Role of SG2NA Variants in Cell Cycle Regulation

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CERTIFICATE

The research work embodied in this thesis entitled **"Role of SG2NA Variants in Cell Cycle Regulation"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This work is original and has not been submitted so far in part or full, for the award of any degree or diploma of any other university.

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Content

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APS	Ammonium persulphate
ATP	Adenosine Tri Phosphate
bp	base pairs
BSA	Bovine Serum Albumin
CBB	Coomasie Brilliant Blue
cm	centimetre
D	days
dATP	deoxy Adenosine Tri Phosphate
DMEM	Dulbecco Modified Eagle's medium
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleoside Triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminscence
EDTA	Ethylene diamine tetra acetic Acid
FBS	Fetal Bovine Serum
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilo Dalton
LB	Luria Bertani
M	Molar
MALDI-TOF	Matrix Assisted Laser Dissorption- Time of Flight
MCS	Multiple Cloning Site
ml	milliliter
mM	milli Molar
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	messenger RNA

m.w.	molecular weight
N	Normal
NE	Norepinephrine
OD	Optical Density
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
p.m.f.	peptide mass fingerprinting
PMSF	Phenyl Methyl Sulfonyl Fluoride
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	rotation per minute
RT-PCR	Reverse Transcription PCR
SDS	Sodium Dodecyl Sulphate
TAE	Tris, Acetate, EDTA buffer
TBS	Tris Buffered Saline
TEMED	Tetramethylenediamine
Tris	Tris hydroxymethylaminomethane
UTR	Untranslated Region
UV	Ultra Violet
v/v	volume/volume
wt.	Weight
w/v	weight/volume
μg	micro gram
μl	micro liter
μM	micromolar

Introduction

The Striatin family constitutes a group of highly homologous metazoan proteins belonging to the WD-40 repeat superfamily of scaffolding proteins which include striatin, SG2NA and Zinedin. Striatin was the first identified member of this family that was purified from rat brain synaptosomes. These group of proteins mainly having four characteristic domain namely, i) The caveolin-binding domain, ii) The coiled- coil domain, iii) Ca^{2+}CaM-binding domain and iv) WD40 repeat domain. SG2NA, one of the members of this family, with potential scaffolding functions, was originally identified as a tumour antigen with increased expression during S to G2 phase of cell cycle. It was originally identified as a tumour auto antigen with augmented expression in S to G2 phase of the cell cycle. From our laboratory it was reported previously that mouse SG2NA has at least five novel splice variants of which two are devoid of the carboxyl terminal WD-40 repeats. The variants of SG2NA are generated by alternative splicing at the Exxon 7–9 regions and differ in their expression in tissues. Also, the expression of 35 kDa variant is more in neonatal than in adult tissues. Western analysis suggests that the SG2NA isoforms differentially respond to growth stimuli. Upon serum stimulation, while the 35 kDa variant is increased, the 78 kDa form is diminished. Splicing variation of SG2NA is conserved in metazoan evolution. In embryonic chicken there are at least four variants of which one is present in brain but absent in heart. Taken together, splicing variation of SG2NA might have some critical roles in differentiation and maturation in metazoan cells. Although it was identified that SG2NA is a WD-40 repeat protein with probable scaffolding function its potential role in cell cycle regulation, cell signalling, its exact function in metazoan biology is unknown. To explore the exact function of the vital protein in metazoan biology we here planned to identify the interacting proteins of SG2NA variants in different phases of cell cycle regulation and the probable effect of the over expression of the variants of this protein in the context of cell cycle progression.

Review of Literature

After the genome sequencing projects successfully completed for many organisms, determining the functions of the genes is being the next challenge of biologists; proteomics, the key concept of deciphering the functions of a gene, promises to bridge the gap between genome sequence and cellular behaviour. There is no correlation between mRNA level and the protein level in a eukaryotic organism (Gygi *et al.*, 1999 and Anderson and Seilhamer 1997). The one gene one protein concept is now obsolete especially in the case of eukaryotes where one gene gives rise to atleast 6 to 8 proteins (Stohman 1994). This disparity is caused by many processes viz. Alternative splicing, RNA editing, Post Translational Modification (PTMs) etc. Striatin family member SG2NA, the scaffolding protein of unknown function, uses all the above mentioned strategies to diversify its coding potential.

STRIATIN FAMILY OF PROTEINS:

The Striatin family constitutes a group of highly homologous metazoan proteins belonging to the WD-40 repeat superfamily of scaffolding proteins. Striatin was the first identified member of this family that was purified from rat brain synaptosomes (Castets *et al.*, 1996). Although striatin doesn't directly involved in cAMP synthesis (Castets *et al.*, 1996) it was originally identified during the investigation of adenylyl cyclase containing Post Synaptic Density (PSD) proteins of neurons to find out what basically gives rise to the high specific activity of neuronal adenylyl cyclase (Orlando *et al.*, 1992). Striatin, the very name indicates its very high level of expression in striatal region of brain. It is expressed in a few subsets of neurons specifically neurons of mammalian basal ganglia, cranial and spinal motor nuclei (Castets *et al.*, 1996). Electron Microscopy analysis reveals its presence in the somato-dendritic compartment of neurons, especially in dendritic spines (Castets *et al.*, 1996). Down-regulation of striatin in vivo by injecting an anti-sense oligodeoxynucleotide(OND) impairs the growth of dendrites as well as nocturnal locomotor activity in rat signifies its importance as signalling molecule (Bartoli *et al.*, 1999). The other two members of Striatin family are SG2NA and Zinedin. The SG2NA (S to G2 Phase Nuclear Autoantigen) was discovered during the analysis of autoimmune serum of a cancer patient that revealed reactivity to a nuclear antigen in a cell cycle specific manner (Muro *et al.*, 1995). Flow cytometric dual parameter analysis reveals that its expression is augmented in S to G2 phase of the cell cycle and thus named as SG2NA (Muro *et al.*, 1995). It shares

structural similarity with striatin family member proteins in terms of identical protein-protein interaction domains and the overall domain structure (Castets *et al.*, 2000). The phylogenetic analysis (Fig.1) of this family of proteins reveals that the members derived from an ancestral gene, expressing in divergent species like filamentous fungi, *C. elegans*, Drosophila, Xenopus, *Gallus gallus*, *Danio rerio*, insects and mammals (Poggeler and Kuck, 2004). The protein sequence of SG2NA is 80% similar to and 66% identical to sequences of striatin (Castets *et al.*, 1996). Zinedin is mainly expressed in the central nervous system. All the three proteins, Striatin, SG2NA and Zinedin, are both cytosolic and membrane-bound and all three bind to calmodulin in Ca^{2+} dependent manner (Castets *et al.*, 2000).

Molecular Structure:

Striatin family proteins are multimodular proteins characterized by four protein-protein interaction domains from N terminus to C terminus, i) a caveolin-binding motif ii) a coiled-coil structure, iii) a calmodulin-binding domain , and iv) a WD repeat domain (Castets *et al.*, 2000) (Fig.2).

The caveolin-binding domain:

The consensus sequence LHFIQHEWARF which denotes the caveolin-binding domain is located at N terminal part of all the striatin family members. It is a very well conserved domain present in all eukaryotes and also in fungus (Castets *et al.*, 2000; Poggeler and Kuck, 2004). Caveolins are the main components of caveolae. These are small proteins, ranging between 18-24 kDa, having a hairpin loop conformation with both the amino and carboxyl termini exposed to the cytoplasm. Apart from contributing towards the structure of caveolae, they also interact with cholesterol and a variety of signalling proteins such as receptors, Src-family of Tyrosine kinases, G proteins ($G\alpha$ subunit), Ha-Ras, MEK/ERK kinases and eNOS, EGFR, PKC isoforms. (Lisanti *et al.*, 1994; Couet *et al.*, 1997; Carman *et al.*, 1999).

The coiled- coil domain:

The coiled-coil domain, a highly conserved (94% homology amongst members) domain present at the amino terminus, is another characteristic structural feature of the Striatin family members (Castets *et al.* 2000). The α-helical coiled-coil motif frequently occurs in scaffolding proteins which promote homo- or hetero or multimerization via this domain (Lupas, 1996; Kammerer,
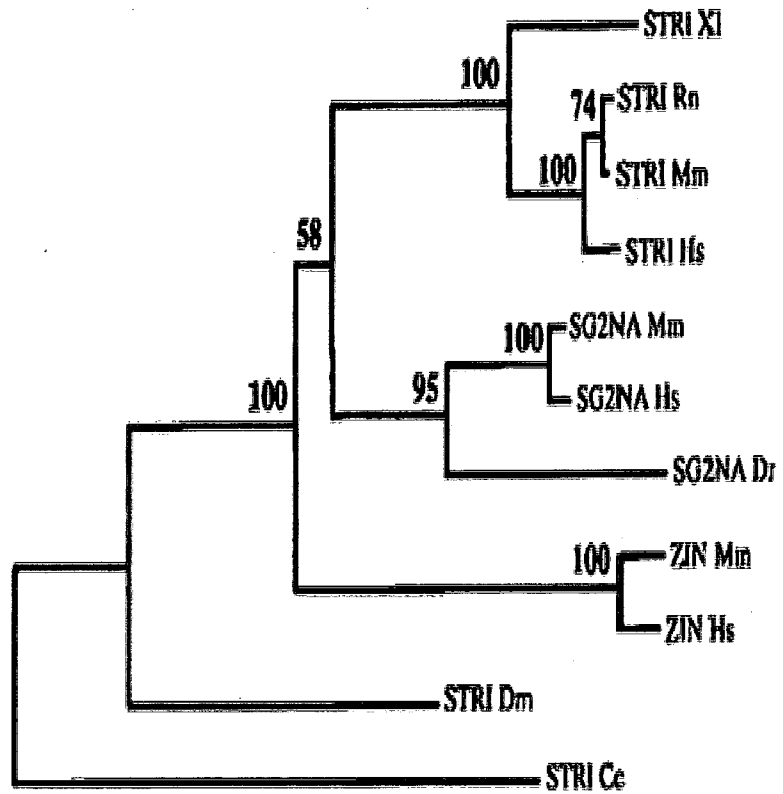
Figure 1: Phylogenetic analysis supports the hypothesis that the present members of the striatin family derive from an ancestral gene, expressing in divergent species as wide as filamentous fungi to mammals. (Castets *et al.*,2000)
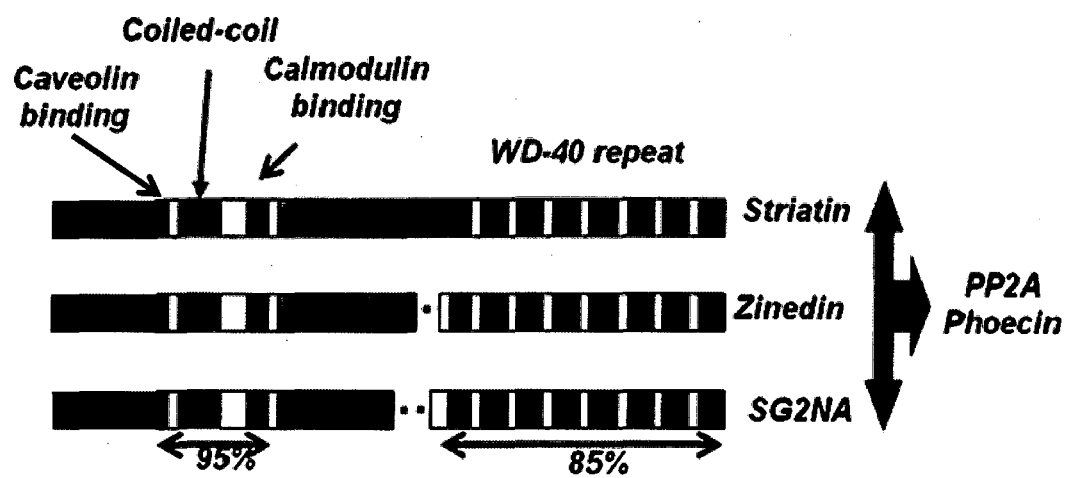
Figure 2. Domain organization of the three known members of the striatin family. Grey box indicates non conserved region. Green boxes are of conserved caveolin binding domain and calmodulin binding domain which with blue boxes of coiled-colied domain share 95% similarity within the striatin family members. The C terminal WD-40 repeat region share 85% similarity (Castets *et al.,* 2000).

1997). In agreement, it has recently been demonstrated that this coiled-coil domain is necessary for the homo- and hetero-oligomerization of striatin and SG2NA, an obligatory step for their correct routing to the dendritic spines (Gaillard *et al.*, 2006). It is possible that the coiled-coil domain of Striatin proteins help in their association with membrane, in a way γ subunits of G heterotrimeric protein direct the membrane attachment of β subunits (Fishburn *et al.*, 2000).

$Ca^{2+}CaM$-binding domain:

Striatin family members have a highly homologous Calmodulin (CaM) binding domain at their amino termini and show calcium-dependent CaM binding activity (Bartoli *et al.*, 1998; Castets *et al.*, 2000; Moreno *et al.*, 2000). The CaM binding domains of SG2NA (166-183 aa) and Zinedin (165-182 aa) have 94% and 79% identity with that of Striatin. The calmodulin-binding site of Striatin (149 and 166 aa) forms a basic amphiphilic helix, a characteristic of CaM-binding sites. Striatin undergoes shift in its cellular localization with changes in Ca^{2+} concentration and its binding equilibrium with calmodulin is highly dynamic with the fluctuations of intracellular Ca^{2+} concentration and thus believed that striatin might play a key role in Ca^{2+} sensing and signaling (Petrozzino *et al.*, 1995; Bartoli *et al.*, 1998). Since other members of the striatin family also have similar Ca^{2+}–CaM-binding domains, they might also be acting as Ca^{2+} sensors in specific cellular context. They may thus be considered as nodal points for signal transduction, regulation of nitric oxide activity, endocytosis, protein trafficking, intracellular Ca^{2+} regulation and cholesterol transport (Krajewska and Maslowska, 2004).

WD40 repeat domain:

One of the characteristic features of the Striatin proteins is the presence of ~40 minimally conserved amino acids with a pair of glycine-histidine (GH) at the amino terminus and a pair of tryptophan-aspartic acid (WD) residues at the carboxy terminus (Castets *et al.*, 1996; Neer *et al.*, 1994). Of the four conserved domains, the WD-40 repeats are the least conserved amongst these three members (Castets, *et.al.* 2000) that occurs in tandem repeats. Their number generally varies from 4 to 10, minimum two and maximum sixteen repeats (Van Nocker *et al.*, 2003,). Striatin is the first identified protein containing WD-40 repeat binds to calmodulin in Ca^{2+} dependent manner (Castets *et al.* 1996). While Striatin has eight WD repeats at its carboxy terminus, of which the second repeat conforms to the consensus while others vary slightly (2-8 amino acid mismatch) (Castets *et al.* 1996). SG2NA contains 6 copies of the WD-40 repeat motifs

homologous to that of Striatin (Muro *et al*. 1995). These repeating units organize into circular, propeller-like structure with seven blades made up of beta sheets, forming a stable platform (Yu *et al.,* 2000; Cheng *et al.,* 2004). Members of WD 40 repeat family have been attributed to biological functions as diverse as RNA transcription and processing (De La Cruz *et al.,* 2005), vesicular trafficking (Fritzuis *et al.,* 2007), cytoskeleton assembly (Pollmann *et al.,* 2006), cell signaling (Fritzius *et al.,* 2006), cell cycle, and cell death (Yoon *et al.,* 2004; Ogawa *et al.,* 2003).

Such diverse roles of these proteins is explained by the observations that WD40 repeat domains do not have any catalytic function rather they provide sites for interaction between different proteins (Li and Roberts, 2001). It may be thus anticipated that all WD-repeat proteins have similar structures that contribute towards their scaffolding functions.

CELL CYCLE REGULATION:

Cell cycle is a highly regulated phenomenon involving a series of macromolecular events in periodic fashion leading to division of a single cell into two daughter cells each containing identical nuclear material similar to that of parental cell. This process is brought about in four major phases. During the G1 phase, replicating somatic cells, synthesize RNAs and proteins, during S phase it prepares for DNA synthesis and chromosome replication. After progressing through the G2 phase, cells begin the complicated process of mitosis, also called the M (mitotic) phase, which is further divided into several stages. Most non-proliferating cells in vertebrates leave the cell cycle in G1, entering the G0 state (Fig.3).

To minimize the occurrence of error during the process, a cell progression is regulated at different phases through several checkpoints (Cooper 2006).

G1 Checkpoint: The G1 Checkpoint ensures that (1) cells are large enough to warrant a new round of DNA synthesis, (2) any damage caused in DNA has been repaired, and (3) external conditions are favourable for mitotic cell division. The responsible pathway of this checkpoint is ATM (ATR)/CHK2 (CHK1)–p53/MDM2-p21 (Kastan MB and Bartek J, 2004).
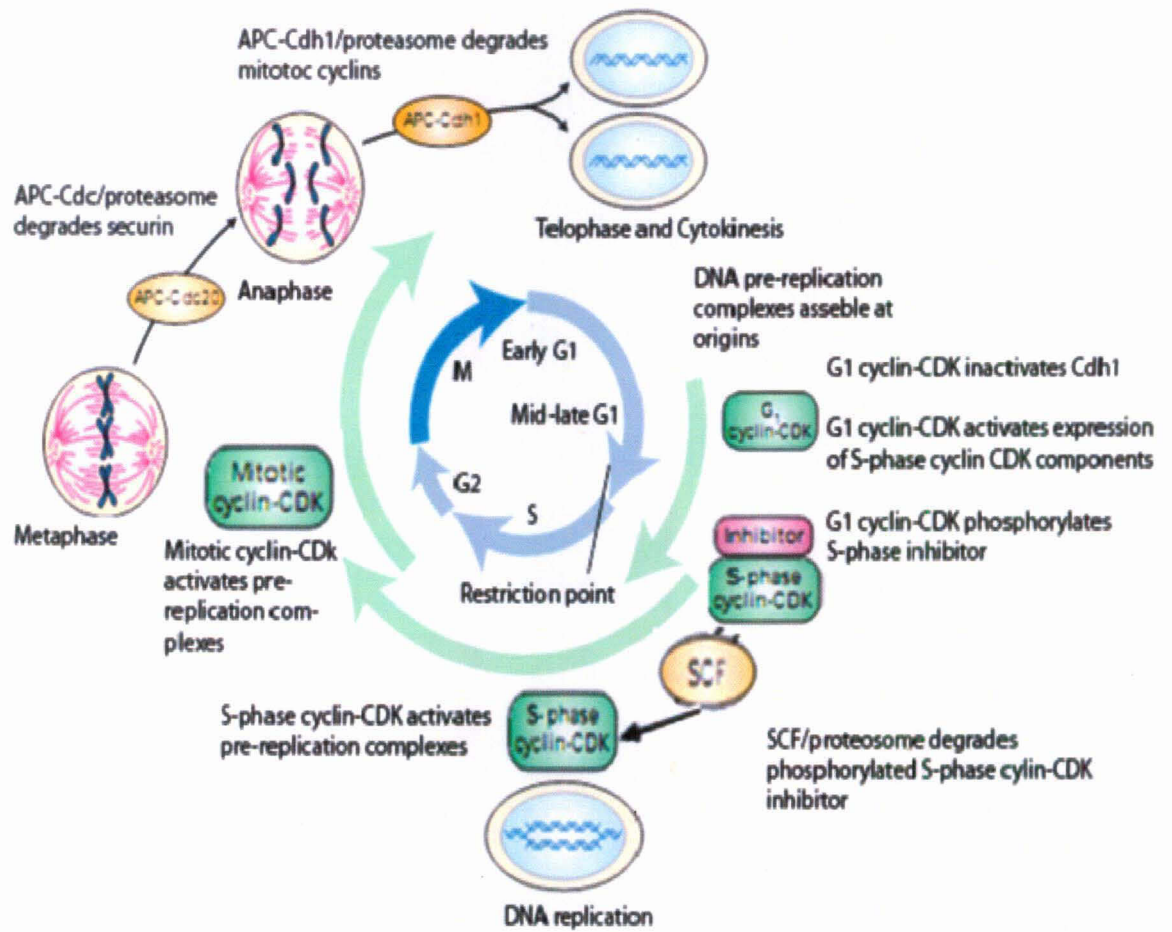
Figure 3. Different phases and checkpoints of mammalian cell cycle regulation.

(Lodish *et al.,* 2006)

S Phase Checkpoint: With the inhibition of replication-origin firing, another one critical function provided by S-phase checkpoint or replication checkpoint is to protect the integrity of the stalled replication forks. Mediator of this checkpoint is NBS1, SMC1, etc.

G2 Checkpoint: The G2 checkpoint / G2/M checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2, or when they progress into G2 with some unrepaired damage inflicted during previous S or G1 phases. Effectors of this checkpoint are ATM/ATR, CHK1/CHK2 and/or p38-kinase etc.

A **morphogenetic checkpoint** in budding yeast arrests cells in **G0** if a bud fails to form (Zimmerman and Kellogg 2001).

Proteins Involved in Cell Cycle Regulations; more with Cyclins and CDKs:

The mechanism and molecular details of the cell cycle regulation has been elucidated only after the discovery of the periodically cycling protein cyclins (Rosenthal *et al.,* 1980).The pattern of cyclin expression varies with a cell's progression through the cell cycle, and this specific cyclin expression pattern defines the relative position of the cell within the cell cycle. There are many cdks have been found in eukaryotic cell cycle regulation which includes cdk1-9. Till date the function of many cdks is yet to be elucidated. Progression of a cell through the cell cycle is promoted by a number of CDKs which, when complexed with specific regulatory proteins called cyclins, drives the cell cycle progression in a regulated mechanism. Another level of regulation of the cell cycle progression is attributed by the CDK inhibitory proteins (CKIs). Based on their evolutionary origins, structure, and CDK specificities there are two CKI gene families have been defined in metazoans (Besson *et al.*, 2008). The INK4 gene family encodes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. These CKIs bind to CDK4 and CDK6 and inhibit their kinase activities by interfering with their association with D-type cyclins (Sherr and Roberts 1999). In contrast, CKIs of the Cip/Kip family bind to both cyclin and CDK subunits and can modulate the activities of cyclin D-, E-, A-, and B-CDK complexes (Sherr and Roberts, 1999). This class of proteins share a conserved N-terminal domain that mediates binding to cyclins and CDKs but diverge in the remainder of their sequence, suggesting that each of these proteins could have distinct functions and regulation (Sherr and Roberts 1999). Comparative gene expression study in mammalian cell reveals that in different phases of cell cycle different genes are periodically

expressed. Functions of many of such genes are not known (Cho *et al.*, 2001, Whitfield *et al.*, 2002).

Scaffolding Protein in the Cycle of Cell Divisions:

Scaffolding proteins represent the structural platform on which multiprotein signaling complexes are assembled (Kolch 2005), thereby conferring required specificity and speed that would otherwise be compromised due to diffusion. Scaffolds thus provide an interface for receptors, signalling kinases, phosphatases and other adaptor proteins. Although the very role of scaffolding proteins in cell signalling is very extensively studied, their role in cell cycle regulation has been neglected. Of the well known scaffolding proteins having significant functions in cell cycle regulation, 14-3-3 has been shown to regulate the cell cycle by binding and sequestering Cdc25 (Cyclin B phosphatase) from nucleus to cytoplasm in resting cells. In active cells, the phosphorylated forms of vimentin and keratin recruit 14-3-3 and releases active Cdc25 which in turn dephosphorylates Cyclin B leading to cell cycle progression. (Hermeking and Benzinger 2006). Over the last decades, the centrosome has been proposed to play a key role in the cell cycle. The matrix proteins of the pericentriole containing large coiled-coil proteins anchor and cluster components of the signaling pathways as well as components of the motor protein complexes (Keryer *et al.*, 2003). Human Cep192, a scaffolding protein has also been analysed for its role in mitotic centrosome and spindle assembly (Gomez-Ferreria *et al.*, 2007). Recently it has been shown that the scaffolding subunit of protein phosphatase 2A (PP2A) interacts with ATM protein and regulate its phosphorylation (Goodarzi *et al.*, 2004). ATM activation by phosphorylation triggers the cell cycle arrest thus implicating any modulation of ATM activation modulates the cell cycle regulation as well. Apart from eukaryotes, scaffolding proteins have been shown to have role in bacterial cell division. Dajkovic *et al.* demonstrated that bacterial cytokinesis, a scaffolding protein mediated process, where cytokinetic ring termed as the Z ring composed of FtsZ filaments forms a scaffold in recruiting other cell-division proteins.

PHYSIOLOGICAL FUNCTION OF STIATIN FAMILY; A SIGNALING SCAFFOLD AND CELL CYCLE REGULATOR?

Striatin family proteins, due to their multi-modular protein-protein interaction domains, are speculated to function as signalling scaffold in many cellular processes (Fig.4). Striatin and SG2NA have been shown to make a stable complex with the PP2A A/C heterodimer and are predicted as B'' type subunits of PP2A (Moreno *et al.*, 2000). Literature study reveals that PP2A plays an important role in Ca^{2+} signalling but the mechanism was unknown. As Striatin and SG2NA are both calmodulin binding proteins that bind to calmodulin in the calcium dependent manner (Castets *et al.*, 2000), they are speculated to sequester the PP2A in calcium signalling pathways. Striatin family members directly interact with phocein, an intracellular protein highly expressed in neurons, has been implicated in vesicular trafficking acting in particular in the endocytic process due to its association with dynamin via direct interactions with nucleotide diphosphate kinase(NDPK) and EPS15 (Bailly and Castets 2007). Striatin, SG2NA and Zinedin interacts with caveolin, the main components of caveolae (Gaillard *et al.*, 2001). Caveolae, are nonclathrin-coated plasma membrane localized, specialized lipid rafts that contain numerous proteins involved in cell signaling such as G-protein coupled receptors, integrins, Src-family kinases, G-proteins, Ras-related GTPases, AC, PKA, PKC isoforms, phospholipases and nitric oxide synthases. (Okamoto *et al.*, 1998). These are highly dynamic structures, anchored by actin cytoskeleton, capable of internalization (Pelkmans *et al.*, 2002; van Deurs *et al.*, 2003). Very recently it has been shown that in vascular endothelial cells, caveolin-binding protein striatin (also most propably with SG2NA as the domain involved in this association, i.e. the N-terminal part of stiatin, is largely conserved in SG2NA) acts as a molecular anchor for estrogen receptor α (ER α) that target it to membrane caveolae and thus serves as a scaffold for the formation of an ERα-Gαi complex that enables estrogen to rapidly activate the mitogen-activated protein kinase and Akt kinase pathway, and endothelial NO synthase (eNOS). However, it has no effect on ER-dependent genomic effect of estrogen (Lu *et al.*, 2004). The *Drosophila* homologue of Striatin, the Connector Kinase of Activator Protein-1 (CKA), a single representative of the Striatin family in *Drosophila* bearing 56% homology with mammalian Striatin, mediates 'dorsal closure', during fly morphogenesis through AP-1 phosphorylation in *Jun* N-Terminal Kinase (DJNK) pathway (Chen *et al.*, 2002). CKA knockout induces developmental abnormalities with dorsal-open cuticle phenotype, head defects and death in the fly which can be rescued by *C. elegans* or mammalian orthologues of CKA, suggesting that the latter are not just structural
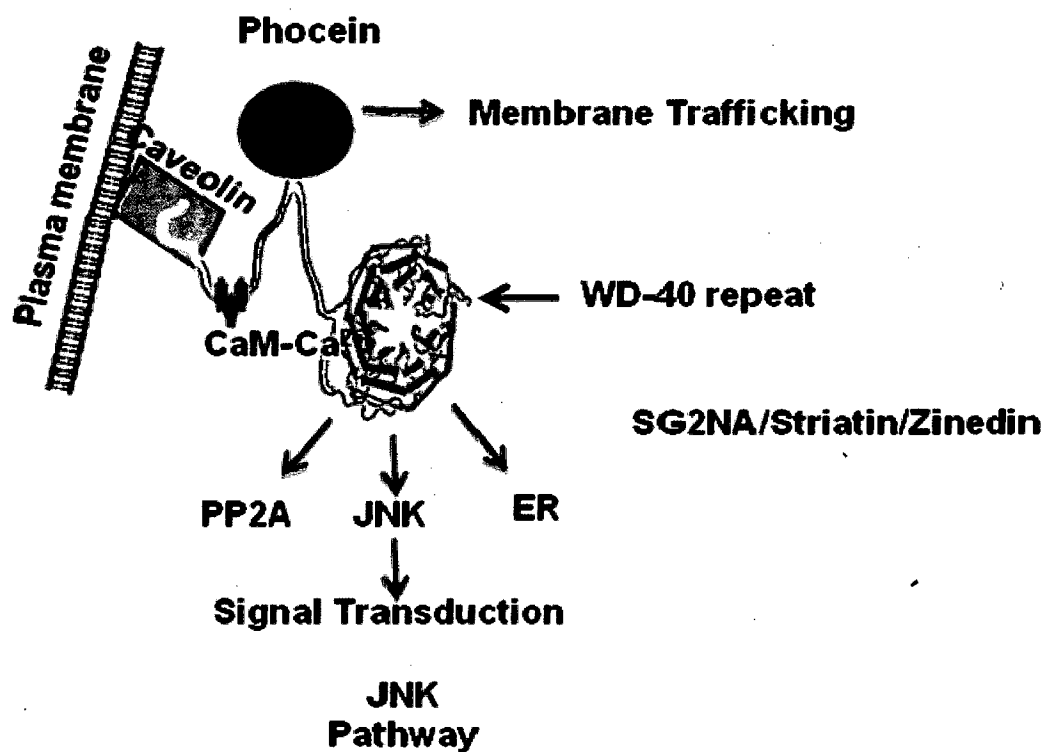
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Figure 4. The scaffolding nature of Striatin family of proteins: they modulate endocytosis via interaction with phocein, caveolin and Ca^{2+}-CaM complex. They also interact with PP2A, ERα, and JNK modulating cell signaling.

homologues but their functions are also conserved in various species. The *pro11* gene product, a Striatin homolog in filamentous fungus *Sordaria macrospora,* is known to play an essential role in the development of fruiting bodies during its development and mouse Striatin gene is able to rescue the sterile phenotype of pro11 knockout mutant, strongly suggesting functional conservation of this gene in higher eukaryotes (Poggeler and Kuck, 2004). Although SG2NA was initially identified as a cell cycle regulated protein with enhanced expression in S to G2 phase transition (Landberg and Tan 1994) its exact mechanism in cell cycle regulation is unexplored. It is still under investigation whether this protein itself a novel cell cycle regulator or its expression is regulated by cell division cycle.

MECHANISM OF DIVERSIFYING THE PROTEINS AND ITS FUNCTION:

"How can the genome of *Drosophila melanogaster* contain fewer genes than the undoubtedly simpler organism *Caenorhabditis elegans*? The answer must lie within their proteomes." (Graveley 2001). Proteomes may be two to three orders of magnitude more complex (>1000 000 molecular species of proteins) than the encoding genomes would predict (Walsh *et al.*, 2005). With the significant advances in genomics and proteomics and getting insight into genome project, it now appears that organismal complexity is not achieved by the linear increase in gene number but by the sophistication in its regulation. Furthermore, contrary to the initial perception that gene expression is primarily controlled at the level of transcription, recent studies reveals mRNA metabolism *i.e.,* processing, splicing, RNA editing, transport, translation, and further post translational modifications (PTMs) of proteins significantly add up towards regulation of gene function, especially in metazoan organisms.

Alternating Splicing:

Alternative splicing is one of the most imperative mechanisms utilized by eukaryotic organisms to expand their protein-coding capacity (Fig.5). However, its potential exploits in diversifying cellular proteome has only lately been appreciated (Blaustein *et al.*, 2007). It now appears that about three fourth of human protein coding genes undergo alternative splicing and certain genes may have as many as >10,000 splice variants (Blaustein *et al.*, 2007; Zipursky *et al.*, 2006). e.g.
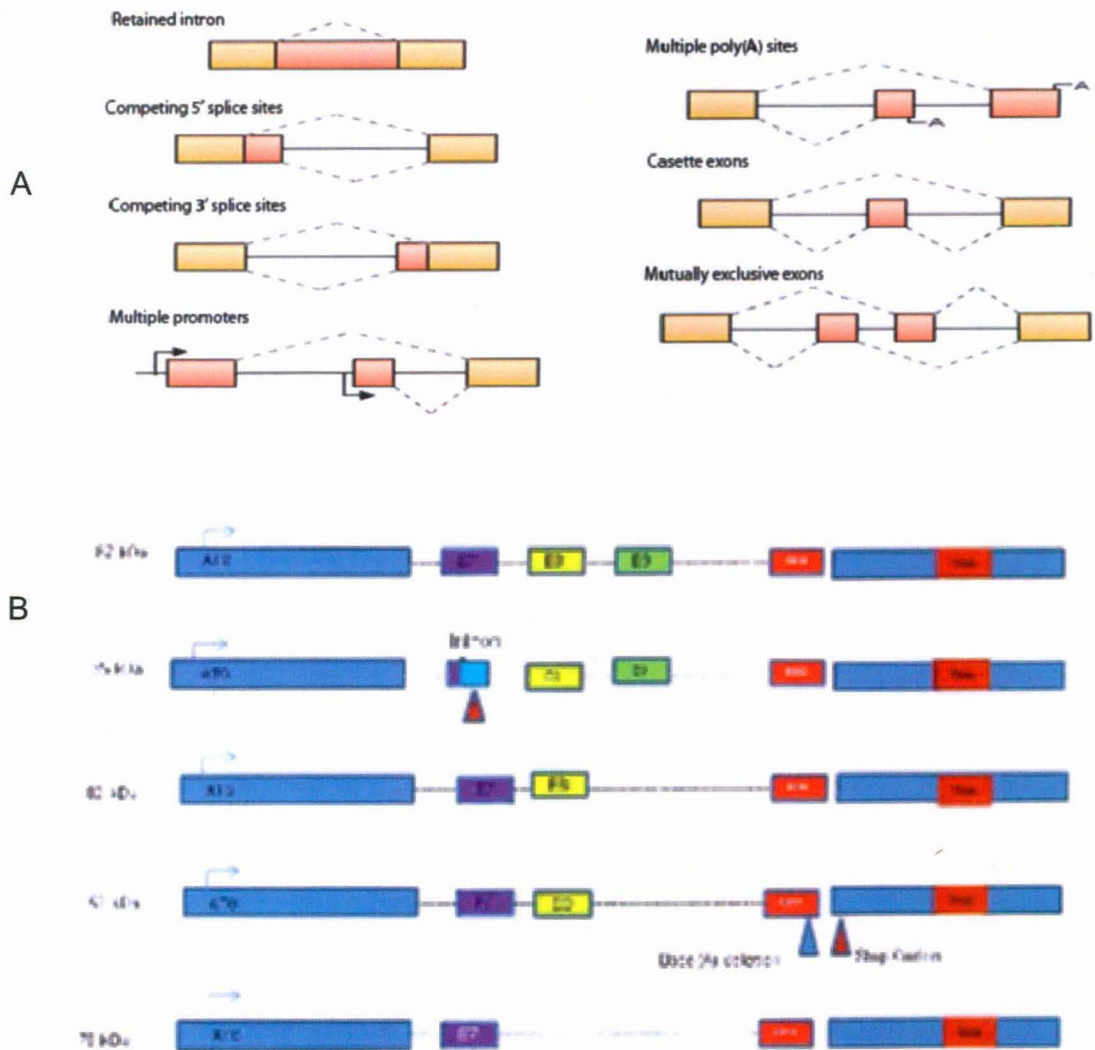
Figure 5. Alternative Splicing Events. (A) Splicing patterns are indicated above and below by the diagonal dashed lines. Constitutively spliced exons are in orange, while alternatively spliced segments (which are treated as exons or introns according to the splicing pattern) are shown in red (Roberts and Smith 2002). (B) Alternative Splicing pattern of the SG2NA and resulting variants. Five variants are shown here which are the result of alternative splicing between exon 7- 10.

Dscam, a cell-surface recognition protein of the immunoglobulin superfamily, encodes 39,936 potential isoforms in *D. virilis* (as compare to 38,016 isoforms in *Drosophila melanogaster*), have been reported to be generated by alternative splicing of mRNA (Graveley *et al.*, 2004). Taken together, alternative splicing appears to be the most versatile tool for regulating gene function in higher eukaryotes, including plants. Very recently, it has been shown that metazoan SG2NA exists in multiple isoforms (Fig.5) generated out of alternative splicing (Mishra *et al.*, 2008). It has been also demonstrated that the splice variants of SG2NA are differentially expressed in various tissues and their expression profile changes with the state of differentiation and the proliferative potential of the cognate cell type.

RNA Editing:

RNA editing describes the alteration of RNA's informational capacity other than by splicing, 5'- and 3'-end formation, and the creation of hypermodified bases. It can be divided into base insertion or deletion editing in which the RNA is cleaved; bases added or removed (Simpson and Thiemann, 1995), base substitution or modification editing in which the RNA is not cleaved (Scott 2005). RNA editing was first discovered in 1986 in *Trypanosoma brucei* and *Crithidia fasciculata*, where it was shown that transcripts of the cytochrome c oxidase subunit II (COII) gene contain four uridines near their 5' ends that are not encoded by the gene (Benne *et al.*, 1986). Followed by the discovery of RNA editing, flurries of articles and reviews came out in very high profile journals showing the importance and mechanism of this highly complicated process in trypanosome. The existence of this type RNA processing phenomena has also been identified in mammals where it had been shown that apo-B48 is collinear with the amino-terminal half of hepatic apo-B100 (Powell *et al.*, 1987) which is resulted by an in-frame UAA stop codon due to C to U base change in the codon CAA coding for Gln^{2153} in apoB-100 mRNA (Chen *et al.*, 1987). In spite of enormous study on RNA editing by base modifications occurring in mammals, very few or negligible reports are there for RNA editing by insertion/deletion. Lately, it has been shown that in human RNA editing by U insertion at the 5' UTR of the mRNA resulting 5' extended proteins variants (Zougman *et al.*, 2008). High throughput murine miRNA transcriptome analysis reveals mature miRNA with 14.8% U insertion modifications and 1.5% U deletion modifications, thus these data implicates the existence of TUTase-mediated dUTP-dependent U-insertion/U-deletion cycle in mammals (Reid *et al.*, 2008). Thus the majority of RNA editing events that has been studied involves changes in mRNA sequences that create new

start and stop codons by uridine insertion and cytidine to uridine (C-to-U) conversions which results in production of altered protein products. Editing also creates essential structural elements at the primary, secondary and tertiary levels, involving both loop nucleotides and base-paired stems in tRNA (Gott and Emeson 2000).

Post Translational Modifications of Proteins:

Biological complexity cannot be simply defined by the number of genes an organism possesses. Advanced species evolved additional regulatory features that allow for an increased number of functions with increased adaptability. Ample evidence suggests that these additional modes of functional indexing are not only diverse, but together they accomplish a specific biological outcome. One of such important regulatory features is post-translational modifications (PTMs) of proteins. Post-translational modification (PTM) of protein is known as any covalent modification at any amino acids of a protein. The modifications can be both reversible and irreversible. The major types of protein covalent modifications like phosphorylation, acetylation, glycosylation, methylation, ubiquitylation, sumoylation have been reported. Very recently, the novel type of post translational modification has been reported that is the addition of AMP to the threonine residue of protein (Yarbrough *et al.*, 2009). The role of protein modification is implicated in a very broad-spectrum. Post-translational modifications of proteins can regulate the protein functions by modulating protein activity due to conformational changes of the protein, their cellular locations, dynamic interactions with other proteins and their stability. Protein modifications have been shown to have impact on chromatin modifications and thus imply its role in chromatin dynamics and gene expression. At least 60 different types of histone modifications have been detected by biochemical techniques like antibodies and mass spectrometric techniques which define the possible numbers of histone modifications (Kouzarides *et al.*, 2007). The functions of histone modifications are as high as the types of modifications present in histone including regulation of gene expression, DNA repair, DNA replication, chromosome condensation and epigenetic memory formation etc. For the widespread function and mechanism of histone modifications scientists are speculating the existence of 'histone code' read by regulatory proteins to affect cell processes such as transcription, replication and chromosome condensation during mitosis. In recent times, with the advanced and improved proteomic technologies, the importances of PTMs are emerging. With variety of proteins

modifications occurring, expertises of this field speculate/hypothesize encrypted protein code embedded in the protein (Sims *and* Reinberg 2008).

POST TRANSLATIONAL MODIFICATIONS AND EUKARYOTIC CELL CYCLE REGULATION:

PTMs, involved in cell cycle regulations, include reversible and irreversible phosphorylation, ubiquitation and sumoylation etc. The role of phosphorylation in cell cycle regulation is very well studied *e.g.,* the kinases, specially the Cyclin Dependent Kinases (CDKs) which are the main regulatory molecules of cell cycle regulation, phosphorylate their respective target molecules to carry cell cycle progression. Recent studies have, however, brought to light additional mitotic kinases like the Polo family, the Aurora family and the NIMA (never in mitosis A) family, as well as kinases implicated in mitotic Checkpoints, mitotic exit and cytokinesis (Nigg 2001). Not only kinases, the reverse counter part of the kinases *i.e.,* the phophatases, are also involved in the cell cycle regulations. Proteosomal degradation processes that define the stability of the protein through ubiquitination and deubiquitination, have also been identified as a crucial regulator of both the core cell cycle machinery and cell cycle checkpoints. Ubiquitinated proteins are often targeted for degradation by the proteasome, the irreversible nature of which is at the heart of the unidirectional progression through the cell cycle program (Song and Rape 2008). The protein Ubc9 (ubiquitin-conjugating enzyme 9) is the classical E2 enzyme associated with the SUMO conjugation process. In budding and fission yeasts, cells that are mutant in Ubc9 or in SUMO itself show defects in progression through the G2/M transition, in addition to defects in chromosome integrity, telomere length, kinetochore function, chromosome segregation, proper checkpoint responses to DNA damage and survival mechanisms under stress conditions (Gutierrez and Ronai 2006). It has been also suggested that, at least in budding yeast, the SUMO system is also required for the APC/C-mediated degradation of cyclin B and Pds1/securin that is controlled by the spindle assembly checkpoint (Dieckhoff *et al.,* 2004). Other post translational modifications like acetylation, methylation, etc also having role in cell cycle regulation. Glycosylation is also thought to be involved in cell cycle regulation of yeast.

Aims & Objectives

SG2NA was originally identified as a nuclear autoantigen whose expression is augmented during S to G2 phase of cell cycle (Landberg and Tan 1994). It has a WD-40 repeat domain present in its carboxyl terminus and in addition, a caveolin binding motif, a coiled–coiled structure and a calmodulin binding domain are present in the amino terminus in the same order. It is highly homologous to Striatin and Zinedin, constituting the striatin subfamily of WD-40 repeat proteins. Striatin, the prototype member of the family, is involved in differentiation of the dendritic spines. SG2NA and striatin interact with phocein (Baillat *et al.*, 2002) and Protein Phosphatase 2A (PP2A) (Moreno *et al.*, 2000), attributing them to vesicular trafficking and cell signaling respectively. Drosophila has only one striatin/SG2NA homologue i.e., CKA, which acts as a platform for organizing the components of JNK signaling and the transcription factor AP-1, indicating a common functional ancestry between the three members of the family (Chen *et al.*, 2002).

We have recently reported that vertebrate SG2NA exists in multiple (87, 83, 82, 78, 38, 35 kDas) isoforms generated out of alternative splicing (Mishra *et al.*, 2008). We also demonstrated that the splice variants of SG2NA are differentially expressed in various tissues and their expression profile changes with the state of differentiation and the proliferative potential of the cognate cell type.

Taken together, although two independent lines of research over the past decade have identified SG2NA as a WD-40 repeat protein with potential roles in cell cycle regulation, cell signaling, its exact function in metazoan biology is unknown. Variants of SG2NA might thus be the critical mediator of cell differentiation and maturation in context specific manner.

The broad objective of our laboratory is to study the possible role of SG2NA variants in cell cycle regulation and cell differentiation. Towards that objective, I have undertaken preliminary characterization of mouse SG2NA variants by

    (i)   Identifying their possible interacting partners.

    (ii)   Studying the possible effect of their over expression on the cell cycle progression.

    (iii)   Testing the possibility of their post-translational modification, especially in the context of their functions.

Materials & Methods

Plasmid Constructs:

GST vector pGEX4T series were purchased from Amersham Pharmacia Biotech, USA. The Myc tagged eukaryotic expression vector pcDNA 3.1 Myc/His series was kind gift from Dr. Debashis Mitra, NCCS, Pune, India.

Biochemical and Molecular Biology Reagents:

All biochemicals were procured from Sigma-Aldrich, USA and Qualigens, India unless mentioned otherwise. All restriction enzymes and DNA modifying enzymes were procured from MBI Fermentas, Inc. USA and NEB, USA. Bacterial growth media (LB and Agar) was purchased from HiMedia. Pre-stained protein molecular weight marker and 1 kB and 100 bp DNA marker were obtained from MBI Fermentas, Inc. USA. Isopropyl ß -D-1thiogalactopyranoside IPTG, was obtained from Amresco, RNAse inhibitor was from New England Biolabs, USA. ECL detection reagent was purchased from Sigma Aldrich, USA. Gel extraction kit, Express midi (DNA preparation) kit were from MDI, India. GSH-Sepharose was from Amersham, ProteinA-Agarose beads, Freund's Complete and Incomplete adjuvant, were from Sigma Aldrich, USA. All plastic wares were from Tarsons, Germany.

Cell Culture:

Tissue culture plastic ware was purchased from Greiner, Germany. Filter membranes (0.22μm pore size) were from Millipore. All Tissue culture reagents including Dulbecco's Modified Eagle's Medium (DMEM), Penicillin/Streptomycin solution, Amphotericin B, Sodium bicarbonate, were purchased from Sigma Aldrich, USA, unless mentioned otherwise. Foetal Bovine Serum (FBS) was from Hyclone, USA. Lipofectamine Transfection reagent and Trypsin-EDTA was from invitrogen. NIH3T3, CHO-K1 cell lines were obtained from National Centre for Cell Science, Pune, India. Tissue culture grade water was prepared in the laboratory by using Quartz distillation system (Bhanu Scientific, Bangalore, India). DMEM was prepared in tissue culture grade water and sterilized by filtration through 0.22μ m filter. Phosphate buffered saline (PBS), pH 7.2- 7.4, was prepared in tissue culture grade water and autoclaved.

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Antibodies:

Monoclonal (mouse) SG2NA (S68) antibody was from Cell Signalling Technology. Secondary antibody Horseradish peroxidase (HRP) conjugated anti-mouse IgG was from Santa Cruz Biotechnology Inc, USA.

Filters and Films:

PVDF membrane purchased from Amersham and millipore. X-Ray Films were purchased from Kodak. Filter Papers were purchased from Whatman Ltd., Madistone, England.

Oligonucleotide primers were synthesized from Sigma Aldrich, India.

Primers Sequences:

Nhe-1 mSG2NAf 5' TACCGCTAGCATGGACGAGCTGGCCGGA 3'

Kpn-I mSG2NAr 5' GCGGTACCGCCACAAATACTTTGGCAAG 3'

m35rKpn-I 5' GCGGTACCGCTCTAAAATTA 3'

BGH Reverse Priming 5' TAGAAGGCACAGTCGAGG 3'

MSRTf 5' GGTGGTGAATCTCCTAAGC 3'

MSRep 5'CCTGTGAGGAAAGCATCAC 3'

Cell Culture Methods:

All cell lines were cultured as monolayers in DMEM (4500mg/L Glucose, 2 mmol/L Glutamine) supplemented with 10% FBS, 90U/ml Penicillin, 90 µg/ml Streptomycin and 5 µg/ml Amphotericin B in humidified, 5% CO_2 containing incubator at 37°C to 60-70% confluence. NIH3T3 is maintained in 10% New Born Calf Serum (NBCS). Subculturing was done by Trypsination with 0.025% Trypsin EDTA solution.

Preparation of Total Cell Extract:

Cells after treatment were washed once with ice cold PBS in culture plates, scraped in 1ml PBS and harvested by centrifugation at 3000 rpm, 4°C for 10min. Cell pellet was lysed in (100µl for pellet of each 100mm dish) RIPA buffer (50mM Tris.Cl; pH 7.4, 150mM NaCl, 1mM EDTA, 1% TritonX100, 1% Sodium Deoxycholate, 0.1% SDS, 1mM PMSF, 1mM DTT, PI cocktail) on ice for 1 hour with intermittent tapping. The lysate was centrifuged at 10,000rpm, 4°C for 10min. The supernatant (total cell extract) was stored at −80°C.

Preparation of Tissue extracts:

Mouse tissues were snap frozen immediately after dissection in Liquid Nitrogen and homogenized on ice in RIPA buffer in a Potter Elvejhem homogenizer. The homogenates were centrifuged at 1,50,000g, 4°C for 40min.

Protein Estimation:

Protein concentrations were estimated by modified Bradford method (Bradford 1976; Kruger, 1994) in a Cary, Eclipse spectrophotometer.

Isolation of Plasmid DNA by Alkali Lysis Method:

Plasmid DNA was prepared by Alkaline Lysis Method from 2ml (Mini Preparation) or 100ml (Midi Preparation) as required (Sambrook *et al.*, 1989).

Isolation of DNA by kit:

Plasmid DNA, for transfection into NIH3T3 and CHO K1 cells were prepared using Express Midi Kit MDI, India according to manufacturer's protocol.

Restriction Digestion:

All Restriction digestions were carried out in the appropriate buffer according to the manufacturer's instructions.

Ligation Reaction:

Restriction digested, vector and insert DNA were purified from agarose gel and ligated according to their molar ratio (Amount of insert = (Size of insert/Size of vector) X 50ng vector) in a 20µ l reaction volume in 1X Ligase buffer, 5U of T4 DNA ligase (MBI Fermentas) and 5% w/v PEG 4000 (for blunt end ligations) at 16°C overnight.

Preparation of Chemically Competent DH5 Competent Cells:

Chemically competent *E. coli* strains DH5α and BL21DE3 were prepared by $CaCl_2$ method. After selection with appropriate antibiotic containing medium, cells from 100ml of a secondary culture (OD =0.4) were collected by centrifugation at 3500 rpm for 15 minutes at 4°C and

resuspended in 100ml of chilled $CaCl_2$ Buffer (100 mM $CaCl_2$) and incubated on ice for 45 minutes. Competent cells were then collected by low speed centrifugation at 3500 rpm for 15 minutes at 4°C and the pellet was resuspended in 5ml of ice-cold Trituration buffer containing 15% glycerol and stored in aliquots of 100 µl at -70°C.

Transformation of Competent *E. coli* with Plasmid DNA:

Approximately 5-6 ng of plasmid DNA or 50 ng equivalent of ligation reaction mix was added to 100 µl of competent cells, mixed well by gentle tapping and incubated on ice for 20 minutes. Heat shock was given for 90 seconds at 42°C followed by a rapid chilling for 5 minutes. LB medium (900 µl) was added and the transformation mix was allowed to revive for 1hour at 37°C and appropriate volume (100µ 1 for plasmid and entire volume for ligation mix) was plated on LB agar plate supplemented with appropriate antibiotic. Plates were incubated at 37°C for 12-16 hours until the colonies were apparent.

DNA Sequencing:

Automated sequencing for confirming the clone was done in the sequencing facility available at the Department of Biochemistry, University of South Campus, New Delhi.

Bacterial Expression of SG2NA:

Isolated colonies of *E. coli* BL-21DE3 containing bacterial expression construct of GST fusion 35kDa SG2NA, 4T3MS were grown overnight in 5ml LB Ampicillin (100µg/ml) at 37°C, 220 rpm. Secondary cultures were inoculated from the overnight primary culture (1%), induced with 1mM IPTG at OD =0.4 and grown at 37°C for 4 hours. Bacterial lysates of equivalent OD_{600} prepared in Laemmli's Buffer (60 mM Tris-HCl, pH 6.8; 10% glycerol, 100 mM DTT 2% sodium dodecyl sulfate (SDS); 1% β-mercaptoethanol and 0.002% bromophenol blue (Laemmli, 1970) were resolved on 10% SDS-PAGE and observed by Coomasie Brilliant Blue staining (0.1% W/V CBB dissolved in 25% V/V methanol and 10 % V/V acetic acid).

Purification of Recombinant Protein:

Recombinant GST mSG2NA (GST fusion 35, 78, and 52 kDa SG2NA) was purified from the insoluble fraction of bacterial lysate using ionic detergent, sodium *N*-lauroyl sarcosinate (Sarkosyl), protocol adapted from Frangioni and Neel, 1993. Bacterial cell pellet was obtained

after centrifugation of IPTG induced 100mL *E. coli* (BL21 DE3) culture containing GSTMS construct at 5000rpm for 10min. Cell pellet was washed, resuspended in 9 mL ice cold STE (100 mM NaCl, 10 mM Tris.Cl; pH 7.6, 1mM EDTA) and Lysozyme was added to a final concentration of 100 µ g/mL followed by incubation at 30°C for 15min. After lysis, protease inhibitors (5 mM DTT, Amresco and bacterial PI cocktail) were added followed by addition of Sarkosyl to a final concentration of 1.5%. The lysate was vortexed and sonicated at power level 4, 50% duty cycle 1min followed by centrifugation at 10,000rpm, 4°C, for 10min. Triton X100 was added to supernatant thus obtained to a final concentration of 2%. Swollen GSH beads, Sigma Aldrich, equilibriated with PBS containing protease inhibitors were added to the mixture and kept for binding overnight at 4°C. Bound beads were collected by pulse centrifugation, washed with wash buffer (10 mM Tris.Cl; pH7.6, 400 mM NaCl, 1mM EDTA and PI) and loaded onto SDS-PAGE after denaturation in Laemmli's Buffer.

GST Pull down assay:

The confluent 100 mm dishes which was synchronised were scraped and the cells were harvested by centrifugation at 3000 rpm for 10 min. Resuspend the pellet in 100 µl of lysis buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 200 mM NaCl, 1mM EDTA, 0.1% β-mercapto ethanol, 1mM PMSF and PI of 5 µl for 1 ml). To lyse the cells incubate it for 10 min in ice in the lysis buffer followed by brief vortex. The lysed cells were then centrifuged at 12000 rpm for 10 min. The sup was collected and the protein was estimated by Bradford method. The purified GST fusion protein which was not eluted form the bead. 50µgm of this protein (50 µl) was incubated with 1 mg of cell lysate for each protein binding. The binding carried out at 4°C in a rocker for 4 hour to overnight followed by 2 hour incubation at room temperature. The bead was washed with lysis buffer twice and then eluted with elution buffer (10 mM reduced glutathione in 50 mM Tris pH 8.0). The elutes were loaded in 12% SDS-PAGE for separation.

Silver Staining for MALDI TOF/TOF Analysis:

Silver staining was carried out according to Blum *et al.*, 1987, the gel is kept in fixative (50% CH3OH, 12% Glacial Acetic acid 100 µl HCHO for 200 ml solution) for 45 min to overnight. Then keep the gel in 50% ethanol for 30 min followed by wash once with double distilled water. The gel was kept in Hypo solution (40 mg Sodium thiosulfate in 200 ml double distilled water) for 1 min. The gel was washed three times of 20 sec each with double distilled water. The gel

was kept in silver nitrate solution (400 mg silver nitrate and 150 µl HCHO in 200 ml double distilled water) for 20 min followed by 3 washes with double distilled water each of 20 seconds. Then the gel was transferred to the developer solution (12 gm Na_2CO_3, 4 ml Hypo solution and 100 µl HCHO in 200 ml double distilled water) till the band appears. As the band appears the gel is immediately washed off with double distilled water. The staining is stopped with stop solution (40% ethanol, and 12% acetic acid) and then the gel is washed with washing solution (50% methanol). Finally, the gel is kept in double distilled water.

Sample Preparation for Mass Spectrometric Analysis:

The required protein band was excised from the gel and destained (for silver stained) by 30µl-50µl of 1:1 working solution of 30mM potassium ferrocyanide and 100mM of sodium thiosulphate solution and vortexed occasionally for 5 minutes. The process was continued until the gel pieces turn transparent. The gel was rinsed three times with occasional vortexing with HPLC Grade H_2O to stop the reaction. The gel pieces were then incubated with 200mM of ammonium bicarbonate (ABC) for 20 minutes, vortexed briefly in between and supernatant was discarded. The protein band was cut in to small pieces approximately of 1-2mm in size and washed with HPLC Grade H_2O. 40% of 100mM ammonium bicarbonate and 60% acetonitrile (100%) was added to the gel pieces and kept at 37°C in sonicator for 30 minutes. The solution was discarded and gel pieces dehydrated by adding Dehydrating solution (1:1 ratio solution of 100mM ammonium bicarbonate and acetonitrile) and kept at room temperature for 5 minutes after vortexing. Supernatant was removed after pulse spin and rehydration solution (50 mM ammonium bicarbonate) and kept at room temperature for 3-5 minutes at room temperature. The rehydration and dehydration steps were repeated twice. Sample was rehydrated. Cover the gel pieces with 10 mM DTT in 100 mM NH_4HCO_3, and reduce at 56°C for 1h. The tubes were kept to room temperature; DTT was removed and equal volume of 55 mM Iodoacetamide in 100 mM NH_4HCO_3 was added. It was incubated in dark at room temperature for 45 min. The gel pieces washed with 100 uL of 100 mM NH_4HCO_3 for 10 min. It was dehydrated with acetonitrile and reswell in 100 mM NH_4HCO_3. Again it was dehydrated with acetonitrile, the liquid was pipetted off and dried in speedvac. Rehydrate gel pieces for 5 minutes at room temperature in 20ul [20ng/µl] trypsin (Promega Sequence Grade Modified) in 25 mM $(NH_4)HCO_3$ for 16-24 hours at 37°C. The rehydrated gel particles were overlaid with a minimum amount of 25mM $(NH_4)HCO_3$ to keep them immersed throughout the digestion. Then the trypsin was removed from the gel

pieces and digestion buffer was added and kept on incubation for 16 hours at 37°C. The supernatant was removed and saved in the microcentrifuge tube. 20µL of 25mM ABC was added and incubated for 15 minutes. 20µL of acetonitrile was added to make the solution 50% and incubated for 15 minutes. Supernatant was removed and added to the previously saved supernatant. 20µL of 5% TFA was added and incubated for 15 minutes. 20µL of acetonitrile was added to make the solution 50% and incubated for 15 minutes. Supernatant was removed and added to the previously saved supernatant. The extracted peptide was dried in the speedvac and store at -20°C till spotting in MALDI plate.

Mass Spectrometric Analysis:

Mass Spectrometric Analysis of peptide fragments obtained by Tryptic digestion of purified recombinant GST tagged SG2NA variants and bands corresponding to interacting partners were excised from CBB stained SDS-PAGE and Silver stained SDS-PAGE respectively were done using Autoflex II MALDI-TOF/TOF, Bruker Daltonics, Germany.

Immunoblot Analysis:

Equal amount of total protein of each sample were resolved on 10% or 12% SDS PAGE, with Prestained protein molecular weight marker and the region of required molecular weight was transferred onto charged PVDF membrane (Amersham, or Millipore) in Towbin's buffer (25 mM Tris, 192 mM glycine and 20% methanol). After transfer, membranes were blocked in 3% BSA prepared in TBST (10 mM Tris.Cl; pH 7.4, 150 mM NaCl, 0.05% Tween-20) at 37^0 C for 3 hour on a rocker. Immunoblotting was done with mouse monoclonal antibodies (dilution of 0.1 µg/mL) at 4^0C overnight. Blots were washed in TBST 5 times; each wash lasting 5min followed by incubation with HRP conjugated antimouse IgG at the dilutions suggested by manufacturer (Santacruz Biotechnology, Inc, CA, USA) at 37^0 C for 40min. Blots were washed in TBST 5 times, followed by one 10 min wash in TBS (10 mM Tris.Cl; pH 7.4, 150 mM NaCl) prior to ECL detection. Immunocomplexes were visualized with ECL detection reagents.

Polymerase Chain Reaction:

Polymerase chain reaction was performed in a 50µl reaction volume with 0.2 mM dNTPs each, 15 picomoles of each primer, 1U Taq or 2.5U Pfu Polymerase and 1ng template DNA in 1X buffer with 3 mM $MgCl_2$

Cycling condition was maintained as follows: (for screening of the clones using internal primers of SG2NA sequences e.g MSRTf and MSRep)

1. 95° C for 10 minutes Initial Denaturation
2. 95° C for 1minute   Denaturation
3. 50° C-55° C for 1minute Annealing
4. 72° C for 1-4 minutes   Extension
    Step 2-4 30times.
5. 72° C for 10 minutes Final Extension

For Amplification to clone the fragment in myc tagged vector

1. 95° C for 10 minutes Initial Denaturation
2. 95° C for 1minute   Denaturation
3. 50° C for 1minute Annealing
4. 72° C for 6 minutes   Extension
    Step 2-4 for 10 times.
5. 95° C for 1minute   Denaturation
6. 56°C for 1 min   Annealing
7. 72°C for 6 min Extension
    Step 5-7   20 times
8. 72° C for 10 minutes Final Extension

The annealing temperature was set according to primer annealing conditions and the extension time was maintained depending on the length of the fragment and DNA polymerase used (1 kb/min for Taq DNA Polymerase and 0.5kb/min for Pfu DNA Polymerase).

Agarose Gel Electrophoresis of DNA:

Agarose gel electrophoresis was done as described in Sambrook et al., 1989. Routinely 1% (or as indicated in Figure legends) Agarose gel prepared in 1X TAE   (40mM Tris-Acetate, 1mM EDTA, pH 8.0) buffer containing 0.5µg/ml Ethidium bromide was used.

Nucleotide/Amino Acid Sequence Analysis:

The sequences of mouse SG2NA variants were analysed by several online available softwares for searching motifs present in the SG2NA variants the software http://elm.eu.org/ was used (Puntervoll *et al.*, 2003). The motifs present in SG2NA as predicted by this software are also

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verified by the new software http://mnm.engr.uconn.edu/MNM/SMSSearchServlet which is more sophisticated and can pick up very small motif present in the eukaryote proteins (Balla *et al.*, 2006). The Exon intron boundaries of SG2NA transcript were determined according to "Ensembl" Genome Browser (http://www.ensembl.org/). Alignments of peptides were made using "MultAlin" (http://bioinfo.genopoletoulouse.prd.fr/multalin/) and 'Clustal W' (http://www.ebi.ac.uk/Tools/clustalw2/index.html) program. Open Reading Frame of cDNA sequences were translated using "ExPASy" (http://au.expasy.org/) for the prediction of molecular weight of the SG2NA variants.

Transient Transfection:

Transient transfection was done in 12 well plate and 60 mm dishes. Cells were transfected at 90% confluence with the reporter construct (GFP vector) alone or cotransfected with expression plasmids (2.5 µg/60 mm dishes for each plasmid) with Lipofectamine 2000 (10µl/60 mm dish) (Invitrogen, USA) according to manufacturer's protocol. After transfection, cells were kept in serum free medium for 4-5 hours followed by addition of 2X (20% Serum, 180U/ml Penicillin, 180 µg/ml Streptomycin and 10 µg/ml Amphotericin B) media to the serum free media and incubated for 24 hours followed by harvesting the cells.

Stable Transfection:

The myc tagged constructs of SG2NA 35 kDa and 87 kDa variant expression plasmids (pcDNA 3.1 myc/his A -) were linearised by restriction digestion with ScaI and transfected into NIH3T3 cells using Lipofectamine 2000, invitrogen, USA, as per manufacturer's protocol. Cells stably expressing myc SG2NA variants were selected with 400µg/ml G418 containing complete medium. Isolated pools of cells were picked using sterile vacuum Grease and 7mm hollow glass tubes and maintained separately in complete medium containing 100µ g/ml G418. Cells were observed under Inverted Phase Contrast Microscope (Nikon).

Cell Synchronisation:

The NIH 3T3 cells were synchronised for the making lysate for GST pull down experiment by double thymidine block. At 50-60 % confluency the NIH3T3 cells were treated with thymidine (from Sigma Aldrich, USA) to a final concentration of 2 mM and incubated at 37°C CO_2 incubator for 12 hour. Then the thymidine block was released by changing the media and

incubated for 6-10 hour. The second thymidine block was given with a final concentration of 2 mM thymidine for 12 hours. After releasing from thymidine block by changing the media the cells were harvested after 2 hour (S phase) and 4 hour (G2 phase).

Staining the Cells and FACS analysis:

After transfection cells were trypsinised and harvested by centrifugation at 1000 rpm for 5 min in 4°C. Then the cell pellet was resuspended in 1 ml cold PBS. The cells were counted and $2*10^6$ cells were taken from each set of experiment. The cells were fixed with chilled absolute alcohol (kept in -20°C) adding drop by drop while overtaxing the tube. The cells can be stored at -20°C for 15 min to 2 weeks. Then the cells are centrifuged at 1000 rpm for 5 min at 4°C. The cells were again resuspended in 1 ml of cold PBS. Then 2 µl of RNAse (10mg/ml) was added to each tube and incubated at 37°C for 30 min.100 µl of Propidium Iodide (PI, 1 mg/ml) was added to each tube and incubated at RT for 10 min. 500 µl of each samples were run on FACS Calibur, BD Biosciences and acquired and analysed through cell quest pro software. The samples were acquired in FL2 using red laser.

Results

General objective:

Despite extensive research on cell cycle regulation worldwide, the precise mechanism of progression through each phase of cell cycle and the roles of various cell cycle regulated proteins are not well-understood. Our laboratory had developed a molecular approach for isolating a repertoire of cis-regulatory DNA sequences involved in embryonic chick heart development (Sindhu *et al.*, 2004). A partial cDNA clone of chicken SG2NA was then isolated while screening the 72 hour embryonic chick heart cDNA library using a novel sequences as probe (unpublished results). The DNA binding and transactivation potential of SG2NA have been reported earlier (Zhu *et al.*, 2001; Landberg and Tan, 1994). Although, we started with the elucidation of the function of SG2NA in cardiovascular system, it turned out to be having multiple isoforms generated by alternative splicing (Mishra *et al.*, 2008), and also undergoes alternative polyadenylations (unpublished data). Since SG2NA was originally reported to be a cell cycle regulated protein (Muro *et al.*1994), we are interested to find out its role in cell cycle regulation. Hence, the present study was aimed towards analyzing the structure-function analysis of SG2NA in the context of cell cycle.

Specific objective:

SG2NA being a WD-40 repeat protein, it is expected to act as a scaffolding platform assembling multiple proteins with catalytic function. Therefore, the immediate objective of the study was identifying its interacting partners in various phases of cell cycle progression.

Experiments and Results:

A. Cloning of mSG2NA Variants:

We had procured a full length cDNA for mouse SG2NA that later turned out to be the 35 kDa isoform that is devoid of the carboxy terminal WD-40 domain arising out of intronic retention after a part of (4 nucleotides) exon 7 (Mishra *et al* 2008). This cDNA was initially cloned in frame (GST) into the bacterial expression vector pGEX 4T3 (Fig.1). RT PCR was then performed with NIH3T3 mRNAs and primer pairs (MSRTf   5' GGTGGTGAATCTCCTAAGC 3' and   MSRep   5'CCTGTGAGGAAAGCATCAC   3') encompassing the common region of all the variants i.e. exon 5 and exon 15 respectively (Fig.2). At least 3 bands representing the three other isoforms (78kDa, 82kDa and 87kDa) of SG2NA were obtained as the PCR products (Fig.2). Those bands were gel purified and cloned into pBlue Script vector at the Sma-I site (PCR products were blunt ended as *pfu* polymerase was used for amplification). The respective pBlue Script constructs were then used as a source of the variable regions unique to individual isoform for subsequent cloning by replacing the corresponding region of pGEX 4T3-35kDa (Figure 1). The variable regions of the cDNAs of all the isoforms are flanked by one unique Xho-I (exon 6) and Blp-I (exon 14). Accordingly, recombinant pBlue Script plasmid DNAs were digested with Xho-I & Blp-I and the DNA fragments were ligated to pGEX 4T3-35kDa DNA also digested with Xho-I & Blp-I. The recombinants were first confirmed by restriction digestion (Fig. 3) and then nucleotide sequencing.

For mammalian expression of the SG2NA variants, we then used the pCDNA 3.1 myc/his A (-) [invitrogen]. The entire ORF of each variants in the pGEX vector (as described above) were amplified using primers flanked by the restriction sites Nhe-I (5' end of the ORF) and Kpn-I (3' end of the ORF) and pfu polymerase. The PCR products were digested with Nhe-1 and Kpn-1 and cloned into the corresponding sites of pCDNA 3.1 myc/his A (Fig.4).

Expression of mSG2NA Variants:

Bacterial Expression of 87, 78 and 35 kDa, variants: The pGEX 4T3 constructs for the respective variants were then transformed into *E. coli* BL21 (DE3) cells and grown in large volume (100-250ml) followed by induction with 1mM IPTG (at 0.5 OD_{600} for 4 hours at 37^0C).
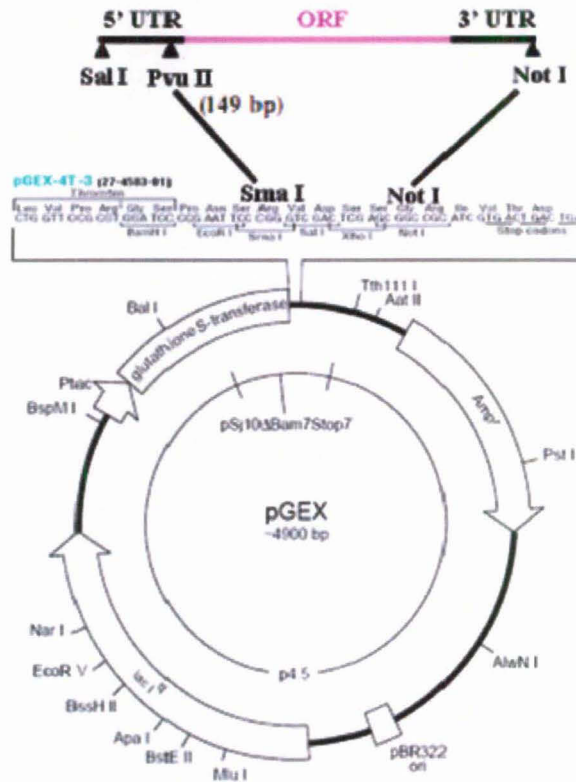
Figure 1. The 35 kDa isoform of SG2NA was purchased from Open Biosystem USA and cloned inframe into the pGEX4T3 vector generating 35 kDa- pGEX4T3 (Mishra *et al.,* 2008)
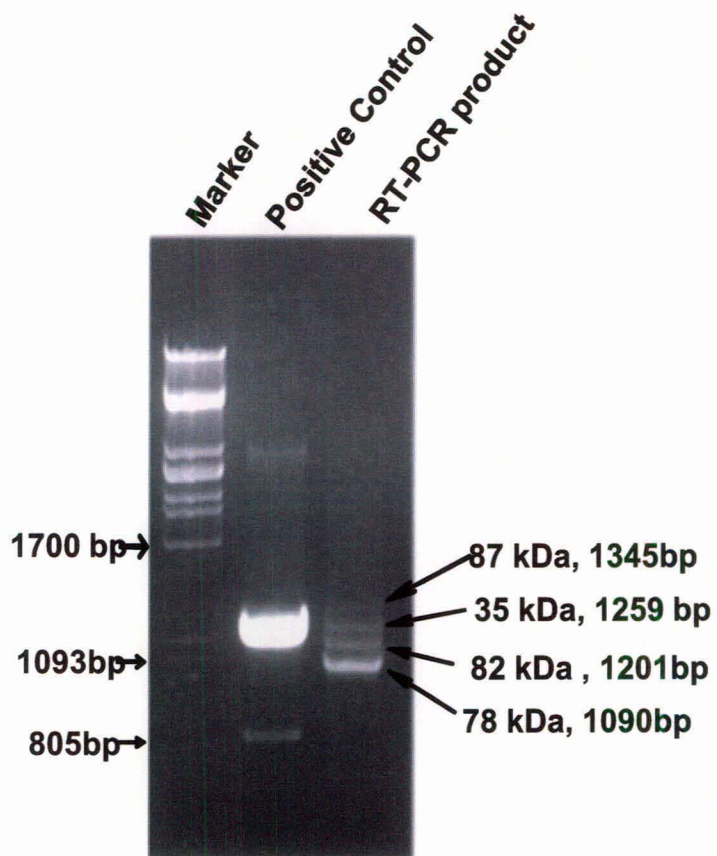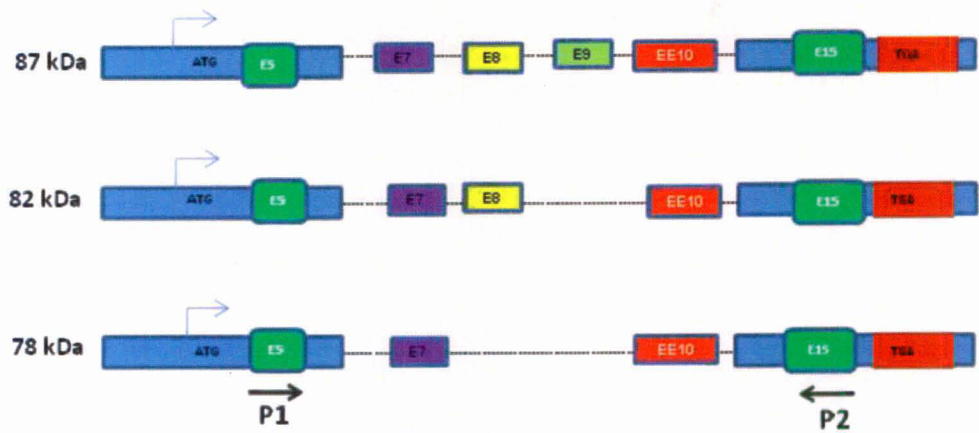
Figure 2. Amplification of SG2NA variants by RT-PCR:. Different isoforms of SG2NA were amplified by RT-PCR using variable region (exon 7-10) spanning primers MSRTf and MSRep (P1 and P2 respectively) encompassing exon 5 and 15. 5µg total RNA from Neuro 2A cells was used for reverse transcription.

A

pBS plasmid harboring regions of 78, 82, 87 kDa SG2NA → Digest with Xho-1 and Blp1 → 700-800 bpfragments

35 kDa- pGEX4T3 → Digest with Xho-1 and Blp1 → 4.9 kb vector containing 5' and 3' regions of 35 kDa SG2NA

Ligation

B

1   2   3   4

←935 bp
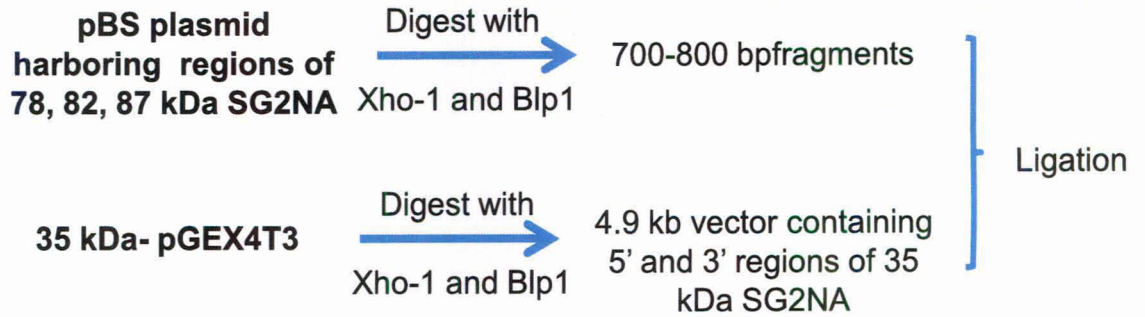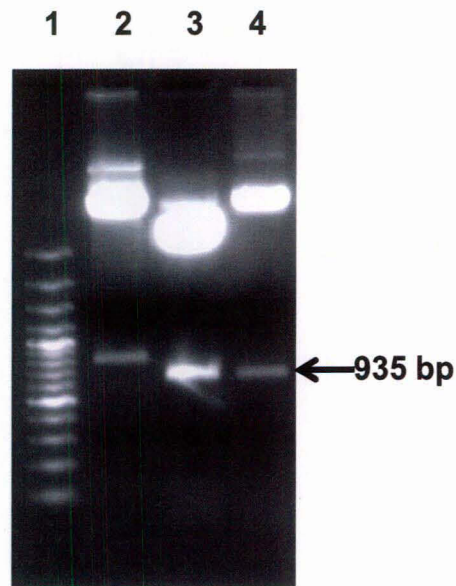
Figure 3. (A) The Xho-I and Blp-I fragments generated from the respective pBS clones (upper panel) were ligated to the vector generated by digestion of 35kDa SG2NA in pGEX 4T3 (Mishra et al, 2008) generating 78, 82 and 87 kDas SG2NA-pGEX 4T3(lower panel). (B) Representative Xho-1-Blp-1 restriction pattern for the 35kDa SG2NA- pGEX 4T3. Lane 1: 100 bp ladder, Lane 2: 35 kDa GST-SG2NA , Lane 3: 82 kDa-pBS SG2NA and Lane 4: 82 kDaSG2NA- pGEX 4T3 plasmid.
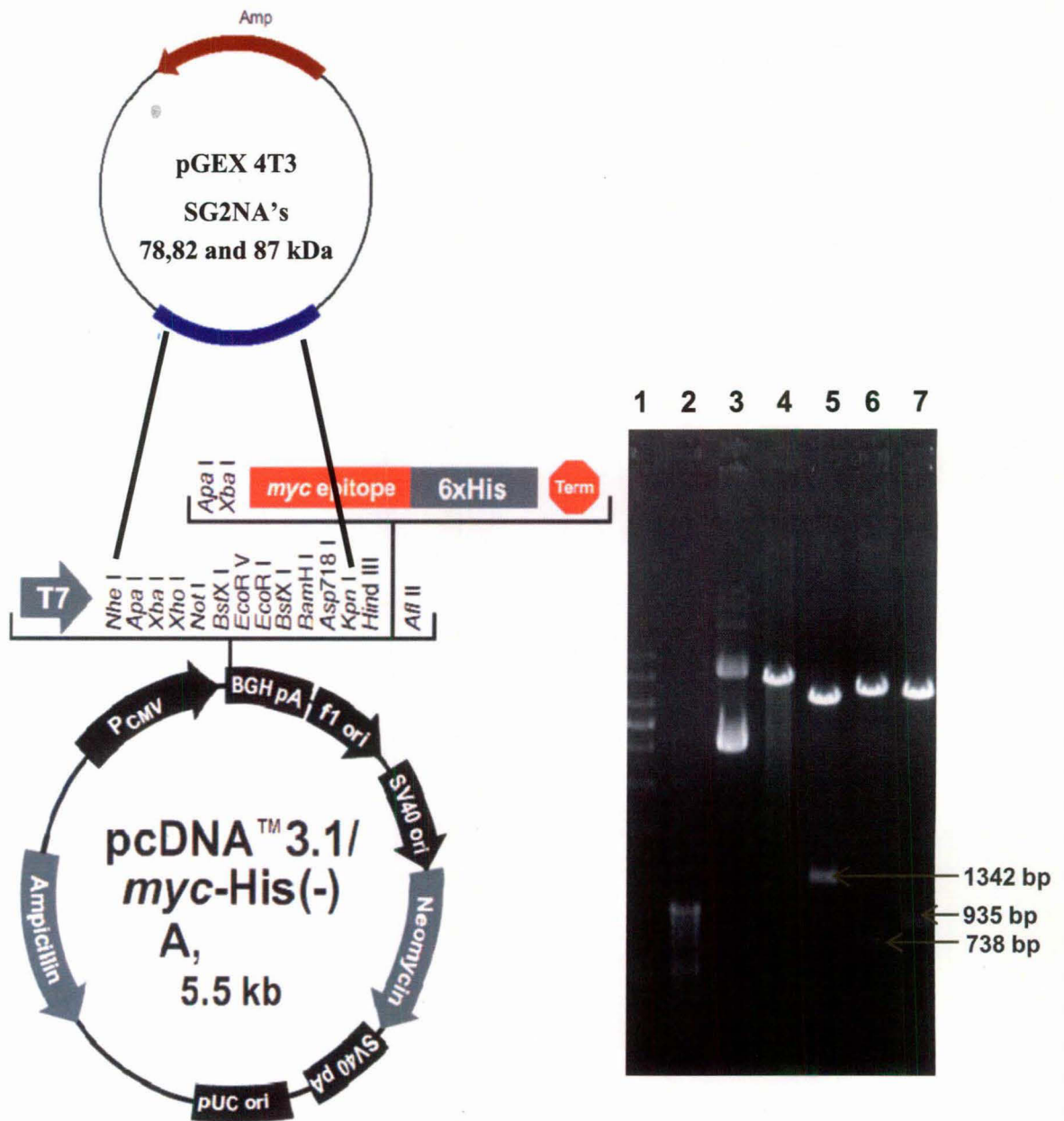
Figure 4. Strategy for cloning SG2NA variants into pcDNA Vector for mammalian expression. Left Panel: The respective ORF were amplified by primers flanking the Nhe-I and Kpn-I sites in 35, 87, 82, 78 kDa SG2NA- pGEX 4T3 and cloned into the Nhe-I and Kpn-I site of pcDNA 3.1 myc/ His (-) vector. Right Panel: Representative 82 kDa myc-SG2NA construct were digested by different restriction enzymes for cloning confirmation. Lane 1: 1 kb ladder, Lane 2: 100 bp ladder, Lane 3: undigested plasmid, Lane 4: Kpn-I digested plasmid, Lane -5 EcoR-I and Hind-III digested plasmid, Lane 6: Nhe-I and Xho-I digested plasmid and Lane 7: Xho-I and Blp-I digested plasmid.

The lysates from uninduced (-IPTG) and induced (+IPTG) bacterial cells were resolved on 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). Although the predicted molecular mass of 35 kDa variant of GST-SG2NA was 60Kda (26kDa for GST plus 35kDa for SG2NA), the induced protein was ~67 kDa in SDS-gel (Fig 5). Similarly, the other isoforms, 78kDa & 87kDa also gave larger size bands of ~125kDa and ~134kDa respectively (Fig.5). Such deviation from the predicted mass might be due to the presence of highly charged N-terminal residues (Muro *et al.*, 1995). Unexpectedly, GST-82 kDa gave a band corresponding to ~90kDa in SDS-PAGE implying it to be a smaller isoform than 82 kDa as predicted from the sequence of RT-PCR products (Fig 2B). In order to check the expression of recombinant SG2NA in mammalian cells, recombinant pCDNA 3.1 myc/his A plasmids were transiently transfected in to CHO-K1 using lipofectamine transfection reagent (Invitrogen). Cells were harvested after 24hours and lysates were prepared in RIPA buffer. One hundred and fifty microgram of lysates was resolved on SDS PAGE followed by Western blotting using mSG2NA antibody. Expression of all the isoforms with appropriate molecular weight, except for the 82 kDa variant were thus confirmed (Fig.6).

SG2NA Undergoes RNA Editing:

As described in the previous section, the molecular weights of the splice variants were first predicted from the EST analysis and then validated by sequencing the RT-PCR products encompassing the variable regions of respective cDNAs. Therefore, the substantially decreased molecular weight of the 82kDa variant expressed both in *E coli* and CHO cells was unexpected. Such discrepancy raised question about the authenticity of the GST-82kDa (as well as the pCDNA 3.1 myc/his A) constructs which was the further sequenced multiple times. Sequencing results revealed the absence of one adenosine at 1571 position leading to the formation of premature stop codon at 473 in the place of 750 (Fig.7). Such deletion caused a frame-shift giving 52 kDa SG2NA rather than 82 kDa as predicted earlier (derived based on the presence of exons and introns in the RT-PCR products).

EST database analysis of the 52 kDa variant carrying a deletion of one adenosine residue was not found in the EST data base for SG2NA. We thus analysed the genome data base to check whether the sequence is present in the intron and the deletion of single A residue is due to intronic retention followed by splicing as in case of other variants (Mishra *et al.*, 2008). This analysis suggested that the base deletion is a characteristic of the 82/52 kDa variant of SG2NA.
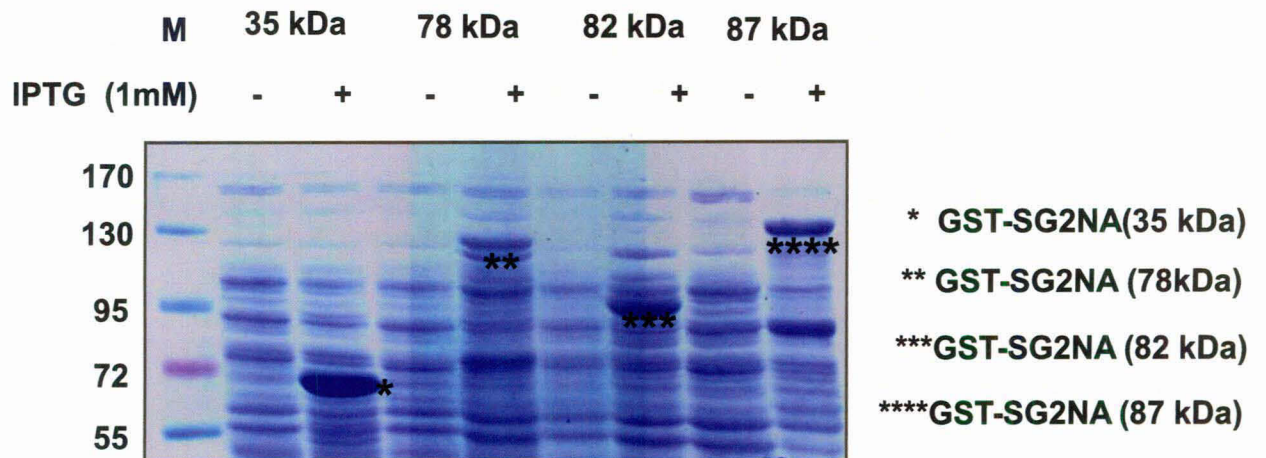
Figure 5. Bacterial Expression of SG2NA variants: The 35, 87, 82, 78 kDa SG2NA-pGEX 4T3 plasmids in *E. coli* BL21 (DE3) were induced with 1mM IPTG for 4 hours at 37 C at 0.5 OD_{600} . The lysates from both uninduced (-IPTG) and induced (+IPTG) cells were resolved on 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). 35 kDa SG2NA gave expression of a ~61 kDa (26kDa GST plus ~35kDa SG2NA), 78 kDa SG2NA gave expression of a ~104 kDa (26 kDa plus 78 kDa) and 87 kDa SG2NA gave expression of ~113 kDa (26 kDa plus 87 kDa) as expected. Surprisingly the 82 kDa SG2NA gave a product of ~78 kDa indicating it to be of 52 kDa rather than 82 kDa.
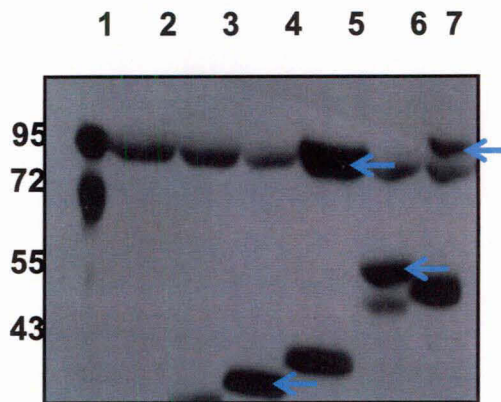
Figure 6. Mammalian expression of SG2NA: The 35, 87, 82, 78 kDa SG2NA- pc DNA plasmids were transfected into CHO K1 cells and kept for 24 hours. Lysates were then Western analyzed with SG2NA monoclonal antibody (NEB). Lane1:Marker, Lane 2: Untransfected Control, Lane 3: Vector Transfected, Lane 4: 35kDa myc-SG2NA transfected, Lane 5: 78 kDa myc-SG2NA transfected, Lane 6: 82kDa myc-SG2NA transfected and Lane 7: 87kDa myc-SG2NA. All the variants showed expression at expected molecular weight except the 82 SG2NA which showed expression of a smaller protein of ~52 kDa. The additional immunoreactive bands found in some lanes are presumably due to cleavage of the original protein.

B

A

Applied Biosystems

GSTm82_MsREP_H04
GSTm82_MsREP
KB_3730_POP7_BDTv3.mob
Pts 2300 to 12500 Pk1 Loc:2103
Version 5.3.1   HiSQV Bases: 697

Inst Model/Name 3730/3730Analyzer-17121-005
Jun 05,2009 05:24PM, IST
Jun 05,2009 06:05PM, IST
Spacing:14.8 Pts/Panel1600
Plate Name: plate-819

S/N G:110 A:65 T:53 C:64
KB.bcp
KB 1.2   Cap:9

One T is missing

```
Query  1561  GAACCTGCAAA-GACAGTTCCTGCCAAAGAGAGTGCCTCTTTAGATGTAGAACCTATCTA  1619
             GAACCTGCAAAAGACAGTTCCTGCCAAAGAGACAGTGCCTCTTTAGATGTAGAACCTATCTA
Sbjct  1561  GAACCTGCAAAAGACAGTTCCTGCCAAAGAGACAGTGCCTCTTTAGATGTAGAACCTATCTA  1620
```

Figure 7. (A) Sequence analysis of the original 82 kDa variant. Sequencing was carried out by the reverse primer of the 82 kDa SG2NA DNA sequence . Two different colonies were sequenced which gave same result. (B) Comparison of the derived sequence (query) to that of the sequence from NCBI data base shows the absence of one "A". (C) ORF analysis reveals that deletion of one A in original cDNA of 82 kDa (Left Panel) variant (Mishra *et al*.,2008) leads to a new truncated 52 kDa ORF (Right Panel) due to insertion of a stop codon.

The RNA Editing is Tissue Specific:

Then we tested whether the 52 kDa variant is expressed endogenously. Extracts werte thus prepared from NIH3T3 cells, CHO-K1 cells and CHO-K1 cells transfected with myc tagged mSGNA (52 kDa) and were analysed by western blot using SG2NA antibody. As shown in Fig. 8, CHO K1 cells does not express a 52 kDa variant of SG2NA (whereas it expresses the transfected myc/his tagged SG2NA), NIH 3T3 cells express the 52 kDa variant. This finding prompted us to check the expression pattern of 52 kDa isoform in different mouse tissues. As shown in Fig 8, we observed that 52 kDa isoform is expressed in brain, lung, ovary, liver and kidney but does not express in heart, spleen and skeletal muscle.

Purification of SG2NA Variants:

Striatin family members are primarily membrane localized and they have a conserved caveolin binding domain at the amino termini. Therefore as expected, preliminary experimentation suggested that upon bacterial expression, GST-SG2NA primarily remains the insoluble fraction. Extensive optimization of culture conditions such as reduced culture temperature, extended period of incubation etc., were largely ineffective in enriching the soluble fraction. Thus the recombinant proteins were extracted from the insoluble fraction by sarkosyl (sodium N-lauryl sarcosine), an ionic detergent, essentially as described by Frangioni and Neel (1993) and as detailed in the "Methods". The purified fractions (bound to GSH-Sepharose beads) were directly loaded onto SDS-PAGE followed by staining with Commassie Brilliant Blue (Fig.9). The corresponding bands were then excised and subjected to mass spectrometric analysis. The spectrum of the peptide mass fingerprinting (p.m.f.) for tryptic digests of GST-SG2NAs (52, 78 and 87 kDa respectively) were taken and matched with gi|148704832 (mouse strn3 56 kDa), gi|148704832 (Mouse strn3 78 kDa) and gi|148704832 (Mouse Strn3 87 kDa, Fig.10) with significant scores (p<0.05).

Identification of Interacting Partners of mSG2NA (35, 52 and 78 kDa) variants:

Presence of WD-40 repeats indicated that SG2NA might be a scaffolding protein for certain yet unknown cellular function. Previous reports also suggested the presence of caveolin binding motif and calmodulin binding domain. Whether or not any other interacting motifs are present in
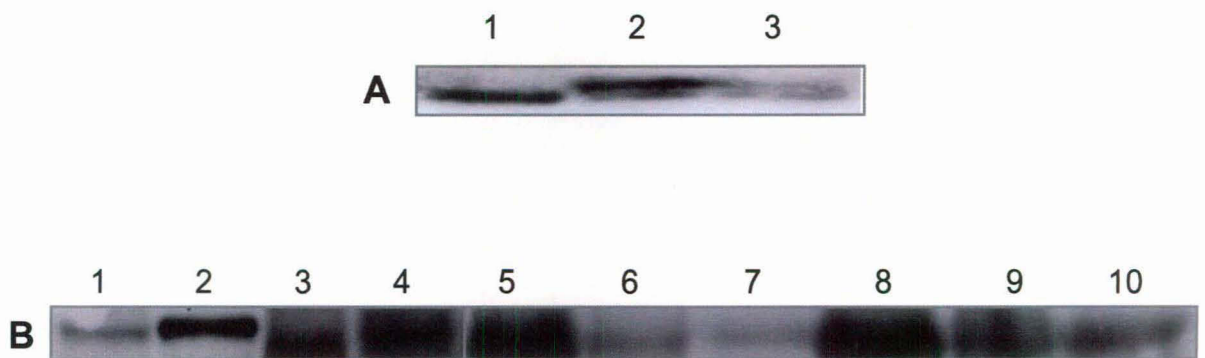
Figure 8. Tissue specific expression of 52 kDa variant: (A)Western analysis was done using lysates from NIH3T3 cells (lane 1), CHO cells transfected with 52kDa-SG2NA-pcDNA (lane 2) and untransfected CHO cells (lane3) using monoclonal SG2NA antibody. The reason for the small discrepancy in the size of the endogenous protein in NIH3T3 cells and the recombinant protein in CHO cells could be due to differential post translational processing. (B) Expression of the 52 kDa variant in mouse tissues were analyzed by western blotting using extracts from (1) Normal NIH 3T3 cells, (2) CHO cells transfected with 52kDa-SG2NA-pcDNA, and mouse (3) heart, (4) brain (5) Lung, (6) Spleen, (7) Skeletal Muscle (8) Ovary (9) Liver and (10) Kidney. 200μgm of cell lysate and 300 μgm of tissue extracts were loaded.

GST SG2NA 35 kDa   GST SG2NA 78kDa   GST SG2NA 52kDa

1 2 3 4 5 6 7 8 9   1   2   3   4   5   6

170
130
95
72
55

A   B
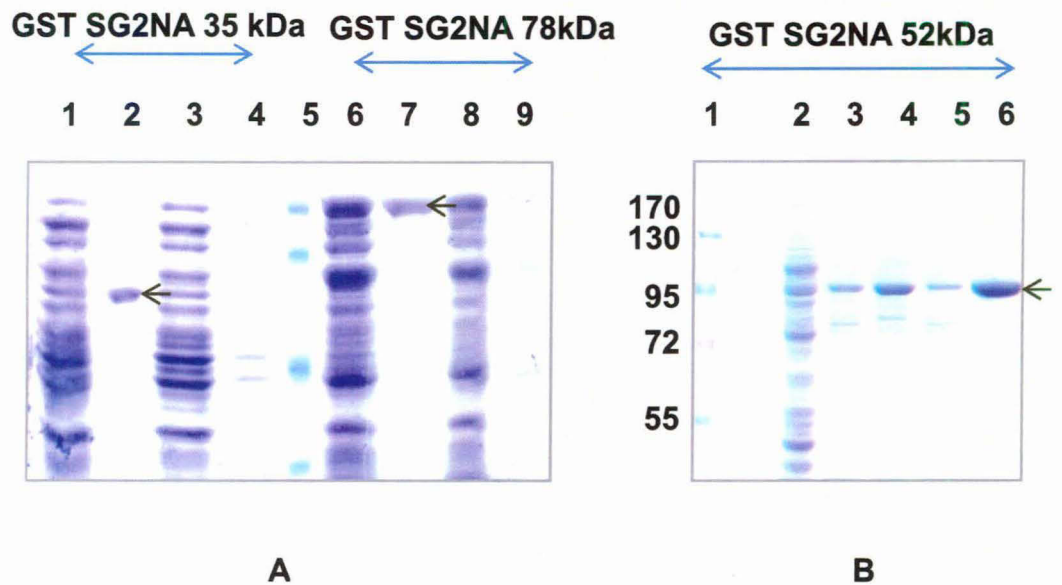
Figure 9. Purification of 35/78/52 kDa GST-SG2NA: Recombinant GST-SG2NA was expressed in E coli and the insoluble fraction were extracted using ionic detergent 'Sarkosyl' as described in 'Methods'. Fractions from various steps of purification were loaded on SDS-PAGE as in (A) Lane 1 & 6: induced (1mM IPTG) bacterial lysate , Lane 2: purified 35 kDa GST -SG2NA, Lane 3 & 8 : Flow Through; Lane 4 and 9 : Wash; Lane 7: purified 78 kDa GST-SG2NA (B) Lane 1: Molecular Weight Marker, Lane 2: Induced bacterial lysate, Lane 3: flow through, Lane 4: bound beads before wash. 5: Wash and Lane 6: purified 52 kDa GST-SG2NA. After separation, the gel was stained with CBB.

Figure 10. Spectrum obtained from the peptide mass fingerprinting (p.m.f) of the purified GST-mSG2NA (A) 52 kDa (B) 78 kDa and (C) 87 kDa variants. Insets are showing their respective matched sequences.
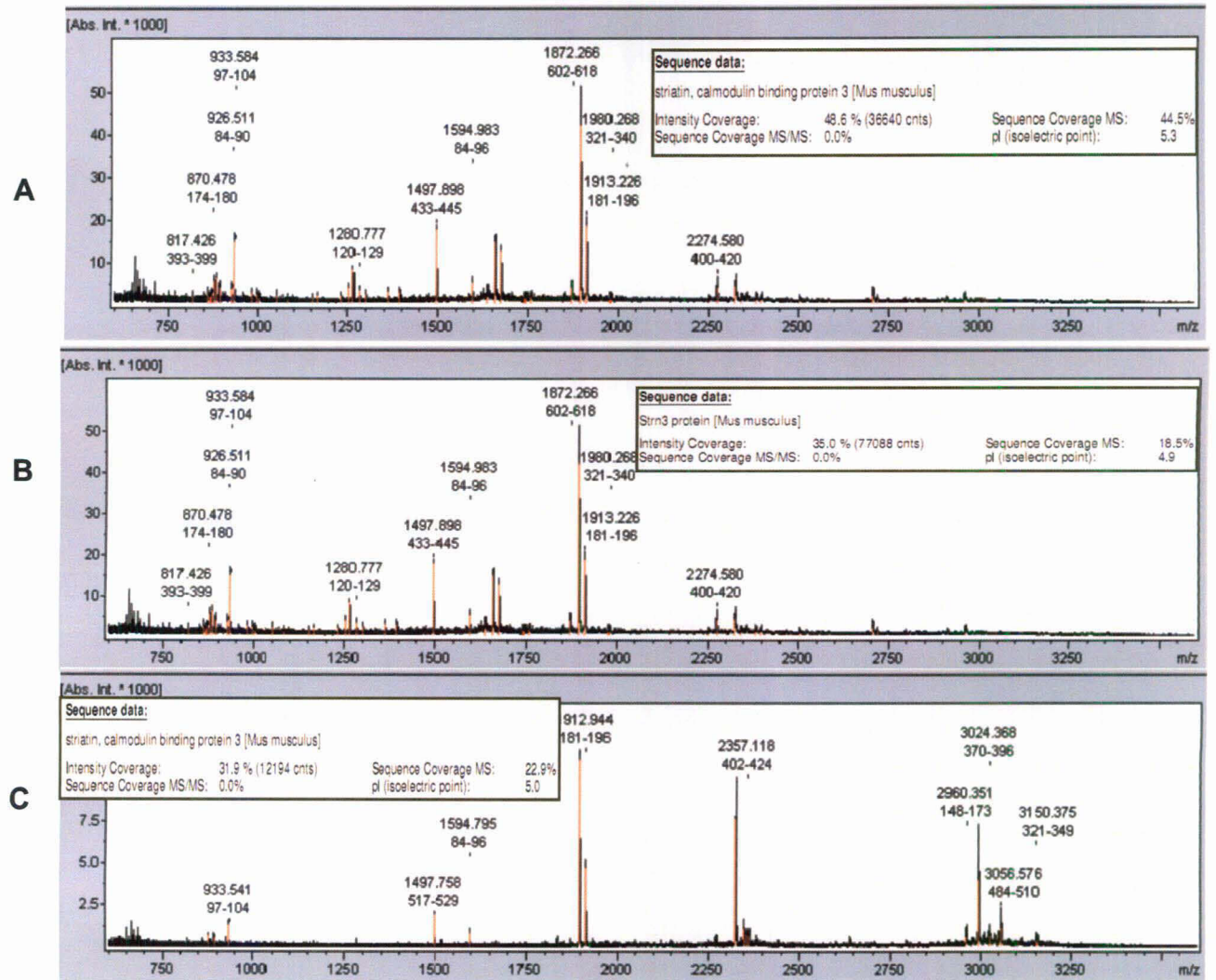
SG2NA, were first checked by motif search tool like ELM and minimotif minar (Fig.11). Such analysis revealed a large number of interacting motif spreading all over from amino to the carboxy terminals. Details description of those is given in Table 1.

Keeping in view that SG2NA was initially identified as a tumour autoantigen whose level is augmented during S to G2 phases of cell cycle; we first attempted to look for its interacting partner in S phase of cell cycle. NIH 3T3 cells were synchronised by double thymidine block and then released to entering into the S phase (2 hours). Recombinant GST mSG2NA (35 and 78 kDas) immobilized on glutathione sepharose beads were incubated with lysates from NIH3T3 cells in S phase. The unbound proteins were washed off and then the bound material was eluted (details given in "Methods". The eluted material were then resolved on SDS PAGE followed by silver staining. Proteins that were eluted with GST-SG2NA (35 and 78 kDas) but not with GST alone were excised (Fig. 12) and analyzed by MALDI-TOF/TOF. Such analyses were done twice for each of GST-35 and 78 KDa SG2NAs while it was done once for the GST-52 kDa SG2NA (Fig.13). The putative interacting partners of three variants of SG2NA are shown in figure 14 and 15.

The Effect of over expression of the mSG2NA Variants on Cell Cycle Progression and Growth.

The effect of over expression of SG2NA on cell cycle progression was also tested in CHO-K1 cells. CHO-K1 cells were grown in 10% FBS followed by transfection of myc-SG2NA (35, 52, 78 and 87 kDas) plasmids (2.5 ug/60mm dish) using lipofectamine (Invitrogen) transfection reagent. Propidium iodide staining were done after 24 hours followed by flow cytometric analysis. Efficacy of transfection was confirmed by parallel tansfection with GFP expression plasmid. From the FACS analysis, it appeared that while the 35 kDa variant restricts the cells at G2/M phase of cell cycle, 78, 82 and 87 kDa variants did not show any detectable effect (Fig. 16). We also attempted to make stable NIH3T3 cell lines expressing myc tagged 35 and 87 kDa SG2NAs. After 48 hours of transfection, a large number of cells had undergone apoptosis (Fig. 17).

Evidences of Post Translational Modification of SG2NA variants:

All along while working with SG2NA we found that the predicted molecular weight is less than the determined molecular weight of the protein. There could be many possibilities: one
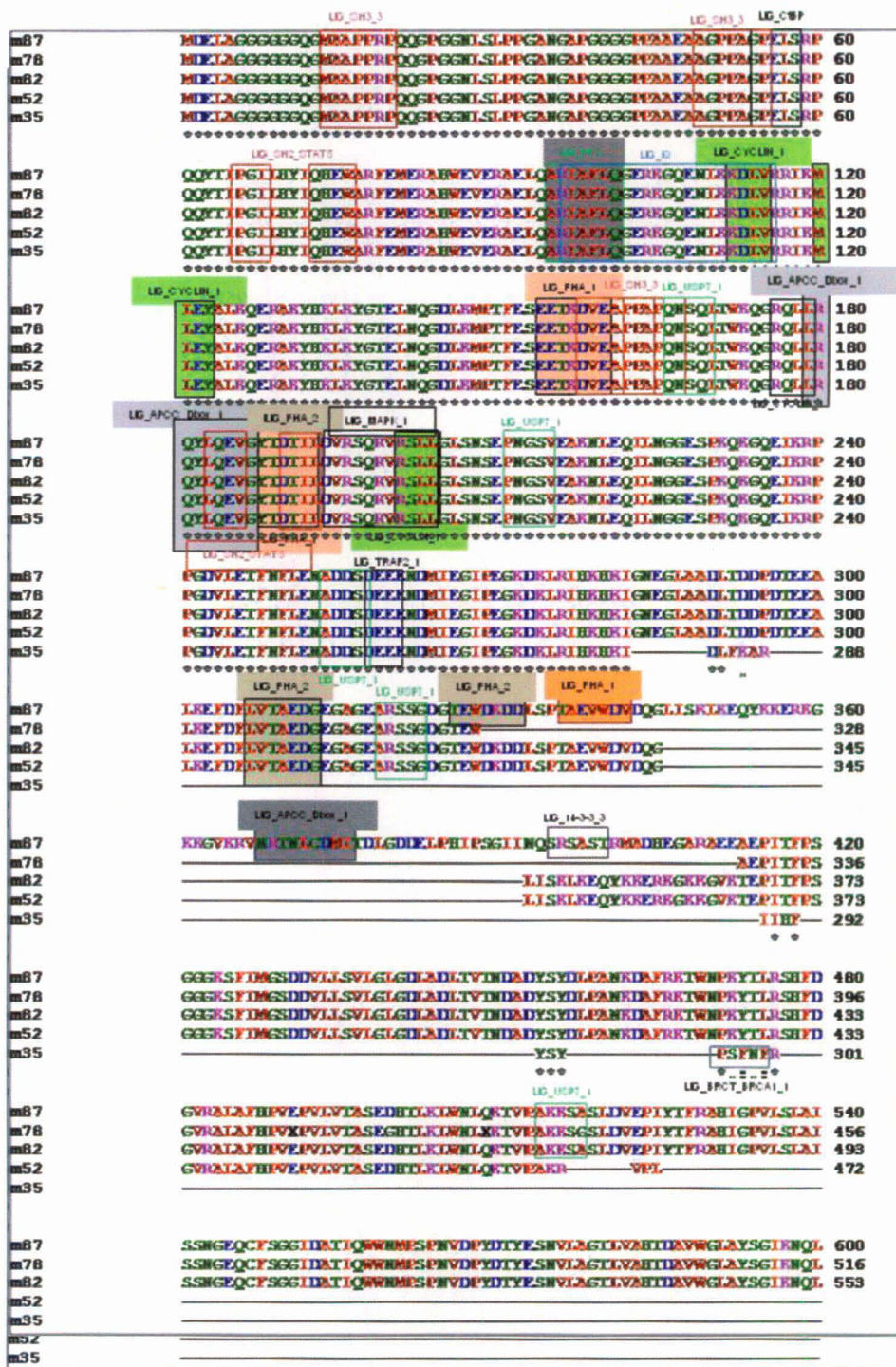
Figure 11. The interacting motifs found in different variants of SG2NA using bioinformatic programs viz., ELM, (see text and table 1 for details).

Figure 12. GST Pull Down Assay with 35 and 78 kDa SG2NA: purified GST mSG2NA immobilized on GST beads were incubated with NIH3T3 lysate synchronised for S and G2 phases of cell cycle (4 hour at 4^0C and half an hour at room temperature). Beads were then washed twice with binding buffer and the eluted materials were separated on 12% SDS PAGE. Lane 1: Protein Molecular Weight Marker; Lane 2: Purified GST alone, Lane 3: Purified 35 kDa GST-SG2NA, Lane 4: Purified 78 kDa GST-SG2NA, Lane 5; NIH 3T3 Cell Lysate (S Phase,) Lane 6: Eluent from GST alone, Lane 7: eluent from 35 kDa GST-SG2NA; Lane 8: eluent from 78 kDa GST-SG2NA. After silver staining the bands which are present in material eluted from 35/78 kDa GST-SG2NAs were excised and sequenced by MALDI TOF/TOF for MS/MS.
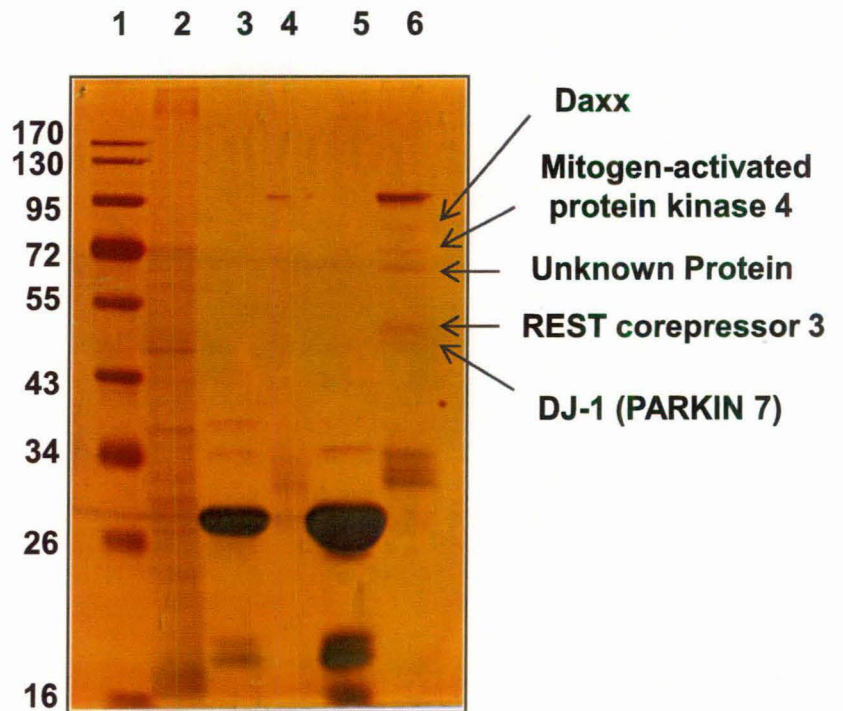
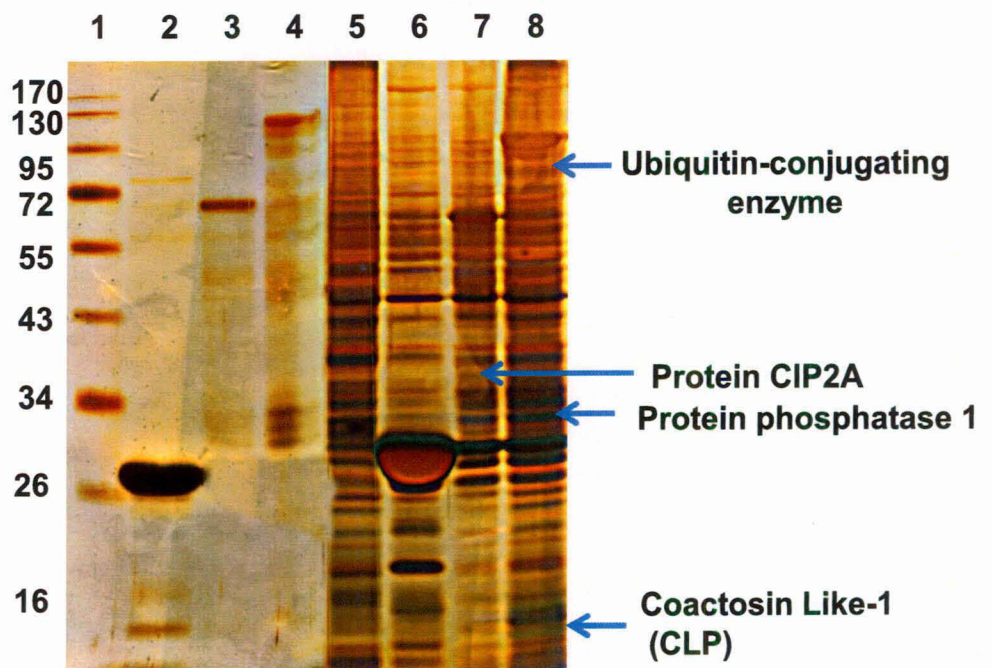Figure 13. GST Pull Down Assay with 52 kDa SG2NA: purified GST mSG2NA immobilized on GST beads were incubated with NIH3T3 lysate synchronised for S phase of cell cycle (4 hour at 4^0C and half an hour at room temperature). Beads were then washed twice with binding buffer and the eluted materials were separated on 12% SDS PAGE. Lane 1: Protein Molecular Weight Marker; Lane 2: NIH 3T3 Cell Lysate (S phase) , Lane 3: Purified GST alone, Lane 4: Purified 52 kDa GST-SG2NA, Lane 5: Eluent from GST alone Lane 6:, eluent from 52 kDa GST-SG2NA. After silver staining the bands which are present in material eluted from 52 kDa GST-SG2NAs were excised and sequenced by MALDI TOF/TOF for MS/MS.
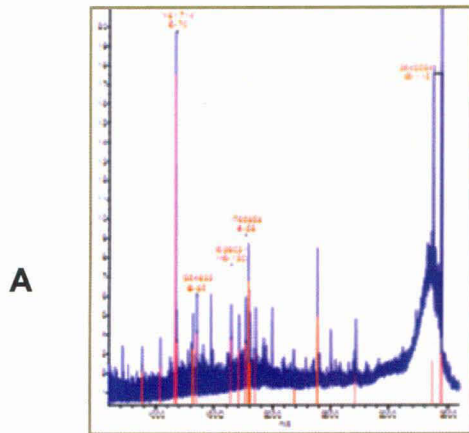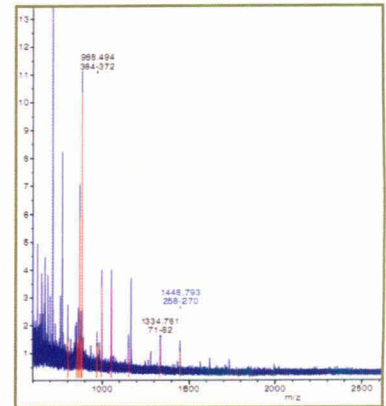
Sequence data:

coactosin-like 1 (Dictyostelium), isoform CRA_a [Mus musculus]

Intensity Coverage:	24.6 % (17178 cnts)	Sequence Coverage MS	42.3%
Sequence Coverage MS	0.0%		
		pI (isoelectric point):	9.9

Sequence data:

Protein phosphatase 1K, mitochondrial OS=Mus musculus GN=Ppm1k PE=1 SV=1

Intensity Coverage:	10.8 % (3493 cnts)	Sequence Coverage MS:	9.1%
Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	5.9
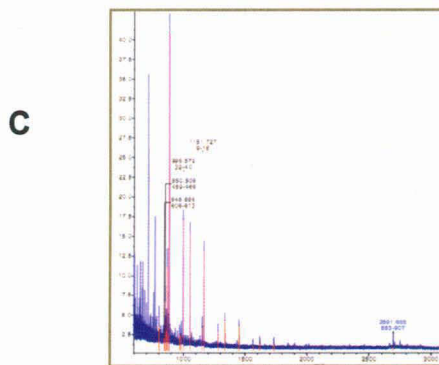
Sequence data:

Protein CIP2A OS=Mus musculus GN=Kiaa1524 PE=2 SV=2

Intensity Coverage:	21.8 % (28893 cnts)	Sequence Coverage MS:	6.6%
Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	5.9
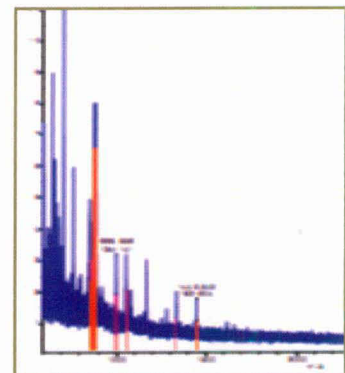
Sequence data:

Ubiquitin-conjugating enzyme E2 T OS=Mus musculus GN=Ube2t PE=2 SV=1

Intensity Coverage:	14.6 % (2881 cnts)	Sequence Coverage MS:	10.3%
Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	8.9

Figure 14. Spectrum obtained from the peptide mass fingerprinting (p.m.f) of the tryptic digested peptides identified as interacting partners of 35,78, 52 kDa SG2NA by GST pull down assays. (A) Coactosin Like Protein (CLP-1); (B) Protein phosphatase 1K; (C) Protein CIP2A, and (D) Ubiquitin-conjugating enzyme E2. Their corresponding matched sequence data is shown just below of the spectrum. Protein Phosphatase binds to both 35 and 78 kDa SG2NA whereas CIP2A exclusively binds to 35 kDa. CLP-1 and Ubiquitin Conjugationg enzyme E2 bind exclusively to 78 kDa SG2NA.

Sequence data:

Protein DJ-1 OS=Mus musculus GN=Park7 PE=1 SV=1

Intensity Coverage:	5.0 % (1553 cnts)	Sequence Coverage MS:	19.8%
Sequence Coverage MS/MS:	0.0%	pI (isoelectric point):	6.4

Sequence data:

death-domain associated protein [Mus musculus]

Intensity Coverage:	6.1 % (8638 cnts)	Sequence Coverage MS9.5%	
Sequence Coverage MS0.0%		pI (isoelectric point):	4.6

Sequence data:

Mitogen-activated protein kinase 4 - Mus musculus (Mouse)

Intensity Coverage:	0.7 % (1003 cnts)	Sequence Coverage MS8.9%	
Sequence Coverage MS0.0%		pI (isoelectric point):	5.1

Sequence data:

REST corepressor 3 - Mus musculus (Mouse)

Intensity Coverage:	45.8 % (139779 cnts)	Sequence Coverage MS11.5%	
Sequence Coverage MS0.0%		pI (isoelectric point):	9.2

Figure 15. Spectrum obtained from the peptide mass fingerprinting (p.m.f) of the tryptic digested peptides peptides identified as interacting partners of 52 kDa SG2NA by GST pull down assays. (A) Protein DJ-1 (PARK-7); (B) Death-domain associated protein. (Daxx); (C) Mitogen Activated Protein Kinase 4,; and (D) REST corepressor 3. Their corresponding matched sequence data is shown just below of the spectrum.
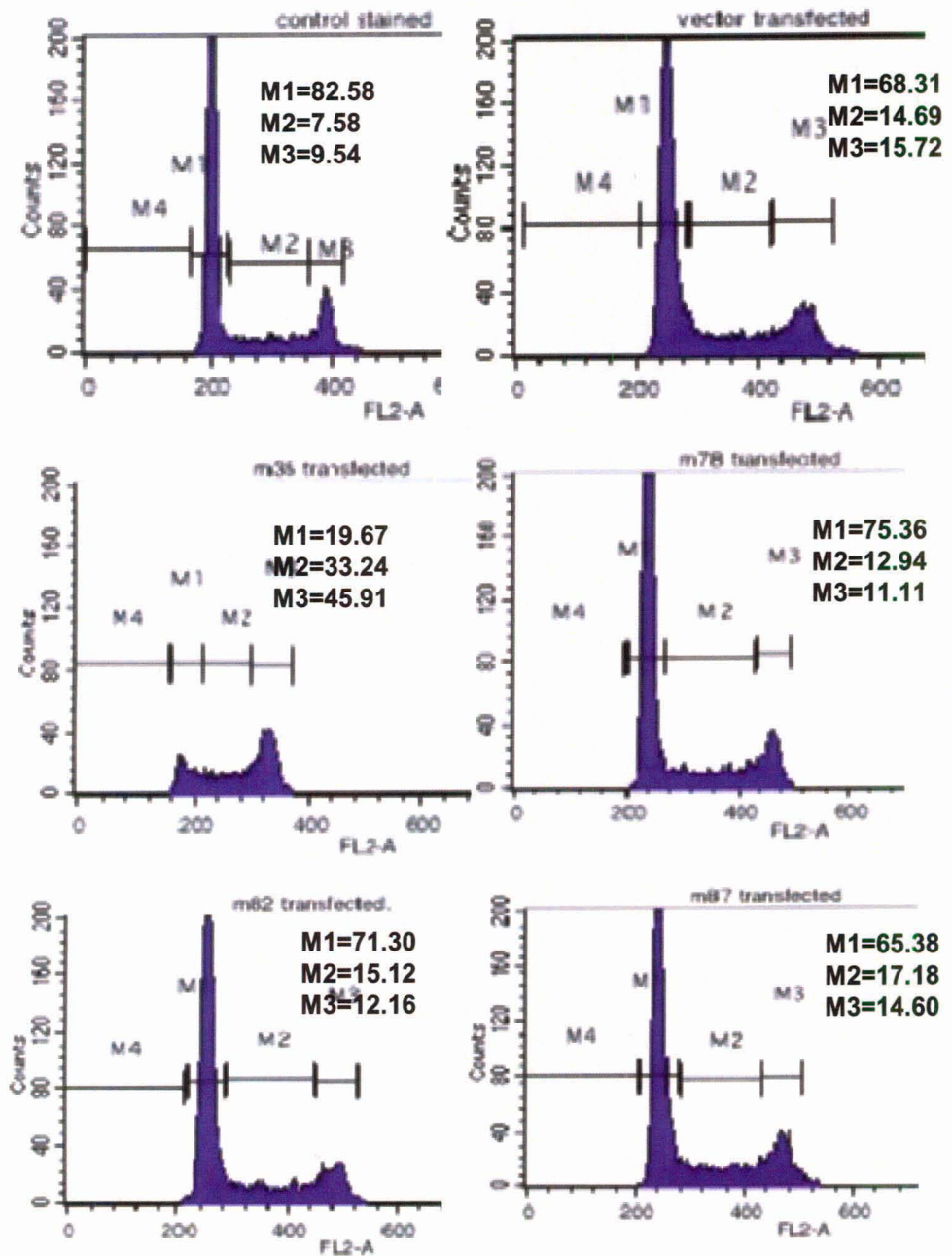
M1= G_1/G_0, M2=S, M3=G2+M, M4= Apoptotic + Necrotic Cells

Figure 16. Effects of over-expression of SG2NA variants on cell cycle progression. CHO-K1 cells were transiently transfected with 35, 52, 78 and 87 kDa variants of SG2NA. Cells were harvested after 24 hour and flowcytometric analysis were carried out.

A

B

C

Figure 17. Phenotypic effects of over expression of SG2NA variants. NIH 3T3 cells expressing 35 and 87 kDa variants underwent apoptosis as tested after 48 hours. Phase contrast photograph of (A) untransfected cells (B) Cells expressing 35 kDa myc-SG2NA (C) Cells expressing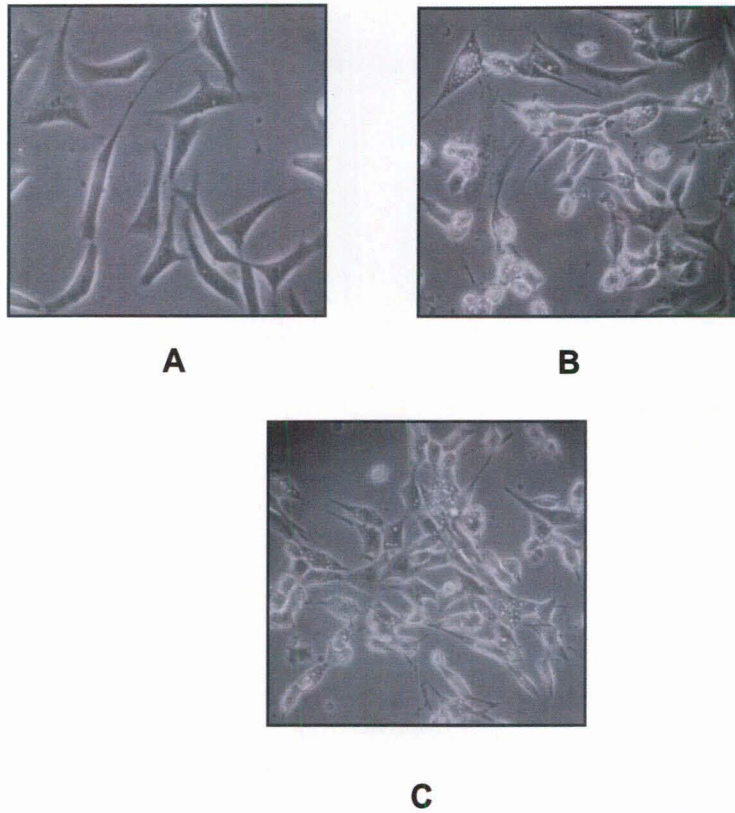 87 kDa myc-SG2NA. Most of the cells became highly vacuolated and rounded upon over expression of SG2NA vis-à-vis untransfected cells.

Table 1: The various motifs foundt in the SG2NA sequences

Interacting Motifs	Position (aa)	Description of the motif	SG2NA variants
LIG_SH3_3	14-20, 49-55, 156-162, 159-165 and 382-388	this motif is recognized by those SH3 domains with a non-canonical class I recognition specificity,	All the variants
LIG_CtBP	54-58	this motif interacts with the CtBP protein	All the variants
LIG_SH2_STAT5	63-66, 71-74, 182-185 and 188-191	this is STAT5 Src Homology 2 (SH2) domain binding motif,	All the variants
LIG_PP1	94-100	Protein phosphatase 1 catalytic subunit (PP1c) interacting motif	All the variants
LIG_CYCLIN_1	112-115, 119-123, 176-179 and 201-204	Substrate recognition site that interacts with cyclin and thereby increases phosphorylation by cyclin/cdk complexes.	All the variants
LIG_FHA_1	154-160, 187-193and 334-340	Phosphothreonine motif binding a subset of FHA domains that show a preference for a large aliphatic amino acid at the pT+3 position,	All the variants
LIG_USP7_1	165-169, 211-215, 254-258, 319-323 and 514-518	This is the USP7 NTD domain binding motif variant based on the MDM2 and P53 interactions.	78, 82 and 87 kDa variants contain five of this motifs, 52 kDa variant contains 4 whereas 35 kDa variant contains 3 of this motif
LIG_APCC_Dbox_1	179-187 and 368-376	An RxxL-based motif that binds to the Cdh1 and Cdc20 components of APC/C thereby targeting the protein for destruction in a cell cycle dependent manner.	Only 87 kDa variant contains two of this motifs others contain only one,
LIG_FHA_2	189-195, 307-313 and 324-330	Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position.	All variants contain two of this motif except 35 kDa variant which contains one of this motif.
LIG_MAPK_1	(196-206)	MAPK interacting molecules (e.g. MAPKKs, substrates, phosphatases) carry docking motif that help to regulate specific interaction in the MAPK cascade. The classic motif approximates (R/K)xxxx#x# where # is a hydrophobic residue and x is any residue,	
LIG_TRAF2_1	257-260	Major TRAF2-binding consensus motif. Members of the tumour necrosis factor receptor (TNFR) superfamily initiate intracellular signalling by recruiting the C-domain of the TNFR-associated factors (TRAFs) through their cytoplasmic tails	
LIG_14-3-3_3	(396-401)	which consensus derived from reported natural interactors	87 kDa variant
LIG_BRCT_BRCA1_1	296-300	this phosphopeptide motif directly interacts with the BRCT (carboxy-terminal) domain of the Breast Cancer Gene BRCA1 with low affinity	35 kDa variant contains motif at its C terminal end which

possibility is that the N terminal part of the protein is highly positively charged (Muro *et al.*, 1995), there is no any other peculiarity we found. These prompted us to check whether this protein undergoes post translational modification. We express the bacterial and mammalian constructs of SG2NA (78 and 87) were expressed in *E .coli* BL21 and CHO-K1 respectively. And the extracts were resolved in SDS-PAGE viz-a-viz for bacterial and mammalian extracts followed by western blotting with SG2NA antibody. It appeared that in case of mammalian expression, at least one isoform of SG2NA variants i.e 78 kDa, migrates very slowly and hence it appears to be of higher molecular weight in SDS-PAGE in comparison to its apparent molecular weight and also it moves slower than its bacterial counterpart (Fig.19).
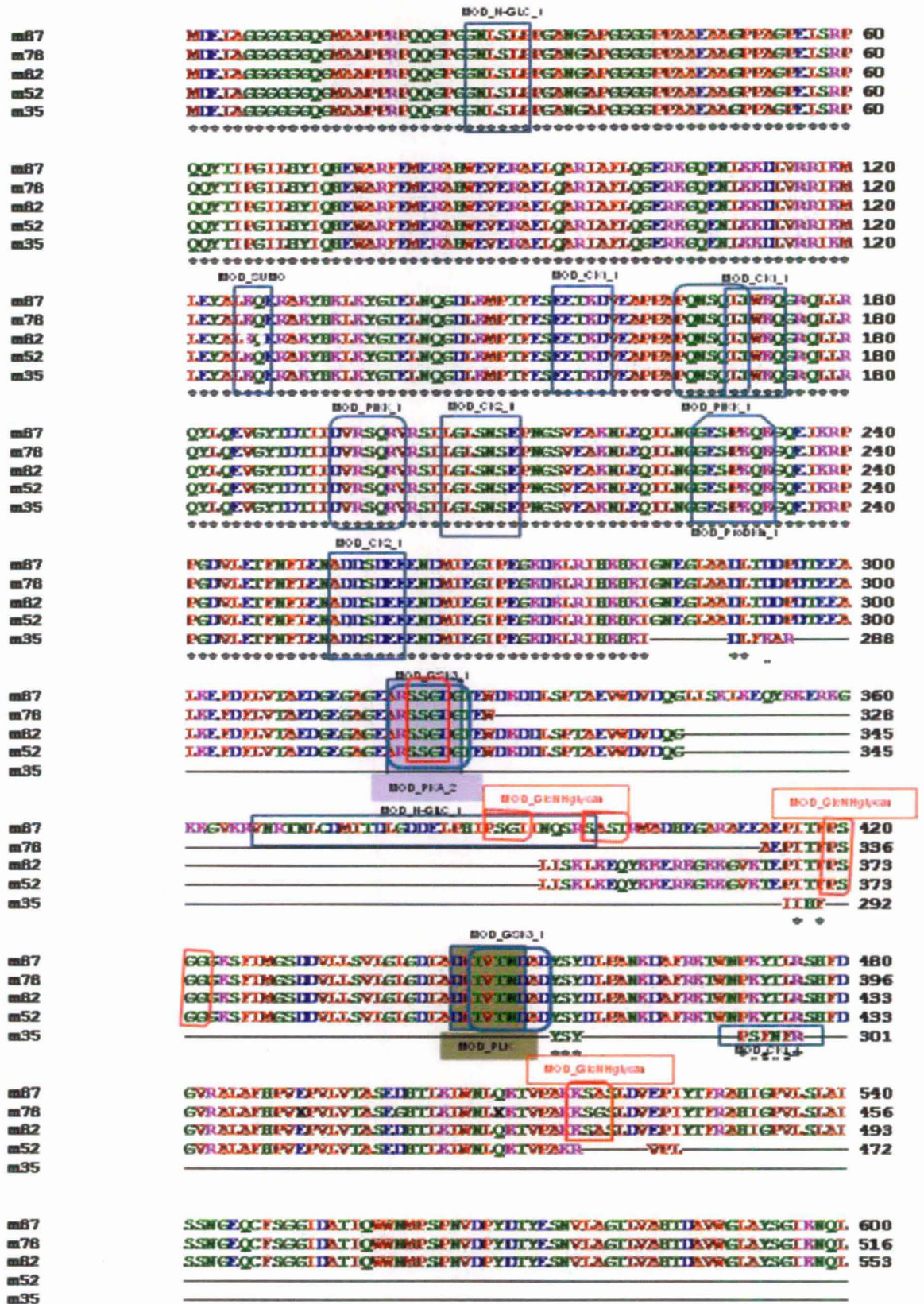
Figure 18. Different Post Translational Modifications predicted for SG2NA variants by ELM program (see text and Table 2 for details).
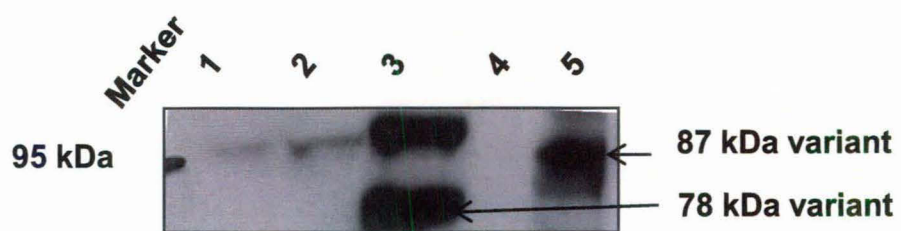
Figure 19. Evidence of posttranslational modifications of SG2NA: Comparative Western analysis of SG2NA variants expressed in bacterial and mammalian cells. (1) Lysates from NIH3T3 cells transfected with 78 kDa myc mSG2NA. (2) Lysates from CHO-K cells transfected with 78 kDa myc mSG2NA. (3) Lysate from 78 kDa His-SG2NA expressing in *E coli* cells (4) Lysates from CHO-K cells transfected with 87 kDa myc mSG2NA (5) Lysate from 87 kDa His-SG2NA expressing *E coli* cells. 100 µgm of protein of each sample was loaded. The blot shows that SG2NA variants expressing in mammalian cells were ~10 kDa larger than the bacterial counterpart indicative of post translational modifications.

Table 2. The probable Post Translational Modification of SG2NA variants

Modification Site	Position (aa)	Description
MOD_CK1_1	(153-159, 168-174, 397-403);	CK1 phosphorylation site,
MOD_CK2_1	(204-210, 254-260);	CK2 phosphorylation site,
MOD_GSK3_1	(319-326, 392-399);	GSK3 phosphorylation recognition site,
MOD_PIKK_1	165-171, 194-200, and 660-666.	(ST)Q motif which is phosphorylated by PIKK family members,
MOD_PKA_2	319-325	PKA phosphorylation site,
MOD_PLK	444-450	Site phosphorylated by the Polo-like-kinase.
MOD_GlcNHglycan	321-324, 388-391, 396-399, 419-422, and 516-519.	Glycosaminoglycan attachment site,
MOD_N-GLC_1	26-31, 211-216, 367-372, and 392-397.	Generic motif for N-glycosylation.
MOD_SUMO	125-128.	Motif recognised for modification by SUMO-1.

Discussion

In metazoan organisms, the functional complexity is achieved by multiple layers of regulation of gene expression. Thus in culmination, a large variety of proteomes is found in different cell types in a given condition and in same cell type under different conditions. Now, it is fully appreciated that regulation of gene expression is not only confined to transcription, rather it is attained at each level multitudes of post-transcriptional events like maturation of mRNA, splicing, polyadenylation and translocation from nucleus to cytosol. Another novel layer of gene regulation i.e., RNA editing was initially thought to be restricted only in parasitic protozoa e.g. Trypanosome, has subsequently been described in mammalian cells. Finally, numerous post-translational modification is also significantly adds to the regulation of gene function in eukaryotes. Noticeably, SG2NA uses almost all of the above mentioned tactics to diversify its function as it undergoes alternative splicing (Mishra *et al.*, 2008), alternative polyadenylation (unpublished data), editing and posttranslational modifications (as shown in this dissertation).

With an aim to explore the scaffolding functions of SG2NA, we had attempted to identify its interacting partners. Three isoforms of SG2NA viz., the 78kDa, 82kDa and 87kDas were expressed in *E coli* as GST fusion proteins. While the 78 and 87 kDa variants gave expected size products on SDS-PAGE, the 82 kDa variant was much smaller than expected. To identify the discrepancy, two rounds of DNA sequencing revealed a shift in the open reading frame due to a single nucleotide (A) deletion at position 1573, thus acquiring a premature stop codon with a translation product of a 52 kDa (instead of 82 kDa). It thus might be a case of serendipity that we identified a type of RNA editing hitherto unknown in mammals. This edited variant expresses in brain, lung, ovary, liver and kidney but not in heart, spleen and skeletal muscle. Striatin and SG2NA are abundantly expressed in brain (while SG2NA variants are also expressed in other tissues). The expression of the 52 kDa edited version of SG2NA in brain is thus in line with the identification of its putative interacting partners DJ-1 (Parkinson's disease 7 autosomal recessive early onset, PARK-7) and Death domain containing protein Daxx as identified by GST pull down followed by MALDI-TOF/TOF analysis. The PD-7 protein is produced due to mutation in *DJ-1* gene. It has been attributed to Parkinson's disease, early onset of which is autosomal recessive; although it's exact function is still unknown. Deletions and point mutations in *DJ-1* have been found worldwide, and loss of function of DJ-1 causes autosomal recessive Parkinson's disease (Lev *et al.*, 2006). Daxx protein was first identified as a signalling protein that specifically binds to Fas Death domain and induces apoptosis by activating JNK (Yang *et al.*,

1997). Subsequently it was shown that DJ-1 has antioxidant function and by interacting with Daxx it retains Dxxn the nucleus and prevents its translocation to the cytoplasm (Junn.*et al.*, 2005). Taken together, apparent connection between 52 kDa SG2NA, DJ-1, Dxx and JNK provide an excellent opportunity to explore the biology of Parkinson's disease in future. Furthermore, Drosophila has only one ortholog of Striation/SG2NA family, i.e., CKA. CKA interacts with the dJNK pathway during morphogenesis (Chen *et al.*, 2002). In our laboratory, we also observed that while H9c2 cardiac myoblasts are induced to apoptosis by treatment with 100 μM NE (Gupta *et al.*, 2006), cell death can be minimized by attenuation of SG2NA by Si RNA (Mishra *et al.*, unpublished results). Taken together, SG2NA appear to be a key modulator of JNK signalling in multiple cell types (neuronal and cardiac muscle cells, in the present context). The 52 kDa variant also interacts with MAPK 4/ERK4 an atypical MAP Kinase with unknown function (Coulombe and Meloche 2007). The only known substrate of the ERK4 is MK5 (Kant *et al.*, 2006) and thus the relevance of its interaction with 52 kDa SG2NA is unclear as yet.

Unlike the 52 kDa, the 78 kDa variant interacts with CLP-1 (mouse coactosin like protein-1 binding to F actin, Provost *et al.*, 2001), Protein phosphatise 1 K and Ubiquitin-conjugating enzyme E2. Earlier, Moreno and co-workers had reported that SG2NA interacts with Protein Phosphatase 2A (PP2A) and can be its substrate (Moreno *et al.*, 2001). In this study, we observed that 35 and 78 kDa SG2NA variants interact with another phosphatase that is protein phosphatise 1. *In silico* analyses suggested that all isoforms of SG2NA have interacting motif for the c subunit of protein phosphatise 1. Also, 35 kDa SG2NA might interact with CIP2A, the small inhibitory protein of PP2A (Junttila *et al.*, 2007) whose function is still unknown.

During the identification of the interacting partners' of SG2NA (78 and 35 kDas), one of its already known binding partner of Striatin, i.e. Adenomatous Polyposis Coli (APC), was also isolated; thereby validating our approach. The armadillo repeat domain of APC directly binds to the WD-repeat region of Striatin (Breitman *et al.*, 2008) and co-localize with tight junction (TJ) protein ZO-1. APC with the help of Striatin contributes to the cell-cell adhesion through the Tight Junction.

Although, based on its augmented expression in S to G2 phases of cell cycle and the presence of cyclin binding sites, a role of SG2NA in cell cycle progression has long been predicted. However, over expression of variants of SG2NA did not suggest any significant alteration in cell

cycle progression, except that in case of the 35 kDa which showed significant increase in S and G2/M phase cells. Since 35 kDa variant is devoid of the entire WD40 repeat domain (carboxyl terminus), it might be a naturally occurring dominant negative form of the other larger variants. It is thus apparent that the 35 kDa isoform might be associated with G2/M checkpoint signalling. Taken together, this study highlights that variants of SG2NA might play important roles in cell signalling and cell cycle regulation.

During the progression of this work, we observed that the predicted (from the ORF) molecular weights of all the isoforms were less than the found in SDS-PAGE. Amongst number of possibilities for such discrepancy, one is that the N terminal part of the protein is highly positively charged (Muro *et al.*, 1995). Another reason could be post translational modifications of all the variants. As predicted by ELM server (Fig.18), all the variants have numerous post-translational modification sites viz., phosphorylation, glycosylation and sumoylation. Among the phosphorylation sites the most important sites are that by casein kinase 1, casein kinase 2, PIKK, Protein Kinase A (PKA) and GSk 3 β. Such possibility of post translational modifications is also corroborated by our observation that SG2NA variants interact with PP1K, CIP2A and MAPK kinase 4. Although extensive analysis of the post translational modification is yet to be done, evidence of the modifications of SG2NA came from the western blot analysis wherein bacterially expressed proteins for all the isoforms were smaller than their counterparts expressing in CHO cells. However, further verifications of the types of modifications could not be done due to time constraints.

Summary & Conclusion

SG2NA was originally identified as a nuclear autoantigen whose expression is augmented during S to G2 phase of cell cycle (Landberg and Tan 1994). It has a WD-40 repeat domain present in its carboxyl terminus and in addition, a caveolin binding motif, a coiled–coiled structure and a calmodulin binding domain located in the amino terminus in the same order. SG2NA is highly homologous to striatin and Zinedin constituting the striatin family of WD-40 repeat proteins. Striatin is the prototype member of the family involved in the differentiation of the dendritic spines. SG2NA and striatin interact with phocein and Protein Phosphatase 2A (PP2A), attributing them to vesicular trafficking and cell signalling respectively. Drosophila has only one striatin/SG2NA homologue i.e., CKA, which acts as a platform for organizing the components of JNK signalling and the transcription factor AP-1, indicating a common functional ancestry between the three members of the striatin family. In our laboratory it recently has been reported that vertebrate SG2NA exists in multiple (87, 83, 82, 78, 38, 35 kDas) isoforms generated out of alternative splicing (Mishra S et al., 2008). We also demonstrated that the splice variants of SG2NA are differentially expressed in various tissues and their expression profile changes with the state of differentiation and the proliferative potential of the cognate cell type. It is thus anticipated that SG2NA might be a critical regulator of cell differentiation and cell cycle regulation. The research work for this dissertation was aimed to assess the function of SG2NA variants in cell cycle regulation. To fulfil that objective the probable interacting partners of the SG2NA variants and the effect of overexpression of the protein variants have been investigated. During this study quite serendipitously it was found that SG2NA may undergo RNA editing and hence adds another layer of complexity to metazoan biology. We for the first time showed that RNA editing may occur in SG2NA and the resulting protein's expression is tissue specific which may play critical role in JNK mediated apoptosis pathway.

Cell Cycle regulation is a very complicated process and orchestrated functions of many proteins regulate this complex process. The existence of different checkpoints in this process ensures the appropriate segregation and transmission of genetic material by cell cycle regulation followed by cell division. We for the first time showed here that the SG2NA specially 35 kDa variant may play critical role in G2/M check point regulation.

Taken together this study thus contributes to our understanding of the function of SG2NA variants in cell cycle regulation. However, further analysis would be required to determine their precise roles in the context of cell cycle regulation.

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