

# Recent Patents of Gene Sequences Relative to the Phosphatidylinositol 3-kinase / Akt Pathway and their Relevance to Drug Discovery

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Received: September 25, 2006; Accepted: November 20, 2006; Revised: November 21, 2006

**Abstract:** Phosphoinositide 3-kinases (PI3Ks) play an essential role in the signal transduction events initiated by the binding of extracellular signals to their cell surface receptors. There are eight known PI3Ks in humans, which have been subdivided into three classes (I-III). The class I<sub>A</sub> of PI3K comprises the p110 $\alpha$ , p110 $\beta$  and p110 $\delta$  isoforms, which associate with receptor tyrosine kinases (RTKs). On the other hand, the class I<sub>B</sub> PI3K p110 $\gamma$  is regulated by G-protein-coupled receptors (GPCRs). Gene targeting studies in mice have revealed specific biological functions for the class I<sub>A</sub> p110 $\delta$  in lymphocyte activation, and the class I<sub>B</sub> p110 $\gamma$  in inflammatory cell responses. In human cancer, recent reports have described activating mutations in the *PIK3CA* gene encoding p110 $\alpha$ , and inactivating mutations in the *PTEN* gene, a tumor suppressor and antagonist of the PI3K pathway. Thus, individual PI3K isoforms are potential drug targets for a variety of human diseases, including allergies, cancer, rheumatoid arthritis and arterial thrombosis. In this review, we will discuss recent patents relating to class I PI3Ks, including patents on the cDNA sequences of p110 $\alpha$  and p110 $\beta$ . Moreover, we will review patents on novel pharmacological PI3K inhibitors and on methods of manipulating T cell responses through PI3K.

**Keywords:** Phosphatidylinositol 3-kinase, Akt, receptor tyrosine kinase, G-protein-coupled receptor, cancer, inflammation, rheumatoid arthritis, allergy.

## INTRODUCTION

### PHOSPHOINOSITIDE 3-KINASES

The phosphoinositide 3-kinases (PI3K) are a family of evolutionary conserved lipid kinases, which play a crucial role in controlling a wide variety of intracellular signaling events. PI3Ks phosphorylate phosphatidylinositol (PI) on the D-3 position of the inositol ring, producing distinct second messengers such as PI(3)P, PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> (PIP<sub>3</sub>) [1]. These second messengers are known to activate diverse target proteins involved in complex signaling cascades, ultimately resulting in the activation of cellular responses including growth, proliferation, survival and motility.

The family of PI3Ks identified in various species can be subdivided into three main classes (class I-III), based on structural similarity and *in vitro* substrate specificity [1] (overview given in Fig. (1)). Class I<sub>A</sub> includes p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$ , which are known to form a heterodimeric complex with a p85, p55, or p50 ( or ) regulatory subunit. This adapter subunit contains two Src-homology 2 (SH2) domains mediating their association with activated tyrosine kinase-coupled growth factor receptors [1]. PIP<sub>3</sub> produced by class I<sub>A</sub> PI3Ks activates the protein kinase phosphoinositide-dependent protein kinase-1 (PDK1), inducing the recruitment and activation of the key signal transducer protein kinase B (PKB)/Akt [2]. Akt is involved in regulating the cell cycle and glucose metabolism

through glycogen synthase kinase-3 (GSK3) [3] and in modulation of cell growth and survival. Moreover, Akt controls the translational machinery through the mammalian target of rapamycin (mTOR), the ribosomal protein S6 kinase (S6K) and the 4E-binding protein (4E-BP) [4]. Downstream events controlled by Akt further include the control of apoptosis through the regulation of proteins such as forkhead (FKHR), BAD, NF- $\kappa$ B and murine double minute gene-2 (MDM-2) [5] (overview given in Fig. (2)). Expression studies of the class I<sub>A</sub> PI3K isoforms have revealed a ubiquitous distribution of the subunits p110 $\alpha$  and p110 $\beta$  in human tissues, whereas p110 $\delta$  appears to be selectively expressed in leukocytes [6-8].

The class I<sub>B</sub> of PI3Ks is composed of one enzyme only, p110 $\gamma$ , which functions through heterodimer formation with a regulatory subunit p101 [9] or p84 [10]. Activation of p110 $\gamma$  is controlled by receptors capable of activating heterotrimeric guanine nucleotide-binding proteins, termed G-protein coupled receptors (GPCRs) [9]. The PI3K p110 $\gamma$  is thought to link GPCR signaling to PIP<sub>3</sub> production, which controls cell motility in inflammatory cells such as macrophages and neutrophils [11] (Fig. 3).

The human class II of PI3Ks comprises the three isoforms PI3KC2 $\alpha$ , PI3KC2 $\beta$  and PI3KC2 $\gamma$  [1]. The hallmarks of class II family members are a substrate specificity restricted to PI and PI(4)P *in vitro* and a C-terminal C2 domain. Although the precise cellular function of these enzymes remains generally poorly understood, recent reports have described class II PI3Ks as downstream transducers of activated polypeptide growth factor receptors [12,13]. The class III of PI3K includes a homolog of the yeast vesicular

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Class I	Subunits Regulatory Catalytic	Substrate Specificity	Activator	Tissue Distribution
<b>Class IA</b> 	p85 $\alpha$ p85 $\beta$ p55 $\alpha$ p55 $\gamma$ p50 $\alpha$	p110 $\alpha$ p110 $\beta$ p110 $\delta$	PI PIP PIP <sub>2</sub>	Receptor tyrosine kinases Ras  p110 $\alpha$ , p110 $\beta$ : ubiquitous p110 $\delta$ : leukocytes
<b>Class IB</b> 	p101 p84	p110 $\gamma$	PI PIP PIP <sub>2</sub>	G-protein-coupled receptors (G $\beta\gamma$ ) Ras  leukocytes
<b>Class II</b>				
		PI3KC2 $\alpha$ , $\beta$ , $\gamma$	PI PIP	Receptor tyrosine kinases G-protein-coupled receptors  PI3KC2 $\alpha$ ,C2 $\beta$ : ubiquitous PI3KC2 $\gamma$ : liver
<b>Class III</b>				
	p150	Vps34p analogues	PI	Constitutively active G-protein-coupled receptors (G $\alpha$ )
ABD – Adaptor Binding Domain RBD – Ras Binding Domain C2 – C2 Domain Helical – Helical Domain	Kinase – Kinase Domain SH3 – Src Homology Type 3 Domain Pr – Proline-rich Domain GAP – Bcr/Rac GAP Homology Domain	SH2 – Src Homology Type 2 Domain G $\beta\gamma$ – G $\beta\gamma$ -binding Site		

**Fig. (1).** Overview of PI3K isoforms. The PI3K family consists of eight isoforms, which are grouped into three classes, based on sequence homology and *in vitro* substrate specificity. The class I isoforms are further subdivided into class I<sub>A</sub> and class I<sub>B</sub> isoforms based on their mechanism of activation. While certain PI3K isoforms are expressed ubiquitously, isoforms such as p110 or p110 have been found to be more tissue-specific.

protein-sorting protein Vsp34 [14] and its major function is in intracellular trafficking events [15].

### INHIBITION OF PI3K SIGNALING

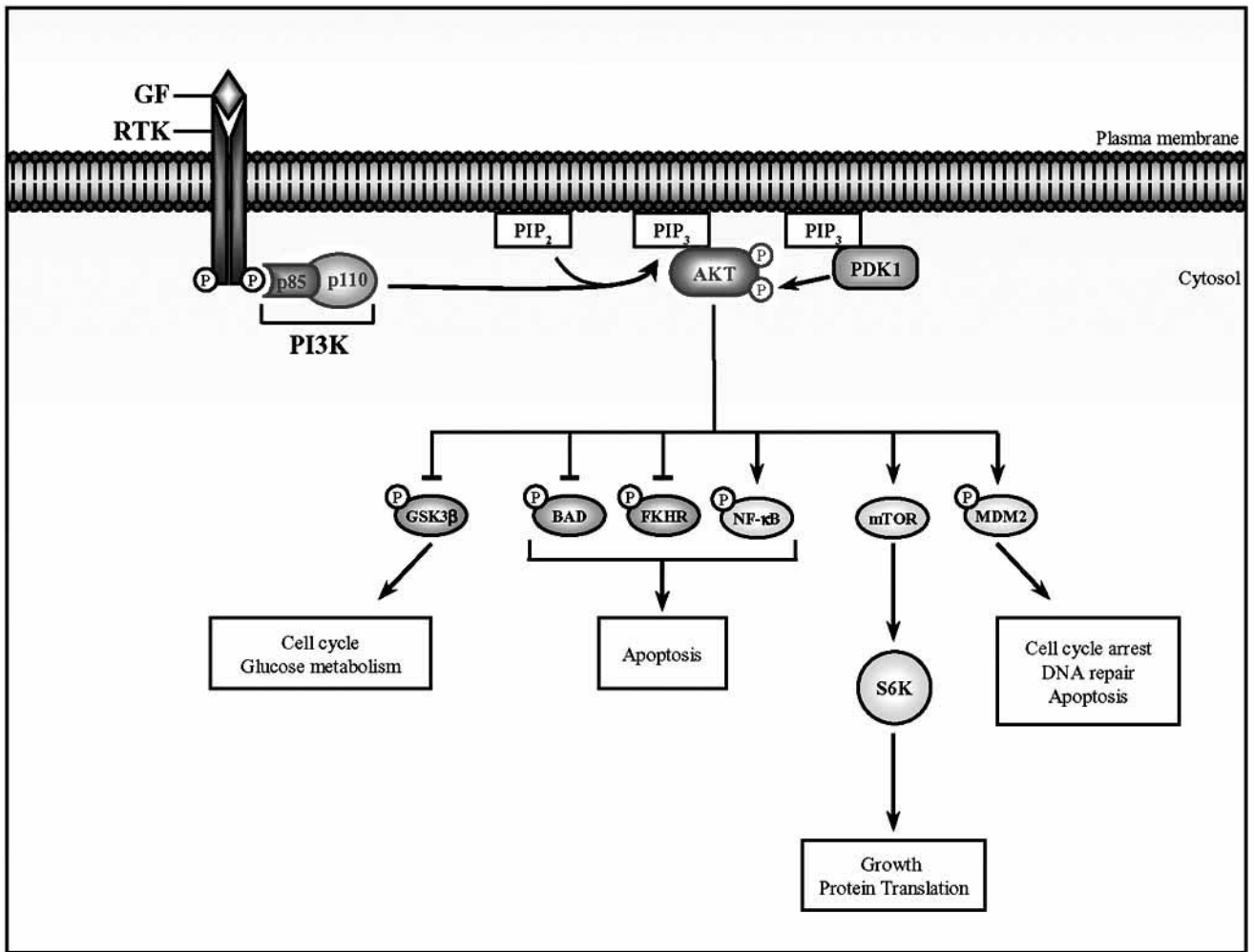
As PI3Ks have been identified to play critical roles in distinct cellular signaling processes, a precise understanding of these kinases, their substrates and effectors is of particular interest. Anomalies in signaling cascades have been described in various human diseases and thus a better understanding of these events is of high importance in the search for therapeutic, diagnostic and screening applications. A number of attempts have been made to better understand PI3Ks in general, as well as to gain insight into the specific functions of the different isoforms. The first selective pharmacological PI3K inhibitors to have been described are wortmannin [16,17], a compound that was originally isolated from soil bacteria and is toxic to fungi, and LY294002 [18], a morpholino derivative of quercetin, a naturally occurring bioflavonoid and broad spectrum kinase inhibitor. Both compounds have been shown to inhibit cell growth at concentrations that would be expected to inhibit class I PI3Ks. However, as these pharmacological inhibitors

display little selectivity within the PI3K family and might also affect other kinases, further research has been aimed at developing compounds with improved specificity and pharmacokinetic properties. Various PI3K inhibitor prodrugs and their possible pharmaceutical applications have been patented by Garlich *et al.* [19]. In addition, patents exist on various PI3K polypeptides, nucleic acid sequences encoding PI3Ks, antibodies that are specifically immunoreactive with PI3Ks and methods for using these reagents in screening and therapeutic applications.

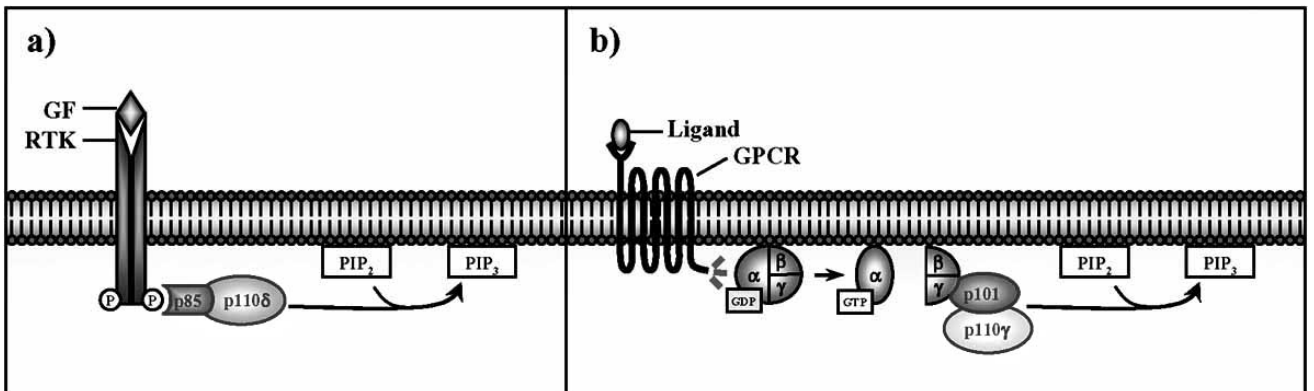
### GENE-TARGETING STRATEGIES

Gene-targeting strategies have been aimed at deleting specific PI3K isoforms and have uncovered key roles of the different enzymes in immunity, metabolism, cardiac function, cell proliferation and cancer susceptibility. Different knock-out mice with targeted deletions of genes encoding PI3K regulatory and catalytic subunits have been generated and phenotypically analyzed [20].

A knock-out mouse with a deletion in the gene of the PI3K class I<sub>A</sub> catalytic subunit p110 has been generated by



**Fig. (2).** Overview of the phosphoinositide 3-kinase (PI3K) signaling pathway. Upon growth factor (GF) binding, transmembrane receptor tyrosine kinases (RTK) are activated by autophosphorylation. PI3K activation occurs when the regulatory p85 subunit is recruited to specific phosphotyrosine sites located in the intracellular domain of the activated RTK. Key downstream signaling mediators include phosphoinositide-dependent kinase 1 (PDK1) which is essential for activation of protein kinase B (PKB/Akt). Effector molecules include glycogen synthase kinase-3 (GSK-3), BAD, forkhead (FKHR), nuclear factor kappa B (NF- B), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (S6K) and murine double minute gene 2 (MDM2).



**Fig. (3).** Activation of p85/p110 and p101/p110 occurs via different receptors. While the p85/p110 complex is recruited to activated receptor tyrosine kinases (RTK) following growth factor (GF) binding (a), the p101/p110 complex is activated by the G subunit following binding of a specific ligand to G protein-coupled receptors (GPCR) (b).

targeting the p85-binding domain of the *PIK3CA* gene, leading to loss of expression of this specific isoform [21]. The *PIK3CA<sup>del/del</sup>* embryos showed a clear developmental delay and died between days 9.5 and 10.5 of embryonic development [21]. In embryos lacking the p110 enzyme, a profound proliferation defect could be observed, which was further supported by a failure in replication of p110-deficient fibroblasts in culture medium, even when supplemented with growth factors. The developmental period between E9.5 and E10.5 is known for increased cellular proliferation, growth, and differentiation. It has thus been hypothesized that the intrauterine death of *PIK3CA<sup>del/del</sup>* embryos at this stage was caused by the inability to meet the increased demand in proliferative signals maintained by PI3K signaling through the p110 isoform [21]. Moreover, mice deficient in the p110 isoform displayed multiple vascular defects [22]. A role for p110 in the control of cell growth was further highlighted by an increase in heart size in transgenic mice expressing a constitutively active mutant of p110 [23]. Conversely, a decrease in heart size was observed upon expression of a dominant-negative mutant of this PI3K isoform in the heart [23]. The interpretation of knock-out data, however, has been complicated by the observation of an upregulation of other PI3K subunits after the deletion of one specific isoform. In *PIK3CA<sup>del/del</sup>* homozygous embryos there was an apparent increase in the expression of the PI3K class I<sub>A</sub> regulatory subunit p85/p55, raising the question of a contributing phenotypical effect by these adapter proteins. Further insight into the function of p110 has been given by the generation of mice carrying a knock-in mutation (D933A) that abrogates the p110 kinase activity [24]. Homozygosity for this mutation resulted in embryonic lethality, while heterozygosity led to impaired signaling via the insulin-receptor substrate (IRS) proteins, which are key mediators of insulin, insulin-like growth factor I (IGF-I) and leptin action. As a result, the mutant mice displayed reduced somatic growth, hyper-insulinaemia, glucose intolerance, hyperphagia, and increased adiposity. Another study recently defined the p110 subunit as the critical lipid kinase required for insulin signaling in adipocytes and myotubes [25]. The discovery of the role of p110 as a key intermediate in metabolic signaling raises concerns about potential side-effects of PI3K inhibitors.

The biological functions of the PI3K class I<sub>A</sub> catalytic subunit p110 have been studied by a partial deletion allele knock-out mouse [26]. Targeting of this specific isoform resulted in very early embryonic lethality in the homozygous state. Zygotes with the *PIK3CB<sup>del/del</sup>* genotype were non-viable very early after fertilization, leading to a deficiency at the blastocyst stage [26]. Crossbreeding studies of p110 knock-out mice with p110-deleted mice did not reveal any possible redundant functions of these two class I<sub>A</sub> catalytic isoforms. However, a possible overlap in functions that might be manifested in more subtle phenotypical abnormalities could not be ruled out so far [26].

Mice deficient in the class I<sub>A</sub> PI3K catalytic subunit p110 have been described by three different groups [27-29]. These studies revealed a major function of p110 in B and T lymphocytes and a failure of the knock-out mice to mount

normal immune responses. The mice lacking p110 showed a reduction in the amount of B1 and marginal zone B cells, as well as lowered serum levels of immunoglobulins [27]. Furthermore, antigen receptor signaling in B and T cells was impaired and the mice were prone to the development of inflammatory bowel disease [28]. However, despite the high levels of p110 expression in hematopoietic tissues, no significant differences were found in either the number and morphology of erythrocytes, peripheral leukocytes and lymphocytes, or hemoglobin levels between wild-type and p110-deficient mice [29].

Knock-out mice with a targeted deletion of the PI3K class I<sub>B</sub> catalytic subunit p110 have been characterized by three different groups [11,30,31]. These studies described phenotypes mainly in components of the innate immune response. Mice lacking p110 showed an accumulation of defective neutrophils, which exhibited a failure in their migratory capacity and a reduced thymic cellularity. Moreover, chemoattractant-stimulated signal transduction was inhibited, because of an impairment of PIP<sub>3</sub> production in p110-deficient cells [30]. In contrast to p110, the deletion of p110 resulted in no effect on B cells. Instead, this isoform appeared to regulate proliferation and cytokine production of T lymphocytes [30]. In view of the impaired migration capacity of p110 knock-out macrophages towards a wide range of chemotactic stimuli, a crucial role for macrophage accumulation in the inflammatory response was furthermore suggested [11]. Phenotypical analysis of mice expressing a kinase-dead p110 only partially reproduced the phenotype of knock-out animals [32]. Whereas both mice exhibited an identical impairment in innate immune reactions, the p110-deficient mice additionally showed a basal enhancement of cardiac contractility and cardiac tissue damage upon pressure overload. It was therefore suggested that p110 possesses a kinase-independent function in the control of cardiac responses. A role of p110 in the control of heart function was also described by Crackower *et al.* [33]. These studies described a role for p110 as a negative regulator of cardiac contractility through the inhibition of cAMP production.

Regarding the regulatory PI3K subunits, gene-targeting strategies have been aimed at disrupting the adapter subunits p85, its splice variants p55 and p50, as well as p85. Ablation of the whole *PIK3R1* gene, which encodes p85 and the mentioned splice variants, resulted in perinatal lethality [34]. However, disruption of the *p85* gene only led to impaired B cell development, a reduction in the number of mature B cells, reduced B cell proliferative responses and a lack of T cell-independent antibody production [35]. Homologous deletion of the gene encoding the adapter subunit p85 resulted in growth reduction of the knock-out mice, compared to their wild-type littermates [36]. Surprisingly, muscle and adipose tissues of both the p85 and p85 knock-out mice exhibited increased insulin-stimulated PI3K activation, enhanced translocation of the glucose transporter isoform-4 to the plasma membrane, as well as hypoglycemia with decreased plasma insulin [34, 36]. Moreover, the mice showed enlarged muscle fibers, brown fat necrosis and calcification of cardiac tissue [34].

## PHOSPHOINOSITIDE 3-KINASES AND HUMAN DISEASE

As the PI3Ks are known to be involved in a wide spectrum of control mechanism in the cell, deregulation of their function has been linked to various human diseases. Key roles of the PI3Ks have so far been described in tumor formation and metastasis, chronic inflammation, allergy and cardiovascular disease.

The importance of PI3K signalling in human cancer is highlighted by the fact that there are numerous oncogenes and tumor suppressor genes whose deregulation activates PI3K signaling and enhances the malignant properties of cells [37]. A prominent finding in this context were mutations in the tumor suppressor gene phosphatase and tensin homologue (*PTEN*), which have been described in various human tumors [1, 37-39]. *PTEN* is a phosphatase that antagonizes the action of PI3K by de-phosphorylating the D-3 position of polyphosphoinositides [40]. The DNA sequence copy number of *PIK3CA*, the gene encoding the p110 catalytic subunit of PI3K located on chromosome 3q26, is frequently increased in ovarian cancers [41]. Moreover, recent reports have described activating mutations in the *PIK3CA* gene in a variety of other human cancers, including, medulloblastoma, breast and colon cancer [42, 43]. Although mutational alterations have predominantly been characterized for p110 so far, an increase in p110 activity was also found in human colon cancer biopsies and adenocarcinoma cell lines [44]. Furthermore, a knock-down analysis of p110 in a prostate cancer mouse model suggested a role for this PI3K isoform in the formation of metastases [45].

Gene-targeting strategies have furthermore uncovered a role for p110 in colorectal adenocarcinoma [46]. Lack of p110 significantly increased the incidence rate of spontaneous development of multifocal carcinomas and invasive adenocarcinoma in the colon of mice. In humans, protein expression analysis of p110 revealed a loss of this protein in a high number of colon cancer cell lines, as well as in primary adenocarcinomas isolated from the colon of human patients [46].

As shown by different studies [30, 47], p110 also plays a pivotal role in mediating leukocyte chemotaxis and activation, as well as mast cell degranulation, thus suggesting an involvement in inflammatory diseases. A specific contribution of the p110 PI3K isoform to inflammation was supported by a recent study demonstrating that p110-deficient mice were protected from rheumatoid arthritis [48]. These mice were essentially protected against collagen II-specific antibody-induced arthritis, correlating with the defective neutrophil chemotaxis observed in this knock-out model [48]. Moreover, treatment of different rheumatoid arthritis mouse models with orally active small-molecule inhibitors of p110 suppressed the progression of joint inflammation and damage in these mice [48]. Other studies, however, have described a positive role for the PI3Ks in the inflammatory response. In rheumatoid arthritis, the anti-inflammatory cytokine IL-10 is spontaneously produced by macrophages and infiltrating blood lymphocytes in the rheumatic joint [49]. An involvement of PI3K signaling in the regulation of IL-10 is known [50] and further analysis

demonstrated that inhibition of PI3K signaling suppressed the production of this cytokine [51].

The PI3K isoform p110 has also been shown to be involved in the inflammatory response [27]. Recent studies further supported this finding and described a contribution to allergen-IgE-induced mast cell activation and vascular permeability, which are common characteristics of chronic inflammations, such as asthma [52]. Pharmacological inhibition of p110 in a murine asthma model resulted in a significant reduction in IgE serum levels and in an attenuation of airway inflammation and hyper-responsiveness, by preventing vascular leakage [52, 53].

Gene knock-out studies of the class I<sub>A</sub> regulatory subunits in skeletal muscle of mice revealed a significant reduction in muscle weight and fiber size. Moreover, these mice exhibited insulin resistance in the muscle and whole-body glucose intolerance [54]. The p85 regulatory subunits are therefore thought to act as critical mediators of PI3K signaling in the regulation of muscle growth and metabolism. Furthermore, they are thought to make an important contribution to the symptoms of hyperlipidemia associated with human type 2 diabetes [54]. Diabetes is associated with vascular complications, including the impairment of vascular function and alterations in the reactivity of blood vessels to vasoactive agents [55]. It has been shown that PI3K signaling plays a role in vascular growth, proliferation and apoptosis and is implicated in modulating vascular smooth muscle cell contractility [56]. A recent study demonstrated that selective inhibition of PI3K attenuated the development of diabetes-induced abnormal vascular reactivity in the carotid arteries of diabetic rats [57].

The growing understanding of the biological functions of PI3Ks has provided new insights into the links between signaling events and cellular responses. As a result, these kinases have become interesting targets in clinical research, driving the urge to patent. Patents were deposited on various components involved in, or targeting PI3K signaling, such as polynucleotides encoding PI3Ks [58], constitutively active PI3Ks and uses thereof [59], cDNA sequences of different PI3K isoforms [60, 61], signaling pathway transducers [62], pharmacological inhibitors [63], and various derivatives [64-68].

## PATENTS

### Cloning, Expression and Characterization of a Novel Form of Phosphatidylinositol-3-Kinase [60, 69]

Stoyanov *et al.* reported for the first time the cloning and characterization of a G protein-activated human phosphatidylinositol 3-kinase [70]. Here we will discuss the identification of the novel isoform of the PI3K family, termed p110 and the cloning, expression and characterization of this enzyme. These findings were patented by the United States Patent Office and by the European Patent Office [60, 69]. The invention concerns a nucleic acid sequence, encoding p110, an antibody directed against the protein, and discusses the diagnostic and therapeutic use of the protein, the nucleic acid sequence and the antibody. The invention comprises seven nucleic acid/protein sequences

(SEQ ID NO: 1-7) and eleven claims regarding the use of these sequences.

The major subject matter of the invention is a nucleic acid sequence encoding a novel PI3K isoform, p110 . In order to isolate the cDNAs, a human bone marrow cDNA library was screened using the polymerase chain reaction. A human bone marrow cDNA library was chosen based on the findings by two other groups, which identified a novel PI3K activity in purified neutrophils and platelets stimulated by G protein subunits [71,72]. For the library screening, oligonucleotide primers were generated based on the highly conserved amino acid sequences KNGDDLRL (termed SEQ ID NO.6) and HIDFG (termed SEQ ID NO.7), which were reported for the catalytic subunit of the first cloned and characterized PI3K (residues 803-809 and 932-936 on p110 ) [73]. Using these primers, a 402 bp-long fragment was obtained and subsequently subcloned and sequenced. The PCR product showed a 57% homology to the corresponding region of the gene encoding the bovine p110 . These PCR fragments were further used as a probe for the identification of a set of overlapping clones from a human U937 cDNA library. These overlapping clones were isolated and analysed, which led to the identification of the largest clone, containing the nucleic acid sequence termed by the authors SEQ ID NO.1. This cDNA sequence contained an open reading frame which codes for a protein of 1049 amino acids (termed SEQ ID NO.2). The new protein sequence was termed p110 and displayed a molecular weight of approximately 120 kDa, similar to the previously published catalytic isoforms p110 and p110 [73,74]. Furthermore, two other highly similar nucleic (SEQ ID NO.3) and amino acid (SEQ ID NO.4) sequences were obtained from the cDNA library, which code for p110 with 1050 amino acids. Thus the invention encompasses two nucleic (SEQ ID NO.1,3) and two amino acid (SEQ ID NO. 2,4) sequences coding for human p110 . It further includes naturally occurring allelic human variations of p110 , as well as proteins produced by recombinant DNA technology, which correspond to the proteins encoded by these sequences.

Furthermore, sequence homology analysis revealed that the amino acid sequence of p110 has a homology of 36% to the human p110 , 33.5% to the human p110 and 27.7% to the yeast PI3K Vps34. The 400 C-terminal amino acids comprised a highly conserved region in these PI3Ks, which encodes a putative catalytic domain. The authors reported moreover that there was no significant homology in the amino-terminal regions of these PI3Ks, which in p110 is known to be responsible for the binding to the regulatory subunit p85 and p85 [75]. This finding raised the question of a different regulatory mechanism for p110 .

A further subject of the invention is the detection of p110 at the protein and transcriptional level. The availability of an antibody that recognizes epitopes in specific regions of p110 would be a big advantage for the detection of the protein in biological samples and may be applied in diagnostic techniques, where patient samples could be tested for abnormal levels of this protein. In order to detect the expression of p110 at the protein level, a polyclonal rabbit

antiserum against p110 was produced by immunization with a 15 amino acid-long peptide corresponding to a unique sequence of p110 NSQL PESFRVPYDPG (SEQ ID NO.5). The expression of p110 in mammalian tissues was analyzed by immunoprecipitation with this specific antibody and subsequent Western blot analysis using the same antibody. In the human leukemic cell lines U937 and K562, p110 was detected as a 110 kDa protein. Hence, a further subject matter of the invention is an antibody specific for p110 , which does not show any cross-reaction with other PI3Ks.

Furthermore, the invention encompasses the detection of p110 at the transcriptional level under stringent hybridization conditions. Multi-tissue Northern blot analysis was performed with random prime-labeled PCR fragments encompassing the sequences encoding amino acids 1 to 233 of p110 . This analysis revealed different expression levels of a 5.3 kb-long mRNA in human tissue from pancreas, kidney, skeletal muscle, liver, lung, placenta, brain and heart.

The invention further encompasses the recombinant expression of p110 . The cloning of the nucleic acid sequence coding for p110 in principle allows the construction of an expression vector enabling the production of the p110 protein in a suitable host cell. The authors presented the use of a baculovirus expression system for the expression of p110 . The cDNA encoding p110 was cloned from codon 4 into the pAcG2T baculovirus transfer vector (BD Biosciences), which allows foreign genes to be expressed as glutathione S-transferase (GST) fusion proteins. Sf9 cells were co-transfected with pAcG2T-p110 and linearized baculovirus DNA prior to expression and purification of the recombinant protein according to standard protocols [75]. This method further allowed the characterization of the substrate specificity of p110 , which was found to phosphorylate PI, PI(4)P and PI(4,5)P<sub>2</sub> on the D-3 position of the inositol ring and was inhibited by wortmannin at nanomolar concentrations. However, recombinant p110 failed to bind the p85 or p85 regulatory subunits. Furthermore, the catalytic activity of the recombinant p110 could be stimulated when incubated with the G subunits, or by Ras-GTP.

Based on the findings described above, the authors further presented a pharmacological model based on the p110 protein, the antibody targeting it and the nucleic acid sequence coding for the active protein. These tools could be used for analyzing the expression and modulating the activity of p110 , which could lead to alterations in cell proliferation, histamine release, differentiation, or glucose transport.

We will now present further insight into the importance of this patent, the increasing evidence of the biological importance of p110 in human diseases and their treatment with novel specific inhibitors. After the purification of the catalytic p110 and the regulatory p101 subunit from pig neutrophils [76], the crystallographic structure of the highly conserved catalytic subunit of the porcine p110 was reported and revealed in detail the structure of the catalytic domain and the Ras-binding domain (RBD) of p110 [77]. Since pharmacological inhibition of the PI3K activity by

wortmannin [16], or LY 294002 [18] was useful to demonstrate their role in a variety of leukocyte responses, but failed to discriminate between distinct PI3K isoforms, neutralizing, isoform-specific antibodies were used for this purpose. For the first time, such an inhibitory antibody was used to determine the role of p110 in leukocyte function, which indicated that this enzyme is required for natural killer cell migration upon chemokine stimulation [78]. The fact that p110 is the sole PI3K isoform activated by GPCRs *in vivo* has been definitively shown in p110 knockout-mice. Three groups reported the generation of p110-deficient mice, which revealed that inactivation of p110 allows normal embryonic and adult development, but causes defects in the immune system [11,30,31]. p110-deficient neutrophils showed defects in migration and oxidative burst in response to GPCR agonists. Furthermore, it was reported that p110 controls thymocyte survival and activation of mature T cells, but has no role in the development or the function of B cells. Moreover, it was observed that p110-deficient macrophages have significantly reduced migration capacity towards chemotactic agents [11]. Taken together, these data demonstrate that p110 has a crucial function in linking GPCR signaling to PI(3,4,5)P<sub>3</sub> production and hence controls the motility of neutrophils and macrophages. Since abnormal macrophage infiltration causes chronic inflammatory diseases, Hirsch *et al.* further proposed, that p110 could be a suitable target for the development of novel specific inhibitors to modulate the function of inflammatory cells [11]. A subsequent study revealed a novel role for p110 in the modulation of mast cell activation and the control of allergic and inflammatory responses [47]. Despite the crucial role of mast cells in the response to infectious agents and parasites, abnormal mast cell activation can cause allergy, asthma and ultimately anaphylactic shock. The release of histamine-containing granules and the increase in intracellular calcium concentration were both attenuated by the loss of p110. Furthermore, the existence of an autocrine activation loop leading to enhanced mast cell activation was proposed [47]. These observations pointed out that inhibition of p110 could result in a therapeutic advantage by diminishing the development of chronic inflammatory diseases and the migration of inflammatory cells, since multiple chemokine agonists activate GPCRs which signal through p110. Based on the emerging evidence that leukocyte motility is crucial in inflammatory disease and that signal transduction occurs through p110, Fuchikami *et al.* presented the first high-throughput *in vitro* screen for p110 activity. The assay made use of recombinantly expressed His-tagged human p110 and had the potential for high-throughput screening for inhibitors of lipid kinase activity [79]. Such an assay could be very useful in identifying novel specific p110 inhibitors, due to fact that the catalytic activity of p110 is crucial for leukocyte migration. It has indeed been reported that mice expressing catalytically inactive p110 show the same chemotactic defects as those observed in p110<sup>-/-</sup> mice [32]. The application of novel specific p110 inhibitors identified by improved screening approaches resulted in the successful treatment of two chronic inflammatory diseases: systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in mice [48, 80]. These results revealed for the first time the high efficacy of targeting signaling molecules in chronic

inflammatory disorders and raise hope for developing therapies with diminished side effects and increased life quality for patients with chronic inflammatory diseases. The use of these specific p110 inhibitors further revealed the contribution of this isoform to thrombus stability [81]. Hence the development of anti-thrombus therapies based on recent findings that p110 modulates the formation and stability of multi-platelet aggregates will be of interest [81-83].

Surprisingly, recent studies have also revealed an oncogenic potential for wild-type p110. It was shown that over-expression of this isoform induced oncogenic transformation in chicken embryo fibroblasts and moreover, that the lipid kinase activity of p110 was essential for transformation [84]. However, cancer-specific mutations in p110 have not been reported to date [84].

More recently, a study revealed a crucial role of p110 in electrotaxis-regulated wound healing. Targeting the p110 gene in mice led to a decrease in electrical field-induced signaling and attenuated the directed migration of healing epithelium [85]. Thus, the first gene involved in cell movement and wound healing in response to electrical signals was identified, making p110 a very promising tool to modulate the wound healing process.

Ten years after the discovery of p110, studies on this key enzyme still uncover new biological functions and the initial hypothesis that targeting this enzyme could have a significant therapeutic benefit could be confirmed by studies using novel specific pharmacological inhibitors.

### Phosphatidylinositol 3-Kinase p110 Catalytic Subunit [61, 86] and Inhibitors of Phosphatidylinositol 3-Kinase p110 [64, 87]

The class I<sub>A</sub> PI3K catalytic subunit p110 was identified and isolated as the last isoform within this class of PI3Ks [6]. A patent on polynucleotides encoding p110 and recombinant p110 polynucleotides, antibodies to p110 and assays for identifying inhibitors of p110 was issued in 1999 by Chantry *et al.* [61, 86]. Their invention was initially based on the identification and isolation of a novel lipid kinase closely related to the other class I PI3K isoforms. The enzyme displayed substantial sequence homology with p110 (72%), p110 (49%) and p110 (45%) in the carboxy-terminal catalytic domain. The newly identified cDNA sequence encoding the lipid kinase was designated p110 and classified in the class I<sub>A</sub> of the PI3K family.

The cloning and characterization of p110 was based on a strategy of amplification of conserved PI3K cDNA sequences. In a PCR analysis, degenerate oligonucleotide primers based on conserved sequences in the catalytic domain of known PI3Ks were used to screen a human cDNA library [6]. The identified full length human p110 cDNA comprised an open reading frame of 3135 nucleotides, encoding a protein of approximately 114 kDa. Similarly to p110, the catalytic domain of p110 was found to be located in the C-terminus of the protein. The functionality of the newly identified PI3K was shown by a lipid kinase activity assay after transfection of cells with epitope-tagged

p110. Production of PI 3-phosphates demonstrated a functional PI3K enzyme activity, which was sensitive to the PI3K inhibitor wortmannin. Similarly to the other class I<sub>A</sub> PI3Ks, which are known to form heterodimers with a p85 subunit, co-immunoprecipitation studies revealed the ability of p110 to associate with the adapter protein p85. Expression analysis in various human tissues revealed an abundant distribution of p110 in lymphocytes and lymphoid tissue, suggesting a role in the immune system [6].

The identification of p110 as another member of the PI3K family opened an interesting avenue for further investigations. The whole family of PI3Ks has been shown to be of high importance in the control of various cellular responses, which has made them interesting targets for research and clinical applications. The described patent includes various of these possible applications.

As a protein involved in signal transduction, the p110 polypeptide is an interesting target for interaction studies. Its use involves the generation of fragments modulating the binding of p110 to a binding partner, or analogs with additions, substitutions or deletions in order to increase or decrease the binding affinity of p110 to a binding partner. Furthermore, modification of the polypeptide might facilitate its passage into the cell and help control its localization. Antibodies specifically immunoreactive with p110 provide another useful tool to modulate the binding affinity of p110 and can further be used for purification of the protein, its detection and quantification in biological samples. Another aspect is that the understanding of the polypeptide provides a base for the identification of modulators that affect the binding affinity, or the functionality of p110, as well as the expression levels of the protein.

With respect to human diseases, the chromosomal localization of certain genes is of high interest to identify deregulated expression of their transcripts. As elevated function of PI3Ks has been shown to play a role in carcinogenesis, the polynucleotides described in the invention may be useful tools for chromosomal localization studies. Furthermore, the above described antibodies could constitute valuable diagnostic tools.

A patent on methods for inhibiting p110 activity, and methods for treating diseases was deposited by Sadhu *et al.* in 2003 [64, 87]. The methods employ active agents that selectively inhibit p110, while not significantly inhibiting other PI3K isoforms. The applications of these p110 inhibitory compounds are of high interest for the treatment of diseases such as disorders of immunity and inflammation. These inhibitors could also be used to inhibit cancer cell growth and proliferation. Furthermore, methods for the inhibition of p110-mediated processes *in vitro* and *in vivo* were included in the described patent.

Generic PI3K inhibitors such as LY294002 [18] or wortmannin [16, 17] have contributed a lot to the understanding of these kinases. The identification of specific functions of the different PI3K isoforms, however, requires more targeted approaches and forced the quest for substances acting on only one specific enzyme. The described invention provides the structure of several compounds displaying

selective inhibitory activity towards p110. The compounds were selected to be at least 10-fold more selective for p110, when compared to other PI3K isoforms. The described compounds inhibit the biological activity of human p110 and represent a useful tool to further characterize the functions of p110. Another aspect of the invention is that the compounds provide methods to selectively modulate p110 activity, which represent useful tools for the treatment of diseases caused by p110 dysfunction.

An embodiment for the use of the described patent includes methods for inhibiting the growth and proliferation of cancer cells. The PI3K p110 has been suggested to play a role in cancer development and a study has shown that p110 enhances the radiation-induced tumor control in murine cancer models [88]. Treatment of mice with Lewis lung carcinoma or hind limb tumors with a p110-specific inhibitor in combination with radiation abrogated the radiation-induced phosphorylation of Akt and significantly reduced the tumor volume [88]. A recent study further underlined the potential of p110-specific inhibitors in cancer treatment by describing a reduction in cell proliferation of acute myeloid leukemia (AML) cells [89]. Treatment of AML cells with a p110-specific inhibitor decreased the constitutive phosphorylation of Akt found in AML cells and furthermore was synergistic in reducing cell viability in combination with the topoisomerase II inhibitor VP16 [89]. Another field of application results from the known role of p110 in B cells. Studies have shown an involvement of p110 in the recruitment and activation of certain inflammatory cells [90]. Inhibition of p110 impaired the movement of neutrophils across inflamed venules [90]. Furthermore, specific inhibition of p110 activity attenuated the allergic airway inflammation and hyper-responsiveness in a murine asthma model [52]. Treatment of model mice with a p110-specific inhibitor significantly reduced antigen-induced airway infiltration of inflammatory cells, secretion of T<sub>H</sub>2 cytokines in the lungs, as well as vascular permeability [53].

### **Mutations of the *PIK3CA* Gene in Human Cancers [91], Condensed Heteroaryl Derivatives and their Use as Inhibitors [65]**

The p110 isoform was described as a retroviral oncoprotein that can transform chicken embryo fibroblasts *in vivo* [92]. Its key role in tumorigenesis was further supported by accumulating evidence indicating that p110 gain-of-function by over-expression or by somatic missense mutations is common in many human cancers. The *PIK3CA* gene consists of 20 exons encoding a protein of 1068 amino acids and 124 kDa size. The 3q26 locus where it is located is amplified in several human cancers, including head and neck cancers [93], cervical cancers [94], gastric cancers [95] and lung cancers [96]. In a landmark study, Samuels *et al.* performed a large-scale sequence analysis of 8 PI3K and 8 PI3K-related genes in human cancers and discovered that *PIK3CA* was the only gene harboring somatic mutations [43]. Furthermore, they reported a surprisingly high proportion of colorectal cancers (32%), gastric cancers (25%) and glioblastomas (27%) carrying somatic mutations in *PIK3CA*. These mutations also occurred in a smaller fraction of lung cancers (4%) and breast cancers (8%). Interestingly,

all mutations were apparent activating missense mutations that were found to cluster primarily in two major “hot-spots”, E545 in the helical phosphatidylinositol kinase homology domain, and H1047 in the catalytic domain (exon 9 and exon 20, respectively). Clearly, the clustering of mutations implies that *PIK3CA* sequence analysis may prove useful for diagnostic purposes, as a marker for early detection of cancers, or for monitoring tumor progression. Furthermore, these data provide a reasonable case for considering p110 as a potential target for pharmacological intervention. Subsequent studies expanded these findings and collectively found *PIK3CA* mutations in a wide range of tumors, such as modulloblastoma (5% and 27%), medulloblastoma (5%), breast cancer (18%, 25%, 27% and 40%), lung cancer (4% and 1%), gastric cancer (6.5%), ovarian cancer (6.6% and 12%), liver cancer (36%) and acute leukemia (1%) [43, 97-100].

Samuels *et al.* patented their discovery [91], which comprises methods for assessing cancer on body samples from a human suspected of having cancer based on the detection of intragenic mutation in a *PIK3CA*-coding sequence. The invention also includes several methods for inhibiting progression of a tumor in a human by reducing the expression, or inhibiting the activity of p110, a method for delivering an appropriate chemotherapeutic drug to a patient in need thereof, and a set of one or more primers for amplifying and/or sequencing *PIK3CA*.

The discovery of non-random somatic mutations in the *PIK3CA* gene in a wide range of human tumors strongly pointed to an oncogenic role for the mutated enzyme. In order to elucidate the consequences of *PIK3CA* alterations, Kang *et al.* determined the growth-regulatory and signaling properties of the three most frequently observed PI3K mutations: E542K, E545K, and H1047R. When expressed in chicken embryo fibroblasts, all three mutants induced oncogenic transformation with a high efficiency and led to constitutive increases in the phosphorylation of Akt, p70 S6 kinase, and 4E-binding protein-1 [42]. This transforming ability correlated with elevated catalytic activity in *in vitro* kinase assays. The expression of these mutant *PIK3CA* enzymes conferred Akt activation in the absence of growth factor stimulation, promoted cell growth and invasion [42, 101]. Moreover, treatment with the PI3K inhibitor LY294002 abrogated PI3K signaling and preferentially inhibited growth of *PIK3CA* mutant cells.

This remarkable progress in our understanding of the role of the PI3K p110 in tumorigenesis allowed the development of new therapeutic strategies for the treatment of cancers harboring *PIK3CA* gene amplifications or mutations. Recently Hayakawa *et al.* carried out a high-throughput screen to identify novel p110 inhibitors and 4-Morpholino-2-phenylquinazolin was discovered as having p110 -inhibitory activity [102]. A series of derivatives were synthesized from this lead compound. Introduction of a 3-hydroxy group on the phenyl group and replacement of the quinazoline ring with a thieno [3,2-*d*]pyrimidine ring resulted in 3-(4-Morpholinothieno[3,2-*d*]pyrimidin-2-yl) phenol hydrochloride, also designated YM024. This compound is a highly selective inhibitor of p110, when compared to other kinases and PI3K isoforms, with an IC<sub>50</sub>

value of 2.0 nM, making it the first example of a p110 -selective PI3K inhibitor. These series of compounds were patented in 2003 by the same group [65], claiming a pharmaceutical composition for a PI3K inhibitor comprising a fused heteroaryl derivative, or a salt thereof and a pharmaceutical acceptable carrier. Moreover, patents exist for their use in the manufacturing of medicaments for use in the treatment of a disorder in which PI3K plays a role, and for use in the treatment of cancer. They also describe a method to treat disorders which are associated with PI3K and to treat cancer by administering an effective amount of the fused heteroaryl derivative to a patient [65]. According to the invention, the pharmacological effects of the compounds have been verified by several pharmacological tests. Compounds of the present invention exhibited an excellent p110 -inhibitory activity, with IC<sub>50</sub> values below 1 μM, whereas others were confirmed to have inhibitory activities against other PI3K isoforms, such as PI3KC2. Several compounds exhibited an excellent growth-inhibitory activity *in vitro* against colon cancer, melanoma, lung cancer, glioma, ovary cancer, prostate cancer and pancreas cancer. Furthermore, inhibition of cancer cell growth was assessed *in vivo*. HeLaS3 cells, a human cervix cancer cell line, were inoculated into the flank of female BALB/c nude mice by subcutaneous injection. When the tumor reached 100-200 mm<sup>3</sup> in volume compounds were administered intraperitoneally once a day for 2 weeks and tumor volume after treatment was determined. The test compounds exhibited superior anti-tumor activities as compared the control group.

YM024 has also already proven to be a valuable research tool for the investigation of isoform-specific p110 functions, not only in cancer, but also in other PI3K-mediated processes, such as inflammation [103].

*PIK3CA* represents one of the most frequently mutated oncogenes identified to date and one of the few established human oncogenes that is commonly activated through either gene amplification or point mutations. Notably, no mutations in the other PI3K catalytic subunits have been reported in human cancer until the present date, suggesting that the PI3K p110 harbors a selective oncogenic potential. The oncogenic transforming activity of the mutant p110 proteins makes them promising targets for small molecule inhibitors that could be further developed into effective and highly specific anticancer drugs.

#### Methods for Modulating T cell Responses by Manipulating Intracellular Signal Transduction [104]

T lymphocytes are cells involved in the adaptive immune response (secondary response), which in case of infection are able to provide a more specific response than the innate immune system (primary response). T cell precursors produced by the bone marrow, migrate to the thymus, where they undergo complete differentiation and selection. In the thymus, T cells come in contact with self MHC-peptide complexes expressed on antigen-presenting cells (APCs) to which the T cells should not react. T cells that show the appropriate affinity for the self MHC-peptide complexes are allowed to migrate to the secondary lymphoid organs (spleen and lymph nodes), while the others are eliminated [105]. Immature T lymphocytes express both the CD4 and CD8 co-

receptor molecules on their surface, but after the selection and maturation process they express only one of them. While CD4<sup>+</sup> cells recognize non-self antigen presented on the surface of APCs by MHC class II molecules and become cytokine-secreting cells, CD8<sup>+</sup> cells recognize non-self antigen presented by MHC class I and become cytotoxic effector cells. APCs include cells such as dendritic cells, Langerhans cells and natural killer cells (NK).

In the secondary lymphoid organs, the lymphocytes are in a state of anergy, until they encounter APC cells, which present a non-self peptide in complex with MHC class I or II molecules on their cell surface (either from a pathogen or a cancer cell) [106]. Mature T cells recognize MHC-non self peptide complexes via their transmembrane T cell receptor (TCR). However, this interaction is not sufficient to generate a proper immune response against the antigen. For complete T cell activation, an interaction with co-stimulatory ligands, such as CD80 and CD86 expressed on the surface of the APCs is necessary. These co-stimulatory ligands are able to bind to the CD28 receptor on the surface of T cells. The signals from this co-stimulatory receptor, in combination with those from the TCR, activate the cell and initiate gene transcription, cell growth and mitosis. One of the most important effects of the co-engagement of TCR and CD28 receptors is the production of IL-2, a cytokine that provides strong proliferative and survival signals.

Several studies have provided clear evidence for a rapid recruitment and substantial activation of PI3K in T cells, which drives the production of D-3 phosphoinositides as signal mediators. Activation of T cells induces a time-dependent elevation of the described second messengers, which remain high until nine hours after activation and then return to normal levels. Subcellular localization studies revealed a major presence of these phosphoinositides in the contact area between the T cell and the APC [107].

Further evidence for an involvement of PI3Ks in proliferation signals in T cells was provided by the fact that mouse T lymphocytes stimulated with an anti-CD3 antibody showed a decrease in their proliferation rate in the presence of the PI3K inhibitor LY294002. In T cells from mice carrying a mutation in the PI3K catalytic subunit p110 (p110<sup>D910A/D910A</sup>), a reduction in Akt/PKB phosphorylation, as well as in CD4<sup>+</sup> T cell proliferation in response to CD3 stimulation was observed [28,108]. The proliferation capacity of CD4<sup>+</sup> cells was restored upon co-stimulation with CD28. These results supported other studies describing an important role for PI3K in TCR signalling independently of CD28, even though the molecular mechanism by which the TCR is coupled to PI3K is still unclear [109,110]. Similar experiments performed in p110 knock-out mice yielded less clear results, which might be caused by a greater capacity of p110 and p110 to compensate for the absence of p110 expression [29].

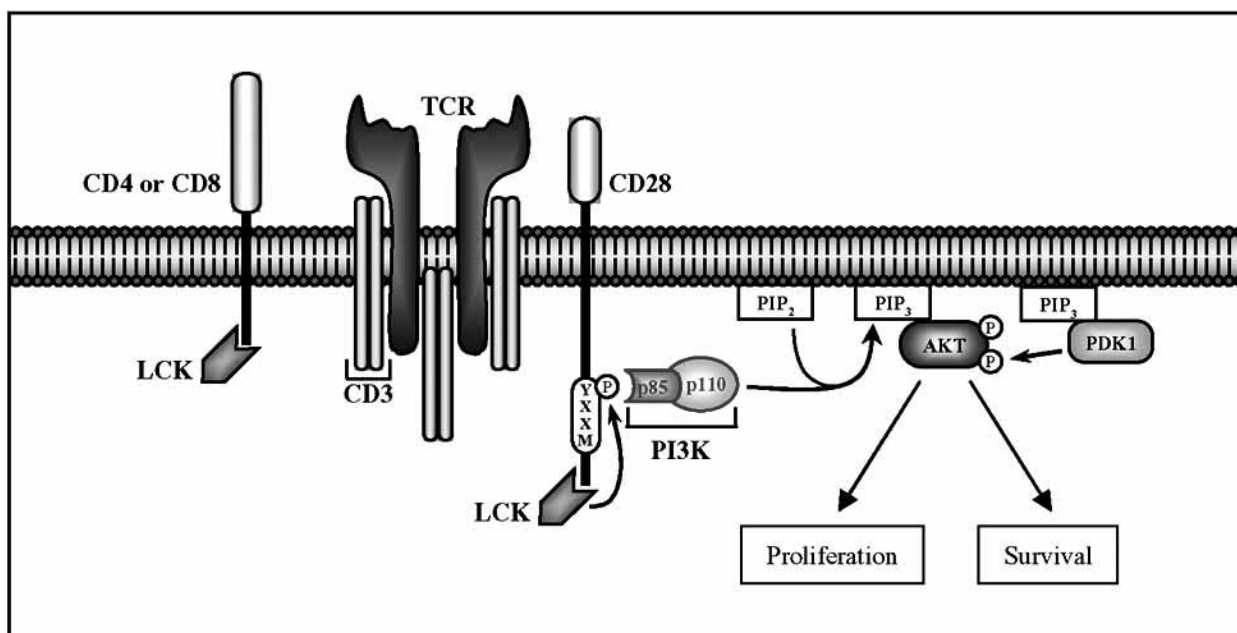
Interestingly, it was shown that CD28 can activate PI3K independent of the TCR and that CD28 contains a Tyr-X-X-Met motif in its cytoplasmic domain that can bind the SH2 domain of p85 [107,111,112]. However, the exact role played by CD28 and its cytoplasmic domain in the activation of PI3K is still not totally understood. CD28-deficient mice expressing a CD28-Tyr170Phe mutant

(Phenylalanine to Tyrosine substitution in the PI3K binding motif of CD28), were still able to co-stimulate T cell proliferation and IL-2 production independently of CD28 association with PI3K [113]. Instead, a defect in Bcl-Xt activation and T cell survival was observed [113, 114]. On the other hand, the capacity of CD28-deficient mice to proliferate and produce IL-2 was also restored by expression of an activated mutant of Akt/PKB, which seems to be in conflict with the previous observation [115]. Despite the fact that the ability of CD28 to recruit PI3K might be dispensable for T cell proliferation and IL-2 production, it is still possible that CD28 can influence the capacity of the TCR to couple to PI3K and activate Akt/PKB independently of the CD28-Tyr170. Furthermore, the cytoplasmic domain of CD28 contains two Proline-rich regions (PYAP) that are able to facilitate the recruitment of p85 through its SH3 domain [116,117]. Taken together, it appears that the activation of PI3K due to CD28 triggering can be *direct*, through the involvement of the YXXM region (Fig. (4)), or *indirect*, through the involvement of the PYAP region and consequent involvement of proteins such as Lck and ZAP-70.

A patent on methods for modulating T cell responses by manipulating intracellular signals associated with T cell co-stimulation was deposited by June in 2004 [104]. The invention focuses on the inhibition or the activation of the production of D-3 poly-phosphoinositides in T cells, which can be achieved by manipulating PI3K signaling, or other intracellular signals associated with co-stimulation. The patent describes useful tools for clinical applications, including situations where it is desirable to inhibit an immune response to an antigen(s), e.g. organ or bone marrow transplantation and autoimmune diseases.

The evidence that CD28 activation plays a crucial role in T cell activation and that PI3K plays a central role in the CD28 cascade focused the attention of research on the possibility of suppressing the immune system by modulating PI3Ks and the signal cascade controlling its activation. The best described PI3K inhibitors are wortmannin [16,17] and the quercetin derivative LY294002 [18]. However, their lack of isoform specificity, as well as their high overall toxicity limits their utility with respect to clinical applications. In view of the limitations of generic pharmacological inhibitors, there is a need to find other agents that can be used alone, or in combination with these PI3K inhibitors. A main result of combining different drugs that act by different mechanisms is that a very powerful immunosuppressive effect can be achieved. This makes it possible to administer low doses of each single drug, which reduces drug-related toxicity. The use of compounds displaying higher specificities, such as inhibitors of the PI3K isoforms p110 or p110 would also have great advantages and are described in this review [60, 64]. An important benefit of specifically suppressing the immune system is that the body is still able to maintain a sufficient level of host defense to protect itself against infections and malignancies.

A new strategy directly connected to the suppression of CD28 activation was derived from the evidence that the cytotoxic T lymphocyte antigen 4 (CTLA4) receptor binds to



**Fig. (4).** Involvement of PI3K in T cell receptor (TCR) signaling. The cytoplasmatic tail of the CD28 co-receptor contains a YXXM motif, to which the PI3K regulatory subunit p85 can bind upon tyrosine phosphorylation. While PI3Ks have the potential to regulate different signaling pathways in T cells, the exact role they play is not yet clear.

the same APC ligand (CD80-CD86) as CD28 [118,119]. The interaction results in an inhibition of IL-2 production, cell-cycle progression and the activation of TCR-induced cyclins through blockage of JNK, Erk and NF- $\kappa$ B activation [120-122]. It would thus be possible to block CD28 engagement and induce CTLA4 triggering by treating patients with a B7-specific fusion protein CTLA4-Ig. The use and toxicity of this molecule is, however, a controversial issue and thus it was used in different approaches depending on the disease. In human bone marrow transplantation, CTLA4-Ig has an *in vitro* use to prevent graft-versus-host disease (GVHD). Its use *in vivo* is common in case of allograft rejection of kidney and heart, as well as for the treatment of psoriasis [123,124]. The effect of CTLA4-Ig might further be improved by combined use with a CD40L-specific monoclonal antibody that induces CTLA4 over-expression [125,126].

Several other drugs/agents are already under study, some of which directly target the different steps of the extracellular signal-regulated kinase (Erk) cascade, Jun N-terminal kinase (JNK), or p38 MAPK and are commonly used *in vitro* for interference with cytokine production. Current immune suppressive therapies which are used mainly in organ and bone marrow transplantation and occasionally in rheumatoid arthritis are Cyclosporine A (a fungal metabolite extracted from *Tolypocladium inflatum gams*) and Tacrolimus (derived from the soil fungus *Streptomyces tsukubaensis*). These two molecules are able to block the translocation of the nuclear factor of activated T cells (NFAT) to the nucleus. In this way these compounds block IL-2 production and cell proliferation, which are the first steps in T cell activation [127-129].

All these different inhibitory approaches can be combined with the PI3K inhibitors described before. In this way, the

problems resulting from the use of wortmannin or quercetin-derived inhibitors alone could potentially be overcome.

There are two main types of applications for inhibitors of T cell activation, which are *in vivo* and *in vitro*. In the first case, the PI3K inhibitor, together with the other agents chosen (especially in the case of antibodies), are directly injected into the patient. For example, in case of organ transplantation, the agents are injected together with the transplant and maintained by follow-up injections. However, this approach is not always successful, depending on the toxicity of the agents and possible unspecific collateral effects on other cells. In the case of transplantation, an *in vitro* approach is therefore often preferred. In this case, a state of anergy has to be induced in order to avoid the GVHD which happens in allogeneic transplantation. This approach is based on cells from a donor cultured together with a CD3-triggering agent and a CD28 inhibitor. At the time when the T cells are totally anergic, they can be transplanted into the patient together with the cells needed.

Most of what has been described above relates to the capacity to trigger CD28 and its related proteins in order to induce T cell anergy. However, these approaches can also be used for immune system stimulation, which is especially beneficial to induce tumor rejection. The basis of this approach is the possibility to produce a stronger co-stimulation for specific T cells that have already received a primary activation signal through TCR triggering. The most important application of PI3K activation/co-stimulation is in cancer immune therapy. In general, chemoresistant cancer cells that express specific antigens on their surface are able to induce T cell activation. However, this activation is often too slow and too low for a complete elimination of the cancer cells [130]. An approach to boost the signal for the immune system is the implementation of a co-stimulation,

independently of the presence of APCs. Some drugs already commonly used in the clinic are thalidomide analogs such as ImiDs and SelCIDs. Their role in co-stimulation of T cells has already been demonstrated [131]. As already discussed in the case of immune system tolerance, the use of these compounds can be planned *in vitro* or *in vivo*.

*In vitro* cancer immunotherapy can be divided into passive and adoptive methods, depending on the characteristics of the lymphocytes generated and injected into the patient. In the case of the adoptive immunotherapy, lymphocytes are cultured in the presence of a specific antigen and a CD28 stimulator (or agents that induce co-stimulation). In this case, the T lymphocytes generated and re-injected into the patient are able to directly attack the tumor cells [132, 133]. In contrast, lymphocytes generated for the active immunotherapy are not themselves specific for the antigen, but are able to stimulate the patient's immune system in order to respond better and faster to the antigen. The latter method can also be used in patients with a temporary immune deficiency in order to generate a temporary immune protection against common pathogens [134].

*In vivo* immunotherapy relates to the possibility of inducing a stronger and faster immunity without any cellular vaccination. Patients are treated with direct injections of agents which provide a stronger and longer co-stimulatory signal by acting directly on specific T cells that have already received a signal through *in vivo* TCR engagement.

The growing understanding of PI3Ks and their possible role(s) in the immune response opened an interesting field of research regarding the identification of better and safer immunosuppressive or immunostimulatory drugs. However, an improvement in short and long term outcome of the desired immune response is still needed. Multiple potential PI3K modulators have recently been described and screening assays will hopefully reveal their potential uses in immunotherapy. However, *ex vivo* studies still have to be performed for a better understanding of the capacity of drugs targeting PI3K activity to modulate immune responses. Systematic studies in animals and human patients will then be required to bridge the transition in drug development from target enzymes to the modulation of lymphocyte function.

## CURRENT & FUTURE DEVELOPMENTS

The patents described in the present review have a wide range of potential applications in medical research, including the development of novel anti-cancer drugs and anti-inflammatory agents. This is the consequence of the various biological functions of distinct PI3K isoforms, which have been characterized in the past years. Gene targeting and transgenic mouse models have been instrumental in describing the distinct roles of the p110 and p110 isoforms in the immune system. These studies have been confirmed by the use of isoform-specific pharmacological inhibitors, which are now further being developed as novel drugs for diseases such as rheumatoid arthritis and allergies. In the case of p110, the discovery of somatic mutations in the *PIK3CA* gene in a substantial number of different human cancers has made this PI3K isoform a validated target for the development of anti-cancer agents. However, in view of the

complexity of the signaling networks involving PI3K isoforms [24,32,135], it remains unclear how soon drugs targeting these enzymes will successfully achieve the transition from the bench to the bedside.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Swiss National Science Foundation (Grant 3100A0-105321), Oncosuisse (OCS-01501-02-2004), the Forschungskredit der Universität Zürich and the Krebsliga Zürich to AA.

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