα. GTP-Gα and Gβγ dissociate and together or independently they can modulate effector activity. The spontaneous hydrolysis of GTP to GDP can be accelerated by various effectors as well as by regulators of G protein signalling (RGS) proteins. GDP-bound Gα then reassociates with Gβγ.

In the basal state, the βγ-dimer and the GDP-bound Gα-subunit are associated, and the heterotrimeric complex can be recognized by an appropriate activated receptor. Coupling of the activated receptor to the heterotrimeric complex promotes the exchange of GDP for GTP on the Gα-subunit, which then dissociates from the activated receptor as well as from the βγ-complex, and both the α-subunit and the βγ-dimer can modulate the activity of various effectors, such as ion

channels or enzymes (e.g. adenylyl cyclase, phospholipase C-β). Signalling is terminated by the hydrolysis of GTP promoted by the GTPase activity which is intrinsic to the Gα-subunit. The resulting GDP-bound Gα-subunit re-associates with the βγ-complex and can enter a new cycle of activation.

Based on the observation that the GTPase activity of isolated G proteins is much lower than that observed under physiological conditions, the existence of mechanisms accelerating GTPase activity had been postulated. Various effectors have indeed been found to enhance GTPase activity of the α-subunit, thereby contributing to deactivation and allowing rapid modulation of G protein-mediated signalling (e.g. phospholipase C-β 1[15]). More recently, a family of proteins able to increase the GTPase activity of Gα-subunits[16] have been identified, i.e. the "regulators of G protein signalling" (RGS proteins). Currently, 30 different RGS proteins have been identified. The physiological role of these molecules is currently under investigation. Besides their role in the modulation of G protein kinetics of activation/deactivation, RGS also influence the specificity of the signalling processes and in some cases can also exert effector functions[17].

G Protein α-subunits and βγ-complexes

The functional versatility of the G protein-mediated signalling system is based on its modular architecture and on the fact that there are numerous subtypes of G proteins. The α-subunits, that define the basic properties of a heterotrimeric G protein, can be divided into four families, which are Gαs, Gαi/Gαo, Gαq/Gα11, and Gα12/Gα13 (Table 3, pag.11).

Name	Gene	Expression	Effector(s)
α-Subunits			
$G\alpha_s$ class			
$G\alpha_s$	*GNAS*	Ubiquitous	AC (all types) ↑
$G\alpha_{sXL}$	(*GNASXL*)	Neuroendocrine	AC ↑
$G\alpha_{olf}$	*GNAL*	Olfactory epithelium, brain	AC ↑
$G\alpha_{i/o}$ class			
$G\alpha_{i1}$	*GNAI1*	Widely distributed	AC (types I,III,V,VI,VIII,IX) ↓ (directly regulated) various other effectots are regulated via $G\beta\gamma$ released from activated G_{i1-3} (see below)
$G\alpha_{i2}$	*GNAI2*	Ubiquitous	
$G\alpha_{i3}$	*GNAI3*	Widely distributed	
$G\alpha_o$	*GNAO*	Neuronal, neuroendocrine	VDCC ↓, GIRK ↑ (via $G\beta\gamma$; see below)
$G\alpha_z$	*GNAZ*	Neuronal, platelets	AC (e.g., V,VI) ↓ (directly regulated); Rap1GAP
$G\alpha_{gust}$	*GNAT3*	Taste cells, brush cells	PDE ↑ ?; other effectors via $G\beta\gamma$?
$G\alpha_{t\text{-}r}$	*GNAT1*	Retinal rods, taste cells	PDE 6 (γ-subunit rod) ↑
$G\alpha_{t\text{-}c}$	*GNAT2*	Retinal cones	PDE 6 (γ-subunit cone) ↑
$G\alpha_{q/11}$ class			
$G\alpha_q$	*GNAQ*	Ubiquitous	PLC-β1-4 ↑
$G\alpha_{11}$	*GNA11*	Almost ubiquitous	PLC-β1-4 ↑
$G\alpha_{14}$	*GNA14*	Kidney, lung, spleen	PLC-β1-4 ↑
$G\alpha_{15/16}$	*GNA16* (*Gna15*)	Hematopoietic cells	PLC-β1-4 ↑
$G\alpha_{12/13}$ class			
$G\alpha_{12}$	*GNA12*	Ubiquitous	PDZ-RhoGEF/LARG, Btk, Gap1m, cadherin
$G\alpha_{13}$	*GNA13*	Ubiquitous	p115RhoGEF, PDZ-RhoGEF/LARG, radixin
β-Subunits			
β_1	*GNB1*	Widely, retinal rods	AC type I ↓ AC types II,IV,VII ↑ PLC-β (β3>β2>β1) ↑ GIRK1–4 (Kir3.1–3.4) ↑ receptor kinases (GRK 2 and 3) ↑ PI-3-K, β, γ ↑ T type VDCC ($Ca_v3.2$) ↓ ($G\beta_2\gamma2$) N-,P/Q-,R-type VDCC ($Ca_v2.1$–2.3) ↓
β_2	*GNB2*	Widely distributed	
β_3	*GNB3*	Widely, retinal cones	
β_4	*GNB4*	Widely distributed	
β_5	*GNB5*	Mainly brain	
γ-Subunits			
γ_1, γ_{rod}	*GNGT1*	Retinal rods, brain,	
γ_{14}, γ_{cone}	*GNGT2*	Retinal cones, brain	
γ_2, γ_6	*GNG2*	Widely	
γ_3	*GNG3*	Brain, blood	
γ_4	*GNG4*	Brain and other tissues	
γ_5	*GNG5*	Widely	
γ_7	*GNG7*	Widely	
γ_8, γ_9	*GNG8*	Olfactory/vomeronasal epithelium	
γ_{10}	*GNG10*	Widely	
γ_{11}	*GNG11*	Widely	
γ_{12}	*GNG12*	Widely	
γ_{13}	*GNG13*	Brain, taste buds	

Table 3 - List of Gα-, Gβ- and Gγ- subunits [4]-
AC, adenylyl cyclase ; PDE, phosphodiesterase; PLC, phospholipase C; GIRK, G protein-regulated inward rectifier potassium channel; VDCC, voltage-dependent Ca^{2+} channel;PI-3-K, phosphatidylinositol 3-kinase; GRK, G protein-regulated kinase; RhoGEF, Rho guanine nucleotide exchange factor.

Each family consists of various members that often show very specific expression patterns. The members of a family are structurally similar and often share some of their functional properties.

The G proteins of the **Gi/Go subfamily** are widely expressed, and especially the α-subunits Gi1, Gi2 and Gi3 have been shown to mediate receptor-dependent inhibition of various types of adenylyl cyclases[18]. Because the expression levels of Gi and Go are relatively high, their receptor-dependent activation results in the release of relatively high amounts of βγ-complexes. Activation of Gi/Go is therefore believed to be the major coupling mechanism that results in the activation of βγ-mediated signalling processes[19]. The function of members of the Gi/Go family has often been studied taking advantage of a toxin from *Bordetella pertussis* (pertussis toxin, PTX) that is able to ADP-ribosylate most of the members of the Gαi/Gαo subfamily close to their C-ter. ADP-ribosylated Gαi and Gαo are unable to interact with the receptor, hence PTX treatment results in receptor uncoupling from the G-protein mediated signalling. The structural similarity between the three Gαi isoforms suggests that they may have partially overlapping functions. A less widely expressed member of the Gαi/Gαo family is Gαz[20], that, in contrast to Gi and Go, is not a substrate for PTX. Gαz is expressed in various tissues including the nervous system and platelets. It shares some functional similarities with Gi-type proteins, but has recently been shown to interact specifically with various proteins including Rap1GAP and certain RGS proteins[20].

The **Gq/11 subfamily** members couple GPCRs to the β-isoforms of phospholipase C (PLC-β) hence initiating phosphoinositol signalling.

PLC-β enzymes catalyze the hydrolysis of the membrane phosphatidylinositol 4,5-bisphosphate (PIP2), to release inositol trisphosphate (IP3) and diacylglycerol (DAG)[21]. These second messengers propagate and amplify the Gα-mediated signal through calcium mobilization following release from IP3-regulated intracellular stores and DAG-mediated stimulation of protein kinase C (PKC)[22]. Inositol lipids, DAG, PKC and calcium each participate in multiple signalling networks linking Gq subfamily members to different cellular events [23]. Although established models indicate that the activity of Gq members is mediated by inositol lipid signalling, growing evidences suggest that these pathways alone do not account for many Gq-mediated responses which are more complex and not yet fully understood.

The biological significance of the diversity among the Gαq members is currently unclear. While the importance of Gq and G11 in various biological processes has been firmly established, the roles of Gα14 and Gα15/16, which show very specific expression patterns, are not clear. For instance mice carrying inactivating mutations in Gα14 and Gα15 genes show none or very minor phenotypical changes[24]. In contrast, mice lacking Gαq or both Gαq and Gα11 have multiple defects [25-26].

G12/G13 subfamily. G12/G13 are expressed ubiquitously and are often activated by receptors coupling to Gq/G11, thus initiating parallel signalling events[27]. The analysis of cellular signalling processes regulated through G12 and G13 has been difficult, since specific inhibitors are not available. In addition, G12/G13-coupled receptors usually also activate other G proteins. Therefore most

information on the cellular functions regulated by G12/G13 has come from indirect experiments employing constitutively active mutants. These studies showed that G12/G13 can induce a variety of signalling pathways leading to the activation of various downstream effectors including heat shock protein (e.g. HSP90), A-kinase anchoring proteins, Bruton's tyrosine kinase (Btk) and radixin[28]. These proteins can represent effectors, modulators or regulators of G12/G13-mediated signalling, but the physiological importance of these interactions is, in many cases, still unclear. Until now the best-characterized downstream signalling pathway regulated by G12/G13 remains the activation of the small GTPase protein RhoA (Ras homolog gene family, member A) mediated by a subgroup of guanine nucleotide exchange factors (GEFs) for Rho which include p115-RhoGEF, PDZ-Rho-GEF, and LARG[29-30]. Finally an interesting link between G12/G13 and cadherin-mediated signalling was recently described, as both Gα12 and Ga13 are shown to interact with the cytoplasmatic domain of some type I and type II class cadherins, causing β-catenin release[31].

The ubiquitously expressed **Gs** protein couples many receptors to adenylyl cyclase and mediates receptor-dependent adenylyl cyclase activation, resulting in the intracellular accumulation of cyclic-AMP (cAMP). The α-subunit of Gs (Gαs), is encoded by the GNAS gene, that gives rise to several products due to the presence of various promoters and splice variants. In addition to Gαs, two transcripts encoding XLαs and Nesp55 are generated by upstream promoters. While Nesp55 is structurally and functionally unrelated to Gαs, XLαs is structurally identical to Gαs but it has an extra long N-terminal

extension that is encoded by a specific first exon[32]. In contrast to Gαs, XLαs has a limited expression pattern being mainly expressed in the adrenal gland, heart, pancreatic islets, brain, and the *pars intermedia* of pituitary[33]. However, XLαs shares with Gαs the ability to bind βγ-subunits and to mediate receptor-dependent stimulation of cAMP production[34].

The βγ-complex of mammalian G proteins is assembled from a repertoire of five G protein β-subunits and twelve γ-subunits (Table 3 pag.11). The βγ-complex was initially considered as a more passive partner of the Gα-subunit. However, it has become clear that βγ-complexes freed from the Gα-subunits can regulate various effectors[19]. These βγ-mediated signalling events include the regulation of ion channels[35], and of particular isoforms of adenylyl cyclase and phospholipase C[36], as well as of phosphoinositide-3-kinase isoforms[37]. With a few exceptions, the ability of different βγ-combinations to regulate effector functions does not dramatically differ[19], therefore much of G protein signalling depends on the identity of the Gα-subunit.

Secondary structure of α-, β- and γ-subunits

Crystallographic studies of Gα-subunits and heterotrimeric complexes[38-39], provide significant insights into the characterization of these proteins. Structural studies of Gα-subunits have focused on Gαt (transducin, the α-subunit involved in vertebrate vision)[40-42], Gαi1[43], and Gαs[44].

Gα-subunits contain two domains: a GTPase domain which contains a six-stranded β-sheet surrounded by six α-helices and is involved in GTP binding and hydrolysis, and a helical domain, comprising a long central helix surrounded by five shorter helices, which bury the GTP molecule within the core of the protein. The helical domain is the most divergent domain among Gα families and may play a role in directing the specificity of receptor- and effector- G protein coupling. Comparison of Gαt-GDP with Gαt-GTP crystal structures has revealed the presence of three flexible regions, designated switches I, II, and III, which become more rigid and well ordered in the GTP-bound active conformation[40-41]. Little is known about the structure of the N- and C-ter domains of Gα-subunits because in the isolated G protein crystal structures solved thus far, the N- and C- ter of Gα-subunits were either removed from the protein or disordered[40-43]. However, in two separate crystal structures of the heterotrimeric complex, the N-terminal helix is ordered by its interaction with the β-propeller domain of Gβ-subunit[38-39]. Biochemical studies suggest that these terminal regions play a key role in the activation process and in directing specific protein-protein interactions.

The Gβ-subunit of heterotrimeric G proteins has a long N-ter helix followed by a repeated module of seven β-sheets, each with four anti-parallel strands, forming a β-propeller[38].

The Gγ-subunit contains two helices: the N-ter helix interacts with the N-ter domain of Gβ, whereas the remaining polypeptide chain of Gγ interacts with the β-propeller structure of the β-subunit[38-39, 43]. Similarly to the C-tail of the α-subunit, the C-tail of the γ-chain

structure is unstructured in the presence of an inactive receptor but forms an amphipathic helix upon rhodopsin activation[45].

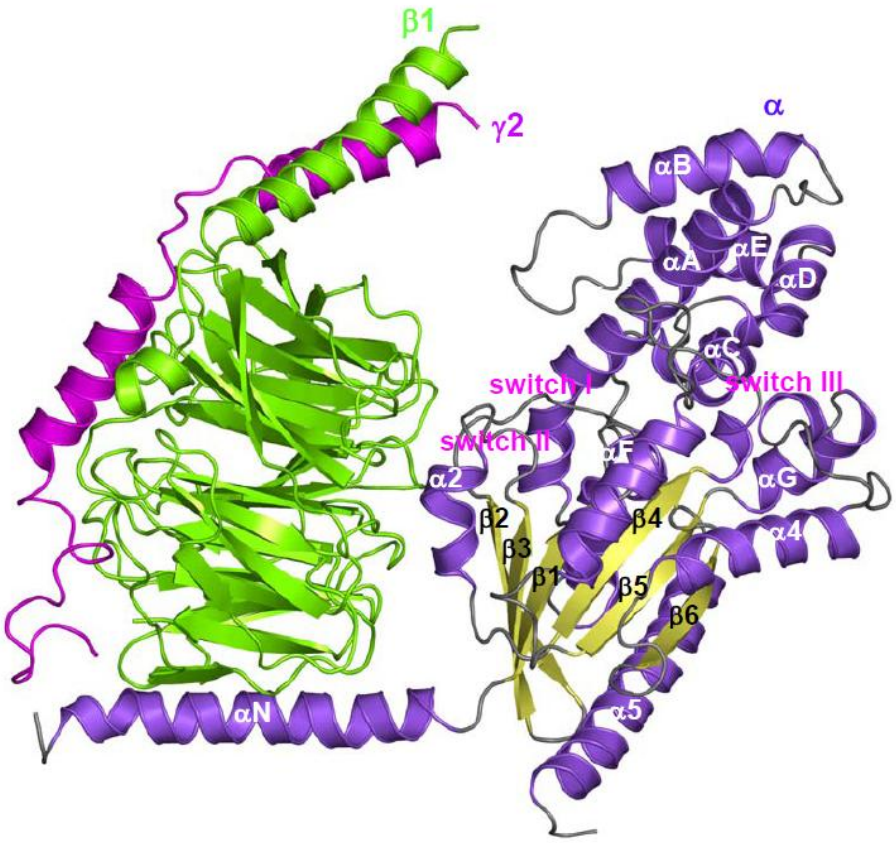

Figure 3 - Tridimensional structure of the Gαi1-β1-γ2 heterotrimer. The Gα domains are labeled according to the Noel's nomenclature[40]. The α-subunit is colored according to the secondary structure (i.e. α-helices in violet and β-strands in yellow), whereas the β- and γ-subunits are colored in green and magenta, respectively.

Diversity of Gq family members and uniqueness of G15/16

Tissue distribution

Each member of the Gαq/11 subfamily (Gαq, Gα11, Gα14 and Gα15/16) has a very different tissue and cell expression pattern (Table 3, pag.11). Gαq and Gα11 mRNAs and proteins are ubiquitously

distributed across tissues, and one or both have been detected in every cell type screened[46-47].

The distribution patterns of Gα14 is more limited, as Gα14 expression has been demonstrated in spleen, lung, kidney, pancreas, liver, testis and bone marrow adherent stromal cells[47-48].

Tissue expression patterns of Gα15 and Gα16 are the most restricted, being uniquely found in tissues rich in hematopoietic cells and in cell types of hematopoietic origin [49] (BOX1, pag.21) especially at the earlier stages of differentiation (Table 4, pag.20).

As a matter of fact Gα16 protein expression is high in normal $CD34^+$ cells (CD34 is the surface marker for hematopoietic progenitor cells), and decreases sharply upon differentiation into granulocytes and erythrocytes[50]. Tenailleau at al. have reported that Gα16 expression remains high during monocytic differentiation of the myeloid precursor cell line HL60 during stimulation with phorbol 12-myristate 13-acetate (PMA) or interferon-γ[50]. However, when the same cell line undergoes to terminal differentiation upon exposure to 1.3% dimethylsulfoxide (DMSO) Gα16 protein is down-regulated[49], suggesting that Gα16 expression can be differentially regulated depending on the nature of the stimulus.

Expression of Gα16 protein has been detected also in human primary myeloid leukemoblasts, as well as in other leukemic cell lines such as KG-1[49], HEL[49], THP-1[49], and U-937[51] (Table 4, pag.20).

Also lymphoid cells in the progenitor stages express Gα15/16 at high levels, and similarly these amounts decrease sharply as the cells become differentiated[47-50]. Most notably Gα16 expression is restricted to progenitor B cells and poorly differentiated B-cell malignancies,

such as pre-B acute lymphocytic leukemia, but not in its differentiated counterparts such as peripheral mature B cells [52]. Moreover when a pre-B-cell line (BLIN-1) is stimulated toward maturation, Gα16 expression was found to disappear during the transition from pre-B to B-cell differentiated stages[52].

Cell Type	Disease	Organism	Ref.
$CD34^+$cells		Human	[50]
HL60	Acute promyelocytic leukemia cell line	Human	[49-50]
NB4	Promyelocytic leukemia cell line		[50]
Primary myeloid leukemia blasts		Human	[50]
KG-1	Acute myelogenous leukemia cell line	Human	[49]
HEL	Erythroleukemia cell line	Human	[49]
THP-1	Acute monocytic leukemia cell line	Human	[49]
U-937	Monocytes of histiocytic lymphoma line	Human	[51]
BLIN-1	Pre-B leukemic cell lines	Human	[52]
MB02	Megakaryocytic leukemia cell line	Human	[53]
REH	Progenitor B-cell line	Human	[48, 52]
NALM-6	Pre-B cell line	Human	[48, 52]
Thymocytes		Human Murine	[54] [48]
Raji	Burkitt's lymphoma cell line	Human	[48]
Daudi	Burkitt's lymphoma cell line	Human	[48]
Cess	Myelomonocytic leukemia cell line	Human	[48]
Jurkat	Acute T cell leukemia cell line	Human	[48]

Table 4 - Cells expressing Gα15/16.

T cells originate from the hematopoietic stem cells in the bone marrow and develop into mature naive T cells ($CD4^+$ or $CD8^+$) in the

BOX 1

Hematopoiesis

Through a series of well-orchestrated divisions, pluripotent hematopoietic stem cells give rise to all the blood elements (i.e. mature lymphocytes, granulocytes, monocytes, erythrocytes, and megakaryocytes/platelets). Functionally, these early progenitors are capable of self-renewal as well as to give rise to a succession of highly proliferative cells with more restrictive capacity for self-renewal.

Self-renewal occurs when a cell enters the cell cycle giving rise to daughter cells which have the same stage of development as the mother. While differentiation defines the sequence of events by which cells mature and acquire more specialized function.

When pluripotent hematopoietic stem cells differentiate, they give rise to: (1) common lymphoid progenitors that give rise to natural killer cells, T lymphocytes and B lymphocytes, and (2) common myeloid progenitors that give rise to different types of leukocytes (monocytes, dendritic cells, neutrophils, eosinophils and basophils), erythrocytes and megakaryocytes/platelets. The different types of blood cell and their lineage relationships are summarized in Figure 4 (pag.22).

In the bone marrow hematopoietic progenitor cells proliferate and become committed to differentiate within a specialized environment that provides a supporting stroma on which the stem cells bind and receive regulatory signals required for stem cell growth.

Similarly development, homeostasis, trafficking, and response capacity of mature hematopoietic cells are tightly regulated by a complex communication network that is mediated by intercellular signals. These signals are triggered by direct cell-to-cell or cell-to-matrix contact or by the release of soluble cytokine mediators.

Cytokines act on cells by interacting with specific receptors on the cell surface. This interaction promotes signal transmission across the cell membrane and activates intercellular signalling cascades that are integrated at the gene expression level. The vast majority of the soluble cytokine ligands can elicit a wide spectrum of biologic responses, but at the same time, there is a considerable overlap in function between many of these cytokines.

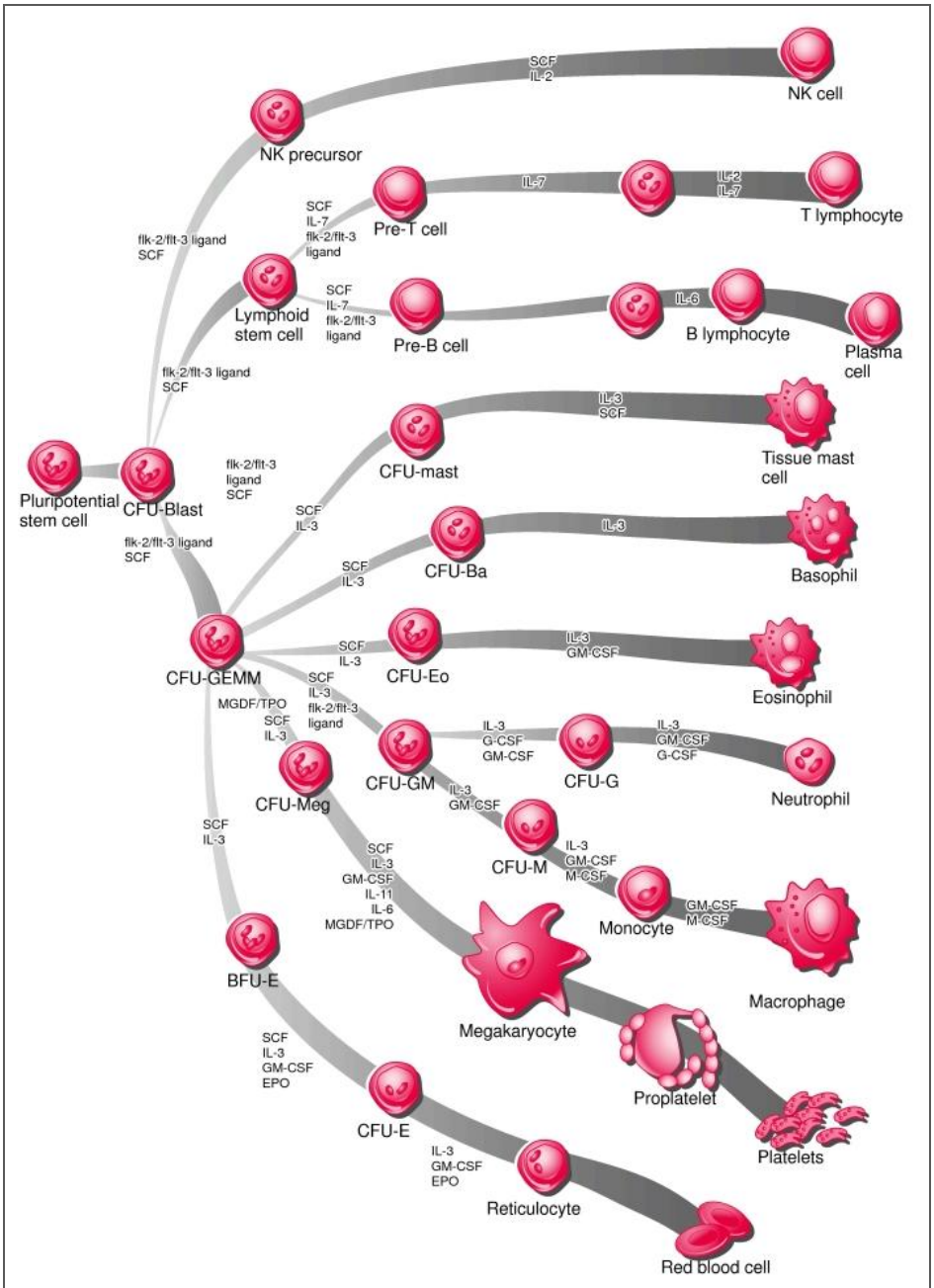

Figure 4 - Hematopoiesis and its growth factors. Pluripotent stem cells divide to produce more specialized types of stem cells: a common lymphoid progenitor (lymphoid stem cell) that gives rise to the T and B lymphocytes responsible for adaptive immunity, and a common myeloid progenitor (CFU-GEMM) that gives rise to different types of leukocytes, erythrocytes, and megakaryocytes that produce platelets.

thymus, where Gα16 have been identified in human thymocytes[54]. In T cells it has been suggested that a coordinated up/down-regulation of Gα16 expression is required for optimal T cell activation, suggesting that under special conditions Gα16 may play a role also in mature cells[55].

The unique distribution patterns of each Gq family member suggests that in their respective endogenous cellular environment, each members is likely exposed to different subsets of receptors, effectors, and other potential protein binding partners, affording many opportunities for selective, tissue- and cell-specific signalling for Gαq, Gα11, Gα14, and Gα15/16. This seems particularly relevant to Gα14 and Gα15/16 which have the most restricted expression patterns.

Sequence diversity and genomics

Gαq/11 subfamily proteins exhibit notable differences in primary structure. Among all families of heterotrimeric G proteins with shared effectors, α-subunits of the Gq family are the most divergent in amino acid sequence. Human Gα11, Gα14, and Gα16 compared with Gαq share 90%, 80%, and 57% amino acid sequence identity, respectively (Table 5, pag.24). While most of the sequence diversity across G proteins occurs outside the highly conserved nucleotide binding region and the conformational switch domain, differences among the sequences of Gαq/11 subfamily exist both outside and within these important regions[47]. For istance Gα15/16 sequence has several amino acid substitutions in the GTP-binding pocket and GTPase domain, and two multiple amino acid inserts are present in the C-ter[47] (Figure 5, pag.24).

In addition, the N-ter domains of the Gαq family (amino acids from 1 to 40) are particularly different, with as little as 35% identity between Gαq and Gα16 (Table 5).

Gα property	**Gαq**	**Gα11**	**Gα14**	**Gα15/16**
Effector coupling	PLC-β	PLC-β	PLC-β	PLC-β
Receptor coupling	Selective	Selective	Limited selectivity	Non-selective
Tissue distribution	Ubiquitous	Ubiquitous	Kidney, liver, lung	Hematopoietic cells
aa sequence identity *	100%	90%	80%	57%
N-ter aa sequence identity * (first 40 aa)	100%	83%	65%	35%

Table 5 - Properties of Gq family members[56]. (aa) amino acid; (*) compared with Gαq

```
sp|P50148|GNAQ_HUMAN      PQRDAQAAREFILKMFVDL------NPD-SDKI-----IYSHFTCATDTE 329
sp|P29992|GNA11_HUMAN     PQRDAQAAREFILKMFVDL------NPD-SDKI-----IYSHFTCATDTE 335
sp|O95837|GNA14_HUMAN     PKQDVRAARDFILKLYQDQ------NPD-KEKV-----IYSHFTCATDTD 331
sp|P30679|GNA15_HUMAN     PKQDAEAAKRFILDMYTRMYTGCVDGPEGSKKGARSRRLFSHYTCATDTQ 350
sp|P30678|GNA15_MOUSE     PRRDAEAAKSFILDMYARVYASCAEPQDGGRKGSRARRFFAHFTCATDTQ 350
                          *::**.**: ***.*:    .              : :::*:******:
```

Figure 5 - Partial sequence alignment between the Gq family members. Stars (*) below the alignment stand for identity, whereas colons (:) and dots (.) indicate high and moderate physico-chemical similarity, respectively.

Despite considerable differences in primary structure, species orthologues of Gαq family members are mainly conserved, with the exception of Gα15 and Gα16[57-58], the mouse and the human proteins respectively, which share only 85% identity[59]. This sequence variability between Gα15 and Gα16 is thought to indicate an unusual rate of evolutionary gene divergence, thereby suggesting a distinct functional importance of the encoded proteins[59].

Genomics studies suggest that Gα11 and Gα15/16 evolved through a tandem gene duplication, and, based on the close linkage of the

encoding genes on the murine chromosomes, the same mechanism of gene divergence is suspected for Gαq and Gα14[59-60]. The evolutionary conservation of these independent Gαq family genes implies that each encoded protein is functionally essential[60-61].

Biochemical diversity

Heterotrimeric G proteins typically localize at the cytoplasmic face of the plasma membrane, where they interact with hepta-helical receptors. For G α-subunits, multiple membrane targeting signals, including long chain lipid modifications occurring at their N-ter (myristoylation and/or palmitoylation) and interaction with βγ subunits, facilitate membrane localization and anchoring (Figure 6, pag.26).

Recent models also suggest that Gα subunits may utilize multiple positively charged residues present at their N-ter as a third signal for membrane targeting and attachment[62]. For example, three-dimensional molecular modeling of Gqα, Gα11, Gα14, and Gα16 predicts the existence of a cluster of N-ter basic amino acids which fold in such a way to form a positively charged patch on the protein surface[63] (Figure 6, pag.26). These residues are expected to align on one face of the Gα N-ter α-helix opposite to residues that contact with the βγ-dimer, and in a position favorable for ionic interactions with anionic phospholipids of the plasma membrane.

A defect in the membrane localization of Gαq was observed when nine basic residues (amino acids 19,20,27,30,31,33,34,37 and 38) inside this patch were mutated to glutamine or when other three basic residues (at position 16,19 and 20) were mutated to glutamic acid.

This loss of plasma membrane localization coincided with defects in palmitoylation, and in this condition also $\beta_1\gamma_2$ co-expression could only partially rescue these effects[62].

The N-ter of Gα14 contains nine basic residues at identical positions as basic residues in Gαq. Fusion of the N-ter 34 amino acids of Gα14 to GFP was sufficient to target GFP to the plasma membrane. But when all nine basic residues (at positions 12,16,23,26,27,29,30,33 and 34) of Gα14 were changed to glutamines, plasma membrane localization of the fusion protein was not disrupted, hence suggesting that the basic residues were not required for plasma membrane localization of Gα14[64]. However, these mutations were not examined in the context of full-length protein[64].

```
CLUSTAL W (1.83) multiple sequence alignment

sp|P29992|GNA11_HUMAN       -MTLESMMAC--CLSDEVKESKRINAEIEKQLRRDKRDARR 36
sp|P50148|GNAQ_HUMAN        -------MAC--CLSEEAKEARRINDEIERQLRRDKRDARR 30
sp|O95837|GNA14_HUMAN       -----MAGCC--CLSAEEKESQRISAEIERQLRRDKKDARR 32
sp|P30679|GNA15_HUMAN       MARSLTWRCCPWCLTEDEKAAARVDQEINRILLEQKKQDRG 39
sp|P30678|GNA15_MOUSE       MARSLTWGCCPWCLTEEEKTAARIDQEINRILLEQKKQERE 39
                                     .*  **: : * : *:. **:: * .:*:: *
```

Figure 6 - The amino acid sequence alignment of the N termini of the Gq family members- The sequences are annotated by their Swissprot ID. Conserved residues are indicated below the alignment with an asterisk (*). Palmitoylated cysteine residues for Gαq[65] (GNAQ), Gα11[66](GNA11), Gα14[64] (GNA14) and Gα16[64](GNA16) are marked in yellow. The putative sites for palmitoylation are marked in pink. The amino acids whose side chains contact βγ dimer are highlighted in blue. In red are the amino acids of the polybasic region involved in ionic interactions with anionic phospholipids of the plasma membrane.

Gα16 contains N-terminal basic residues too. Mutations of five of the basic residues in the N-ter of Gα16 (at positions 19,23,30,36 and 37) did not cause a defect in plasma membrane localization of a GFP fusion construct, but the same mutations in full-length Gα16 resulted in a markedly reduced plasma membrane expression and in an

impaired functionality of the protein, even if it was still palmitoylated[64].

All these results suggest that the role of N-ter polybasic motifs can differ among the members of Gαq subfamily. For instance these differences may be due to the fact that Gα14 and Gα16 have three cysteines that can serve as sites of palmitoylation.

Palmitoylation. Palmitoylation is a post-translational modification that consists in the covalent attachment of the saturated fatty acid palmitate to a protein via a thioester bond (S-acylation). In contrast to other covalent lipid modifications, palmitoylation is a very dynamic modification, because this bond is chemically labile and has been shown to rapidly turning over *in vivo* [67-68]. This lipid modification also occurs on one or several cysteine residues within the first 20 amino acids of the N-ter region of the Gα-subunits, but the specific amino acid sequence required to facilitate palmitoylation has not yet been defined in detail. Generally, with the exception of Gαt (transducin) and Gαgust (expressed in taste cells and belonging to the Gi/Go family) all Gα-subunits undergo palmitoylation, but neither the specific palmitoyltransferase that catalyzes the lipidation nor the exact cellular site where this occurs have been discovered thus far.

Both Gαq and Gα11 are dually palmitoylated at adjacent cysteines (C9 and C10)[69-70] (Figure 6, pag.26), and non-palmitoylated Gαq and Gα11mutants (C/A or C/S site mutants) are cytosolic and cannot transduce signalling compared with wild type forms[65, 71].

These cysteines are conserved in Gα14 and Gα15/16 (C5, C6 and C9, C10, respectively), with a third cysteine supposed to be palmitoylated (C6 and C13 respectively). Recent data suggest that Gα14 is

palmitoylated at all the three putative cysteines while Cys 9 and Cys 10 but not Cys 13 are palmitoylated[64] in Gα16. However, mutating these three residues as individual C/S (cysteine to serine) in the full-length Gα16 protein reduces membrane localization and signalling capacity of G16 suggesting that also Cys 13 is of functional importance for G protein signalling[64].

Receptor coupling

Although all activated Gαq family members can stimulate PLC-β isoforms leading to the production of IP3 and DAG, numerous reports indicate that GPCRs can differently couple to each member.

For example, by measuring inositol lipid signalling in heterologous expression systems it was shown that while all Gαq family members can couple to α_{1B} adrenergic receptors (α_{1B}-AR), only Gαq and Gα11 can link to α_{1D}-AR, and Gαq, Gα11 and Gα14, but not Gα15, can couple to α_{1A}-AR[72-73]. Other studies demonstrated that the neurokinin-2 receptor shows a ligand-mediated response in membranes co-transfected with Gαq, Gα11 and Gα14 but not with Gα16, and that the muscarinic M1 receptor is able to activate PLC through Gαq/11 but not Gα14 and Gα16[74]. As a final example, in COS7 cells, inositol lipid signalling stimulated by the chemokine interleukin-8 receptor occurs through Gα14 and Gα15/16, but not Gαq or Gα11[75].

Surprisingly, Gα15/16 and to a lesser extent Gα14 exhibit an unexpected capacity to couple to GPCRs that are not reported to be naturally linked to inositol lipid signalling (Table 6, pag.29). Various heterologous expression studies have shown that Gα15/16 indiscriminately interacts with a wide variety of Gs- and Gi/o-linked

Receptor category	Subtype	$G_{15/16}$-coupled?
G_i-coupled receptors		
Adenosine	A_1	$G_{15/16}$
Adrenergic	α_2	G_{16}
Cannabinoid	CB_1	G_{16}
Chemoattractant	C3a, C5a, fMLP	$G_{15/16}$
Chemokine	CCR1, CCR2b, CCR3, CXCR1, CXCR2	G_{16}
	CCR2a, CCR5, CCR7, CXCR4	No
Dopamine	D_2	G_{16}
γ-Aminobutyric acid, metabotropic	$GABA_{B1a}$ + $GABA_{B2}$, $GABA_{B1b}$ + $GABA_{B2}$	G_{16}
Glutamate, metabotropic	*Drosophila*, $mGlu_2$	$G_{15/16}$
	$mGlu_{4a}$, $mGlu_{7a}$, $mGlu_{8a}$	G_{15} only
Melatonin	MT_1, MT_2	G_{16}
	Xenopus	No
Muscarinic acetylcholine	M_2	$G_{15/16}$
	M_4	G_{15} only
Neuropeptide	AF/FF	G_{16}
Opioid	μ	$G_{15/16}$
	δ, κ, nociceptin	G_{16}
Purinergic	UDP-glucose	G_{16}
Serotonin	$5\text{-}HT_{1A}$	$G_{15/16}$
Somatostatin	SST_1, SST_2	$G_{15/16}$
Tastant/L-amino acids	mouse $T2R_5$, human $T2R_{16}$	No
	$T1R_1$ + $T1R_3$, $T1R_2$ + $T1R_3$	G_{15} only
Smoothened	–	G_{15} only
G_s-coupled receptors		
Adenosine	A_{2A}	$G_{15/16}$
Adrenergic	β_1	G_{16}
	β_2	$G_{15/16}$
Dopamine	D_1	$G_{15/16}$
	D_5	G_{16}
Glucagon		No
Histamine	H_2	G_{16}
Luteinizing hormone		G_{16}
Parathyroid hormone		G_{16}
Prostaglandin	IP	G_{16}
Secretin		G_{16}
Thyrotropin-releasing hormone		G_{16}
Vasoactive intestinal peptide		G_{15} only
Vasopressin	V_2	$G_{15/16}$
G_q-coupled receptors		
Adrenergic	α_{1A}, α_{1C}	No
	α_{1B}	G_{16}
Bombesin		G_{16}
Metabotropic glutamine	$mGlu_{1a}$	No
Muscarinic acetylcholine	M_1, M_3, M_5	G_{16}
Serotonin	$5\text{-}HT_{1C/2C}$	$G_{15/16}$
Thrombin		$G_{15/16}$
Thromboxane	TXA_2	$G_{15/16}$
Vasopressin	V_{1A}	$G_{15/16}$

Table 6 - Receptor-coupling of Gα15/16

GPCRs, such as subtypes of adrenergic, muscarinic, vasopressin, adenosine, serotonin, opioid, various chemotactic receptors and metabotropic glutamate receptors[76-78] (Table 6, pag.29).

Gα14 also mediates inositol lipid signalling when co-expressed with certain Gs- and Gi/o-linked GPCRs[79], although the profile of receptors that can activate Gα14 is not as extensive as that for Gα15/16.

Whether the promiscuous coupling capacities of Gα15/16 and Gα14 in transfected systems reflect physiological GPCR-Gα interactions is currently unclear. Although chemotactic receptors and Gα15/16 are predominantly expressed in hematopoietic cells, the effects of chemokines on calcium signalling in myeloid cells are largely PTX-sensitive, indicating that these responses are mediated through the Gβγ dimer derived from resident Gαi proteins rather than Gα15/16[80]. Moreover in HL-60 cells the expression levels of chemokine receptors and Gα16 protein are not synchronous, since Gα16 is highly expressed in progenitor cells and decreases during differentiation[49], whereas chemokine receptors are expressed at the highest levels in mature cells[81].

Although the real physiological significance of such a promiscuous coupling it is yet unclear, this interesting feature, observed in heterologous system especially for Gα15/16, has been widely exploited to facilitate the de-orphanization of novel GPCRs, and a number of successful cases have been reported[3, 82-83]. In spite of that, Gα15/16 is far from being the ideal 'universal adaptor' for all kinds of GPCRs, as some GPCRs are found to be incapable of recognizing Gα15/16 (e.g. muscarinic M1[74], neurokinin-2[74], melatonin MT_{1c} [84]

receptors) or to interact weakly with it (e.g. somatostatin SST_1 [85], dopamine D2[85] and μ-opioid[85] receptors). For this reason the quest for a 'better coupler' has evolved into the generation of chimeric Gα subunits obtained by replacing the C-ter residues of α5 helix of Gα16 with those of either Gαi2[86], Gαo1[86], Gαz[87] or Gαs[88]. Using this approach the coupling with Gαi- or Gαs-linked receptors was greatly enhanced so that these chimeric Gα16 subunits have been successfully employed in high throughput screening platforms[89-90].

Although both Gα15 and Gα16 are described as promiscuous, many studies show that certain receptors can activate Gα15 but not Gα16, implying that the mouse Gα subunit is more functionally promiscuous than its human orthologue[78, 91-92]. Because of this observation many efforts have been done using chimeric constructs and three-dimensional modeling to decipher the minimal requirements for switching specificity. Individual residue lying on the exposed surface of the α5 helix of Gα16, as well as a stretch of six

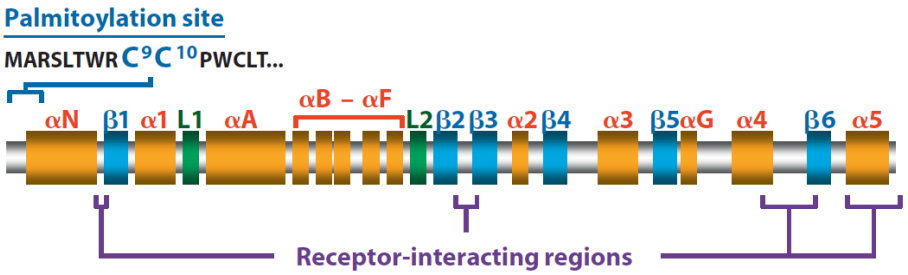

Figure 7 - Covalent modifications and receptor-interacting regions of Gα16[93]. A schematic diagram shows the linear arrangement of the secondary structures of Gα16. α-Helices and β-strands are colored in orange and blue, respectively. The two green linker regions (L1 and L2) connect the two major tertiary structural domains – helical and GTPase domains – of Gα16. The amino acid sequences corresponding to the four known receptor-interacting regions on Gα16 are marked below the schematic structural diagram.

amino acids between the α5 helix and the extreme C-ter tail (residues 62-367), have been shown to play an important role in determining the coupling specificity[94]. Other potential receptor-interacting regions on Gα16 have also been identified in the β2/β3 loop[94],αN/β1 loop[91], as well as the uniquely long α4/β6 loop[95] (Figure 7, pag.31).

On the other hand, multiple N- and C-ter sites and internal residues of Gαq and Gα11 impose the selectivity of these proteins in receptor interaction. Unique to Gαq and Gα11 is a six amino acid N-ter extension that lies upstream of the translation initiating methionine. A truncation mutant of Gαq lacking this extension mediates receptor-stimulated PLC-β1 activation similarly to wild type Gαq[65, 96], but it also productively couples normally Gi/o- and Gs-linked receptors to PLC-β with varying efficiencies[97]. One possible explanation is that the N-ter extension structurally constrains Gαq and Gα11 in a favorable conformation for interaction with PLC-β-linked GPCRs[97-98]. Alteration of this N-terminal extension at any of the six amino acids introduces promiscuity in receptor coupling of Gαq[98]. Perhaps the lack of a similar structural constraint on Gα14 and Gα15/16 contributes to their non-selective receptor coupling patterns.

GPCR DESENSITIZATION

GPCR activity is the result of a coordinated balance between molecular mechanisms governing receptor signalling, desensitization, and resensitization. The desensitization of a GPCR response can be described as the loss of responsiveness subsequent to prolonged or repeated administration of an agonist[99], and represents an important physiological "feedback" mechanism that protects cells against both acute and chronic receptor over-stimulation.

The major mechanism underlying desensitization is GPCR phosphorylation [100]. Until the mid-1980s, GPCR phosphorylation by second messenger-dependent protein kinases, such as protein kinase A (PKA) and protein kinase C (PKC) was regarded as the principal mechanism of GPCR desensitization[101]. However, the observation that the β2-adrenoceptor (β2-AR) could be phosphorylated and desensitized in cells lacking functional PKA[102], pointed to the existence of other kinases that could phosphorylate GPCRs. The identification of a novel second messenger independent protein kinase, with the ability to phosphorylate the agonist-occupied β2-AR[103], was a landmark in the understanding of GPCR desensitization. This kinase, originally called β-adrenoceptor kinase (β-ARK), was soon found to be just one member of a family of kinases, subsequently termed G protein-coupled receptor kinases (GRKs), with the original β-adrenoceptor kinase assuming the name of GRK2. The GRKs have since been shown to play a central role in the agonist-induced phosphorylation and desensitization of many GPCR responses[104]. However, it was found that GRK phosphorylation of GPCRs was by

itself insufficient to produce extensive desensitization of the receptor response[105]. Accordingly, another family of regulatory proteins was identified and called arrestins[106]. The arrestin family comprises four members in mammals: two visual arrestins (the visual or rod arrestin, and the cone arrestin or X-arrestin) and two non-visual arrestins called β-arrestin1 and β-arrestin2. β-arrestins are able to bind with high affinity agonist-occupied and GRK-phosphorylated GPCRs, thus uncoupling them from G-protein activation and inducing desensitization of the receptor-generated response.

These evidences led to the development of a 'classical' model for the agonist-induced desensitization of GPCRs[107-108], considered to be generally applicable to most GPCRs.

B-arrestin mediated desensitization

According to the 'classical' model of GPCR desensitization the agonist-occupied receptor becomes a substrate for phosphorylation by members of the GRK family. The GRK-phosphorylated receptor exhibits a high affinity for arrestins, which bind to the GPCRs and inhibit further coupling to G proteins, hence desensitizing the response (Figure 8, pag.35). In addition to GRK-mediated phosphorylation, recent studies have underlined also the importance of agonist occupation of the GPCRs for β-arrestin binding. For instance using Fluorescence Resonance Energy Transfer (FRET) to detect interactions between the β2-AR and arrestins, Krasel et al. have shown that upon agonist removal, arrestin dissociated rapidly from the β2-AR, even though the GPCR was still phosphorylated by GRK[109]. Furthermore there are some GPCRs, such as the leukotriene B4

receptor[110], to which arrestins can associate in an agonist-dependent manner without the requirements of receptor phosphorylation.

In addition to their role in desensitizing GPCR response, arrestins have also a central role in GPCR trafficking. In fact arrestins are able to target activated GPCR to clathrin-coated pits, which are specific invaginated membrane structure (buds or pits) involved in receptor endocytosis. Upon GPCR internalization the receptor can be either dephosphorylated and recycled to the plasma membrane, or targeted to lysosomes for down-regulation[107-108] (Figure 8).

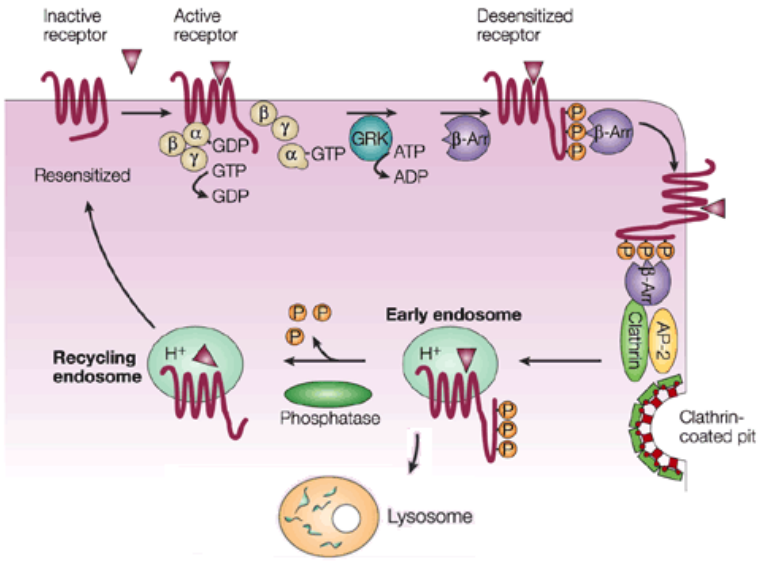

Figure 8- Desensitization, endocytosis and resensitization/down-regulation of GPCR. After agonist stimulation the phosphorylation of GPCRs by GRKs inhibits receptor coupling to heterotrimeric G proteins at the plasma membrane (desensitization). Interaction of phosphorylated receptors with β-arrestins (β-Arr) promotes receptor endocytosis through binding of GPCR-β-arrestin complex to clathrin[111] and/or AP-2[112] (proteins which play a major role in the formation and internalization of endocytic coated vesicles respectively). Endocytosed receptors can be either dephosphorylated by an endosome-associated phosphatase and then recycled back to the plasma membrane (resensitization) or sorted to lysosomes for degradation. [modified from Sorkin et al.[113]]

The process followed by the internalized receptor often depends upon the length of agonist treatment, with many GPCRs undergoing significant down-regulation only following hours of agonist treatment. However, according to Oakley et al. the fate of an internalized GPCR also may be influenced by the stability of the interaction between the receptor and β-arrestin.

These authors have described two characteristic patterns of agonist-induced β-arrestin interaction that allow to categorize GPCRs in two distinct classes[114]. One class (represented by β2 and α1B adrenergic receptors, μ-opioid, endothelin A, and dopamine D1A receptors), binds to β-arrestin2 with higher affinity than β-arrestin1. For these receptors, the interaction with β-arrestin is transient. β-Arrestin is recruited to the receptor at the plasma membrane and then translocates with it to clathrin-coated pits. Upon internalization of the receptor, the receptor-β-arrestin complex dissociates, and while the receptor proceeds to an endosomal pool, the β-arrestin recycles to the cytosol. The second class (represented by angiotensin AT1a receptor, vasopressin V2 receptor (V2R), and neurotensin 1, thyrotropin-releasing hormone, and neurokinin NK-1 receptors) binds β-arrestin1 and β-arrestin2 with equal affinity. These receptors form stable complexes with β-arrestin, therefore the receptor-β-arrestin complex internalizes as an unit and is targeted to endosomes. The β2AR, which binds β-arrestin transiently, is rapidly dephosphorylated and recycled to the plasma membrane, whereas the V2R, which binds β-arrestin stably, recycles slowly. Moreover exchanging the C-terminal tails of these two receptors not only reverses the pattern of β-arrestin binding, but also reverses the pattern of receptor dephosphorylation and

recycling[115], suggesting that the stability of the receptor-β-arrestin interaction also influences the fate of the internalized GPCR.

Recent observations have suggested new advances in the classical model of GPCR desensitization. First, it is clear that, apart from mediating desensitization, GRKs and arrestins are also able to act as signal initiators by acting as multi-protein scaffolds, leading for example to G protein-independent and arrestin-dependent activation of mitogen-activated protein kinases[116-117]. Second, in some cases GRKs are able to mediate phosphorylation-independent as well as arrestin-independent desensitization. This is seen for GRK2 and GRK3 particularly with Gq-coupled receptors, where the kinase binds to the GPCR and also to Gq via the N-terminal RGS-like region of the GRK, thus preventing coupling between GPCRs and G proteins[118]. For some GPCRs, such as the group I metabotropic glutamate receptors (mGluRs)[119] and parathyroid hormone receptor (PTHR)[120], this type of phosphorylation-independent desensitization by GRK2/3 probably represents an important regulatory mechanism in vivo[118]. Third, even the idea of GPCR internalization being necessary for dephosphorylation and resensitization of the receptor needs reassessment in light of recent findings that the β2-AR[121], and the thyrotropin-releasing hormone (TRH) receptor[122] can undergo dephosphorylation at the cell surface in the absence of internalization. Finally, the phenomenon of dimerization must be considered. Thus, although agonist occupancy is normally required for GRK phosphorylation of a GPCR, a recent study shows that a novel Ca^{2+} signal generated by a D1–D2 dopamine heterodimer can be

desensitized in a GRK-dependent fashion by pretreatment with agonists able to activate only one receptor in the heterodimer[123]. The classical view of GPCR regulation is thus being regularly updated, and it is evident that there is much GPCR subtype-dependent variation in the mechanisms involved, also because another important variable is the cell type in which the GPCR is expressed.

Constitutively desensitized receptor mutants in congenital disorders

Rhodopsin, which is covalently bound to its inverse agonist 11-cis-retinal, is unique among GPCRs because its background noise (constitutive activity) is virtually zero. It has been calculated that one rhodopsin molecule can undergo spontaneous activation once in 2,000 years[124]. Otherwise most GPCRs have detectable levels of constitutive (agonist-independent) activity[125] and certain naturally occurring mutations enhancing their constitutive activity have been shown to underlie a variety of human congenital disorders, ranging from stationary night blindness to various forms of cancer (reviewed in Seifert et al. [125]).

Because the receptor conformations preferred by G proteins, GRKs, and arrestins are those assumed by the activated receptor, in theory one would expect these constitutively active mutants to be subjected to GRK- and arrestin-dependent desensitization. The first experimental proof of that was found in the visual system. Several rhodopsin mutants that constitutively activate transducin in biochemical assays, and cause night blindness or retinal degeneration in humans, were shown to be constitutively phosphorylated by rhodopsin kinase and bind visual arrestin, suggesting that the disease

phenotype may be the result of either their uncontrolled signaling or persistent desensitization[126]. On the same lines, two forms of constitutively active luteinizing hormone receptor were found to internalize faster than the wild-type receptor via an arrestin-dependent pathway[127]. Also the naturally occurring vasopressin receptor mutation R137H associated with familial nephrogenic diabetes insipidus, that was originally described as a loss-of-function mutation, actually is described as a constitutive arrestin-mediated desensitized receptor. In fact, contrary to the wild-type vasopressin receptor, this "non-signaling" R137H receptor is phosphorylated and sequestered in arrestin-associated intracellular vesicles even in the absence of agonist. By eliminating molecular determinants on the receptor that promote high-affinity arrestin–receptor interaction re-establishes plasma membrane localization and the ability of the mutated receptors to signal[128]. Thus, in the case of constitutively active GPCRs, persistent desensitization can overwhelm persistent signaling and directly contribute to the etiology of a mutation-induced disease.

SCOPE OF THE THESIS

The aim of this research work is to further understand the unique features of the heterotrimeric G protein Gα15/16, with greater attention to its expression pattern profile and its signalling properties.

CHAPTER 2 presents new experimental data showing that the promiscuous coupling to G15 makes the signalling of three different GPCRs more resistant to β-arrestin dependent desensitization.

CHAPTER 3 describes all the peculiarities of Gα15/16 thus far identified, and highlights some aspects that could foresee a role for this protein beyond hematopoiesis.

CHAPTER 4 draws the possible future developments of this research project and presents some preliminary data demonstrating that Gα15/16 is expressed in human tumor cell lines and that Gα15/16 mRNA could undergo alternative splicing.

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Chapter 2

“Heterotrimeric G proteins demonstrate differential sensitivity to β-arrestin dependent desensitization”

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Abstract

G15 is a heterotrimeric G protein of the Gq/11 family. In this study, we describe its exceptional poor sensitivity to the general regulatory mechanism of G protein-coupled receptor (GPCR) desensitization. Enhancing β2 adrenergic receptor desensitization by arrestin over-expression, did not affect signalling to G15. Similarly, increased levels of arrestin did not affect G15 signalling triggered by the activation of V2 vasopressin and δ opioid receptors. Furthermore, co-immunoprecipitation experiments showed that G15 α-subunit (as opposed to Gαq and Gαs) is recruited to a V2 vasopressin receptor mutant that is constitutively desensitized by β-arrestin. Interestingly, co-expression of Gα15 partially rescued cell surface localization and signalling capabilities of the same mutant receptor and reduced β2 adrenergic receptor internalization. Taken together, these findings provide evidence for a novel mechanism whereby GPCR desensitization can be bypassed and G15 can support sustained signalling in cells chronically exposed to hormones or neurotransmitters.

Introduction

Heterotrimeric G proteins, formed by the α and βγ subunits, support cellular signalling by coupling seven trans-membrane receptors to intracellular effectors like adenylyl cyclase, phospholipase C (PLC) and small GTPases. Virtually all aspects of physiology are affected by their activity including neurotransmission, immunity, hemodynamics, metabolism and proliferation, growth, differentiation and death of multiple cell types. G15 is a Gq/11-like G protein that couples GPCRs

to PLC and it is expressed in hematopoietic precursor cells[56]and in activated lymphocytes[55]. Gα15 is the most divergent member of the Gq/11 subfamily. It has been acquired relatively late during evolution, likely by gene duplication[24] and possesses the unique ability to couple to a wide variety of GPCRs in a number of cell systems[76]. Although its physiological role remains unclear, this striking property made G15 suitable in high throughput screenings as an almost universal adapter that conveniently couples orphan GPCRs to a measurable downstream signalling pathway[129]. G proteins accurately transmit extracellular signals relying on sophisticated regulatory mechanisms. The strength of their signalling is timely regulated by receptor desensitization, i.e. the loss of response subsequent to prolonged or repeated administration of an agonist[99]. A group of kinases, termed GPCR kinases (GRKs), initially phosphorylate the receptor carboxyl-terminal tail. Phosphorylation is followed by the interaction with two conserved and ubiquitously expressed arrestin isoforms: β-arrestin 1 and β-arrestin 2. As a result, the receptor is withheld from its cognate G protein and it is targeted to endosomal vesicles[130]. Arrestins follow internalizing GPCRs inside the cell but, depending on the receptor subtype, the interaction may be transient (e.g. β2 adrenergic receptor (β2AR)) or stable, with β-arrestin escorting the GPCR to endosomal compartments (e.g. V2 vasopressin receptor (V2R))[131]. The impact of desensitization on the coupling between GPCRs and G15 has yet to be investigated. In this study we report that G15-mediated signalling of β2AR, V2R, and δ-opioid receptor (DOR) is markedly resistant to arrestin-induced desensitization.

Results

GPCRs signalling to G15

To verify that G15 couples multiple GPCRs to various downstream signalling pathways, we co-expressed its α-subunit (Gα15) in COS-7 cells with three different GPCRs. The β2AR is normally coupled to Gs and Gi and therefore it does not efficiently couple to PLC. In fact, as shown in Figure 9a (pag.80), addition of 10 µM Isoproterenol (Iso) to cultured COS-7 cells did not induce any significant increase in inositol phosphate production. The expression of Gα15, (with or without the β2AR), produced only a slight increase of the inositol phosphate concentration, while addition of Iso induced a dramatic increase above the background. Thus, Gα15 transfection made PLCβ responsive to Iso in COS-7 cells.

A similar result was obtained with the DOR, that is primarily coupled to Gi[85]. Like in the case of the β2AR, inositol phosphate production could be stimulated by an opioid agonist (10 µM [d-Pen2,5]-enkephalin (DPDPE)) provided that COS-7 cells were previously co-transfected with Gα15 (Figure 9b, pag.80).

The V2R is naturally coupled to Gs and adenylyl cyclase[132] and also activate PLCβ [133-134]. Accordingly, there was a significant increase in inositol phosphate production in response to vasopressin (AVP). However the expression of Gα15 significantly ($p<0.01$) enhanced V2R mediated PLC signalling (Figure 9c, pag.80).

These experiments demonstrate that all three GPCRs can efficiently couple to G15.

β2AR signalling to G15 is poorly affected by β-arrestin

We next determined whether G15 sensitivity to GPCR desensitization was similar to other G proteins. β-arrestin opposes prolonged GPCR activity by preventing the stimulatory interaction between receptor and G protein. We utilized COS-7 cells, which express low levels of endogenous β-arrestins [135], to over-express β-arrestin 1 with β2AR and Gα15 and directly assess the efficiency of arrestin dependent desensitization. As expected, β-arrestin 1 significantly ($p<0.01$) attenuated β2AR coupling to Gs reducing cAMP accumulation induced by 1 h stimulation with 10 μM Iso (Figure 10a, pag.81). In striking contrast, β2AR coupling to G15 was substantially insensitive to β-arrestin 1 over-expression, leaving unaffected agonist promoted inositol phosphate accumulation (Figure 10b, pag.81).

To confirm that what observed for Gα15 was not the result of a differential sensitivity of downstream effectors to the increased β-arrestin levels, we monitored intracellular Ca^{2+} mobilization utilizing a protocol of desensitization based on repeated agonist stimuli in HEK-293T. Upon transfection with β2AR and either Gα15 or Gαq, we quantified the extent of the transient increase in free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) following Iso stimulation (challenge, Figure 11, pag.82). After washing away the ligand, the cells were stimulated again and this second $[Ca^{2+}]i$ increase was also quantified (re-challenge). The response to the second challenge of 10 μM Iso was significantly ($p<0.01$) attenuated (~50%) as compared to the first stimulation in the presence of Gαq, but not in the presence of Gα15. Therefore we demonstrated by two different approaches that agonist induced signalling of β2AR to G15 was resistant to GPCR desensitization.

We hypothesized that Gα15 interacts with the receptor so effectively to compete out not only β-arrestins, but possibly also other G proteins. If this is the case, G15 presence should reduce the ability of GPCRs to couple to other G proteins. Indeed, the effect of Iso on cAMP accumulation was significantly ($p<0.01$) reduced when Gα15-EE was co-transfected with β2AR (Figure 12, pag.82).

The poor sensitivity to β-arrestin dependent desensitization was not unique to the signalling triggered by the β2AR. Inositol phosphate production stimulated by DPDPE in COS-7 cells co-transfected with Gα15 and DOR was also unaffected by β-arrestin 1 over-expression (Figure 13a, pag.83).

Unlike the β2AR and the DOR, in the case of the V2R the efficiency of β-arrestin dependent desensitization could not be analyzed without keeping into account coupling toward endogenous Gq and G11 (Figure 9c, pag.80). In the absence of Gα15, the over-expression of ubiquitous β-arrestin 1 and 2 isoforms reduced to 35±9% and 36±11% (respectively) the inositol phosphate production induced by 100 nM arginine vasopressin (AVP) via endogenous Gq/11 (Figure 13b, pag.83, inset table). When Gα15 was co-transfected, a β-arrestin dependent reduction was still observed, however, a large fraction of the stimulation was left intact. This result appears consistent with β-arrestin dependent desensitization being effective only on the Gq/11 component of the signal while leaving unaffected the G15 component. As an approximation, to separate the G15 component from the Gq component, the values obtained in the absence of G15 were subtracted from the value obtained in the presence of Gα15 (net of the basals). This operation was repeated for the control samples and for the two

arrestin samples. The estimated G15 contribution to V2R signalling was totally unaffected by both β-arrestin 1 and β-arrestin 2 over-expression (Figure 13b, pag.83, inset table).

Phosphorylation is known to stabilize β-arrestin binding to GPCR[136].Therefore we sought to rule out the possibility that only the phosphorylated form of the V2R was signalling through G15 by using a phosphorylation-resistant mutant. We previously demonstrated that shortening the V2R carboxy terminal tail, by the insertion of a stop codon at position 345 (V2R-S345ter), eliminates all phosphorylation sites without affecting the affinity for AVP or the coupling to Gs[137]. When Gα15 was co-expressed with V2R-S345ter or with V2R WT the inositol phosphate accumulation induced by AVP was similar (Figure 13c, pag.83). This ruled out a preferential interaction between G15 and the phosphorylated state of the receptor, suggesting that the protein complex forms regardless of the presence of the carboxy-terminus and its multiple phosphorylation sites[137].

G15 reveals the activity of a fully desensitized GPCR

A mutant form of the V2R (V2R-R137H) has been proposed to be constitutively desensitized even in the absence of ligand. The substitution to histidine of the central arginine of the DRY motif stabilizes the receptor in a conformation that remains stably associated with β-arrestin[128]. As a consequence, the V2R-R137H is incapable of activating Gs and adenylyl cyclase as demonstrated by heterologous expression in HEK-293T[128] and L- [132] cells and by the occurrence of this mutation in patients suffering of nephrogenic diabetes insipidus. Accordingly, the V2R-R137H transfected in COS-7 cells did not

promote inositol phosphate accumulation, neither in the presence of endogenous Gαq/11 (data not shown) nor of over-expressed Gαq tagged with the epitope EYMPTE (EE) (Figure 14, pag.84).

We hypothesized that Gα15 would be able to circumvent β-arrestin induced desensitization and recognize the hyper-phosphorylated mutant. The V2R-R137H was thus co-expressed with Gα15 and inositol phosphate accumulation was measured. Gα15-EE, and Gαq-EE as a control, were expressed to comparable levels. The presence of Gα15-EE restored V2R-R137H signalling toward PLC. The effect was not due to an increase in receptor expression, since comparable receptor levels were present regardless of the type of α-subunit transfected (Figure 14, pag.84). Supporting the hypothesis that the V2R-R137H represents a receptor fully locked in an active conformation, AVP could not further increase V2R-R137H activity. Similar results were obtained utilizing equivalent amounts of cDNAs of the untagged versions of Gαq and Gα15 (data not shown). Gα15 appears therefore capable of circumventing the steric hindrance created by the stable interaction between β-arrestin and V2R-R137H[128].

Co-immunoprecipitation experiments were set up to analyze the possibility that G15 displays a stronger affinity for the V2R-R137H as compared to other G proteins. All Gα subunits were expressed to similar levels (as assessed by direct comparison in western blot) (Figure 15a, pag.84). When equal amounts of the constitutively active receptor were precipitated (Figure 15b, pag.84), only Gα15-EE was found associated to the V2R-R137H, and β-arrestin over-expression did not prevent its interaction with V2R-137H (Figure 15b, pag.84).

One of the consequences of the stable interaction with β-arrestin[138],[139] is that most of the V2R-R137H is sequestered intracellularly[128, 140]. We hypothesized that, by interacting with the mutant receptor, G15 could revert the constitutive internalization of the V2R-R137H and rescue it to the cell surface. The amount of receptor on the plasma membrane was monitored under non permeabilizing conditions by flow cytometry. An HA epitope placed at the amino-terminus of the receptor was utilized for this purpose (Figure 16a-c, pag.85). Gα15 expression restored a significant fraction of V2R-R137H to the cell surface (Figure 16d, pag.85). We also determined if β-arrestin over-expression could reverse this effect and β-arrestin had no effect on V2R-R137H expression on the cell surface. Moreover Gα15 over-expression significantly reduced Iso dependent β2AR internalization (Figure 16e, pag.85).

Discussion

GPCR desensitization is a fundamental regulatory mechanism that finely tunes receptor activity and matches cellular responsiveness to the intensity of agonist stimulation. Such signal integration is highly relevant not only under normal conditions but also under pharmacological treatment of many diseases, when GPCR agonists typically induce receptor desensitization.

Our experiments demonstrate that the coupling of distinct GPCRs (i.e. β2AR, DOR, V2R) to G15 is unexpectedly resistant to this phenomenon. The resistance to desensitization was verified on a rapid recycling receptor like the β2AR (that immediately detaches from arrestin upon internalization) as well as on a slow recycling receptor

like the V2R (that brings arrestin to endosomal compartments). Furthermore, the effect was confirmed by analyzing GPCR desensitization either by focusing on arrestin dependent regulation or by inducing pharmacological desensitization upon repeated agonist exposure. The poor sensitivity of G15 to β-arrestin dependent desensitization therefore adds a novel feature to its atypical signalling properties.

The process of receptor desensitization is not strictly limited to agonist-occupied receptors, since homologous phosphorylation and interaction with β-arrestin also occur when the receptor becomes active spontaneously[141] or when specific mutations lock it in an active conformation[128]. One example is the V2R-R137H, a mutant V2R uncoupled from both Gs and Gq by desensitization. Similar to other mutations found in rhodopsin[142], in the alpha 1B adrenergic receptor and in the angiotensin II type 1A receptor[138], the constitutive activity promoted by the mutation of a conserved arginine[143] residue is associated with a receptor that is highly phosphorylated, constitutively bound to β-arrestin and mostly localized inside the cell. We found that G15 circumvents V2R-R137H desensitization behaving differently from other heterotrimeric G protein subtypes, such as its homolog Gq (Figure 14 and Figure 15). G15 ability to resist GPCR desensitization better than other G proteins was further substantiated by the observation that, as compared to Gαs or Gαq, Gα15 interacts with higher affinity with the immunoprecipitated V2R-R137H.

Alternative explanations are available to justify G15 remarkable resistance to β-arrestin action. G15 could recognize a receptor activation domain that remains unshielded by β-arrestin docking.

Otherwise a tight interaction of Gα15 with the receptor could displace β-arrestin and other G proteins from a common binding site. In fact, for rhodopsin, a competition between transducin and cone arrestin for the same docking region underlies desensitization[144]. The V2R-R137H mutant maintains high affinity binding for AVP[140], however, both, G15 and arrestin interactions are only marginally affected by the addition of AVP (Figure 14 and Bernier et al.[145]). As well, the phosphorylation of the V2R-R137H C-terminus is poorly increased by the interaction with the agonist[128, 137]. Despite these similarities, we did not observe a cause-effect relationship between phosphorylation and the interaction with G15, as the latter was not prevented by eliminating the C-terminus.

Further work is required to identify the molecular determinants that allow G15 to recognize so many different GPCRs under 'non desensitizing' conditions[56, 146-147] and now this analysis should also keep into account the desensitized form of the GPCR.

We offer a novel perspective to unravel the physiological significance of G15. The apparently normal hematopoiesis and inflammatory response observed in Gα15 knock-out mice[24] is likely due to the good level of inter-change ability among Gq family members. However, compensatory responses could become inadequate when the stimulation becomes particularly protracted or intense. 'Sustained' G15 signalling could also be particularly relevant to explain enduring and ligand-independent signals generated by GPCRs with high constitutive activity. One example is represented by the pertussis toxin-resistant constitutive signalling generated by human cytomegalovirus-associated pUS28[148]. Signalling generated by this

viral receptor is reminiscent of what observed with the V2R-R137H as it occurs despite the fact that the carboxyl-terminus of the receptor is constitutively phosphorylated by GRK and while agonist-independent receptor endocytosis traps part of the protein in perinuclear endosomes[149]. G15 is expressed within a short time window during early hematopoiesis[56] or upon lymphocytes activation[55]. Its expression could be related to the need for producing potent and durable signalling under specific circumstances, such for instance antigen-induced lymphocytes activation and expansion. Given its promiscuous nature, G15 could sustain prolonged and intense stimulations amplifying the signalling generated by the many GPCRs present in hematopoietic cells or in lymphocytes (including the vasopressin[150], adrenergic[151-152] and opioid receptors[153-155]). A poorly regulated signalling could become relevant also to pathological conditions implying GPCR hyperstimulation, as for instance in cancer development[156].

Conclusion

We showed that the promiscuous coupling to G15 makes the V2R, β2AR and DOR signalling more resistant to β-arrestin dependent desensitization. This may provide a novel mechanism by which GPCRs generate sustained stimulation, in particular under physiological and/or pathological conditions requiring very intense signalling activity such as inflammation and cancer.

Materials and methods

Materials

pCIS-Gα15 plasmid was kindly donated by Dr Stefan Offermanns (Universitat Heidelberg, Heidelberg, Germany). β2-adrenergic and δ-opioid receptors cloned in pcDNA3 expression vectors were generous gifts of Dr Tommaso Costa (Istituto Superiore Sanità, Rome). pcDNA3-Gα15-EE plasmid was purchased from the Guthrie cDNA Resource Center (http://www.cdna.org). Expression vectors for β-arrestins were generous gifts of Dr Antonio De Blasi (Istituto Neurologico Mediterraneo, Neuromed, Pozzilli, Italy). The pcDNA3-V2 vasopressin receptor plasmids were previously described[157].

Cell culture and transient transfection

COS-7 and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. Cells (30–50% confluent) were transfected using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) and routinely analyzed 48 h after transfection. The total amount of transfected DNAwas kept constant by compensation with control plasmid.

Phosphoinositol accumulation

Accumulation of inositol phosphate was measured by a modification of the method by Hung et al. [133]. COS-7 cells were grown in 12-well tissue culture plates and 36 h after transfection, each well was supplemented with 2 μCi/ml of myo-[^{3}H]inositol. Following overnight

labeling, cells were rinsed three times at room temperature with 1 ml of washing buffer [Dulbecco's phosphate buffered saline (D-PBS), supplemented with 5.5 mM glucose, 0.5 mM $CaCl_2$, and 0.5mM $MgCl_2$]. Cells were then incubated at 37°C for 30min in 0.5ml D-PBS supplemented with 5 mM LiCl to inhibit inositol monophosphatase. The incubation was continued for 1 h at 37°C. Agonists were added 10 min after LiCl. At the end of the incubation, the supernatant was removed and 0.75 ml ice-cold 20 mM formic acid was added to each well to extract the produced IP. Inositol phosphate was separated from myo-[^{3}H]inositol by a simplified ion exchange chromatographic procedure[133]. Briefly, after 1 h on ice, the 20 mM formic acid extracts were applied to Dowex AG 1-X8, 100–200 mesh, formate form columns (0.6 cm diameter, 1.0 ml bed volume; BioRad, Hercules, CA) that had been sequentially pre-rinsed with 2 M ammonium formate/0.1 M formic acid, water, and 20 mM ammonium hydroxide adjusted to pH 9.0 with formic acid. Immediately after sample loading, 3 ml of 40 mM ammonium hydroxide, pH 9.0, were added to each column and the eluates collected into vials containing 10 ml of scintillation fluid (ULTIMA-FLO AF; Packard Instruments Inc., Palo Alto, CA). These first eluates were previously shown to recover the vast majority (98%) of myo-[^{3}H]inositol present in the samples[133]. The columns were then washed three times with 4 ml of 40 mM ammonium formate and inositol phosphates were eluted with 5 ml of 2 M ammonium formate/0.1 M formic acid into scintillation vials containing 15 ml of scintillation fluid. To normalize the accumulation of inositol phosphate over total [^{3}H]inositol incorporated, the c.p.m. of [^{3}H]IP

(last eluate) were divided by the sum of c.p.m. of myo-[^{3}H]inositol (first eluate) plus c.p.m. of [^{3}H]IP
and expressed as percentages.

Western immunoblotting

COS-7 cells were transfected as described in the previous paragraph. After removal of culture medium cells were lysed in Nonidet P-40 (NP40) buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% v/v NP40) containing protease inhibitor cocktail (Sigma Aldrich, Saint Louis, MO) and spun in a microcentrifuge at 11,000 ×g. Protein contents of supernatants were measured by the BCA assay (Pierce, Rockford, IL) and equal amounts of proteins were resolved by SDS-PAGE (12% acrylamide) and transferred to a Hybond-P membrane (GE Healthcare-Amersham, Piscataway, NJ). Membranes were blocked by incubation with 5% (w/v) non fat dry milk and hybridized with primary anti-EE monoclonal Ab (Covance, Princeton, NJ) or anti-HA rabbit polyclonal Ab and horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary Abs (Pierce, Rockford, IL). Antigen–antibody complexes were detected using SuperSignal West Dura chemiluminescent substrates (Pierce, Rockford, IL) according to the manufacturer's instructions, and visualized with Kodak Image Station 440.

cAMP accumulation

48 h after transfection cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS, incubated 15 min at 37°C in PBS supplemented with 4 mM EDTA, and scraped. After centrifugation cell pellets were resuspended

at a density of 10^6 cells/90 µl in D-PBS supplemented with 5.5mM 3-isobutyl-1-methylxanthine. Samples were equilibrated for 15 min at 37°C and treated for 1 h with agonists or assay buffer (basal) at 37°C. cAMP accumulation was stopped by placing the tubes in liquid nitrogen and subsequent boiling for 5 min. Samples were then spun for 8 min at 12,000 rpm in a microcentrifuge and supernatants were immediately used for the assay. cAMP content was quantified by means of a competitive binding cAMP assay kit (GE Healthcare-Amersham) following manufacturer's instructions.

Determination of cytosolic free Ca^{2+} levels

Determination cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]i$) was performed as previously described[158]. Briefly, HEK-293T cells were seeded on sterile coverslips coated with poly-D-lysine. After an incubation for 30 min at 30°C in the dark with 2 µM Fura 2/AM, the dye was removed and the cells were further incubated for 30 min at 30°C to complete the Fura 2/AM hydrolysis. After loading, cells were washed twice with PBS and transferred to the spectrofluorimeter, where fluorescence was monitored at 37°C (505 nm emission, 340 and 380 nm excitation). To extrapolate Ca^{2+} concentration from the fluorescence recording, the system was calibrated as follows: F_{max} was obtained by adding 2 µM ionomycin and 100 µM digitonin, and F_{min} was obtained by adding 5 mM EGTA and 60 mM Tris base.

Co-immunoprecipitation

COS-7 cells were grown and transfected in 100 mm tissue culture dishes using 4 µg of plasmid DNA encoding for HA tagged V2R-

R137H, EE-tagged Gα subunits and β-arrestin 1. 48 h post transfection, cells were lysed in 1 ml of 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, supplemented with protease inhibitor cocktail (Sigma Aldrich). After 1 h at 4°C, the particulate was removed from the samples by centrifugation at 21,000 ×g. Immunoprecipitation was performed for 16 h at 4°C using anti-HA monoclonal Ab previously crosslinked to CNBr-activated Sepharose 4B beads (GE Healthcare- Amersham). Immune complexes were washed three times with 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% (v/v) NP-40 and eluted in Laemmli sample buffer. Samples were separated by SDS-PAGE (12% acrylamide) and analyzed by immunoblotting as described.

Receptor internalization

HA epitope tagged receptors in 12-well dishes were incubated with or without agonist for 30 min in serum-free medium at 37°C. Cell surface receptors were labeled with 12CA5 mAb, and Alexa 488-conjugated goat antibody against mouse IgG as a secondary antibody. Receptor internalization was quantified as loss of cell surface receptors as measured by fluorescence-assisted cell sorting.

Statistical analysis

Data were evaluated using GraphPad Prism version 4. Statistical comparison of multiple groups was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Data are expressed as means±S.E.M unless otherwise indicated.

Legend of figures

Figure 9 - G15 signalling promiscuity in COS-7 cells.

COS-7 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with myo-[^{3}H]inositol overnight. Accumulation of [^{3}H]inositol phosphates was measured in the presence of 5 mM LiCl as described. The inositol phosphate isolated was normalized for the total amount of myo-[^{3}H]inositol incorporated (see Materials and methods). Values are expressed as fold increase over the basal PLC activity level of cells transfected with an irrelevant plasmid. Data represent average ± S.E.M. of three independent experiments performed in triplicate (**p<0.01). In the right side panels, immunoblotting analysis of the expression levels of the GPCR, Gα15 and endogenous caveolin 1. a) Cells co-expressing Gα15 and β2AR stimulated by 10 µM Iso. b) Cells co-expressing Gα15 and DOR stimulated by 10 µM DPDPE. c) Cells co-expressing Gα15 and V2R stimulated by 100 nM AVP.

Figure 10 - β2AR signalling through G15 resists β-arrestin dependent desensitization.

The β2AR was transfected in COS-7 cells with or without β-arrestin 1. a) Stimulation of cAMP accumulation was compared by setting to 100% the maximal stimulation (10 µM Iso) obtained in control transfected cells (with β Gal cDNA instead of β-arrestin 1 cDNA). Stimulated β2AR dependent accumulation of cAMP was significantly dampened in cells co-expressing β-arrestin 1 (**p<0.01). Data represent average±S.E.M. of three independent experiments

performed in duplicate. b) The cotransfection of β-arrestin 1 did not produce a statistically significant effect on the stimulated β2AR dependent accumulation of inositol phosphate. Data represent average±S.E.M of four independent experiments performed in triplicates. In the right side panels, immunoblotting analysis of the expression levels of β2AR (anti-HA antibody), Gα15-EE (anti-EE antibody), β-arrestin 1 and endogenous caveolin 1.

Figure 11 - β2AR signalling through G15 resists desensitization induced by repeated stimulation.

$[Ca^{2+}]i$ elevation was induced by 10 μM Iso in HEK-293T cells co-expressing β2AR and similar levels of either Gα15 or Gαq carrying the same EE tag (western blot at the right side illustrating also the expression levels of endogenous caveolin 1 as a reference). The peaks of $[Ca^{2+}]i$ transient were measured after the first agonist challenge (set as 100%) and upon re-addition of agonist after 5-min washout (rechallenge). The response to the first Iso stimulation in cells transfected with Gα15-EE was 90%±13% than in cells transfected with Gαq-EE. Data represent means of $[Ca^{2+}]i$ stimulation over basal±S.E.M. of at least three independent experiments performed in triplicate (**$p<0.01$).

Figure 12 - G15 competes Gs signalling

Co-transfection of Gα15 significantly reduced (**$p<0.01$) cAMP accumulation stimulated by 10 μM Iso in cells expressing the β2AR (see immunoblot in Figure 9a). Data represent average±S.E.M. of three independent experiments performed in duplicates.

Figure 13 - G15-mediated signalling of DOR and V2R is not affected by β-arrestin 1 over-expression.

a) In COS-7 cells transiently transfected with DOR and Gα15, 10 μM of DPDPE stimulated inositol phosphate accumulation to the same extent in the absence or in the presence of over-expressed β-arrestin 1. Values are expressed as fold increase over the basal PLC activity level of cells transfected with an irrelevant plasmid. Data represent average±S.E.M. of three independent experiments performed in triplicates. b) COS-7 cells expressing V2R, with or without Gα15 and β-arrestin 1 or 2 as indicated (western blot in the inset panels illustrate the expression levels of proteins as indicated), were stimulated with 100 nM AVP. Data represent one representative experiment performed in triplicate (average±S.D.). The same experiment was repeated four times and averaged (table below). In each experiment, the Gq/11-dependent stimulation measured in the absence of over-expression of Gα15 and β-arrestin (Control) was set as 100%. β-arrestin-resistant signalling was calculated as the residual inositol phosphate accumulation following β-arrestin over-expression. To estimate the G15 component, the endogenous Gq/11 contribution was algebraically subtracted for each condition (average±S.E.M.). c) Inositol phosphate accumulation stimulated by 100 nM AVP was not different in COS-7 cells transfected with V2RWT or with the phosphorylation-defective V2R-S345ter mutant. Data represent average±S.E.M. of three independent experiments performed in triplicates.

Figure 14 - G15 couples to the constitutively desensitized V2R-R137H mutant.

EE-tagged Gαq and Gα15 were individually co-expressed with HA tagged V2R-R137H in COS-7 cells. Receptor dependent inositol phosphate accumulation is observed in the presence of Gα15-EE (**$p<0.01$) but not Gαq-EE. Values are expressed as fold increase over the basal PLC activity level of cells transfected with Gαq and an irrelevant plasmid instead of V2R-R137H. Data represent average±S.E.M. of four independent experiments performed in triplicates. In the right side panels, immunoblotting analysis of the expression levels of HA-V2R-R137H (anti-HA antibody), Gαq-EE and Gα15-EE (anti-EE antibody).

Figure 15 - Gα15 stably interacts with constitutively desensitized V2R-R137H.

a) EE-tagged Gαq, Gα15 and Gαs were individually co-expressed with the HA tagged V2R-R137H in COS-7 cells as indicated. Gα proteins total expression levels were compared by western blot in whole cell lysate (upper panel). The V2R-R137H was immunoprecipitated utilizing a monoclonal antibody against the HA epitope and immunoblotted with polyclonal anti-HA (middle panel) or anti-EE antibodies (lower panel). b) As described above, Gα15-EE, but not Gαq-EE, was co-immunoprecipitated with the HA-V2R-R137H. The same amount of Gα15-EE was immunoprecipitated (lower panel) in the absence or in the presence of FLAG tagged β-arrestin 1 over-expression (upper panel).

Figure 16 - G15 affects GPCR intracellular trafficking.

Cell surface GPCRs expression was measured by flow cytometry in COS-7 cells transiently transfected with the indicated combinations of constructs. Fluorescent labeling of the receptor was achieved by an antibody directed to the amino-terminal HA epitope in un-permeabilized cells. a–c) FACS profiles representative of four experiments (averaged in d), the black trace represents cells transfected as indicated, the gray trace represents mock transfected cells. d) Specific surface fluorescence was quantified as % of the HA-V2R-WT (a) and plotted as means±S.E.M of three independent experiments (*$p<0.05$, **$p<0.01$). e) β2AR internalization was quantified as the % of surface fluorescence lost upon treatment of the cells with 10 μM Iso, in the presence or in the absence of G15 (**$p<0.01$). Values are plotted as means±S.E.M of five independent experiments.

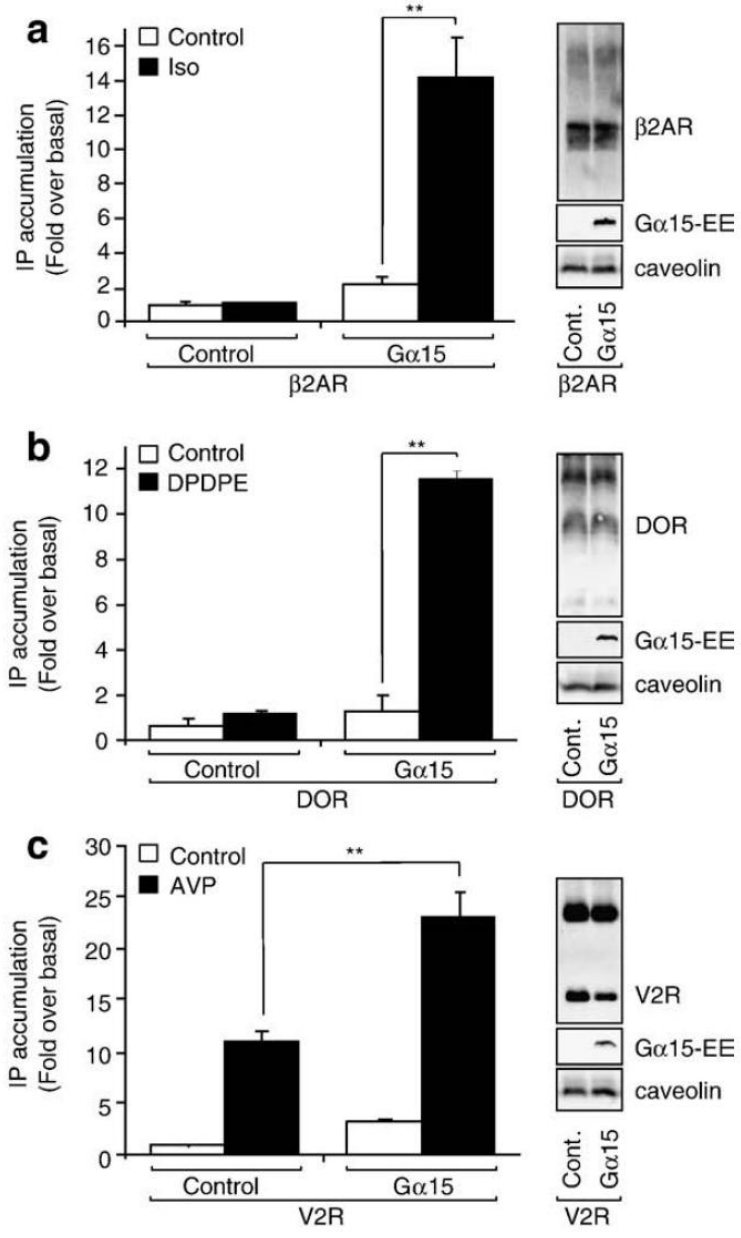


Figure 9 - G15 signalling promiscuity in COS-7 cells.

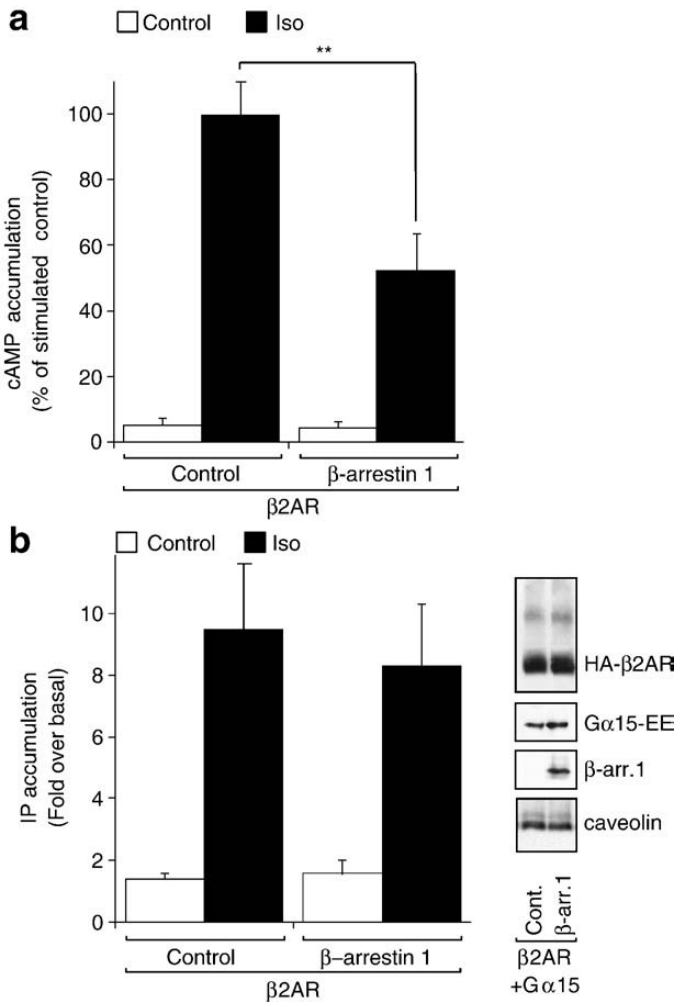


Figure 10 - β2AR signalling through G15 resists β-arrestin dependent desensitization.

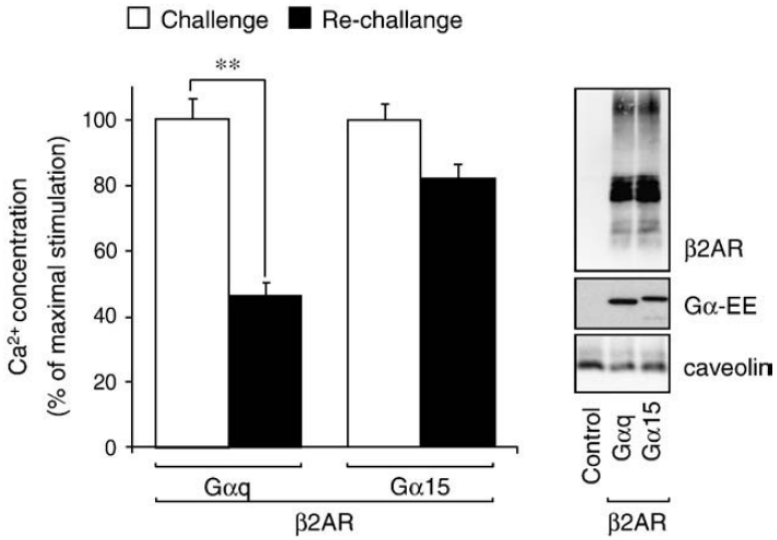


Figure 11 - β2AR signalling through G15 resists desensitization induced by repeated stimulation.

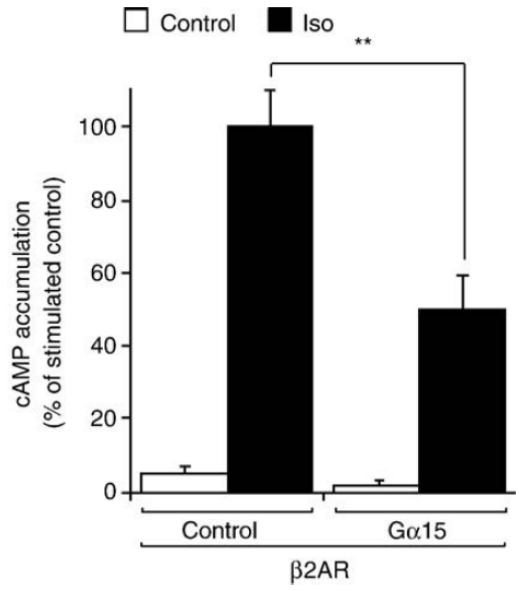
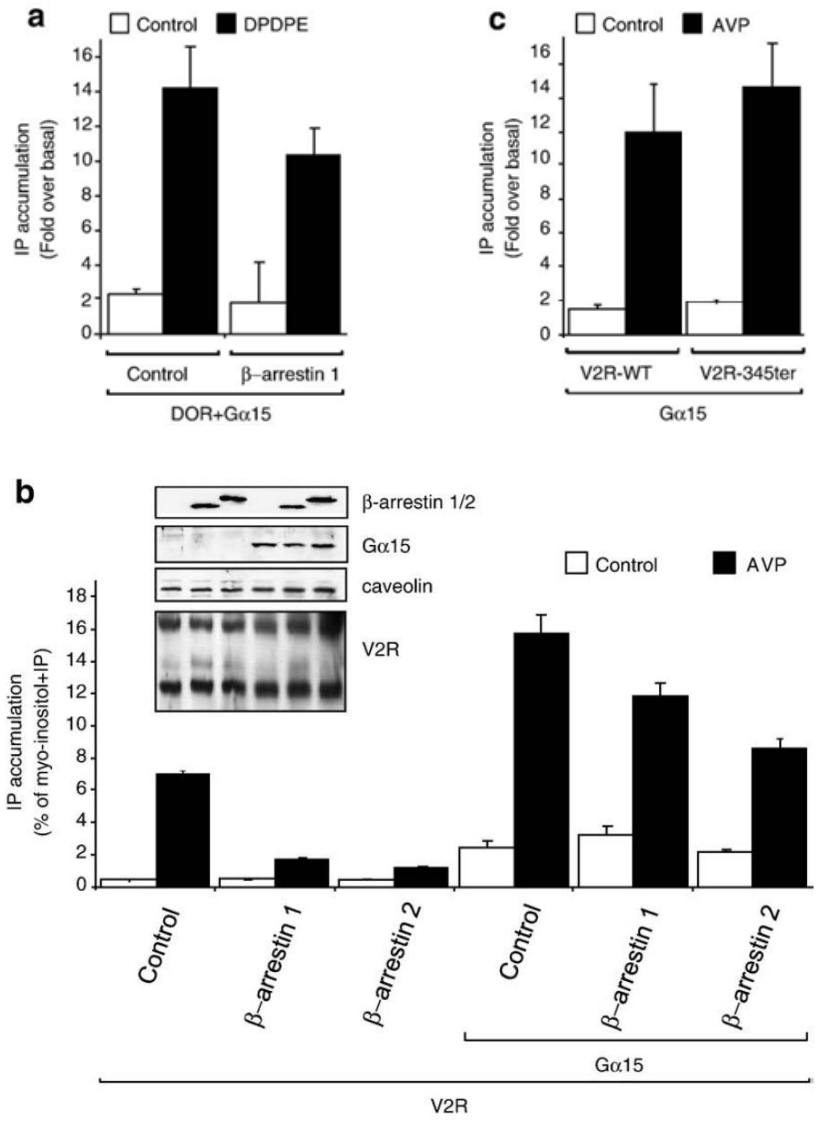


Figure 12 - G15 competes Gs signalling.



	Gq/11			G15		
	average	± S.E.M.	p-val.	average	± S.E.M.	p-val.
Control	100%			119%	± 14%	n.s.
β-arrestin 1	35%	± 9%	<0.001	114%	± 4%	n.s.
β-arrestin 2	36%	± 11%	<0.001	102%	± 14%	n.s.

Figure 13 - G15-mediated signalling of DOR and V2R is not affected by β-arrestin 1 over-expression.

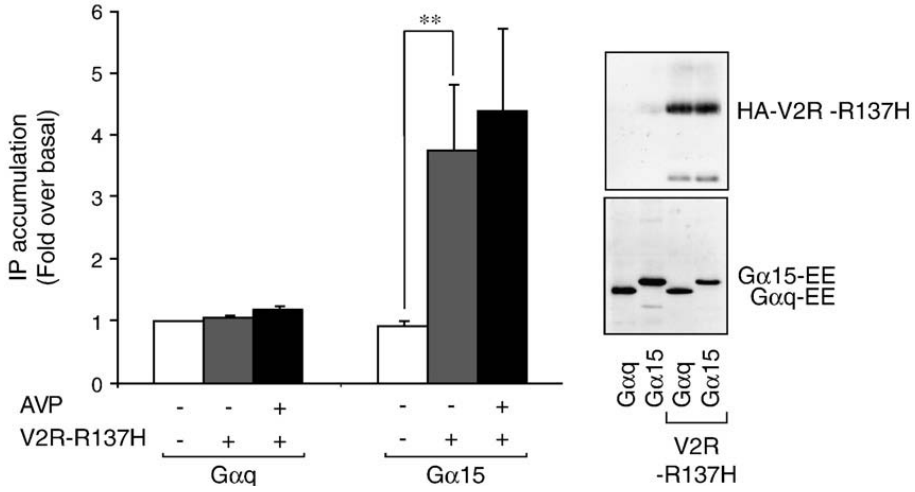


Figure 14 - G15 couples to the constitutively desensitized V2R-R137H mutant.

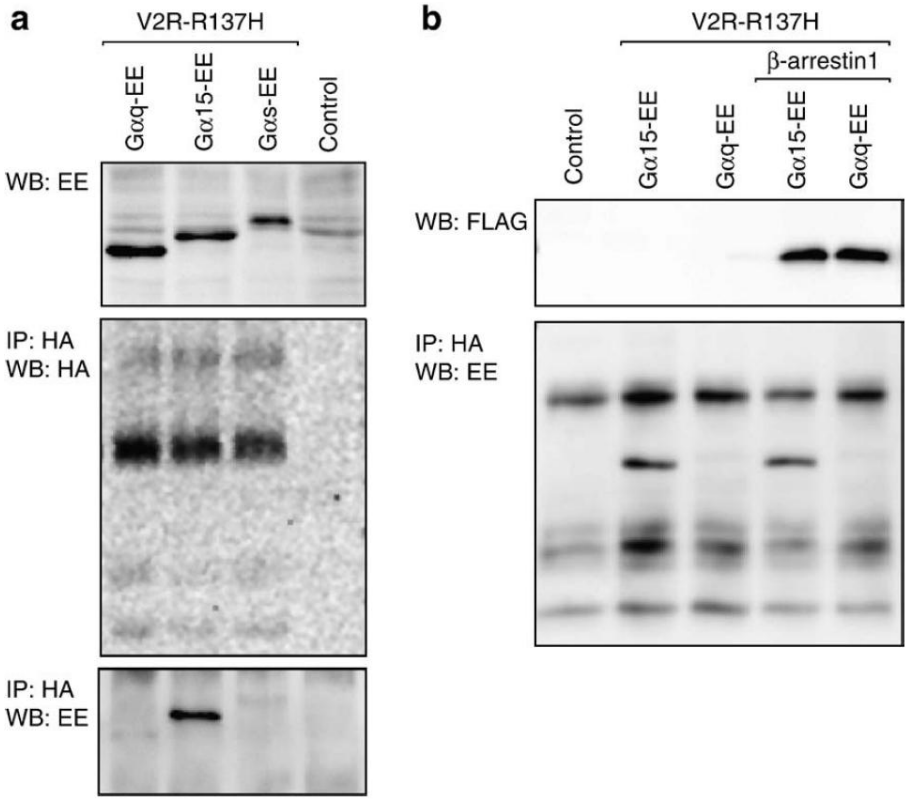


Figure 15 - Gα15 stably interacts with constitutively desensitized V2R-R137H

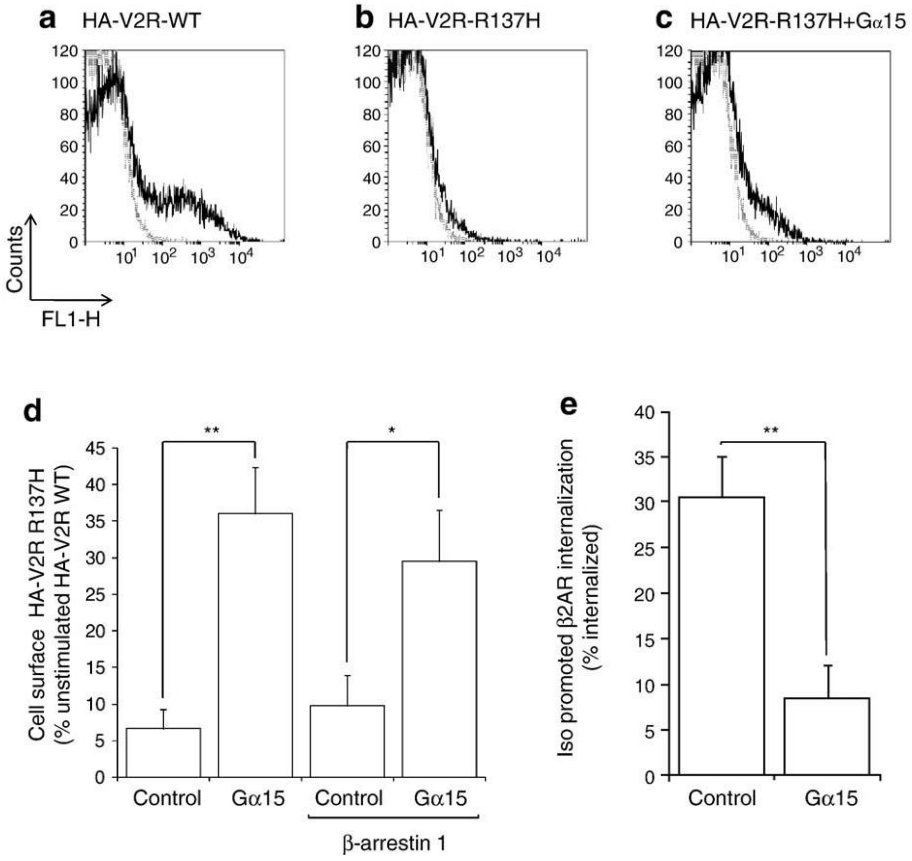


Figure 16 - G15 affects GPCR intracellular trafficking.

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Chapter 3

"The puzzling uniqueness of the heterotrimeric G15 protein and its potential beyond hematopoiesis"

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Abstract

Heterotrimeric G proteins transduce the signals of the largest family of membrane receptors (G protein coupled receptors, GPCR) hence triggering the activation of a wide variety of physiological responses.

G15 is a G protein characterized by a number of functional peculiarities that make its signalling exceptional:

- it can couple a variety of Gs-, Gi/o-, Gq-linked receptors to PLC activation;
- relatively to other G-proteins, it is poorly affected by β-arrestin-dependent desensitization, the general mechanism that regulates GPCR function;
- at the protein level, its expression is only detected in highly specific cell types (hematopoietic and epithelial cells).

G15 α-subunit displays unique structural and biochemical properties and is phylogenetically the most recent and divergent component of the Gαq/11 subfamily. All these aspects shed a mysterious light on G15 biological role, which remains substantially elusive. Thus far, G15 signalling has been analyzed in the context of hematopoiesis. Here, we highlight observations supporting the view that G15 functions may extend further beyond the immune system. In addition, we describe puzzling aspects of G15 signalling that offer a novel perspective in the understanding of its physiological role.

Introduction

The α subunit of the heterotrimeric G15 protein (Gα15) is the most divergent member of the Gαq family sharing 57% amino acid sequence identity with Gαq (for a sequence alignment see Hubbard et

al. 2006[56]). Gα15 was originally cloned in mouse[47]. The human isoform was named Gα16 assuming it represented a novel subtype[49], but later on it was recognized as the poorly conserved ortholog of Gα15 sharing only 85% sequence identity. G15 is best known for its ability to create a functional link between hundreds of different GPCRs and the β isoform of phospholipase C (PLCβ)[129]. For this reason, G15 has often served as a versatile readout, particularly, in the preliminary characterization of orphan GPCRs when the lack of an agonist precludes any functional characterization of the downstream signalling pathway (and viceversa). Powerful cellular platforms for the screening of specific ligands have been created by co-expressing G15 together with orphan GPCRs. Furthermore, thanks to G15, it was confirmed that unconventional GPCRs like OA1[141] and smoothened[159] are indeed G protein-coupled.

Despite the restricted expression profile (Table 7, pag.114) and the great success as a pharmacological tool, a number of gene knockout studies (in animal or cellular models) revealed relatively little about G15 physiological activity (see below). Part of the mystery surrounding the role of this G protein may derive from assumptions drawn perhaps too prematurely after the cloning, such as an exclusive link with hematopoiesis.

The evasive nature of Gα15 expression

Gα15 distribution profile

Hematopoiesis:

In the original characterization, the murine Gα15 was found to be selectively expressed by hematopoietic cells and therefore in the bone

marrow, thymus (where it declines in the adult), spleen and embryonic liver[47, 49]. Consequently, a wealth of attention was focused on early maturation stages of hematopoiesis[56, 93] such as CD34 positive hematopoietic stem cells (HSCs)[50, 160], erythroid precursors[53], megakaryocytic[161] and B cells progenitors[52]. The expression of Gα15 is subsequently lost upon cell maturation. This was reproduced in vitro using inducible cellular models, such as HL60 or WB4 cells[48-50], where the protein expression progressively declines upon acquisition of a neutrophil-like phenotype. On the other end, Gα15 expression may be transiently restored by committed hematopoietic cells upon specific stimulation, as shown by activating quiescent T cells with Leuco A[55].

Based on data collected in four different cell lines, Wilkie et al. concluded that Gα15 is absent in stromal cells lines. In good agreement, we found only minimal mRNA traces and no corresponding protein signal in mesenchymal stem cells from bone marrow or thymic stromal cells, either freshly isolated or cultured (Figure 17a e b, pag.115). It appears therefore that, besides HSCs, other stem cells including more immature stages like embryonic stem cells and yolk do not express Gα15[47]. In summary, G15 expression cannot be generalized to all stem cells but overlaps with the CD34 marker for stem and progenitor cells populations[160, 162].

Epithelia:

Despite broadly described as hematopoietic specific[49, 56, 93], G15 expression was occasionally reported in tissues that are not part of the immune system, particularly in a variety of epithelia. While analyzing by in situ hybridization baboon skin, Rock et coll. [163] reported the

presence of Gα15 in hair follicular epithelium. In the hair follicle bulge of murine skin[164] reside CD34-positive cells that may serve as a reservoir for Langerhans cells as well as other immune cell precursors[165-166].The existence of a population of slowly cycling immature cells originated from hematopoietic precursors could thus explain the presence of Gα15 signal. On the other hand, CD34 was also specifically associated with keratinocyte stem cells characterized by high in vitro clonogenic potential[167] and Gα15 mRNA was found in cultured human keratinocytes from neonatal foreskin (that originate from ectoderm rather than mesoderm) but not in fibroblasts, melanocytes or endothelial cells[163]. Consistent with this finding, we report robust expression in the skin and in the epidermoidal A431 cell line (Figure 17, pag.115). Furthermore, Northern blot analysis detected Gα15 mRNA in rat tongue epithelia[168].

Since mature cutaneous epithelium is maintained by an unknown number of progenitor populations[169], it would be interesting to define in deeper details the cellular localization of Gα15 in these tissues.

Consistent with an expression profile extended to epithelial cells, transcriptional levels comparable to HSCs are reached in cells of an internal epithelium, namely thymic epithelial cells (TECs, Figure 17, pag.115). By contrast, mesenchymal stem cells derived from the same organ resulted negative.

HSCs, TECs and progenitor cells in epidermis share functional and phenotypic characteristics. For instance, epidermal keratinocytes can recruit hematopoietic precursors and support the development of a thymic microenvironment[170]. Thus, one could wonder whether a common functional signature links these cell types to the expression

of Gα15. A very intriguing possibility is that Gα15 is expressed at intermediate stages of maturation, when cells are in the process of leaving quiescence to generate lineages that can be stimulated to rapid proliferation. If demonstrated, this aspect could be relevant in pathological processes.

Although there is no direct indication pointing to an involvement of G15 in tumor cell growth[171], Gα15 was co-immunoprecipitated to the M1 muscarinic receptor in prostate adenoma[172-173]. Normal tissue was not analyzed but we found the immunoblot of healthy prostate negative for Gα15 expression, thus suggesting that its appearance may relate to initial phases of the transformation process.

or else?:

By performing Southern blotting analysis of PCR products, Wilkie et al. showed a weak Gα15 expression in several organs[47]. We confirmed by quantitative PCR (Figure 17a, pag.115) that very low, albeit significant, levels of Gα15 are present in heart, kidney and almost all tissues analyzed. Though abundance was much reduced as compared to HSCs or TECs, a small number of Gα15 mRNA copies is therefore present in most tissues, nonetheless the translational level remains below the detection limit of the antibodies available for immunoblotting (estimated as less than 1 ng in HeLa cells[174]). An antibody suitable for immune-histochemistry would allow analyzing different epithelia present in various organs and pinpoint positive cell lineages. Unfortunately, a similar tool has not yet been described in the literature, possibly because of the limited choice of epitopes presenting specific immunogenicity without cross-reaction with other members of the Gq/11 protein subfamily members.

Under these circumstances, there is a strong possibility that small subpopulations of cells derived from specialized epithelia may contribute the low signal detected in most human organs.

Two Gα15 isoforms?

Another puzzling aspect about Gα15 expression is that, in addition to the most commonly observed 43 kDa form, a 46 kDa form was repeatedly described[50, 55, 161]. During megakaryocytic maturation[161] and T lymphocyte activation the transient appearance of the heavier band anticipates the similarly transient expression of the 43 kDa form[55]. The down modulation of both forms by five specific shRNA sequences (G Innamorati et al., unpublished observation) proves that both represent Gα15 rather than a cross-reacting α-subunit, or any other protein sharing a common epitope.

For other G protein α-subunits, alternative splicing causes the appearance of similar doublets in acrylamide gels[175-176]. However, in the case of Gα15, neither PKC phosphorylation[95], nor cysteine palmitoylation at position 9 and 10 of the N-terminus [64] significantly affected the migration of the recombinant protein. The main gene transcript (NM_002068) of human GNA15 (the gene encoding for Gα15) consists of 7 exons. An alternative splice variant (AK300481) was found in NCBI database by SpliceMiner software[177], however, this mRNA variant contains the first two and a larger third exon not compatible with the 46 kDa protein.

Further investigation is needed to unravel the molecular details that differentiate these two species and to verify if they fulfill specific roles.

The evasive nature of G15 coupling

G15 coupling to GPCRs appears as characterized by low selectivity but high efficiency.

G15 promiscuity and its physiological coupling

Many authors documented the peculiar promiscuity of G15 by showing functional interactions with a wide variety of different receptors in a large number of transfected cell lines[76-77]. Such versatility would predict that dozens of different GPCRs expressed by any given cell[178] may act as physiological upstream activators of G15. In addition to various chemokine receptors, HSCs express other GPCRs such as the β2AR[151], opioid receptors[179] and smoothened[159]. Needless to say, these receptors were proved to be good couplers of G15 in recombinant systems[76-77, 85, 146, 180]

However, promiscuity remains to be demonstrated under naïve conditions. The identification of specific receptor-G15 interactions represents a very challenging task as PLC is also activated by the ubiquitous Gq/11 or by other G proteins via release of βγ-subunit, or by indirect activation through other intermediate effectors (see for instance the activation of PLCε by Gs via PKA[181]). The lack of pharmacological inhibitors specific for Gα15 further complicates the analysis. Thus, it is not surprising that only very few examples describe GPCR signalling through naïve G15.

Knocking out Gα15 expression in transgenic mice reduced the coupling of C5a to calcium release in macrophages while leaving intact the coupling to other GPCRs[24], including P2Y2 receptor stimulated by UTP[24]. Surprisingly, in erythroleukemia cells, silencing Gα15 led to reduced mobilization of intracellular Ca^{2+} upon

stimulation of the same P2Y2 purinoceptor[182]. These results may be reconciled considering that cell specific components contribute to define the specificity of receptor-G protein interaction[183] and hence specialized cells may dictate specific coupling profiles.

Olfactory G proteins and transducins are exclusively expressed in sensory neurons to mediate the signalling of dedicated GPCRs (olfactory receptors and opsins respectively). Likewise, the selective expression of Gα15 in HSCs suggested that it may serve as a specific effector of GPCRs involved in immunity[184]. In this context, Sphingosine 1-Phosphate Receptor 4 (S1PR4) is a good candidate as a physiological activator of G15. The lymphoid tissue specific S1PR4 is part of a family of receptors responding to lysophospholipids or lysosphingolipids[185]. It has been reported that the genes encoding for S1PR4 and GNA15 are located in tandem, likely under the control of the same promoter[186]. Consistently, according to microarray data, both genes are simultaneously expressed in mouse fetal liver cells and are both silenced during erythroid differentiation (A Ronchi, personal communication, 2007). An interaction between these two proteins could explain why, in the presence S1P containing serum[187], the inhibition or downregulation of Gα15 affects erythroid cells growth and differentiation[53]. Nonetheless, when tested with GTP photo-affinity label in CHO cells[188], S1PR4 was shown to be coupled to Gi and G12/13 but not to Gq/11 and G15.

Another good candidate as a specific G15 activator would be CXCR4, the receptor responsible for retaining HSC in the bone marrow[189]. Bafflingly, among the very few GPCRs that upon exogenous expression refrain to couple with G15, are indeed several chemokine

receptors including CXCR4[146, 190] in addition to CCR5[191], CCR7[191] and CCR1[190, 192]. Again, the cellular context could make a difference since silencing Gα15 mRNA in monocytic THP1 cells partially reduced chemotactic ability in response to CCR1[193]. Another exception to G15 promiscuity is CCR2A[192-193], but not its splice variant CCR2B that only differs in the carboxyl-terminus. Yet, also CCR2A coupled to G15 when ectopically expressed in HEK-293 cells[190] instead of COS-7 cells. If the cellular context influences the specificity of the interaction, many molecular mechanisms could be involved. Although the GPCR carboxyl-terminus is not generally considered to directly determine G protein specificity (G15 included), it could act indirectly by bridging GPCRs to preassembled signalling complexes. Likewise, post-translational modifications targeting the G protein to specific plasma membrane microdomains could physically restrict G15 interactions with GPCRs partitioned within these discrete areas.

Another largely underestimated cause for coupling discrimination is the identity of the β- and γ-subunits forming the heterotrimeric complex. Five β- and twelve γ-subunits assemble in multiple combinations with the α-subunit. If the composition of the heterotrimer narrows the number of upstream GPCR partners[194], the cellular repertoire of β- and γ-subunits[195] could be crucial to modulate specificity. In a similar manner, the formation of receptors heterodimers could provide an additional mechanism for discrimination[196].

Discrepancies observed in different cellular systems are not unraveled by transgenic animal models that lacked to provide a clear indication

about which GPCRs are upstream G15. Gα15 knockout mice display normal maturation of all cell lineages and mount a normal response to the immune challenges[24]. Unfortunately, very limited data is available in non hematopoietic tissues. For instance, the increase of cyclic GMP occurring upon activation of the muscarinic M3 receptor in membrane fractions of tracheal smooth muscle was inactivated by a Gα15 specific antibody[197].

Expanding research focus beyond immunity could result critical. In addition, newly emerged data suggests that G15 action may become particularly relevant under exceptional conditions, i.e. in the case of prolonged stimulation (see below).

Differential sensitivity of G15 to receptor desensitization

There is an additional feature that makes G15 different from other G proteins, i.e. its relatively enduring activity under conditions in which the coupling efficiency of other G-proteins is reduced by GPCR desensitization. GPCR desensitization is a general regulatory mechanism operated by cytosolic adaptor proteins, named β-arrestins, that rapidly translocate to hundreds different GPCRs after agonist stimulation[116]. The term "arrestin" derives from the protein ability to dampen receptor signalling by steric hindrance of G protein coupling. Moreover, β-arrestins promote receptor endocytosis (internalization) by recruiting endosomal adaptor proteins. The interaction with β-arrestin is stabilized by receptor phosphorylation. Seven isoforms of GPCR kinases (GRKs) are responsible for phosphorylating multiple sites of the receptor carboxylterminus in response to ligand binding.

In respect to other G proteins, G15 signalling is poorly affected when desensitization is either induced by repeated GPCR activation or it is emphasized by β-arrestin over-expression[198]. G15 is the only Gq/11 family member that does not interact with GRK2, whereas for other G proteins the interaction prompts GRK translocation from the cytosol to the plasma membrane. As a consequence, GRK2 is not recruited to GPCR upstream G15[199]. In addition to a reduced receptor phosphorylation, the missing interaction with GRK2 could have additional consequences since the negative modulation that GRK2 exerts on G protein signalling goes beyond its kinase activity. In fact, a kinase-dead GRK2 mutant was reported to equally modulate the activities of Gq, G11 and G14 while leaving unaffected the activity of G15. GRKs contain RGS (Regulator of G protein Signalling) domains believed to accelerate G protein inactivation by stabilizing the transition state of Gα-catalyzed GTP hydrolysis. It is thus tempting to explain G15 enduring signalling with a prolonged permanence in the GTP bound state. However, the RGS domain of GRK2 was described as particularly weak[200] and other GRKs and RGS containing proteins (such as RGS2), known to interact with G15[199], could easily compensate. Further investigation is required to explain the mechanistic base of G15 refractoriness to β-arrestin-dependent desensitization that nevertheless implies the permanence of Gα15 in the complex assembled around the desensitized GPCR. This was shown with a V2 vasopressin receptor mutant constitutively stabilized in a desensitized state by a mutation in the conserved DRY sequence (R137H)[128]. The R137H-V2R co-immunoprecipitates with Gα15, but not with Gαq or Gαs, as if Gα15 possesses a better capability to

remain in direct contact with activated receptors, possibly by competing with β-arrestin[198].

The desensitization process modulates GPCR function by adjusting receptor efficiency to the intensity and persistence of the stimulation. In immune cells GRKs and arrestins are dynamically regulated[201] thus the specificity of GPCR response can drastically vary depending on the cell activation state. In the case of stimuli particularly intense (as possibly during commitment to high proliferation states) G15 could represent a key element that, by better resisting to arrestin-dependent desensitization, takes over when other G proteins become ineffective. In this model, G15 would determine a qualitative evolution of the signal with the final result of reprogramming the cell.

G15 Sensitivity

The interactions occurring between GPCR and G15 may result more stable than the interaction with other G proteins. A steady interaction of wild type P2Y2 receptor with Gα15 was assessed by FRET even in the absence of ligand[202]. The presence of G15 biased the pharmacological profile of the κ opioid receptor[93], again suggesting the existence of preformed receptor-G protein complexes. A similar interaction with G15 may even interfere with the activation of other G protein subtypes as shown for pUS28[203], a viral GPCR characterized by elevated ligand-independent constitutive activity and by increased phosphorylation[204]. This effect was unmasked because Gq/11 promotes serum response factor (SRF) dependent transcriptional activity much more effectively than G15 or G14, and the over-

expression of G15 reduced SRF effect by directly competing with Gq/11 for the chemokine- activated pUS28[203].

Exogenous Gα15 expression at physiological levels (promoted by a tetracycline induced transactivation system) exerted a similar effect by blunting the Ca^{2+} transient induced through the Gq/11-coupled TRH receptor[92]. G15 expression also inhibited the signalling of β2AR to Gs[198].

If the ability of G15 to compete for activated GPCRs relates to its resistance to GPCR desensitization remains to be addressed.

Getting further insights on G15 biological function

The high degree of promiscuity, combined with the functional redundancy within the Gq/11 subclass and with the absence of specific pharmacological inhibitors, restricts the number of approaches that can be used to define G15 function. For this reason, many researchers took advantage of Gα15-Q212L, a constitutively active mutant unable of efficient GTP hydrolysis. By this approach direct activation of downstream effectors is achieved bypassing the GPCR. Gα15-Q212L promoted the activity of transcription factors like NFκB and STAT3 (Signal Transducer and Activator of Transcription 3) via PKC[51, 205] and c-Src/MAPK dependent pathway[206-207](Figure 18, pag.115). As a member of a family of latent cytoplasmic transcription factors, STAT3 has long been implicated in cell growth and development relaying signals from the plasma membrane to the nucleus. It is therefore tempting to speculate that G15 promotes quiescence and initiates differentiation programs in transient amplifying cells. Experiments produced in various cell lines suggested that G15 regulates cell

maturation but, at the same time, revealed several contradictory aspects. In a neuronal maturation model (PC12 cells) G15 promoted cell differentiation[208] and similar results were observed in a model of erythroid differentiation (MB-02 erythroleukemia cells)[53]. However, in the latter case, over-expression or down-modulation of Gα15 sorted out the same effect. Likewise, in lymphoid Jurkat cells, both sense and antisense DNAs produced a similar reduction in CD69 and IL2 expression[55]. Reduced cell growth was obtained in MB-02 and in "small cell lung carcinoma" (SCLC) cells[171] upon over-expression of Gα15-Q212L. In vascular smooth muscle cells, only Gα15 did not produce pro-apoptotic effects among the Gq/11α family members that were tested[209]. In SCLC cell lines, constitutively active Gα15 inhibited cloning efficiency but no effect was observed in "non cell lung carcinoma" clones[171].

Thus, it is far too premature to draw any conclusion. In particular, results based on constitutively active Gα15 are particularly questionable because the signal triggered by a permanently active Gα subunit is clearly different from the signal triggered by an activated receptor.

First, it does not support transient events, such as the acute increase of the intracellular Ca^{2+} concentration that is normally produced by a receptor.

Second, signals elicited by constitutively active G proteins lack parallel coordinated pathways initiated by GPCRs that sometimes are even G protein-independent[210] and, anyway, always include βγ. For example, when G15 is stimulated by the adenosine A1 receptor in HEK cells, the βγ subunit activates NF-κB[206].

Third, the sustained basal inositol phosphate turnover achieved in clones expressing Gα15-Q212L produced loss of responsiveness to agonist dependent Ca^{2+} mobilization[171, 211-212], probably due to a partial depletion of Ca^{2+} stores together with a reduction of IP3 receptor number. Exogenous expression of Gα15-Q212L inhibited cell growth in NIH-3T3[211] and Swiss 3T3 fibroblasts[213] but at the same time inhibited the responsiveness to PDGF, TPA and bombesin towards effectors like PKC, Raf, MEK, thus indicating a general distortion of the signalling network.

Fourth, cells tonically exposed to G protein signalling might compensate by counteracting downstream signalling steps or even suffer undesired long-term consequences as shown for the same G15[211]. Constitutively active Gq/11 produces PLCβ signals capable of inducing cell transformation at low levels of expression but it becomes eventually noxious at higher levels[214].

The type of response to G15 signalling is also likely to depend upon the intensity of the stimulus, and activity levels promoted by over-expression of Gα15-Q212L exceed what is normally effective in the cell.

In summary, G15 physiological activity certainly relies on coordinated multi-branched signals that are flawed by the chronic activation of a single pathway.

As mentioned above, the assumption that G15 biological role strictly relates to hematopoietic cells growth/differentiation and to lymphocytes activation[93] is mostly inferred on its distribution and poorly supported by knockout mice that are substantially normal and

capable of responding to several inflammatory challenges[24]. Normal hematopoiesis was also observed in Gα15 and Gαq double-knockout mice that in most hematopoietic cells only express Gα11. Gα11 knockout mice exhibit as well normal hematopoiesis suggesting functional redundancy in Gq/11 subclass signalling[24] (double Gαq and Gα11 knockout is lethal). G15 function remains therefore substantially unknown.

G15 activity may become specifically important when GPCR stimulation is particularly intense and prolonged. Under these conditions, desensitization is expected to silence other pathways that are instead more strictly regulated. Retroviral transduction of silencing RNA and conditional knockout models will probably turn out to be determinant in the near future to clarify G15 specific functions: by this mean compensatory mechanisms should be avoided shutting off only G15-dependent branches.

Conclusions

A number of experimental observations support the hypothesis that G15 appeared late in evolution (Figure 17c, pag.115) to fulfill highly specialized functions. A loose selectivity combined to high affinity and atypical resistance to GPCR desensitization could provide a strategy to deliver stimuli that are particularly intense. Such powerful action is likely to develop along specific intracellular pathways. For instance, only G15 among Gq/11 family members efficiently activates NF-κB in HeLa cells (in response to fMLP, C5a, C3a, receptors, CCR8 and CXCR2)[215] and in HEK (in response to adenosine A1

receptor)[206]. More in general, different genes were transcribed upon transfection of the constitutively active Gα15[209]. Unfortunately, thus far, no clear physiological outcome has convincingly been associated to G15 activity.

Treasuring on indications provided by studies in signal transduction, future research will identify circumstances where G15 atypical signalling is matched by evident phenotypic outcomes. G15 is expressed in tissues characterized by a high rate of cell turnover (bone marrow and epithelia[55]). We suggest that research focus should be extended beyond the immune response (epithelial and other intermediate maturation stages) and that experimental conditions should highlight G15 function peculiarities so that its effects emerge over the redundant functions of the other Gq family members (i.e. under prolonged/intense GPCR stimulations).

Legend of figures

Table 7 - List of organs and cells from various organisms (H=human, b=baboon, m=mouse, r=rat) showing Gα15 expression according to literature data.

The word "Traces" reported in parenthesis means that mRNA expression is low and the protein is undetectable by immunoblotting. Other tissues where reported as substantially negative: yalk (m), uterus (m), testis (m), liver (m, H) (Figure 17)[47]. In the last two columns the approximate expression patterns inferred from EST sources as reported by UniGene (NCBI). Reference numbers are Hs.73797 and Mm.1546 for human and mouse respectively. Symbols are present when data are available and refer to the number of transcripts per million of ESTs (- absent; + 1-9 copies;++ 10-29 copies;+++ 30-99 copies; ++++ more than 100 copies).

Figure 17 - Gα15 expression in human tissues and cells as assessed by quantitative RT-PCR (A) or immunoblotting (B).

HSC is for hematopoietic stem cells, TEC for thymic epithelial cells, TMSC for mesenchymal stem cells. A) GNA15 (Gα15 gene) expression analysis was performed by quantitative RT-PCR using the TaqMan assay Hs_00157720_m1 (Applied Biosystems). As an endogenous reference, Glyceraldehyde-3-phosphate dehydrogenase transcript level was measured in parallel. GNA15 copy number (means of three measures ± SD) was assessed by the standard curve method, according to Lai et al. 2003[216]. B) Immunoblotting detected a positive signal in TEC, consistent with the robust Gα15 mRNA

presence, while TMSC resulted negative. Gα15 was also present in the epithelioid cell line A413 as opposite to the melanoma cell line IPC298. C) Phylogenetic tree of Gαq/11 family members obtained at http://www.phylogeny.fr, according to Dereeper et al. 2008[217]. GNA15 appears as the most distant member within the Gαq family.

Figure 18 - Generalized scheme of G15 signalling.

This scheme represents an overlay of signalling pathways that were pointed out by specifically modulating G15 activity. Different approaches were used to achieve this objective including promoting gain of function (by recombinant expression of Gα15 as the wild type form or as a constitutively active mutant) or inducing loss of function in cells endogenously expressing Gα15 (by RNA interference or by competing deficient mutant). In many cases, a signalling knot represents more than one protein isoform. Like any other Gq/11 family member, G15 activates different isoforms of PLCβ promoting PIP2 hydrolysis in response to GPCR stimulation[75-76]. The activation of CaMKII[206, 218] and Ca^{2+}-dependent[92] or independent (PKCμ in COS cells unpublished) PKC isoforms triggers several pathways, including those stimulating NFκB[215], ERK[218], JNK[208] JAK[205, 219], cSrc[51]. Small GTPases, such as Ras and Rho are indirectly modulated by G15, via TPR1[93, 220] and p63RhoGEF[221] respectively. The best characterized effector of Ras is the MAPK pathway that proceeds through ERK1/2[222]. MAPKs activated by G15[193, 223] lead to the activation of transcription factors such as STAT[205, 218], NFκB[51, 215] and SRE[221, 224]. G15 phosphorylation by PKC modulates GPCR coupling providing feedback regulation[95, 225]. In addition, like other Gq/11 family

members[226], G15 was shown downstream the large signalosoma assembled upon T cell receptor activation[227], G15 was reported as an intermediate effector towards the activation of Lck and Fyn. As a result, the inhibition of G15 function reduced lymphocyte activation in response to T cell receptor engagement. In addition, G15 may affect other physiological functions such as transcription, proliferation[213], differentiation[53], secretion[55, 92].

	Organ, cells	Specie	Ref.	EST (m)	EST (H)
Immune system	Tonsil: B and T cells	H	[48]		-
	Thymus (decreasing in adult)	M	[47]	++	++++
	Lymph nodes	M	[24]	-	-
	Blood			-	+++
	Activated peripheral blood cells	H	[55]		
	Lymphocytes				
	preB cells	H	[52]		
	HSC	H	[55]		
	γδ	H	[55]		
	activated T cells	H	[55]		
	Megakaryocytes	H	[161]		
	Platelets	H	[50]		
	Neutrophils	m	[24]		
	Monocytes	H	[50]		
	Bone marrow, HSC and erythroid cells	H	[50]	++++	++
Epithel.	Hair follicle	b	[47]		
	Skin: keratinocytes	b	[163]	+++	+
	Tongue: taste bud	R	[168]	-	+++
	Thymus: epithelial cells	H	Figure 17		
Other organs	Brain (traces)	m	[47]	+	+
	Heart (traces)	m	[47]	+++	-
	Lungs (traces)	m	[47]	-	++
	Kidney (traces)	m	[47]	-	+

Table 7 - List of organs and cells from various organisms (H=human, b=baboon, m=mouse, r=rat) showing Gα15 expression according to literature data.

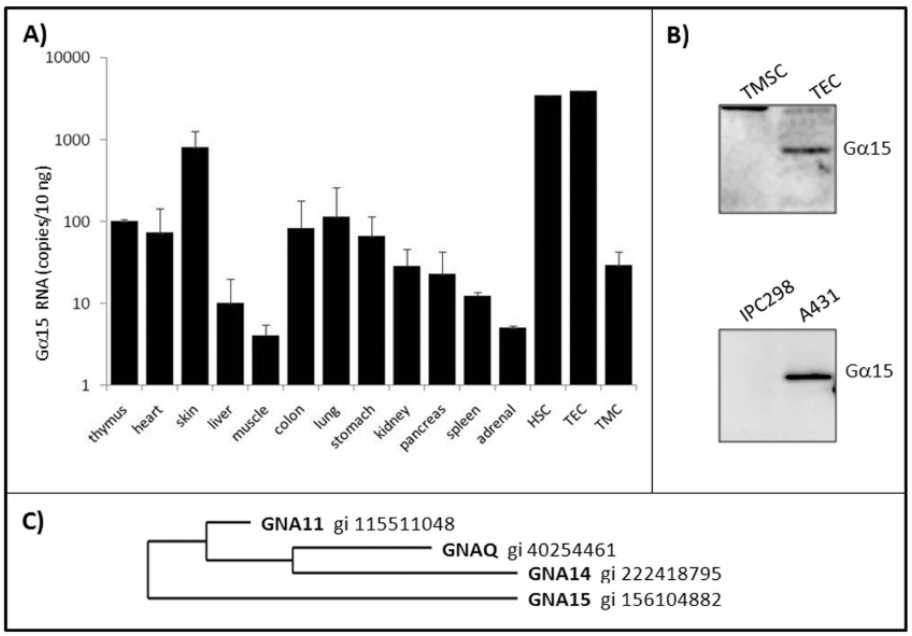

Figure 17 - Gα15 expression in human tissues and cells as assessed by quantitative RT-PCR (A) or immunoblotting (B).

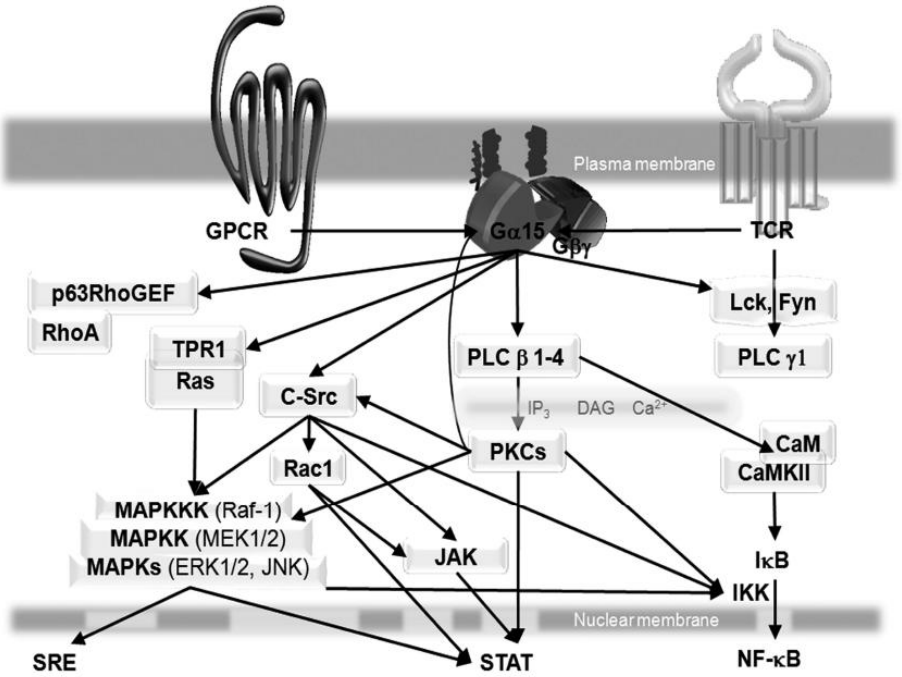

Figure 18 - Generalized scheme of G15 signalling.

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Chapter 4

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

This dissertation highlights the peculiar features of G15/16 protein among the members of the Gq/11 family of heterotrimeric G proteins. These unique characteristics of G15/16 have been mainly discovered, characterized and exploited in heterologous expression systems, whereas very little is known about the physiological and pathological role of the endogenously expressed protein.

Human tumor cell lines express the α-subunit of G16

The expression of Gα15/16 seems to be tightly regulated, since unlike most ubiquitous Gα-subunits, it is restricted to specific cell types (e.g. hematopoietic stem cells and progenitors, and keratinocites) and/or to specific conditions (e.g. T cell activation). This expression pattern profile is probably related to the higher binding affinity of Gα15/16 to different GPCRs as compared to other α-subunits. This feature makes Gα15/16 capable to successfully compete with other Gα-subunits (e.g Gαq and Gαs), as well as to make GPCRs unusually resistant to β-arrestin-mediated desensitization.

All these properties could provide cells with a strategy to generate sustained signalling over time, such as that necessary to stimulate proliferation. This hypothesis prompted us to investigate whether the expression of Gα15/16 might be enhanced in human tumor cell lines of different tissue origins.

Intriguingly, by western immunoblotting analysis, we detected Gα16 immunoreactivity in the following cell lines (Figure 19):

- PT45 and Su-86-86 (pancreatic tumors);
- HT29 (colon-rectal tumor);
- MDA-MB-231 (mammary tumor);

- J82 (bladder tumor);
- 7951 (melanoma);
- Caov3 (ovarian carcinoma);
- A-431 (epidermoid carcinoma);

Other tumor cell lines resulted negative for Ga16 expression but positive for the ubiquitous Gαq/11 protein.

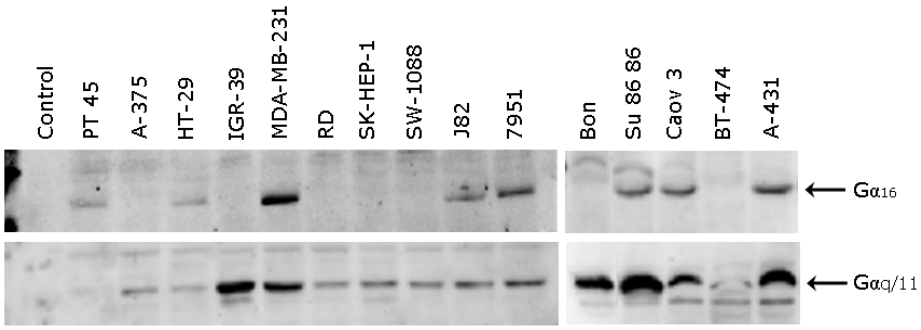

Figure 19 – Gα16 expression in human tumor cell lines. The human tumor cell lines indicated were lysed and protein extracts were resolved by SDS-PAGE (10% acrylamide). Proteins were transferred to nitrocellulose membranes and immunoblotted with anti-Gα15/16 antibody (dilution 1:500, Torrey Pines Biolabs) or anti-Gαq/11 antisera (dilution 1:500 ,CQ antiserum[228]).

We are still unable to say whether endogenously expressed Gα16 displays the same characteristics observed following its ectopic expression in transfected cells.

The first step toward this goal would be to identify at least one endogenous GPCR capable to signal through G16 in these tumor cell lines. However, GPCRs selectively coupled to endogenous G16 are unknown even in hematopoietic cells where the protein is known for long to exist. Moreover such ideal GPCR would have to stimulate PLC-β activity only through G16, without any interference from the ubiquitous Gq/11 proteins that usually couples GPCR to PLC-β.

We are actively searching for a G16 coupled GPCR because it would allow us to ascertain the signalling properties of the endogenous G16 protein and check whether any correlation exists between its expression and cell growth and/or neoplastic transformation.
In addition, we shall verify whether the ability of Gα15/16 to confer GPCRs resistance to β-arrestin induced desensitization, also applies to cells of hematopoietic origins, for instance in HEL cells (where Baltensperger et al. have demonstrated that P_{2U} purinoceptor obligatorily engages Gα16 to mobilize intracellular Ca^{2+} [182]).

Existence of two isoforms of Gα16 in human pancreatic carcinoma QGP1 cell line

Interestingly, in the QGP1 human pancreatic carcinoma cell line we detected by western immunoblotting two specific immunoreactive Gα16 bands respectively at 43 and 46 kDa. This finding is not entirely new , since the presence of two bands has been already detected[50, 55, 161], but no one has yet established the identity of the heavier form.
In order to demonstrate that the 46 kDa band corresponds to a high molecular form of Gα16 we individually transduced QGP1 cells with four batch of lentiviral particles containing different shRNAs directed against Gα16. Few days post-infection we tested Gα16 silencing through western immunoblotting. Both the bands were down-regulated in cells transfected with all four shRNAs as compared to cells transfected with the empty vector or with the same vector carrying GFP as control (Figure 20).
Thus we are confident that also the 46 kDa band represents a Gα16 isoform.

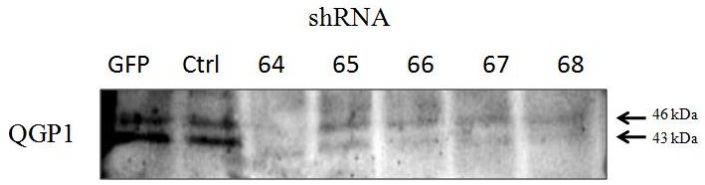

Figure 20 - Knockdown of Gα16 by lentiviral-directed shRNAs in QGP1 cells. QGP1 cells were individually transduced with different batches of lentiviral particles each containing four different shRNAs specific for Gα16 (64,65,66,67,68), the empty vector (Ctrl) or the same vector carrying GFP as control (GFP).

To verify whether the 46 kDa form could derive from an alternative splicing we performed Reverse Transcription (RT)-PCR using three different pairs of primers:

1. GNA15-1_FW: 5'-GGTGTTTCAGGCAAGGAAGT-3'
   GNA15-1_RW: 5'-GAGGATGACGGATGTGCTTT-3'
   The FW primer matches with the 5'UTR (untranslated region) of Gα16 mRNA, whereas the RW is complementary to a sequence of exon 6;
2. GNA15-2_FW: 5'-ACGTGATCGCCCTCATCTAC-3'
   GNA15-2_RW: 5'-TTTTCCAGCGGTCTGTTACC-3'
   This FW primer corresponds to a sequence of exon 5, whereas the RW matches with the 3'UTR of Gα16 mRNA;
3. GNA15_FW: 5'-CACCACGCTAGCCTGGTCATG-3'
   GNA15_RW: 5'-GCGCCCTTCTTGCTGCCCTCGGG-3'
   These primers match with two highly specific regions of Gα16 mRNA and were previously utilized by other authors[52].

According to the unique Gα16 mRNA sequence deposited in the NCBI database (Figure 21, pag.131), the three expected PCR products are of 996, 985 and 671 bp respectively.

>gi|156104882|ref|NM_002068.2| Homo sapiens guanine nucleotide binding protein (G protein), alpha 15 (Gq class) (GNA15), mRNA

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GGGGAGCCCT GGCCTCCCCA CCTCCTCCCG TCCCCACCCT GTTCCCAGCA CTCAAGCCTT 60
GCCACCGCCG AGCCGGGCTT CCTGGGTGTT TCAGGCAAGG AAGTCTAGGT CCCTGGGGGG 120
TGACCCCCAA GGAAAAGGCA GCCTCCCTGC GCACCCGGTT GCCCGGAGCC CTCTCCAGGG 180
CCGGCTGGGC TGGGGGTTGC CCTGGCCAGC AGGGGCCCGG GGGCGATGCC ACCCGGTGCC 240
GACTGAGGCC ACCGCACCAT GGCCCGCTCG CTGACCTGGC GCTGCTGCCC CTGGTGCCTG 300
ACGGAGGATG AGAAGGCCGC CGCCCGGGTG GACCAGGAGA TCAACAGGAT CCTCTTGGAG 360
CAGAAGAAGC AGGACCGCGG GGAGCTGAAG CTGCTGCTTT TGGGCCCAGG CGAGAGCGGG 420
AAGAGCACCT TCATCAAGCA GATGCGGATC ATCCACGGCG CCGGCTACTC GGAGGAGGAG 480
CGCAAGGGCT TCCGGCCCCT GGTCTACCAG AACATCTTCG TGTCCATGCG GGCCATGATC 540
GAGGCCATGG AGCGGCTGCA GATTCCATTC AGCAGGCCCG AGAGCAAGCA CCACGCTAGC 600
CTGGTCATGA GCCAGGACCC CTATAAAGTG ACCACGTTTG AGAAGCGCTA CGCTGCGGCC 660
ATGCAGTGGC TGTGGAGGGA TGCCGGCATC CGGGCCTACT ATGAGCGTCG GCGGGAATTC 720
CACCTGCTCG ATTCAGCCGT GTACTACCTG TCCCACCTGG AGCGCATCAC CGAGGAGGGC 780
TACGTCCCCA CAGCTCAGGA CGTGCTCCGC AGCCGCATGC CCACCACTGG CATCAACGAG 840
TACTGCTTCT CCGTGCAGAA AACCAACCTG CGGATCGTGG ACGTCGGGGG CCAGAAGTCA 900
GAGCGTAAGA AATGGATCCA TTGTTTCGAG AACGTGATCG CCCTCATCTA CCTGGCCTCA 960
CTGAGTGAAT ACGACCAGTG CCTGGAGGAG AACAACCAGG AGAACCGCAT GAAGGAGAGC 1020
CTCGCATTGT TTGGGACTAT CCTGGAACTA CCCTGGTTCA AAAGCACATC CGTCATCCTC 1080
TTTCTCAACA AAACCGACAT CCTGGAGGAG AAAATCCCCA CCTCCCACCT GGCTACCTAT 1140
TTCCCCAGTT TCCAGGGCCC TAAGCAGGAT GCTGAGGCAG CCAAGAGGTT CATCCTGGAC 1200
ATGTACACGA GGATGTACAC CGGGTGCGTG GACGGCCCCG AGGGCAGCAA GAAGGGCGCA 1260
CGATCCCGAC GCCTCTTCAG CCACTACACA TGTGCCACAG ACACACAGAA CATCCGCAAG 1320
GTCTTCAAGG ACGTGCGGGA CTCGGTGCTC GCCCGCTACC TGGACGAGAT CAACCTGCTG 1380
TGACCCAGGC CCCACCTGGG GCAGGCGGCA CCGGCGGGCG GGTGGGAGGT GGGAGTGGCT 1440
GCAGGGACCC CTAGTGTCCC TGGTCTATCT CTCCAGCCTC GGCCCACACG CAAGGGAGTC 1500
GGGGGACGGA CGGCCCGCTG CTGGCCGCTC TCTTCTCTGC CTCTCACCAG GACAGCCGCC 1560
CCCCAGGGTA CTCCTGCCCT TGCTTGACTC AGTTTCCCTC CTTTGAAAGG GAAGGAGCAA 1620
AACGGCCATT TGGGATGCCA GGGTGGATGA AAAGGTGAAG AAATCAGGGG ATTGAGGACT 1680
TGGGTGGGTG GGCATCTCTC AGGAGCCCCA TCTCCGGGCG TGTCACCTCC TGGGCAGGGT 1740
TCTGGGACCC TCTGTGGGTG ACGCACACCC TGGGATGGGG CTAGTAGAGC CTTCAGGCGC 1800
CTTCGGGCGT GGACTCTGGC GCACTCTAGT GGACAGGAGA AGGAACGCCT TCCAGGAACC 1860
TGTGGACTAG GGGTGCAGGG ACTTCCCTTT GCAAGGGGTA ACAGACCGCT GGAAAACACT 1920
GTCACTTTCA GAGCTCGGTG GCTCACAGCG TGTCCTGCCC CGGTTTGCGG ACGAGAGAAA 1980
TCGCGGCCCA CAAGCATCCC CCCATCCCTT GCAGGCTGGG GGCTGGGCAT GCTGCATCTT 2040
AACCTTTTGT ATTTATTCCC TCACCTTCTG CAGGGCTCCG TGCGGGCTGA AATTAAAGAT 2100
TTCTTAGAGG CTGCGTCGCC AGCGTCCTGT TT                               2132
```

Figure 21- mRNA sequence of the Homo sapiens Gα15 (GNA15) deposited in the NCBI database. The start codon (ATG) and the stop codon (TGA) of the coding sequence are highlighted in yellow. The sequence of the 5' UTR region is written in blue, whereas the sequence of the 3' UTR region is in green. The Fw and Rw primers of the first, second and o third PCR are highlighted in blue, green and pink respectively.

However, the second PCR generated two products of amplification: one of the expected size and the second of about 660 bp (Figure 22, pag.132).

If this second product is really the result of an alternative splicing which can give rise to the heavier form of Gα16, we could imagine

that a latent intron can be eliminated, resulting in an alternative termination with a stop codon located further downstream from the other.

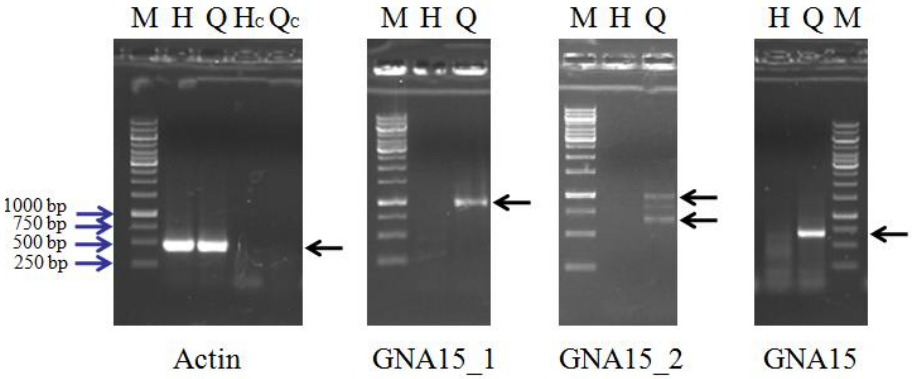

Figure 22 - RT-PCR analyses of Gα16 and β-actin mRNAs on total RNA extracted from QGP1 and HEK 293T cells. Total RNA extraction from QGP1 (Q) and HEK 293T (H) cells (the latter used as negative control) was performed with TRIzol reagent using a standard protocol. Total RNA was treated with DNAse I (RNAse-free), and a part of sample was used to perform PCR reactions with β-actin specific primers to make sure that no contamination from genomic DNA (Hc,Qc) is present. Reverse transcriptase (RT) reactions were performed on the remaining part of the samples, the then PCR reactions were performed with 1 µl of cDNA, 1X buffer, 4 mM MgCl2, 200 µM of each dNTPs, and 0.2 µM of each GNA15_1, GNA15_2, GNA15 and β actin specific primers. M= DNA ladders.

Further investigations on the nature of the heavier Gα16 isoform will be carried out after the resolution of it sequence, which is currently in progress.

Our future work will be devoted to challenge the hypothesis that G15/16 could play a role in oncogenic signalling.
More specifically our studies will be aimed at:

- Studying the effects of shRNA Gα16 silencing on cell proliferation, differentiation, neoplastic transformation, invasivity and migration of Gα16-expressing human cancer cell lines;
- clarifying the signalling properties of G16 in cancer cell lines, using a proteomic-based approach to identify specific interacting proteins, which can justify G16-induced refractoriness to GPCR desensitization;
- performing immunological screening for G16 expression in human tumor biopsies, to confirm that Gα16 is expressed not only in tumor cell lines but also in cancer tissues from patients;
- identifying the nature and the role of the higher mass isoform of Gα16.

In conclusion we believe that an increased understanding of G16 biology could have potential therapeutic drawbacks. In particular, the characterization of G16 as an oncogene could add a novel potential drug target, as well as a new diagnostic and prognostic tumor marker, even if more research is needed to validate this attractive hypothesis.

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Publications

1. The puzzling uniqueness of the heterotrimeric G15 protein and its potential beyond hematopoiesis.

   Giannone F, Malpeli G, Lisi V, Grasso S, Shukla P, Ramarli D, Sartoris S, Monsurrò V, Krampera M, Amato E, Tridente G, Colombatti M, Parenti M, Innamorati G.

   J Mol Endocrinol. 2010 Feb 11. [Epub ahead of print]dsghahdsv

2. Heterotrimeric G proteins demonstrate differential sensitivity to beta-arrestin dependent desensitization.

   Innamorati G, Giannone F, Guzzi F, Rovati GE, Accomazzo MR, Chini B, Bianchi E, Schiaffino MV, Tridente G, Parenti M.

   Cell Signal. 2009 Jul;21(7):1135-42.