Identification of PI3Kγ in Endothelial Cells and its Involvement in Sphingosine 1-Phosphate Mediated Endothelial Cell Migration

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Identification of PI3Kγ in Endothelial Cells and its Involvement in Sphingosine 1-Phosphate Mediated Endothelial Cell Migration

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Zusammenfassung

Das aus Thrombozyten freigesetzte Sphingosin-1-Phosphat (S1P) akkumuliert während entzündlicher Vorgänge. Es aktiviert Zielzellen über G-Protein-gekoppelte Rezeptoren der “Endothelial differentiation gene” (Edg) -Familie. S1P induziert Proliferation sowie Migration in Endothelzellen und ist ein wichtiger angiogener Faktor. Die Signalwege, die nach Bindung an Edg-Rezeptoren die einzelnen S1P-stimulierten Antworten vermitteln, sind nicht vollständig aufgeklärt.

Zellmotilität wird unter anderem auch durch Phosphoinositid-3-Kinasen (PI3K) gesteuert. Vor allem die an ein Gi-Protein gekoppelte PI3Kγ scheint für die durch Chemokine stimulierte Migration von Leukozyten verantwortlich zu sein und daher in Entzündungsreaktionen eine große Rolle zu spielen. Die PI3Kγ besteht aus einer katalytischen Untereinheit mit sowohl Lipidkinase- als auch Proteinkinase-Aktivität (p110γ) und aus einer nichtkatalytischen Adaptor- oder Regulator-Untereinheit (p101).

In der hier vorgelegten Arbeit wurde die Expression der PI3Kγ in Endothelzellen mit Hilfe einer sensitiven Reversen Transkriptase-PCR und in Immunoblotanalysen untersucht. Wir konnten erstmals zeigen, daß p110γ in Endothelzellen verschiedenen Ursprungs sowohl auf mRNA- als auch auf Proteinebene exprimiert ist, während die mRNA der bisher als Adaptor- oder Regulatorprotein angenommenen Untereinheit p101 nicht nachweisbar war. Unsere Daten lassen daher vermuten, daß PI3Kγ in Endothelzellen unabhängig von p101 aktiv sein kann.

Die Stimulation von Endothelzellen aus Nabelschnurvenen (HUVEC) mit S1P führte in der vorgelegten Studie zu einer zeit- und konzentrationsabhängigen Aktivierung der „Extracellular signal regulated kinase“ (ERK), der Proteinkinase B/Akt und der niedrigmolekularen GTPase Rac-1. Diese S1P-induzierten Antworten konnten durch Pertussistoxin und durch die PI3K-Inhibitoren Wortmannin und LY294002 reduziert werden, was auf die Einbeziehung eines Gi/PI3K-gekoppelten Signalweges hindeutet. Dagegen waren Tyrosinkinasen und

Transaktivierung von Wachstumsfaktor-Rezeptoren vermutlich nicht an der S1P-stimulierten Aktivierung von ERK, Akt oder Rac-1 beteiligt. Weder Genistein, ein genereller Tyrosinkinase-Inhibitor, noch Inhibitoren der Rezeptortyrosinkinasen für „Vascular endothelial growth factor“ (VEGF), „Platelet-derived growth factor“ (PDGF) oder „Epidermal growth factor“ (EGF) waren in der Lage, diese S1P-vermittelten endothelialen Antworten zu hemmen.

S1P führte zu einer zeit- und konzentrationsabhängigen Stimulation der Migration von Endothelzellen (HUVEC), die möglicherweise über die Proteinkinase Akt und/oder Rac-1 vermittelt wird. Für beide Signalmoleküle wurde bereits die Beteiligung an Signalwegen, die Zellmotilität steuern, beschrieben. Die S1P-induzierte Migration der Endothelzellen wurde durch Pertussistoxin, Wortmannin und LY294002 verhindert, was auf die Beteiligung einer Gi-gekoppelten PI3K, vermutlich der PI3Kγ, schließen läßt.

Die transiente Überexpression einer katalytisch inaktiven Mutante der PI3Kγ (PI3Kγ KR) mit Hilfe einer neuen, auf Elektroporation beruhenden Transfektions-Technologie führte zu einer Verminderung der S1P-induzierten Migration. Erste Ergebnisse von Kotransfektionsexperimenten mit PI3Kγ KR und „Green fluorescent protein“ (GFP) als Indikator, zeigen zudem, daß die Migration in Zellen, in denen die dominant negative Mutante der PI3Kγ überexprimiert ist, blockiert ist. Die Untersuchungen der vorgelegten Studie weisen damit auf eine wichtige Rolle der PI3Kγ bei der Vermittlung Gi-Protein-gekoppelter endothelialer Funktionen hin.

Summary

Platelet-derived sphingosine 1-phosphate (S1P) is a potent angiogenic factor that accumulates at high levels during inflammation. Via activation of G-protein-coupled receptors belonging to the endothelial differentiation gene (Edg) family, S1P has been demonstrated to mediate proliferatory and migratory responses in endothelial cells. However, the signaling molecules and pathways activated downstream of Edg receptors are not fully defined.

Phosphoinositide 3-kinases (PI3K) have been implicated in cell motility. In particular, Gi-coupled PI3Kγ has emerged as a key mediator of the chemoattractant- stimulated leukocyte migration and hence, plays a pivotal role in inflammation. Structurally, PI3Kγ consists of a p110γ catalytic subunit, which exhibits both lipid and protein kinase activities, and a recently identified non-catalytic p101 adaptor subunit.

The present study investigated the expression of PI3Kγ and its adaptor protein p101 in endothelial cells and their involvement in S1P-mediated cellular signaling. Using a sensitive reverse-transcriptase PCR and immunoblotting analysis, we demonstrate for the first time that p110γ is expressed in vascular endothelial cells of different origins. Remarkably, expression of the putative adaptor subunit p101 was not detected at the RNA level, suggesting that p101 is dispensable for PI3Kγ function in endothelial cells. The existence of p110γ in the absence of p101 has not been described previously.

S1P stimulation of endothelial cells isolated from umbilical cord veins (HUVEC) resulted in a time- and concentration-dependent activation of the extracellular signal regulated kinase (ERK), the protein kinase B/Akt, and the low molecular weight GTPase Rac-1. These cellular responses were inhibited by pertussis toxin and by pharmacological PI3K inhibitors (wortmannin or LY294002), providing evidence for the involvement of a Gi/PI3K-coupled pathway. In contrast, tyrosine kinase activity and growth factor receptor transactivation do not contribute

significantly to S1P-stimulated ERK and Akt phosphorylation, since these processes were neither inhibited by genistein, a general tyrosine kinase inhibitor, nor by specific receptor tyrosine kinase (RTK) inhibitors against vascular endothelial cell growth factor (VEGF), platelet-derived growth factor (PDGF) or epidermal growth factor (EGF).

S1P induced a time- and concentration-dependent stimulation of endothelial cell migration. This probably occurs via a mechanism involving Rac-1 and Akt, as both have been implicated in S1P-stimulated migratory responses. S1P-stimulated endothelial chemotaxis was abrogated by pertussis toxin, wortmannin, and LY294002 treatment, suggesting an involvement of the Gi-coupled PI3Kγ.

The role of PI3Kγ in S1P-induced migration was assessed in transient transfection studies using a novel, electroporation-based transfection technique. Overexpression of a catalytically inactive form of PI3Kγ, PI3Kγ KR, significantly reduced S1P-induced chemotaxis. Furthermore, co-transfection of the green fluorescent protein (GFP) confirmed that migration was abolished in cells that specifically express the dominant negative form of PI3Kγ. In conclusion, the present study describes the identification of PI3Kγ in endothelial cells and provides strong evidence for an essential role of PI3Kγ in Gi-protein mediated endothelial chemotaxis.

Table of Contents

1. Introduction

1.1 Angiogenesis and the vascular endothelium

The vascular endothelium consists of simple squamous epithelium lining the lumen of all blood vessels. Located at the interface between the circulation and underlying tissues, the vascular endothelium has been long thought to be an inert layer which functions only to separate the blood from tissues. In recent years, it has become clear that the endothelium not only provides a structural barrier but is a dynamic tissue that is essential for a wide variety of biological processes, including the regulation of vascular tone, inflammation, hemostasis and angiogenesis. In adults, the endothelium is normally quiescent despite constant exposure to circulating blood cells and plasma components. In its physiological state, the endothelium is tightly regulated in order to maintain a non-thrombogenic, non-adherent surface, which is capable of regulating macromolecule/nutrient exchange and production of vasoactive substances and growth factors. During vascular injury and wound healing, the endothelium becomes activated and is responsible for the recruitment of immune cells to sites of inflammation and the regulation of blood coagulation.

The endothelium plays a central role in angiogenesis. Angiogenesis is the biological process by which new blood vessels are formed from the pre-existing vasculature. Under physiological conditions, angiogenesis is tightly regulated and plays an essential role in development (vasculogenesis), reproduction, inflammation and wound healing. However, unregulated angiogenesis can occur under pathological conditions and contributes to numerous diseases, including tumor growth and metastasis, and diabetic retinopathy and rheumatoid arthritis.

Angiogenesis is a complex, multistep process involving the dynamic interplay between cells, soluble factors and the extracellular matrix. Despite this complexity, angiogenesis can be broken down into several discrete steps: degradation of the extracellular matrix (ECM) by proteolytic enzymes, migration and proliferation of endothelial cells, and morphological differentiation into three dimensional tube-like

structures. In recent years, it was found that each of these activities can be stimulated or inhibited by specific molecules. Folkman, the pioneer of angiogenic research, proposes that angiogenesis is regulated by a balance between positive and negative mediators, constituting the “angiogenic switch”. Furthermore, Folkman proposes that an imbalance of these regulators determines the angiogenic or anti-angiogenic phenotype, resulting in a “turning on” or “turning off” of the angiogenic switch (Folkman et al., 1997).

The field of angiogenesis has advanced rapidly in the last decade with the discovery of specific molecules which promote angiogenesis. Although many factors have been identified, the molecular mechanisms by which they regulate angiogenesis remain poorly characterized. Until recently, most studies have focused on polypeptide growth factors, such as basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF), which are mitogenic for endothelial cells *in vitro* and promote angiogenesis *in vivo*. These factors have been shown to physically bind and activate receptor tyrosine kinases, thereby initiating intracellular signaling pathways which drive the angiogenic process. A number of membrane lipid metabolites, such as prostaglandin E1 and E2, have been shown to induce angiogenesis *in vivo* (Jackson et al., 1997). Recently, Pinedo found that platelets contribute to angiogenesis via the secretion of platelet derived angiogenic factors (Pinedo et al., 1998). One potent factor is the lysophospholipid sphingosine 1-phosphate (S1P), which is abundantly stored in platelets and accumulates at high levels at sites of vascular injury. Although S1P is observed to potently contribute to angiogenesis, the molecular mechanisms by which S1P exerts its effects remain poorly characterized.

1.2 Sphingosine 1-Phosphate (S1P)

S1P, a polar sphingolipid metabolite, is stored and released from activated platelets. Platelets are the primary source of S1P in plasma and serum, but S1P can be also synthesized by many cell types in response to extracellular stimuli (growth factors, G-protein coupled receptor agonists, cytokines, phorbol esters, vitamin D_3 and antigen). Concentrations of S1P can reach micromolar levels in

serum and account for much of the cellular effects of serum. S1P is generated from sphingomyelin by the sequential action of sphingomyelinase, ceramidase, and sphingosine kinase, which catalyzes the phosphorylation of sphingosine to sphingosine 1-phosphate. Conversely, S1P can be degraded by two mechanisms: S1P can be cleaved by S1P lyase to produce hexadecanal and phosphoethanolamine or dephosphorylated by S1P phosphatase back to sphingosine. S1P regulates a wide variety of biological functions in many mammalian and other vertebrate cell types, including proliferation, migration, apoptosis, cytoskeletal organization and differentiation. Therefore, it is not surprising that S1P has been implicated in many physiological and pathophysiological disease states, such as cancer, angiogenesis, wound healing and inflammation.

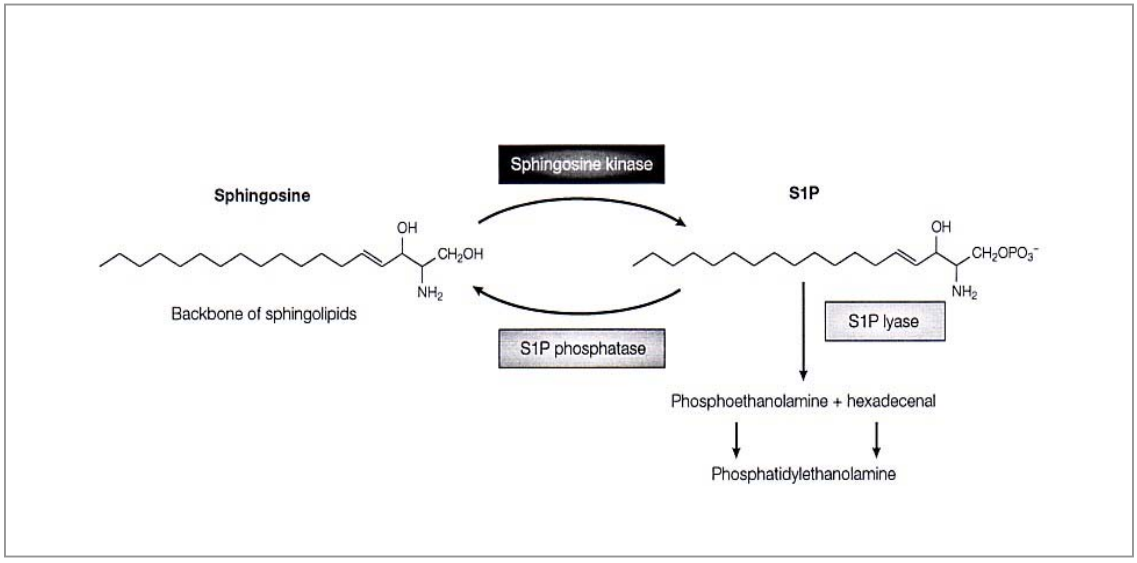

Figure 1 | S1P synthesis and degradation (adapted from Spiegel et al. 2003)

S1P was originally thought to function as a second messenger based on the ability of extracellular growth factors, such as PDGF and tumor necrosis factor-α (TNF-α), to activate sphingosine kinase and to increase intracellular S1P levels. However, specific intracellular targets proposed to mediate the intracellular actions of S1P have not yet been identified. Furthermore, the recent cloning and characterization

of a family of plasma membrane G-protein coupled receptors, known as Endothelial Differentiation Genes (Edg), which possess high affinity for S1P has strengthened the belief that S1P exerts its action through an extracellular, receptor-dependent mechanism.

1.2.1 EDG receptors

The Edg family of G-protein coupled receptors can be divided into two major groups based on ligand specificity. One receptor subgroup exhibits high affinity for the lysolipid lysophosphatidic acid (LPA) and consists of three members: Edg-2, Edg-4, Edg-7. The second subgroup comprises five isoforms which display high affinity for S1P: Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8. The distinct ligand affinities, expression pattern, and G-protein coupling account for the ability of the individual Edg receptor isoforms to differentially affect basic cellular processes.

EDG-1

The first Edg receptor discovered, Edg-1, is abundantly expressed on endothelial cells. Edg-1 was originally cloned as an immediate-early gene product in phorbol ester-differentiated human umbilical vein endothelial cells (HUVEC) (Hla and Maciag, 1990). Edg-1, a 380 amino acid protein with seven hydrophobic, transmembrane spanning domains, was subsequently identified as a high affinity receptor for S1P (K_d 8-20 nM). A variety of structurally similar sphingolipids and lysolipids (sphingosine, ceramide 1-phosphate, LPA, sphingosylphosphorylcholine) failed to displace S1P bound to Edg-1 (Lee et al., 1996) (Kon et al., 1999) and to induce receptor internalization (Liu et al., 1999). Edg-1 was found to couple exclusively to the pertussis toxin-sensitive heterotrimeric G-protein, G_i.

Edg-1 is widely expressed in different cell types, tissues and at different stages of development. The broad expression pattern of Edg-1 highlights its general importance in the regulation of diverse physiological cell functions. In endothelial cells, many different cellular effects of S1P have been proposed to be mediated by Edg-1 activation. Deletion of Edg-1 in mice leads to embryonic death resulting from incomplete blood vessel maturation.

EDG-3

Edg-3, like Edg-1, is expressed in endothelial cells. Though in HUVEC, Edg-3 is expressed at lower levels as compared to Edg-1 (Wang et al., 1999). Edg-3 was cloned from a human genomic library during a search for human cannabinoid receptors. Exhibiting approximately 50% sequence homology to Edg-1, it is thus not surprising that Edg-3 exhibits high affinity for S1P (K_d 23-26 nM). Unlike Edg-1, Edg-3 couples to multiple heterotrimeric G proteins, G_i, G_q, G_{12} and G_{13}.

Edg-3 exhibits a wide tissue expression pattern, with highest levels in endothelial cells, leukocytes and cardiovascular tissue. Suramin, a polycyclic anionic compound, selectively antagonizes the interaction of S1P with Edg-3 and is frequently used in studies. Surprisingly, deletion of Edg-3 in mice results in no visual phenotypic abnormality, suggesting that other receptors may be able to compensate for Edg-3 function.

1.2.2 S1P/EDG-mediated cellular effects in endothelial cells

S1P mediated Edg-receptor activation has been demonstrated to be essential in many aspects/components/phases of the endothelial cell angiogenic response, including cell survival, proliferation, migration, and differentiation (capillary tube formation).

S1P has been shown to protect endothelial cells from cell death induced by serum withdrawal. The effect of S1P on cell survival is sensitive to pertussis toxin treatment, suggesting the involvement of the Gi-coupled Edg-1 receptor (Kimura et al., 2001). In support of this, reduction of Edg-1 expression by antisense targeting inhibits endothelial cell proliferation (Lee et al., 1999), while reduction of Edg-3 levels showed no effect. Recently, S1P has been proposed to enhance endothelial cell survival by increasing endothelial nitric oxide synthase (eNOS) activity and subsequent nitric oxide production. Inhibition of NO production with N-monomethyl-L-Arginine (L-NAME) impaired S1P-mediated endothelial survival (Kwon et al., 2001). Alternatively, intracellular generation of S1P, by intracellular injection or by increasing sphingosine kinase activity, has been observed to promote DNA

synthesis and survival (Pyne and Pyne, 2000). Intracellular targets of S1P have not yet been identified and hence, the mechanism of S1P intracellular action is not clear.

Although S1P has been observed to inhibit migratory responses in many cell types (fibroblasts, VSMC), S1P promotes endothelial cell migration. Based on receptor overexpression studies, the effect of S1P on endothelial migration appears to be receptor-mediated. Wang et al. demonstrates that S1P stimulates migration of both bovine and human endothelial cells in a pertussis toxin sensitive manner, implicating the involvement of a Gi-coupled receptor (Wang et al., 1999). Furthermore, overexpression of Edg-1 and Edg-3 in human embryonic kidney (HEK-293) and Chinese hamster ovary (CHO) cells, which do not migrate normally, stimulates migration in response to S1P (Wang et al., 1999) (Kon et al., 1999). Moreover, antisense targeting of Edg-1 and Edg-3 impairs S1P-induced motility (Lee et al., 1999) (Paik et al., 2001) (Wang et al., 1999). Similarly, S1P-stimulated migration was impaired in Edg-1 null cells (Liu et al., 2000).

In recent years, it has become evident that S1P signaling is essential for complete vessel formation. S1P stimulates endothelial tube formation *in vitro* (Wang et al., 1999). This appears to be mediated by Edg-1 signaling, as deletion of Edg-1 in mice results in embryonic lethality due to the incomplete maturation of blood vessels (Lee et al., 1999). Moreover, antisense reduction of Edg-1 expression inhibits endothelial tube formation.

Via activation of receptors belonging to the Edg family, S1P elicits profound cellular effects in endothelial cells. However, the events which occur downstream of receptor activation have been poorly characterized. In particular, how S1P signals are transduced inside the cell, the specific molecules involved, and how these signals are translated into cellular effect are all unknown. More in-depth examination of these questions will certainly lead to a more complete understanding of the angiogenic process and possible therapeutic strategies.

1.3 Phosphoinositide 3-kinases (PI3-kinases)

The transduction of signals across the cell surface to various intracellular targets is essential for the regulation of virtually all aspects of cell behavior, such as cellular metabolism, movement, proliferation and differentiation. Extracellular signals manifest in various forms: physical (mechanical stress, light) and molecular (growth factors, cytokines, lipids). Molecular signals can be transmitted across the plasma membrane by directly penetrating the membrane, in the case of steroid hormones, or by binding to specific cell surface receptors (e.g. receptor tyrosine kinases, cytokine, adhesion and G-protein coupled receptors). Signals are subsequently transduced intracellularly by a sequence of biochemically defined processes such as protein phosphorylation, protein-protein interactions, and by the generation of second messengers.

An important family of second messengers include those derived from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2). In response to a variety of hormones and growth factors, PIP_2 is hydrolyzed by phospholipase C (PLC) to generate the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG), which stimulate intracellular Ca^{2+} release and protein kinase C (PKC) activity, respectively. Although PIP_2 cleavage is one of the most well characterized phospholipid signaling pathways to date, other phospholipid derived second messengers play important roles. PIP_2, in addition to being hydrolyzed by PLC, can be alternatively phosphorylated at the 3´-OH position of the inositol ring by the enzyme phosphoinositide 3-kinase (PI3K) to generate phosphatidylinositol 3,4,5- triphosphate (PIP_3). In quiescent cells, PIP_3 is normally absent but levels can rise sharply as a result of PI3K activity. PIP_3 then functions as a second messenger in mediating the activation of many protein kinases (PKC, Akt) in response to cellular stimulation. The relevance of PI3K signaling is illustrated by the recent discovery of the phosphatase and tensin homolog (PTEN) tumor suppressor protein. PTEN, a 3´-phosphatase, has been shown to dephosphorylate $PI(3,4,5)P_3$ to $PI(4,5)P_2$, which contributes to its tumor suppressor function. Mutations of PTEN, which result in elevated PIP_3 levels and constitutive PI3K

signaling, have been shown to be common in diverse human cancers (Maehama and Dixon, 1999).

PI3K comprise a family of enzymes that catalyze the transfer of the γ-phosphate group of ATP to the D3 position of phosphoinositides and have been demonstrated to be important regulators of mitogenesis, cell survival, metabolism, cytoskeletal rearrangements, and vesicular trafficking. The PI3K family can be divided into three main classes, based on differences in substrate specificity, structure and regulatory mechanisms.

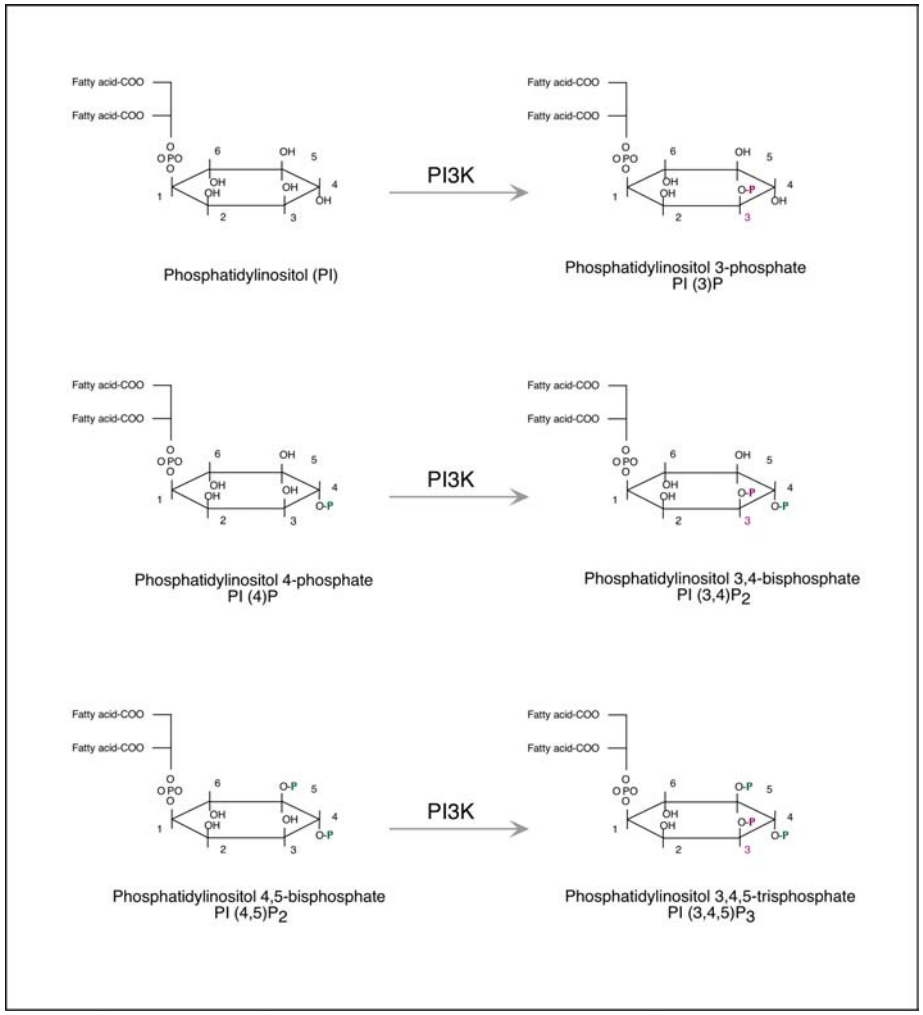

Figure 2 | The phospholipids generated by the action of phosphoinositide 3-kinases (PI3K)

1.3.1 Class I PI3-kinases

Class I PI3K specifically phosphorylate PI, PI(4)P, and PI(4,5)P_2 *in vitro* but exhibit selectivity towards PI(4,5)P_2 *in vivo*. Class I PI3K can be further divided into two subclasses, IA and IB, based on adaptor association and mode of regulation.

PI3K Class IA

Three class IA PI3K isoforms have been identified to date: PI3K α, β and δ. Class IA PI3K family members are heterodimers with a total molecular weight of approximately 200 kDa. They consist of a catalytic subunit of 110 kDa (p110 α, β, δ) constitutively associated to a 50-85 kDa regulatory subunit (p85 α, β, p55γ). PI3K α, β isoforms are expressed ubiquitously, while the expression of PI3K δ is restricted to cells of haemopoietic origin.

Class IA PI3K are activated in response to a variety of extracellular agonists; in particular, by agonists that stimulate receptors with intrinsic tyrosine kinase activity, such as epidermal growth factor (EGF), b-FGF, PDGF, and insulin. In fact, PI3K activity was first observed in anti-phosphotyrosine immunoprecipitates from PDGF-stimulated fibroblasts (Kaplan et al., 1987). In addition, class IA PI3K have been shown to be activated by non-receptor tyrosine kinases, such as Janus activated kinases (JAK) and the Src-family kinases, which are able to interact with receptors lacking intrinsic kinase activity, such as B/T cell antigen receptors, cytokine receptors, and co-stimulatory molecules (e.g. CD28).

Structurally, class 1A (and also IB) catalytic subunits of PI3K consist of four homology regions (HR), HR1-HR4. HR1, the C-terminal homology region, corresponds to the catalytic domain and contains several motifs that are conserved in protein kinases, such as the DFG (Asp-Phe-Gly) motif that forms part of the ATP binding site and the Lys residue (e.g. Lys^{802} for p110α), which is the binding site for the potent PI3K inhibitor wortmannin. HR2, also known as the phosphoinositide kinase (PIK) domain, is conserved in all lipid kinases; however, the function of this region is unknown. HR3, also called the C2 domain, is conserved in PI3K and is

suggested to mediate interaction with phospholipid membranes. HR4, the Ras-binding domain (RBD), is unique to class I PI3K.

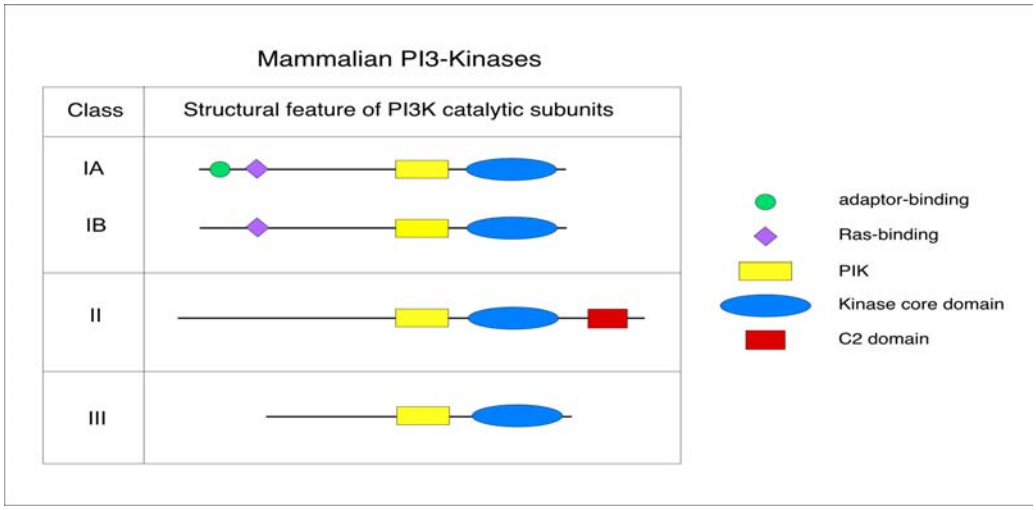

Figure 3 | Structural features of mammalian PI 3-kinases
PIK, phosphoinositide kinase. Figure adapted from Vanhaesbroeck et al. 1999.

The p85 regulatory subunit supports a Src-homology-3 (SH3) domain, a breakpoint cluster region (BCR) surrounded by two proline rich regions and two C-terminal Src homology-2 (SH2) domains separated by an inter-SH2 (i-SH2) domain. The i-SH2 domain is supposed to contribute to the tight binding of the p85 regulatory subunit to the N-terminus of the p110 catalytic subunit. The SH2 domains of the regulatory subunit enable PI3K to bind phosphorylated tyrosine motifs that are generated by activated tyrosine kinases on cytoplasmic tails of growth factor receptors and various adaptor proteins. Class IA PI3K then become activated by the translocation from the cytosol to the membrane where it can readily access its lipid substrate, leading to a local increase in PIP_3 production. This translocation is believed to be vital for PI3K activation *in vivo*, but other mechanisms may contribute to activation. For example, it was observed that the binding of the p85 SH2 domains to phosphotyrosine peptides leads to an increase in lipid kinase activity (Rordorf-Nikolic et al., 1995). This would change the relative localization of the SH2 domains and exert stress on the i-SH2 domain. In support of this theory, Klippel et

al. show that a fusion protein of iSH2-p110 was constitutively active and exhibited enhanced catalytic activity (Klippel et al., 1993).

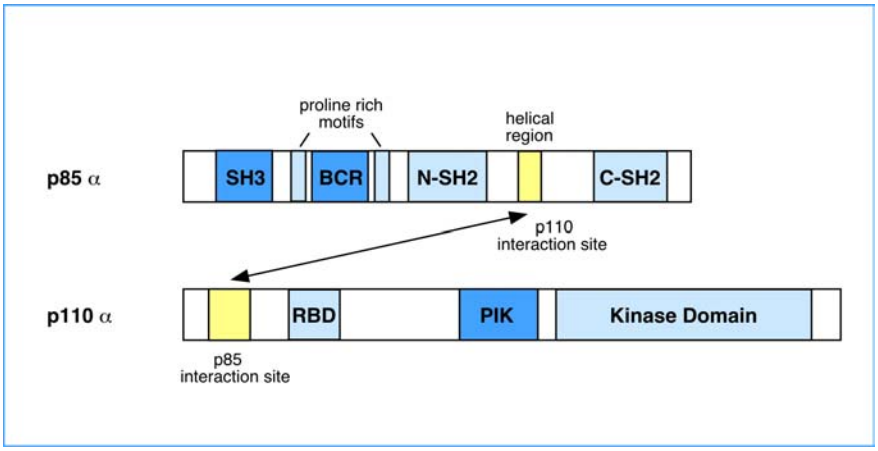

Figure 4 | Structure of the regulatory p85α subunit and the catalytic p110α subunit of the heterodimeric Class IA PI3K
BCR, breakpoint cluster region; SH3, Src-homology 3; SH2, Src-homology 2; RBD, Ras-binding domain; PIK, phosphoinositide kinase
Adapted from Panayotou G., Signal Transduction, 1998.

In addition to lipid kinase activity, class IA catalytic subunits exhibit an intrinsic protein serine/threonine kinase activity, capable of trans-phosphorylating adaptor subunits and/or auto-phosphorylating *in vitro*. It has been demonstrated that p85α adaptor subunits can be phosphorylated (at Ser^{608}) by p110α, which results in the down-regulation of p110α lipid kinase activity (Dhand et al., 1994). There is additional evidence showing that class IA PI3K associate with and phosphorylate various adaptor molecules, such as the insulin receptor substrate-1 (IRS-1) in insulin-stimulated adipocytes (Lam et al., 1994) and focal adhesion kinase (FAK) in PDGF-stimulated fibroblasts (Chen and Guan, 1994). In contrast to p110α, p110δ does not phosphorylate its regulatory subunit but autophosphorylates *in vivo*, which leads to a complete downregulation of its lipid kinase activity (Vanhaesebroeck et al., 1999).

PI3K Class IB

PI3Kγ is the only existing Class IB PI3K known to date. Class IB PI3Kγ consists of a catalytic subunit (p110γ) which differs markedly from class IA p110α, β, δ in both structure and regulation. The existence of this unique PI3K isoform became evident upon observing that stimulation of neutrophils with a formylated tripeptide, N-formyl-Met-Leu-Phe (fMLP), resulted in a striking increase in PIP_3 levels (Stephens et al., 1993). Since fMLP binds to a seven transmembrane G-protein coupled receptor, it was initially unclear how the known PI3K could be activated. Screening a human bone marrow cDNA library, Stoyanov et al. subsequently cloned and characterized a novel PI3K isoform, PI3Kγ, which associates with and becomes activated by heterotrimeric G-protein $\beta\gamma$ subunits (Stoyanov et al., 1995). PI3Kγ does not associate with either p85α or p85β, consistent with the absence of a p85-binding domain at the NH2-terminus of p110γ. Instead, PI3Kγ was found in association with a novel 101 kDa adaptor protein, p101 (Stephens et al., 1997).

Despite being termed an adaptor/regulatory subunit, the functional relevance of the p101 subunit is still controversial. Since p101 was shown to bind G$\beta\gamma$ more tightly than p110γ, it was thought that p101 was required to couple p110γ to G$\beta\gamma$ subunits upon activation of G-protein coupled receptors. Stephens et al. demonstrated that co-expression of p110γ/p101 in Sf9 insect cells and in Cos-7 cells resulted in a significant increase in PI(3,4)P_2 and PI(3,4,5)P_3 production as compared to p110γ expression alone(Stephens et al., 1997). Based on these findings, it was concluded that p101 functions as a regulatory subunit that is necessary to amplify the effects of G$\beta\gamma$ on p110γ lipid kinase activity. In reconstitution assays (*in vitro*), p101 was shown to be essential for maximal activation of p110γ lipid kinase activity; however, it is unclear whether this is relevant *in vivo*. Furthermore, subsequent reports demonstrated that p110γ (both N- and C-terminal sequences) can associate with G$\beta\gamma$ directly and that p110γ lipid kinase activity was substantially activated by G$\beta\gamma$ even in the absence of the p101 subunit (Stoyanov et al., 1995) (Stoyanova et al., 1997) (Tang and Downes, 1997) (Leopoldt et al., 1998), arguing against an indispensable role of p101 in G$\beta\gamma$ stimulation of p110γ.

Maier et al. proposed that p101 is responsible for the PI(4,5)P2 substrate selectivity of p110γ, by sensitizing p110γ towards Gβγ in the presence of PI(4,5)P_2 (Maier et al., 1999). Moreover, Metjian et al. found that overexpression of a mutant form of p110γ, which was defective in p101 binding, resulted in a constitutive localization of p110γ to the nucleus (Metjian et al., 1999). Based on these findings, the authors proposed that p101 regulated the translocation of p110γ to the nucleus and suggested a previously unidentified role for p110γ at the nuclear membrane. In addition, Lopez-llasaca et al. suggested a role for p101 in the activation of JNK by PI3Kγ (discussed below). Thus, further investigations are required to elucidate the physiological function of p101 and its significance in PI3Kγ signaling.

Interestingly, PI3Kγ has been shown to mediate Gβγ- dependent activation of the mitogen activated protein kinase (MAPK) (Lopez-llasaca et al., 1997). Lopez-llasaca et al. demonstrated that overexpression of PI3Kγ in Cos-7 cells activated MAPK in a Gβγ-dependent manner. Overexpression of a catalytically inactive mutant of PI3Kγ (PI3Kγ KR) markedly attenuated MAPK activation in response to stimulation of muscarinic (m2) G-protein coupled receptors or Gβγ stimulation. Furthermore, the signaling of PI3Kγ to MAPK was dependent on a pathway involving tyrosine kinase activity, Shc, Grb2, Sos, Ras and Raf proteins.

It was initially unclear if the lipid kinase or protein kinase activity of PI3Kγ was involved in the pathway. Utilizing PI3Kγ mutants with aborted lipid kinase activity, Bondeva et al. demonstrated that MAPK can be activated in the complete absence of PI(3,4)P_2 and PI(3,4,5)P_3 production, indicating that PI3Kγ lipid kinase activity and thus, production of 3-phosphorylated phosphoinositides, is not vital for MAPK activation (Bondeva et al., 1998). Based on these findings, Bondeva et al. concluded that PI3Kγ is capable of activating two distinct pathways: the lipid kinase activity of PI3Kγ being responsible for 3-phosphorylated lipid production and Akt activation, while PI3Kγ protein activity contributes to MAPK stimulation and propagation of growth signals.

In addition, PI3Kγ has also been shown to mediate Jun Kinase (JNK) activation (Lopez-Ilasaca et al., 1998). In Cos-7 cells, overexpression of PI3Kγ stimulated JNK activity, in a Gβγ-dependent manner. However, this activation was significantly enhanced by co-expression of the non-catalytic subunit p101, whereas the activation of MAPK was unaffected by p101 co-expression. Furthermore, dominant negative mutants of Ras, Rac and p21-activated kinase (PAK) suppressed the activation of JNK induced by co-expression of PI3Kγ and p101. Hence, optimal activation of JNK requires on PI3Kγ and p101, via a pathway involving Ras, Rac and PAK. The distinct effects of PI3Kγ on MAPK and JNK activation, and the differential requirement for p101, highlight the complexity of PI3Kγ signaling.

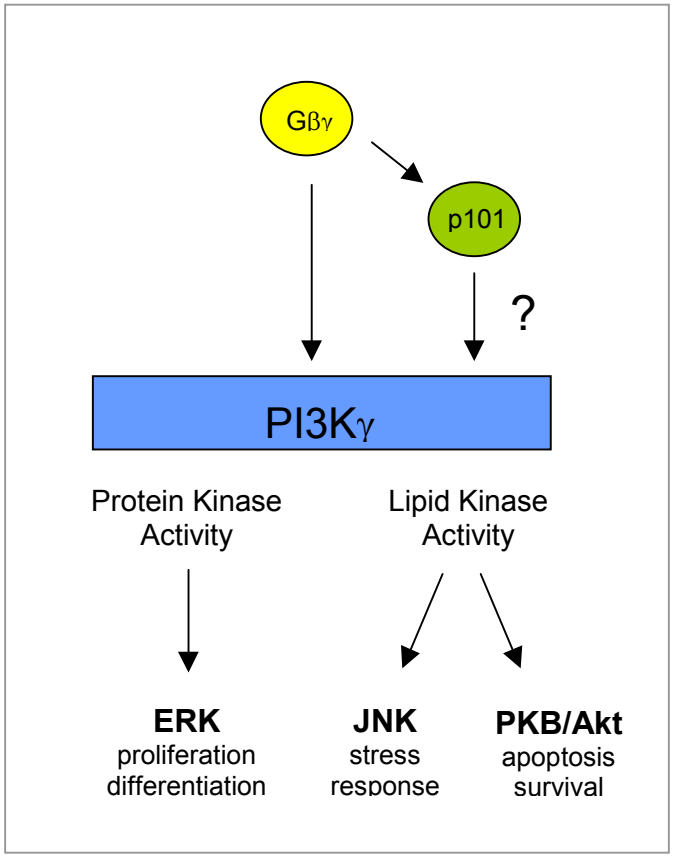

Figure 5 | Different activities of PI3Kγ PI3Kγ is activated upon stimulation of Gi-protein coupled receptors in a Gβγ-dependent way. The adaptor protein p101 supports enzyme activation. PI3Kγ exhibits lipid kinase activity and protein kinase activity. Using overexpression of wild-type and different mutants, activating effects of PI3Kγ on extracellular signal regulated kinase (ERK), Jun kinase (JNK) and protein kinase B (PKB)/Akt as well as autophosphorylation and transphosphorylation of p101 have been observed Only little data on the role of endogenously expressed PI3Kγ is available.

PI3Kγ is activated in response to stimulation of G-protein coupled receptors by agonists, such as fMLP, complement factor 5a (C5a), muscarinic receptor, and interleukin-8 (IL-8). PI3Kγ has been shown to directly associate with Gβγ subunits, although the mechanism by which Gβγ subunits activate PI3Kγ is not well understood. Gβγ could activate PI3Kγ by allosteric interactions or alternatively, by localizing PI3Kγ to the membrane, allowing easier access to lipid substrates. In support of the latter, Bondeva et al. observed that targeting PI3Kγ to the membrane by fusing PI3Kγ to the COOH-terminal isoprenylation signal of K-Ras (PI3Kγ-CAAX) results in the constitutive production of PIP_3 (Bondeva et al., 1998). Along with lipid kinase activity, PI3Kγ possesses an intrinsic serine kinase activity, which renders p110γ capable of auto-phosphorylation *in vitro* (Stoyanova et al., 1997). Furthermore, p110γ has been demonstrated to trans-phosphorylate p101 and the protein kinase, MEK-1, *in vitro* (Bondev et al., 1999). However, the physiological relevance of p110γ protein kinase activity is not well understood.

Due to the relatively recent discovery of PI3Kγ, the functional significance of PI3Kγ is only beginning to be defined. To investigate the role of PI3Kγ in biological responses, mice lacking p110γ have been generated. Leukocytes lacking p110γ were unable to produce PIP_3 in response to activation by fMLP, C5a, or IL-8. Subsequent signal transduction events mediated by PIP_3 production were also disturbed in p110γ -/- leukocytes. The PIP_3-dependent phosphorylation and activation of Akt was impaired in response to chemoattractant stimulation, whereas PIP_3 production and Akt activation were both normal in response to the activation of receptor tyrosine kinases (e.g. GM-CSF). These results confirm that p110γ is activated by G-protein coupled receptors and not by receptor tyrosine kinases. Furthermore, loss of p110γ exhibited dramatic effects on the haemopoietic system. Thymocyte survival and T cell activation was impaired in p110γ deficient mice (Sasaki et al., 2000b). PI3Kγ -/- neutrophils displayed impaired respiratory burst and motility, while both PI3Kγ-deficient neutrophils and peritoneal macrophages exhibited an impaired migration in response to chemotactic stimuli and in various mouse models of peritonitis (Hirsch et al., 2000) (Sasaki et al., 2000b) (Li et al.,

2000). Taken together, these studies demonstrate that PI3Kγ is an important mediator of chemotactic responses and plays a crucial role in inflammation.

Based on early reports, the PI3Kγ was initially believed to be mainly expressed in cells of haemopoietic origin (Ho et al., 1997). However, recent studies provide evidence for a wider expression pattern of PI3Kγ than previously thought. For instance, PI3Kγ has been shown to inhibit the proliferation of colon cancer cells and suppresses the development of colorectal carcinoma *in vivo* (Sasaki et al., 2000a). The broad tissue distribution of PI3Kγ illustrates its importance in the regulation of diverse cell functions. Taken together, these findings indicate that the current state of knowledge concerning the expression profile and functional significance of PI3Kγ is incomplete and inspires further investigation.

1.3.2 Classes II, III and IV PI3-kinases

Class II PI3K utilize primarily PI and PI(4)P as substrates and are the largest in size of the PI3K family (170-210 kDa). The first class II PI3K was cloned in drosophila (PI3K_68D), while mammals have been shown to possess 3 class II PI3K isoforms, PI3K-C2 α, β, γ. Class II PI3K are characterized by a C-terminal C2 domain, which bind weakly to phospholipids in a Ca^{2+}-independent manner and are essential for the catalytic activity of the enzyme (MacDougall et al., 1995). As of yet, Class II PI3K have not been found to associate with any adaptor proteins or Ras, their N-terminus being quite different from those of the class I enzymes. Class II PI3K can be activated by EGF, PDGF, insulin, integrins and the chemokine monocyte chemotactic protein-1 (MCP-1). However, the mechanism of activation is unclear. In immunoprecipitations of drosophila PI3K_68 kDa, tyrosine phosphorylated proteins of 90 and 190kD have been shown to be associated with PI3K_68kDa, suggesting that protein tyrosine kinases and protein-protein interactions play an important role in the activation of class II PI3K (Molz et al., 1996).

Class III PI3K utilize phosphatidylinositol (PI) as a substrate, producing exclusively PI(3)P. However, the relatively constant levels of phosphatidylinositol 3-phosphate

PI(3)P in cells suggests that class III PI3K are not activated to a significant degree by cellular stimulation or alternatively, are regulated in a spatio-temporal manner. The best example of a class III PI3K is the Saccharomyces cerevisiae VPS34 gene product, Vps34p. The complete absence of PI(3)P in yeast cells, as a consequence of VPS34 deletion or in cells expressing catalytically inactive Vps34p, results in impaired vacuolar protein sorting. Vps34p has been shown in association with Vps15p protein serine/threonine kinase (p150 in mammals), which is essential for the recruitment of Vps34p to the Golgi membranes and activation of its lipid kinase activity. Vps34p is resistant to inhibition by wortmannin treatment (Takegawa et al., 1995).

Class IV PI3K-related kinases have been identified based on their homology to the kinase domains of PI3- and PI4- kinases. Members of this family include TOR (target of rapamycin), DNA-PK (DNA-dependent protein kinase) and ATM (ataxia telangiectesia mutated). Because no lipid substrates have been identified, it is hypothesized that the activity of class IV PI3K derives from serine/threonine protein kinase activity, rather than lipid kinase activity.

1.4 Downstream effectors of PI3-kinase activation

In quiescent cells, baseline levels of PI(3,4)P_2 and PI(3,4,5)P_3 are normally very low but rise rapidly upon cellular stimulation, due to PI3K activity. Numerous targets for PI(3,4)P_2 and PI(3,4,5)P_3 have so far been identified, most of which directly interact with PI(3,4)P_2 and PI(3,4,5)P_3 via a distinct lipid binding domain, the Pleckstrin homology (PH) domain. PH domains are conserved globular protein domains of approximately 110 amino acids which mediate protein-protein and protein-lipid interactions. PH domains which bind PI(3,4)P_2 and PI(3,4,5)P_3 are contained within many proteins with diverse functions, including protein kinases (Akt), nucleotide exchange factors (Vav, cytohesin-1, Sos1), GTPase-activating factors (GAP1), phospholipases (PLCγ2, PLCδ1) and adaptor proteins.

1.4.1 PKB/Akt activation

The best studied effector of PI3K is the protein serine/threonine kinase Akt, also known as protein kinase B (PKB). Akt targets numerous effector proteins and has been shown to regulate diverse processes, including cell survival and glycogen metabolism. Structurally, Akt consists of an N-terminal PH domain, a kinase domain and a C-terminal regulatory domain. Binding of $PI(3,4)P_2$ to Akt results in a 3-5 fold stimulation of Akt activity *in vitro*. However, full activation of Akt *in vivo* requires the phosphorylation of two specific sites, Thr^{308} in the kinase domain and Ser^{473} in the C-terminal tail region. Phosphoinositide-dependent kinase 1 (PDK-1) was found to be responsible for phosphorylation of Thr^{308} and Ser^{473}. Similarly to Akt, PDK-1 possesses a PH domain, which facilitates high affinity binding to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ lipids and subsequent recruitment to the membrane. The proposed mechanism of Akt activation involves the recruitment of Akt to the membrane, whereby lipid binding induces a conformational change which facilitates phosphorylation of Thr^{308} and Ser^{473} residues. Additionally, PDK-1 has been shown to phosphorylate and activate PKC isoforms (PKC ζ and δ) and $p70^{S6k}$, a kinase involved in regulation of the G_1 cell cycle progression.

Akt is a multifunctional protein which has been shown to control cell survival, glycogen metabolism and cellular transformation. Targets of Akt include metabolic regulators glycogen synthase kinase-3 (GSK-3) and Phosphofructo-2-Kinase (PFK-2), proteins involved in apoptosis and transcription factors (Fig. 6). With respect to apoptosis, Akt has been demonstrated to phosphorylate BAD, releasing the anti-apoptotic proteins Bcl-2 and Bcl-X_L which then function to counteract apoptosis. Caspase-9, a protease involved in the initiation of apoptosis, can also be phosphorylated and inhibited by Akt. Alternatively, Akt has also been shown to promote cell survival by phosphorylating transcription factors belonging to the Forkhead (FH) family. The phosphorylation of FH transcription factors is believed to promote their export from the nucleus to the cytoplasm where they are retained by binding to cytosolic 14-3-3 proteins. This, in turn, isolates them from the pro-apoptotic Fas ligand. Furthermore, Akt has also been shown to associate with and

phosphorylate I-kB kinase (IKK), leading to the nuclear translocation of the nuclear factor kB (NF-kB), which activates the transcription of anti-apoptotic proteins.

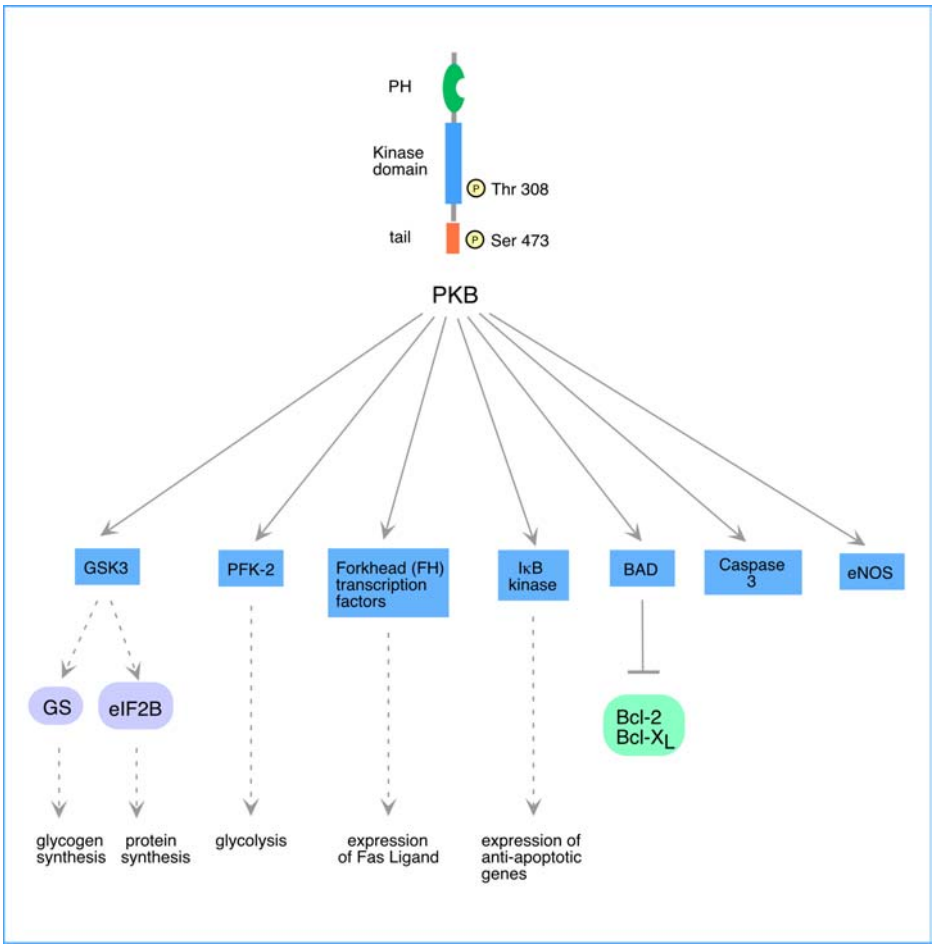

Figure 6 | Targets of Protein Kinase B/Akt
Figure adapted from Vanhasesbroeck et al. 1999) (Vanhaesebroeck and Waterfield, 1999). GSK3, glycogen synthase kinase-3; eIF2B, elongation initiation factor 2B; PFK-2, Phosphofructo-2-Kinase; eNOS, endothelial nitric oxide synthase; PKB, protein kinase B

Recently, Akt has been shown to be required for migratory responses. In leukocytes and *Dictyostelium* slime mold, chemoattractants activated Akt through G-protein coupled PI3K pathway. Chemoattractant stimulation of neutrophils and *Dictyostelium* resulted in a transient activation and localization of green fluorescent protein (GFP)-tagged PH domain of Akt to the plasma membrane (Meili et al., 1999). In particular, membrane localization of GFP-PH-Akt fusion protein was

restricted to the leading edge of cells (Servant et al., 2000) (Parent et al., 1998). Furthermore, PI3Kγ -/- leukocytes, in which migration was abrogated, displayed an impaired PIP_3-dependent phosphorylation and activation of Akt, suggesting a role for PI3Kγ in the regulation of Akt-mediated migratory responses (Sasaki et al., 2000b) (Hirsch et al., 2000).

1.4.2 Rho GTPases

The regulation of the actin cytoskeleton is essential for cell motility, chemotaxis, and cell division. The Rho family of small GTPases (Rho, Cdc42, Rac) has been demonstrated to be key regulators of the actin cytoskeleton. Microinjection of active forms of Cdc42 leads to the formation of filopodia, while active Rac induces lamellipodia and membrane ruffles, and active Rho causes stress fiber and focal contact assembly. Expression of a dominant negative Rac mutant has been shown to inhibit chemoattractant-induced migration (Anand-Apte et al., 1997) (Leng et al., 1999). On the other hand, overexpression of active Rac and Tiam-1, a guanine nucleotide exchange factor for Rac, has been shown to promote cell motility (Sander et al., 1998) (Leng et al., 1999).

Rho GTPases belong to the Ras family of monomeric 20-30 kDa GTP-binding proteins, which cycle between inactive, GDP-bound and active GTP-bound states. Only in the GTP-bound state can Rho GTPases interact with downstream effector molecules. The interconversion between inactive and active states is influenced by three types of proteins: GTPase-activating proteins (GAP) which stimulate intrinsic GTPase activity, guanine nucleotide dissociation inhibitors (GDI) which inhibit the exchange of GDP for GTP, and guanine nucleotide exchange factors (GEF) which catalyze the exchange of GDP for GTP.

PI3K have been shown to be important in the activation of Rac. The activation of Rac by both tyrosine kinase and G-protein coupled receptors appears to be PI3K-dependent, as assayed using specific inhibitors of PI3K and dominant negative mutants. Activation of Rac by PDGF and insulin is completely dependent on PI3K activity (Nobes et al., 1995). In addition, the PI3K inhibitor wortmannin inhibits binding of GTP to Rac1 in response to PDGF stimulation (Hawkins et al., 1995).

Remarkably, constitutively active PI3K leads to membrane ruffling in the absence of growth factor stimulation (Rodriguez-Viciana et al., 1997). In addition to tyrosine kinase receptors, G-protein coupled receptors have been shown to induce cytoskeletal rearrangements which have been shown to be dependent on PI3K activity. In particular, PI3Kγ has been shown to be important for Rac-induced actin rearrangements; *in vitro* studies propose that Rac is downstream of p110γ because activation of fMLP receptor stimulated cytoskeletal rearrangements via a pathway involving G$\beta\gamma$, p110γ, Rac exchange factor Vav, and Rac (Ma et al., 1998). The mechanism is not yet fully understood but Han et al. suggested that the production of PI(3,4,5)P_3 in response to PI3K activation leads to the phosphorylation and activation of the Rac exchange factor Vav (Han et al., 1998).

The exact function of PI3K in the context of Rac activation is not well understood. In fact, it is still unclear whether PI3K functions upstream or downstream of Rac. Based on studies showing that membrane ruffling induced by overexpression of constitutively activate Rac variant is not inhibited by PI3K inhibitors, PI3K was proposed to act upstream of Rac. However, in epithelial cells, it was observed that inhibitors of PI3K blocked cell migration induced by constitutively activate Rac, suggesting that PI3K acts downstream of Rac activation (Keely et al., 1997). Another area of debate is the molecular mechanism by which PI3K activates Rac. Both Cdc42 and Rac have been reported to bind directly to the p85 subunit of PI3K (Zheng et al., 1994) (Tolias et al., 1995) (Bokoch et al., 1996). Alternatively, it was proposed that PI3K instead affects GEF activity. Phosphoinositide-binding PH domains are present in GEF specific for the Rho family of GTPases. In particular, Tiam-1 and Vav-1, guanine nucleotide exchange factors for Rac, have been shown to bind PIP_3, which stimulates GEF activity (Fleming et al., 2000). Moreover, PI3-kinase products, PIP_3 and PIP_2, have been found to co-localize with Rac at the leading edge of migrating neutrophils and *Dictyostelium* (Haugh et al., 2000) (Servant et al., 2000). While the importance of PI3K in Rac signaling is evident, the exact mechanisms require further investigation.

1.5 Mitogen-activated protein kinases (MAPK)

MAPK are key mediators involved in transduction of proliferatory signals from activated cell surface receptors to the nucleus. The general mechanism of MAPK activation by classical receptor tyrosine kinases has been extensively studied and characterized. Growth factors stimulate the activation of a protein kinase cascade via recruitment of adaptor proteins (Shc/Grb-2) and the sequential activation of Ras, Raf kinase, MAP kinase kinase (MEK), and extracellular regulated signal kinase (ERK). In contrast, the pathway linking G-protein coupled receptors to MAPK activation is not well understood.

Recent evidence suggests that a unique pathway linking G-protein coupled receptors to MAPK activation does not exist; but instead, G-protein coupled receptors “transactivate” receptor tyrosine kinases and utilize already known pathways leading to MAPK activation. This mechanism of transactivation has been shown for many different G protein ligands, including thrombin, endothelin-1, and angiotensin II. In particular, the transactivation of the EGF (Daub et al., 1997) (Daub et al., 1996) (Gutkind, 1998) is a favored mechanism downstream of G-protein coupled receptor activation. Thrombin and endothelin-1 have been demonstrated to transactivate the EGF receptor in Rat-1 fibroblasts (Daub et al., 1996). Similarly, LPA activates MAPK via transactivation of the EGF receptor in COS-7 fibroblasts, through a mechanism requiring Src tyrosine kinase activity (Luttrell et al., 1997).

In addition to regulating gene transcriptional events associated with proliferation, ERK activation has also been implicated in the regulation of cell motility. Prolonged activation of ERK has been associated with cytoskeletal rearrangements and increased motility. Inhibition of ERK by the specific MEK inhibitor PD98059 blocked BAEC migration stimulated by basic fibroblast growth factor (FGF-2) (Rikitake et al., 2000). In addition, inhibition of ERK signaling by lysophosphatidylcholine (LPC), a component of oxidized-LDL, led to a reduction in endothelial migration (Rikitake et al., 2000). However, molecular mechanisms by which ERK regulate cell motility are not fully understood. Klemke et al. have demonstrated that ERK 1/2 activity controls cell motility by regulating the myosin light chain kinase/myosin light

chain pathway. The authors showed that ERK phosphorylates and activates myosin light chain kinase (MLCK), leading to myosin light chain (MLC) phosphorylation and increased cell motility. Overexpression of a constitutively active MEK resulted in an activation of MAPK, which led to the phosphorylation of MLCK, MLC and elevated levels cell migration. Conversely, inhibition of MAPK activity resulted in decreased MLCK function, MLC phosphorylation and cell motility (Klemke et al., 1997).

1.6 Project Aim

The regulated migration of endothelial cells plays an important role in the angiogenic process. Platelet-derived sphingosine 1-phosphate (S1P) is a potent angiogenic factor, eliciting diverse effects in endothelial cells. Via activation of G-protein coupled receptors belonging to the endothelial differentiation gene (Edg) family, S1P has been shown to be important for mediating proliferatory and migratory responses in endothelial cells. However, the downstream signaling pathways mediated by Edg receptor activation remain largely undefined.

Members of the phosphoinositide 3-kinase (PI3K) family have been implicated in cell motility. In particular, the Gi-coupled PI3Kγ was recently demonstrated to play a central role in the migration of monocytes and neutrophils stimulated by chemoattractants. However, little is known concerning the expression and function of PI3Kγ in the vascular system and specifically, in the motility of endothelial cells.

The aim of the present study is to investigate PI3Kγ, its expression and role in S1P-induced endothelial motility. PI3Kγ expression will be assessed using sensitive RT-PCR and immunoblotting techniques. In addition, the expression of the PI3Kγ adaptor subunit p101 will be examined at the RNA level. Specific inhibitors of Gi/o proteins and PI3-kinases will be employed to evaluate possible involvement of PI3Kγ in S1P-induced cellular responses related to cell motility, including ERK, Akt and Rac-1 activation, as well as in S1P-stimulated chemotaxis. Furthermore, transient overexpression of a catalytically inactive form of PI3Kγ will be used to assess the involvement of PI3Kγ in S1P-induced endothelial cell migration.

2. Materials and Methods

2.1 Materials

2.1.1 Primary cell culture

HUVEC	human umbilical vein endothelial cells
BREC	bovine microvascular retinal endothelial cells

2.1.2 Established cell lines

A549	human lung carcinoma (ATCC CCL 185)
BA/F3	mouse pro-B lymphoid cells (DSMZ ACC 300)
COS-7	monkey kidney fibroblast cells (ATCC CRL 1651)
EA.hy926	fusion cell line of HUVEC and A549
HBMEC	human brain microvascular endothelial cells
HMEC-1	human microvascular endothelial cells
U937	human myeloid leukemia cells

2.1.3 Antibodies

Anti-PI3Kγ mAb, directed against the N-terminus, 1:100

Anti-phospho p42/p44 ERK MAP Kinase mAb (Cell Signaling), 1:1000

Anti-pan ERK mAb (Cell Signaling), 1:1000

Anti-vinculin mAb (Serotec LTC, UK), 1:10,000

Anti-phospho Akt/PKB pAb (Cell Signaling), 1:500

Goat anti-mouse IgG antibody, HRP-conjugated (Santa Cruz), 1:10,000

Goat anti-rabbit IgG antibody, HRP-conjugated (Santa Cruz), 1:10,000

Anti- Rac1 mAb (Upstate Biotechnology), 1:1000

2.1.4 Oligonucleotides

Oligonucleotides for PI3Kγ-specific RT-PCR:

Forward Primer 5'- CAT ATT GAC TTC GGG CAC ATT CTT G -3'

Reverse Primer 5'- GTC TCT GCA AAC TTC GAT CTG ATC -3'

Oligonucleotides for p101-specific RT-PCR:

Forward Primer 5'- GAT CTC CTA CCA GAG ACT GGT GAG G -3'

Reverse Primer 5'- ATG CCA GAT CGG AGC TCC TGT GCC T -3'

Oligonucleotides for β-actin-specific RT-PCR:

Forward Primer 5'- GAC TAC CTC ATG AAG ATC -3'

Reverse Primer 5'- GAT CCA CAT CTG CTG GAA -3'

Oligonucleotide for p110γ WT/KR sequencing:

CTG CAG AAT TCT CAA CTC CCC

Oligonucleotide for CAAX sequencing:

GGG ATG CCC TCA CAG TGG GGA AAA ATG

2.1.5 Plasmids

GFP-N1	(green fluorescent protein, Clontech)
pcDNA3	(mammalian expression vector, Invitrogen)
p110γ WT	(encoding full length PI3Kγ)
p110γ KR	(kinase dead; Lys^{833} to Arg mutation in catalytic domain)
p110γ CAAX	(constitutively active; membrane associated form of PI3Kγ)

2.1.6 Inhibitors

LY294002 (Calbiochem, Schwalbach)

Wortmannin (Sigma, Deisenhofen)

Pertussis Toxin (Alexis, Grünberg)

Genistein (Calbiochem, Schwalbach)
SU5614 (Calbiochem, Schwalbach)
Tyrphostin AG1296 (Calbiochem, Schwalbach)
Tyrphostin AG1478 (Calbiochem, Schwalbach)
Protease Inhibitor Cocktail (PIC), (Roche, Basel)

2.1.7 Cell culture reagents

Dulbecco's Modified Eagle Medium (Life Technologies, Eggenstein)
Endothelial Basal Medium (EBM), (Clonetics, San Diego)
Medium 199 (M199), (BioWhittaker, Walkersville)
RPMI 1640 (Sigma, Deisenhofen)
Fetal Calf Serum (Life Technologies, Eggenstein)
Human Serum (Life Technologies, Eggenstein)
Endothelial Cell Growth Supplement (ECGS), (Sigma, Deisenhofen)
Heparin-Sodium (Roche, Basel)
L-Glutamine (ICN Biomedicals, Eschwege)
Trypsin/ EDTA (ICN Biomedical, Eschwege)
Penicillin/Streptomycin (Gibco BRL, Eggenstein)
Gelatin (ICN Biomedicals, Eschwege)
Bovine Serum Albumin (BSA), fatty acid-free (Sigma, Deisenhofen)

2.1.8 Other reagents and materials

XL-1 Blue *Escherichia Coli*
Ampicillin (Sigma, Deisenhofen)
Kanamycin (Sigma, Deisenhofen)
Dimethyl Sulfoxide (DMSO), (Sigma, Deisenhofen)
D-erythro Sphingosine 1-Phosphate (Biomol, Plymouth)
Isopropyl-β-D-thiogalactopyranoside (IPTG), (Sigma, Deisenhofen)
Protein A-Sepharose (Amersham Pharmacia, Uppsala)
Hoefer Electrophoresis Chamber (Hoefer Scientific, San Francisco)
Millipore Semi-Dry Transfer Apparatus (Millipore, Bedford)

Immobilon-polyvinyldifluoride (PVDF) membrane (Millipore, Bedford)

Whatmann 3M paper (Whatmann GmbH, Göttingen)

NEN Renaissance Chemiluminescence Reagent (NEN Life Sciences, Brussels)

BioMax MR-1 film (Kodak, New York)

Qiagen RNeasy Mini Kit (Qiagen, Düsseldorf)

QIA-shredder column (Qiagen, Düsseldorf)

Qiagen OneStep RT-PCR Kit (Qiagen, Düsseldorf)

Qiagen Endofree Maxi Kit (Qiagen, Düsseldorf)

Amaxa Nucleofector Kit (Amaxa, Köln)

Falcon Cell Culture Inserts/Companion Plates (Becton Dickinson, USA)

Hematoxylin (Dako, Hamburg)

Glutathione-Sepharose 4B (Amersham Pharmacia, Uppsala)

Bio-Rad Poly-Prep Chromatography Columns (Bio-Rad Laboratories, München)

Bio-Rad DC Microassay Reagent (Bio-Rad Laboratories, München)

Eukitt Mounting Medium (Merck, Darmstadt)

Fluoromount Medium (Sigma, Deisenhofen)

SuperFrost Slides and Coverslips (Menzel, Braunschweig)

Leitz Fluorescent Microscope (Leitz, Wetzlar)

Zeiss Inverted Microscope (Zeiss, Jena)

Zeiss Laser Scanning Microscope (Zeiss, Jena)

Casy-1 Cell Counter (Schärfe Systems, Reutlingen)

Casyton Isotonic Solution (Schärfe Systems, Reutlingen)

2.2 Methods

2.2.1 Cell culture

Human umbilical cord vein endothelial cells (HUVEC) were isolated using 0.05% collagenase and cultured on 0.2% gelatin-coated 75-cm^2 flasks in M199 supplemented with 20% heat-inactivated serum (15% fetal calf serum, 5% human serum), 7.5 μg/ml endothelial cell growth supplement (ECGS), 7.5 U/ml heparin-Na, 100 U/ml penicillin-streptomycin. Cells were maintained at 37 °C in 5% CO_2. Confluent cultures were detached by brief treatment with trypsin-EDTA (0.05 %/0.02 %) and plated onto 60-mm, 90-mm dishes, or tissue culture inserts for stimulation and migration experiments. All experiments were performed with HUVEC monolayers of the second passage.

BREC were cultured in endothelial basal medium (EBM) with 10% fetal calf serum, 50 μg/ml heparin-Na and 50 μg/ml ECGS. A549, Cos-7 and HMEC-1 were cultured in Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal calf serum. Bone marrow-derived IL-3-dependent BA/F3 cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10 μg/ml gentamycin and 6% Wehi-3B cell-conditioned medium, as a source of IL-3. EA.hy926 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum and 1% HAT supplement (5 mM hypoxanthin, 20 μM aminopterin, 0.8 mM thymidine). HBMEC were cultured in RPMI medium supplemented with 10% heat-inactivated serum, 10% Nu-serum, 2 mM L-glutamine, 1 mM pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml). U937 cells were cultured in RMPI 1640 medium containing 10% fetal calf serum.

2.2.2 Sphingosine 1-phosphate (S1P)

S1P (D-erythro, MW = 379.5, $C_{18}H_{38}NO_5P$) was dissolved in pure ethanol (1 mg/ 1.32 ml). The mixture was vortexed and when needed, warmed to 65 °C to

dissolve the S1P. The ethanol was evaporated under a cold stream of nitrogen and aliquots of S1P were stored at -20 °C. Before use, S1P was reconstituted with hepes-albumin buffer, pH 7.4 and vortexed vigorously.

Hepes-albumin buffer:	20 mM Hepes 150 mM NaCl 0.25 mM BSA

2.2.3 Inhibitors

Protease inhibitor cocktail (PIC) was prepared by dissolving 1 tablet in 500 µl ddH_2O and used in lysis buffers (10 µl/ml lysis buffer). Wortmannin, LY294002, genistein, SU5614, AG1296 and AG1478 were dissolved in pure DMSO. Inhibitors were stored as single-use aliquots at -20 °C. Pertussis toxin was used to treat cells for 3 h; whereas, all other inhibitors were used for 30 min. Control cells received the same concentration of solvent. For migration assays, inhibitors were added to both upper and lower wells of the migration chamber.

2.2.4 RNA isolation

Total RNA was isolated from cells using the Qiagen RNeasy Mini Kit according to the instructions in the kit. Briefly, 5×10^6 cells were lysed in RLT lysis buffer containing the denaturing agent guanidinium isothiocyanate (GITC). To shear genomic DNA and to reduce the viscosity of the lysate, samples were homogenized using the QIAshredder column (Qiagen). Ethanol was added to the lysate to create conditions that promote selective binding of the RNA to the RNeasy column. Samples were applied to the RNeasy mini-spin column for absorption of RNA to the membrane. Contaminants were washed away with wash buffers and RNA was eluted from the RNeasy column using 50 µl of Rnase-free water. RNA was stored at -20 °C until use. The concentration of RNA was determined as the absorbance at 260 nm in spectrophotometer, the absorbance of 1 unit at 260 nm corresponding to 40 µg of RNA per ml (A_{260nm} = 1 unit = 40 µg/ml).

2.2.5 RT-PCR

RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (Qiagen), which allows for reverse transcription and polymerase chain reaction to take place sequentially in the same tube. For each reaction, 0.5 µg RNA and 50 pmol forward/reverse primers were used. The thermal cycler conditions used for PCR reaction are shown below:

PI3Kγ -specific RT-PCR

Reverse Transcription	30 min	50 °C
Initial PCR Activation	15 min	95 °C
3 Step Cycling:		
Denaturation	30 sec	94 °C
Annealing	1 min	56 °C
Extension	45 sec	72 °C
Number of Cycles	30 cycles	
Final Extension	10 min	72 °C

p101 -specific RT-PCR

Reverse Transcription	30 min	50 °C
Initial PCR Activation	15 min	95 °C
3 Step Cycling:		
Denaturation	30 sec	94 °C
Annealing	45 sec	59 °C
Extension	30 sec	72 °C
Number of Cycles	30 cycles	
Final Extension	5 min	72 °C

RT-PCR products, mixed with five-fold concentrated DNA loading buffer, were separated by electrophoresis on 2% agarose gels in TAE buffer containing 0.5 µg/ml ethidium bromide and visualized using a UV illuminator.

TAE buffer:
40 mM Tris-acetate
1 mM EDTA
pH 8.3

DNA loading buffer (5X):
30% Glycerol
0.25% Bromophenol Blue
0.25% Xylene Cyanol

2.2.6 Cell stimulation

HUVEC seeded onto 60-mm or 90-mm dishes were 80-90% confluent on the day of stimulation. Prior to stimulation, cells were washed once and serum-starved with M199/0.2% BSA (fatty acid-free) for 1 h. Inhibitors were administered in M199/0.2% BSA (fatty acid-free) for the appropriate amount of time. Cell stimulations were performed in the presence of inhibitors. After stimulation, cells were washed twice with cold wash buffer and immediately placed on ice. Lysis buffer was given to the cells (60 µl/60-mm or 250 µl/90-mm dish) and cells were scraped from the dish using a cell scraper. Lysate was collected in an eppendorf tube and centrifuged at (15,000 x g, 15 min, 4 °C) to remove the Triton-insoluble material. The cleared lysate was transferred to a new eppendorf tube and 10 µl was set aside for protein determination. Lysates were diluted with 3-fold concentrated Laemmli buffer, heated at 100 °C for 5 min, and stored at -20 °C until use. Protein concentration was determined using the Lowry method (Biorad DC Assay) and bovine serum albumin as standard.

Wash buffer:
50 mM Tris, pH 7.4
2 mM EDTA
1 mM EGTA
50 mM NaF
10 mM $Na_4P_2O_7$
1 mM Na_3VO_4
1 mM DTT

Lysis buffer:
50 mM Tris, pH 7.4
2 mM EDTA
1 mM EGTA
50 mM NaF
10 mM $Na_4P_2O_7$
1 mM Na_3VO_4
1 mM DTT

0.1 % SDS
1 % Triton X-100
+ 10 μl/ml PIC

Laemmli buffer (3X): 186 mM Tris, pH 6.8
10 mM EDTA
9 % SDS
15 % Glycerol
6 % β-mercaptoethanol
0.03 % Bromophenol Blue

2.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Cell lysates (50 μg/lane) were separated by SDS-PAGE (15% for Rac and 10% for other experiments) and transferred to PVDF membrane using a semi-dry transfer apparatus. Briefly, six pieces of Whatmann 3MM paper and 1 PVDF membrane were cut to the exact size of the gel and were assembled on the transfer apparatus in the following order (from anode (+) to cathode (-): 3 pieces of Whatmann 3MM paper (pre-wet in transfer buffer): PVDF membrane (pre-wet in methanol, water, transfer buffer): SDS-gel: 3 pieces of Whatmann 3MM paper (pre-wet with transfer buffer). With a glass pipette, air bubbles were carefully rolled out and excess buffer was removed using a paper towel. Semi-dry transfer was carried out at a constant voltage (25V) and current of 2.0 mA/cm^2 for 1.5 h. After transfer, the PVDF membrane was stained with Ponceau-S solution to ensure equal transfer of proteins, washed briefly in Tris-buffered saline/Tween-20 solution (TBS-T) and blocked with 5% nonfat dry milk in TBS-T for 1.5 h. Blots were probed with the appropriate primary and secondary antibodies in 1% bovine serum albumin/TBS-T solution overnight or for 1 h, respectively, and detection was carried out using ECL chemiluminescence reagent (Amersham) and exposure onto BioMax film (Kodak). For counterstaining, immunoblots were stripped by incubating blots in stripping buffer at 56 °C for 30 min, with agitation and reprobed with appropriate antibodies.

Tris-buffered saline (10X): 137 mM NaCl
2.68 mM KCl
25 mM Tris base
pH 7.4

TBS-Tween (TBS-T):	1X TBS + 0.1 % Tween-20
Electrophoresis buffer (1X):	25 mM Tris 250 mM Glycine (pH 8.3) 0.1 % SDS
Transfer buffer (1X):	48 mM Tris base 39 mM Glycine 0.037 % SDS 20 % Methanol
Ponceau S stain:	2 % Ponceau-S 30 % Trichloroacetic acid 30 % Sulfosalicylic acid
Stripping buffer:	62.5 mM Tris-HCl, pH 6.7 2 % SDS 0.7 % β-mercaptoethanol

2.2.8 DNA transformation in Escherichia coli

Competent *Escherichia coli* cells

A small scale culture of XL-1 Blue *Escherichia coli* was prepared by inoculating 2 ml of LB medium containing antibiotics and grown O/N, 37 °C. Half of the overnight culture was used to inoculate 150 ml LB medium and growth was continued until an OD_{600}= 0.6 (2-3 h) was obtained. The culture was centrifuged (500 x g, 10 min) and the cell pellet was resuspended in 1/10 volume TSB solution (15 ml). Cell suspension was kept on ice for 10 min, divided in 100 µl aliquots, and stored at -80 °C until ready to use.

TSB:	1X LB medium 5 % DMSO 10 mM $MgCl_2$ 10 mM $MgSO_4$ 10 % PEG-4000
LB medium:	10 g/L Bacto-Tryptone 5 g/L Bacto-yeast extract 10 g/L NaCl pH 7.0

LB-agar plates: LB medium + 1.5 % agar (15 g/L)

KCM transformation

Plasmid DNA (100 pg-10 ng) was mixed with 5-fold concentrated KCM Buffer (20 μl) and ddH_2O (80 μl). XL-1 blue competent *Escherichia coli* were thawed on ice and 100 µl was added to the DNA/KCM mixture and incubated for 30 min on ice. Next, the mixture was incubated for 10 min at room temperature. Sample was mixed with LB medium (1 ml) without antibiotics and further incubated for 1 h at 37 °C with vigorous shaking. 10-200 µl of the mixture was spread onto LB-agar plates and incubated overnight at 37 °C. Single colonies were selected and amplified in 200 ml LB medium containing the appropriate antibiotics (100 μg/ml ampicillin or 30 μg/ml kanamycin). DNA was isolated and purified using Qiagen Endofree Kit. DNA concentration and purity was determined by measuring the absorbance at 260/280 nm ($OD_{260/280}$) using a spectrophotometer.

KCM buffer (5X): 0.5 M KCl
0.15 M $CaCl_2$
0.25 M $MgCl_2$

2.2.9 Endothelial cell transfection

Endothelial cells were transfected using the AMAXA biosystems nucleofection procedure and according to the manufacturer's instructions (www.amaxa.com). Briefly, cells were washed twice with Hepes-Na buffer and detached using warm trypsin-EDTA. After trypsinization, cells were neutralized with M199/10% FCS. Cells were pelleted by centrifugation (500 x g, 6 min), resuspended in Hepes-Na buffer and counted in a Neubauer hemacytometer. For each transfection, 1 x 10^6 cells were used. The required number of cells was pelleted by centrifugation (500 x g, 6 min) and resuspended in room temperature Nucleofector solution (100 μl per transfection). The cell suspension was mixed with plasmid DNA (3 μg/ 100 μl) and transferred to an electroporation cuvette. Electroporation was carried out using different nucleofection programs (U-01, S-01, T-01). After electroporation, cells were covered with warm growth (400 μl) medium and plated immediately onto 60-mm dishes containing 3 mls of growth medium. After six hours, growth medium

was replaced. Cells were analyzed for protein expression or harvested for cellular assays 24 h post-transfection.

Hepes-Na buffer:	10 mM Hepes-Na 145 mM NaCl 5 mM KCl 1 mM $MgSO_4$ 10 mM Glucose pH 7.4

2.2.10 Migration assay

Cell migration assays were performed using tissue culture inserts (polyethylene terephthalate (PET) membrane, 8 µm pores; BD Falcon Inserts) situated in 12-well plates. Inserts were coated with 0.2% gelatin solution overnight, 4 °C. On the day of experiment, the gelatin solution was removed by aspiration and inserts were allowed to dry. HUVEC were cultivated on 0.2% gelatin coated 75-cm^2 flasks and grown to sub-confluency. For transfection experiments, cells were maintained on 30-mm dishes and harvested 24 h post-transfection. Cells were washed twice with pre-warmed Hepes-Na buffer and detached using trypsin/EDTA solution (1.4 ml/75-cm^2 flask or 1 ml/ 30-mm dish) for 1-3 min at 37 °C. Trypsinization was stopped using M199/10% FBS and plates were rinsed twice with Hepes-Na buffer. Cells were pelleted by centrifugation (500 x g, 6 min) and resuspended with M199/0.2% BSA (fatty acid-free). Cell suspension was diluted to 5.0 x 10^5 cells/ml in M199/0.2% BSA (fatty acid-free) and 0.5 ml of the cell suspension (2.5 x 10^5 cells) was applied to the upper chamber of the insert.

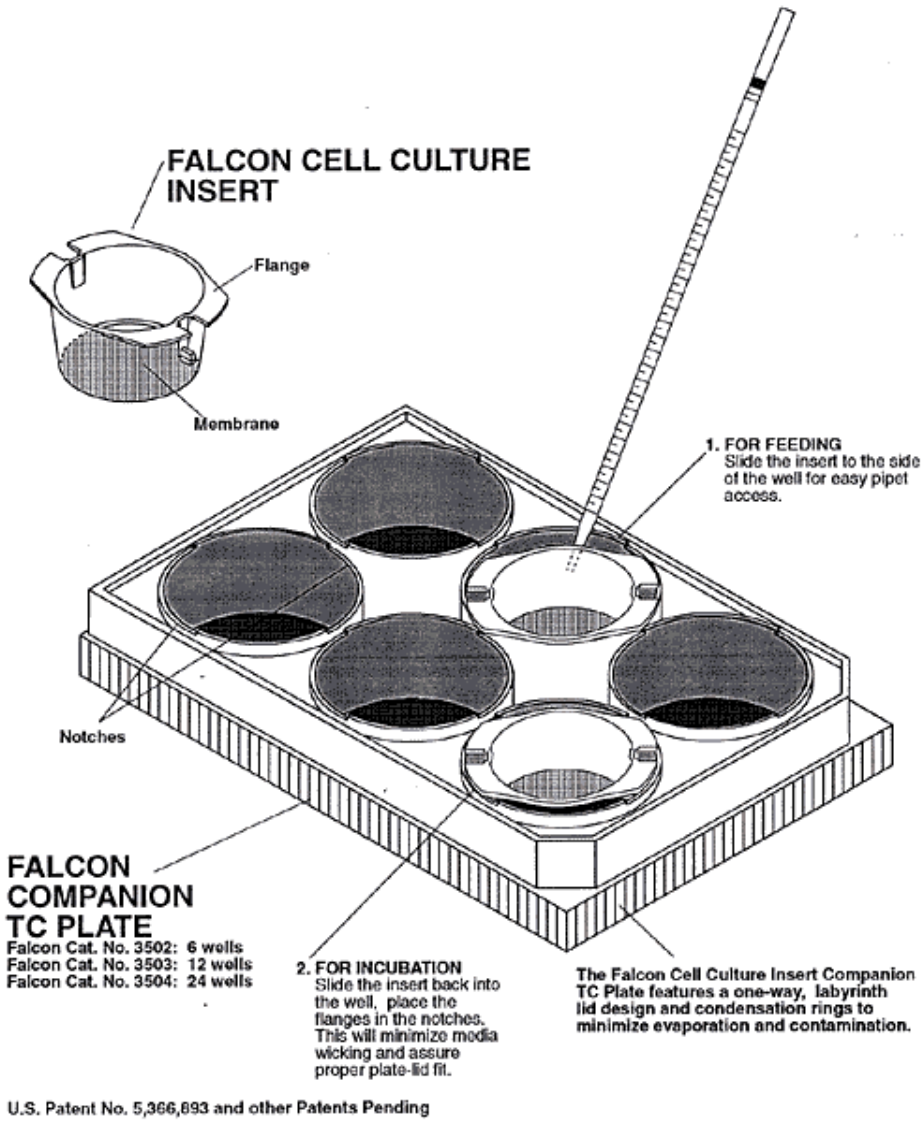

Figure 6 | Migration Chamber (6 well format)
Figure adapted from Becton Dickinson product brochure.

To the lower chamber, 1 ml of M199/0.2% BSA (fatty acid-free) was added, with or without inhibitors. Lastly, agonist was added to the lower chamber and the migration apparatus was gently agitated to ensure even distribution of the chemoattractant. Migration was allowed to proceed for 4 h at 37 °C and 5% CO_2. Inserts were stained with hematoxylin and number of migrated cells was determined. To visualize green fluorescent protein (GFP) expression, inserts were directly embedded using Fluoromount, without prior fixation.

Hematoxylin staining

Inserts were washed twice using cold Hepes-Na buffer/1.5 mM $CaCl_2$ and fixed with 4% paraformaldehyde for 10 min. The fixing solution was removed by aspiration and inserts were washed twice with ddH_2O. Inserts were stained with hematoxylin (Dako) for 5 min, washed twice with tap water and incubated for 10 min to allow for color development. Afterwards, the tap water was removed by aspiration and the non-migrated cells were removed by wiping the upper face of the insert with a cotton-tip. Inserts were allowed to air-dry overnight in the dark. Using a clean scalpel, inserts were carefully excised from their support and embedded onto tissue culture slides using Eukitt mounting medium. The number of migrated cells was counted using an inverted microscope (Leitz) at 25-fold magnification. The number of migrated cells was determined as the mean of 10 randomly selected fields.

2.2.11 Rac-1 GTPase activation assay

The activation of Rac was measured in a pull-down assay, in which the CRIB (Cdc42- and Rac-interacting binding) domain of p21-activated kinase (PAK) was used to isolate the active GTP-bound form of Rac (Bagrodia et al., 1995). A Glutathione S-Transferase (GST)-tagged form of the p21-activated kinase (PAK) CRIB domain was employed in pull-down assays. Briefly, 1 x 10^6 cells were seeded on 0.2% gelatin-coated 90-mm dishes and grown until almost confluent. On the day of experiment, cells were serum-starved for 5 h and stimulated with S1P (1 μM, 5 min). Without washing, cells were lysed using cold lysis buffer (1 ml/90-mm plate), containing GST-PAK (20 μg/ml), protease inhibitors (10 μl PIC/ml) and GDP (100 μM). Cells were immediately placed on ice and scraped using a cell scraper. Lysates were collected, vortexed for 10 sec and cleared by centrifugation (15,000 x g, 15 min, 4 °C). GSH-Sepharose beads were washed three times in lysis buffer and 40 μl (1:1 suspension) was given to the tubes. Samples were incubated on a rotator for 30 min, 4 °C. Afterwards, the beads were pelleted by centrifugation, washed three times with cold lysis buffer (500 μl), resuspended with 3-fold concentrated Laemmli buffer (45 μl) and heated at 100 °C

for 5 min. Samples were separated by 15% SDS-PAGE, transferred to PVDF membrane and immunoblotted using anti-Rac1 monoclonal antibody (1:1000 dilution in 5% TBS-Tween). To avoid transferring the GST-PAK protein (approx. 30 KDa), gels were cut slightly below the 30 KDa marker band and only the lower part was transferred and immunoblotted against Rac (approx. 20 KDa). The upper gel was stained with coomassie blue to control for equal amounts of GST-PAK protein.

Lysis buffer:
50 mM Tris, pH 7.5
100 mM NaCl
5 mM $MgCl_2$
1 mM EGTA
1% NP-40
10% Glycerol
(+ 100 μM GDP)
(+ 20 μg/ ml GST-PAK)
(+ 10 μl/ ml PIC)

Recombinant expression of GST-PAK CRIB domain

GST-PAK CRIB domain was produced in *Escherichia Coli.* An overnight culture was used to inoculate 500 ml of LB containing 100 μg/ml ampicillin . The culture was grown until an OD_{600nm} = 0.7 was obtained (approx. 2 h). Protein expression was induced by the addition of IPTG (100 μM) and growth was continued for an additional 5 h at room temperature. Cells were pelleted by centrifugation (500 x g, 10 min) and resuspended with TBN-150 buffer (10 ml). Cells were lysed by three cycles of freezing and thawing. Lysozyme (5 mg/100 μl TBN-150 buffer) was given to lysate, mixed well and incubated for 20 min at room temperature. A Triton X-100/EDTA solution (280 μl of 500 mM EDTA, 350 μl of 10% Triton X-100) was added to the lysate and further incubated for 20 min at room temperature. Lysate was cleared by ultra-centrifugation (30,000 rpm, 30 min, 4 °C). In the meantime, Glutathione-Sepharose 4B beads (1 ml) were washed three times using TBN-150 buffer and used to pack a column (Biorad Poly-Prep Chromatography Columns). Cleared lysate was applied to the column and allowed to run through. The column was washed four times with EQM buffer and eluted with 5 ml Elution buffer. Eluate was dialyzed overnight against phosphate-buffered saline (PBS) with a minimum of three buffer changes. Successful expression and isolation of GST-PAK was

assessed by 15% SDS-PAGE and coomassie staining. GST-PAK protein was stored as single-use aliquots at -80 °C.

TBN-150 buffer:
25 mM Tris, pH 7.5
150 mM NaCl
10 mM β-mercaptoethanol

add fresh to 10 ml:
2 µl Leupeptin (1 mg/ml)
10 µl Pepstatin (1 mg/ml)
100 µl PMSF (100 mM in Ethanol)
55 µl EGTA (0.36 M, pH 8)
10 µl Pefabloc (100 mg/ml)
4 µl EDTA (500 mM, pH 8)

EQM buffer:
50 mM Tris, pH 8.0
100 mM NaCl
10 mM β-mercaptoethanol

Elution buffer:
50 mM Tris, pH 8.0
100 mM NaCl
10 mM β-mercaptoethanol
10 mM Glutathione

Phosphate-buffered saline:
1 mM KH_2PO_4
10 Mm Na_2HPO_4
137 mM NaCl
2.7 mM KCl
pH 7.4

Coomassie stain:
0.25 % Coomassie Brilliant Blue R-250
25 % Ethanol
10 % Acetic acid

Destaining solution:
45 % Methanol
10 % Acetic acid

3. Results

3.1 Expression of PI3Kγ in endothelial cells

3.1.1 Expression of p110γ in endothelial cells

The expression of the PI3Kγ catalytic subunit, p110γ, was evaluated in endothelial cells using a sensitive RT-PCR to detect the messenger RNA of p110γ. Gene-specific primers were designed against human p110γ sequences in exons 10 and 11, which are separated by 18.8 kilobases of intron sequence (Fig. 7).

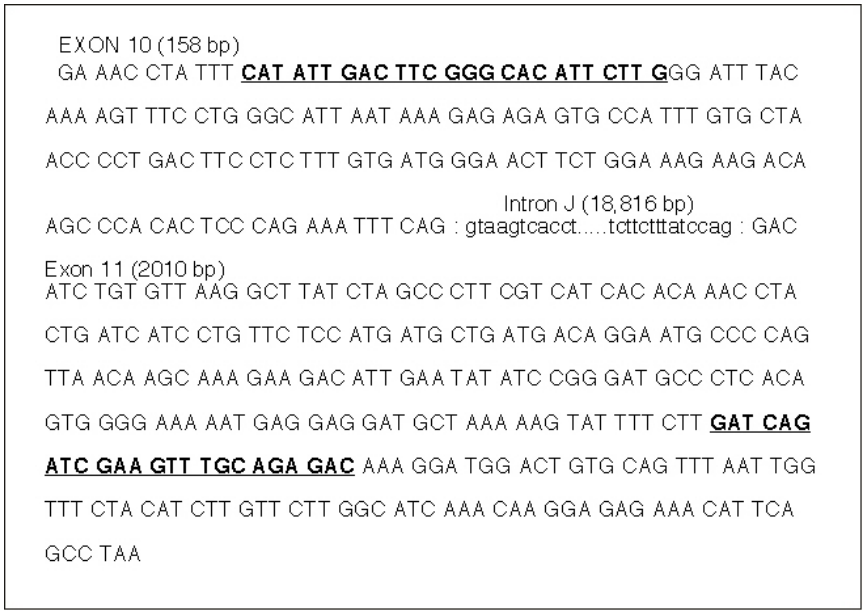

Figure 7 | Exon 10/11 of human p110γ Sequences used for primer selection are highlighted.

Due to this large intron sequence, it was possible to exclude the amplification of genomic DNA. The size of the expected product was approximately 348 base pairs as detected by 2% agarose gel electrophoresis. β-actin primers, which amplify a 513 base pair product, were used to control for the equal loading of RNA. Several different endothelial cell types were examined for the presence of p110γ mRNA using RT-PCR, including HUVEC, EA.hy926 (fusion cell line of HUVEC and A549),

BREC, HBMEC and HMEC. U937, a myeloid leukemia cell line, which is known to express high levels of p110γ was employed as positive control for p110γ expression. Regardless of source, most endothelial cell types examined, with the exception of the HBMEC, were found to express p110γ mRNA (Fig. 8).

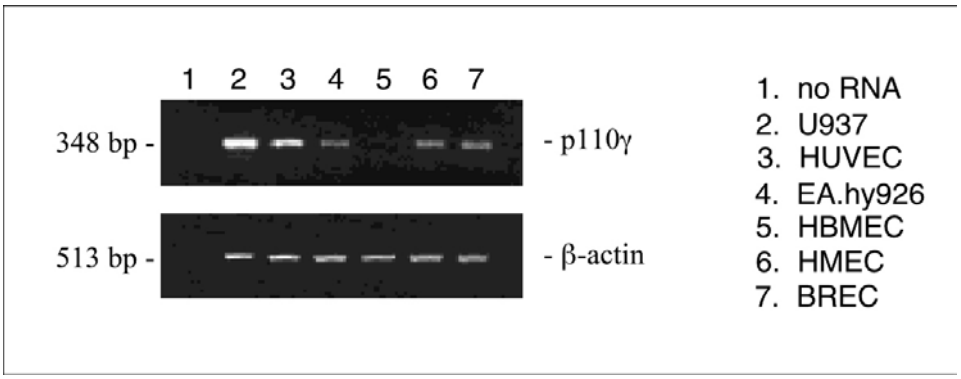

Figure 8 | p110γ-specific Reverse Transcriptase -Polymerase Chain Reaction (RT-PCR). RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (0.5 μg RNA and 50 pmol forward/reverse primers). RT-PCR products were separated on 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized using UV light. One typical experiment out of 3 is shown.

Protein expression of p110γ was investigated in HUVEC and EA.hy926. Employing an antibody which is directed against the N-terminus of p110γ, we found that p110γ was also expressed in endothelial cells HUVEC and EA.hy926 at the protein level (Fig. 9).

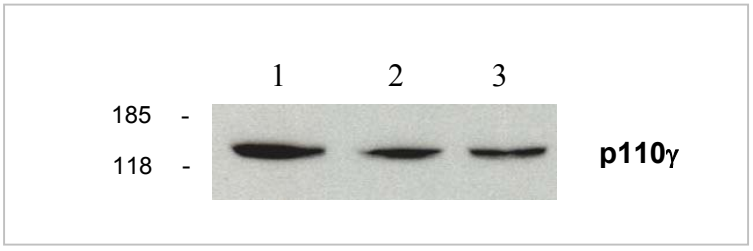

Figure 9 | Expression of PI3Kγ protein in (1) U937, (2) EA.hy926 and (3) HUVEC. Cell lysates were prepared from U937, EA.hy926 and HUVEC and separated using 10% SDS-PAGE. Immunoblotting analysis of cell lysates was performed using specific antibodies against p110γ.

Since EA.hy926 is formed by the fusion of HUVEC and A549 lung carcinoma cells, the contribution of A549 to the expression of p110γ was investigated. Cell lysate from BA/F3 (pro-B cell line) was used as positive control for the expression of p110γ. A549 cells appeared not to express p110γ protein, confirming that the expression of p110γ in the endothelial fusion cell line EA.hy926 derives solely from HUVEC (Fig. 10).

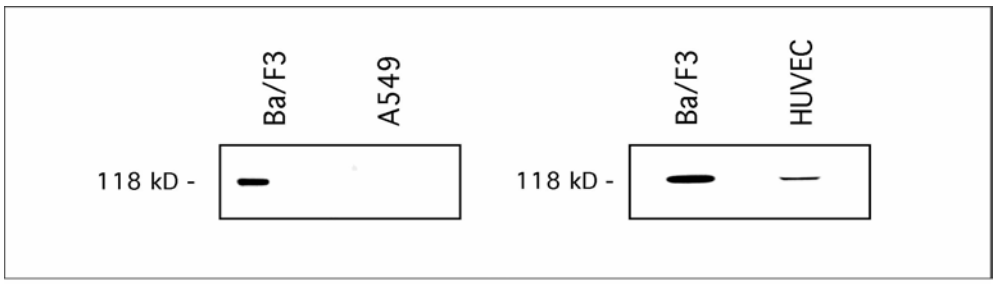

Figure 10 | Expression of p110γ protein in A549 and HUVEC. Cell lysates were prepared from U937, EA.hy926 and HUVEC and separated using 10% SDS-PAGE. Immunoblotting analysis of cell lysates was performed using specific antibodies against p110γ. Ba/F3 (pro-B cell line) served as positive control for p110γ protein expression.

3.1.2 Expression of p101 in endothelial cells

Recently, p101 was described as an essential adaptor subunit of p110γ. However, investigations of p101 have been hindered by the absence of a commercially available antibody. Thus, we examined the expression of p101 messenger RNA in endothelial cells using the highly sensitive RT-PCR.

We designed oligonucleotide primers according to the human sequence of p101. The size of the expected product was approximately 315 base pairs, as detected by 2% agarose gel electrophoresis. β-actin primers were used to control for the equal loading of RNA. A myeloid leukemia cell line, U937, which is known to express abundant amounts of p101 protein, was employed as positive control for p101 expression. Despite expression at high levels in U937 cells, p101 RNA was not detected in HUVEC, EA.hy926, HBMEC, HMEC and BREC (Fig. 11).

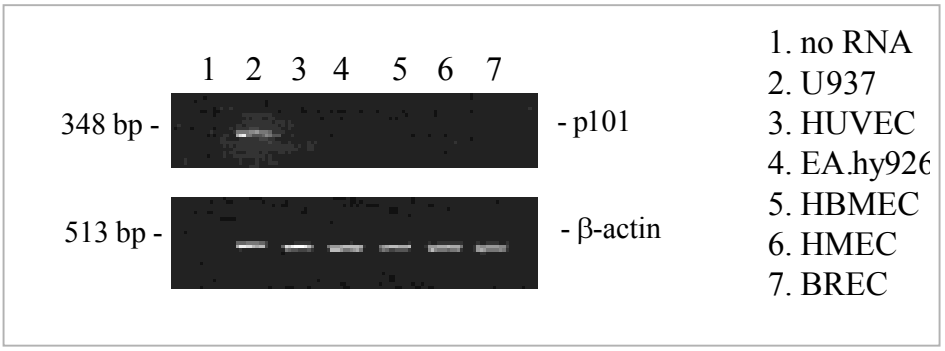

Figure 11 | p101-specific RT-PCR. RT-PCR was performed using the Qiagen OneStep RT-PCR Kit (0.5 μg RNA and 50 pmol forward/reverse primers). RT-PCR products were separated on 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized using UV light. One typical out of 3 is shown.

3.2 S1P-induced MAPK activation in HUVEC

3.2.1 Time and concentration dependence

We have investigated the effect of S1P on the activation of the extracellular regulated kinase (ERK) MAP Kinase in HUVEC. In brief, HUVEC monolayers were stimulated with various concentrations of S1P (0.01-10 μM) for different times (0, 15, 30 sec, 1, 2, 5, 10, 20 min). Cell lysates (50 μg protein/ lane) were subjected to immunoblot analysis using phospho-specific antibodies against ERK, which specifically recognizes the activated, phosphorylated form of ERK. The equal loading of protein was monitored by measuring levels of total ERK using a pan-ERK antibody.

We observed that S1P treatment led to a striking activation of ERK in HUVEC. ERK phosphorylation occurred in a time-dependent manner (Fig.12). Following stimulation with S1P (1 μM), phosphorylation of ERK was detected as early as 2 min and reached a maximum after 5 min. ERK activation was sustained for up to 20 min.

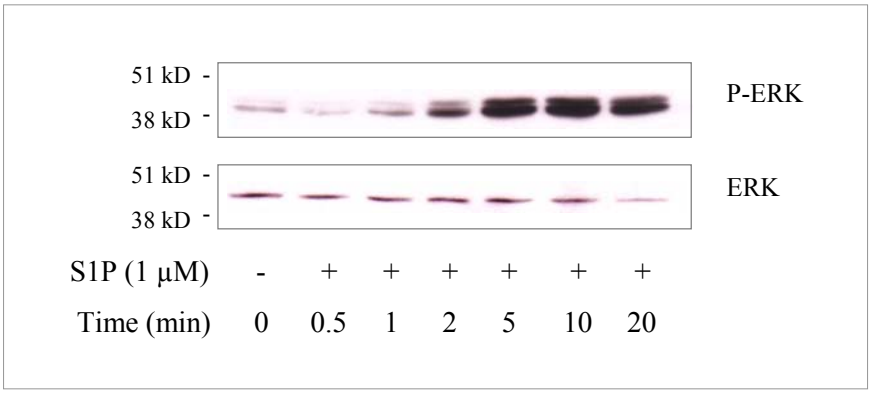

Figure 12 | Time-dependent phosphorylation of ERK MAP kinase by S1P in HUVEC. HUVEC were stimulated with 1 µM S1P for 0.5, 1, 2, 5, 10, 20 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK or, after stripping, total ERK. One of three similar blots is shown.

Additionally, phosphorylation of ERK appeared to be dependent on S1P concentration. We observed a slight activation of ERK already with nanomolar concentrations of S1P, although higher doses were required to achieve significant activation of ERK (Fig. 13). A potent activation was also seen with 10 µM S1P; however, this dose ceases to be in the physiological range.

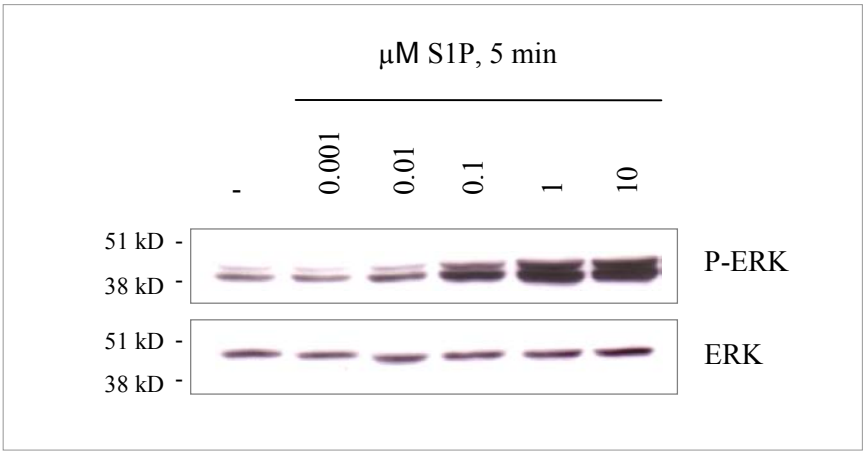

Figure 13 | Concentration-dependent ERK activation by S1P stimulation in HUVEC. HUVEC were stimulated with 0.001, 0.1, 1 and 10 µM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK or, after stripping, total ERK. One of three similar blots is shown.

3.2.2 Effect of PI3K and G–protein inhibitors on MAPK activation

The involvement of PI3K in S1P-induced ERK activation was assessed using a general PI3K inhibitor. Wortmannin, originally isolated from *Penicillium wortmannii*, irreversibly binds a critical lysine residue in the active site of PI3K and thereby, functions as a competitor for ATP-binding. Wortmannin inhibits all isoforms of PI3K, though the effective concentration required for inhibition varies for the different PI3K classes. When used in the nanomolar range, wortmannin has been demonstrated to specifically inhibit the class I PI3K (Walker et al., 2000). As shown in Figure 14, stimulation of HUVEC with S1P (1 or 10 μM, 5 min) led to a potent phosphorylation of ERK, which was significantly diminished by pretreatment with wortmannin (100 nM, 30 min).

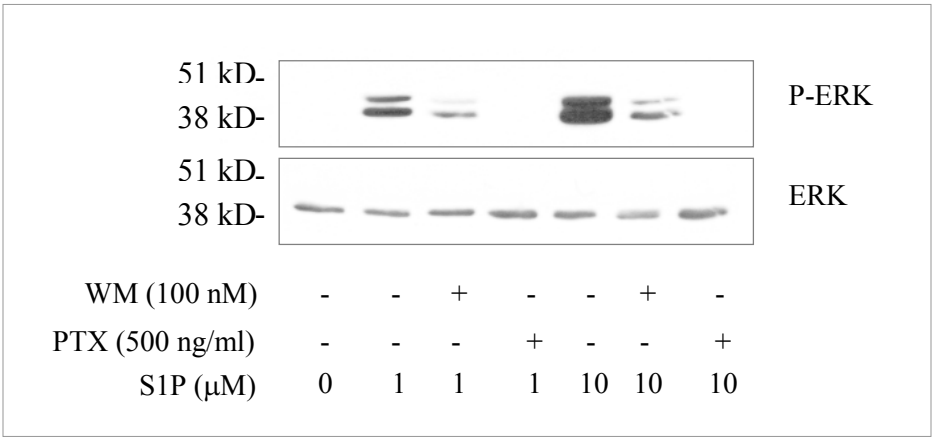

Figure 14 | Effect of wortmannin and pertussis toxin on S1P-induced ERK activation in HUVEC. HUVEC were pretreated with wortmannin or pertussis toxin for 30 min or 3 h, respectively, and subsequently stimulated with 1 μM or 10 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK or, after stripping, total ERK. One of three similar blots is shown. WM, wortmannin; PTX, pertussis toxin.

Furthermore, the involvement of Gi/o-proteins was evaluated using pertussis toxin, which ADP-ribosylates and inactivates Gi/o-proteins. Treatment of HUVEC with pertussis toxin (500 ng/ ml, 3 h) prior to stimulation with S1P resulted in a complete inhibition of ERK phosphorylation. Immunoblotting against total ERK was used to control for equal protein loading.

3.2.3 Effect of tyrosine kinase inhibitors on MAPK activation

Many G-protein coupled receptors have been demonstrated to transactivate receptor tyrosine kinases, such as the EGF, PDGF and VEGF receptors. To characterize the signaling pathways involved in S1P-induced ERK activation, we examined the effects of tyrosine kinase inhibitors, genistein and SU5614. Genistein is a general inhibitor of tyrosine kinases, while SU5614 inhibits specifically the VEGF receptor tyrosine kinase. Treatment of HUVEC with tyrosine kinase inhibitors genistein (10-100 μM, 30 min) and SU5614 (1-50 μM, 30 min) seemed not to affect the phosphorylation of ERK stimulated by S1P (1 μM, 5 min), as shown in Fig. 15.

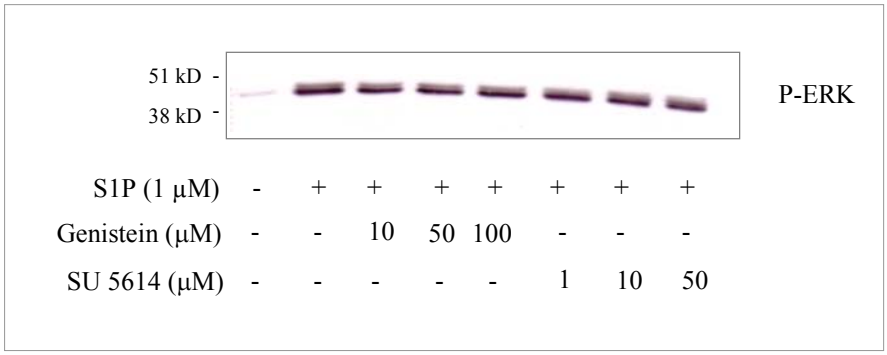

Figure 15 | Effect of tyrosine kinase inhibitors genistein and SU5614 on S1P-induced ERK activation in HUVEC. HUVEC were pretreated with genistein or SU5614 for 30 min, and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK. One of three similar blots is shown.

Next, we looked at the effect of tyrphostin inhibitors AG1296 and AG1478 on S1P-induced ERK activation. AG1296 inhibits the tyrosine kinase activity associated with the PDGF receptor. AG1478, on the other hand, is a specific inhibitor of the EGF receptor family (ErbB family) tyrosine kinases. We found that micromolar concentrations of both AG1296 (1, 10, 25 μM, 30 min) and AG1478 (1, 10, 25 μM, 30 min) did not affect ERK activation in response to S1P treatment (Fig. 16). Based on these results, it appears that EGF, PDGF and VEGF receptor transactivation are not involved in S1P-induced ERK phosphorylation in human endothelial cells.

Figure 16 | Effect of receptor tyrosine kinase inhibitors AG1296 and AG1478 on S1P-induced ERK activation in HUVEC. HUVEC were pretreated with AG1296 or AG1478 for 30 min and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK. One of three similar blots is shown.

3.2.4 Effect of PI3Kγ KR overexpression on MAPK activation

The introduction of foreign genes or altered forms of an endogenous gene into eukaryotic cells has proven useful in studying the in vivo functions of endogenous genes. Many types of cells are able to automatically take up DNA which is added to the medium. However, primary cells are known to be particularly difficult to transfect and are refractory to most classical transfection methods.

A novel transfection method based on electroporation (AMAXA Nucleofection) was used for the transfection of HUVEC with p110γ wildtype or mutant DNA. We used p110γ KR, a catalytically inactive form of p110γ in which Lys-833 was mutated to Arg, to examine different amounts DNA (1.5, 3.0 μg) and different programs (U-01, T-01, S-01), withU-01 being strongest and S-01 the weakest. Immunoblotting analysis with specific antibodies against p110γ showed that 3 μg DNA and program U-01 were the most effective (Fig. 17). Thus, we used these conditions for further experiments.

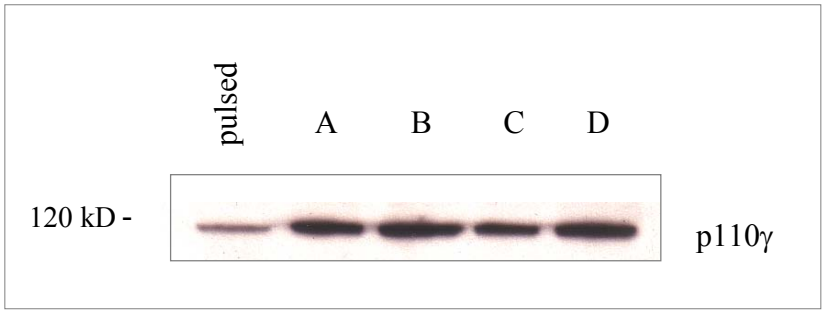

Figure 17 | AMAXA Nucleofection. HUVEC were transfected without DNA 'pulsed', (A) 1.5 μg p110γ KR, U-01, (B) 3 μg p110γ KR, U-01, (C) 3 μg p110γ KR, S-01, (D) 3 μg p110γ KR, T-01. Twenty-four hours post-transfection, cells were lysed. Immunoblotting analysis of cell lysates was performed using specific antibodies against p110γ.

To directly assess the role of G-protein coupled PI3Kγ in S1P activation of MAPK, we introduced into HUVEC plasmid DNA encoding for wildtype or mutant forms of p110γ and analyzed their effect on the activation of ERK in response to S1P. Two different mutants were employed: p110γ KR (kinase dead), and p110γ CAAX (constitutively active; localized to membrane by fusion with Ras isoprenylation signal –CAAX). DNA encoding for empty vector (pcDNA3) served as control.

Twenty-four hours post-transfection, cells were analyzed for the expression of the transfected DNA using a p110γ-specific monoclonal antibody. As shown in Fig. 18 (upper panel), p110γ WT and KR plasmids were overexpressed at high levels, confirming that HUVEC transfection using the novel AMAXA nucleofection technique was both successful and efficient.

The effect of the p110γ mutants on S1P-induced ERK activation was evaluated. Transfected cells were stimulated with S1P (1 μM, 5 min) and subsequently, lysates were immunoblotted against phospho-ERK.

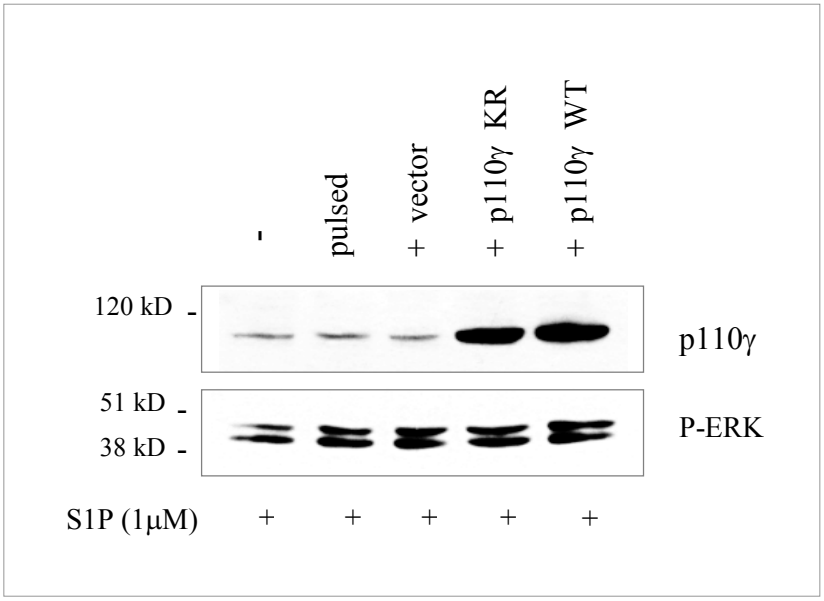

Figure 18 | Effect of p110γ wildtype and p110γ KR overexpression on S1P-induced ERK activation in HUVEC. HUVEC were transfected without DNA 'pulsed', pcDNA3 vector, p110γ KR or p110γ wildtype. Twenty-four hours post-transfection, cells were serum starved for 1 h and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against p110γ or phosphorylated ERK. One of three similar blots is shown.

To assess the effects of the AMAXA nucleofector solution and the electroporation procedure on ERK activation, we included an electroporation control ("pulsed"). In addition, we transfected vector DNA to control for the effects due to the expression of vector alone. As seen in Fig. 18 (lower panel), ERK phosphorylation in response to S1P was not affected by electroporation alone ("pulsed") or by transfection with empty vector. Furthermore, overexpression of p110γ WT and p110γ KR constructs did not attenuate S1P activation of ERK. Despite inhibitory effects of PI3K and Gi/o-protein inhibitors (Fig. 14) on ERK phosphorylation, which implicate the involvement of a Gi/o-protein sensitive PI3-kinase, PI3Kγ appears not to be involved in S1P-induced ERK activation in HUVEC.

3.3 S1P-induced Akt activation in HUVEC

3.3.1 Time and concentration dependence

Akt is a well known effector of PI3K. Via its PH domain, Akt has been shown to bind phosphorylated lipids, PIP_2 and PIP_3, which facilitates its recruitment to the membrane and subsequent activation by phosphorylation. We have investigated the effect of S1P treatment on the phosphorylation of Akt in HUVEC. Cells were stimulated with various concentrations of S1P (0.01-10 μM) for different times (0, 15, 30 sec, 1, 2, 5, 10, 20, 60 min) and cell lysates were immunoblotted using an anti-phospho-Akt polyclonal antibody, which recognizes Akt when phosphorylated at Ser^{473}.

We observed that S1P treatment of HUVEC led to a rapid activation of Akt, which was both time- and concentration- dependent (Fig. 19). Following stimulation with S1P (1 μM, 5 min.). Akt phosphorylation was detected as early as 1 min and reached a maximum at 5 min. The activation of Akt was sustained for up to 60 min.

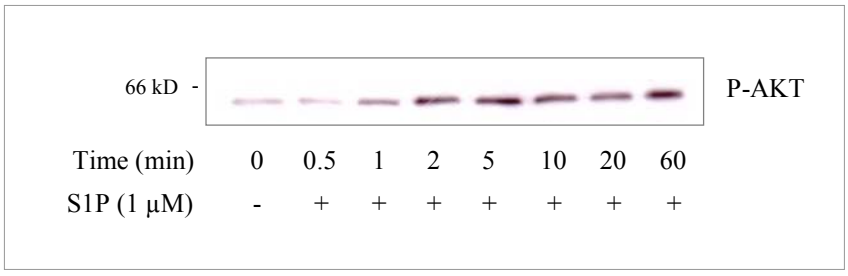

Figure 19 | Time-dependent activation of Akt by S1P in HUVEC. HUVEC were stimulated with 1 μM S1P for 0.5, 1, 2, 5, 10, 20, and 60 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated Akt. One of three similar blots is shown.

In addition, we observed that the activation of Akt was dependent on S1P concentration (Fig. 20). A slight activation of Akt was observed even with concentrations of S1P in the low nanomolar range. However, maximal activation of

Akt was required micromolar concentrations of S1P. Interestingly, Akt appeared to be phosphorylated in its basal state, in the absence of stimulation.

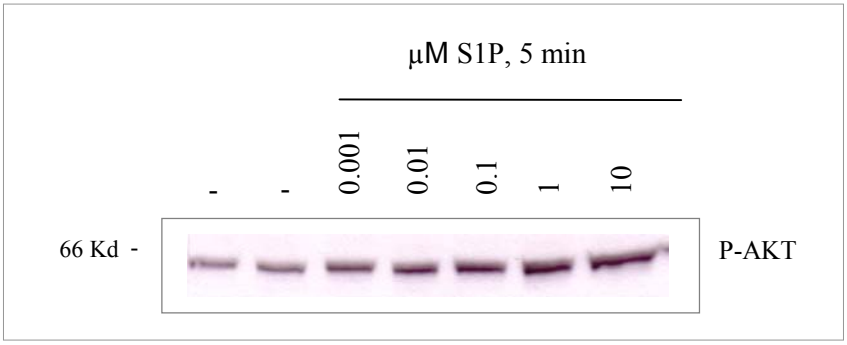

Figure 20 | Concentration-dependent activation of Akt/PKB by S1P stimulation in HUVEC. HUVEC were stimulated with 0.001, 0.001, 0.1, 1 and 10 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated Akt. One of three similar blots is shown.

3.3.2 Effect of PI3K and G -protein inhibitors on Akt activation

The involvement of PI3K in S1P-induced Akt activation was explored using two different PI3K inhibitors, wortmannin and LY294002. LY294002, a synthetic compound and like wortmannin, competes with ATP binding. Inhibition by LY294002, however, is reversible. S1P (1 μM, 5 min) stimulated Akt phosphorylation was abrogated by both wortmannin (100 nM, 30 min) and LY294002 (10 μM, 30 min) pretreatment. The involvement of Gi/o-proteins was evaluated using pertussis toxin, which specifically inhibits Gi/o-proteins. As illustrated in Fig. 21, pretreatment of HUVEC with pertussis toxin (500 ng/ml, 3 h) resulted in a complete attenuation of S1P-induced Akt phosphorylation, suggesting that Gi/o-proteins are critical for this process.

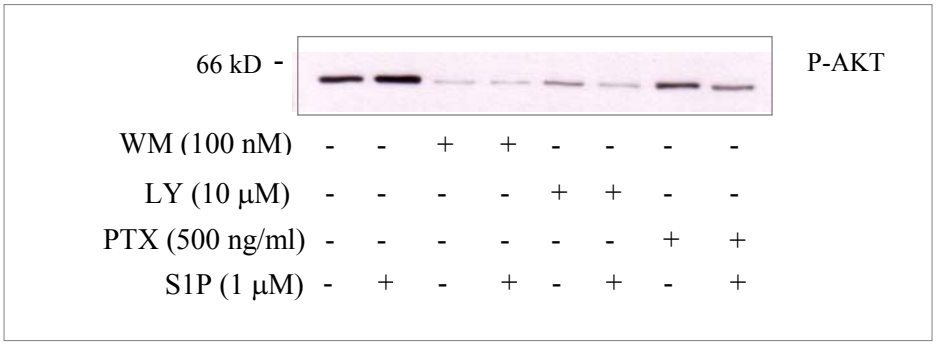

Figure 21 | Effect of wortmannin, LY294002 and pertussis toxin on S1P-induced Akt activation in HUVEC. HUVEC were pretreated with 100 nM wortmannin or 10 μM LY294002 for 30 min or 500 ng/ml pertussis toxin for 3 h and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated ERK or, after stripping, total ERK. One of three similar blots is shown. LY, LY294002; WM, wortmannin; PTX, pertussis toxin.

3.3.3 Effect of tyrosine kinase inhibitors on Akt activation

The contribution of tyrosine kinase activity to S1P-stimulated Akt phosphorylation was determined using the inhibitor genistein. Pretreatment of HUVEC with different concentrations of genistein (10-100 μM, 30 min), a general inhibitor of tyrosine kinases activity, showed no significant effect on S1P-stimulated Akt phosphorylation (Fig. 22). Thus, tyrosine kinase activity does not to contribute to Akt activation by S1P.

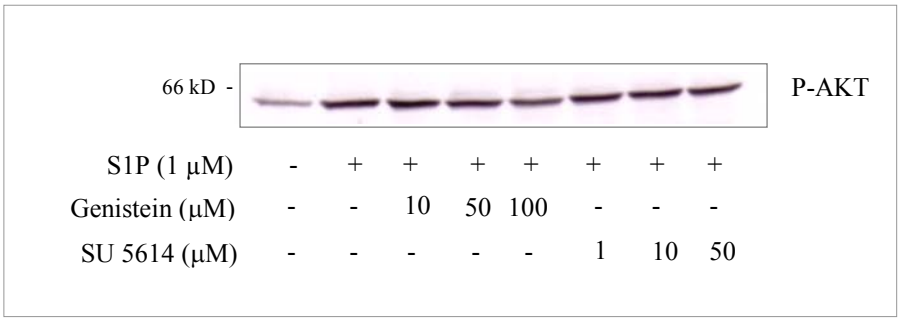

Figure 22 | Effect of tyrosine kinase inhibitors genistein and SU5614 on S1P-induced Akt activation in HUVEC. HUVEC were pretreated with genistein or SU5614 for 30 min, and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated Akt. One of three similar blots is shown.

Next, we evaluated the involvement of receptor tyrosine kinase transactivation in Akt activation by S1P in HUVEC. Pretreatment with SU5614 (1-50 μM, 30 min), an inhibitor of VEGF receptor tyrosine kinase activity, exhibited no effect of S1P-induced Akt phosphorylation. Similarly, pretreatment with inhibitors specific for the PDGF receptor tyrosine kinase, AG1296 (1-25 μM, 30 min), and EGF receptor tyrosine kinase, AG1478 (1-25 μM, 30 min), did not significantly affect Akt activation in response to S1P stimulation in HUVEC (Fig. 23). Based on these observations, the transactivation of VEGF- , PDGF- and EGF-receptor tyrosine kinases appears to be irrelevant for S1P-induced Akt activation in human endothelial cells.

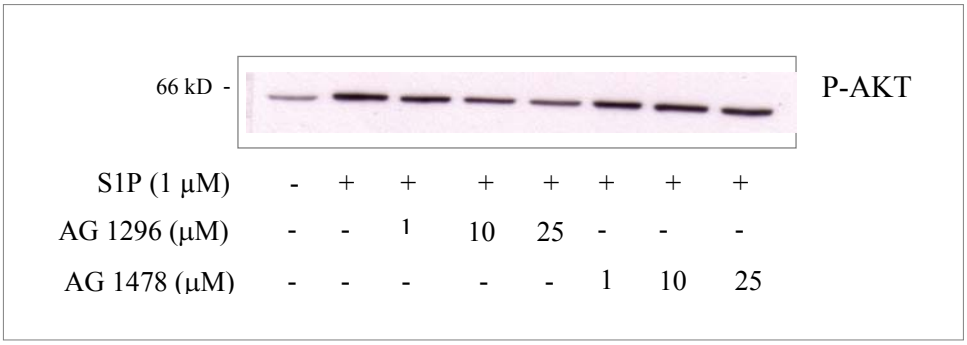

Figure 23 | Effect of receptor tyrosine kinase inhibitors AG1296 and AG1478 on S1P-induced Akt activation in HUVEC. HUVEC were pretreated with AG1296 or AG1478 for 30 min and subsequently stimulated with 1 μM S1P for 5 min. Immunoblotting analysis of cell lysates was performed using specific antibodies against phosphorylated Akt. One of three similar blots is shown.

3.4 S1P-induced Rac-1 GTPase activation in HUVEC

3.4.1 Time and concentration dependence

S1P has been implicated in the regulation of cell motility and cytoskeletal reorganization in several cell types. Members of the Rho family small GTPases, in particular Rac-1, have been demonstrated to be important mediators of actin cytoskeleton activities. Therefore, we investigated the effect of S1P stimulation on endogenous Rac-1 activity in HUVEC. The activation of Rac was measured in a pull-down assay, in which the CRIB (Cdc42- and Rac- interacting binding) domain of p21-activated kinase (PAK) was used to isolate the active, GTP-bound form of

Rac (Bagrodia et al., 1995). Using a GST-PAK fusion protein, the active form of Rac-1 was precipitated from HUVEC following S1P treatment.

Stimulation of HUVEC with S1P (1 μM) for different times (0.5, 1, 2, 5, 10 min) led to a rapid and potent activation of Rac-1 (Fig. 24). Rac-1 was activated 30 sec after S1P stimulation and levels of GTP-bound Rac-1 were sustained for up to 10 min.

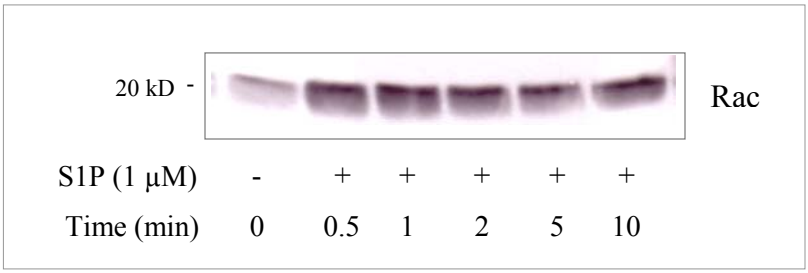

Figure 24 | Time dependent activation of Rac-1 small GTPase in S1P-stimulated HUVEC. HUVEC were stimulated with 1 μM S1P for 0.5, 1, 2, 5, and 10 min. Cells were lysed in the presence of GST-PAK domain and GSH beads were used to pull down GTP-loaded Rac. Immunoblotting analysis of cell lysates was performed using specific antibodies against Rac-1. One of three similar blots is shown.

3.4.2 Effect of PI3K and G-protein inhibitors on Rac-1 activation

The involvement of PI3K in S1P-stimulated Rac-1 activation was examined using two structurally distinct PI3K inhibitors, wortmannin and LY294002. Cells were treated with either wortmannin (100 μM, 30 min) or LY294002 (10 μM, 30 min) prior to S1P stimulation (Fig. 25). We observed that Rac-1 activation by S1P (1 μM, 5 min) was markedly reduced by both wortmannin and LY294002, implicating the involvement of PI3-kinases. Furthermore, S1P-induced activation of Rac-1 appeared to be Gi/o-mediated, as pertussis toxin pretreatment (500 ng/ml, 3 h) completely abrogated Rac-1 activation. Taken together, these findings suggest the involvement of a Gi/o-sensitive PI3-Kinase in the activation of Rac-1 by S1P in HUVEC.

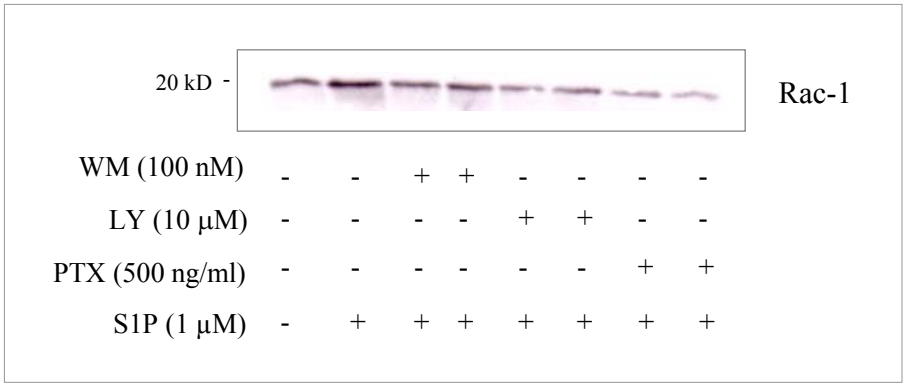

Figure 25 | Effect of PI3K inhibitors (wortmannin and LY294002) and Gi/o-inhibitor pertussis toxin on S1P-induced Rac activation in HUVEC. HUVEC were pretreated with 100 nm wortmannin or 10 μM LY294002 for 30 min or 500 ng/ml pertussis toxin for 3h and subsequently stimulated with 1 μM S1P for 5 min. Cells were lysed in the presence of GST-PAK domain and GSH beads were used to pull down GTP-loaded Rac. Immunoblotting analysis of cell lysates was performed using specific antibodies against Rac-1. One of three similar blots is shown. WM, wortmannin; LY, LY294002; PTX, pertussis toxin.

3.5 S1P-induced HUVEC migration

3.5.1 Time, cell number and concentration dependence

S1P has been demonstrated to both stimulate and inhibit migration, depending on cell type. The effect of S1P on the migration of human endothelial cells was examined. To establish assay conditions, we evaluated the effect of different migration times (2, 4 h), cell numbers (0.25, 0.5, 1.0 x 10^6) and S1P concentrations (0.01, 0.1, 1, 10 μM) on S1P-stimulated HUVEC migration. After 2 h, the migratory response induced by 1 μM S1P was low but increased by 2.5-fold after 4 h (Fig. 26, panel A). Thus, we decided to use 4 h as our standard incubation time.

Next, we evaluated three different cell concentrations: 0.25 x 10^6, 0.5 x 10^6, 1.0 x 10^6/ insert. Upon stimulation with 1 μM S1P, we observed the highest level of HUVEC migration with 0.5 x 10^6 cells/insert (Fig. 26, panel B). Surprisingly, the

levels of migration decreased significantly using 1.0 x 10^6 cells/insert. This reduction was probably due enhanced cell-cell aggregation and hence, reduced adherence to the insert. To avoid effects due to cell aggregation, we decided to use 0.25 x 10^6 cells/insert.

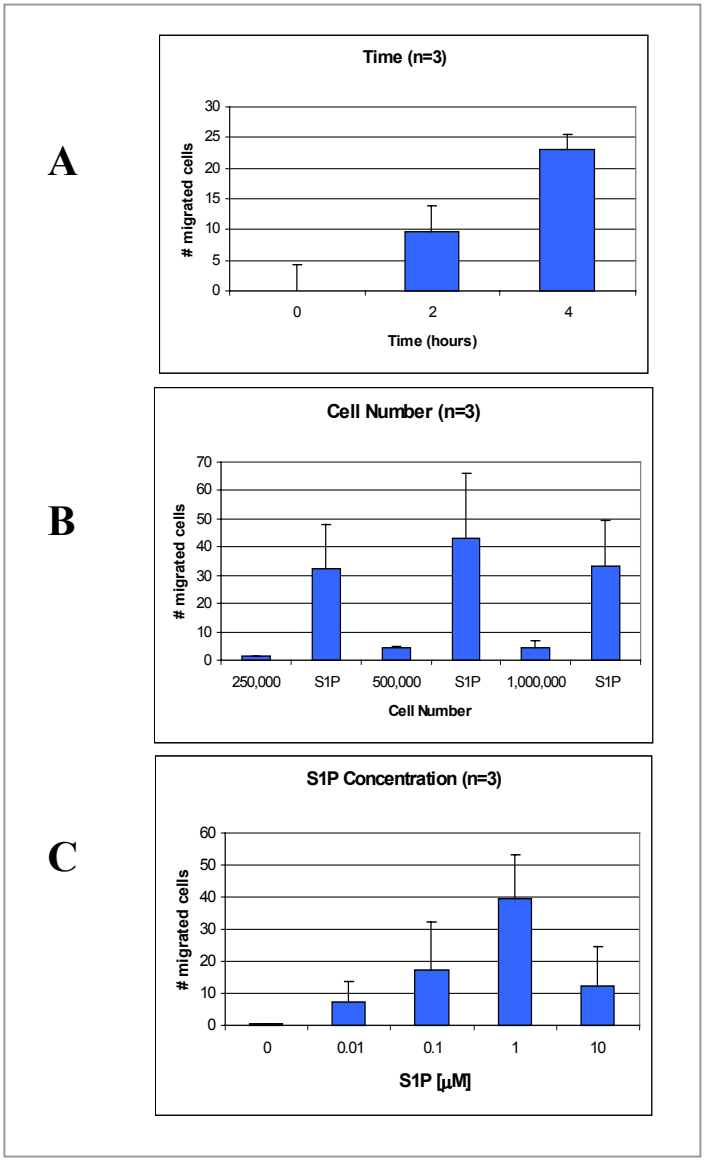

Figure 26 | S1P-induced HUVEC migration. Dependence on (A) time, (B) cell number and (C) S1P concentration.

(A) HUVEC (2.5 x 10^5) were seeded onto tissue culture inserts. S1P (1 μM) was applied to the lower chamber and cells were allowed to migrate for 2 or 4 h.

(B) HUVEC (2.5 x 10^5, 5 x 10^5, 1 x 10^6) were seeded onto tissue culture inserts. S1P (1 μM) was applied to the lower chamber and cells were allowed to migrate for 4 h.

(C) HUVEC (2.5 x 10^5) were seeded onto tissue culture inserts. S1P (0.01, 0.1, 1, 10 μM) was applied to the lower chamber and cells were allowed to migrate for 4 h.

(A-C) Inserts were stained with hematoxylin and the number of migrated cell was counted. Data are given as mean value ± SEM (n=3).

Furthermore, the effect of S1P concentration on HUVEC migration was examined. We tested four different concentrations of S1P: 0.01, 0.1, 1, and 10 μM. As shown in Fig. 26 (panel C), HUVEC migration increased in a concentration- dependent manner, reaching a maximum at 1 μM S1P. In response to stimulation with 10 μM

S1P, HUVEC migration was markedly reduced, by approximately 75%. This is consistent with reports by Wang et al. who show that high concentrations of S1P (1-10 μM) inhibited chemotaxis of both vector and Edg-1 overexpressing breast cancer cells (Wang et al., 1999). These conditions (0.25 x 10^6 cells/insert, 1 μM S1P, 4 h) provided optimal HUVEC migration and were used in subsequent experiments.

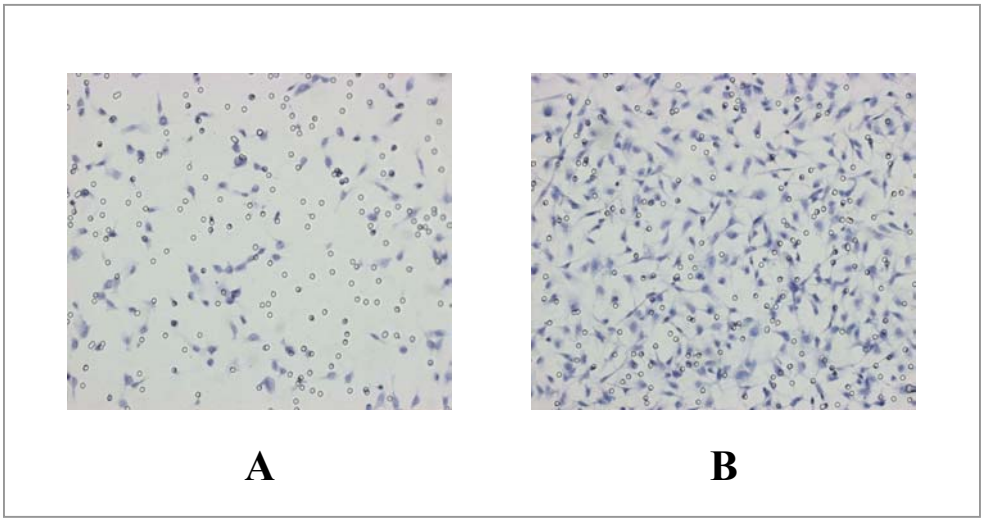

Figure 27 | HUVEC migration (A) control and (B) stimulated with 1 μM S1P. HUVEC were seeded in tissue culture inserts situated in 12-well plates. In B, S1P was applied only to the lower chamber. Cells were allowed to migrate for 4 h. Thereafter, inserts were stained with hematoxylin and migration was visually assessed using microscopy.

3.5.2 Effect of PI3K and G-protein inhibitors on HUVEC migration

Members of the PI3K family of lipid kinases and Gi/o-proteins have been implicated in motility of many different cell types. Therefore, we investigated the effect of PI3K inhibitors and the Gi/o-protein inhibitor pertussis toxin on S1P-stimulated HUVEC migration. Three different strategies were used to administer the inhibitors: (1) inhibitor pre-treatment, (2) no pre-treatment, inhibitors added to the both upper and lower chambers of the migration apparatus and (3) combination of 1 and 2. The addition of inhibitors during the 4 h migration period almost completely abolished S1P stimulated migration (Fig. 28). Similar results were obtained when cells were

pretreated with inhibitors or when pretreatment and addition during assay were combined (data not shown).

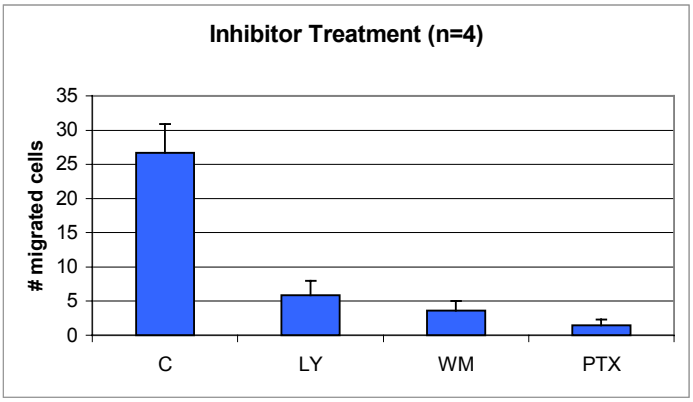

Figure 28 | Effect of PI3K inhibitors (wortmannin and LY294002) and Gi/o-inhibitor pertussis toxin (PTX) on S1P- induced HUVEC migration. HUVEC were seeded in tissue culture inserts situated in 12-well plates. Inhibitors were added to both upper and lower chamber, whereas S1P was applied only to the lower chamber. Cells were allowed to migrate for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cell was counted. Data are given as mean value ± SEM (n=4). C, control; LY, LY294002; WM, wortmannin; PTX, pertussis

3.5.3 Effect of tyrosine kinase inhibitors on HUVEC migration

The involvement of tyrosine kinase activity in S1P-induced HUVEC motility was evaluated using inhibitors of tyrosine kinases. Pretreatment with genistein (50, 100 μM, 30 min) did not significantly affect HUVEC migration, although a slight inhibition was observed with 100 μM genistein (Fig. 29, upper panel). Similarly, an inhibitor specific for the VEGF receptor tyrosine kinase, SU5614 (1, 10 μM, 30 min), did not profoundly affect HUVEC migration (Fig. 29, lower panel). Thus, tyrosine kinase activity and in particular, VEGF receptor transactivation, are dispensable for S1P-induced motility responses in HUVEC.

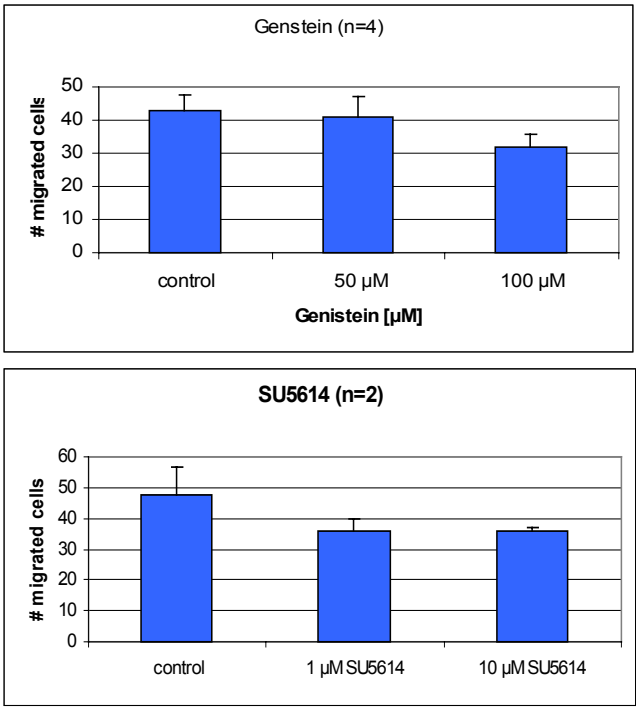

Figure 29 | Effect of tyrosine kinase inhibitor genistein (upper panel) and VEGF receptor tyrosine kinase inhibitor SU5614 (lower panel) on S1P-induced HUVEC. HUVEC were seeded in tissue culture inserts situated in 12-well plates. Inhibitors were added to both upper and lower chamber, whereas S1P was applied only to the lower chamber. Cells were allowed to migrate for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cell was counted. Data are given as mean value ± SEM (n=4 for genistein experiments), (n=2 for SU5614 experiments)

3.5.4 Effect of PI3Kγ KR overexpression on HUVEC migration

PI3Kγ has been shown to be required for the migration of monocytes and neutrophils in response to chemotactic stimuli (Hirsch et al., 2000). The ability of PI3K inhibitors and pertussis toxin to potently block S1P-stimulated HUVEC motility suggests the involvement of a Gi/o-protein coupled PI3K. To directly assess the role of PI3Kγ in HUVEC motility, we overexpressed a catalytically inactive mutant of PI3Kγ, PI3Kγ KR, in HUVEC.

Before evaluating the effects of PI3Kγ KR on HUVEC migration, several important control experiments were performed. The individual effects of the (1) nucleofector

reagent and electroporation process and (2) plasmid DNA on HUVEC migration were evaluated. To ensure that nucleofected cells exhibited the same migratory abilities as non-transfected cells, HUVEC were transfected with a vector encoding for GFP. GFP, isolated from the Pacific jellyfish *Aequoria victoria,* transduces blue chemiluminescence into green fluorescent light by energy transfer. Due to its non-

invasive nature, GFP is frequently used in a wide range of applications where it functions as a reporter of gene expression or as an indicator of protein-protein interactions. Since primary cell transfection is often limited by low transfection efficiency, expression of GFP allowed us to specifically identify and evaluate the activity of the transfected cells, as demonstrated in Fig. 30.

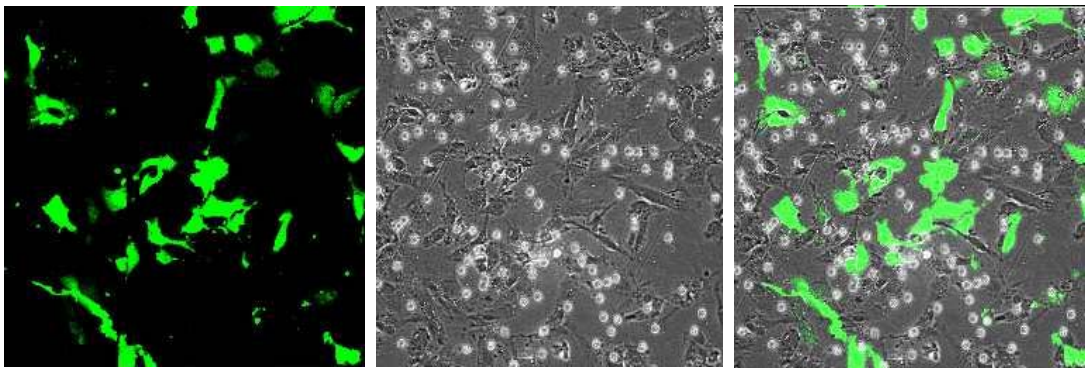

Figure 30 | S1P-induced migration of GFP-transfected HUVEC
HUVEC were transfected with 3 µg GFP plasmid using Amaxa nucleofection. Cells were harvested 24 h post-transfection and seeded in the upper chamber of tissue culture inserts. S1P (1 µM) was applied to the lower chamber and migration was allowed to proceed for 4 h. Thereafter, inserts were stained with directly embedded using Fluoromount and the number of fluorescent migrated cells was visualized using a laser scanning microscope. Shown above are images using fluorescence filter (left), light microscope (center) and overlapping images (right). GFP, green fluorescent protein.

Using a laser scanning microscopy, we quantitated the number of fluorescent GFP-expressing cells in relation to the total number of cells, on both upper and lower sides of the insert. GFP-expressing cells migrated with similar efficiency as “pulsed” control, cells nucleofected without DNA (Fig. 31). Hence, transfected HUVEC retained the ability to migrate in response to S1P stimulation and migration was comparable to normal, untransfected cells.

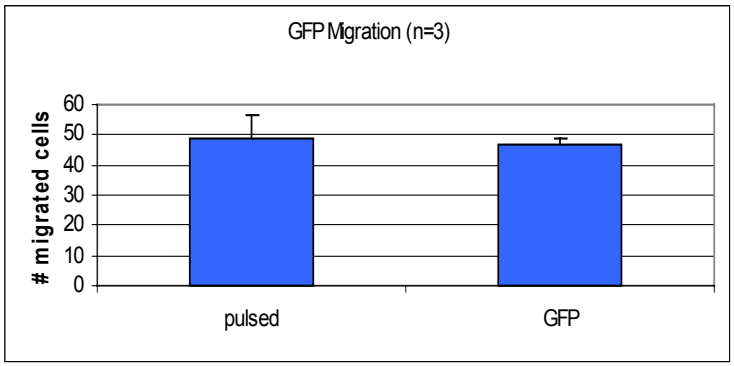

Figure 31 | S1P-induced HUVEC migration of pulsed versus GFP-transfected cells. HUVEC were transfected without DNA 'pulsed' or with GFP. Cells were harvested 24 h post-transfection and cell were seeded in the upper chamber of tissue culture inserts. S1P (1 μM) was applied to the lower chamber and migration was allowed to proceed for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cells was counted. Data are given as mean value ±SEM (n=3). GFP, green fluorescent protein.

The involvement of PI3Kγ in S1P-induced HUVEC migration was examined by overexpressing the kinase inactive form of PI3Kγ, p110γ KR. Strikingly, we observed that S1P-activated migration of HUVEC was significantly impaired (Fig. 32).

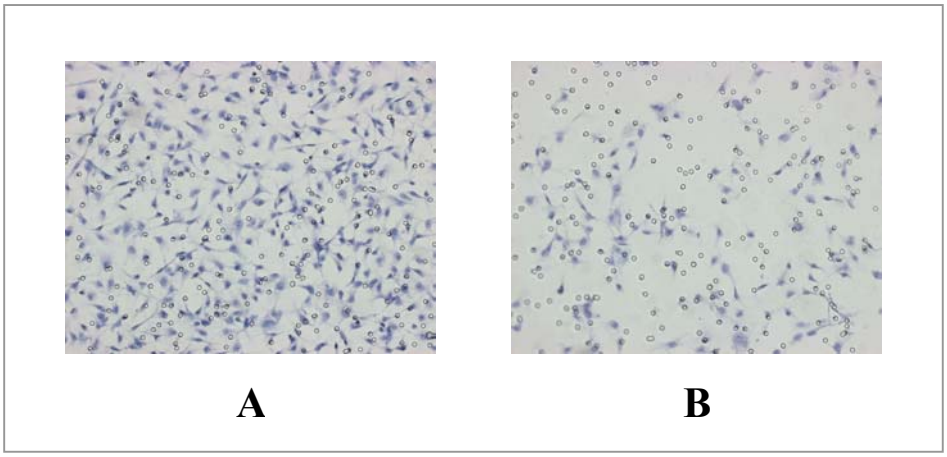

Figure 32 | S1P-induced HUVEC migration (A) pulsed and (B) p110γ KR
HUVEC were transfected without DNA 'pulsed' or with 3 μg p110γ KR. Cells were harvested 24 h post-transfection and seeded in the upper chamber of tissue culture inserts. S1P (1 μM) was applied to the lower chamber and migration was allowed to proceed for 4 h. Thereafter, inserts were stained with hematoxylin and migratory responses were visually assessed by microscopy.

Compared to pulsed control, the overexpression of p110γ KR resulted in a 30% reduction in S1P-induced migration (Fig. 33).

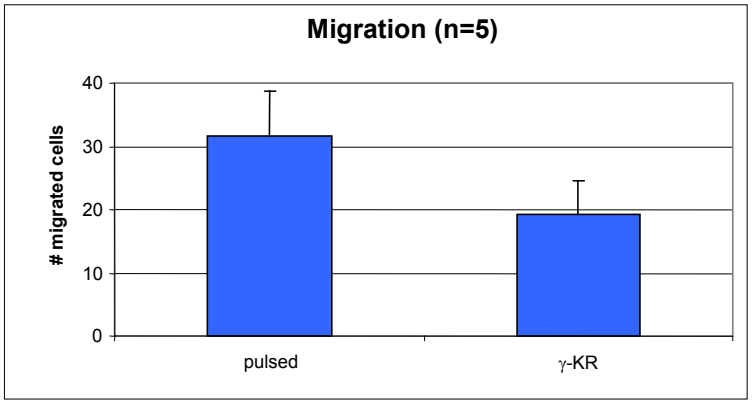

Figure 33 | Effect of p110γ KR overexpression on S1P-induced HUVEC migration. HUVEC were transfected without DNA 'pulsed' or with 3 μg p110γ KR. Cells were harvested 24 h post-transfection and cell were seeded in the upper chamber of tissue culture inserts. S1P (1 μM) was applied to the lower chamber and migration was allowed to proceed for 4 h. Thereafter, inserts were stained with hematoxylin and the number of migrated cells was counted. Data are given as mean value ±SEM (n=5). γ-KR, p110γ KR mutant.

To prove that the decreased migration of the whole cell population was due to reduced response of transfected cells, co-transfection experiments were performed in which HUVEC were transfected with both p110γ KR and GFP constructs. Transfection experiments were carried out as previously described, except that 1.5 μg GFP and 1.5 μg p110γ KR DNA were used. HUVEC transfected with 1.5 μg GFP served as control. Migration was assessed as described previously, except that inserts, without prior fixation, were embedded directly using Fluoromount medium, to allow visualization of green fluorescence. The number of fluorescent cells that have migrated was quantitated using a fluorescent microscope.

In preliminary experiments, control GFP-transfected cells exhibited pronounced migration in response to S1P. Remarkably, migration was completely abolished in cells expressing both GFP and p110γ KR (Fig. 34). Thus, co-transfection of GFP/ p110γ KR demonstrates that migration is abrogated only in those cells expressing

p110γ KR. Taken together, our results provide an additional hint for the involvement of G-protein coupled PI3Kγ in S1P- stimulated migration of HUVEC. However, further study is required to confirm these observations.

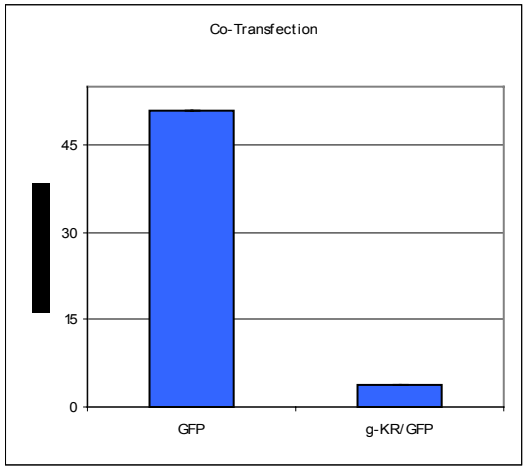

Figure 34 | Co-transfection of p110γ KR (1.5 μg) and GFP (1.5 μg). HUVEC were transfected with 1.5 μg GFP or 1.5 μg GFP/1.5 μg p110γ KR. Cells were harvested 24 h post-transfection and cell were seeded in the upper chamber of tissue culture inserts. S1P (1 μM) was applied to the lower chamber and migration was allowed to proceed for 4 h. Thereafter, inserts were embedded using Fluoromount and the number of fluorescent migrated cells was counted.

4. Discussion

4.1 Expression of PI3Kγ in endothelial cells

4.1.1 Expression of the p110γ catalytic subunit in endothelial cells

Based on initial reports, Gi-coupled PI3Kγ was thought to be primarily expressed in leukocytes. Consequently, investigations of PI3Kγ function have been restricted to haemopoietic cells. PI3Kγ has been shown to play a central role in the migration of macrophages and neutrophils (Hirsch et al., 2000).

Recently, a broader expression profile of PI3Kγ has been postulated. Go et al. suggested a role for PI3Kγ in endothelial cells (Go et al., 1998). Treatment of bovine aortic endothelial cells (BAEC) with the PI3K inhibitor wortmannin abrogated JNK activation in response to shear stress. Furthermore, overexpression of an epitope-tagged form PI3Kγ (vsv-PI3Kγ) led to a shear stress dependent activation of PI3Kγ, whereas overexpression of the kinase inactive PI3Kγ (PI3Kγ KR) inhibited the shear stress-dependent activation of JNK, but not ERK. Despite these findings, the authors failed to convincingly demonstrate endogenous PI3Kγ expression in endothelial cells.

Our study is the first to show endogenous PI3Kγ expression in human endothelial cells. The expression of PI3Kγ was observed at both the RNA and protein level. Compared to human myeloid leukemia cell line U937, PI3Kγ was expressed in endothelial cells at lower levels. PI3Kγ was detected in human umbilical cord vein endothelial cells, fusion cell line EA.hy926, human microvascular endothelial cells, but not in human brain microvascular endothelial cells. The absence of p110γ in the latter, though surprising, could be accounted for by several possible explanations. For example, p110γ might be expressed in HBMEC, but at levels below the limits of detection. Alternatively, p110γ may be of minor importance in functions in brain endothelial cells and perhaps other PI3K isoforms, possibly

PI3Kβ, are sufficient to substitute for p110γ functions in these cells. The immortalization procedure or the extended periods of passaging could have also resulted in the loss of p110γ in HBMEC. Although PCR primers were constructed based on the human sequence, p110γ was nevertheless detected in bovine endothelial cells, providing evidence for significant homology between human and bovine p110γ sequences.

4.1.2 Expression of p101 in endothelial cells

Unlike class IA PI3-kinases, class IB PI3Kγ does not associate with p85 adaptor subunits but instead has been found to bind a non-catalytic p101 subunit. Interestingly, p101 shows no homology to any protein characterized to date. Recent efforts have focused on elucidating the physiological function to p101, although progress has been severely hindered by the lack of p101-specific tools (e.g. antibodies against p101).

Since its discovery, there has been much discrepancy in the literature concerning the physiological function of p101. In initial reports, p101 was termed an 'adaptor' protein required to couple p110γ to Gβγ subunits upon activation of G-protein coupled receptors. A regulatory function was then later assigned to p101; Stephens et al. showed that p101 is essential for Gβγ-induced activation of p110γ lipid kinase activity (Stephens et al., 1997), although this was not confirmed by others (Maier et al., 1999). Instead, Maier et al. proposed that p101 is responsible for the PI(4,5)P_2 substrate selectivity of PI3Kγ, by sensitizing p110γ toward Gβγ subunits in the presence of PI(4,5)P_2. A later report even revealed a novel activity of p101 suggesting that p101 regulated the localization of p110γ within cells (Metjian et al., 1999). On the other hand, studies from our group showed that p101 is required for the PI3Kγ-dependent activation of JNK (Lopez-Ilasaca et al., 1998). In light of recent data, evidence for a physiological role of p101 has been so far inconclusive and requires further investigation.

In this study, the expression of p101 in vascular endothelial cells was evaluated. The absence of p101-specific antibodies prompted the generation of p101 specific

PCR primers. Oligonucleotide primers were designed according to human p101 sequences and employed in reverse-transcriptase-PCR, a highly sensitive method for detecting messenger RNA. Surprisingly, p101 was not detected in endothelial cells, despite expression of p110γ RNA and protein. If p101 were indeed an essential adaptor/regulator of p110γ, as many claim, a coinciding expression pattern p110γ and p101 would be expected. Thus, our results question the current belief that p101 is always required for p110γ and significantly affects p110γ function. Studies by Maier et al. demonstrated that p101 binding protects p110γ from degradation (Maier et al., 1999). According to our findings, p110γ protein appears to be stable in endothelial cells, even in the absence of p101. The lack of p101 expression in endothelial cells also raises the possibility that other adaptors or regulatory proteins may couple to PI3Kγ in endothelial cells.

In conclusion, we report the novel finding that PI3Kγ is expressed in the absence of p101 in human endothelial cells. These results conflict with current data which indicate an integral role for p101 as an adaptor/regulatory protein of p110γ. Our findings suggest that p101 is dispensable for p110γ function in endothelial cells. However, the impact on p110γ function, compared to a situation in which both p110γ and p101 are expressed, awaits further clarification.

4.1.3 Cellular system: Human umbilical vein endothelial cells (HUVEC)

Most studies of endothelial cells have been performed using established cell lines. Many prefer using immortalized endothelial cell lines (EA.hy926, HBMEC, HMEC-1) because they are easier to cultivate and handle. Additionally, cell lines are less resistant to transfection and due to their long lifespan in culture, the establishment of stable transfectants is possible. However, numerous reports show that transformation often results in phenotypic changes, possibly due to altered protein expression and thus, deviates from the physiological situation. We, and others, have observed that HUVEC fusion cell line EA.hy926 cells are less responsive to stimulation as compared to primary HUVEC. Even responses of the spontaneously

transformed HUVEC cell line, ECV 304, have been shown to differ significantly from non-transformed donor cells (Hughes, 1996; Stannard et al., 1997).

Thus, to circumvent problems associated with the immortalization process, primary HUVEC were employed in our investigations. HUVEC retain many characteristics of the *in vivo* endothelium and are widely used to study the molecular mechanisms of the angiogenic process (Kvietys and Granger, 1997). On the other hand, HUVEC culture, as any primary culture, was associated with more complicated growth conditions, limited lifespan, donor variations and resistance to transfection procedures.

4.2 S1P-induced MAP kinase (ERK) activation in HUVEC

S1P has been implicated in the regulation of cell proliferation and survival of diverse cell types but the underlying signaling pathways are not completely understood. Since ERK is a central molecule involved in the transmission of proliferatory signals and, more recently, migratory signals, the effect of S1P on ERK activation in HUVEC was examined.

S1P induced a potent activation of ERK, which occurred in both a time- and concentration- dependent manner. This process appears to be Gi-mediated, as S1P-stimulated ERK phosphorylation in HUVEC was completely attenuated by pertussis toxin. In support of this, Rakhit et al. demonstrated that in airway smooth muscle cells (ASMC), ERK activation by S1P was sensitive to pertussis toxin (Rakhit et al., 1999; Rakhit et al., 2000). Since ASMC do not express Edg-3, these results, as well as our data from endothelial cells, clearly point to the involvement of the Gi-coupled Edg-1 receptor in S1P-induced ERK activation. In addition, we show that S1P-stimulated ERK phosphorylation in HUVEC involves PI3-kinases, based on the inhibitory effect of the general PI3K inhibitor wortmannin. A role for PI3K in ERK activation has also been proposed previously (Varticovski et al., 1994). In particular, PI3Kγ protein kinase activity has been implicated in the regulation of ERK (Bondeva et al., 1998). Furthermore, the activation and phosphorylation of ERK-1 and -2 in PI3Kγ -/- neutrophils treated with fMLP or C5a was severely impaired (Sasaki et al., 2000). The effects of wortmannin and

pertussis toxin in our study, taken together, imply the involvement of a Gi/PI3K-coupled pathway in ERK activation by S1P in HUVEC.

One possible mechanism by which signals emanating from GPCRs are transduced intracellularly is by transactivation of tyrosine kinase receptors. Indeed, S1P has been reported to increase tyrosine phosphorylation of the VEGF receptor Flk/KDR, which is important for eNOS activation in BAEC (Tanimoto et al., 2002). To evaluate the involvement of tyrosine kinases and receptor tyrosine kinases in S1P-mediated ERK activation in our study, specific tyrosine kinase inhibitors were employed. HUVEC pretreatment with general tyrosine kinase inhibitor genistein and specific receptor tyrosine kinase inhibitors against VEGF, PDGF and EGF (SU5614, AG1296, AG1478, respectively) exhibited no effect on S1P-stimulated ERK phosphorylation. Thus, tyrosine kinase activity does not play a significant role in S1P-stimulated phosphorylation of ERK. These findings are inconsistent with reports by Kim et al., in which S1P was shown to transactivate the EGF receptor (Kim et al., 2000). Since this study was performed in Rat-1 fibroblasts, we are unable to exclude the possibility that mechanisms of S1P-mediated ERK activation are cell-type specific. The differential expression of Edg receptors and G proteins could account for the use of different signaling pathways in diverse cell types.

Since both pertussis toxin and PI3K inhibitors reduced S1P-mediated ERK phosphorylation, we specifically addressed the role of PI3Kγ in ERK activation by overexpressing a catalytically inactive form of PI3Kγ. The general difficulty of achieving high level transfection in primary cell cultures is well documented. We evaluated many different methods of transfection, ranging from classical (calcium phosphate, DEAE-Dextran) to more sophisticated liposome-based methods (Fugene, Superfect, Lipofectin, Lipofectamine). With all of these methods, HUVEC transfection was not only unsuccessful, but significant levels of cell death were observed. Thus, of particular interest to us was the AMAXA nucleofection method, a technique based on electroporation, which was reported to be effective in primary cell transfection. With this method, high levels of transfection were achieved in HUVEC, with only minimal cell death.

Using AMAXA nucleofection, the effect of a dominant negative mutant of PI3Kγ (PI3Kγ KR) on S1P-induced ERK activation was evaluated. PI3Kγ KR is a catalytically inactive form of PI3Kγ, in which a lysine-833 residue in the catalytic site was mutated to arginine. We observed that high level expression of p110γ KR had no effect on S1P-induced ERK activation in HUVEC. Surprisingly, the overexpression of p110γ wild-type protein did not enhance ERK activation. This observation is in conflict with earlier data from Cos-7 cells, in which wildtype p110γ overexpression significantly increased ERK phosphorylation (Bondeva et al., 1998; Lopez-Ilasaca et al., 1997), suggesting that cell-specific differences in PI3Kγ-mediated signaling pathways occur. Based on our findings, it seems that S1P-induced ERK activation in endothelial cells is independent of PI3Kγ activity, although our data point to the involvement of both Gi-proteins and PI3K activity in this process.

Interestingly, PI3Kβ has been also shown to be linked to G-protein coupled receptors in addition to its role in classical receptor tyrosine kinase pathways. Activity of p110β has been shown to elevate upon binding to Gβγ subunits (Murga et al., 2000). Moreover, Igarashi et al. demonstrate the involvement of PI3Kβ in S1P signaling in bovine aortic endothelial cells (Igarashi and Michel, 2001). Thus, the possible involvement of PI3Kβ in S1P-mediated ERK activation in endothelial cells requires further clarification.

4.3 S1P-induced Akt activation in HUVEC

In this study, the effect of S1P stimulation on Akt in human endothelial cells was examined. S1P treatment led to a rapid phosphorylation of Akt, which occurred in a time- and concentration- dependent manner. These findings agree with data from Morales-Ruiz et al. who report that S1P stimulated Akt phosphorylation in bovine lung microvascular endothelial cells (BLMVEC), which is important for the activation of endothelial cell nitric oxide synthase (eNOS) and NO production. In the same study, overexpression of the dominant negative form of Akt (AAA-Akt)

abrogated S1P-induced eNOS phosphorylation and nitric oxide production (Morales-Ruiz et al., 2001).

Akt is a critical downstream effector of PI3K. Akt contains a PH-domain, which enables Akt to directly bind to lipid products generated by PI3K activity. This association facilitates the translocation of Akt to the plasma membrane, where it becomes activated by upstream kinases, namely PDK-1. Our study confirms that PI3K inhibitors (wortmannin, LY294002) reduce Akt activation thus underlining that S1P is activating Akt via a mechanism involving PI3K activity. Additionally, an inhibitory effect of pertussis toxin was shown in this study, and by others, suggesting that a Gi/PI3K-coupled pathway is critical for S1P-induced Akt phosphorylation (Igarashi et al., 2001). Inhibitors of tyrosine kinase activity and RTK activity, on the contrary, exhibited no effect, suggesting that receptor transactivation is most probably irrelevant. Thus, our results point to an involvement of a $G\beta\gamma$-activated PI3K in S1P-mediated Akt activation, although it remains to be clarified if PI3Kγ or PI3Kβ play a role.

Akt activation may be involved in S1P-mediated endothelial cell migration. Recently, Akt has been implicated in endothelial motility responses and angiogenesis (Kureishi et al., 2000; Murohara et al., 1998). Overexpression of a constitutively active myristylated form of Akt, Myr-Akt, has been demonstrated to induce angiogenesis in the chorioallantoic membrane (CAM) of the chicken embryo, suggesting that PI3K and Akt signaling is required for normal embryonal angiogenesis (Jiang et al., 2000). Several reports have implicated Akt as a downstream effector of angiogenic growth factors that promote endothelial cell migration. Inhibition of PI3K or Akt blocks endothelial cell migration stimulated by VEGF (Dimmeler et al., 2000; Dimmeler and Zeiher, 2000). Furthermore, overexpression of activation deficient Akt (AA-Akt) markedly attenuated VEGF-induced cytoskeletal rearrangement and cell migration of BLMVEC (Morales-Ruiz et al., 2000).

The mechanism by which Akt activation leads to chemotaxis is not known. In a recent study, Hla et al. demonstrated that Akt directly associates with and

phosphorylates the third intracellular domain of the Edg-1 receptor. This phosphorylation was found to be necessary for Rac activation, cortical actin assembly and cell migration. A targeted mutation of the Akt phosphorylation site of Edg-1 phosphorylated residue (T236A Edg-1) sequestered Akt and impaired S1P-induced Rac activation, cortical actin assembly and subsequent migration. Thus, Akt-mediated S1P receptor phosphorylation appears to play an important role in transducing migratory signals, although pathways activated downstream of Akt-mediated Edg-1 phosphorylation still need to be defined (Lee et al., 2001).

4.4 S1P-induced Rac-1 GTPase activation in HUVEC

The regulated assembly of the actin cytoskeleton in endothelial cells is vital to the angiogenic process and is mediated by low molecular weight GTPases, such as Rac-1. Thus, the involvement of Rac-1 in S1P-stimulated HUVEC was evaluated. Until recently, the activity of Rho family GTPases was evaluated using bacterial toxins that either activate or inactivate Rho GTPases. However, most of these toxins are relatively unspecific and target multiple GTPases. The most specific inhibitor that is widely used is C3 transferase (C3), an enzyme from *Clostridium botulinum* that ADP-ribosylates and inhibits Rho. Other toxins exhibit a wider range of inhibition; Toxin B from *Clostridium difficile* inactivates Rho, Rac and Cdc42, whereas Cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli* activates Rho, Rac and Cdc42.

In this study, a novel strategy was used to directly assess the activation state of Rac-1. Since Rac-1 is activated by GTP exchange, the activation of Rac-1 was assessed by measuring the amount of the active, GTP-bound form of Rac in cells. This was accomplished using a GST-fusion protein containing the Rac-binding domain of PAK kinase, an effector of Rac. Using this method, a rapid and time-dependent activation of Rac-1 was observed in response to S1P treatment. Pretreatment of HUVEC with pertussis toxin completely inhibited S1P-induced Rac-1 activation in our study, suggesting the involvement of the Gi-coupled receptors, most likely Edg-1 and Edg-3. Accordingly, overexpression of Edg-1 and Edg-3 has been shown to stimulate Rac activation in CHO cells (Okamoto et al.,

2000). In Edg-1 null embryonic fibroblasts, S1P stimulation failed to activate Rac or stimulate migration as compared to wild-type cells (Liu et al., 2000). Furthermore, an involvement of Rac in S1P-induced signaling has already been observed in HUVEC, since antisense inhibition of Edg-1 and Edg-3 expression attenuated S1P-induced Rac activation and migration (Paik et al., 2001)

Our results show that both PI3K inhibitors wortmannin and LY294002 attenuated S1P-induced Rac activation. LY294002 appeared to be slightly more effective, which could be explained by the fact that it is more stable than wortmannin in solution (Walker et al., 2000). These findings suggest that PI3K activity is essential for GTP-loading of Rac stimulated by S1P. Our data agree with a previous report from endothelial cells transfected with an epitope-tagged p21 rac. In these cells, Hawkins et al, showed that GTP-loading of Rac is blocked by PI3K inhibitors, suggesting that p21 rac is downstream of PI3K (Hawkins et al., 1995). In addition, studies performed in human neutrophils showed that PI3K is required upstream of Rac in chemoattractant-activated signaling for lamellipodium formation and cell migration (Akasaki et al., 1999; Hooshmand-Rad et al., 1997).

The specific PI3K isoform involved S1P-induced Rac-1 activation in HUVEC remains unknown; however, the results presented in this study suggest a role for a Gi-coupled PI3K. Recently, PI3Kγ has been implicated in the regulation of Rac activity. Ma et al. show that cytoskeletal reorganization induced by fMLP is dependent on PI3Kγ, Rac guanine exchange factor Vav, and Rac. Expression of an inactive form of PI3Kγ blocked Rac-dependent actin rearrangement (Ma et al., 1998). The mechanism is not yet fully understood but recent evidence indicates that the production of $PI(3,4,5)P_3$ in response to PI3K activation leads to the activation of the guanine nucleotide exchange factor Vav (Han et al., 1998) and subsequently, GTP-loading and activation of Rac-1. Probably, similar events occur in endothelial cells, which however, require further examination.

Interestingly, the Edg-5 receptor has been shown to negatively regulate Rac and inhibit cell migration. In CHO cells overexpressing Edg-5, S1P treatment completely abolished membrane ruffling and chemotaxis (Okamoto et al., 2000).

Surprisingly, S1P stimulated PI3K activity in these cells to the same extent as in cells overexpressing Edg-1 but the basal and stimulated activity of Rac was abrogated. It remains to be elucidated, thus, how receptors from a single family with similar signaling pathways elicit seemingly opposite effects on cellular functions and at which point in the pathway these signals diverge.

4.5 S1P-induced HUVEC Migration

Despite inhibiting migration of most cell types, S1P potently stimulated endothelial cell migration. The downstream signaling molecules and pathways activated by S1P, however, are not clearly defined. Our study confirms that S1P activated the migration of endothelial cells in a time- and concentration-dependent manner. We also found that S1P-induced HUVEC migration is significantly reduced by pertussis toxin treatment, which points to the involvement of a Gi-coupled pathway, most likely mediated by Gi-coupled Edg-1 and Edg-3 receptors. Our findings are consistent with previous investigations. For example, Wang et al. demonstrated that S1P stimulated migration of both human and bovine endothelial cells, via a pertussis toxin dependent mechanism (Wang et al., 1999). Furthermore, Lee et al. provided evidence for the involvement of the Gi-coupled phospholipase C (PLC) pathway in S1P-induced endothelial cell motility (Lee et al., 2000), although this was not confirmed by others (Meyer zu Heringdorf et al., 2002).

We observed that S1P-induced HUVEC migration is not significantly affected by tyrosine kinase inhibitors. Pretreatment of HUVEC with the general tyrosine kinase inhibitor genistein and RTK (VEGFR, EGFR, PDGFR) -specific inhibitors did not profoundly affect S1P-stimulated HUVEC migration. Our findings are thus, inconsistent with Vouret-Craviari et al., who demonstrated that S1P-induced cell spreading and migration were dependent on Src tyrosine kinase activity (Vouret-Craviari et al., 2002). The role of Src activity in this study was examined in S1P-induced wound closure of endothelial monolayers using the Src inhibitor PP2. PP2 treatment completely inhibited the extension of membrane structures and cell migration in the presence of S1P, implying that Src is required for the cell movement. In addition, English et al. reported that S1P-induced chemotaxis of

bovine pulmonary artery endothelial cells (BPAEC) is inhibited by tyrosine kinase inhibitors, genistein (50 μM) and herbimycin A (10 μM) and PP2 (10 μM), but not by PI3K inhibitors (English et al., 1999). We were also not able to confirm a mechanism proposed by Endo et al. who showed that S1P migration occurs via a mechanism involving transactivation of the VEGF receptor and CrkII adaptor protein phosphorylation (Endo et al., 2002). CrkII has been demonstrated to bind DOCK180, an SH3 binding protein that activates Rac to induce membrane ruffling (Kiyokawa et al., 1998). In our study, however, the VEGFR tyrosine kinase inhibitor SU5614 had no effect on HUVEC migration in response to S1P. This may be due to fundamental differences in experimental conditions. Generally, data from the literature concerning downstream signals of S1P has been extremely conflicting. Results may differ profoundly depending on endothelial cell type, culture conditions, substances used to coat membranes (gelatin, collagen, fibronectin). In addition, some assay migration of cells in suspension, while others allow cells to first adhere to filter before application of stimulus. Inconsistencies could also arise depending on the method by which migration is assayed, ranging from wound healing assays to classical Boyden chamber studies.

Our study indicates that the activity of PI3K is required for S1P-stimulated HUVEC motility. Both wortmannin and LY294002 dramatically reduced HUVEC chemotaxis activated by S1P. In addition, our results suggest that the PI3K isoform involved in S1P-induced HUVEC migration could be PI3Kγ. Overexpression of a catalytically inactive PI3Kγ KR reduced S1P-induced HUVEC migration. Furthermore, in a co-transfection experiment using GFP as a marker, we demonstrated that migration was abolished in the cells that specifically express the dominant negative form of PI3Kγ, indicating a role for PI3Kγ in endothelial cell motility.

However, additional experiments are required to substantiate these results. Moreover, at present we are unable to exclude the potential contribution of other PI3K isoforms in S1P-induced endothelial migration. Particular emphasis should be given to PI3Kβ, which has been described to play a role in S1P-induced motility in bovine endothelial cells; however, in this study, PI3Kγ was not investigated (Igarashi and Michel, 2001). However, a G-protein coupled PI3Kγ has been

demonstrated to play a crucial role in leukocyte motility in response to chemoattractant stimulation (Hirsch et al., 2000). In order to address these issues, future investigations should focus on targeting endogenous PI3K protein using improved antisense strategies, or the novel RNA interference (RNAi) method.

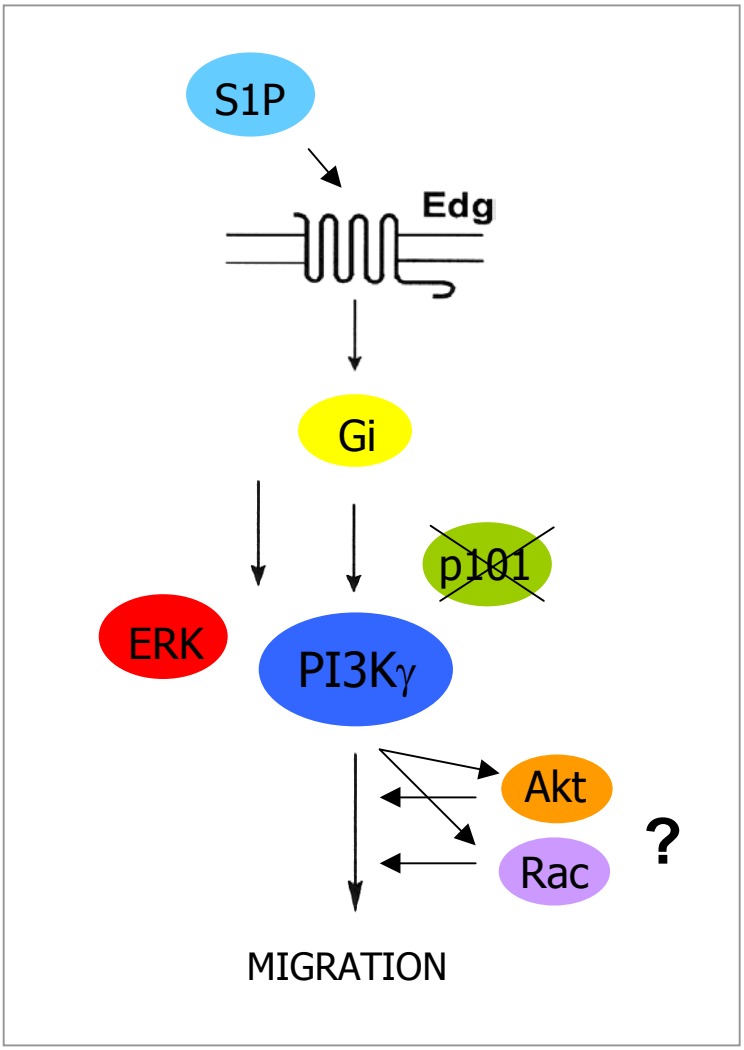

Figure 35 | Proposed role for PI3Kγ in S1P-induced endothelial cell migration. S1P stimulates endothelial cells via binding to a Gi-coupled Edg-receptor. Gßγ subunits of Gi-protein activate endothelial PI3Kγ in an p101-independent manner. PI3Kγ in turn leads to an activation of protein kinase Akt and the small GTPase Rac-1 which both are involved in mediating S1P-induced endothelial migration. S1P also activates ERK in a Gi-dependent but PI3Kγ -independent way. ERK activation does not contribute to S1P-induced migration.

These methods have been proven effective in many cases, however for primary cells, low transfection efficiencies continue to be problematic. Therefore, alternative

methods of transfection, such as adenoviral- and retroviral- based systems, should be evaluated. A more direct approach would be to employ isoform specific PI3K inhibitors, which are currently in development.

In conclusion, our results describe the activation of ERK, Akt and Rac-1, as well as migration of endothelial cells, in respose to S1P treatment. The cellular responses induced by S1P were mediated by Gi-proteins and PI3K, whereas tyrosine kinases were not involved. The results presented in this study point to an important role of a Gi-dependent PI3K in endothelial cell motility and provide compelling evidence for a role of PI3Kγ in these processes. Our current hypothesis is illustrated in Figure 35. S1P-stimulated Rac-1 and Akt are involved in the migratory response, whereas phosphorylation of MAPK seems to play a minor role. In support of this, the MEK-1 inhibitor PD98059 was not able to inhibit S1P-stimulated migration (data not shown). Future studies should aim to confirm involvement of PI3Kγ in S1P-mediated migration of endothelial cells and to identify potential downstream effectors.

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6. Appendix

Abbreviations

A549	Human Lung Carcinoma Cell Line
ASMC	Airway Smooth Muscle Cells
ATM	Ataxia Telangiectesia Mutated
BAEC	Bovine Aortic Endothelial Cells
BCR	Breakpoint Cluster Region
b-FGF	Basic Fibroblast Growth Factor
BLMVEC	Bovine Lung Microvascular Endothelial Cells
BREC	Bovine Microvascular Retinal Endothelial Cells
BSA	Bovine Serum Albumin
C5a	Complement Factor 5a
CAM	Chorioallantoic Membrane
CHO	Chinese Hamster Ovary Cell Line
CNF1	Cytotoxic Necrotizing Factor
COS-7	African Green Monkey Kidney Fibroblast Cell Line
CRIB	Cdc42- and Rac-interacting Binding
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl Sulfoxide
DNA-PK	DNA-dependent protein kinase
EA.hy926	Fusion of HUVEC and A549
EBM	Endothelial Basal Medium
ECGS	Endothelial Cell Growth Supplement
ECM	Extracellular Matrix
EDG	Endothelial Differentiation Gene
EGF	Epidermal Growth Factor
eIF2B	Elongation Initiation Factor 2B
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal Regulated Kinase
FAK	Focal Adhesion Kinase
FH	Forkhead
FMLP	N-formyl-Met-Leu-Phe
GAP	GTPase-activating protein
GDI	Guanine Nucleotide Dissociation Inhibitors
GEF	Guanine Nucleotide Exchange Factors
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophate-Colony Stimulating Factor
GPCR	G-protein Coupled Receptor
GSH	Glutathione Sepharose
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase

HBMEC	Human Brain Microvascular Endothelial Cell Line
HEK-293	Human Embryonic Kidney Fibroblast Cell Line
HMEC	Human Microvascular Endothelial Cell Line
HR	Homology Regions
HUVEC	Human Umbilical Vein Endothelial Cells
IKK	I-kB kinase
IL	Interleukin
IP_3	Inositol Triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
i-SH2	Inter-SH2 Domain
JAK	Janus Activated Kinase
JNK	Jun Kinase
KR	Lysine to Arginine Mutation
L-NAME	N-monomethyl-L-Arginine
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LSM	Laser Scanning Microscopy
LY	LY294002 PI3K Inhibitor
m2	Muscarinic Acetylcholine 2 Receptor
M199	Medium 199
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen Activated Protein Kinase Kinase
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
NF-kB	Nuclear Factor-kB
pAb	Polyclonal Antibody
PAK	p21-activated Kinase
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PDK-1	Phosphoinositide-dependent kinase 1
PET	Polyethylene Terephthalate
PFK-2	Phosphofructo-2-Kinase
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol 3-phosphate
PIC	Protease Inhibitor Cocktail
PIK	Phosphoinositide Kinase Domain
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PTEN	Phosphatase and Tensin Homolog
PTX	Pertussis Toxin
RNAi	RNA Interference
RBD	Ras-binding Domain

RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S1P	Sphingosine 1-Phosphate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SH-2	Src Homology-2
SH-3	Src Homology-3
SOS	Son of Sevenless
SPHK	Sphingosine Kinase
TBS	Tris-Buffered Saline
TGF-α	Transforming Growth Factor-α
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
TOR	Target of Rapamycin
U937	Human Myeloid Leukemia Cell Line
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
WM	Wortmannin
WT	Wildtype

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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Jena, den 30 Juni 2003

Erklärung zur Bewerbung

Hiermit erkläre ich, daß ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad doctor rerum naturalium beworben habe, und daß weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

Jena, den 30 Juni 2003