IDENTIFICATION OF CANDIDATE SUBSTRATES OF SIK2 *IN VITRO*

by

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ABSTRACT

IDENTIFICATION OF CANDIDATE SUBSTRATES OF SIK2 *IN VITRO*

SIK2 is a serine/threonine kinase widely expressed in rat retina and it is postulated to be a potential regulator of FGF signal transduction in retina.

The aim of this work is to explore a link between SIK2 and FGF/ERK pathway. In this context, three novel candidate SIK2 substrates, Gab1, Grb2 and ARaf that involved in this pathway and have consensus SIK2 phosphorylation motifs were identified by bioinformatics tools. Even though the search found no canonical SIK2 phosphorylation motif on Frs2 and Shc1 proteins, these were included in this study, because of their proposed regulatory roles in FGF signal transduction pathway and by having numerous potentially phosphorylatable serine and threonine residues. Subsequently, these candidates were expressed in bacteria as GST fusion proteins, purified through affinity columns and their phosphorylation by SIK2 was assayed using radioactively labeled ATP in vitro.

We have shown two components of FGF signal pathway, Gab1 and A-Raf, that can be phosphorylated by SIK2 in vitro. These proteins are proposed to have major roles in FGF/ERK pathway, determining the level and duration of ERK activity. The duration of ERK activity (transient or sustained) suggested to be a determining factor in proliferation or differentiation responses in some cell types. It is conceivable that the phosphorylation status of these proteins defined by SIK2 may fine-tune the levels and duration of ERK activity, thus SIK2 may be a component of the events leading to proliferation versus differentiation decisions upon growth factor stimulation.

ÖZET

SIK2'NİN ADAY SÜBSTRATLARININ İN VİTRO BELİRLENMESİ

SIK2, gen anlatımı sıçan retinasında yaygın görülen bir serin/threonin kinazdır ve FGF sinyal yolağında potansiyel bir regülatör olduğu savlanmaktadır.

Bu çalışmanın amacı; SIK2 ile FGF/ERK sinyal yolağı arasındaki bağı araştırmaktır. Bu bağlamda, üç aday SIK2 sübstratı, Gab1, Grb2 ve A-Raf bu yolakta yer alıp, aynı zamanda SIK2'ye özgü fosforlama motifini içeren proteinler olarak biyoenformatik yöntemle belirlenmiştir. Bu araştırma kapsamında, FRS2 ve Shc1 proteinleri bu motifi içermemelerine karşın, FGF sinyal yolağında önerilen regülatör rolleri ve çok sayıda fosforlanabilir serin/threonin aminoasitleri nedeniyle irdelenmişlerdir. Belirlenen adaylar GST füzyon proteinleri olarak bakteride üretilmiş, afinite kolonlarından geçirilerek saflaştırılmıştır. Bu proteinlerin sübstrat olma potansiyelleri SIK2 ile in vitro kinaz yöntemi kullanılarak analiz edilmiştir.

Sonuçlarımız, FGF sinyal yolak elemanlarından Gab1 ve A-Raf proteinlerinin SIK2 ile fosforlanabileceğini göstermektedir, buna karşılık Grb2, FRS2 ve Shc1 proteinlerinin SIK2 tarafından fosforlanmadığına işaret etmektedir. Bu proteinler FGF/ERK sinyal yolağında ana rollere sahiptir, ERK aktivitesinin süresi ve düzeyini belirler. ERK aktivitesinin süresi (kısa veya uzun erimli) bazı hücre tiplerinde çoğalma ve farklılaşma cevaplarının belirleyici faktörü olarak ileri sürülmektedir. Verilerimiz, SIK2 tarafından tanımlanan bu proteinlerin fosforlanma durumlarının ERK aktivitesinin düzeyini ve sürekliliğini in vivo düzenleme, dolayısıyla SIK2'nin büyüme faktörü uyarımı sonucu çoğalma veya farlılaşma kararlarına neden olan olayların bir parçası olma olasılığını gündeme getirmektedir.

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AMPK	AMP-activated protein kinase
ACTH	Adrenocorticotropic hormone
AMP	Adenosine 5'-monophopshate
ATP	Adenosine 5'-triphosphate
APS	Ammonium persulfate
bp	Base pair
BSA	Bovine serum albumin
cAMP	cyclic adenosine 5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CR	Conserved regions
CRD	Cystine-rich domain
CRE	cAMP-response element
CREB	cAMP-response element binding
CYP11A	Cholesterol side chain cleavage P450
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithithreitol
dNTP	Deoxynucleosidetriphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated protein kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRS	Fibroblast growth factor receptor substrate
GAB1	Grb2-associated binder 1
GST	Glutathione sepharose transferase
GTC	Guanidium isothiocyanate

HSPG	Heparan sulfate proteoglycan
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-d-thiogalactopyranoside
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	C-Jun N-terminal kinase
Kb	Kilo base
KDa	Kilo Dalton
LB	Luria broth
MAPK	Mitogen-activated protein kinase
MARK	Microtubule affinity regulating kinase
MEK	Mitogen-activated protein kinase kinase
MOPS	Morpholinopropane sulfonic acid
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAK	p-21 activated kinase
PBS	Phosphate buffered saline
PC12	Phenochoromacytoma cells
PCR	Polymerase chain reaction
PH	Plekstrin homology domain
PI3	Phosphoatidylinositol-3
PIP3	Phosphoatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	PhopsholipaseCγ
PMSF	Phenylmethylsulphonylfluouride
PP2A	Protein phosphatase-2A
PTB	Phosphotyrosine binding
RT-PZR	Revers transkriptaz-polimeraz zincir reaksiyonu
PVDF	Polyvinyl difluoride
RBD	RAS-binding domain
RNA	Ribonucleic acid

RPE	Retinal pigmented epithelium
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription coupled polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS	Sodium dodecylsulfate
SH	Src homology domain
SHP2	Src homology phosphatase2
SH3	Src homology 3
SIK	Salt-inducible kinase
SOS	Son-of-sevenless
STAT	Signal transducers and activators of transcription
StAR	Steroidogenic acute regulatory
STK	Serine/threonine protein kinase
TAE	Tris-acetate-EDTA
TBST	Tris buffered saline with Tween 20
TE	Tris-EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
TORC	Transducer of regulated CREB activity
UBA	Ubiquitin-associated domain
UTR	Untranslated region
UV	Ultraviolet

1. INTRODUCTION

Salt-inducible kinase 2 (SIK2) carboxyl-terminal region was obtained in a yeast two-hybrid screen in a retinal cDNA library using cytoplasmic domain of fibroblast growth factor receptor (FGFR) 2 as bait (Özcan, 2003). Full length SIK2 was cloned afterwards on the basis of its high homology with a newly identified candidate gene (Uysal, 2005). Though the interaction between SIK2 and FGF receptor appears to be an artifact, the molecule is postulated to be a potential regulator of fibroblast growth factor (FGF) signal transduction due to the presence of Src homology (SH) 2 and SH3 binding motifs in SIK2 that would allow interaction with a number of signal mediator proteins with SH2 and SH3 domains implicated in this pathway (Schlessinger, 1994). Moreover, serine/threonine phosphorylation is a central mechanism in FGF dependent activation of MAPK pathways. Finally, activity and duration of growth factor induced Ras/ERK pathway has been suggested to be regulated by PKA, a SIK2 upstream kinase, dependent pathways (Pursiheimo *et al*., 2002a).

Serine/threonine kinase SIK2 is an adenosine mono phosphate (AMP) activated protein kinase (AMPK) family member, and so far two molecules, insulin receptor substrate (IRS) and transducer of regulated cAMP-responsive element binding (CREB) activity (TORC), that are involved in metabolic regulation of cells, has been identified as its substrates in vivo and vitro (Horike *et al*., 2003, Screaton *et al*., 2004). In this study ability of SIK2 to phosphorylate selected FGF signal pathway elements is explored. Initially candidate substrates that contain concensus SIK2 phosphorylation motif were identified by bioinformatics tools. Subsequently these candidates were expressed in bacteria, purified and their phosphorylation by SIK2 was assayed *in vitro*.

1.1. Protein Kinases

It is predicted that there are more than 500 protein kinases in human genome (Rubin *et al*., 2000). Transfer of the gamma-phosphoryl group of adenosine 5'-triphosphate (ATP) to tyrosine (Y), serine (S), and threonine (T) residues in protein substrates is the most abundant type of post-translational modification, and besides regulating cellular processes

such as metabolism, membrane transport, the protein kinases are part of the growth factor signaling cascades controlling proliferation, differentiation, and survival (Hunter *et a*l., 2000). Thus, kinase activity in the wrong place and time can have catastrophic consequences, leading to cell transformation, cancer, metabolic disorders and developmental abberations. Overall, 95 per cent of phosphorylations are on tyrosine residues, but only 5 per cent on serine residues and 1 per cent on threonine residues.

In eucaryotes, protein kinases are thought to have evolved from a common ancestral protein. In growth factor signaling, both serine/threonine and tyrosine kinases play important regulatory roles. Phosphorylation events may activate downstream pathway elements or they may be inhibitory. The kinase specificities are sequence dependent.

1.2. Serine/Threonine Kinases

The kinase domains of serine/threonine protein kinases (STK) are highly conserved with two distinct subdomains, or lobes. As an example three-dimensional structure of a catalytic domain of well studied protein kinase A (PKA) is shown in Figure 1.1 (Knighton *et al*., 1991). The larger C-terminal lobe is mainly helical. The smaller N-terminal lobe is composed of a five-stranded β sheet and one α helix, called helix αC. ATP is bound to a site beneath the highly conserved phosphate binding loop (P loop) connecting strands β1 and β2 that positions ATP phosphates via conserved aromatic side chain interactions. Due to its glycine-rich nature, this P loop is very flexible, inhibitors induce large distortions and disables phosphate transfer ability of the kinase (Mohammadi *et al*., 1997). Peptide substrate binding site is in activation loop, centrally located between the two lobes. This loop contains conserved 20-30 residues, invariably it starts with aspartic acid-phenylalanine-glycine residues and ends with alanine-proline-glutamic acid residues. In PKA, as in most kinases, phosphorylation of this loop renders the kinase active. In the unphosphorylated state, the activation loop collapses into the active site, blocking the substrate binding (Hubbard, 1997). Upon phosphorylation, activation loop moves away from the catalytic center and adopts a conformation that allows substrate binding and catalysis. The position and number of phosphorylation sites within the activation loop varies from kinase to kinase. The most important residue for catalytic activity is K72 in PKA that is highly conserved among other serine/threonine kinases. This residue contacts

with the α- and β-phosphate groups of ATP and positions them properly for catalysis. The side chains of the bound peptide substrate are located in specific pockets, which are shared between the two lobes. The chemistry of the amino acid side chains in each pocket provides the molecular basis of the specificity of a protein kinase. For optimal phosphotransfer the precise spatial arrangement of several catalytic residues that are absolutely conserved among all known kinases is required (Kuriyan *et al*., 2002).

The kinase domains of STKs are divided into smaller subdomains, defined as regions not interrupted by large amino acid insertions and containing characteristic patterns of conserved residues. Twelve of these subdomains are recognized as being invariant throughout the STK family and essential for enzyme function. In PKA the invariable residues are G50 and G52 in subdomain I, K72 in subdomain II, G91 in subdomain III, D166 and N171 in subdomain VIB, D184 and G186 in subdomain VII, E208 in subdomain VIII, D220 and G225 in subdomain IX, and R280 in subdomain XI.

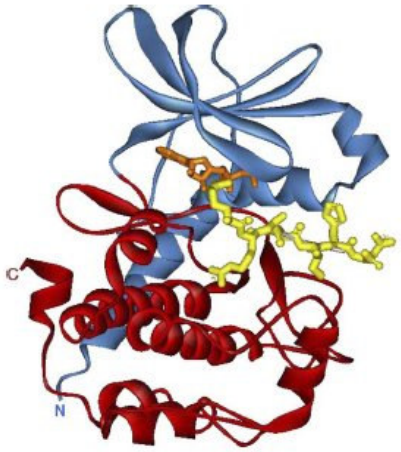

Figure 1.1. Schematic view of PKA catalytic subunit. Blue: N lobe, red: C lobe, orange: ATP molecule, yellow: peptide substrate (Kuriyan *et al.,* 2002)

1.3. AMP-Activated Protein Kinase

AMPK subfamily of STKs are key players in whole-body energy metabolism. The increase of AMP:ATP ratio leads to AMPK activation. AMPK in turn phosphorylates several downstream substrates, the overall effect is to switch off ATP-consuming pathways and to switch on ATP-generating pathways (Carling, 2004). In skeletal muscles in response

to contraction or leptin AMPK activation results in increased glucose uptake and fatty acid oxidation, whereas in liver adiponectin activates AMPK to stimulate glucose usage, fatty acid oxidation and inhibiting glucose production. It also contributes to the regulation of insulin gene expression and insulin secretion in the pancreatic β cell. AMPK itself is regulated by insulin (Carling, 2004). Figure 1.2 summarizes downstream target proteins and pathways regulated by AMPK.

AMPK is a heterotrimeric enzyme composed of catalytic α and β/γ regulatory subunits. There exists two isoforms of catalytic subunit (α1, α2), and two isoforms of β and four isoforms for β/γ regulatory subunit, respectively, each encoded by independent genes. Physiologically relevant complexes among of the 16 possible combinations are not known at this time.

N-terminal half of the α subunit contains a typical serine/threonine protein kinase catalytic domain and carboxyl terminal half is required for association with the β and γ subunits. The β subunit acts as a scaffold for the binding of the α and γ subunits. The γ subunit contains four CBS domains that have the potential for AMP binding. Lkb1 has been identified as an upstream kinase of AMPK, which phosphorylates threonine 172 residue on its activation loop.

Analysis of the human kinome indicates that there are 13 protein kinases closely related to AMPK, hence they were named AMPK-related kinases, also including SIK family (Manning *et a*l., 2002). Unlike microtubule affinity regulating kinase (MARK) these kinases are single-subunit enzymes, they lack AMPK regulatory subunits, and none of them is activated by AMPK agonists or by muscle contraction (Sakamoto *et al*., 2004).

1.4. Salt-Inducible Kinase Family

SIK family of serine/threonine kinases consists of three members. SIK1 that was the first to be cloned from adrenal glands of rats fed with high salt diet (Wang et al., 1999). The other two, SIK2 and SIK3, were identified on the basis of their sequence similarity to SIK1 (Okamoto *et al.,* 2004).

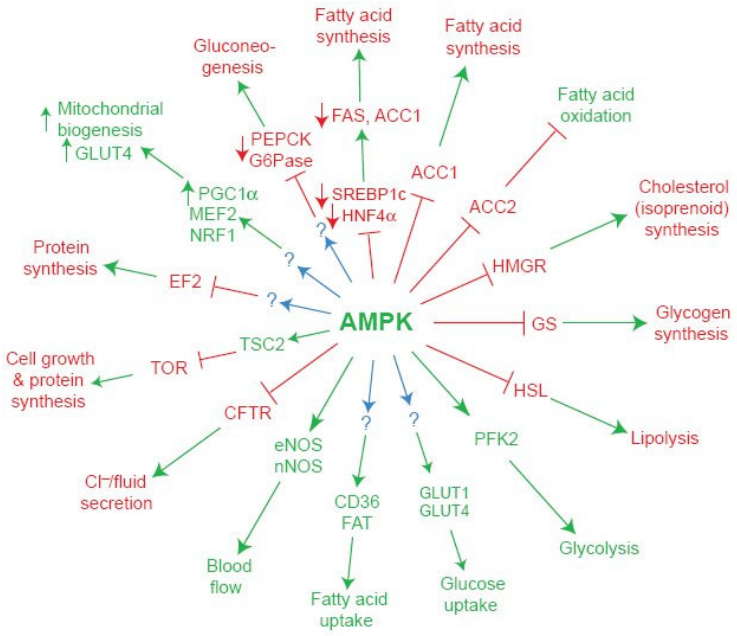

Figure 1.2. AMPK-regulated metabolic pathways. Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression (Hardie, 2004)

1.4.1. SIK1

SIK1 cDNA was independently cloned from adrenal tissue of high salt diet-fed rats (Wang *et al.*, 1999) and from rat phenochoromacytoma cells (PC12) treated with potassium chloride (Feldman *et al.*, 2000). SIK1 expression was also shown in brain, pituitary, ovary, lung, testis and heart (Feldman *et al.*, 2000, Horike *et al.*, 2003, and Lin *et al.*, 2001). SIK1 expression is induced when Y1 mouse adrenocortical tumor cells were treated with adrenocorticotropin (ACTH) via cAMP dependent PKA pathway. When overexpressed, SIK1 represses ACTH-stimulated cAMP-responsive element (CRE)-dependent gene transcription of two steroidogenic genes, cholesterol side chain cleavage P450 (CYP11A) and steroidogenic acute regulatory (StAR) genes (Lin *et al.*, 2001).

It has been proposed (Okamato, 2004) that PKA phosphorylation of SIK1 on S577 leads to its translocation to cytosol, and the decreasing levels of the SIK1 in the nuclei triggers the initiation of CRE-dependent gene transciption (Figure 1.3). SIK1 appear to phosphorylate basic leucine zipper domain of CREB and inhibit the function of the CREB-containing transcription activation complex formed on the CRE-like element in the promoter region of the steroidogenic genes (Takemori *et al*., 2002, Doi *et al*., 2002).

In addition to PKA, Lkb1, an upstream activator of AMPK, has been shown to phosphorylate SIK1 at T182 in the activation loop which creates a 14-3-3 binding site and induce a structural change that stabilizes SIK1 in an active conformation (Lizcano *et al.*, 2004, Al-Hakim *et al.,* 2005). The 14-3-3 binding to the LKB1-phosphorylated T182 on SIK1's activation loop also moves SIK1 from nucleus to the cytosol (Lizcano *et al.*, 2004, Al-Hakim *et al.,* 2005).

Koo *et al.* (2006) observed that SIK1 expression levels increased four-fold in liver via the CREB-TORC2 pathway under fasting conditions and it attenuates the gluconeogenic programme by phosphorylating TORC2 at S171, thus promoting TORC2 export to cytoplasm.

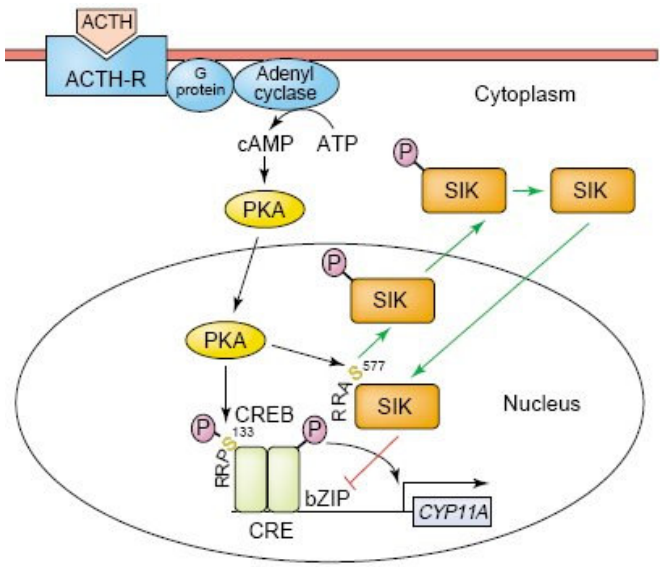

Figure 1.3. Model of SIK1 induced steroidogenic gene repression (Okamoto et al., 2004)

1.4.2. SIK2

SIK2 was first detected in mouse adipose tissue (Horike et al., 2003). Its expression was induced in 3T3-L1 preadipoctes at the onset of differentiation, along with the adipocyte specific transcription factors and present at the highest level in mature adipocytes, suggesting that the protein is important in the early phase of adipocyte differentiation (Yeh *et al.,* 1995). Over-expression of SIK2 in 3T3-L1 cells the forskolin-dependent transcriptional activation of CRE-reporter genes are repressed, and it appears that the S587 phosphorylation by PKA is critical in the cytoplasmic translocation of SIK2 (Horike *et al*., 2003).

First substrate of SIK2 identified is rat insulin receptor substrate 1 (IRS1), an important regulator of insulin pathway (Horike *et al*., 2003). Insulin dependent tyrosine phosphorylation of IRS1 by insulin receptor, a member of the receptor tyrosine kinase family, initiates the activation of the downstream elements of the pathway. But phosphorylation of S789 of IRS is known to attenuate insulin signaling either by interfering its association with the insulin receptor at the plasma membrane, preventing docking of down stream effectors or enhancing IRS degradation (Gual *et al*., 2005). As in insulin resistant rats S789 phosphorylation of IRS is found to be elevated (Qiao *et al*., 2002) and SIK2 expression and activity were induced markedly in the white adipose tissue of diabetic mice (Horike *et al*., 2003), it was suggested that SIK2 might be involved in development of type 2 diabetes (Horike *et al*., 2003).

The only other known SIK2 substrate is TORC2 in pancreatic islet cells (Screaton *et al.*, 2004). Elevation of plasma glucose and gut hormone levels is thought to lead hypophosphorylation of TORC2 and its translocation to the nucleus, where it interacts with CREB to upregulate CREB-dependent transcription, insulin being one of the target genes. In this context, SIK2 was proposed to phosphorylate TORC2 at S171, thus sequesters it in the cytoplasm through interaction with 14-3-3 proteins and inhibits CREB dependent gene transcription (Figure 1.5). In the same work SIK2 was suggested to be inhibited by phosphorylation via PKA.

Other than adipocytes and pancreatic cells, SIK2 expression was detected in testis (Horike et al., 2003), in developing and adult retina (Özcan, 2003; Özmen, unpublished data).

Consensus SIK2 phosphorylation motif was, based on sequence similarity of the two substrates, identified as (Hy)[(B)X or X(B)]XX(S/T)XXX(Hy), where Hy represents hydophobic residues and B basic residues (Horike *et al.*, 2003).

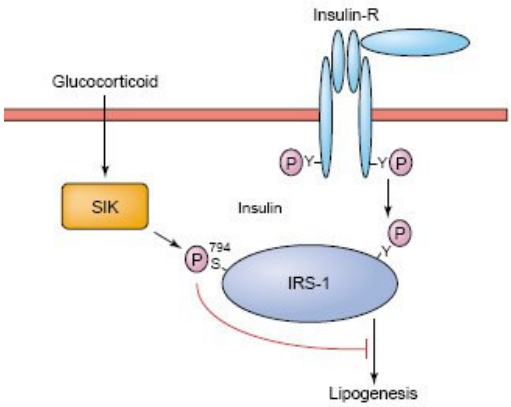

Figure 1.4. SIK2 involvement in insulin signaling (Okamoto *et al.*, 2004)

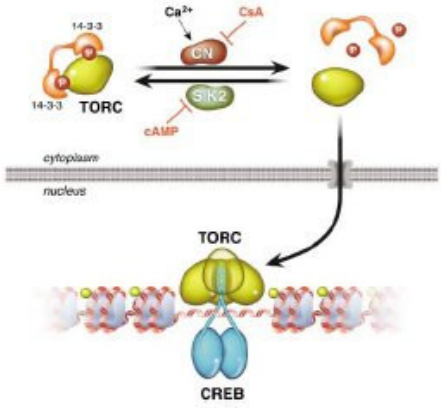

Figure 1.5. SIK2 involvement in TORC2 activity in CREB dependent gene transcription (Screaton *et al.*, 2004)

Mouse SIK2 is predicted to be 103 kDa protein, the kinase domain spans amino acid residues from 20 to 271 at the N-terminal. K49 residue resided in the ATP-binding loop is essential for SIK2's catalytic activity, its replacement with methionine results in kinase-inactive enzyme. SIK2's autophosphorylation activity was shown in transfected COS7 cells, but the critical residue has not been identified yet (Horike *et al.*, 2003). One known upstream kinase of SIK2 is LKB1, phosphorylating T172 residue in the activation loop thereby increasing its activity 30 fold in vitro. Replacement of this residue with glutamic acid (T172E) renders SIK2 constitutively active and the basal activity of this mutant protein is similar to that of wild-type enzyme phosphorylated at this residue (Lizcano et al., 2004). Ubiqitin-associated domain (UBA) of SIK2 spaning amino acid residues 293 and 346 was proposed to directly interact with the kinase domain of SIK2, permitting it to be in a conformation that can be readily phosphorylated and activated by the Lkb1:STRAD:MO25 complex (Jaleel et al., 2006). PKA-phosphorylation domain resides between amino acid residues 577-623 and S587 phosphorylation by PKA marks SIK2 for nuclear export (Horike et al., 2003).

Structure of rat SIK2 cloned from retinal tissue (Uysal, 2005; Özcan, 2003) indicates existence of three isoforms in rat retina (Uysal, 2005). Open reading frame of isoform A that is 36 bp longer than that of isoform B and C, consists of 2799 bp. Isoforms B and C differ only in their 3'-UTR. Although there are three alternatively spliced transcripts, they seem to encode two different proteins with a little difference at their carboxyl-terminal. Northern blot analysis revealed the presence of two transcript species, 7.5-8 kb, in retina, and RT-PCR experiments show that SIK2 expressed in all retinal cell layers (Özcan, 2003, Özmen, unpublished data). Rat SIK2 shows 94 percent overall identities to mouse and 89.3 percent to human SIK2. The highest conservation is in their kinase domains, where the amino acid identity is over 98 per cent between the three orthologues. The highest divergence is observed in the C terminal region (Uysal, 2005).

1.4.3. SIK3

The third member of the SIK family, SIK3, was identified from data base search for SIK1 homologs. It has 68 per cent similarity in kinase domain and 37 per cent in the UBA domain to SIK1. SIK3 is ubiquitously expressed in humans, mice, rats and shares sequence

homology with C. elegans gene Kin-29, that regulates the expression of olfactory receptor (Okamoto *et al*., 2004; Lanjuin and Sengupta, 2002). It was also suggested that Kin-29 regulates the body size through modulation of Sma/Mab pathway (Maduzia *et al*., 2005). However, no functional data about SIK3 are available in vertebrate systems.

1.5. Fibroblast Growth Factors

FGF family comprises 23 polypeptide growth factors that are evolutionarily conserved among different species from nematodes to humans (Ornitz *et al*., 2001). The members vary in size from 17 to 34 kDa in vertebrates and share an internal core domain of 120 amino acids, with 28 highly conserved and six identical amino acid residues (Figure 1.6). They are known to bind heparin and interact with high affinity receptors (FGFRs) on target cell membranes (Eriksson *et a*l., 1991, Zhu X *et al*., 1991, Plotnikov *et al*, 1999)

Most FGF family members have an amino-terminal signal peptide and are secreted classical pathway. FGF1, 2, 9, 16, and 20 lack this peptide, they are secreted by an alternative pathway (Miyake et al., 1998). FGF11-14 lack signal sequences and are intracellular (Smallwood *et al*., 1996). FGF2 and FGF3 contain nuclear localization signals, therefore the proteins can be found in the nucleus (Powell and Klagsbrun, 1991).

FGF family members are differentially expressed in many tissues with different spatiotemporal pattern. FGF3, 4, 8, 15, 17, and 19 are expressed exclusively during embryonic development, whereas others are expressed both in embryonic and adult tissues.

The first member of FGF family is named as FGF1 after it was identified by its ability to stimulate proliferation of fibroblasts. Other than cell proliferation, family members are implicated in gastrulation, embryonic axis formation, mediate differentiation and cell migration during development of vertebrates (Slack, 1994). In the adult organism, they mediate several cellular responses such as tissue repair, wound healing, angiogenesis (Ornitz *et al*., 2001).

Several FGF family members are expressed during vertebrate eye development and also mature retina. FGF3, FGF8, and FGF17 have been identified during development in

the optic stalk of several vertebrate models (Crossley and Martin, 1995; Reifers *et al*., 1998, 2000; Vogel-Hopker *et al*., 2000; Walshe and Mason, 2003). FGF2 mediates neural, but not RPE differentiation in chick embryonic retina (Pittack *et al*., 1997). FGF1 is expressed at high levels in the peripheral retina during the initial stage of chick retinogenesis (McCabe *et al*., 1999). FGF19 has been detected in embryonic human retina (Xie *et al*., 1999). In neuronal cells of postnatal retina was shown to express FGF1, FGF2 and FGF9, levels being highest in the adult (Buğra *et al*., 1993; Buğra and Hicks, 1997; Cinaroglu *et al*., 2005). In the mature chick retina, Müller glia are plastic and can respond to FGF2 by proliferation and subsequent differentiation into neurons (Fischer *et al.,* 2002). FGF1, FGF2 and FGF9 are mitogenic for Müller cells in vitro and FGF1, FGF2 for photoreceptors (Cinaroglu *et al*., 2005, Hicks and Courtois, 1992).

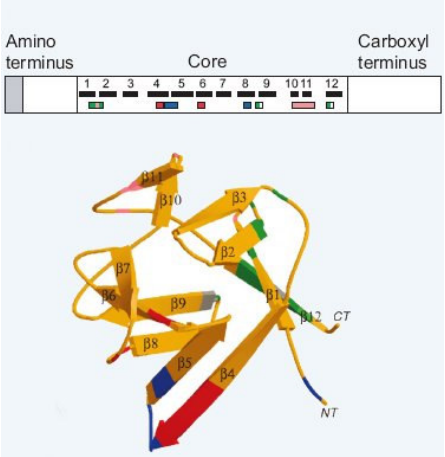

Figure 1.6. The overall structure of the FGF polypeptide. Shaded box: signal peptide, pink: heparin-binding region, green: the region interacting with Ig-domain 2 of high affinity receptors, blue: interacting with Ig-domain 3, red: interacting with alternatively spliced region of Ig-domain 3, gray: interacting with the linker region (Ornitz *et al*., 2001)

1.6. Fibroblast Growth Factor Receptors

FGFs mediate their effects by binding to and activating fibroblast growth factor receptors that are a subfamily of cell surface receptor tyrosine kinases (RTK's) with the help of heparan sulfate or heparan sulfate proteoglycans (HSPG) (Ornitz, 2000). The

FGFR family is composed of four members, FGFR1-4, which share between 55% to 72% homology at the protein level (Johnson & Williams 1993).

FGFRs are transmembrane proteins containing three extracellular immunoglobulin (Ig)-like domains (designated IgI, IgII and IgIII), an acidic region between IgI and IgII domains followed by a heparin-binding region and a cell adhesion homology domain. Following the transmembrane domain, intracellular portion of FGFRs have a juxtamembrane domain that forms a platform for docking proteins, a split tyrosine kinase domain, and a short carboxy-terminal tail (Figure 1.7). FGFR isoforms containing either two or three Ig-like domains, soluble secreted forms, and IgIII domain isoforms can be generated (Figure 1.8) by differential exon usage (Johnson & Williams 1993). While the soluble form has no known physiological function, the IgIII domain isoforms alter ligand specificity profoundly (Powers *et al.*, 2000).

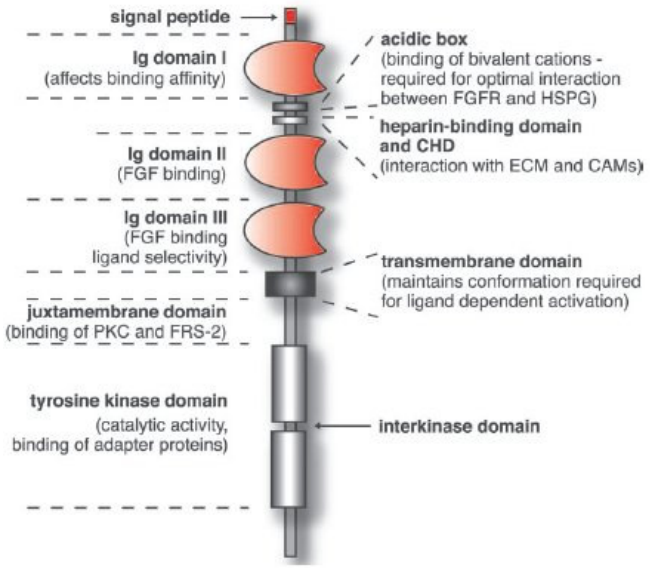

Figure 1.7. The Domain Structure of Generic FGFR Proteins
(Böttcher and Niehrs, 2004)

In vertebrate retina, all FGFR isoforms are expressed, whereas FGFR1, FGFR2 and FGFR4 are preferentially expressed within photoreceptors (Kinkle *et al.*, 2002). FGFR1, FGFR2 and FGFR3 expression has also been shown in Müller cells (Cinaroglu *et al.*, 2005). Transfection of truncated FGFR1 into Xenopus embryos leads to a 50% loss in photoreceptors and amacrine cells, and a parallel increase in Müller glia, suggesting a role

of FGFR in cell fate determination (McFarlane et al., 1996). Also, expression of truncated FGFR1 and FGFR2 in photoreceptors induces progressive retinal degeneration (Campochiaro *et al*., 1996).

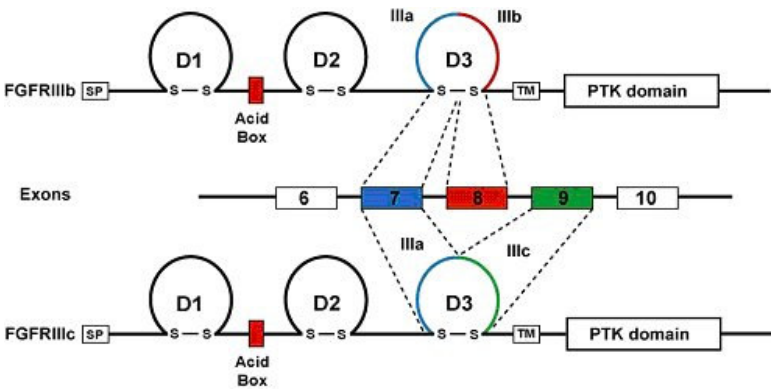

Figure 1.8. Generation of FGFR isoform IIIa, b, and c by differential exon usage (Böttcher and Niehrs, 2004)

1.7. Cell Signaling via FGF-Receptors

1.7.1. Receptor Dimerization and Autophosphorylation

One FGF ligand binds each FGFR monomer region spanning the Ig-like domain II, III, and the linker between the two domains so that a 2 FGF:2 FGFR dimmer is formed and stabilized by the binding of an HSPG across a cavity formed by the FGF-FGFR pairs (Stauber et al. 2000). Even in the absence of a ligand, FGFR can form dimmer on the cell membrane, but only after the cognate ligand binding this dimmer is stabilized with the help of the HSPG. Dimerization sets stage for autophosphorylation of different tyrosine residues in the cytoplasmic portion, creating docking sites for downstream proteins containing SH2 (src homology 2), SH3 (src homology 3) and phosphotyrosine binding (PTB) domains. There are six tyrosine phosphorylation residues that are conserved in all FGFR proteins, differential phosphorylation enables the receptor to interact with alternative downstream elements (Figure 1.9). For example, phosphorylation of the Y724 of FGFR3 initiates src homology phosphatase 2 (Shp2) phosphorylation and activation of mitogen-activated protein kinase (MAPK), phosphotidylinositol-3 (PI3) kinase and signal transducers and

activators of transcription 1/3 (Stat1/3) pathways, and phopsholipase Cγ (PLCγ) binding is through the Y760 residue, fibroblast growth factor receptor substrate 2 (FRS2) binding is through Y577 (Kristen *et al*., 2001).

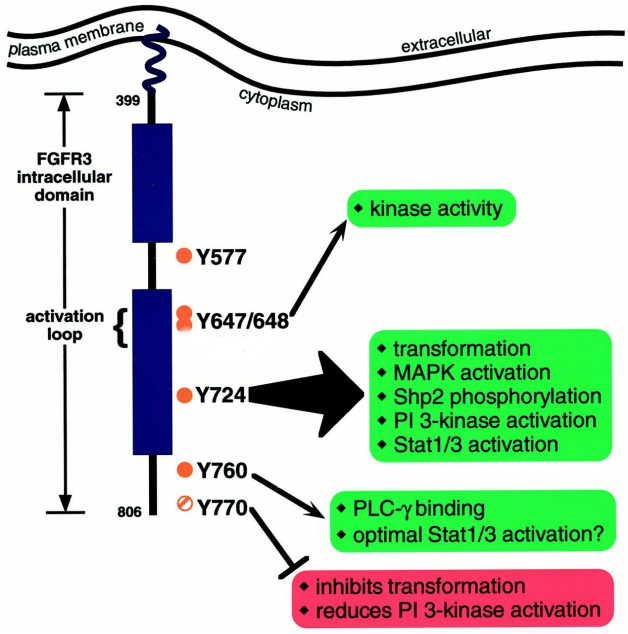

Figure 1.9. Phosphorylated tyrosine residues of FGFR3 (Kristen *et al*., 2001)

1.7.2. FGF/FGFR Signal Transduction Pathways

There are three main pathways activated by FGFRs (Figure1.10): Ras/MAPK pathway, PLCγ/Ca^{2+} pathway and PI3 kinase/Akt pathway (Böttcher and Niehrs, 2003).

1.7.2.1. PLCγ/Ca^{2+} Pathway. PLCγ is known to be a substrate of all FGFRs. Autophosphorylation at Tyr-766 of FGFR1 creates docking site for SH2 domain of PLCγ. After receptor interaction PLCγ is activated by tyrosine phosphorylation. Active PLCγ hydrolyzes phosphotidylinositol-4,5-diphosphate to inositol-1,4,5-triphosphate and diacylglycerol (DAG). In turn, DAG activates protein kinase C (PKC), whereas inositol-1,4,5-triphosphate stimulates Ca^{2+} release from intracellular compartments (Mohammadi *et al.,* 1991). PLCγ via PKC modifies the phosphorylation status of Raf and thereby the Ras/MAPK pathway is activated (Huang *et. al.,* 1995).

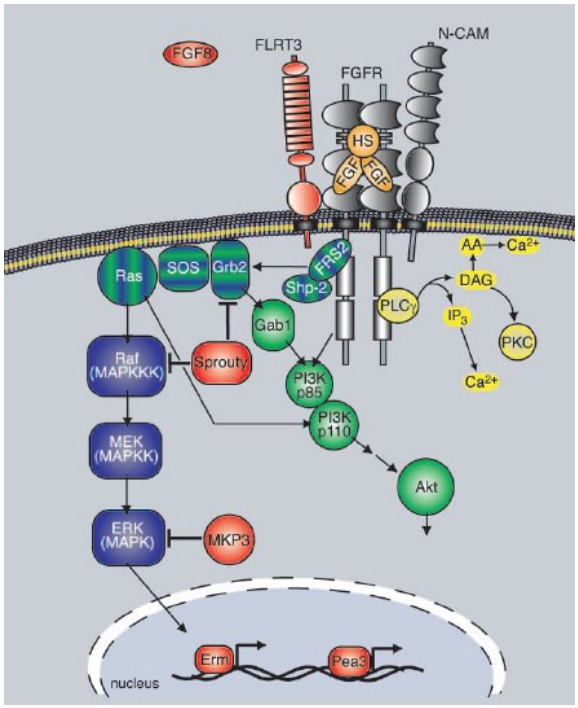

Figure 1.10. Three main pathways downstream of FGFR in response to FGFs. Yellow: PLCγ/Ca^{2+} pathway elements, blue and blue/green: Ras/MAPK pathway elements, green and blue/green: PI3 kinase/Akt pathway elements (Böttcher and Niehrs, 2003)

1.7.2.2. PI3 kinase/Akt Pathway. This is the main pathway that blocks apoptosis and mediates FGF dependent cell survival (Hawkins *et al*., 1997). PI3 kinase can be activated by FGFRs either by binding of PI3 kinase-regulatory subunit p85 to phosphorylated tyrosines, Y724 of FGFR3 for example, or by the recruitment of PI3 kinase catalytic subunit p110 to the membrane by activated Ras (Rodriguez *et al*., 1994). In the third mechanism, Grb2-associated binder 1 (Gab1)-FRS2-Grb2 complex brings PI3 kinase-p85 subunit to the membrane. The active PI3 kinase can activate Akt directly or indirectly via phosphorylation by PDK1/PRK-2 complex at T308 and S473 residues (Hadari *et al*., 2001). Activated Akt phosphorylates Bad and Caspase 9 rendering these apoptotic proteins inactive, and forkhead transcription factors such as FKHRL1, FKHR, and AFX, so that they are sequestered in the cytoplasm due to binding of 14-3-3 protein to the phosphoserine residues.

1.7.2.3. Ras/MAPK Pathway. FGF dependent proliferation and differentiation events are mainly transduced via Ras/ERK pathway. Main modulator of the pathway is FRS2 which upon interaction with the juxtamembrane region of FGFRs is phosphorylated on several tyrosine residues, four of which create docking sites for Grb2-Sos complex and two of them for Shp2 binding. Sos, guanine nucleotide exchange factor, recruitment to membrane into close proximity of small G-protein Ras activates this protein, which in turn activates effector protein Raf. Raf, a serine-threonine kinase, stimulates MAPK-kinase (MEK), MEK phosphorylates MAPK, ERK, on threonine and tyrosine residues. Activated ERK phosphorylate transcription factors and change the catalytic activities of enzymes (*Yang et al.,* 2004). The sustained activation of ERK by FGF stimulation is considered to induce the differentiation, whereas transient activation to induce proliferation of PC12 cells (Yamada and Yoshimura, 2002).

p38 and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) also belong to the MAPK family. These MAPKs are mainly involved in transmitting cytokine production and cytokine stimulated cellular proliferation and survival (Allen et al., 2000, Crawley et al., 1997).

1.7.3. Candidate SIK2 Substrates involved in FGF Signalling Pathway

1.7.3.1. FRS2. FRS2 protein is responsible both for the recruitment of positive regulators to FGFR and is a target for negative feedback mechanism resulting in signal attenuation and fine-tuning of its own activity (Lax *et al*., 2002). FRS2 is a membrane anchored docking protein whose amino-terminal PTB domain binds to the [KSIPLRRQVTVS] motif on the juxtamembrane region of FGFR independent of tyrosine phosphorylation (Ong *et al*., 2000). It has two isoforms, FRS2α that is ubiquitously expressed and FRS2β whose expression is mainly neuronal. Carboxyl terminal region contain several tyrosine and threonine phosphorylation residues. After FGF stimulation, FRS2 tyrosine residues are phosphorylated that creates four binding sites for Grb2-Sos and two for Shp2 (Figure 1.11 and Figure 1.12). Recruitment of Grb2-Sos complexes, directly or indirectly via Shp2, to the membrane and activates Ras/MAPK pathway. FRS2 also mediate PI3 kinase stimulation via forming ternary complex with Gab1-Grb2 (Hadari *et al.,* 2001). Tyrosine phosphorylated FRS2 can also form complexes with negative modulators, such as ubiquitin

ligase Cbl, resulting in ubiquitination of itself and FGFR (Wong *et al.,* 2002). MAPK-dependent threonine phosphorylation FRS2 results in its release from FGFR and eventual attenuation of the MAPK response (Ong *et al.,* 2000). In unstimulated cells FRS2 shows a basal level of threonine phosphorylation, the responsible kinase is unknown.

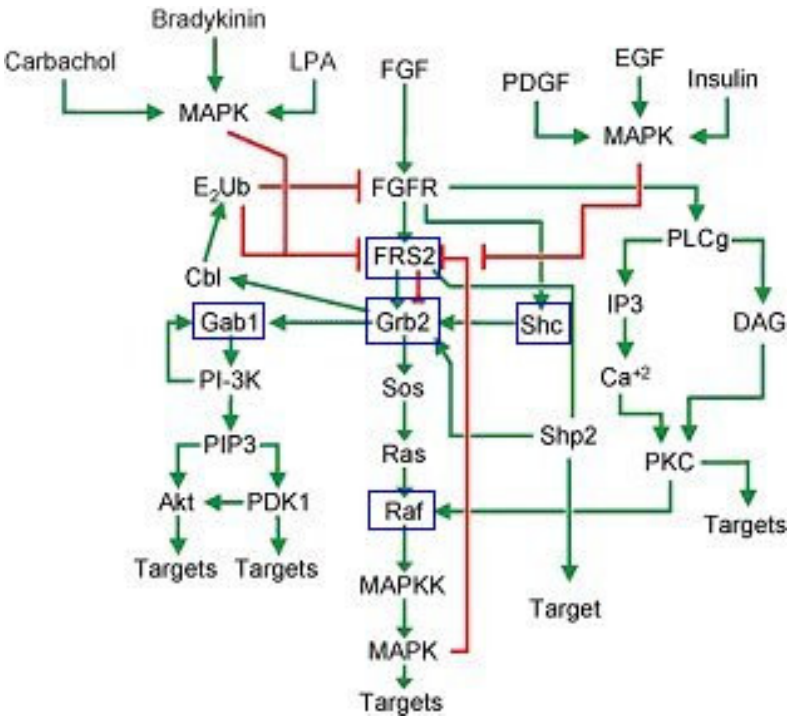

Figure 1.11. Signaling pathways downstream of FGFR. Green arrows: signalling pathways dependent on tyrosine phosphorylation of FRS2 following FGF-stimulation, red arrows: negative signals mediated by FRS2, Blue boxes: key proteins in FGF pathway (Eswarakumar et al., 2005)

1.7.3.2. Grb2. Grb2 is an adaptor protein that has an amino and carboxyl terminal SH3 domains and an internal SH2 domain that binds to FRS2 or Shc in a phosphorylation dependent manner. All identified FGFRs lack direct binding sites for Grb2 (Songyang *et. al*., 1993). It exists in complex with the nucleotide exchange factor Sos, which catalyzes the exchange of GDP for GTP on Ras thereby promoting Ras activation (Pawson, 1995). Another constitutive binding partner of Grb2 is Gab1, the interaction initiates Akt dependent anti-apoptotic pathway (Hadari *et al.,* 2001). Both Sos1 and Gab1, which bind predominantly to the N- and C- terminal SH3 domains of Grb2, respectively, they coexist

in same ternary complex. The concurrent recruitment of both Sos1 and Gab1 to the membrane via FRS2, elicits bifurcating signals by activating the Ras/MAPK and PI3 kinase/Akt pathways simultaneously (Ong *et al.,* 2001). SH2 domain of Grb2 can also bind to FRS2 associated and tyrosine phosphorylated Shp2. Grb2 can specifically interact with Y783 phosphorylated PLCγ. Grb2, in addition to its key function in signaling through Ras, may have a negative regulatory role on EGF-induced PLCγ activation (Choi *et al.,* 2005). Cbl protein binds to SH3 domain of Grb2 that attenuates MAPK signaling (Wong *et al.,* 2002). No serine/threonine phosphorylation on Grb2 has been shown yet.

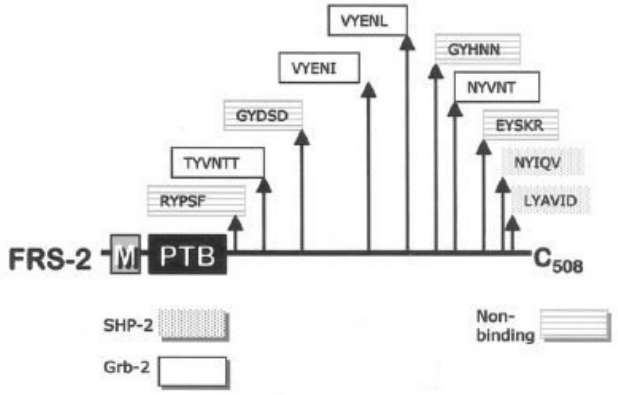

Figure 1.12. FRS2 domain structure. M: mystrylation site, PTB: phosphotyrosine binding domain, blank box: Grb2 binding site, dotted box: Shp2 binding site (Guy *et al.,* 2002)

1.7.3.3. Gab1. Gab1 is a member of PTB domain containing docking protein family. Due to its amino-terminal plekstrin homology domain (PH) it is anchored to the membrane and through its proline rich region it interacts constitutively with SH3 domain of Grb2. Grb2-Gab1 complex is recruited to the tyrosine phosphorylated FRS2 protein and most probably because of its proximity to FGFR, becomes tyrosine phosphorylated on sites that bind to the p85 of PI3 kinase and consequently leads to the activation of cell survival pathways in FGF stimulated cells (Ong *et al*., 2001). Moreover, recruitment of PI3 kinase by Gab1 results in a positive-feedback loop mediated by binding of the PH domain of Gab1 to the product of PI3 kinase activation, phosphatidylinositol-3,4,5-triphosphate on the membrane (Lamothe *et al*., 2004). Association between the phosphorylated Y627 and Y659 of Gab

proteins and the SH2 domains of Shp2 is an essential part of the mechanism that upregulates the phosphatase activity of Shp2, leading to activation of the MAPK cascade (Figure 1.11 and Figure 1.13) and subsequent biological responses (Cunnick *et al.,* 2001). Gab1 directly associates with hepatocyte growth factor receptor (Met receptor), but does not associate with either the insulin receptor (IR), or epidermal growth factor receptor (EGFR). It was recently demonstrated that upon EGFR activation, Shp2 dephosphorylates Gab1 on Ras-GAP interaction motif. Therefore Ras-GAP disengages from the complex; consequently, increasing Ras activity (Montagner *et al.,* 2005). Gab family proteins acting via Shp2 are required for full ERK activation in many signaling pathways. In most of them Gab1-Shp2 complex formation is not absolutely necessary for Ras-ERK activation, but sustained activity is defective in Gab1−/− cells. Thus binding of Gab1 to Shp2 suggested to act as a signal amplifier (Gu *et al*., 2003). Shp2 mediated dephosphorylation of different motifs on Gab proteins also functions in signal termination. Shp2 was reported to dephosphorylate PI3 kinase binding site on Gab1, thus negatively regulate PI3 kinase/Akt upon EGF stimulation (Zhang et al., 2002). Besides Shp2, upon EGF stimulation ERK phosphorylates several serine residues in the vicinity of PI3 kinase binding tyrosine residues on Gab1, that blocks their interaction and inhibits PI3 kinase/Akt pathway (Figure1.14) (Lehr et al., 2004). Binding of Gab1 to Crk, CrkL and PLCγ have also been reported (Schaeper *et al.,* 2000). Experiments with cells derived from Gab1 knockout embryos have demonstrated that Gab1 plays an important role in EGF, PDGF, or HGF induced stimulation of MAPK (Itoh *et al.,* 2000, Yamasaki *et al*., 2003). It has been suggested that, Gab1 hyperphosphorylation on serine/threonine residues and hypophosphorylation on tyrosine residues prevent interaction with partners, possibly due to conformational change that renders kinase domain of growth factor receptors inaccessible (Gual *et al.,* 2001).

1.7.3.4. Shc. Shc is a ubiquitiously expressed member of PTB-domain containing docking protein family. Two of the three known isoforms, p46 and p52 are formed by translation from different in-frame initiation codons and the third isoform, p66, is generated by alternative splicing. Shc isoforms contain an amino-terminal SH2 domain, followed by a proline-rich CH1 domain and a carboxy-terminal PTB domain (Sasaoka *et al*., 2000), p66 isoform contains an additional amino-terminal proline-rich region termed CH2. Shc proteins are recruited by activated FGFRs via its PTB or SH2 domains and Y239, 240 and

317 residues are phosphorylated by the receptor. Tyrosine residue 317, at the YXN consensus sequence responsible for binding the SH2 of Grb2, is necessary for the activation of Ras through Shc (Salcini *et al.,* 1994). The identification of in vivo complex formation between the FGFR-1 and Shc has been failed, but tyrosine phosphorylation of Shc proteins to some extent dependent on Y766 in FGFR-1. This is the only known binding site in the receptor for SH2 domain-containing proteins, and it is also required for binding of PLCγ (Peters *et al*., 1992, Mohammadi *et al*., 1992). p66 Shc is tyrosine phosphorylated after binding to EGFR, it also can be serine/threonine phosphorylated, where the upstream kinase responsible is not known. Serine/threonine phosphorylation of p66 Shc prevents its EGFR association but not its binding to Grb2, so that it sequesters Grb2-Sos complex from the membrane and eliminates Ras activation (Okada *et al*., 1997). p66 Shc association with 14-3-3 proteins can also sequester it from membrane, resulting increased Grb2-Sos levels available to other Shc isoforms (Foschi, 2001). However, in Shc knockout mice, Ras activation can proceed normally (Liu *et al.*, 2000). One possible explanation offered is that Shc may amplify FGF signaling in the Grb2/Sos-Ras/MAPK pathway only when low concentrations of FGF is available.

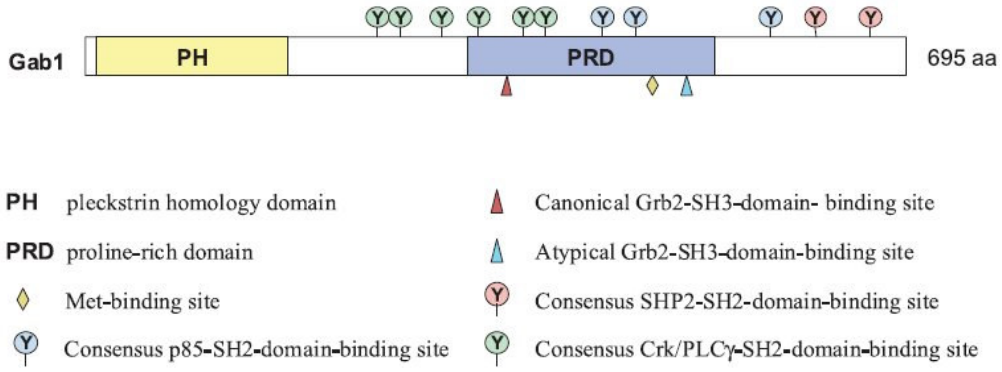

Figure 1.13. Domain structure and binding sites of Gab1. (Liu *et al.,* 2002)

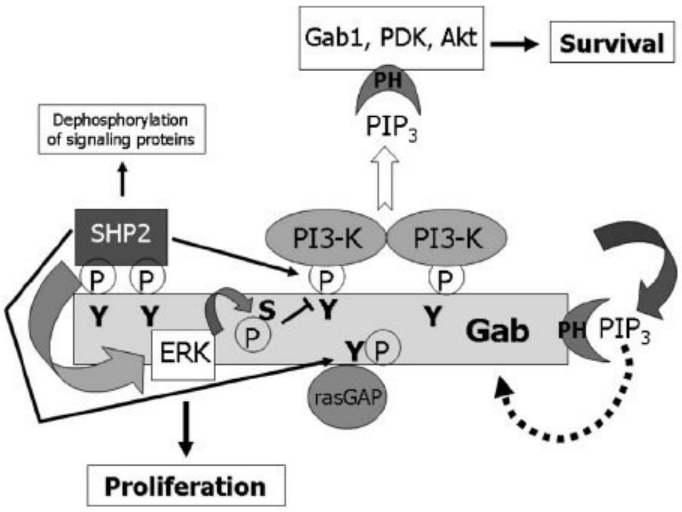

Figure 1.14. Possible molecular interactions of Gab1 (Sarmay *et al*., 2006)

1.7.3.5. Raf. The mammalian Raf family of serine/threonine kinases consists of three highly conserved members: A-Raf, B-Raf and C-Raf (Figure 1.15). Although C-Raf is ubiquitously expressed, A-Raf is mainly expressed in adult testis, epididymis, vas deferens, seminal vesicle, ovary, oviduct, bladder, kidney, intestine, heart, spleen, thymus, cerebellum and B-Raf is restricted to neuronal tissues (Wellbrock *et al*., 2004, Luckett *et al*., 2000). They share a common structure, with three conserved regions, first two CR1 and CR2 in the N terminus and the third CR3, which encodes the kinase domain, in the C terminus. In CR1, RAS-binding domain (RBD) is located upstream of cystine-rich domain (CRD), together they form Ras binding site that is the initiating event of Raf activation. Raf proteins differ in their activation. B-Raf only requires Ras-mediated membrane recruitment for activation. By contrast, A-Raf and C-Raf require further phosphorylation by tyrosine kinases such as Src and p-21 activated kinase (PAK) (Wellbrock *et al.,* 2004). The three Raf isoforms phosphorylate and activate downstream kinases (MEKs and ERKs) with quite different potencies: B-Raf is a strong MEK activator, A-Raf a weak MEK activator, and Raf-1 is intermediate between the two (Marais *et al*., 1997, Pritchard *et al*., 1995). Therefore, C-Raf and A-Raf only signal to fine-tune the duration of ERK activity and determine the cell fate (Wellbrock *et al*., 2004). In basal state Raf-C is phosphorylated by PKA at S43, S233 and S259 residues (S214 in A-Raf), and at S621 (S582 on A-Raf) by

an unknown kinase. At this stage the protein is in a complex with 14-3-3 and sequestered in the cytoplasm. GTP loaded Ras binds only when these residues are dephosphorylated by protein phosphatase-2A (PP2A) and translocated to the cell membrane, where S621 residue of C-Raf is again phosphorylated allowing 14-3-3 protein binding and dimerization of C-Raf. For full activation of C-Raf, Y341 and S338 residues (Y302 and S299 on A-Raf) should also be phosphorylated by Src and PAK. Moreover, phosphorylation of T491 and S494 within the activation loop is also required. The kinases that mediate S621, S404, T491 phosphorylations have not been identified and it is possible that their phosphorylation is due to autophosphorylation (Wellbrock *et al*., 2004). Fully activated Raf can phosphorylate MEK1 and MEK2 on two serine residues (S217 and S221 in MEK1) within their activation segments, they in turn phosphorylate threonine and tyrosine residues within the activation segments of ERK1 and ERK2 (T202/Y204 in ERK1) (Wellbrock *et al*., 2004). In Figure 1.15, the activation model of Raf is briefly illustrated.

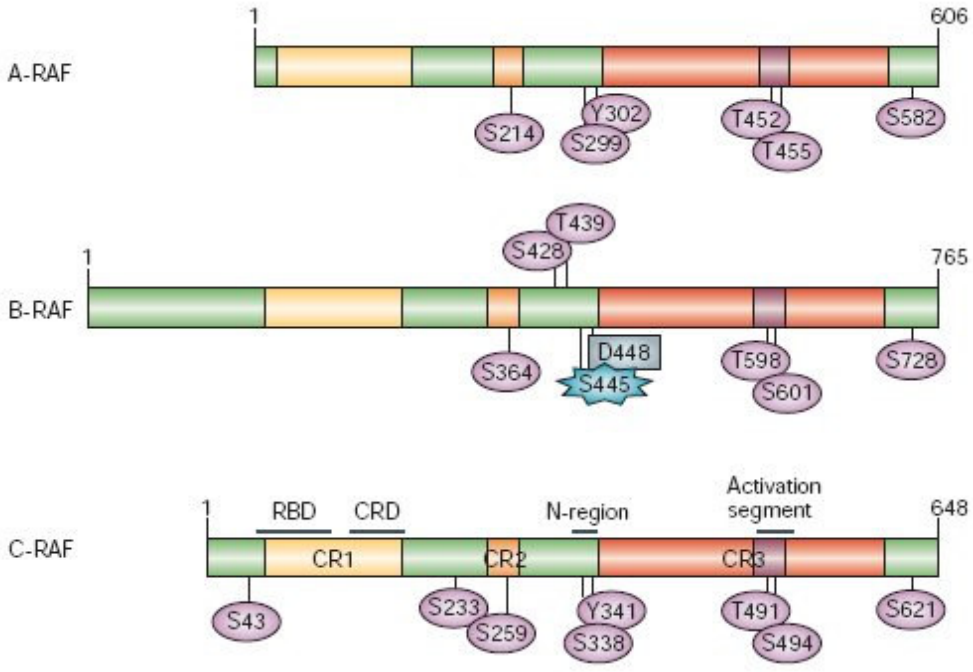

Figure 1.15. Structure of the Raf isoforms (Wellbrock et al., 2004) RBD: Ras Binding Domain, CRD: Cysteine Rich Domain, CR1-3: Conserved Region 1-3 (Wellbrock *et al*., 2004)

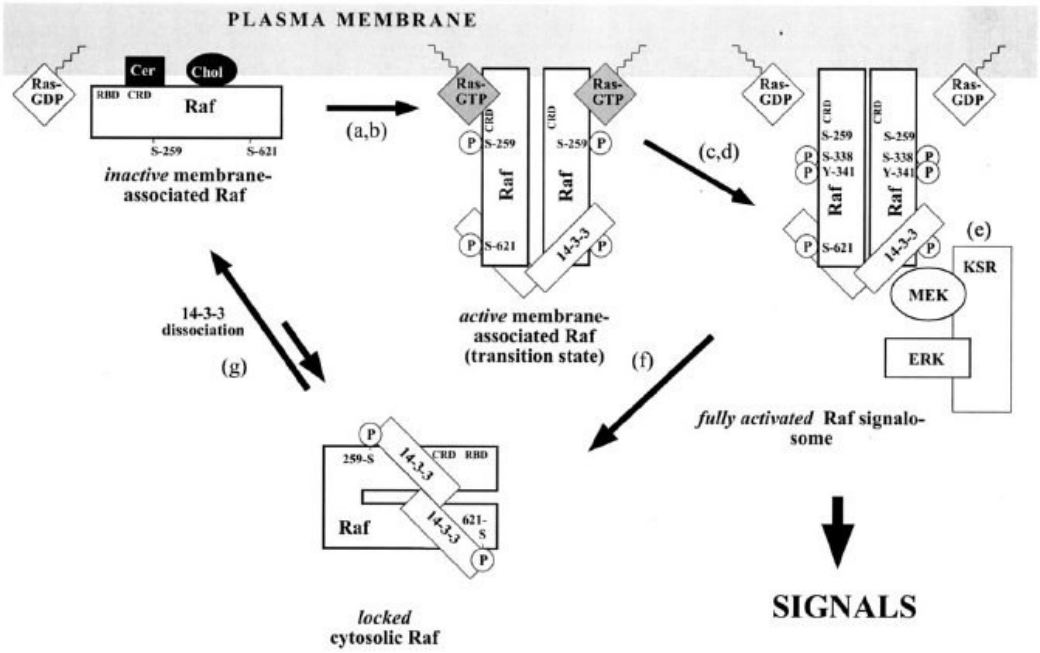

Figure 1.16. Model of Raf1 activation (Hekman, 2004)

2. PURPOSE

SIK2 is a serine/threonine kinase widely expressed in rat retina (Özcan, 2003; Özmen, unpublished data). It has been shown that SIK2 is involved in insulin signaling pathway in adipocytes via IRS1 phosphorylation and regulation of gene expression in pancreatic islet cells in response to blood glucose levels and gut hormones via Torc2 phosphorylation. However, researchers proposed that there should be other SIK2 substrates in different tissues and other signaling pathways (Horike *et al.*, 2003).

Since there are SH2 and SH3 binding motifs that would allow interaction with FGFR and a number of mediator proteins, and serine/threonine phosphorylation is a central mechanism for activation of FGF dependent pathways and their regulation (Eswarakumar *et al*., 2005; Özcan, 2003), we postulate that SIK2 may be implicated in FGF signal transduction pathways. Reports that PKA, an SIK2 upstream kinase, modulates FGF/MAPK activation (Pursiheimo *et al.*, 2002a) and FGF2 dependent transcription in fibroblasts requires PKA cooperation (Pursiheimo *et al*., 2002b) supports the hypothesis.

In order to identify a link between SIK2 and FGF signal transduction pathway we aimed

- To identify proteins containing SIK2 phopshorylation motif and involved in FGF signaling pathway by bioinformatic tools,
- To test whether these identified candidate proteins are the substrates of SIK2 performing in vitro kinase assay.

3. MATERIALS AND METHODS

3.1. Animals and Tissue Preparation

Albino Wistar rats bred at Boğaziçi University Psychology Department were used in this study and were treated in accordance with the University guidelines. Adult and newborn (postnatal day 0, P0) animals were anesthetized by ether inhalation and sacrificed by cervical dislocation. Dissected retinas were rapidly frozen in liquid nitrogen and they were stored at -80^0C.

3.2. Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (USA) or Merck (Germany) unless otherwise stated in the text. All solutions, plastic and glassware were sterilized by autoclaving at 121^0C for 20 minutes when possible. Solutions used in RNA work were treated with 0.1 per cent diethylpyrocarbonate (DEPC) for 1 hour before sterilization, glassware were baked at 150^0C for 4 hours.

3.3. Computational Analysis

3.3.1. Database Search for SIK2 Motif containing Proteins

The motif search program at the website www.scansite.mit.edu/motifscanner was used to identify proteins that contain the SIK2 phosphorylation motif, (Hy)[(B)X or X(B)]XX(S/T)XXX(Hy), in SwissProt database.

3.3.2. Primer Design

SIK2 primers were selected from rat cDNA cloned in our laboratory to generate fragments containing only the kinase domain (SIK2-KD) or containing kinase and UBA domains (SIK2-KD/UBA). Grb2, A-raf, and Shc1 primers were selected from published rat

sequences. The forward and reverse primers for Gab1, FRS2 primers were selected from regions, which show the highest homology between mouse and predicted rat sequences. Primer3 software was used in designing the primers and they were synthesized at Iontek (Istanbul, Turkey). pGEX primers were obtained commercially (Amersham Biosciences, USA) and used for sequencing of cloned fragments at Iontek.

The list of primers, their sequences, predicted product lengths and the experimentally determined annealing temperatures are given in Table 3.1.

Table 3.1. List of primers used in this study and optimal amplification conditions. The restriction enzyme sites included in the primers are indicated in paranthesis and the positions of primers are indicated in paranthesis under gene names.

GENE	GENEBANK ACCESION NUMBER	PRIMER SEQUENCE (5'- 3')		Product Length (bp)	Tm (^{0}C)
SIK2 -KD (1-813 bp)	DQ188032	Forward (BamHI)	AGA GGA TCC TTC ATG GTC ATG GCG GAT	833	56
		Reverse (EcoRI)	AGA GAA TTC TTA CAT CCA CTT GTG TTC CTT GA		
SIK2-KD/UBA (1-1008 bp)	DQ188032	Forward (BamHI)	AGA GGA TCC TTC ATG GTC ATG GCG GAT	1028	50
		Reverse (EcoRI)	AGA GAA TTC TAT TCC ACC AAC AAG AAA TAA AT		
FRS2 (1-1524 bp)	XM_235164 NM_177798	Forward (BamHI)	AGA GGA TCC CTG AAG AAG CCA TGG GTA GC	1544	58.1
		Reverse (SalI)	AGA CGC GGC CGC AGG ATT CCT CTC AGG GCC		
SHC1 (507-1400 bp)	NM_053517	Forward (BamHI)	AGA GGA TCC AGT GGG GAC CCG GAC ACA G	914	60
		Reverse (EcoRI)	AGA GAA TTC TCG ATC CAC AGG TTG CTG TA		
GRB2 (14-653 bp)	BC091144	Forward (BamHI)	AGA GGA TCC GAA GCC ATC GCC AAA TAT GA	635	59.5
		Reverse (EcoRI)	AGA GAA TTC TGC TTC TTA GAC GTT CCG GT		
GAB1 (3540-4124)	XM_341667 NM_021356	Forward (BamHI)	AGA GGA TCC CAT TTT GAC TCT GCC AAA CCC	605	59
		Reverse (EcoRI)	AGA GAA TTC ACG GTG GAA ATG GTG TTA CT		
A-RAF (1580-1775 bp)	NM_001033663	Forward (BamHI)	AGA GGA TCC CCG GAC CTC AGC AAA ATC TTC	215	62
		Reverse (EcoRI)	AGA GAA TTC AAC TCA TCA GCC TGG GTA CG		
pGEX (869-1041)	-	Forward	GGG CTG GCA AGC CAC GTT TGG TG	173	63
		Reverse	CCG GGA GCT GCA TGT GTC AGA GG		

3.4. Cloning of PCR Products into pGEX-2TKP Vector

3.4.1 RNA Isolation

RNA was isolated by acid phenol-chloroform method (Chomczynski and Sacchi, 1987) with slight modifications (Bugra and Hicks, 1997). Briefly, two retinas were homogenized in 800 µl guanidinium thiocyanate (GTC) solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 15 mM sodium N-lauryl sarcosine, and 0.1 M β-mercaptoethanol) using a hand-held tissue grinder. To the homogenate 1:10 volume of 2 M sodium acetate (pH 4.0), 1 volume of water saturated phenol and 1:5 volume of water saturated chloroform:isoamylalcohol (49:1) were added, vortexed after each addition. The samples were kept on ice for 15 minutes and centrifuged at 8000 xg for 20 minutes at 4°C. The aqueous phase, containing RNA, was transferred to a clean tube, equal volume of ice-cold isopropanol was added and RNA was precipitated at –20°C for 2 hours. After centrifugation for 25 minutes at 12000 xg at 4°C, the pellet was washed with 75 per cent ethanol and resuspended in water. To remove contaminating DNA, the samples were treated with 1 unit of RNase free RQ1 DNase (Promega, USA) in a buffer containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$ for 30 minutes at 37°C. With the addition of 10 volumes of GTC DNase was denatured and subsequently RNA was precipitated with equal volume of isopropanol at –20°C for at least 2 hours.

Precipitated RNA was dissolved in water, then reprecipitated with the addition of 1:10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol to remove any residual GTC. The precipitate was pelleted by centrifugation for 20 minutes at 12000 xg at 4°C, the pellet was washed in 75 per cent ethanol, air dried, and resuspended in water.

RNA quantification was made spectrophotometrically, where an OD260 of 1 is equal to 40 µg/ml of RNA. The samples with OD_{260}/OD_{280} ratios of 1.8-2.0 were considered pure.

3.4.2. Formaldehyde Agarose Gel Electrophoresis

To verify the quality of the isolated RNA, formaldehyde agarose gels were used. Approximately 1.5 µg total RNA was mixed with sample buffer (250 µl 100 per cent deionized formamide, 83 µl 37 per cent formaldehyde, 50 µl 10X MOPS (200 mM Morpholinopropane sulfonic acid (pH7.0), 80 mM sodium acetate, 10 mM EDTA), 50 µl Rnase-free glycerol, 10 µl 2.5 per cent bromophenol blue, 57 µl DEPC-treated water), heated to 65°C for 3 minutes and immediately chilled on ice. Then 0.1 µg ethidium bromide was added to the samples and fractionated on a 1 per cent formaldehyde agarose gel. The gel was prepared by melting of 5 g agarose in 127 ml H_2O and 14.19 ml 10X MOPS buffer and addition of 8.1 ml 37 per cent formaldehyde upon cooling to about 55^0C. The gel was run at 50 V. The bands were visualized under UV light and images were captured using GelDoc imaging system and analyzed by Quantity One software (BioRad, Hercules, CA, USA).

3.4.3. cDNA Synthesis

cDNA was synthesized using 1 µg of total RNA and 500 ng of random hexanucleotide primers, 120 nmol of each dNTP, 12 units of RNasin in reverse transcription buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT. The mixture was heated at 95^0C for 1 minute to denature RNA and immediately transferred on ice. The sample volume was brought to 30 µl with DEPC treated water subsequently 200 units of MMuLV-RTase (Promega, USA) and 30 units of RNasin (Promega, USA) was added. After 2 hours of incubation at 42°C, the reaction was stopped by heating the reaction mix to 95^0C for 10 minutes. The reaction volume was completed to 100 µl with water and stored at -20°C until used.

3.4.4. Reverse Transcriptase coupled Polymerase Chain Reaction

Amplifications were carried out using 2µl cDNA, 0.2 mM dNTP mixture, 3mM $MgCl_2$, 0.25 µM primer pairs, 25 mM N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercapthoethanol and 0.5 units of TaKaRa Ex Taq™ (Takara Bio. Inc., Japan) in a total

volume of 25 µl. The amplification reaction was started with initial denaturation at 95^0C for 5 minutes followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at the indicated temperature (Table 3.1) for 45 seconds and extension at 72°C for 90 to 120 seconds depending on the product length.

3.4.5. Agarose Gel Electrophoresis

Amplification products or other DNA samples were mixed with 1/6 volume of 6X loading buffer (250 mg bromophenol blue, 550 mg xylene cyanol in 33 ml 150 M Tris, pH 7.6, 60 ml glycerol and 7 ml H_2O) and loaded onto 1 or 2 per cent agarose gels prepared in 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid) and addition of 0.5 µg/ml ethidium bromide. The gels were run in the same buffer at 120 V. Molecular weight markers, Gene Ruler 1kb DNA ladder and DNA Ladder Mix were purchased from Fermentas (Lithuania). The DNA bands were visualized under UV light and the images were documented with GelDoc imaging system (BioRad, USA).

3.4.6. Preparation of the Vector and the Amplification Products for Cloning

pGEX-2TKP vector (Figure 3.1) and the RT-PCR fragments were incubated with 1 unit BamHI and 1 unit EcoRI (Promega, USA) restriction enzymes per 1 µg of DNA in Buffer E containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 50 mM NaCl at 37^0C overnight to generate ends for directional cloning. Samples were fractionated on 1 or 2 per cent agarose gels and appropriate DNA fragments cut out of the gel under UV light and extracted using QIAQuick Gel Extraction Kit (Qiagen, Germany) according to manufacturers instructions. Briefly, sliced gel was solubilized in a buffer supplied with the kit containing pH indicator, loaded on a spin column, cetrifuged at 10000 xg for 1 minute. Then the column was washed with buffer containing absolute ethanol, DNA was eluted with Tris-EDTA (TE) (pH 8.0) buffer containing 10 mM Tris-Cl (pH 8.0), and 1 mM EDTA. DNA concentrations were obtained by measuring optical density at 260 nm (OD_{260} value of 1 corresponds to 50 µg/ml of DNA).

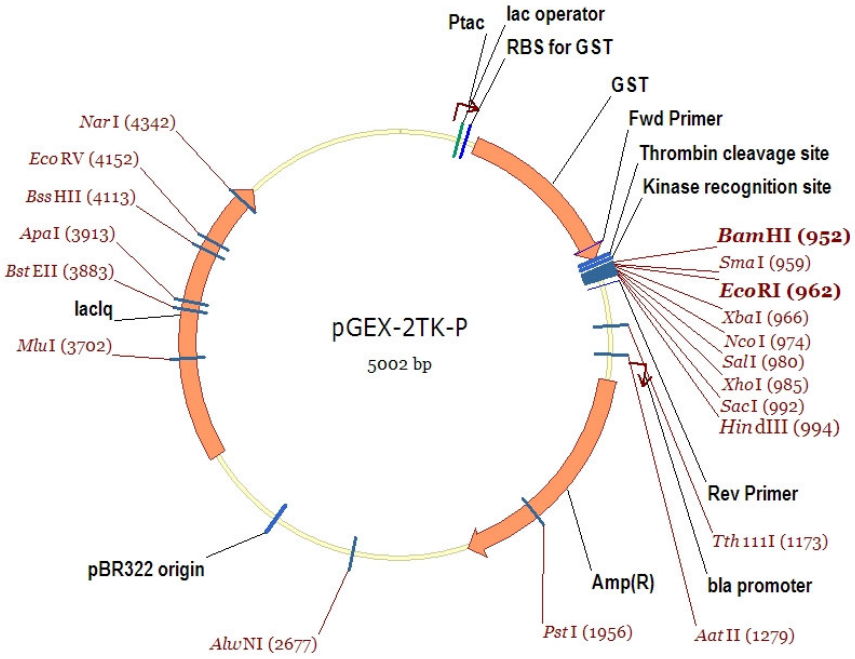

Figure 3.1. pGEX-2TKP vector map (Stratagene, USA)

3.4.7. Ligation

BamHI/EcoRI cut pGEX-2TKP vector and cDNA fragments were ligated in 1:5 end ratio. The reactions were carried out using 50 ng vector in DNA ligase buffer (400 mM Tris-Cl, pH7.8, 100 mM MgCl2, 100 mM DTT, 5 mM ATP), 1 unit of T4 DNA ligase and the appropriate amount of amplification fragments (Promega, USA) at 4^0C overnight.

3.4.8. Competent Cell Preparation

Stock of frozen BL21 bacterial cells were streaken on LB agar plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 18 g/l agar) and incubated overnight at 37^0C. A well isolated individual colony was transferred and grown in 5 ml of liquid LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) overnight at 37^0C. This overnight culture was used to inoculate 50 ml LB medium with 1:100 ratio and grown at 37^0C until OD_{600} reached 0.3-0.4. Then, the cultures were chilled on ice for 30 minutes and centrifuged at

4^0C for 5 minutes at 5000 rpm. The bacterial pellet was resuspended in 12.5 ml of ice-cold 50 mM $CaCl_2$, incubated on ice for 15 minutes. The bacteria were collected as before, resuspended in 2 ml of ice-cold 50 mM $CaCl_2$ containing 15 per cent glycerol. The samples were aliquoted, 200 µl each, stored at -70^0C until used.

3.4.9. Transformation

For efficient BL21 transformation, 100 µl of frozen BL21 competent cells were mixed with 12.5 ng of ligation mixture, and another 100 µl were mixed with uncut pGEX-2TKP vector as a control of transformation efficiency. They were kept on ice for 30 minutes, heat-shocked at 42^0C for 90 seconds and cold-shocked on ice for 2 minutes. Subsequently, 900 µl of SOC medium (2 g tryptone, 0.5 g yeast extract, 1 ml 1 M NaCl, 0.25 ml 1 M KCl, 1 ml 1 M $MgCl_2$, 1 ml 2 M glucose in 1 l dH_2O) was added and cells were allowed to grow at 37^0C for an hour with shaking at 250 rpm. Subsequently, 100 µl and also the residual bacterial suspensions were plated on LB plates containing 100 µg/ml ampicillin and grown overnight at 37^0C.

3.4.10. Colony Polymerase Chain Reaction

To verify that the colonies grown upon transformation contain the desired gene fragments, individual colonies were transferred using sterilized toothpicks into PCR tubes with 10 µl dH_2O, boiled for 10 minutes to lyse the cells and the amplifications were carried out in the presence of 0.2 mM dNTP mixture, 3mM $MgCl_2$, 0.25 µM pGEX primer pair (Table 3.1), 1X Taq polymerase buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8 per cent Nonidet P40) and 0.5 units of Taq polymerase (Fermentas, Lithuania) in a total volume of 20 µl. The amplification reaction was started with initial denaturation at 95^0C for 5 minutes followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 63^0C for 45 seconds and extension at 72°C for 90 to 120 seconds depending on the expected product length.

3.4.11. Plasmid DNA Isolation and Sequencing

Selected colonies were grown overnight in LB medium containing 100 μg/ml ampicillin at 37^0C with shaking at 225 rpm and plasmids were isolated with MiniPrep kit (Qiagen, USA). Briefly 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 minutes. Supernatant was applied to the QIAprep spin columns where DNA binds to the silica gel membrane in the presence of high salt. Impurities were removed by washing with buffer containing absolute ethanol and plasmid DNA was eluted with TE buffer (pH 8.0). Plasmids were analyzed in 1 per cent agarose gels, and were sequenced at Iontek (Turkey) using pGEX primers (Table 3.1).

Stocks of bacteria carrying plasmids with the desired fragments were prepared by mixing overnight bacterial cultures with 15 per cent glycerol and stored -80^0C until used.

3.5. Expression of GST Fusion Proteins in E.coli BL21

E.coli BL 21 strain transformed with the pGEX recombinant plasmids grown overnight in LB with 100 μg/ml ampicillin were inoculated into 12 ml LB containing 100 μg/ml ampicillin with 1:100 ratio and allowed to grow until OD_{600} of 0.5 reached. The fusion protein expression was induced by adding IPTG to a final concentration of 0.2 mM, subsequently bacteria was allowed to grow at 28°C for 2 hours. Bacteria were sedimented by centrifugation at 10000 xg for 2 minutes. The bacterial pellet was resuspended in 600 μl of ice-cold resuspension buffer containing protease inhibitors, 10 μg/ml leupeptin, and 5 μg/ml aprotinin and 1mM phenylmethylsulphonylfluouride (PMSF) in 1X phosphate buffered saline (PBS), composed of 140 μM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, (pH 7,3). For lysis, cells were sonicated 3 times for 3 minute duration each at 95 volt on ice, 1 per cent Triton X-100 was added and incubated on ice for 30 minutes. After solubilization of proteins, cell debris was pelleted with centrifugation at 10000 xg at 4^0C for 45 minutes. The supernatant containing the expressed fusion proteins was transferred to fresh tube and stored -80^0C until purification by GST- affinity column chromatography.

3.6. Affinity Purification of GST Fusion Proteins

GST fusion proteins were purified from bacterial cell extract using glutathione Sepharose 4B MicroSpin affinity columns (Amersham, USA). Bacterial sonicates were loaded onto the columns and incubated at room temperature with delicate shaking, to allow GST fusion proteins bind glutathione sepharose 4B resin, for 10 minutes. Then, to remove residual unbound proteins the column was centrifuged at 3000 rpm for a minute and the column was washed with 600 µl of 1X PBS three times. Bound proteins were released with the addition of elution buffer, 10mM glutathione in 50 mM Tris-Cl (pH 8.0), and collected by centrifugation for a minute at 3000 rpm.

3.7. SDS-PAGE and Western Blotting

Affinity purified fusion proteins fractionated on 10 per cent polyacrylamide gels (10 per cent acrylamide:bisacrylamide (29:1), 375 mM Tris.HCl (pH 8.8), 0.1 per cent SDS, 0.1 per cent ammonium persulfate, 0.1 per cent N,N,N',N'-tetramethylethylenediamine) with 5 per cent stacking gel (5 per cent acrylamide:bisacrylamide (29:1), 125 mM Tris.HCl (pH 6.8), 0.1 per cent SDS, 0.1 per cent ammonium persulfate, 0.1 per cent N,N,N',N'-tetramethylethylenediamine). Samples were boiled in 3X sample buffer (150 mM Tris-Cl, pH 6.8, 300 mM DTT, 6 per cent SDS, 0.3 per cent bromophenol blue, 30 per cent glycerol) at 95^0C for 5 minutes before loading. The gel was run in 25 mM Tris-HCl, 250 mM glycine and 0.2 per cent SDS buffer at 100-120 V.

Coomassie Blue staining was done after incubation of the gel in fixing solution (50 per cent methanol, 10 per cent acetic acid in water) for 1 hour. Subsequently, gel was gently shaken in Coomassie Blue solution (50 per cent methanol, 0.05 per cent Coomassie R250, 10 per cent acetic acid) for at least 2 hours. Gels were rinsed with fixing solution for 5 minutes, then, washed extensively with destaining solution (5 per cent methanol, 7 per cent acetic acid in water). The gel was dried in the gel dryer for 1 hour at 80^0C and scanned for documentation.

For Western blotting, the samples fractionated on polyacrylamide gels were electroblotted to polyvinyl difluoride (PVDF) membranes (Roche, Germany) in transfer

buffer (200 mM glycine, 25 mM Tris.HCl, 15 per cent methanol) at 100 V for 1-1.5 hours. To equilibrate the membrane, it was washed in tris buffered saline-tween (TBST) solution (150 mM NaCl, 20 mM Tris.HCl, pH 8.0, 0.1 per cent Tween 20) three times for total of 30 minutes. The membranes were incubated in blocking solution (1 per cent bovine serum albumin (BSA), 0.5 per cent skimmed milk powder in TBST) for 1 hour at room temperature with gentle shaking. The membrane was left overnight in the blocking solution containing horse radish peroxidase (HRP) conjugated anti-GST antibody (Santa Cruz Biotechnology, USA) at 4^0, where the antibody was diluted in 1:1000 ratio. Subsequently, the membrane was washed with TBST three times for 10 minutes each to remove unbound antibody and incubated in Lumi-light Western blotting substrate (Roche, Germany) for 5 minutes, exposed to Lumi-light chemiluminescence detection film (Roche, Germany) for varying times. The bands were visualized by immersing the film in the developer solution (Kodak, USA) for 30 seconds to 3 minutes, then in tap water, and finally in the fixative solution (Kodak, USA) for 3 minutes.

3.8. Bradford Assay

For determination the concentration of proteins Bradford assay (BioRad, USA) was used. Unknowns and BSA dilutions ranging from 0.2 to 1.4 mg/ml were mixed with 5 ml of 1:5 diluted Bradford dye reagent. After a period of 5 minutes, absorbance measured at 595 nm. Unknown sample concentrations were read from the standard curve.

3.9. *In vitro* Kinase Assay

The *in vitro* autophosphorylation assay and kinase assays were performed according to the method of Feldman et al., 2000. Briefly, reactions containing approximately 2 µg of purified GST-fusion proteins, 1µl of radioactive ATP cocktail containing 100 µM cold ATP, 1 µCi [γ^{32}P]ATP (3000 Ci/mmol) and kinase buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$) were set up at room temperature. The reactions were initiated by the addition of 0.25 µg of purified GST-SIK2 (KD) and for autophosphorylation assay, 2 µg of GST fused SIK2 proteins were added into reaction buffer without any GST-fusion protein, incubated at room temperature for 30 minutes. The reactions were terminated by the addition of 3X sample buffer (150 mM Tris-HCl, pH 6.8),

300 mM DTT, 6 per cent SDS, 0.3 per cent bromophenol blue, 30 per cent glycerol) and boiling at 95^0C for 5 minutes. The reaction mixtures were run on 10 or 12 per cent SDS-PAGE, gel was fixed, and dried as described before. The gels were exposed to X-ray film for varying times at -70^0C.

4. RESULT

4.1. Database Search for SIK2 Motif containing Proteins

Using the motif search program at the web site www.scansite.mit.edu proteins that contain the SIK2 phosphorylation motif, (Hy)[(B)X or X(B)]XX(S/T)XXX(Hy) and involved in FGF/ERK pathway were searched for. Results indicated that A-Raf, Gab1 and Grb2 contain the motif (Table 4.1), therefore considered to be strong candidates as SIK2 targets. FRS2 and Shc1 have been proposed to be the main regulatory elements in feedback regulation of the FGF signal transduction pathway via serine/threonine phosphorylation (Okada *et al.,* 1997, Wu *et al*., 2003, Yayon *et al*., 1999). Even though the search identified no canonical SIK2 phosphorylation motif in these proteins, because of their proposed regulatory roles and presence of numerous serine and threonine residues, they were included in our study.

Table 4.1. Candidate Proteins containing SIK2 phosphorylation motif. Red: conserved hydrophobic residues in motif which are L, V, I, M, G, A, green: conserved basic residues that are K, R, H, blue: serine or threonine residues, black: any amino acid

Canonical SIK2 phosphorylation Motif			
(Hy)[(B)X or X(B)]XX(S/T)XXX(Hy)			
Protein Name	**Motif**	**Position**	**SwissProt No.**
A-Raf	**IERSASEPSL**	575-584	NP_071977
Gab1	**LPRSYSHDVL**	1242-1251	XP_341668
Grb2	**VVKFNSLNEL**	122-131	NP_110473

4.2. RNA Isolation

Total RNA isolated from adult rat retina tissue was run on 1 per cent formaldehyde gel to control RNA quality and the bands were visualized in GelDoc imaging system. An example of the RNA preparations is shown in Figure 4.1. Only the RNA samples with the same profile as in the figure were consired intact and used in further cloning experiments.

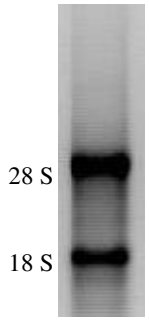

Figure 4.1. Total RNA from adult rat retina

4.3. Cloning of SIK2 and the Candidate Target Genes

4.3.1. Generation of Cloning Fragments

Isolated total rat retinal RNA was reverse transcribed with random hexamers and the cDNA population was used as template to generate SIK2, Gab1, Raf, Grb2, Shc1 and FRS2 fragments. The primer sequences and the annealing temperatures are given in Table 3.1, the size of the amplification products including 20 bp of BamHI and EcoRI linkers obtained correspond to the predicted sizes from the sequence data (Figure 4.2). Shc1, Grb2 and FRS2 fragments (914 bp, 635 bp, and 1544 bp, respectively) spans most of the coding regions. On the other hand, the Gab1 and Raf fragments of 605 bp and 215 bp, respectively, are partial. Raf itself is a kinase and has autophosphorylation activity, where the target site coincides with the putative SIK2 phosphorylation site (S580). Therefore, its carboxyl terminal region spanning a small part of the kinase domain and the SIK2 motif containing region (ORF: 1580-1775 bp) was chosen in this study to eliminate its kinase activity which would be a serious handicap in the later kinase assays. Our attempts to clone the full length Gab1 fragment failed, thus we resorted the clone, a region containing the motif (ORF: 3540-4124 bp). The kinase domain of SIK2 protein shown not to have catalytic activity on its own, but when expressed with the UBA the ability of substrate phosphorylation is similar to the full-length protein (Jaleel et al., 2006). On the basis of these reports, a 833 bp fragment containing the kinase domain SIK2-KD was chosen to be used as kinase-inactive form; and a 1028 fragment, SIK2-KD/UBA, spaning the kinase and the UBA domains as the kinase- active form of the protein.

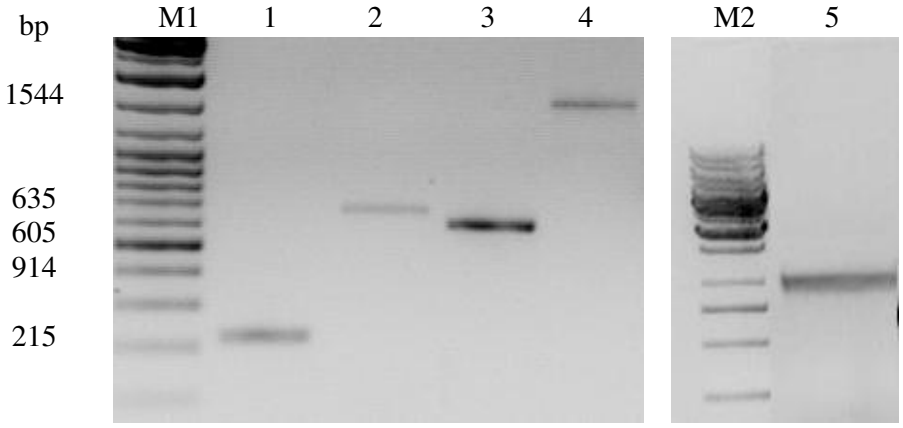

Figure 4.2. RT-PCR fragments of candidate substrates. Lane 1: A-Raf, lane 2: Grb2, lane 3: Gab1, lane 4: FRS2, lane 5: Shc1, M1: Gene Ruler DNA Ladder Mix, M2: Gene Ruler 1kb DNA ladder

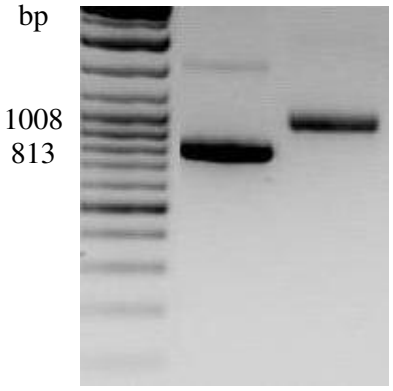

Figure 4.3. RT-PCR fragments of SIK2. Lane 1: SIK2-KD, lane 2: SIK2-KD/UBA, M: Gene Ruler 1 kb DNA ladder

4.3.2. Cloning of the Fragments

BamHI/EcoRI double digested amplification products of SIK2-KD, SIK2-KD/UBA, Gab1, A-Raf, Grb2, Shc1 and FRS2 DNA fragments and pGEX-2TKP vector were ligated in 1:5 end ratio. Ligation products were transformed into BL21 bacterial cells and the recombinant plasmid containing colonies were selected with colony PCR using pGEX

primers (Table 3.1). Empty pGEX-2TKP vectors give nearly 150 bp bands when amplified with these primers. SIK2-KD, SIK2-KD/UBA, Gab1, A-Raf, Shc1, FRS2 and Grb2 ligated to pGEX-2TKP plasmids are expected to give 963, 1158, 735, 345, 1044, 1674, and 765 bp products, respectively. Therefore colonies containing the predicted size inserts were selected as positive clones (Figure 4.4). Plasmids isolated from these colonies (figure 4.5) were sequenced to verify their identity (data not shown).

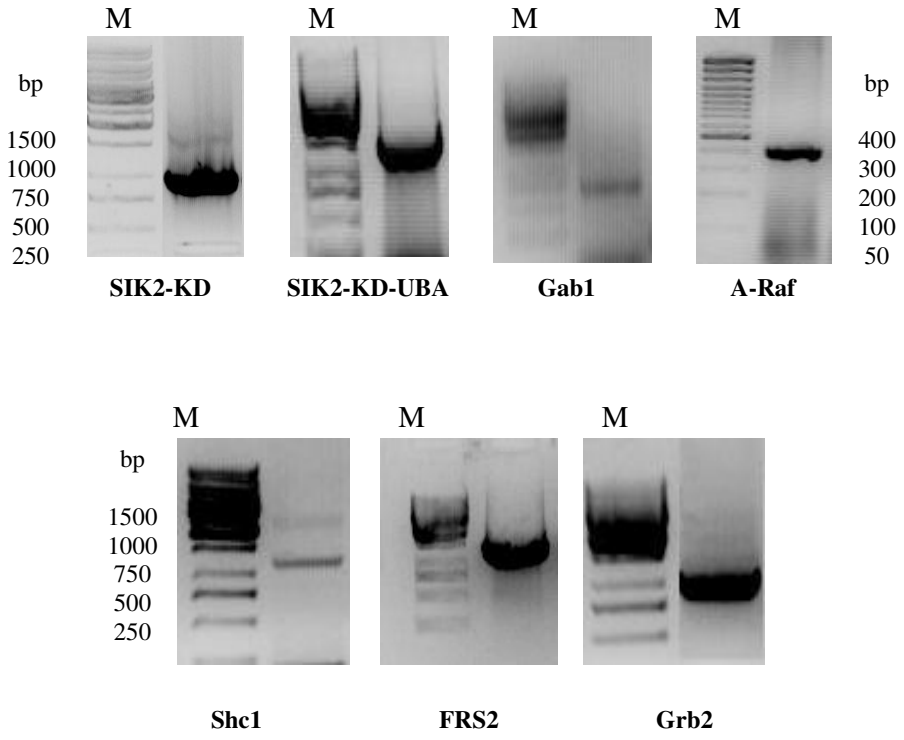

Figure 4.4. Colony PCR fragments. Markers (M) are GeneRuler 1 kb DNA ladder except GeneRuler 100 bp ladder for A-Raf

4.4. Expression and Affinity Purification of GST-Fusion Proteins

Recombinant pGEX-2TKP plasmid containing BL21 strains were induced using 0.2 mM IPTG for the fusion protein expression. Subsequently the bacterial lysate was passed through Glutathione Sepharose 4B affinity columns to purify the fusion proteins. The column eluates fractionated on SDS-PAGE and Western blotts carried out using HRP conjugated anti-GST antibody and detection was made with chemiluminescence. In the

lysates of bacteria transformed with the empty pGEX-2TKP a 27 kDa band was detected as expected (Figure 4.6, Lane1). The lysates of the bacteria carrying Grb2 recombinant plasmid and the one harboring Gab1 each gave single band at 50 kDa and Raf a band of 38 kDa, in agreement with the predicted sizes (Figure 4.6, Lane 2, 3 and 5, respectively). We estimated that the size of the GST-FRS2 fusion protein would be 61 kDa and that of Shc1 83 kDa. The highest bands in these lysates conform to the expected sizes, however we also detected degradation products (Figure 4.6, Lane 6 and 4). Western blot analysis of the SIK2-KD and SIK2-KD/UBA is shown in Figure 4.7. Both SIK2-KD and SIK2-KD/UBA have two distinct bands corresponding 57, 35 kDa and 64, 60 kDa, respectively. Upper bands have the expected sizes, and lower molecular weight ones are likely to be degradation products.

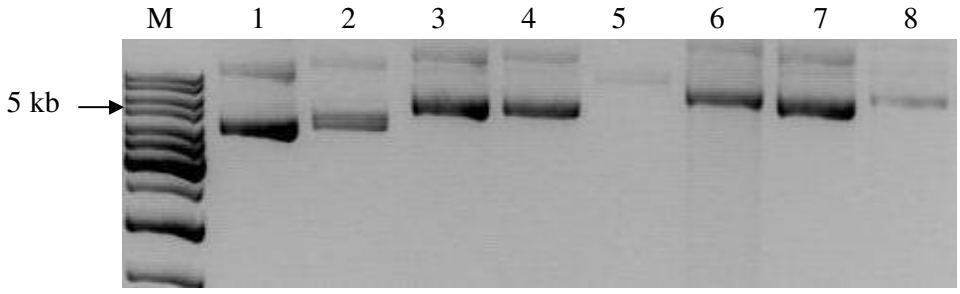

Figure 4.5. Recombinant plasmids. Lane1: Plasmid corresponding to empty pGEX-2TPK and lane 2-8: pGEX ligated to A-Raf-C', Grb2, Gab1, FRS2, Shc1, SIK2-KD, and SIK2-KD/UBA, M: Gene Ruler DNA Ladder Mix

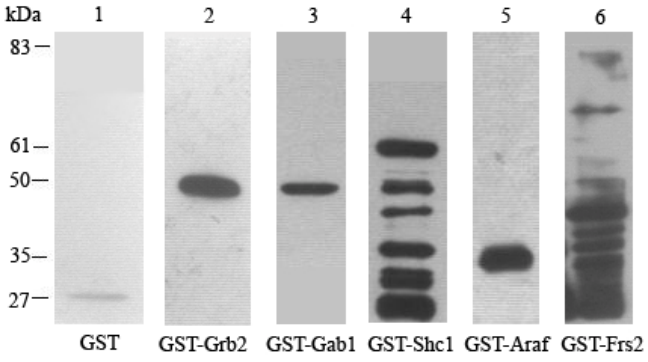

Figure 4.6. Western blot analysis of affinity purified GST-fusion proteins. Lane1: GST, lane2-6: GST fused Grb2, Gab1, Shc1, A-Raf and FRS2, respectively

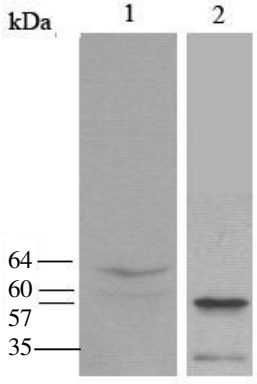

Figure 4.7. Western blot analysis of affinity purified GST fused SIK2 proteins. Lane 1: SIK2-KD/UBA, lane 2: SIK2-KD

4.5. In *vitro* Kinase Assay

4.5.1. Autophosphorylation Assay of SIK2-KD and SIK2-KD/UBA

In literature SIK2's autophosphorylation ability and the increase of its kinase activity via autophosphorylation has been reported (Horike *et al.,* 2003). Therefore, autophosphorylation assays were done with SIK2-KD and SIK2-KD/UBA in the presence of [γ^{32}P] ATP at 30^0C for an hour, followed by SDS-PAGE fractionation and autoradiography. Results indicate that SIK2 kinase domain alone show no autophosphorylation activity (Figure 4.7A, lane 2). When SIK2-KD/UBA was used we detected two bands with approximately 60 and 64 kDa (Figure 4.7A, lane 3) as in the Western blots (Figure 4.7). These results confirm the earlier reports that kinase activity of SIK2 is enhanced by UBA domain (Jaleel *et a*l., 2006). Therefore SIK2-KD/UBA protein is used in the later experiments for phosphorylation assays.

In negative controls GST purified from empty vector harboring bacteria was used, and as expected no phosphorylation activity was detected on autoradiograms (Figure 4.7, panel A, lane 1).

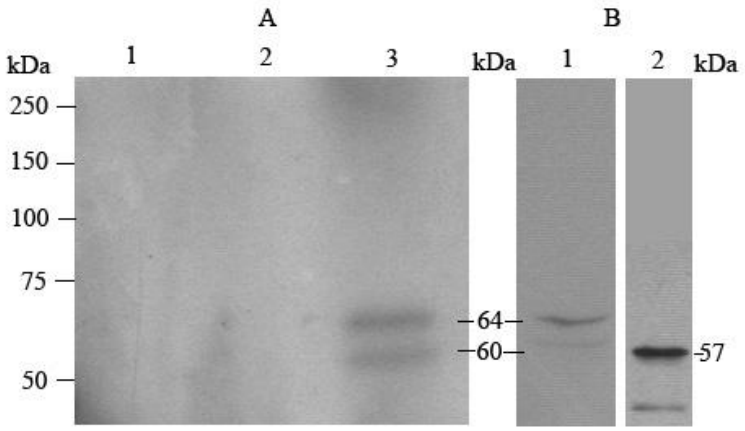

Figure 4.7. Autophosphorylation of SIK2. Panel A. Autoradiogram following the kinase assay. Lane1: GST, lane2: GST-SIK2-KD, lane 3: GST-SIK2-KD/UBA. Panel B. Western blot analysis with anti-GST antibody. Lane 1: GST-SIK2-KD/UBA, lane 2: GST-SIK2-KD

4.5.2. *In vitro* Kinase Assay of GST-Fusion Proteins with SIK2-KD/UBA

To test whether GST-FRS2, Shc1, Grb2, Gab1 and A-Raf proteins can be substrates of SIK2, *in vitro* kinase assay was done using GST-SIK2-KD/UBA and the candidate substrates in the presence of [γ^{32}P] ATP at 30^0C for 1 hour.

We detected kinasing of the GST-Gab1 and GST-A-Raf (Figure 4.8, lane 1 and Figure 4.9 lane 1) in the presence of SIK2-KD/UBA protein. Sizes of these bands, 50 kDa for Gab1 and 35 kDa for A-Raf, correspond to the Western blot data (Panel B in Figure 4.8 and 4.9). In samples where SIK2 was omitted (Figure 4.9 and 4.10, lane 3) no Gab1 or A-Raf phosphorylation was observed, indicating that these proteins showed no autophosphorylation activity. In the same vein with the SIK2 autophosphorylation experiments, when SIK2-KD was used in place of SIK2-KD/UBA (Figure 4.9 and 4.10 lane 2) kinasing of these candidate proteins was not achieved.

Parallele experiments done using GST-Grb2, GST-Shc1 and GST-FRS2 proteins did not result in their phosphorylation (Figure 4.10).

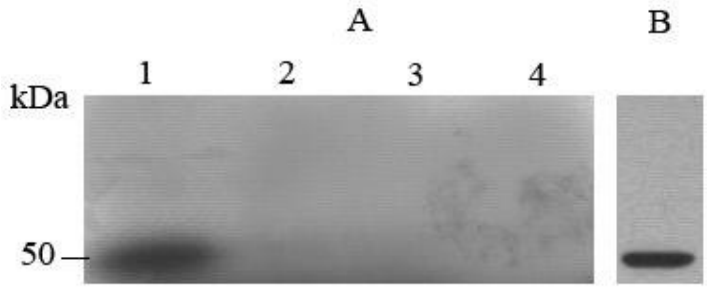

Figure 4.8. Analysis of Gab1 as potential SIK2 substrate. Panel A. Autoradiogram was obtained following the kinase assay. Lane1: SIK2-KD/UBA and GST-Gab1, lane 2: SIK2-KD and GST-Gab1, lane 3: GST-Gab1, lane 4: SIK2-KD/UBA and GST. Panel B. Western blot analysis of GST-Gab1 with anti-GST antibody

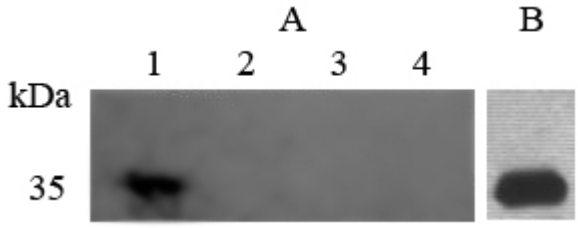

Figure 4.9. Analysis of A-Raf as potential SIK2 substrate. Panel A. Autoradiogram was obtained following the kinase asay. Lane1: SIK2- KD/UBA and GST-A-Raf, lane 2: SIK2-KD and GST-A-Raf, lane 3: GST-A-Raf, lane 4: SIK2-KD/UBA and GST. Panel B. Western blot analysis of GST-A-Raf with anti-GST antibody

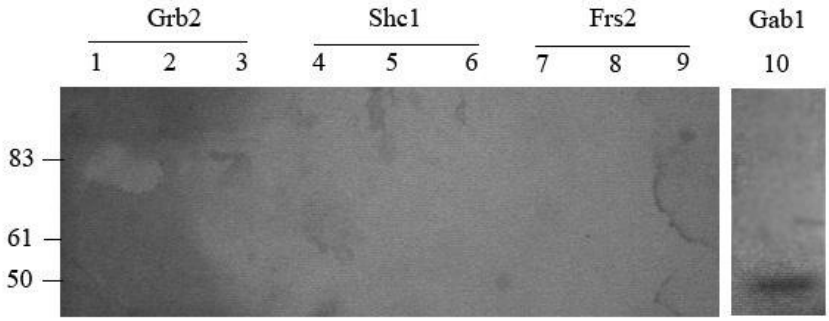

Figure 4.10. Analysis of Grb2, Shc1 and FRS2 as potential SIK2 substrates. Autoradiogram was obtained following in vitro kinase assays. Lanes 1, 4, 7 and 10: assays were done in the presence of SIK2-KD/UBA, lanes 2, 5, 8: GST-Grb2, GST- Shc1 and GST-FRS2 proteins assayed with kinase inactive SIK2-KD, lanes 3, 6, 9: GST-Grb2, GST- Shc1 and GST-FRS2 proteins assayed alone as autophosphorylation controls

5. DISCUSSION

SIK2 is a recently identified serine/threonine kinase. So far two SIK2 substrates have identified, IRS1 in mature adipocytes and TORC2 in pancreatic cells. It has been proposed to contribute to insulin resistance in diabetic mice adipocytes by phosphorylating IRS1 S789 residue, known to attenuate insulin signaling either by interfering its association with the insulin receptor at the plasma membrane, or preventing docking of downstream effectors such as PI3 kinase (Gual *et al*., 2005, Horike *et al*., 2003). Second known SIK2 substrate is TORC2 is implicated in regulation of CREB dependent gene expression in pancreatic islet cells in response to blood glucose levels and gut hormones (Screaton *et al.*, 2004). In this system SIK2 was proposed to phosphorylate TORC2 at Ser171, allowing its interaction with 14-3-3 proteins, thus TORC sequestered in the cytoplasm and is unable to stimulate CREB dependent gene transcription (Screaton *et al*., 2004). PKA and LKB1 have been experimentally shown as the upstream SIK2 kinase (Horike *et al*., 2003, Screaton *et al*., 2004). It has been suggested that there might be other SIK2 substrates in different tissues that act in other signalling pathways (Horike *et al*., 2003).

SIK2 carboxyl-terminal region was obtained in a yeast two-hybrid screen of a retinal cDNA library using cytoplasmic domain of FGFR2 as bait (Özcan, 2003). Subsequently, full-length clone was obtained and the analysis showed that a direct interaction between the two proteins is an artifact (Uysal, 2005). However, the following issues makes it plausible that SIK2 may interact with the FGF pathway components and regulate signal transduction: 1) The presence of SH2 and SH3 binding motifs in SIK2 would allow interaction with a number of signal mediator proteins with SH2 and SH3 domains, e.g., Src, FRS2, Grb2, PI3 kinase, and Shp2 (Schlessinger, 1994). 2) Serine/threonine phosphorylation is a central mechanism in FGF depent activation of MAPK pathways and differential threonine phosphorylation of components, especially FRS2, has been suggested to modulate the kinetics of this cascade and/or instrumental in feedback regulation (Eswarakumar *et al*., 2005; Özcan, 2003). 3) Activity and duration of growth factor induced Ras/ERK pathway has been suggested to be regulated by PKA, a SIK2 upstream kinase, dependent pathways (Pursiheimo *et al*., 2002a) and in fibroblasts FGF2 dependent transcription from FIRE requires cooperation of PKA pathways (Pursiheimo *et al*., 2002b).

In order to identify a link between SIK2 and FGF signal transduction pathway components, canonical SIK2 phosphorylation motif was searched in subsite in SwissPort database. The computational analysis revealed three proteins Grb2, Gab1 and A-Raf with this motif. In addition to these three proteins, due to the presence of several potential serine and threonine residues, and their importance as regulation or bifucation point in FGF pathways, FRS2 and Shc1 were included in this study. The fragments of coding regions of all the candidate proteins were generated by RT-PCR, cloned into pGEX-2TKP vector and expressed in bacteria as GST fusion proteins to be used in *in vitro* kinase assays. Due to the problems of amplification of full length Gab1, only a peptide containing SIK2 phosphorylation motif was cloned. Also, because A-Raf has been proposed to have an autophosphorylation activity, its carboxyl terminal region spanning a small part of the kinase domain and SIK2 motif-containing region was cloned. For the other substrate candidates cloned fragments were almost full length. A recent study indicated that kinase domain of SIK2 by itself has no catalytic activity, but together UBA domain substrate phosphorylation is restored (Jaleel et al., 2006). Therefore, a fragment spaning kinase and UBA domains (SIK2-KD/UBA), as well as the kinase domain (SIK2-KD) fragments were cloned.

We confirmed in this study that the kinase domain alone has no catalytic activity as it failed to show autophosphorylation. In the same vein, none of the candidate substrates were phosphorylated in vitro by the SIK2-KD peptide. However, UBA domain containing construct efficiently autophosphorylated, veryfying the importance of this domain in SIK2 kinase activity.

Among the candidates that contain the SIK2 consensus phosphorylation sequence, Gab1 and A-Raf was kinased by SIK2-KD/UBA, but not Grb2. These results suggest that Gab1 and A-Raf are potential in vivo targets of SIK2 and phosphorylation sites are likely to be S266 and S580 residues, respectively. We also have to keep in mind that Grb2 assumes a three-dimentional structure in bacteria other than the native form, where SIK2 target sequence is masked.

When the amino acid sequences of the motif found in Gab1 and A-Raf is compared with that of Grb2, different residues detected at positions 1 and 4. Phosphorylated proteins

have L or I as opposed to V in unphosphorylated Grb2. This is in agreement with the IRS1 and TORC2 motif (Horike *et al*., 2003, Screaton *et al*., 2004), therefore SIK2 targets should contain L/I at position 1 of the motif, formerly proposed to be any hydrophobic amino acid, (Hy)[BX or XB]XXS/TXXX(Hy). At position 4 in the motif of all SIK2 phosphorylated proteins S or T residue is seen, Grb2 at this position has phenylalanine. The L at the last position of the motif appears to be conserved in all the above mentioned proteins including Grb2. We think the SIK2 phosphorylation motif may to be restricted to (L/I)[(B)X or X(B)] (S/T)X(S/T)XXXL sequence. We detected no phosphorylation of the tested proteins that do not harbor the motif, FRS2 and Shc1, pointing to the importance of the consensus sequence. However, these bacterially expressed proteins were near full-length, formally there exist the possibility that a phosphorylation site, albeit not identical to the canonical sequence, was present but inaccesible to SIK2.

FGF-induced tyrosine phosphorylation of Gab1 is mediated via FRS2α that recruits Grb2-Gab1 complex to the vicinity of FGFR (Hadari *et al*., 2001). Only known Gab1 phosphorylation sites are Y448, Y479 and Y590 and proposed to be indispensable for FGF1 induced stimulation of PI3 kinase leading to survival response (Lamothe *et al*., 2004). FGF1-induced ERK/MAPK pathway activation in FRS2 knockout cells (Lamothe *et al.,* 2004) indicate that Gab1 also contribute probably by recruitment of the tyrosine phosphatase Shp2. It is known that in EGF pathway, tyrosine-phosphorylated Gab1 through its interaction with Ras-GAP has negative regulatory effect on Ras activation, this tyrosine can be dephosphorylated by Shp2, recriuted by Gab1 at a different phosphorylated tyrosine, leading to Ras-GAP release from membrane thus eleviates inhibition on Ras activation (Lamothe *et al*., 2004). It is conceivable that similar interactions take place in FGF dependent MAPK pathway, Gab1 may enhance Ras activation via recruitment of Shp2-Grb2-Sos complex or inactivation of Ras-GAP. In vitro experiments showed that Gab1 can be serine/threonine phosphorylated by ERK, and suggested to represent a negative feed-back (Lehr et al., 2004). Our study indicate S266 as a potential phosphorylation site by SIK2, it will be important to elucidate if this finding reflects in vivo situation and if it has a regulatory function in FGF pathways.

In this study, we showed that SIK2 can phosphorylate S580 residue on A-Raf *in vitro.* Because A-Raf S580 residue corresponds exactly to S621 on C-Raf, SIK2 is

expected to phosphorylate S621 of C-Raf. These two proteins have 85 % amino acid identity and conserved phosphorylation sites. In contrast to C-Raf, information on A-Raf is scarce, but they have been proposed to carry out redundant functions and can compensate for each other (Wellbrock *et al*, 2004). Phosphorylation of S621 seems to be essential for C-Raf activation, the mutation of S621A resulted in a Raf protein that could no longer be activated even in the presence of Ras and Src (Bonner *et al.,* 1986). The kinase(s) that phosphorylate(s) S621 have not been identified yet, in human protein reference database (http://www.hprd.org/), PKA, AMPK and Raf itself have been proposed. Only S43, S233 and S259 in C-Raf are phosphorylated when cAMP levels increase in cells. This suggested that S621 is not likely to be PKA target. We suggest S580 on A-Raf and S621 on C-Raf phosphorylation, that is necessary and sufficient for growth factor induced Raf dimerization, may be done by SIK2.

In summary, we have shown two components of FGF signal pathway, Gab1 and A-Raf can be phosphorylated by SIK2 in vitro. The duration of ERK activity (transient or sustained) suggested to be a determining factor in proliferation or differentiation responses in some cell types. It conceivable that these phosphorylation status of these proteins defined SIK2 may fine-tune the levels and duration of ERK activity, thus SIK2 may be a component of the events leading to proliferation versus differentiation decisions upon growth factor stimulation.

APPENDIX A: LIST OF AMINO ACID ABBREVIATIONS

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

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