ERK, AKT AND PKA AS REGULATORY KINASES OF SIK2 IN FGF SIGNALING IN MULLER CELLS

by

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To my family

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ABSTRACT

ERK, AKT AND PKA AS REGULATORY KINASES OF SIK2 IN FGF SIGNALING IN MULLER CELLS

FGF2 dependent retinal Müller cell proliferation is elicited mainly via activation of Ras/MAPK pathway. Phosphorylation/dephosphorylation of intermediates is one of the key mechanisms for the transient and rapid regulation of the pathway. Studies from our laboratory suggest serine/treonine kinase SIK2 is involved in this regulatory process. In this study we focused on defining potential upstream kinases of SIK2 in Müller cells in the context of FGF signaling. Presence of ERK phosphorylation motif on SIK2 led us to explore the possibiliy of SIK2 being a substrate of this dual kinase. Through the use of constitutively active ERK we showed phosphorylation of SIK2 *in vitro*. When the FGF dependent activation of ERK blocked by U0126 prior to growth factor stimulation, decrease in SIK2 activity and in its threonine phosphorylation were evident, while no change was observed in serine phosphorylation. Co-immunoprecipitation studies revealed that the two proteins interact *in vivo* in FGF dependent manner. ERK inhibition also led to a decrease in SIK2 protein levels in our cells. Based on these results we propose that ERK acts as an activatory kinase of SIK2, targeting threonine residues and modulate SIK2 stability. Co-immunoprecipitation of SIK2 with FGFR2 and Gab1 raises the possibility that ERK dependent SIK2 activation downregulates FGF signaling through the kinasing of these proteins.In the second part whether PKA and AKT can regulate SIK2 in Müller cells as reported in other cellular contexts was analyzed. Blocking of AKT activation or inhibition of PKA prior to FGF2 induction resulted in decrease of SIK2 serine phosphorylation and its activation. SIK2 serine phosphorylation increased significantly when PKA activator was used. Co-immunoprecipitation analysis confirmed PKA/SIK2 interaction *in vivo*. The results indicate that AKT and PKA act as inhibitory upstream kinases of SIK2 in FGF2 signaling.

ÖZET

ERK, AKT VE PKA MÜLLER HÜCRELERİNİN FGF SİNYAL İLETİMİNDE SIK2'NİN DÜZENLEYİCİ KİNAZLARIDIR

Retinal Müller hücrelerinde FGF2'ye bağlı proliferasyon Ras/MAPK sinyal yolağı aracılığıyla gerçekleşmektedir. Sinyal aracılarının fosforilasyon/defosfarilasyonları yolağın geçici ve hızlı bir şekilde düzenlenmesinde yer alan önemli mekanizmalardan biridir. Laboratuvarımızda yapılan çalışmalar bir serin/threonin kinaz olan SIK2'nin bu düzenleyici sistemde görev aldığını önermektedir. Bu çalışmadaki amacımız FGF sinyal iletiminde SIK2'nin potansiyel üst kinazlarını belirlemektir. ERK fosforlanma motifini içermesi; bizi SIK2'nin bu ikili kinazın ERK'in substratı olma olasılığını araştırmaya yöneltmiştir. Verilerimiz *in vitro* şartlarda, her daim aktif ERK'in SIK2'yi fosforladığını göstermiştir. FGF'e bağlı olan ERK aktivasyonu U0126 ile engellendiği koşullarda SIK2 treonin fosforlanması ve SIK2 aktivasyonunda düşüş gözlenirken serin fosforlanması değişmemektedir. Eş-immunçökeltme çalışmaları FGF'e bağlı olarak SIK2-ERK'in *in vivo* olarak etkileşim içinde olduğuna işaret etmektedir. Ayrıca ERK inhibisyonu SIK2 protein seviyesinde azalmaya yol açmıştır. Bu sonuçlar ışığında, ERK'in SIK2'yi treonin fosforlayarak onu aktive eden ve kararlığını etkileyen bir kinaz olduğu önerilmektedir. SIK2'nin FGFR2 ve Gab1 ile birlikte immunçökeltme göstermesi SIK2 aktivasyonunun ERK'e bağlı olarak FGF sinyal iletimini olumsuz etkilemekte olduğunu düşündürmektedir. Çalışmanın ikinci kısmında, çeşitli hücrelerde gösterilen SIK2 aktivitesinin AKT ve PKA tarafından düzenlenme olasılığı Müller hücre bağlamında irdelenmiştir. FGF uyarımı öncesinde AKT ve PKA aktivasyonunun engellenmesi SIK2 serin fosforlanma seviyesinde azalmaya yol açmaktadır. PKA aktivatörü kullanıldığında ise SIK2 serin fosforlanması anlamlı bir şekilde artmaktadır. *In vivo* olarak PKA-SIK2 etkileşimi eş-immunçökeltme çalışmalarıyla doğrulanmıştır. Bulgular AKT ve PKA'in SIK2'nin engelleyici kinazları olduğunu göstermiştir.

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LIST OF SYMBOLS

^{o}C	Centigrade degree
V	Volt
v	Volume
w	Weight
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μl	Microliter

LIST OF ACRONYMS/ABBREVIATIONS

8BrcAMP	8 Bromo Cyclicadenosine Mono Phosphate
ACTH	Adenocorticotropic Hormone
AKAP	A-kinase Anchoring Protein
AMP	Adenosine Mono Phosphate
AMPK	AMP-Activated Kinase
APS	Amonium PerSulfate
ATP	Adenosine TriPhosphate
BDNF	Brain-Derived Neurotrophic Factor
bp	Base pair
BSA	Bovine Serum Albumine
$CaCl_2$	Calcium chloride
cAMP	Cyclic Adenosine 5'-Monophosphate
CBB	Coommassie Brilliant Blue
cDNA	Complementary DNA
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COP1	Caspase Recruitment Domain-Containing Protein 1
CRE	c AMP-Responsive Element
CREB	CRE Binding Protein
Co-IP	Co-Immunoprecipitation
CO_2	Carbondioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBC	Extraction Buffer C
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Regulated Kinase
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor

FIRE	FGF Induced Response Element
FBS	Fetal Bovine Serum
Frs2	Fibroblast Growth Factor Receptor Substrate 2
GAB1	Grb2-Associated Binder 1
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor-Bound Protein 2
GST	Glutathione S Transferase
H_2O	Water
HBS	HEPES Balanced Salt
HRP	Horse Radish Peroxidase
HSPG	Heparan Sulfate Proteoglycans
IgG	Immunoglobulin G
INF	Interferon
IP	Immunoprecipitation
IRS	Insulin Receptor Substrate
IVK	*In vitro* kinase
kb	Kilobase
kDa	KiloDalton
KI	Kinase Inactive
LB	Luria Bertani Broth
LKB1	Liver Kinase B 1
mA	Miliamper
MAPK	Mitogen-Activated Protein Kinase
MIO-M1	Moorfields/Institute of Opthalmology-Müller 1
mg	Miligram
$MgCl_2$	Magnesium Chloride
min.	Minutes
ml	Mililiter
mm	Millimeter
mM	Milimolar
mt	Mutant
NaCl	Sodium Chloride
Nm	Nanometer

ng	Nanogram
OD	Optical Density
PAGE	Polyacylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PC12	Pheochromocytoma
Pen/Strep	Penicillin/Streptomycin
PH	Plekstrin Homology
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase C-gamma
PTB	Phosphotyrosine binding
PVDF	Polyvinylidene fluoride
RGC	Retina Ganglion Cells
RNA	Ribonucleic Acid
RPE	Retina Pigmented Epithelium
rpm	Rotations Per Minute
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dedocyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec.	Seconds
Ser	Serine
SH	Src Homology Domain
SHP2	SH2-Domain Containing Phosphatase 2
SIK2	Salt Inducible Kinase 2
SNF	Sucrose Nonfermenting
SNH	Sucrose Nonfermenting Homology
Sos	Son of Sevenless
Srebp	Sterol Regulatory Element Binding Proteins
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween

TEMED	Tetramethylethylenediamine
TGF	Transforming Growth Factor
Thr	Threonine
TORC2	Transducer of Regulated CREB Activity 2
TWEEN	Polysorbate
WB	Western Blot
WT	Wild-Type
x g	Times Gravity

1. INTRODUCTION

1.1. Retina

The vertebrate retina is a light sensitive complex tissue located at the back of the eye. Light from the environment initiates series of electrochemical events, which are processed, integrated and relayed to optic centers in the brain. In embryonic development the retina and optic nerve originate from the neural tube, so it is a part of Central Nervous System (CNS). It is a layered structure (Figure 1.1.) comprised of six neural cell types --ganglion, amacrine, horizontal, bipolar, rod photoreceptor and cone photoreceptor cells (Newman and Reichenbach, 1996).

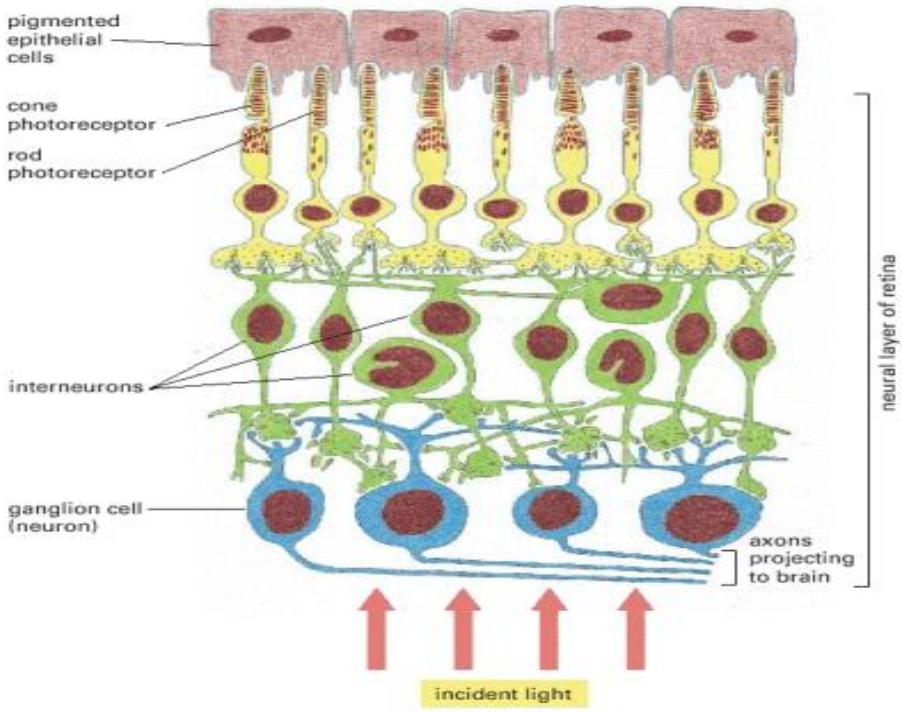

Figure 1.1. Structure of the Retina. The stimulation of the photoreceptors by light is relayed via interneurons to the ganglion cells, which convey the signal to the brain (Alberts *et al.*, 2002).

Mammalian eye contains three types of glial cells. First one is microglial cells; they are blood derived resident immune cells which triggers defense mechanisms such as

inflammatory responses and tissue repair. The other two are macroglial cells; astrocytes which surround the ganglion axons and Müller cells. Müller cells are the principal glial cells of the neural retina which span the entire thickness of retina (Bringmann *et al.*, 2006). They have wealth of ion channels, ligand receptors, membrane transporters and enzymes (Bringmann *et al.*, 2001; Bringmann *et al.*, 2006).

1.2. Protein Kinases

In human genome, it is estimated that there are more than 500 protein kinases (Rubin *et al.*, 2000). Transfer of the gamma–phosphoryl group of adenosine 5'-triphosphate (ATP) to tyrosine (Tyr), serine (Ser) and threonine (Thr) residues to protein substrates is the most important type of posttranslational modification. They have vital role in regulating cellular processes such as metabolism, membrane transport. In addition, the protein kinases participate in signaling cascades that lead to cell proliferation, differentiation and cell survival. For this reason, the kinase activation in wrong time and place may trigger severe results such as cell transformation, cancer, metabolic disorders and developmental abnormalities (Hunter, 2000).

In eukaryotes, protein kinases are thought to have evolved from an ancestral protein. In growth factor signaling, due to kinase specificity, phosphorylation cases can be activatory or inhibitory.

1.3. Serine/ Threonine Kinases

The kinase domains of serine/threonine kinases (STKs) are highly conserved with two distinct sub domains or lobes (Knighton *et al.*, 1991). C terminal lobe is helical; the N terminal lobe is composed of 5 strands of β sheets and one α helix, called helix αC. ATP is bound to near a highly conserved phosphate binding loop (P loop) connecting β1 and β2 that positions ATP phosphates. This P loop is very flexible, inhibitors can cause large distortions and disable phosphate transfer ability of the kinase (Mohammadi *et al.*, 1997). Substrate binding site is in the activation loop, centrally located between the two lobes. In unphosphorylated state, the activation loop collapse into the active site, blocking substrate binding (Hubbard *et al.*, 1994). Upon phosphorylation activation loop moves away from the catalytic center and gains a conformation that allows substrate binding and catalysis.

The number of phosphorylation sites within the activation loop varies from kinase to kinase. For optimal phosphotransfer the precise spatial arrangement of several conserved catalytic residues that are required (Kuriyan *et al.*, 2002).

1.4. AMP-Activated and Related Protein Kinases

AMP-activated protein kinase (AMPK) is a subfamily of STKs with diverse substrates contributing to its wide variety roles in cell metabolism. It is activated by physiological or pathological stimuli that deplete cellular energy levels resulting in an increased AMP: ATP ratio (Hardie, 2003). Besides this, AMPK is regulated by hormones and cytokines that influence whole body metabolism (Kahn *et al.*, 2006). The enzyme is also implicated in the regulation of cell proliferation and establishment of polarity process (Williams *et al.*, 2008).

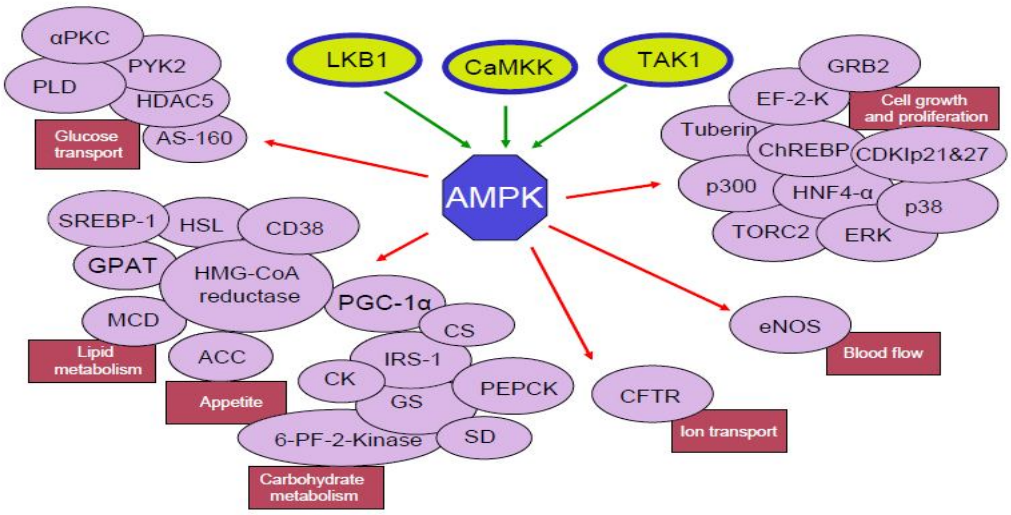

Figure 1.2. Metabolic Pathways and Regulated by AMPK. AMPK directly activated by three kinases, LKB1, TAK1 and CAMKK. When it is activated in various tissues and organs AMPK triggers a variety of cellular and physiological processes by activating cell-specific enzymes (Gruzman *et al.*, 2009).

The enzyme is expressed in all eukaryotic cells as heterotrimeric complex composed of catalytic α and β/γ regulatory subunits. These subunits are encoded by

different genes and several isoforms of each have been discovered: α1, α2, β1, β2, γ1, γ2 and γ3 (Hardie *et al.*, 2003).

The catalytic α subunit includes a classical STK kinase domain close to the N terminal. Free α subunits are usually inactive due to the presence of autoinhibitory domain that is located in the center of this subunit. Carboxyl terminal half is required for β and γ recruitment that elevates autoinhibition.

AMPK activation requires threonine phosphorylation at residue 172 in activation loop by an upstream kinase. To date three AMPK kinases have been identified (Gruzman *et al.*, 2009). First one is LKB1, in a complex with regulatory proteins STRAD and MO25, it activates AMPK (Hawley *et al.*, 2003; Woods *et al.* 2003). In addition to this, Ca^{2+}/calmodulin dependent protein kinase kinase (CaMKKβ), phosphorylate and activate AMPK in response to increase in intracellular Ca^{2+} concentration (Hawley *et al.* 2005; Hurley *et al.*, 2005; Woods *et al.*, 2005). Finally, transforming growth factor-β-activated kinase (TAK1) was implicated in regulation of AMPK activity, but the mechanism is not known (Momcilovic *et al.* 2006; Xie *et al.* 2006).

Recently, 12 AMPK related kinases (BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK2, MARK1, MARK2, MARK3, MARK4 and MELK) were identified by sequence homology to the catalytic domain of AMPK (Bright *et al.*, 2009). They have similar structural organization, including N-terminal catalytic domain, followed by a UBA domain and C terminal spacer with KA1 domain in some cases (Lizcano *et al.*, 2004; Sakamoto *et al.*, 2004). Salt Inducible Kinase 2 **(**SIK2) is a member of AMPK related kinase, but unlike the major AMPK related kinase family (MARK); they are single subunit enzymes including only catalytic subunits and not activated by agonists and muscle contraction (Sakamoto *et al.*, 2004).

1.5. Salt Inducible Kinase Family

1.5.1. SIK1

SIK1 was first isolated from the developing myocardium of mice (Ruiz *et al.*, 1994) and cloned from adrenal glands of high salt diet-fed rats (Wang *et al.*, 1999) and from rat phenochoromacytoma cells (PC12) treated with potassium chloride (Feldman *et al.*, 2000). In mammals SIK1 mRNA is most abundant in the adrenal gland, brain, testes and skeletal muscle with lower expression in adipose, liver, and heart (Horike *et al.*, 2003).

SIK1 expression is increased when Y1 mouse adrenocortical tumor cells treated with adrenocorticotropin hormone (ACTH) via cyclic AMP (cAMP) dependent PKA pathway. SIK1 phosphorylated at Ser-577 by PKA and this phosphorylation leads to its nuclear export (Takemori *et al.*, 2002). The nuclear export of SIK1 allows initiation of CRE-dependent gene transcription (Figure 1.3) including steroidogenic genes (Takemori *et al.*, 2002; Doi *et al.*, 2002).

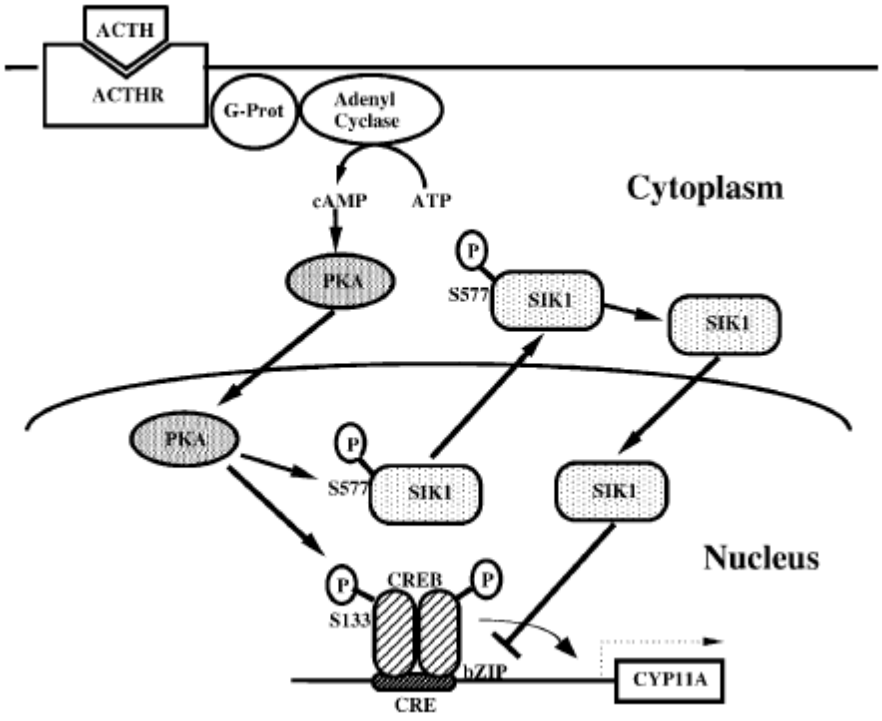

Figure 1.3. Model of SIK1 Induced Steroidogenic Gene Repression. Phosphorylation of SIK1 by PKA plays role in CREB dependent transcription (Okamoto *et al.*, 2004).

LKB1 was shown to phosphorylate SIK1 at Thr-182 residue in the activation loop which allows a 14-3-3 protein binding site and induce structural change that stabilize SIK1 in an active conformation. In addition to this 14-3-3 binding to LKB1 phosphorylate SIK1 on Thr-182 residue triggers SIK1 nuclear export to the cytosol (Lizcano *et al.*, 2004; Al-Hakim *et al.*, 2005).

SIK1 expression is increased four-fold in liver via CREB- Transducer of regulated CREB activity 2 (TORC2) pathway under fasting conditions, it serves as a feedback inhibitor on TORC2 at the end of the fasting period (Koo *et al.*, 2006). In islet cells, SIK1 phosphorylation of TORC2 at Ser-171 residue leads to its nuclear exclusion in hepatocytes; mutation of this residue to alanine makes TORC2:CREB responsive promoters to promote gluconeogenesis (Koo *et al.*, 2006; Shaw *et al.*, 2005).

SIK1 inhibits lipogenesis by phosphorylation of Sterol regulatory element binding proteins (Srebp1-c) on multiple serine residues. In mouse liver, ectopic expression of SIK1 reduce lipogenesis and hepatic triglyceride accumulation (Figure 1.4) (Yoon *et al.*, 2009).

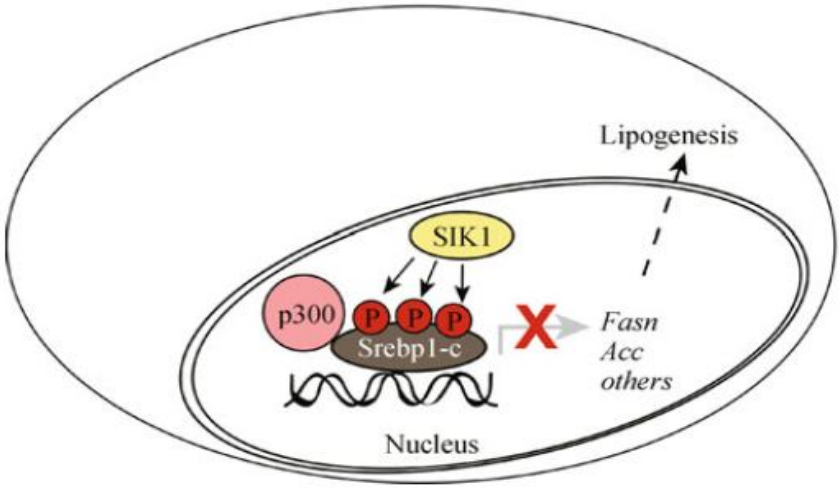

Figure 1.4. Mechanism of SIK1 Inhibition of Hepatic Lipogenesis. SIK1 inhibits lipogenesis via phosphorylation of Srebp1-c in hepatocytes (Berdeaux, 2011).

There are also some non-metabolic functions of SIK1. It participates in survival of neurons that subjected to cerebral ischemia (Cheng *et al.*, 2009; Cheng *et al.*, 2011). In knockdown studies, neuronal cell death is increased after ischemic injury through the

repression of class II HDACs (Cheng *et al.*, 2011). It was shown that SIK1 acts as a tumor suppressor by supporting anoikis (Cheng *et al.*, 2009).

1.5.2. SIK2

SIK2 is a 931 amino acid protein that was first isolated from mouse adipose tissue (Katoh *et al.*, 2004) and proposed to be important at early phase of adipocyte differentiation (Horike *et al.*, 2003). Overexpression of SIK2 in 3T3-L1 cells the forskolin dependent transcriptional activation of CRE-reporter genes are repressed and it seems PKA is critical in the cytoplasmic translocation of SIK2 via S587 phosphorylation (Horike *et al.*, 2003).

The first identified substrate of SIK2 is Insulin Receptor Substrate 1 (IRS1) in adipocytes (Horike *et al.*, 2003). Interaction of IRS1 with insulin receptor (IR) and IRS1 tyrosine phosphorylation triggers the expression genes that are important in glucose uptake and triglyceride synthesis (Gual *et al.*, 2005). SIK2 seems to have a role in negative regulation of this system via phosphorylation of S789 on IRS1, this phosphorylation is known to attenuate the insulin signaling (Gual *et al.*, 2005). Insulin resistant diabetic rats S789 phosphorylation of IRS1 levels are elevated (Qiao *et al.*, 2002) and SIK2 expression in white adipose tissue of diabetic rats is increased (Horike *et al.*, 2003).

SIK2 is one of the key regulators at maintenance of glucose homeostasis. Blood glucose concentration is generally kept within a physiological range by the actions of two counter-regulatory hormones; insulin and glucagon which regulate hepatic gluconeogenesis and peripheral glucose uptake (Moller, 2001). In resting conditions, SIK2 phosphorylates TORC2 (Screaton *et al.*, 2004). This phosphorylation causes nuclear export of TORC2, hampering complex formation with CREB (Figure 1.5a). During fasting conditions PKA phosphorylates SIK2 at S587 that inhibits its activity. SIK2 inhibition triggers dephosphorylation of TORC2, which then translocates to nucleus and forms a ternary complex with CREB/CBP. This complex will promote gluconeogenesis (Dentin *et al.* 2007). During refeeding of mice, insulin stimulates AKT phosphorylation of SIK2 at S358 (Figure 1.5b). This phosphorylation causes SIK2 dependent S171 phosphorylation

and nuclear export of TORC2. Phospho-TORC2 is polyubiquitylated by COP1 E3 ubiquitin ligase complex and undergoes proteosomal degradation (Berdeaux, 2011).

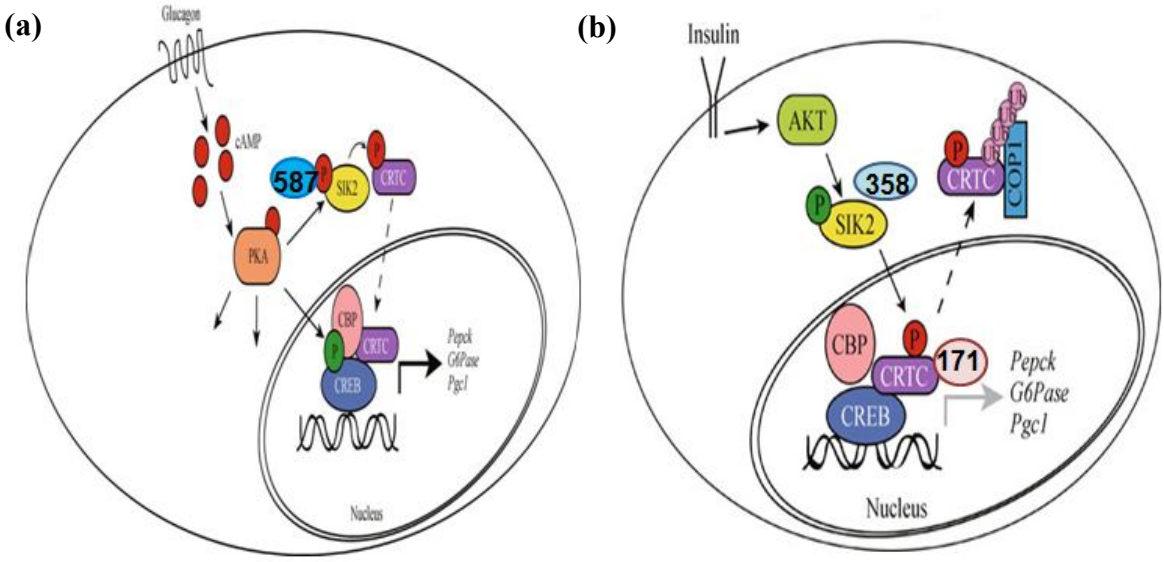

Figure 1.5. SIK2-dependent TORC2 Regulation in Hepatocytes. (a) during resting conditions; SIK2 reduce gluconeogenesis; in fasting PKA prevents SIK2 that gluconeogenesis will re-initiate; (b) during refeeding SIK2 triggers degradation of TORC2 that gluconeogenesis will decrease (Berdeaux, 2011).

Insulin promotes lipogenesis after feeding through a gene expression cascade directed by sterol regulatory element binding proteins (SREBPs) (Raghow *et al.*, 2008). Glucose stimulates activity of the carbohydrate responsive element binding protein (ChREBP), promotes glucose storage by induction of lipogenic genes through interaction with SREBP1-C (Figure 1.6). In response to glucose, p300 acetylates and activates ChREBP. The histone acetyltransferase, p300 has a SIK2 consensus phosphorylation site on (Ser89) was recently shown (Bricampert *et al.*, 2010). This phosphorylation causes inactivation of p300 that reduces lipogenic gene expression downstream of ChREBP (Berdeaux, 2011).

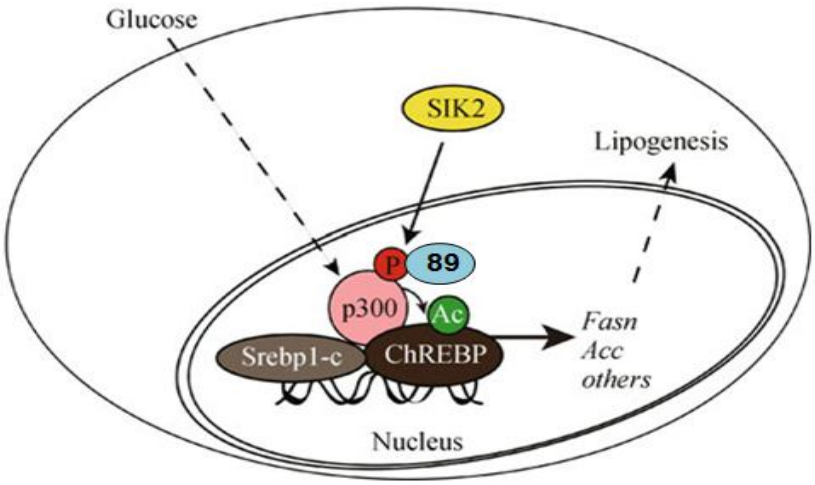

Figure 1.6. Inhibition of Lipogenesis by SIK2. SIK2 phosphorylation of p300 hampers ChREBP acetylation thus prevents its association with the coactivator SREBP leading to reduced lipogenesis (Berdeaux, 2011).

Mouse SIK2 gene is postulated to encode a 103 kDa protein (Figure 1.7), the kinase domain spans amino acid residues 20-271 at the N terminal. K49 residue which located in the ATP binding loop essential for SIK2 autophosphorylation activity, its replacement with methionine results in kinase inactive form of the enzyme. The other known kinase of SIK2 is LKB1; it phosphorylates T172 residue in activation loop that enhances its activity 30 fold *in vitro* (Lizcano *et al*., 2004). UBA domain of SIK2 was suggested to directly interact with the kinase domain to allow SIK2 to gain conformation that can be readily phosphorylated and activated by LKB1: STRAD: MO25 complex (Jaleel *et al*., 2006).

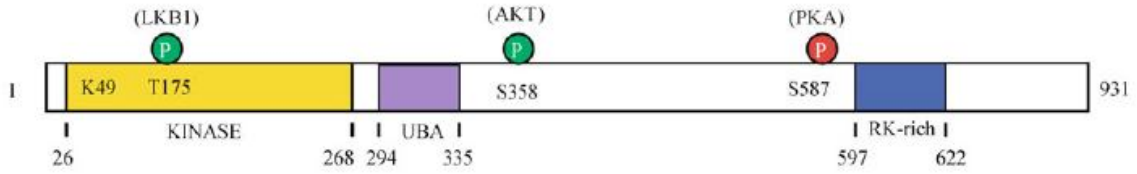

Figure 1.7. Domain Structure of SIK2. The N-terminal kinase domain represented as the yellow box, purple box indicates UBA and RK rich nuclear localization signal is shown in blue. (Berdeaux, 2011).

SIK2 has been suggested to contribute to initiation of centrosome separation in interphase through phosphorylation of centrosome linker protein c-Nap1 (Ahmed *et al.*,

2010). In diffuse large B-cell lymphoma, overexpression and amplification of SIK2 was observed (Nagel *et al.*, 2010).

Structure of rat SIK2 cloned from retinal tissue indicated the presence of 3 alternative splice forms in this tissue (Uysal, 2005; Özcan 2003). RT-PCR results revealed that SIK2 expression are found in all retinal layers (Özcan, 2003; Özmen 2007). Rat SIK2 shows 94% overall identity to mouse and 89.3% to human SIK2. The highest conservation is in the kinase domains reaching 98% and the highest divergence is seen in the C terminal region (Uysal, 2005). SIK2 expression was observed in developing and adult retina (Özcan, 2003; Özmen 2006). Further experiments showed that SIK2 phosphorylation status, activity and cellular localization changed upon FGF2 treatment in Müller cells (Küser, unpublished data; Candas, 2004). Our data indicate that SIK2 activity reaches maxima within 10 min FGF2 induction whereas its activity decrease within 60 min FGF2 induction. Also its threonine phosphorylation is important for its activation (Küser, unpublished data). Two FGF signaling modulators Gab1 and A-Raf1 were shown as SIK2 targets *in vitro* (Kuser, 2006). On going studies in our laboratory indicate an increase in ERK 1/2 phosphorylation levels and proliferation rates in MIO-M1 cells when SIK2 expression is downregulated (Küser, unpublished data).

1.5.3. SIK3

SIK3 has a kinase domain between aminoacids 8-259, a UBA domain between 283-336 residues and finally a phosphorylation domain between 486-518 (Katoh, 2004). It is ubiquitously expressed in human, mice and rats. Knockdown of SIK3 homolog in Drosophila, CG15072, results in mitotic defects such as spindle and chromosome abnormalities (Bettencourt-Dias *et al.*, 2004). A recent paper reported that SIK3 overexpression in ovarian cancer cells and it lends survival advantages to the transformed cells for growth (Charoenfupraset, 2011).

1.6. Fibroblast Growth Factors

The 23 mammalian fibroblast growth factors are grouped into 6 subfamilies based on sequence homology (Ornitz, 2001). The members vary in size from 17 to 34 kDa in vertebrates. They have an internal core domain of 120 aminoacids, where 28 of them are conserved and six are identical (Figure 1.8). The FGF homologous factors (FGF11-FGF14) have high sequence homology with FGF members, but they do not activate FGFRs (Itoh *et al.*, 2004).

FGFs are classified to be paracrine factors and they are generally known for their roles in tissue patterning and organogenesis during development. FGF19, FGF21 and FGF23 subfamily have recently shown that they function in an endocrine manner, dependent on the presence of klotho proteins in their target tissues, regulate cholesterol, glucose, vitamin D and phosphate homeostasis (Fu *et al.*, 2004; Kharitonenekov *et al.*, 2005; Tomlinson *et al.*, 2002).

Several FGF members are expressed during eye development and also in mature retina in vertebrates. FGF3, FGF8 and FGF17 were shown to be present during optic stalk development in several vertebrate models (Crossley and Martin, 1995; Reifers *et al.*, 1998; Vogel-Hopker *et al.*, 2000; Walshe and Mason, 2003). FGF2 initiates neural differentiation in chick retina (Pittack *et al.*, 1997). FGF1 is highly expressed in peripheral retina during the initiation of chick retina development (Xie *et al.*, 1999). It was found that neuronal cells of postnatal and adult retina express FGF1, FGF2 and FGF9 in (Bugra *et al.*, 1993; Buğra and Hicks, 1997; Cinaroglu *et al.*, 2005). In mature chick retina Müller glia respond to FGF2 by proliferation and a subpopulation transdifferentiate into neurons (Fischer *et al.*, 2002). FGF1, FGF2 and FGF9 are mitogenic factors for Müller cells *in vitro* and FGF1 and FGF2 proposed to have roles in the survival of photoreceptors (Cinaroglu *et al.*, 2005; Hicks and Courtois, 1992).

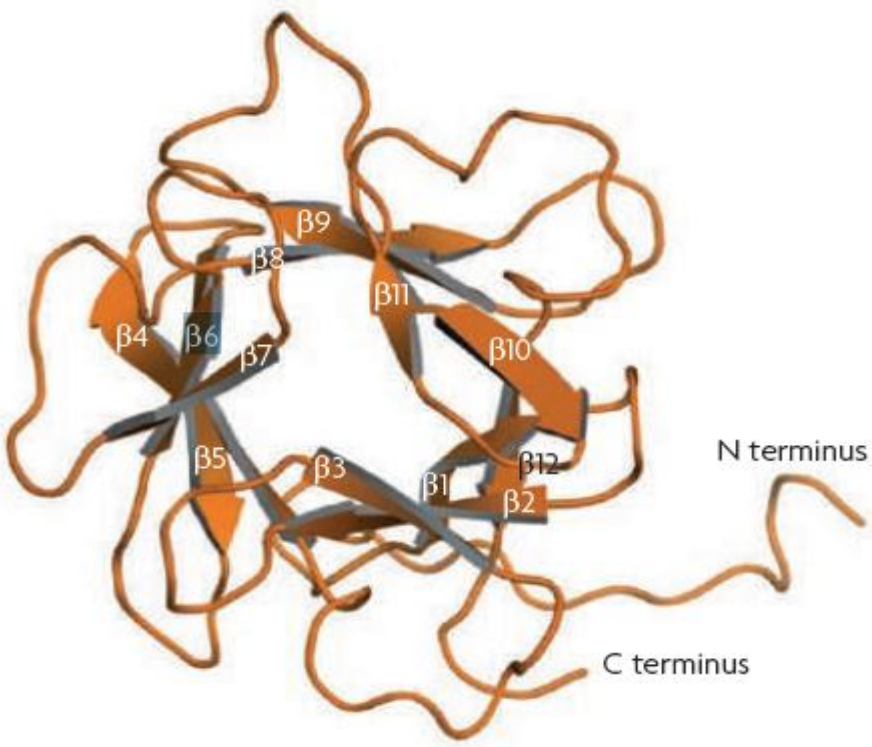

Figure 1.8. The Overall Structure of FGF Polypeptide. FGFs have a homologous core region that spans aminoacids 120-130, ordered into 12 antiparallel β-strands (β1-β12) surrounded by amino and carboxyl termini (Beenken *et al.*, 2009).

1.7. Fibroblast Growth Factor Receptors

FGFs carry out their diverse functions by binding and activating the Fibroblast Growth Factor Receptor (FGFR) family of tyrosine kinase receptors with the help of heparan sulfate proteoglycans (Ornitz, 2000). There are four FGFR genes (FGFR1-FGFR4). FGFRs have three extracellular immunoglobulin like domains (D1-D3), a serine-rich sequence between D1 and D2 named acid box, a single pass transmembrane domain and a cytoplasmic split tyrosine kinase domain (Figure 1.9) (Mohammadi *et al.*, 2005). The D2-D3 domains of FGFR are important for ligand binding and specificity, D1 domain and acid box are implicated in autoinhibition (Wang *et al.*, 1995). Several isoforms of FGFR1-FGFR3 generated by exon skipping involving D1 domain and/or acid box and by alternative splicing in the second half of the D3 domain (Johnson and Williams, 1993).

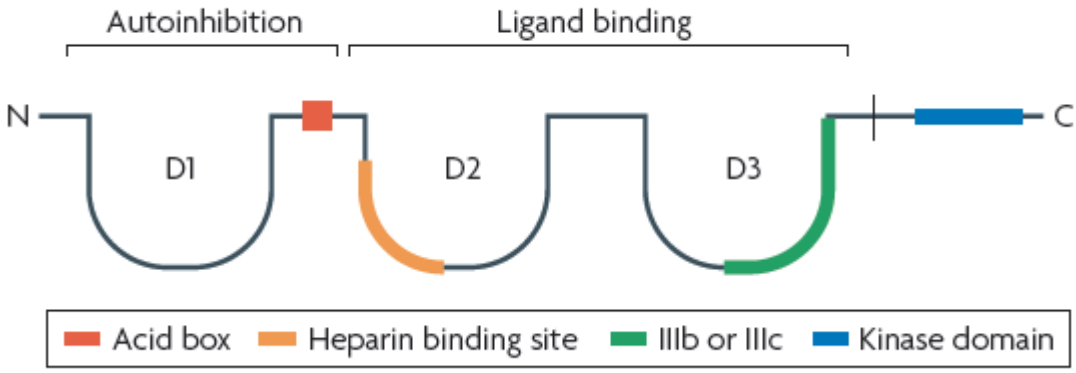

Figure 1.9. Schematic of the FGFR Structure. FGFRs have three extracellular immunoglobulin like domains (D1-D3), an acid box and an intracellular kinase domain (Beenken and Mohammadi, 2010).

In vertebrate retina, all of the FGFR genes are expressed; FGFR1, FGFR2 and FGFR4 are expressed within photoreceptors (Kinkl *et al.*, 2002), FGFR1, FGFR2 and FGFR3 expression has been seen in Müller cells (Cınaroglu *et al.*, 2005). Truncated version of FGFR1 in Xenopus embryo causes 50% loss in photoreceptors and amacrine cells and increase in Müller cells, that refers to a role in determination of FGFR in cell fate (McFarlane *et al.*, 1996). Expression of truncated forms of FGFR1 and FGFR2 in mammalian photoreceptors trigger progressive retinal degeneration (Campochiaro *et al.*, 1996).

1.8. Cell Signaling via FGF Receptors

1.8.1. Receptor Dimerization and Autophosphorylation

FGFs are secreted glycoproteins that are sequestered to the extracellular matrix also by the help of heparan sulphate proteoglycans (HPSGs). FGFs are released from the extracellular matrix by enzymes such as heparinases, proteases or specific FGF binding proteins and the seperated FGFs bind to cell surface HPSGs (Stauber *et al.*, 2000). Cell surface HPSGs stabilize receptor-ligand interaction by forming a complex with FGFR (Zhang *et al.*, 2000; Ornitz *et al.*, 1996).

Upon ligand binding FGFRs dimerize, with the subsequent activatory conformational shift, tyrosine kinase domains and the C-terminal tail are autophosphorylated. These pTyr residues on the receptor function as docking sites for proteins containing SH2 (src homology 2), SH3 (src homology 3) and phosphotyrosine binding (PTB) domains (Kristen *et al.*, 2001).There are six tyrosine phosphorylation residues that are conserved in all FGFR proteins, differential phosphorylation enables the receptor to interact with alternative downstream proteins that leads to activating different signaling pathways (Kristen *et al.*, 2001).

1.8.2. FGF/ FGFR Signal Transduction Pathways

There are three main pathways, which are activated by FGFRs (Figure 10); Phospho lipase C γ (PLC γ) /Ca^{2+}, Phophatidylinositol 3-Kinase (PI3K)/AKT and Ras/Mitogen activated protein kinase (MAPK) pathways (Böttcher and Niehrs, 2003).

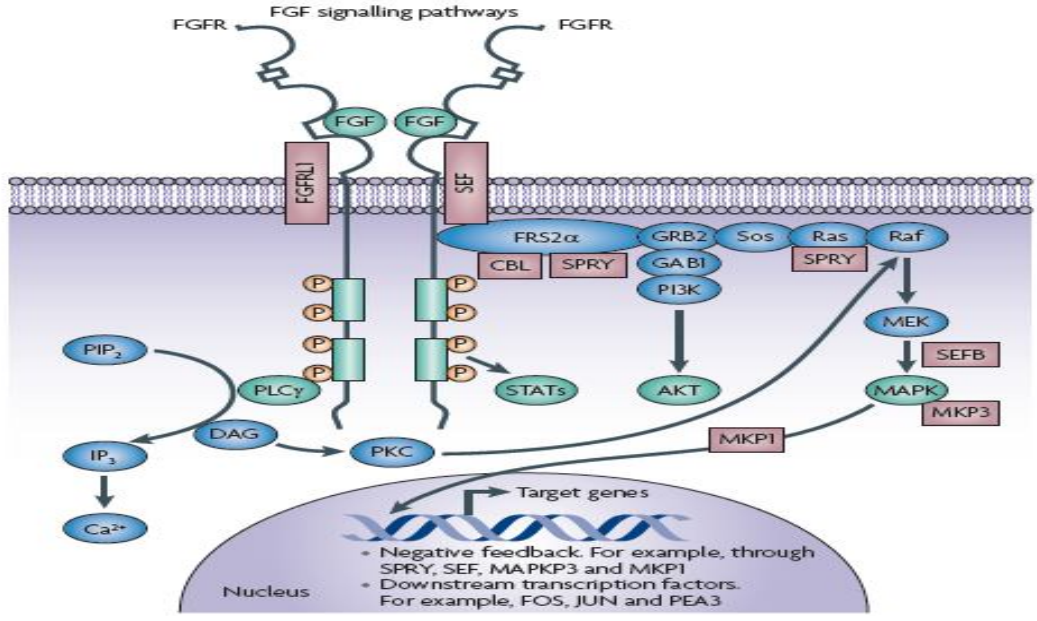

Figure 1.10. The Main Pathways Downstream of FGFR. Ras/MAPK; PI3K/AKT and PLCγ/Ca^{2+} and their elements downstream of FGF-FGFR (Turner *et al.*, 2010).

1.8.2.1. PLCγ/Ca^{2+} Pathway. PLCγ is a target of all FGFRs. The PLCγ/Ca^{2+} pathway involves autophosphorylation of FGFR1 at Tyr-766 creates a binding site for SH2 domain

of PLCγ (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Upon PLCγ activation, phosphatidylinositol-4,5-diphosphate hydrolysis to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG is an activator of protein kinase C (PKC), whereas IP3 stimulates the release of Ca^{+2} from intracellular compartments (Mohammadi *et al.*, 1991). PLCγ is also important for phosphorylation of Raf that activates Ras/MAPK pathway (Huang *et al.*, 1995).

1.8.2.2. PI3K/AKT Pathway. This pathway blocks apoptosis and regulates FGF dependent cell survival (Hawkins *et al.,* 1997). PI3K can be activated via three mechanisms. In the first one, p85 which is the regulatory subunit of PI3K binds to phosphorylated tyrosines. The second one is PI3K catalytic subunit (p110) recruited to the membrane by phosphorylatyed Raf. The third one is; Grb1- associated binder (Gab1) – Fibroblast growth factor receptor substrate 2 (Frs2) – Growth factor receptor-bound protein 2 (Grb2) complex brings PI3K-p85 subunit to the membrane (Kouhara *et al.*, 1997). Activated AKT phosphorylates proapoptotic proteins, Bad and Caspase 9, triggers inactivation (Hadari *et al.*, 2001).

1.8.2.3. Ras/MAPK Pathway. FGF dependent proliferation and differentiation processes are mainly regulated by Ras/ERK pathway. The important modulator of this pathway Frs2, upon interaction with the juxtramembrane region of FGFRs, which is phosphorylated on several tyrosine residues. (Kouhara *et al.*, 1997). Four of these tyrosine residues create docking sites for Grb2-Sos complex and two of them for Shp-2 binding (Kouhara *et al.*, 1997; Hadari *et al.*, 1998; Eswarakumar *et al.*, 2005). Sos, guanine nucleotide exchange factor, recruitment to membrane with interaction with Ras, activates this protein, which in turn activates Raf. Serine-threonine kinase Raf activates MAPK/extracellular signal-regulated kinase (MEK), MEK then phosphorylates MAPK Extracellular Regulated Kinase (ERK), on threonine and tyrosine regions. Activated ERK phosphorylate transcription factors, leading to expression of genes involved in cell proliferation (Yang *et al.*, 2004). p38 and c-Jun N terminal kinase/stress activated protein kinase (JNK/SAPK) are members of the MAPK family. These MAPKs trigger transmitting cytokine production and cytokine mediated cellular proliferation and survival (Allen *et al.*, 2000, Crawley *et al.*, 1997).

1.9. Extracellular Regulated Kinase (ERK)

ERKs are members of MAPK super family that are conserved in all eukaryotes and have been studied extensively in model organisms (Avruch *et al.,* 2007). ERK has functions (Figure 1.11) in diverse cellular processes such as proliferation, cytokinesis, control of transcription, differentiation, senescence, cell death, migration, GAP junction formation, cell adhesion, actin and microtubule networks (Lloyd, 2006). In addition to these, ERK functions in immune system development, and antigen activation, memory formation (Ramos, 2008).

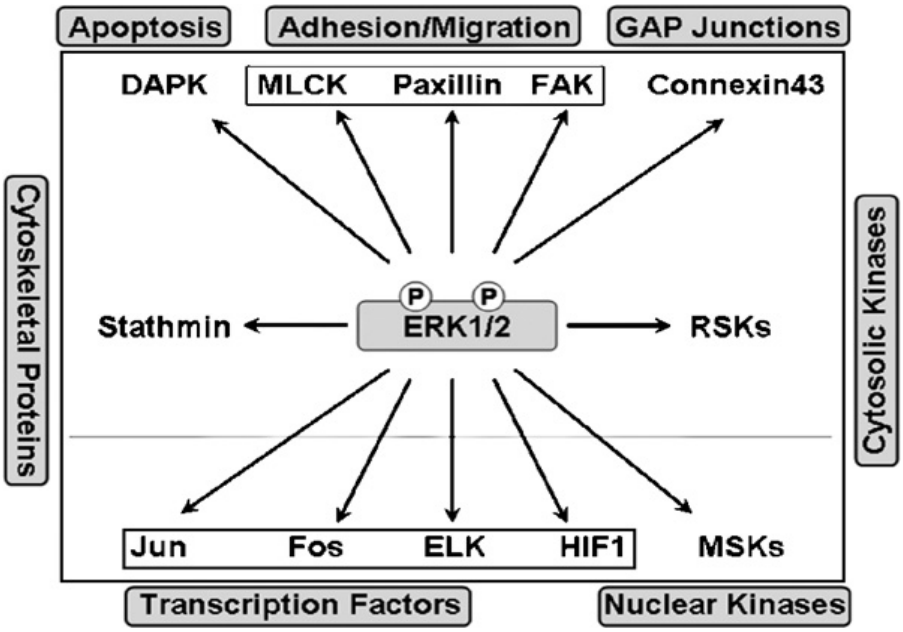

Figure 1.11. The Diversity of ERK1/2 Substrates. ERK has more than 100 known substrates. Examples of these substrates are shown with divergent functions (Ramos, 2008).

Upon activation, ERK can phosphorylate more than 100 substrates with diverse functions (Ramos, 2008). ERKs generally phosphorylate serine/threonine residues followed by a proline (Ser/Thr-Pro). There are two well known docking domains that provide ERK binding to the various substrates (Tanoue *et al.*, 2000; Lee *et al.*, 2004). These are the D-domain that recognize DEJL target motif, and the DEF domain recognize

FxTP motif in the substrates (Biondi *et al.*, 2003; Yoon *et al.*, 2006; Kallunki *et al.*, 1994; Dimitri *et al.*, 2005).

SIK2 has ERK phosphorylation motif (Ser/Thr)Pro residues adjacent to FxFP motif, raising the possibility of threonine 758, 863 and serine 379, 775, 891 residues being targets for this dual kinase. Our data indicate that SIK2 and ERK phosphorylation kinetics are similar in that their activity reaches maxima within 10 min FGF2 induction whereas their activity decrease within 60 min FGF2 induction (Küser, unpublished data). We hypothesize that SIK2 may be activated taking the signal from ERK in response to FGF2 and phosphorylates Gab1 and Raf-1 to decrease their interaction with other signaling intermediates, thereby creating a negative feedback loop.

1.10. AKT

All AKT serine-threonine kinases have a common structure that includes N terminal regulatory domain resembling a PH domain, a region linking the PH domain to the kinase domain (Ahmed *et al.*, 1993), and a C terminal region required for the induction and maintenance of its kinase activity (Chan *et al.*, 1999). In mammals three isoforms of AKT are encoded by distinct genetic loci: AKT (AKT1), AKT2, AKT3. AKT 1 is ubiquitously expressed with the exception of kidney, liver and spleen (Coffer and Woodgett, 1991; Bellacosa *et al.*, 1993). AKT2 expression differs between different tissues with higher expression levels in the muscle, intestinal organs and reproductive organs (Jones *et al.*,1991) AKT3 is expressed in brain and testis at high levels and show low expression in intestinal organs and muscle tissue (Nakatani *et al.*,1999).

AKT was shown to be a target of PI3K and are known to be the key regulators of signal transduction pathways downstream of activated growth factor and cytokine receptors. AKT kinases are critical in various signaling cascades that modulate cell proliferation, survival, cell size and response to glucose metabolism, cell invasiveness, genome stability, angiogenesis (Testa *et al.*, 2005).

AKT 2 is at the critical node in insulin signaling, especially in insulin sensitive organs (Altomore *et al.*, 1998) such as adipocytes, liver and muscle. For instance, it

modulates insulin dependent glucose uptake and glycogen synthesis in skeletal muscle (Sakamoto *et al.*, 2006). It has been suggested that during refeeding insulin activates AKT2 in liver that triggers SIK2 phosphorylation of Ser358 phosphorylation (Dentin *et al.*, 2007). Then SIK2 phosphorylates TORC2 at S171 resulting in its sequesteration in cytoplasm (Figure 1.5) and inhibition of gluconeogenesis (Berdeaux, 2011).

1.11. Protein Kinase A

PKA is an effector of cAMP. PKA has different substrates that varies cell types that affects many processes such as insulin secretion, sperm motility, glycogen and triacylglycerol breakdown, cell survival (Krebs, 1989; Lacroix and Hontela, 2001; Langfort *et al.*, 2003). It also regulates gene expression directly by phosphorylating CREB or indirectly by phosphorylating other proteins that regulate CREB activity (Rosenberg *et al.*, 2002; Screaton *et al.*, 2004).

PKA includes two catalytic subunits and two regulatory subunits (Figure 1.12). The catalytic subunit was defined for the first time based on the conserved structural properties of the protein kinase (Knighton *et al.*, 1991b). The catalytic subunit is globular protein with two lobes; small lobe carries a binding site for ATP. The large lobe acts as a docking scaffold for binding to protein partners that act as substrates or inhibitors (Cheng *et al.*, 2001; Johnson *et al.*, 2001; Knighton *et al.*, 1991a, 1991b). The activation loop generates as a major binding surface for the R subunit (Kim *et al.*, 2005). The regulatory subunit is a modular protein that serves as a receptor for catalytic subunit of PKA in eukaryotic cells. At the N terminus, helical dimerization/docking (D/D) domain localizes, that interacts with scaffold proteins, A-kinase anchoring proteins (AKAPs) (Kinderman *et al.*, 2006; Newlon *et al.*, 2001).

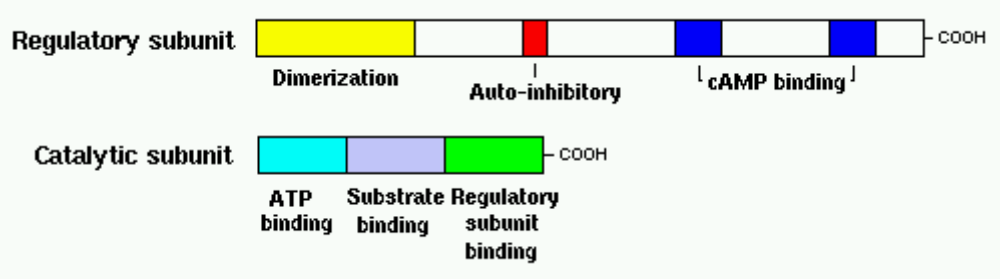

Figure 1.12. Regulatory and Catalytic Subunits of PKA. The catalytic subunit is important for ATP binding; regulatory subunit acts as a receptor for catalytic subunit (Johanson *et al.*, 2001).

Dependent on the cell line and the signal, PKA may have either inhibitory or activatory role in regulating signal transduction. Recent studies suggest that cAMP and PKA has a role in regulation of Ras/ERK pathway (Pursiheimo *et al.*, 2002a). Furthermore, FGF induced FGF inducible Response Element (FIRE) activation requires both Ras/ERK and PKA pathways (Pursiheimo *et al.*, 2002b).

1.12. Müller Cells and Their Roles in Retina

Müller cells constitute 90% of all glial cells in the retina. Their cell bodies are located in inner nuclear layer, their processes spans the entire thickness of the tissue and contact retinal neurons (Reichenbach *et al.*, 1995). They link retinal neurons to retinal blood vessels, vitreous body, subretinal space and facilitate exchange of molecules with these compartments. In the adult Müller cells are involved in maintenance of homeostasis of the retinal extracellular space by balancing K+ ions, pH and by removing neurotransmitters, GABA, glycine and glutamate from the extracellular space (Bringmann *et al.*, 2006).

It is suggested that Müller cells provide trophic factors for neuronal survival (Figure 1.13). Neurotrophic factors secreted from Müller thought to support ganglion cell survival and neurogenesis (Garcia *et al.*, 2002). Müller glia *in vivo* and in culture express Neurotrophic Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT3) and their high-affinity Trk or low-affinity p75 receptors (Chakrabarti *et al.*, 1990; Seki *et al.*, 2005, Taylor *et al.*, 2003, Wahlin *et al.*, 2000 and Oku *et al.*, 2002,

Garcia and Vecino, 2003). FGF2, Ciliary Neurotrophic Factor (CNTF) and Leukemia Inhibitory Factor (LIF) are expressed in these cells (Bugra *et al.* 1997; Gao and Hollyfield, 1992; Cao *et al.*, 1997; Neophytou *et al.*, 1997). These molecules may contribute to different aspects of differentiation, proliferation, neuroprotection and survival of Retinal Gamglion Cells (RGCs) and photopeceptors in the retina (Meyer-Franke *et al.*, 1995, Harada *et al.*, 2000, Peterson *et al.*, 2000, Kawasaki *et al.*, 2000 and Walsh *et al.*, 2001; Bringman *et al.*, 2006).

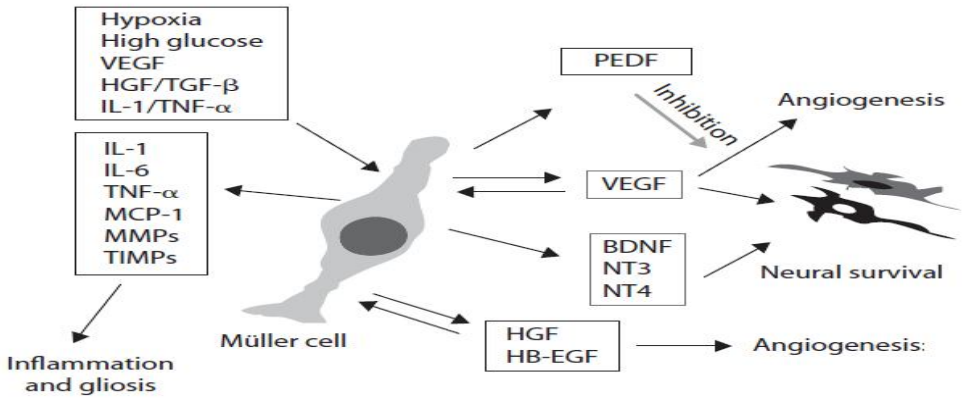

Figure 1.13. Growth Factors Secreted by Müller Cells. Müller glia release pro-angiogenic, anti-angiogenic and neuroprotective factors (Limb and Jayaram, 2010).

Müller cells undergo de-differentiation and abnormal proliferation known as gliosis as a result of retinal injury and diseases such as ischemia, diabetes or glaucoma. The universal early cellular marker for retinal injury is the upregulation of the intermediate filament protein, glial fibrillary acidic protein (GFAP) (Bringmann and Reichenbach, 2001). Early after injury, gliosis thought to be neuroprotective that some neurotrophic factors or antioxidants released (Yasuhara *et al.*, 2004). The de-differentiation of Müller cells contributes to neuronal cell death via impairment of neurotransmitter removal and dysregulation of water and ion homeostasis and decrease in survival factors. Re-entry of Müller cells to proliferation state eventually establish glial scar and lead to retinal detachment (Burke and Smith, 1981).

The molecular mechanism of gliotic response after the diseased states of retina is not fully understood. *In vitro* studies show that FGF1, FGF2, FGF9 evoked signaling elicit the proliferation of immortalized human Müller cell line, MIO-M1 and primary Müller cell cultures (Hollborn *et al.,* 2004, Hicks and Courtois, 1992, Cinaroglu, 2005). Activation of ERK 1/2 and p38 are involved in modulating the mitogenic effect of FGF2 (Hollborn *et al.,* 2004). During experimental detachment, FGF2 is released within minutes from Müller cells, RPE and neurons in the retina, as indicated by the phosphorylation of the FGF receptors, and by activation of ERK1/2 in Müller cells (Geller *et al.*, 2001). This observation suggests FGF2 as one of the signaling molecules that involved in retinal gliosis.

2. PURPOSE

Findings from our laboratory suggest the serine/threonine kinase SIK2, widely expressed in rat retina (Özcan, 2003; Özmen, 2003) and Müller cells (Candas, 2007), takes part in the FGF signaling pathway. Some of the key findings are:

SIK2 has SH2 and SH3 binding motifs that would facilitate interaction with FGFR and a number of downstream proteins, and has serine/threonine phosphorylation sites itself (Uysal, 2005). SIK2 phosphorylates pathway elements Gab1 and Raf1 *in vitro* (Kuser, 2006). SIK2 serine/threonine phosphorylation profiles, activity and cellular localization change in an FGF-dependent manner (Özmen, 2003; Candaş, 2007; Küser, unpublished data). Knock-down of SIK2 expression leads to enhanced FGF-dependent ERK activation and Müller cell proliferation (Küser, unpublished data).

In this work we focused on defining potential upstream kinases of SIK2 in Müller cells in the framework of FGF signaling. PKA and AKT have been shown to act on SIK2 in different cellular contexts (Horike, 2003; Pratsinis and Kletsas, 2007) and here we investigated whether this is the case in our system. Since SIK2 has potential ERK phosphorylation motif (Ser/Thr)Pro adjacent to FxFP motif (DED domain), this dual kinase was also included in the study. Thus, we aimed to investigate;

- Changes in phosphorylation and activation profile of SIK2 upon ERK, AKT inhibition prior to FGF induction; and upon activation as well as inhibition of PKA;

- Whether ERK directly phosphorylate SIK2 *in vitro;*

- Interaction of SIK2-ERK, SIK2-AKT and SIK2-PKA by co-immunoprecipitation.

3. MATERIALS

3.1. Cell Lines

Spontaneously immortalized human Müller glia cell line (MIO-M1) was kindly provided by Prof. Astrid Limb from Moorfields Institute of Ophthalmology, London.

3.2. Chemicals, Plastic and Glass Ware

All solid and liquid chemicals used in this study were purchased from Sigma Aldrich (USA), Merck (Germany) unless stated otherwise in the tables or in the text. Plastic and glassware were sterilized by autoclaving at 121^{o}C for 20 min. before use.

3.3. Buffers and Solutions

3.3.1. Cell Culture

Table 3.1. Cell Culture Materials.

Dulbecco's Modified Eagle Medium (DMEM) GlutamaX	Invitrogen, USA
Complete Medium for MIO-M1	DMEM with Glutamax (Invitrogen, USA) Supplemented with 10% Fetal bovine serum (FBS) 0.1% Penicillin/ Streptomycin (Invitrogen, USA)
FBS	Invitrogen, USA
Freezing Medium	20% FBS 70% DMEM GlutaMAX 10% Dimethyl sulfoxide (DMSO)
0.5% Trypsin-Ethylene diaminetetraacetic acid (EDTA) 1X.	Invitrogen, USA
Penicillin/Streptomycin	Invitrogen, USA
DMSO	
H89 Solution	10 µM H89 in DMEM
8BrcAMP Solution	500 µM 8BrcAMP in DMEM
U0126 Solution	10 µM U0126 (Cell Signaling,USA) in DMEM
LY294002 Solution	50 µM LY294002 (Cell Signaling,USA) in DMEM
Fibroblast Growth Factor 2 (FGF2) Medium	DMEM supplemented with 1 ng/ml FGF2 and 10 µg/ml heparin

3.3.2. Transfection

Table 3.2. Transfection Materials.

2X Hepes Buffered Saline (HBS) Buffer	1.6 g NaCl 0.074 g KCl 0.027 g Na_2HPO_4 0.2g Glucose 5 ml 1 M HEPES Make up to 100 ml with dd H_2O Adjust pH 7.05
Chloroquine	25 µM in dd H_2O
$CaCl_2$	2 M in dd H_2O
Luria Bertani (LB) medium (1 L)	10 g Tryptone 5 g Yeast Extract 5 g NaCl

3.3.3. Protein Isolation

Table 3.3. Protein Isolation Materials.

Protease Inhibitor Cocktail	10 µg/ml leupeptin, 5µg/ml aprotinin (Roche, Germany)
Phosphatase Inhibitor Cocktail	1 mM Na_3VO_4, 1 mM Phenylmethylsulphonylfluoride (PMSF), 20 mM NaF (Roche,Germany)

3.3.4. Western Blot

Table 3.4. Western Blot (WB) Materials.

Acrylamide:Bisacrylamide (37.5:1)	68.1 g acrylamide 1.8 g N`N`-bis-methylene- acrylamide to 100 ml with dd H_2O
Ammonium Persulfate (APS)	10% (w/v) in dd H_2O
Sodium dodecyl sulfate (SDS)	20% (w/v) in dd H_2O
N,N,N,N ,-tetramethylethylenediamine (TEMED)	
Blocking Solutions For; Salt Inducible Kinase 2 (SIK2) phospho AKT, pan AKT	 5% non-fat milk powder with 1% BSA in tris buffered saline tween (TBST) 5% non-fat milk powder in TBST
phospho Extracellular Regulated Kinase (pERK), ERK, Glutathione S Transferase (GST), B actin, Grb2 Associated Binding protein 1 (Gab1), Fibroblast Growth Factor 2 (FGFR2) pThreonine, pSerine	 1% non-fat milk powder in TBST 3% bovine serum albumin in TBST
Coomassie Blue Destaining Solution:	37.5 ml Acetic acid 25 ml Ethanol 100% 437 ml dd H2O

Table 3.4. Western Blot (WB) Materials (continued).

Ponceau Staining Solution	1 g Ponceau (AppliChem, Germany) 50 ml acetic acid Make up to 1000 ml with ddH_2O
Protein Marker	PageRuler^{TM} Plus Prestained Protein Ladder Fermentas (10-170 kDa)
Polyviniyl difluoride (PVDF)	Roche,Germany
Resolving Gel (10%)	10% acryalmide:bisacrylamide (37.5:1) 1.5 M Tris.HCl pH 8.8 0.1% SDS 0.1% APS 0.1 % TEMED
Stacking Gel (5%)	5% acrylamide: bisacrylamide 37.5:1 1 M Tris-HCl (pH 6.8) 0.1 % SDS 0.1% APS 0.1 % TEMED
6x Sample Buffer	1.2 g SDS 0.9 g Dithiothreitol (DTT) 6 mg bromophenol blue 4.7 ml glycerol 1.2 ml Tris 0.5 M pH 6.8 2.1 ml dd H_2O

Table 3.4. Western Blot Materials (continued).

10X Transfer Buffer	72 g Glycine 15,15 g Tris-Base 500 ml dd H_2O
1X Transfer Buffer	70 ml 10X Transfer Buffer 105 ml Methanol (Sigma, USA) 525 ml dd H_2O
5X Tris-Glycine Buffer	7,55 g Tris-Base 47 g Glycine 50 ml 10% SDS 450 ml dd H_2O
10X TBS	24,2 g. Tris-Base 87,66 g. NaCl 1000 ml dd H_2O (pH 8.0
1X TBST	50 ml 10X TBS 450 ml dd H_2O 500 µl Tween 20
Stripping Solution	62.5 mM Tris-HCl, pH 6.8 2% SDS 0.7% β-Mercaptoethanol
Western Blot Luminol Reagent	Santa Cruz

3.3.5. Immunoprecipitation and Co-Immunoprecipitation

Table 3.5. Immunoprecipitation and Co- Immunoprecipitation Materials.

Lysis Buffer 1	1 M Tris, pH 8 7,31 g. NaCl 0.5 M EDTA, pH 8 10% NP40 445 ml dd H_2O
Lysis Buffer 2	20 mM Tris-Cl (pH: 7.5) 150 mM NaCl 1 mM EDTA 1% Triton 2,5 mM Na pyrophosphate before use 1 tablet protease inhibitor cocktail+ phosphatase inhibitor cocktail (Roche) was added
Cell Lysis Buffer	1 M Tris, pH 8 7,31 g. NaCl 0.5 M EDTA, pH 8 10% NP40 445 ml dd H_2O before use 1 tablet protease inhibitor and phosphatase inhibitor cocktail was added
Extraction Buffer with Chaps (EBC)	20mM Tris-Cl; pH: 8, 125 mM NaCl 2 mM EDTA before use 1 tablet protease inhibitor cocktail+ phosphatase inhibitor cocktail (Roche) was added

3.3.6. In vitro Kinase

Table 3.6. *In vitro* Kinase Materials.

Adenosine Tri Phosphate (ATP) cocktail	100 μM cold ATP 1 μCi [γ32P]ATP (3000 Ci/mmol) (Institute of Isotopes Co.Ltd,Hungary)
Kinase Buffer	50 mM Tris-Cl, pH 7.4, 1 mM DTT 10 mM $MgCl_2$ 10 mM $MnCl_2$

3.4. Fine Chemicals

3.4.1. Antibodies

Table 3.7. Antibodies Used in Western Blot, Immunoprecipitation and Co-immunoprecipitation.

Antibody	**Source**	**Company**	**Dilution Used**	**Usage**
Anti- GFP	Rabbit	Abcam, UK	1:250	IP
Anti-Beta actin, Horseradish Peroxidase (HRP) conjugated	Mouse	Santa Cruz	1:2000	WB
Anti-rabbit Immunoglobulin G (IgG)	Goat	Santa Cruz	1:5000	WB
Anti-mouse Immunoglobulin G (IgG)	Goat	Santa Cruz	1:5000	WB
Anti-SIK2	Rabbit	Novus	1:1000	WB
Anti-SIK2	Rabbit	Novus	1:250	IP, Co-IP
Anti pAKT	Rabbit	Cell Signaling	1:2000	WB
Anti panAKT	Rabbit	Cell Signaling	1:1000	WB

Table 3.7. Antibodies Used in Western Blot, Immunoprecipitation and Co-immunoprecipitation (continued).

Anti pERK	Mouse	Santa Cruz	1:1000	WB
Anti ERK	Rabbit	Santa Cruz	1:1000	WB
Anti GST	Mouse	Santa Cruz	1:1000	WB
Anti phosphoThreonine (pThr)	Rabbit	Invitrogen	1:1000	WB
Anti phosphoSerine (pSer)	Rabbit	Invitrogen	1:1000	WB
Anti Gab1	Rabbit	Santa Cruz	1:1000	WB
Anti FGFR2	Rabbit	Santa Cruz	1:1000	WB

3.4.2. Plasmids

Table 3.8. Plasmids.

pEGFP C3 SIK2 KI (K49M)	Retina Lab (Gamze Küser)
pEGFP C3 SIK2 Wild Type	Retina Lab. (Gamze Küser)

3.5. Kits

Table 3.9. Kits

Plasmid Maxiprep	HiPure Plasmid Filter (Invitrogen, USA)
BCA assay kit	Pierce, Thermo (USA

3.6. Equipment

Table 3.10. Equipment Used in This Thesis.

Equipments	Models
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Electronic Balance VA 124, Gec Avery,USA DTBH 210, Sartorius, GERMANY
Carbon dioxide tank	2091, Habaş, TURKEY
CCD camera	CCD Camera, JAI Corporation, JAPAN
Centrifuges	ProFuge, 10K, Strategene, USA Mini Centrifuge 17307-05, Cole Parmer, USA Genofuge 16M, Techne, UK Centurion K40R, UK Centrifuge B5,B.Braun B. Int. GERMANY Centrifuge 5415R, Microfuge tube, USA J2-MC Centrifuge, Beckman Coulter, USA J2-21 Centrifuge, Beckman Coulter, USA
Cold room	Birikim Elektrik Soğutma, TURKEY
Deep Freezers	2021D (-20^{0}C), Arçelik, TURKEY -70^{o}C Freezer, Harris, UK -86^{o}C ULT Freezer, ThermoForma, USA
Documentation System	Gel Doc XR System, Bio Doc, ITALY
Electrophoretic Equipments	Mini-PROTEAN 3 Cell, BIO-RAD, USA Easi-cast system, Hybaid, UK
Heat blocks	DRI-Block DB-2A, Techne, UK StableTemp Dry Bath Incubator, Cole Parmer,USA
Hemocytometer	Improved Neubauer, Weber Scientific International LTD, UK

Table 3.10. Equipment Used in This Thesis (continued).

Equipments	**Models**
Homogenizer	Pellet Pestles Tissue Grinder, Kimble Kontes USA
Hybridization Oven	Shake'n'Stack, Hybaid, UK
Ice Machine	Scotsman Inc., AF20, ITALY
Incubator	Hepa Class II Forma Series, Thermo Electron
Laminar flow cabinet	Class II A Tezsan, TURKEY Class II B Tezsan, TURKEY
Magnetic Stirrer	M221 Elekro-mag, TURKEY Clifton Hotplate Magnetic Stirrer, HS31, UK
Micropipettes	Gilson, FRANCE
Microscopes	CM110 Inverted Microscope, Prior, UK Zeiss, Axio Observer Z1 Inverted Mic., USA
pH meter	WTW, GERMANY
Pipettor	Pipetus-akku, Hirscmann Labogerate, GERMANY

Table 3.10. Equipment Used in This Thesis (continued).

Equipments	**Models**
Power Supplies	EC135-90, Thermo Electron Corporation Power Pac Universal, BIO-RAD, USA
Protein Visualization	Stella, Raytest, Germany
Refrigerators	2082C, Arçelik, TURKEY 4030T, Arçelik, TURKEY
Sealer	Vacuplus FS400A, Electric Petra, GERMANY
Shakers	VIB Orbital Shaker, InterMed, DENMARK Lab-Line Universal Oscillating Shaker, USA Adjustable Rocker, Cole Parmer, USA
Software	Metasystems, GERMANY Quantity One, Bio-Rad, ITALY
Spectrophotometer	CE5502, Cecil, UK NanoDrop ND-1000, Thermo, USA
Vortex	Vortexmixer VM20,Chiltern Scientific, UK
Water Bath	TE-10A, Techne, UK
Water Purification	UTES, TURKEY
X-Ray Film Cassettes	24X30 IMS, ITALY 24X 30 DIA-X, GERMANY

4. METHODS

4.1. Cell Culture

4.1.1. Maintenance of MIO-M1 Cells

Spontaneously immortalized MIO-M1 Müller glia cells were maintained in DMEM with glutamine supplemented with 10% FBS and 0.1% penicillin/streptomycin. When the plates reached 80% confluence, the cells were washed with PBS, treated with 0.05 trypsin solution for 5 min. and scraped. The cells pelleted by centrifugation at 2000 x g for 5 min. and after resuspension in complete medium they were divided into three or five plates twice a week.

4.1.2. Treatment of MIO-M1 Cells

Cells were seeded 10 cm tissue culture dishes and were allowed to grow to subconfluency. The cells were washed with PBS and starved in DMEM and 0.1% penicillin/streptomycin overnight. Subsequently they were treated with 1 ng/ml FGF2 and 10 μg/ml heparin for 0 min., 10, 60 min., they were immediately washed with ice cold PBS with protease and phosphatase inhibitor cocktails. The cells were then scraped and collected into microfuge tubes, pelleted with centrifugation and stored at -70^{o}C until used. In the studies involving ERK inhibition, AKT inhibition, PKA activation/inhibition, the cells were incubated with U0126, LY294002, 8BrcAMP or H89 respectively for 30 min. prior to FGF2 induction. The final concentration of U0126, H89 was 10 μM; LY294002, 50 μM; 8BrcAMP, 500 μM.

4.2. Bicinchoninic Acid Assay

For determination the concentration of proteins BCA Assay kit was used. Unknowns and BSA dilutions ranging from 0.025 to 2 mg/ml were mixed with 170 μl of 50:1 diluted

BCA Working Solution. After 30 min. at 37^{o}C incubation absorbance measured at 595 nm. Unknown sample concentrations were extrapolated from the standard curve.

4.3. SIK2 Immunoprecipitation

The cells grown to 80% confluence on 10 cm^2 cell culture plates washed twice with cold PBS after removing the culture media. PBS supplemented with protease inhibitors and phosphatase inhibitors were added and cells scraped into microfuge tube and centrifuged at 13.200 rpm in microcentrifuge for 5 min at 4^{o}C. Pellets were resuspended in ice cold 500-1000 µl Lysis Buffer and incubated on ice for 1-2 hours with occasional mixing on a shaker. The protein extract transferred to a cold centrifuge tube and sonicated 3 times for 3 sec on ice. Then it centrifugated at 13200 rpm for 20 min at 4^{o}C. The supernatant taken to a fresh tube on ice. The protein concentration of the cell lysate determined. SIK2 (Novus Biologicals) antibody at amount of 4-8 µg added to 500 to 1000 µl of cell lysate. A 40 µl aliquot of Protein A agarose beads was equilibrated with washing the beads with 1 ml lysis buffer and spinned at 13200 rpm for 1 min. Subsequently, 40 µl of Protein A slurry, prepared as indicated, was added to lysate-antibody mixture, and incubation was carried out overnight at 4^{o}C on a rocking platform. The beads were collected as before, washed three times with 1 ml cold lysis buffer, equal volume of 2X protein sample buffer was added. The mixture vortexed, boiled at 95 ^{o}C for 5 min. and spinned at 13200 rpm for 1 min.. The supernatant was collected and run on 10% SDS-polyacylamide gel.

4.4. SDS-PAGE and Western Blot

Immunoprecipitated proteins fractionated on 10% polyacrylamide gels. Samples were boiled in 6X or 2X protein sample buffer at 95^{o}C for 5 min. before loading. The gel was run in 1X Running Buffer at 80 V until stacking the gel then at 120 V.

Coomasie Blue staining was performed after incubation of the gel in the fixing solution for 1 hour. Then the gel was gently shaken in Coomasie Blue solution for at least 2 hours. Gels were rinsed with fixing solution for 5 min, then, and destained.

In Western blotting, the samples fractionated on polyacrylamide gels were electroblotted to PVDF membranes in transfer buffer at 100 V for 1-1,5 h. Subsequently the membranes were washed in TBST solution three times for total of 30 min. The membranes were incubated in appropriate blocking solution (Table 3.4) for 1-2 hour at room temperature with gently shaking. The membrane was incubated overnight in blocking solution containing appropriate primary antibody (pERK, ERK, pAKT, pan AKT, SIK2, PKA, pThreonine, pSerine) at 4^{o}C. Membranes were washed with TBST three times for 10 min. each to remove unbound antibody and incubated with HRP conjugated secondary antibody at room temperature with gently shaking. The previous washing steps were repeated. Western blot luminol reagent was applied onto the membranes for 5 min and exposed to chemoluminiscence detection. Image J programme was used to analyze the protein bands.

4.5. *In vitro* Kinase Assay

The *in vitro* autophosphorylation and kinase assays performed according to method of Feldman *et al.*,2000. Reactions containing IRS1 and immunoprecipitated SIK2 proteins (1 substrate:8 kinase), 1 µl of radioactive ATP cocktail containing 100 µM cold ATP, 1 µCi [γ32P]ATP (3000 Ci/mmol) and kinase buffer were set up at 30^{o}C. For SIK2 activity assay; immunoprecipitated 25 µl SIK2 proteins were added into reaction buffer incubated at 30^{o}C for 30 min. The reactions were terminated by the addition of 6X sample buffer and boiling at 95^{o}C for 5 min. The reaction mixtures were run on 10% SDS-PAGE, gel was fixed and dried in the gel dryer. The gels were exposed to X-ray film the Amersham Hyperfilm (GE Healthcare) for varying times at -70^{o}C. The band were visualized by immersing film in the developer solution (Kodak, USA) for 30 seconds to 2 min., then in tap water and finally in the fixative solution (Kodak, USA) for 5 min. Films were air-dried and densitometric readings were taken.

4.6. Plasmid Isolation

Overnight cultures of 200 ml bacteria carrying the appropriate plasmids were used. Bacterial cells were lysed and chromosomal DNA was denatured under strong alkaline conditions (pH 13.0), cell debris and chromosomal DNA were removed by centrifugation at 10000 xg for 10 min. Supernatant was applied to columns provided in the HiPure Plasmid Maxi kit, where DNA binds to the silica gel membrane in the presence of high salt. Impurities were removed by washing the columns with buffer containing absolute ethanol and plasmid DNA was eluted with TE buffer (pH 8.0). Concentration of the eluted plasmid DNA was determined by absorbance 260 nm with Nanodrop Spectrophotometer. The plasmid DNA was stored at -20°C for further applications.

4.7. Calcium Phosphate Transfection

MIO-M1 cells were seeded at $4.5x10^6$ cells in 10 cm^2 plate and incubated overnight under 5% CO_2 in 10 ml media. On the next day, chloroquine was added to 25 µM final concentration. 10 µg pEGFP C3 SIK2 Wild Type and 10 µg pEGFP C3 SIK2 KI (K49M) plasmids were added to the microcentrifuge tubes and the volume was completed to 439 µl. To this mixture, 61 µl 2 M $CaCl_2$ was added and finally the volume was completed to 1000 µl with 2x HBS buffer. The tubes were incubated at room temperature for 10 min. to precipitate the DNA molecules with $PO4^{-3}$ ions. After incubation, the mixture was added to the cell culture media and incubated at 37°C, under 5% CO_2 for 8-12 hours. The medium was replaced with fresh complete DMEM after 8-12 hours to remove chloroquine.

4.8. SIK2 Co-Immunoprecipitation

After $4,5x10^6$ MIO-M1 cells were harvested in 1XPBS with protease inhibitor cocktail, cells were pelleted by centrifuging at 13200 rpm for 5 min. at 4^0C and then lysed

in five packed-cell volume of EBC buffer. Total protein concentration was determined with BCA kit and 500 µg total protein were precleared in 40 µl of Protein A agarose slurry per 1 ml lysate. After preclearance the lysate was incubated with anti-SIK2 antibody for overnight at 4^{o}C on a rotator then next day 2 hours with 30 µl of protein A agarose slurry. Agarose beads were collected by centrifugation at 800 xg for 3 min. and the supernatant was carefully aspirated. The beads were washed four times with ice-cold EBC buffer with protease inhibitor cocktail. After the last wash, to remove residual wash buffer the tubes were centrifuged one more time at 800 x g for 1 minute at 4^{o}C. The pellet was resuspended in 50 µl of 2X sample buffer and boiled for 5 min at 95^{o}C to dissociate the adsorbed material from the beads. Then the beads were collected by centrifugation and the supernatant fraction was transferred to a new tube and loaded on 10% SDS-polyacrylamide gel.

4.9. Statistical Analysis

All statistical analyses were performed on Statistical Package for Social Sciences (SPSS) software version 11.5. Student's t-test was applied for three times repeated data. Levels of statistical significance is $\alpha = 0.05$ for all tests and error bars represent 1 standard error of the mean (SEM) in all charts.

5. RESULTS

5.1. Modulation of FGF-dependent SIK2 Activity by ERK

In this section possibility of ERK to be an upstream regulatory kinase of SIK2 in FGF2 signaling pathway was investigated. In this context, ERK activation was blocked via MEK inhibitor U0126 in MIO-M1 cells, subsequently modulations in SIK2 phosphorylation, activation and ERK-SIK2 interaction were analyzed. The experiments were performed at 10 min post induction where FGF-dependent ERK activation reaches maxima.

5.1.1. Changes in FGF-dependent ERK Activation and SIK2 Levels in the Presence of MEK Inhibition

To verify that ERK activation can be blocked by the MEK inhibitor U0126, MIO-M1 cells were incubated with the inhibitor for 30 min. prior to 10 min FGF2 induction. The lysates were subjected to Western Blot analysis using pERK primary antibody. The cells subjected to 10 min FGF2 treatment were used as positive controls. Subsequent to the chemoluminiscent detections, the blots were stripped and re-probed with anti ERK antibody. Densitometric readings obtained with pERK antibody were normalized to that of ERK antibody in the same samples (Figure 5.1a). The results indicated ERK activation was blocked 95% in the presence of the inhibitor ($p < 0.05$), as expected (Crews *et al.*, 1998).

In the same samples SIK2 protein levels were analyzed using SIK2 antibody where β actin levels were used as loading control. We observed that SIK2 levels decreased about 33% ($p < 0.1$) upon ERK inhibition as compared to the controls (Figure 5.1b).

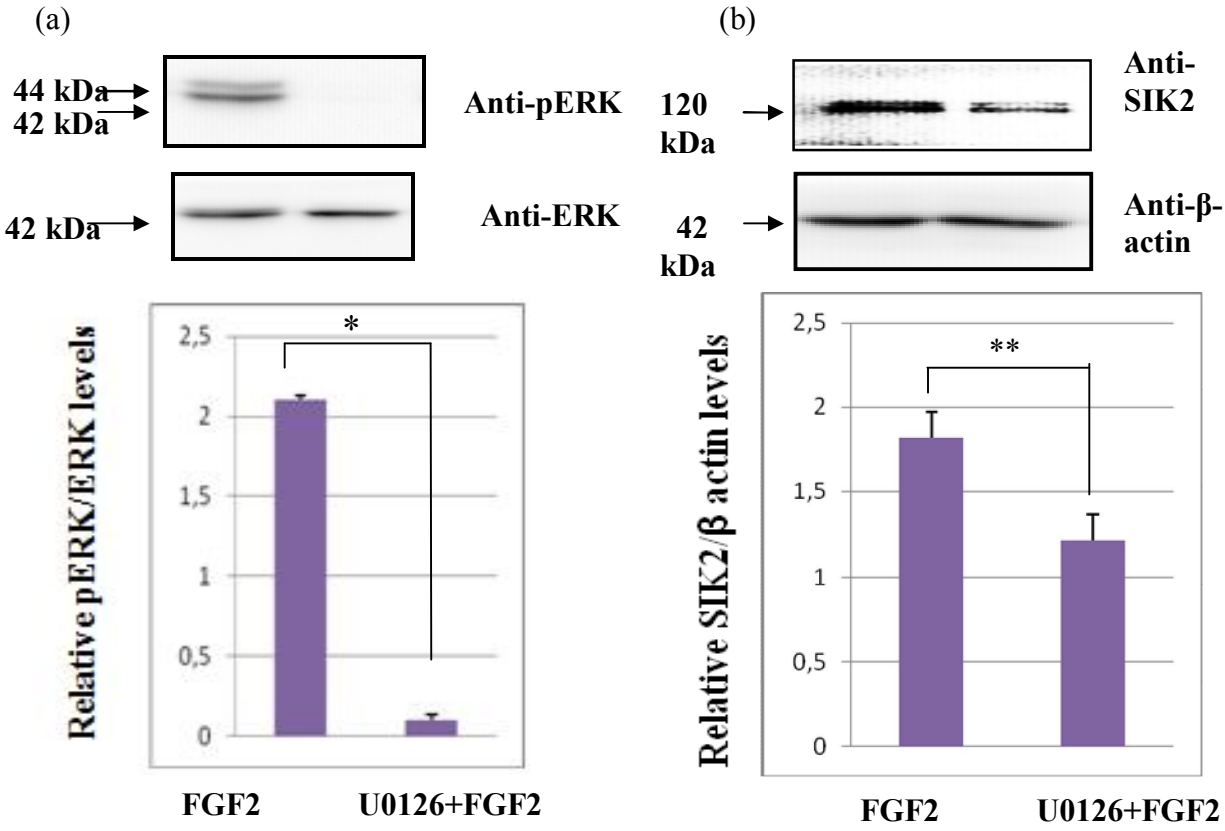

Figure 5.1. Changes in Phosphorylation Profile of ERK and SIK2 Protein Levels upon ERK Inhibition Prior to FGF2 Induction. (a) Relative pERK/ERK levels; (b) Relative SIK2/β actin levels. The experiments were repeated 3 times and student's t-test were performed, *p< 0. 05, **p < 0. 1.

5.1.2. Phosphorylation Profile of SIK2 upon ERK Inhibition prior to FGF2 Induction

To observe changes in FGF-dependent threonine and serine phosphorylation status of SIK2 as a result of ERK inhibition, SIK2 was immunoprecipitated from cells treated with/without U0126 prior to 10 min FGF2 induction using anti-SIK2 antibody. The samples were analyzed by Western blotting using either anti-pThreonine or anti-pSerine primary antibody. After chemoluminiscent detection, the blots were stripped and re-probed with anti SIK2 antibody. The densitometric readings obtained with phospho specific antibodies were normalized to that of internal SIK2 levels. We observed that ERK

inhibition resulted in 50% decrease ($p < 0.05$) in threonine phosphorylation levels of SIK2 (Figure 5.2a). In the same samples serine phosphorylation on SIK2 (Figure 5.2b) increased about 20% ($p < 0.1$). These data supports the possibility of ERK being an upstream kinase of SIK2 in MIO-M1 cells.

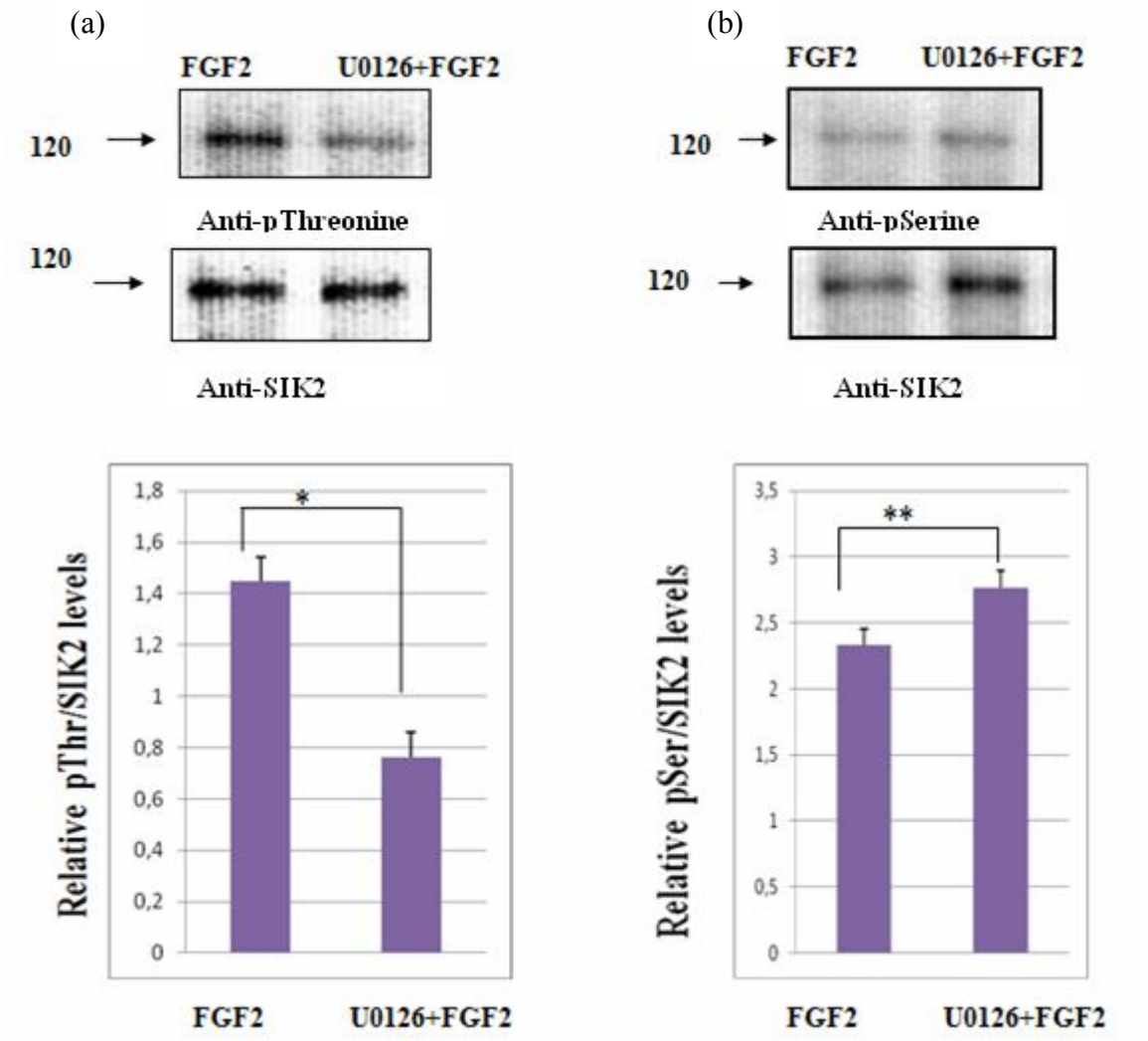

Figure 5.2. The Effect of ERK Inhibition on FGF-dependent SIK2 Phosphorylation. (a), Relative pThr/SIK2 levels; (b), Relative pSer/SIK2 levels. The experiments were repeated 3 times and student's t-test were performed, *$p < 0.05$, **$p < 0.1$.

5.1.3. Modulations in SIK2 Activity upon ERK Inhibition

In order to investigate if ERK inhibition leads to changes in the activation profile of SIK2, the protein was immunoprecipitated from the cells treated with or without ERK inhibitor prior to FGF2 induction for 10 min. The *in vitro* kinase assays were carried out in the presence of [^{32}P] γ-ATP for 30 min., followed by SDS-PAGE fractionation and autoradiography. In positive control samples immunoprecipitated IRS1 protein was used as SIK2 substrate. While autophosphorylation was negligible in untreated cells (Figure 5.3a), FGF2 treatment resulted in significant SIK2 activity increase, approximately 3 times ($p<0.05$). When ERK activation was blocked by the presence of U0126, (Figure 5.3b), the FGF dependent SIK2 kinase activity increase observed was modest ($p<0.1$). This data agrees with our earlier findings that SIK2 activity is increased in FGF dependent manner and it indicates that it can be modulated by ERK.

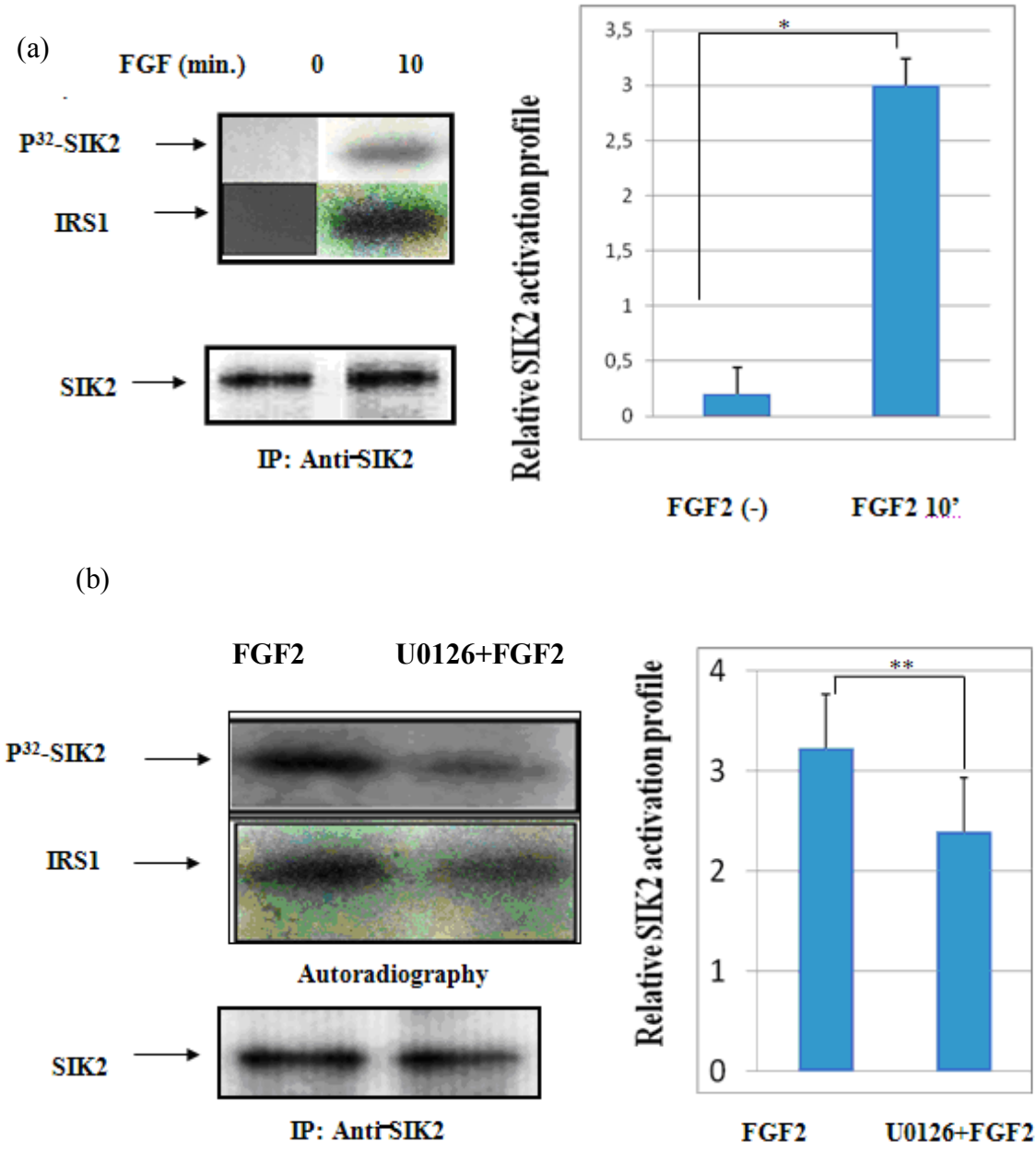

Figure 5.3. Changes in FGF-dependent SIK2 Activity in the Presence of ERK Inhibition. The experiments were repeated 3 times and student's t-test were performed, *p< 0. 05, **p < 0. 1.

5.1.4. In vitro Kinasing of SIK2 by ERK

To test whether ERK directly phosphorylate SIK2, kinase inactive form of SIK2, GFP-SIK2-KI (K49M), was expressed in MIO-M1 cells. The immunoprecipitated proteins were used in *in vitro* kinase assays in the presence of constituvely active human recombinant ERK and [γ^{32}P] ATP at 30^{o}C for 30 min.

In control samples where only ERK or only SIK2 was included, no SIK2 (150 kDa) or ERK (68 kDa) phosphorylations were observed, indicating that neither protein has autophosphorylation activity (Figure 5.4). In samples, where both ERK and GFP-SIK2-KI were present, we detected a 150 kDa band in *in vitro* kinase assay (Figure 5.4). These data indicates that SIK2 is a substrate of ERK; that ERK can directly phosphorylate SIK2 *in vitro.*

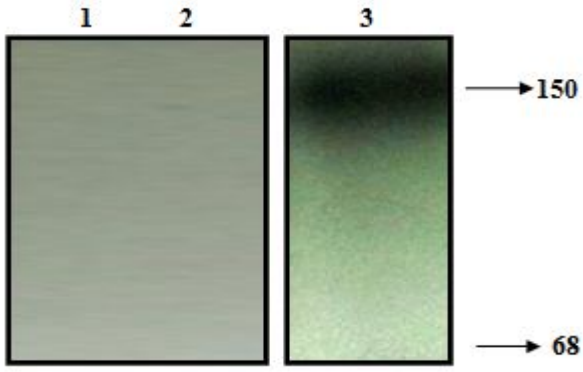

Figure 5.4. Analysis of ERK as a Potential SIK2 Kinase. Autoradiogram was obtained after the kinase assays where in the reaction mixture only active ERK (Lane 1) or only GFP-SIK2-KI (Lane 2) or in the presence of active ERK and GFP-SIK2-KI (Lane 3).

5.1.5 Verification of ERK-SIK2 Interaction in MIO-M1 Cells

Our data raised the possibility of ERK being an SIK2 kinase *in vivo*, thus we investigated their interaction by co-immunoprecipitation studies and tested if the interaction is FGF2 dependent. To this end, wild type SIK2, SIK2-WT, was overexpressed in MIO-M1 cells, the lysates from FGF untreated and treated samples were subjected to

immunoprecipitation using SIK2 antibody. The presence of ERK in the immunoprecipitates was evaluated by Western blotting.

Co-immunoprecipitation of ERK with SIK2 in untreated cells revealed weak bands on Western blots probed with specific antibody (Figure 5.5). Upon 10 min FGF2 stimulation we observed 62% higher levels of ERK in the immunoprecipitates (Figure 5.5).

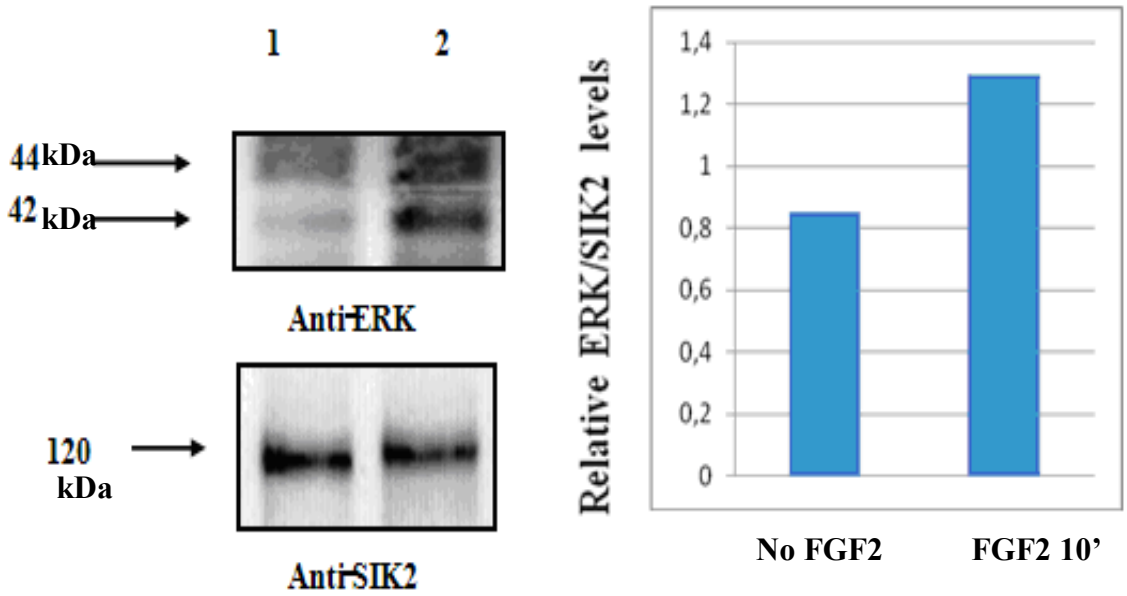

Figure 5.5. Analysis of SIK2-ERK Interaction by Co-immunoprecipitation. Lane1: Lysates of untreated cells, Lane 2: Lysates of 10 min FGF stimulated cells. The experiment was performed twice.

5.1.6 Interaction of FGF Pathway Mediators GAB1-FGFR2 with SIK2 in MIO-M1 Cells

Activated FGFR2 interacts with FRS2α, the main mediator of this pathway, subsequently Grb2-Gab1 recruited to the complex (Hadari *et al.*, 2001). As Gab1 that has been shown to be a subsrate of SIK2 *in vitro* (Küser, 2006), we next tested the Gab1-FGFR2-SIK2 interactions *in vivo* in FGF2 stimulated MIO-M1 cells.

In these experiments SIK2-WT overexpressing MIO-M1 cells were used GAB1-SIK2 and FGFR2-SIK2 interactions were analyzed by Western blotting subsequent to immunoprecipitations carried out by anti-SIK2 antibody. Blots were probed with SIK2 antibody first, stripped membranes were re-probed with anti Gab1 or anti FGFR2 antibody. We observed weak signal in the absence of FGF stimulus, while enhanced levels of both Gab1 and FGFR2 detected with 10 min FGF induction in MIO-M1 cells (Figure 5.6). These data indicate that SIK2 can be found in a complex with FGFR2 and Gab1 in FGF treated cells, thus support its involvement in FGF signaling.

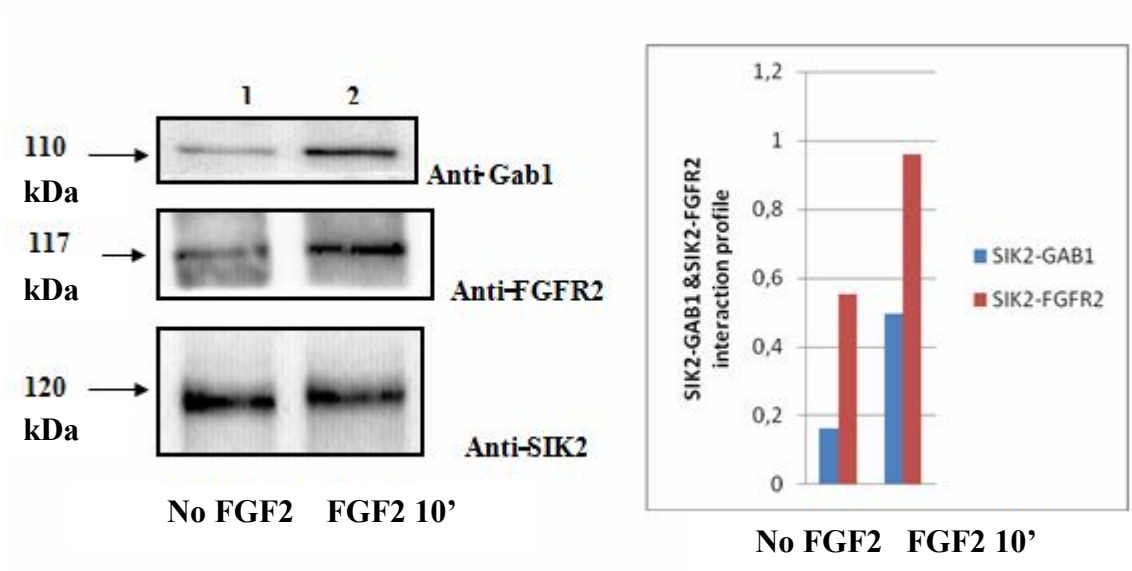

Figure 5.6. Analysis of SIK2-FGFR2-GAB1 Interaction by Co-immunoprecipitation. In lane 1; samples were not exposed to FGF induction, samples in lane 2 were obtained from cells stimulated with FGF for 10 min. The experiments were performed twice.

5.2. Modulation of FGF-dependent SIK2 Activation by AKT

In this section possibility of AKT being an upstream regulatory kinase of SIK2 in FGF2 signaling pathway was investigated. In this context, AKT activation was blocked via PI3K inhibitor LY294002 in MIO-M1 cells, subsequently modulations in SIK2 phosphorylation and activation studies were carried out. The experiments were performed at 60 min post induction where FGF-dependent AKT activation reaches maxima.

5.2.1. FGF-dependent AKT Activation and SIK2 Levels upon PI3K Inhibition

To verify that AKT activation is inhibited by the PI3K inhibitor LY294002, MIO-M1 cells incubated 30 min with the inhibitor prior to 60 min FGF2 induction. The lysates were subjected to Western Blot analysis using pAKT primary antibody. The cells treated with FGF2 for 60 min were used as positive control. Subsequent to the chemoluminiscent detections, the blots were stripped and re-probed with anti panAKT antibody. Densitometric readings obtained with pAKT antibody were normalized to that of pan-AKT antibody in the same samples (Figure 5.7a). The results indicated that AKT activation was blocked 96% in the presence of the inhibitor ($p< 0.05$), as expected (Hartmann *et al.*, 2009).

In the same samples SIK2 protein levels were analyzed using SIK2 antibody where β-actin levels were used as loading control. We observed that SIK2 levels increased about 28% ($p<0.1$) upon AKT inhibition as compared to the controls (Figure 5.7b).

5.2.2. Phosphorylation Profile of SIK2 upon AKT Inhibition Prior to FGF2 Induction

To observe possible changes in SIK2 serine phosphorylation levels as a result of AKT inhibition, SIK2 was immunoprecipitated from cells stimulated with FGF2 with/without prior LY294002 treatment using anti-SIK2 antibody. The samples were analyzed by Western blotting using anti-pSerine primary antibody. After chemoluminiscent detection, the blots were stripped and re-probed with anti-SIK2 antibody. The densitometric readings obtained with phosphoantibody were normalized to that of internal SIK2 levels. We observed AKT inhibition resulted in 30% decrease ($p<0.05$) in SIK2 serine phosphorylation levels (Figure 5.8). These data supports the possibility of AKT being an upstream kinase of SIK2 in MIO-M1 cells.

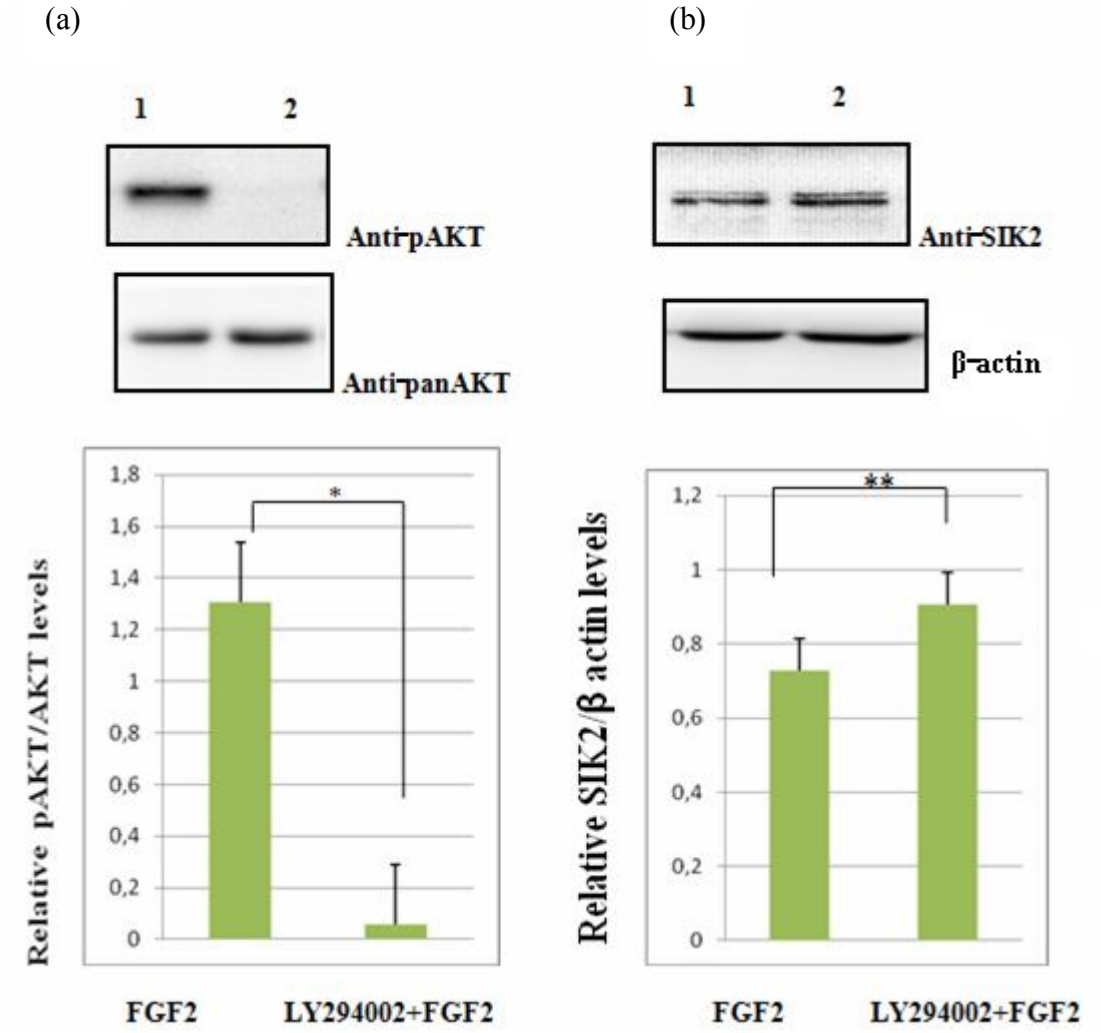

Figure 5.7. Changes in Phosphorylation Profile of AKT and SIK2 Protein Levels upon AKT Inhibition Prior to FGF2 Induction. MIO-M1 cells were treated with LY294002 for 30 min prior to 60 min FGF2 induction. The experiments were repeated 3 times and student's t-test were performed, *p< 0. 05, **p < 0. 1.

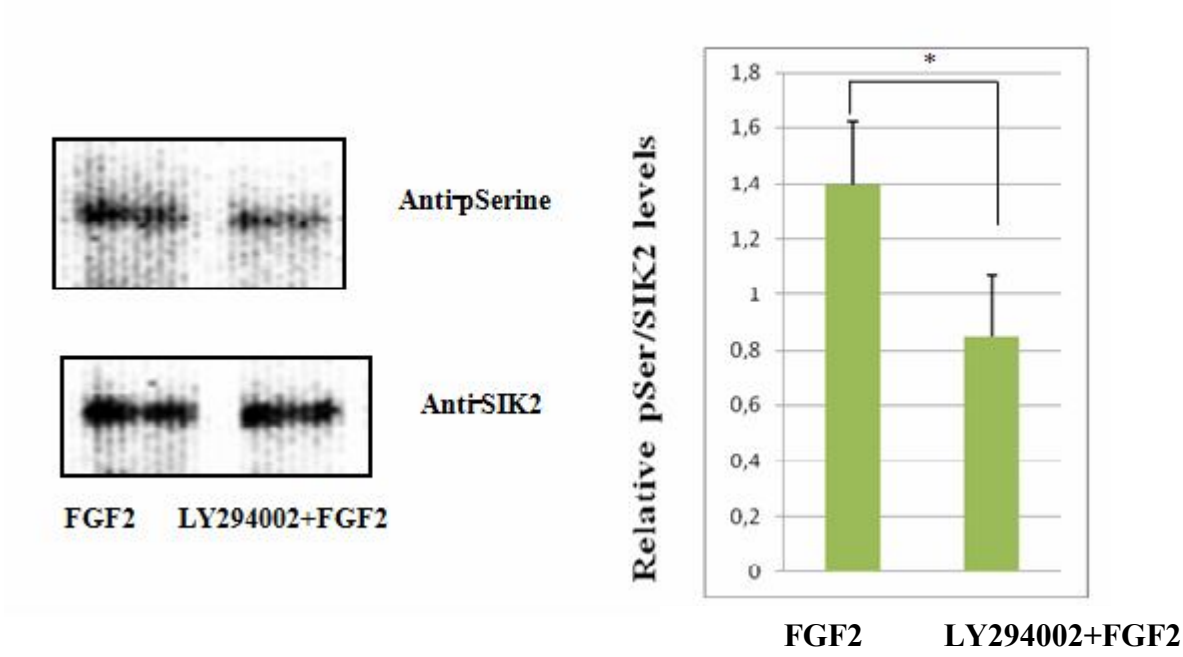

Figure 5.8. The effect of AKT Inhibition on FGF-dependent SIK2 Phosphorylation. The experiments were repeated 3 times and student's-test was performed; *$p < 0.05$.

5.2.3 Modulations in SIK2 Activity upon AKT Inhibition

In order to investigate if AKT inhibition leads to changes in the activation profile of SIK2; the protein was immunoprecipitated from the cells subjected to AKT inhibition prior to FGF2 induction for 60 min, the control samples did not receive the inhibitor. The *in vitro* kinase assays were carried out in the presence of [^{32}P] γ-ATP for 30 min, followed by SDS-PAGE fractionation and autoradiography. As a positive control immunoprecipitated IRS1 protein was used as SIK2 substrate. When AKT activation was inhibited, FGF dependent SIK2 kinase activity increase was significantly more than the controls ($p < 0.05$) (Figure 5.9). The data suggests downregulation of FGF-dependent SIK2 activity at 60 min may be due to AKT activation at this time point.

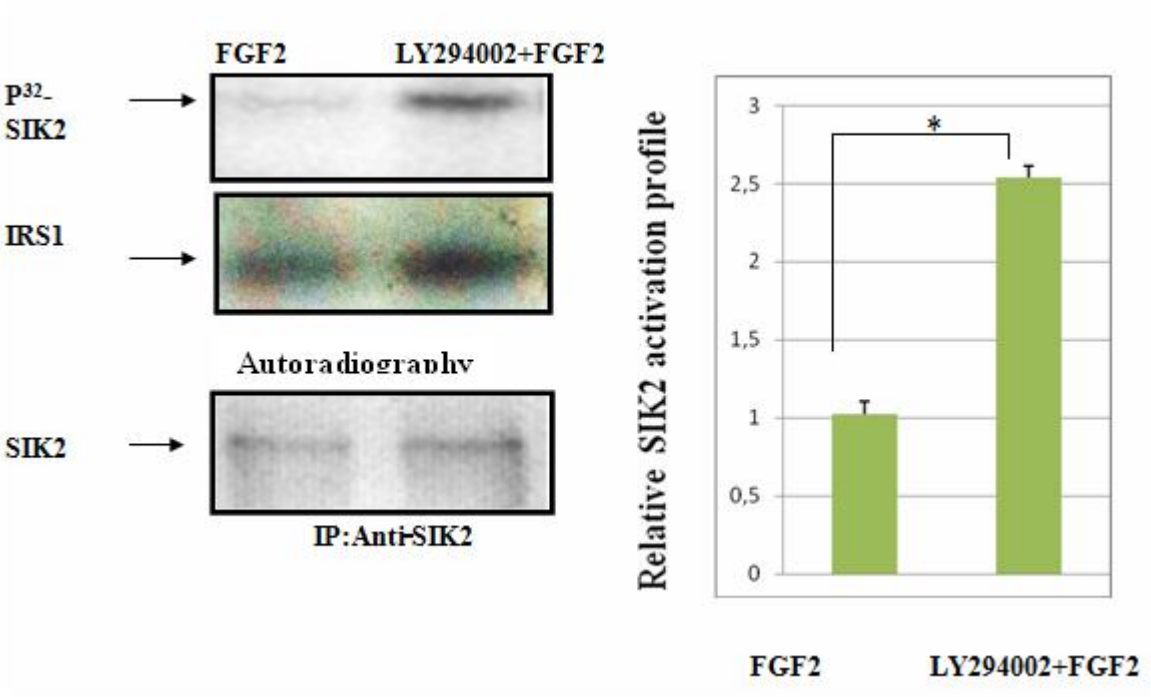

Figure 5.9. Changes in FGF-dependent SIK2 Activity in the Presence of AKT Inhibition. The experiments were repeated 3 times and student's t-test were performed, *p< 0. 05.

5.3. Modulation of FGF-dependent SIK2 Activity by PKA

Previous studies show that PKA is an inhibitory kinase of SIK2 via serine phosphorylation of SIK2 in Y1 mouse adrenocortical and COS-7 cells (Horike *et al.,* 2003; Screaton *et al.*, 2004). SIK2 activation is decreased upon 60 min FGF2 induction. To test whether PKA is also responsible for SIK2 inhibition at this time point of FGF2 induction; MIO-M1 cells treated with PKA inhibitor H89 or PKA activator 8BrcAMP prior to 60 min FGF2 induction. Then SIK2 phosphorylation and activation profiles as well as SIK2-PKA interaction were analyzed.

5.3.1. Changes in FGF-dependent SIK2 Phosphorylation by PKA Inhibition/Activation

To observe possible changes in SIK2 serine phosphorylation levels as a result of PKA inhibition or activation; SIK2 was immunoprecipitated from MIO-M1 cells stimulated with/without PKA inhibitor H89 or PKA activator 8BrcAMP prior to 60 min FGF2 treatment using anti SIK2 antibody. The samples were analyzed by Western blotting using anti-pSerine primary antibody. After chemoluminiscent detection, the blots were stripped and re-probed with anti-SIK2 antibody. The densitometric readings obtained with phosphoantibody were normalized to that of internal SIK2 levels.

We observed that PKA inhibition resulted in 58% decrease ($p<0.05$) in SIK2 serine phosphorylation levels. In contrast to this, PKA activation resulted in 59% increase ($p<0.05$) in SIK2 serine phosphorylation levels (Figure 5.10). This data agrees with the literature (Horike *et al.,* 2003; Screaton *et al.*, 2004) and supports the possibility of PKA to act as an inhibitory upstream kinase of SIK2 in MIO-M1 cells.

5.3.2. Changes in FGF-dependent SIK2 Activity by PKA Inhibition/Activation

In order to investigate if PKA inhibition/activation leads to changes in the activation profile of SIK2; SIK2 was immunoprecipitated from the cells treated as described above and used in *in vitro* kinase assays in the presence of [^{32}P] γ-ATP for 30 min., followed by SDS-PAGE fractionation and autoradiography. As a positive control immunoprecipitated IRS1 protein was used as SIK2 substrate.

When PKA was inhibited, the FGF dependent SIK2 kinase activity was 50% ($p< 0.05$) higher than the controls (Figure 5.11). On the other hand, when PKA is activated, the FGF dependent SIK2 kinase activity is 83% decreased ($p<0.05$) (Figure 5.11). These findings support that FGF-dependent SIK2 activity can be negatively modulated by PKA in the context of MIO-M1 cells.

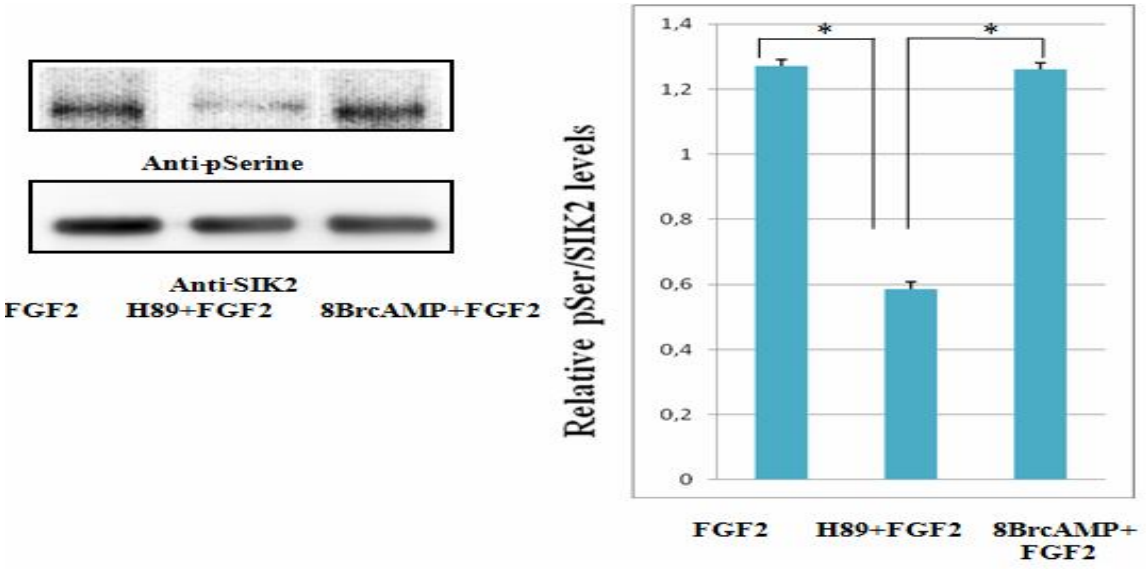

Figure 5.10. The Effect of PKA Inhibition or Activation on FGF-dependent SIK2 Phosphorylation. The experiments were repeated 3 times and student's t-test was performed; *p < 0. 05.

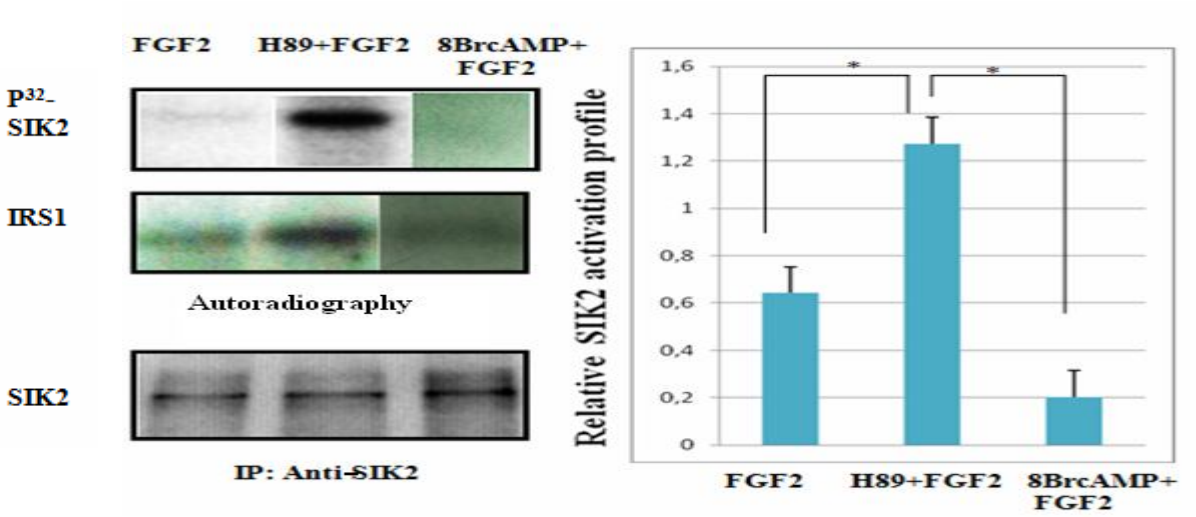

Figure 5.11. Changes in FGF-dependent SIK2 Activity in the Presence of PKA Inhibition or Activation. The experiments were repeated 3 times and student's t-test was performed; *p < 0.05.

5.3.3 Verification of PKA-SIK2 Interaction in MIO-M1 Cells

Our data raised the possibility of PKA being an SIK2 kinase *in vivo*, thus we investigated their interaction by co-immunoprecipitation studies and tested if the interaction is FGF dependent. To this end, wild type SIK2 (SIK2-WT) was overexpressed in MIO-M1 cells, the lysates from FGF untreated and treated samples were subjected to immunoprecipitation using SIK2 antibody. The presence of PKA in the immunoprecipitates was evaluated by Western blotting.

Co-immunoprecipitation of PKA with SIK2 in untreated cells revealed weak bands on Western blots probed with the specific antibodies (Figure 5.12). Upon 60 min FGF stimulation we observed 50% higher levels of PKA in the immunoprecipitates.

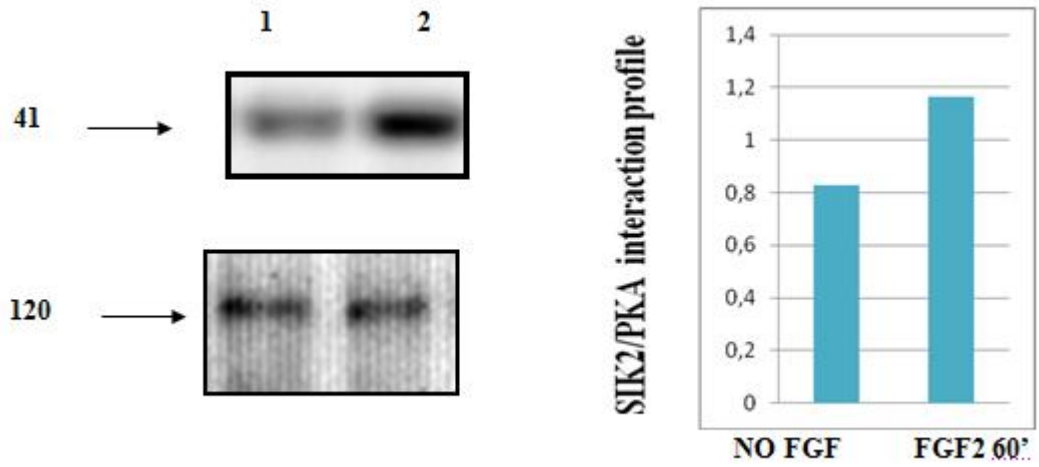

Figure 5.12. Analysis SIK2-PKA Interaction by Co-immunoprecipitation. Lane1: Lysates of untreated cells, Lane 2: Lysates of 60 min. FGF stimulated cells.

6. DISCUSSION

SIK2 localization, phosphorylation and activity appear to be regulated upon FGF2 and FGF9 stimulation of MIO-M1 cells (Çınaroğlu *et al.*, 2005, Özmen, 2006; Candaş, 2007), and two main components of FGF signal pathway, Gab1 and A-Raf were suggested as novel SIK2 substrates *in vitro* (Küser, 2006). In Müller proliferative response to FGF2, ERK is activated rapidly and transiently (Çınaroğlu, 2005). Knock-down of SIK2 expression leads to enhanced FGF-dependent ERK activation and Müller cell proliferation (Küser, unpublished data). In this study we analyzed whether three candidate STKs, namely ERK, AKT and PKA are the upstream kinases of SIK2 in Müller cells.

The presence of ERK phosphorylation motif (Ser/Thr) on SIK2 raised the possibility of threonine 758, 863 and serine 379, 775, 891 residues being targets of this dual kinase. The observation that SIK2 and ERK phosphorylation kinetics are similar in that their activity reaches maxima within 10 min FGF2 induction (Küser, unpublished data) is consistent with this possibility. The use of constitutively active ERK indicated that SIK2 is one of its substrates *in vitro*. *In vivo* studies involved of blocking of ERK activation via MEK inhibitor U0126. Initial experiments verified effectiveness of the inhibitor. Subsequently, threonine and serine phosphorylation status of SIK2 and its activity were analyzed using the protein immunoprecipitated from lysates of cells received FGF2 induction with prior exposure to U0126. Western blotting studies indicated a significant decrease in the level of threonine phosphorylation of SIK2 upon ERK inhibition compared to the controls. In contrast, serine phosphorylation levels didn't change significantly. Also under ERK inhibition FGF dependent SIK2 kinase activity showed modest decrease. These results are in agreement with our previous studies that upon 10 min FGF2 induction SIK2 activity reaches maxima where pThr/pSer ratio is at the highest. The modest activity change observed may be due to the presence other activatory kinases such as LKB1. Co-immunoprecipitation studies confirm SIK2-ERK interacts *in vivo* in FGF2 dependent manner in the same timeframe. The inhibition studies indicating decrease in SIK2 protein suggests ERK might trigger conformational and stability changes of SIK2.

Thus, we propose that ERK acts as an activatory kinase of SIK2, possibly targeting 758 and/or 863 threonine residues.

In liver in response to insulin Akt 2 has been shown to regulate SIK2 by Ser 358 phosphorylation (Dentin *et al.*, 2007). In our system maximal FGF dependent AKT activation observed at 60 min post induction, which coincides with the minimal activity of SIK2 (Küser, unpublished data). Therefore, we examined whether AKT participates in downregulation of SIK2 activity through serine phosphorylation, with the use of PI3K inhibitor LY294002 prior to 60 min. FGF2 induction. We verified effective blocking of FGF dependent AKT activation by the inhibitor. Subsequent analysis demonstrated that AKT inhibition resulted in a significant decrease in SIK2 serine phosphorylation levels significantly. *In vitro* kinase assays showed under AKT inhibition FGF dependent SIK2 kinase activity was increased significantly compared with the controls. The results suggest that AKT may trigger downregulation of SIK2 or contribute to maintaining of the protein in inactive state.

As previous studies suggested PKA is an inhibitory upstream kinase of SIK2 (Horike *et al.*, 2003). In the last part of this study, we examined whether PKA is also an inhibitory kinase of SIK2 in our system. For this purpose, involvement of PKA was studied through its activation or inhibition prior to 60 min FGF2 induction. In these experiments serine phosphorylation of SIK2 was decreased significantly when PKA was inhibited. In contrast, PKA activation resulted in increased pSer-SIK2 levels compared to controls. *İn vivo* SIK2-PKA interaction was confirmed by co-immunoprecipitation. These results indicate that PKA is an upstream kinase of SIK2 and modulate its FGF-dependent activation in MIO-M1 cells.

In parallel to these studies SIK2 interaction with FGFR2 and key modulator of FGF pathway Gab1, were shown by co-immunoprecipitation experiments. The enhanced levels of both Gab1 and FGFR2 were detected with in 10 min FGF induction in the immune complexes strongly supports SIK2 involvement in FGF2 pathway.

The study presented here showed that SIK2 phosphorylation and activation status regulated by three kinases, ERK, AKT and PKA. We suggest these kinases facilitate fine

tuning of SIK2 activity, and the duration of FGF dependent ERK activation. Ongoing studies in our laboratory indicate SIK2 phosphorylates Gab1 and Raf1 on serine residues implicated in downregulation of the Ras/Erk pathway. Therefore, SIK2 as an ERK substrate may be part of the negative feedback loop of the Ras/MAPK pathway, interfering with activating complex formation at the level of the receptor, as well as with Raf1 activation in the context of FGF signaling (Figure 6.1). Negative regulation by AKT and PKA may contribute to maintaining SIK2 in inactive state. Involvement of PKA implicates SIK2 in crosstalk between FGF pathway and the metabolic pathways.

In the context of retina better understanding of FGF pathway will provide new perspectives into Müller gliosis in response to injury and disease states. SIK2 as potential node in proliferation of glia may be a target in new therapeutical approaches in diseases such as diabetic retinopathy.

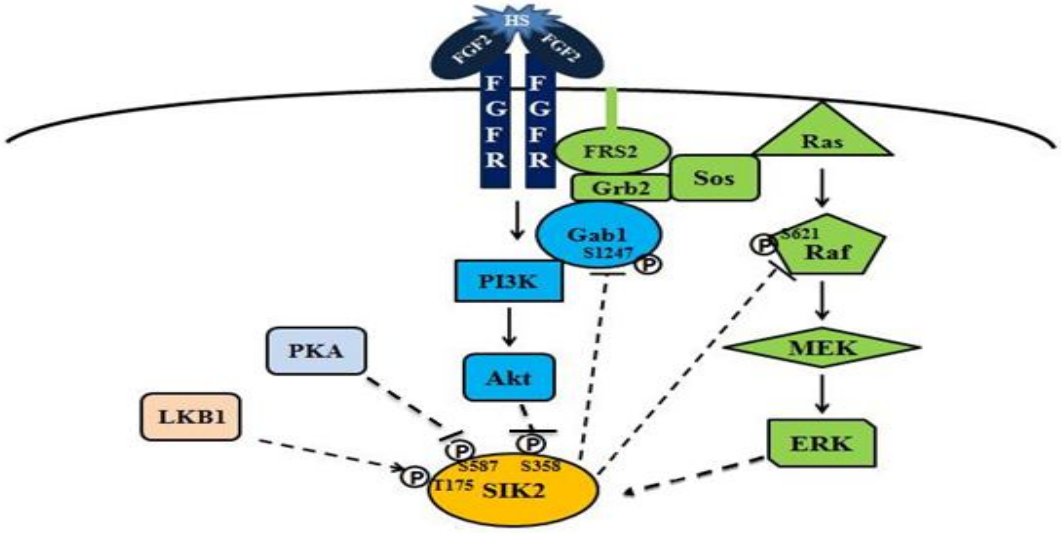

Figure 6.1. Working Model for SIK2 Involvement in FGF Signal Transduction Pathway.

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