Chapter 8

S6 Kinase: A Compelling Prospect for Therapeutic Interventions

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Additional information is available at the end of the chapter

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Abstract

S6 kinase, a member of AGC family of protein kinases and a downstream effector of mTORC1 pathway has over the years found much relevance in maintaining a normal cellular state by virtue of its established role in regulation of cell growth and proliferation. S6 kinase activity has been linked to different cellular processes like glucose homeostasis, translational and transcriptional regulation. Hence any dysregulation in S6K1 leads to the emergence of various pathological conditions like diabetes, cancer and obesity. It is as such S6 kinase has emerged as a potential target for therapeutic interventions employed in curing such diseases. The Present Chapter reviews the regulation of S6K1, its structural organization and functions, besides highlighting its potential to act as an alternative therapeutic target for various cancerous situations exhibiting deranged mTOR signaling so as to overcome the possibility of relapses observed otherwise while using conventional drugs

Keywords: S6 kinase, mTOR, rapamycin, cancer, cell growth and proliferation

1. Introduction

Cell signaling pathways function as cohorts to contribute in maintaining cellular homeostasis-a phenomenon that remains pivotal in regulating cell growth and survival. The unique but immensely coordinated response of these diverse signals is primarily regulated by the availability of nutrients, growth factors and energy status of the cell. The signals destined to activate certain cellular functions may simultaneously be antagonistic for other functions; to strike a much required balance for proper functioning of the cell. Relentless efforts

over the last several decades have led to recognize the ability of these signaling networks to promote cell growth and proliferation by way of their potential to phosphorylate one another to distinguish themselves as kinases. The kinases specifically transfer the phosphoryl group from ATP to their substrates such as to bring about a change in their function that helps transduce the upstream signals for the accomplishment of functions. Intrigued by the properties of these kinases, the scientific community over the years has shown great interest in identifying more kinases that lead to the identification of a family of protein kinases known as ribosomal S6 kinases (RSKs) that respond to nutrients and growth factors. The RSKs came to fore after studying the inducible phosphorylations on ribosomal protein S6 (rps6) and accordingly p90 ribosomal S6 kinase was identified from Xenopus oocytes. The homologous counterparts of p90 S6 kinase in humans were soon identified and grouped into family of four enzymes termed as RSK1-4. It was later on observed that the RSKs may play a minor role in rps6 phosphorylation [1] to give way for the emergence of a major rps6 kinase identified as 70 kDa ribosomal S6 kinase [1–4]. The 70 kDa ribosomal S6 kinase was purified from mitogen stimulated Swiss mouse 3T3 cells [5]. Cloning of 70 kDa ribosomal S6 kinase was soon accomplished after protein sequencing of a rat and rabbit cDNA [6–8]. Subsequent identification and cloning of human orthologs of S6K genes RPS6KB1 and RPS6KB2 encoding S6K1 and S6K2 thus, respectively, led to the emergence of a second family of S6 kinases, a principal kinase of rps6 in somatic cells [2, 9–14].

S6 kinases figure among important signaling molecules required for propagation of cell growth and proliferation. Adding to its importance is the fact that S6 kinase (S6K1) is a downstream effector of mTORC1 (mechanistic target of rapamycin), a master regulator of cell growth and proliferation. Evidences are strong enough to suggest that the mTOR-S6K1 axis controls fundamental cellular processes including transcription, translation and lipid synthesis. Further, this nexus partly regulates glucose homoeostasis, controls insulin sensitivity, adipocyte metabolism, tissue and organ size, learning, memory and aging [15]. S6K1 has therefore been considered to play a pivotal role in regulating cellular physiology. Any dysregulation of this signaling axis has deleterious implications ranging from metabolic disorders to various cancers. mTOR/S6K1 signaling network has hence remained central target for various medical interventions employed over the years for treating the pathological conditions. However, so far the therapeutic interventions involving mTOR as a target molecule for treating cancers, particularly renal carcinomas have reported a fair share of relapses. The resistance shown to these drugs post chronic exposures remain one of the major concerns the scientific community is faced upto. One of the reasons for any such observation would be the incomplete understanding vis-a-vis the regulation of mTOR/S6K1 axis. It is as such conceivable that improved understanding of S6K1 regulation would help develop novel therapeutics that should take care of these challenges. The present chapter reviews the regulation of S6K, its structure and functions.

2. S6K protein family; domain architecture and cellular localization

Based on the sequence alignment of their catalytic domains, S6Ks have been placed in AGC family of protein kinases that mainly comprise of protein kinase A (PKA) protein kinase G (PKG) and protein kinase C (PKC) [16]. The structural resemblance in AGC kinases allows

them to exhibit more or less a similar mode of regulation. These kinases fundamentally exhibit bi-lobal structural organization around the kinase domain. This structural arrangement enables their amino and carboxyl termini to coordinate ATP binding [17, 18]. Further these kinases comprise of an activation loop (commonly known as T-loop) that precedes their C-terminus lobe. The phosphorylation of T-loop brings in conformational changes important for phosphoryl transfer. Two other important phosphorylation sites, the turn motif (TM) (so-named due to its location at the cusp of a structural turn in the PKA tail) and hydrophobic motif (HM) sequentially follow the kinase domain. The phosphorylated HM site engages a hydrophobic pocket within the N-lobe. The phosphorylated TM site stabilizes phospho-HM binding to the N-lobe hydrophobic pocket. Together, these three phosphorylations stand critical in stabilizing the catalytically active state [17, 18].

RPS6KB1, human ortholog of S6K1 genes, encodes two isoforms, p70S6K1 and p85S6K1 (formerly known as p70S6KII and p70S6KI, respectively) through alternative translational start sites [10]. p70S6K1, a 502 amino acid protein and its larger isoform p85S6K1, having nuclear localization sequence (NLS) within the 23 amino acid N-terminal extension, were earlier believed to remain localized in the cytoplasm and nucleus, respectively. However, the data generated of late, contests the nuclear localization of p85S6K1 while showing its sub-cellular localization to be cytoplasmic and at the same time claims p70S6K1 localization to be both nuclear and cytoplasmic [19]. S6K2, encoded by RPS6KB2 gene, constitutes

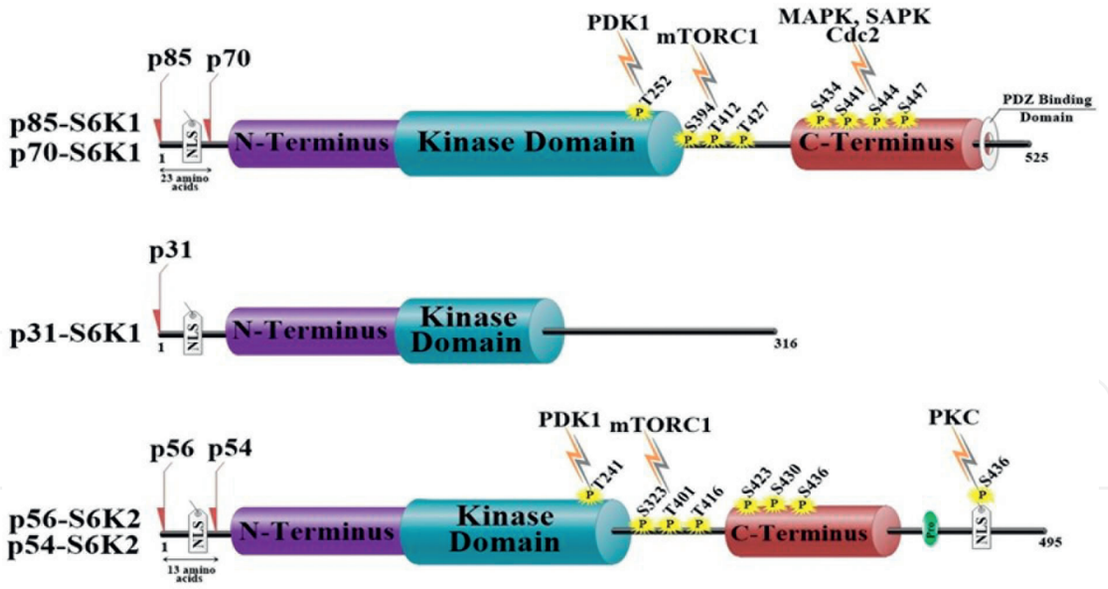

Figure 1. S6K isoforms, structure and domain organization with potential phosphorylating kinases along with their sites of action. S6K1 isoforms include p70-, p85- and p31-S6K1; alternative start site usage lengthens the p85- and p31-S6K1 N-termini by 23 amino acids (note that p31-S6K1 lacks most of the kinase domain). S6K2 isoforms include p54- and p56-S6K2; alternative start site usage lengthens the p56-S6K2 N-terminus by 13 amino acids. NLSs lie within the N-terminal extensions of p85-S6K1 and p56-S6K2, whereas S6K2 additionally contains an NLS within the C-terminus as well as a proline-rich domain (Pro). S6Ks contain an acidic N-terminal domain (NTD), kinase domain (KD), linker region and acidic C-terminal domain (CTD). The N-terminal domain contains the TOS motif, whereas the CTD contains the auto-inhibitory pseudosubstrate domain and RSPRR motif. mTORC1 phosphorylates the HM (hydrophobic motif) site (Thr412) in the linker region and PDK1 phosphorylates the T-loop site (Thr252) within the kinase domain. Other regulatory phosphorylation (P) sites, including the TM (turn motif) site (Ser394), are shown.

another member of S6 kinase family that, via alternative start sites, produces two nuclear isoforms, owing to the presence of nuclear localization sequence at the C-terminus [11]. The longer isoform, p56S6K2 comprises a 13 amino acid extension at N-terminus than its shorter isoform, p54S6K2 [9]. Moreover S6K2 isoforms have been characterized by the presence of proline-rich domain at the C-terminal [20]. S6K1 and S6K2 have been structurally dissected into several regulatory domains, namely an acidic N-terminus that contains the TOR signaling (TOS) motif; the kinase domain that contains the activation/T-loop; a linker region that contains the TM and HM sites; and a basic C-terminus containing an auto-inhibitory pseudosubstrate domain (**Figure 1**). These proteins are homologous to each other within appreciable limits; their catalytic domains share about 83% identity at the amino acid level [9]. However, the differences observed in the extreme N- and C-terminal regions, direct these kinases to distinct compartments or to different molecular targets. Thus, for example, the C-terminal PDZ binding domain in S6K1 allows recruitment to the actin cytoskeleton via binding to neurabin [21] and S6K2 containing a proline-rich region in its C-terminus facilitates interaction with SH3 domain or WW repeat containing protein [9]. Besides full length forms of S6K1 and S6K2, a novel kinase domain truncated splice variant, p31S6K (**Figure 1**) with potential oncogenic properties, has also been reported [22].

3. S6 kinase regulation; a historical perspective for prevalent mode of activation

Research carried out over the past 20 years identified diverse growth factors and mitogens with the ability to activate the S6 kinases [23, 24]. Seemingly among these multiple inputs, insulin/IGF pathway, which signals via phosphoinositide 3-kinase (PI3K) and mTORC1 appears to be the best studied activator of S6K1 [25, 26]. Besides PI3K, other independent signaling pathways like Ras/MAPK (mitogen activated protein kinase) have also been implicated in S6K1 activation [27, 28]. S6K1's contribution in controlling cell growth and proliferation being paramount made the basis for studying its structure and function. The data accumulated over a period of time, hence revealed the molecular insights that govern activation of S6K1 by mitogens and identified, to a large extent, the complex interactions between its specific domains and phosphorylation sites. These observations eventually became the reason for putting forth the models that explain the activation of S6K1 by stepwise multisite phosphorylations [29–33]. These models propose that during inactive state, the basic C-terminal pseudosubstrate domain of S6 K1 interacts with its acidic N-terminus. This interaction obstructs the phosphorylation at kinase domain and renders it inactive [6, 34]. Stimulation by growth factors besides other mitogens result in the phosphorylation of four proline-directed sites at C-terminal auto-inhibitory pseudosubstrate domain (Ser434, Ser441, Ser444 and Ser447) [30, 32]. These phosphorylations induce a conformational change thereby releasing the interaction between the two termini and enables access to the HM and T-loop sites. Phosphorylation at these two critical sites ultimately leads to the full activation of the kinase [6, 34]. Several proline-directed kinases, including ERK1/2, JNK1/2 and CDK1 have been implicated in phosphorylation of these sites; it however remains unclear as to which

kinase(s) play a dominant role in-vivo [32]. Scientific observations during early 1990's propounded the theory that mTOR controls the in-vivo activation of S6K1 [35]. These observations were further augmented during 1998–1999 when mTORC1 was shown to directly phosphorylate the HM site (Thr412) of S6K1 in vitro [36, 37]. The indispensible nature of HM site phosphorylation is further supported by the evidence that mutation of Thr 412 to alanine (T412A) abolishes the S6K1 activity, whereas the phosphor mimicked variant (T412E) enhances basal activity even in absence of mitogens [20, 33, 38]. The findings put forward hence strengthened the notion that phosphorylation of Thr 412 at HM site remains pivotal for S6K1 activation. Co-ordinate phosphorylation of Thr252 in the activation/T-loop and Thr412 at HM site leads to maximal activation of S6K1 [29, 33, 39]. Set out to identify the potential kinases responsible for Thr 252 phosphorylation, in vitro followed by in-vivo studies show PDK1, also corroborated as a kinase for AKT, to directly phosphorylate Thr252 at T-loop site [29, 39, 40]. This was supported by the findings that PDK1 null embryonic stem cells, $PDK1^{-/-}$ or T252A mutation rendered S6K1 enzymatically dead [33, 41]. Further it is observed that PDK1 mediated phosphorylation of C-Terminus truncated variant of S6K1, S6K1-ΔCT remains significantly higher than full length S6K1 while mutation in c-terminal phosphorylation sites to alanine results in poor S6K1 activation [29]. Moreover, PDK1 poorly activates S6K1 T412A-ΔCT or T394A-ΔCT in vitro. All these findings reveal that unphosphorylated c-terminus puts restraint toward full activation of S6K1 by blocking the access of PDK1 to activation loop and the importance of HM and TM sites toward bringing PDK1 mediated S6K1 activation [29]. Phosphorylation of Ser394 (or Ser371 in P70S6K isoform) at turn motif site also remains of much significance for complete activation of S6K1, as S394A substitution of S6K1 renders it completely inactive [42]. Notwithstanding the importance of turn motif site, the data accumulated on regulation and function of this phosphorylation event has progressed, albeit rather slowly. Whatever little the quantum of data may be available for regulation around TM site phosphorylation till date, it concordantly reflects that this site does not represent an autophosphorylation event [42]. Some more interesting observations reveal that even the addition of T412E substitution fails to restore the activity of S394A mutant, thus supporting the notion that this site plays an important yet independent role in regulating the activity of S6K1 [42]. Evidences, though scant, have also emerged which corroborate mTOR in promoting Ser394 phosphorylation in vitro [43]. However, since data accumulated in certain other cases does not correlate phospho-Ser394 well with mTORC1 activity, intermediation of some other kinase besides mTORC1 is also speculated. Reports based on analogy to co-translational phosphorylation of TM site (Thr450) in AKT are tempting to speculate that S6K1 TM site phosphorylation also represents an early event that occurs co-translationally prior to T-loop and HM site phosphorylation [44]. Indeed, a recent report supports such an idea, as Ser394 phosphorylation occurs simultaneously with the production of S6K1 protein from a transfected plasmid [31]. Since the phosphorylations at the HM and T-loop sites exhibit strong cooperativity, the temporal order of these two phosphorylations has not yet been convincingly deciphered. However, the data available till date has put forth two models for S6K1 activation wherein the conventional and widely accepted model suggests mTORC1 mediated phosphorylation at Thr 412 to precede PDK1 mediated phosphorylation at Thr 252 [29, 38, 39]. An alternate model however, suggests that the phosphorylation at T-loop (Thr 252) precedes HM phosphorylation (Thr 412) [31, 33] (**Figure 2**).

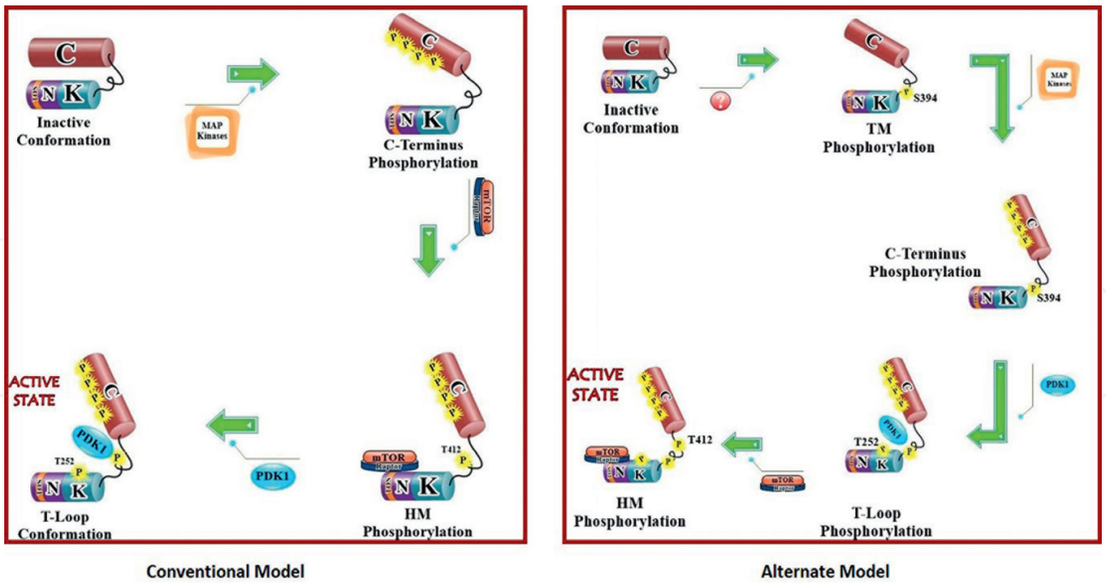

Figure 2. Stepwise activation of S6K1 via multi-site phosphorylation. Conventional model: the interaction of the C- and N-terminal domains results in auto-inhibition of S6K1. Step 1: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 2: the release of the auto-inhibitory C-terminal domain (CTD) enables mTORC1 access to the HM and thus phosphorylation of Thr^{412}. Step 3: the release of the auto-inhibitory CTD and phosphorylation on Thr^{412} enables PDK1-mediated phosphorylation of the T-loop on Thr^{252}, resulting in full activation of S6K1. Phospho-Thr^{412} serves as docking site for PDK1. Owing to insufficient data, the temporal order of TM site phosphorylation (Ser^{394}) is not depicted. Alternative model: Step 1: an unknown kinase phosphorylates the inactive form of S6K on the TM site Ser^{394}. Step 2: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 3: The release of the auto-inhibitory C-terminal domain enables PDK1 access to the T-loop. Step 4: PDK1-mediated phosphorylation of Thr^{252} promotes mTORC1-mediated phosphorylation on the HM site, Thr^{412}. KD, kinase domain; N,N-terminal domain.

S6K1 amino terminus serves a regulatory role in promoting phosphorylation at HM site and thereby at T-loop site that ultimately brings about its complete activation. The regulation by amino terminus is documented to be two pronged. Firstly, it acts as a receptor of an activating input critical for Thr 412 and Thr 252 phosphorylation; and, secondly, it suppresses an inhibitory function mediated by C-terminus. These observations have strong scientific support as the data generated over a period of time shows that amino terminus truncation of S6K1 (ΔNT) abolishes the phosphorylation of rapamycin-sensitive sites Thr 412, Thr 252, Ser 427 and renders it inactive [38, 45] whereas additional deletion of C-terminus (ΔNT/ΔCT) restores all the rapamycin-sensitive phosphorylations as well as the kinase activity of S6K1. The regulatory function of the amino terminus was mapped to a short stretch of amino acids (comprising of 5–9 amino acids and represented by a signature sequence FDIDL) present at extreme end known as TOS motif [20]. It has been shown that S6K1 truncated of TOS motif or its F5A variant (F5A mutation within the FDIDL sequence) abolishes S6K1 kinase activity as well as Thr412 and Thr252 phosphorylation. However, deletion of C-terminus from the F5A mutant (F5A-ΔCT) partially restores kinase activity and Thr 412 phosphorylation [20]. The TOS motif function has been related toward a launch pad required for mTORC1 to engage substrates and to mediate phosphorylation of rapamycin-sensitive sites [46, 47]. The data generated supports the view that mTOR interaction with TOS motif is indirect and

mediated by another member of mTORC1 complex, a scaffold protein called Raptor [46, 47]. For phosphorylation of S6K1 and 4EBP-1, the other mTORC1 effector molecule, binding with raptor has been unequivocally stated to be critical. However, the precise mechanism by which raptor mediates efficient phosphorylation of S6K1 and 4EBP-1 remains largely debatable. Two models have however been proposed to explain this mechanism. The first model suggests that raptor and mTOR associate in two states with varying affinities governed by the nutrient availability. During nutrient starved state Raptor binds tightly to mTOR and renders it inactive. While as the loose-binding complex, formed during nutrient sufficiency, activates mTOR and promotes efficient phosphorylation of mTOR targets [48]. Furthermore, overexpression of raptor increases the amount of mTOR found in the tight-binding complex, thereby explaining the observation that overexpression of raptor inhibits mTOR activity. However, it is interesting to note that rapamycin is able to disrupt the raptor-mTOR interaction regardless of nutrient status [49], but it is phosphate dependent. The second model supports the existence of Raptor as a scaffolding protein for mTORC-1complex, wherein Raptor has been shown to preferentially bind unphosphorylated forms of mTOR targets and recruit the substrates to the mTOR complex for phosphorylation. Role of S6K1 amino terminus in suppressing the inhibitory function of C-terminus remained unclear till identification of an RSPRR motif (a short stretch of 5 amino acids, 433–437) in C-terminus way back in 2005 [47]. This motif has been suggested to negatively regulate S6K1 activation. Evidences, though scant, have propounded that RSPRR motif functions as a docking site for a negative regulator, such as a phosphatase, that is suppressed by mTORC1 [47].R3Amutation of RSPRR motif within the dead ΔNT or TOS motif-mutant (F5A) backbone (NT-R3A or F5A-R3A) has been shown to rescue insulin-stimulated Thr412 phosphorylation and S6K1 activation [15]. These findings though place TOS motif in exhibiting negative control over RSPPR motif inhibition, the exact mechanism behind this regulation still remains largely a mystery.

3.1. Rapamycin, S6K1 inhibition; a chronology of events

In 1970s, a bacterial strain, Streptomyces hygroscopicus was isolated from a soil sample from Easter Island ("Rapa Nui" in the native language) [50]. This strain was found to produce an anti-fungal metabolite [51]. Post purifications, the metabolite was characterized as a macrocyclic lactone and named "Rapamycin" after its birthplace (**Figure 3**) [52].

The strong ability of rapamycin to modulate cellular proliferation besides its promising role in immune suppression for treating various cancers lead to a desperate search in decoding its mode of action and simultaneously initiated hunt for nailing down its targets. The search continued for about two decades and finally culminated in early 1990s with identification of a target protein in yeast, whose mutant conferred resistance to the growth inhibitory effects of rapamycin. The protein was subsequently named as TOR (target of rapamycin) [53–56]. Shortly after, the mammalian counterpart of TOR (mTOR) was identified as physical target of rapamycin by three groups separately in 1994, 1995, respectively [57–59]. However effective mechanism of rapamycin action remained oblivious till intracellular cofactor, the peptidyl-prolyl cis/trans isomerase, immunophilin FK506-binding protein 12 (FKBP12) was shown to bind rapamycin as a gain-of-function component to mediate its inhibitory effect on TOR [56].

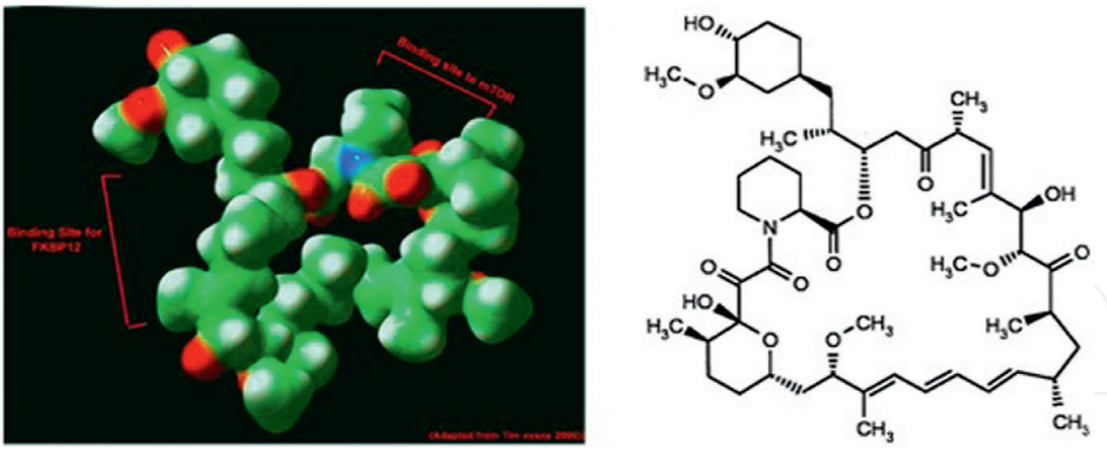

Figure 3. Electron-density model of a molecule of the immunosuppressant drug rapamycin: It is mainly used to prevent rejection in organ transplantation, and is used in kidney transplants. It also has anti-fungal and anti-cancer properties, and was originally derived from soil bacteria found on Easter Island. The chemical formula is C51.H79.N.O13. The atoms are represented here as color-coded blobs: carbon (green), hydrogen (white), nitrogen (blue) and oxygen (red) (adapted from Dr. Tim Evans Science photo library).

Thus, TOR is also referred to as FKBP12 rapamycin associated protein (FRAP) [60].Rapamycin-FKBP12 complex binds to the FKBP12-rapamycin binding (FRB) domain of TOR (**Figure 4**) [55, 60–62] to inhibit its intrinsic kinase activity, including autophosphorylation, thereby inhibiting access of TOR to its substrates [56]. This finding is however in contradiction with the earlier reports wherein it was shown that rapamycin has little effect on mTOR kinase activity [63]. In mammals, rapamycin in complex with FKBP12 acts as an allosteric inhibitor of mTOR complex1 (mTORC1). Only mTORC1 is acutely sensitive to inhibition of rapamycin. However, long term exposure to rapamycin has been shown to inhibit mTORC2 in certain cell types [64]. It is believed that the rapamycin-FKBP12 complex prevents the association between mTOR and raptor; therefore, downstream targets that depend on raptor binding are specifically inhibited [49].

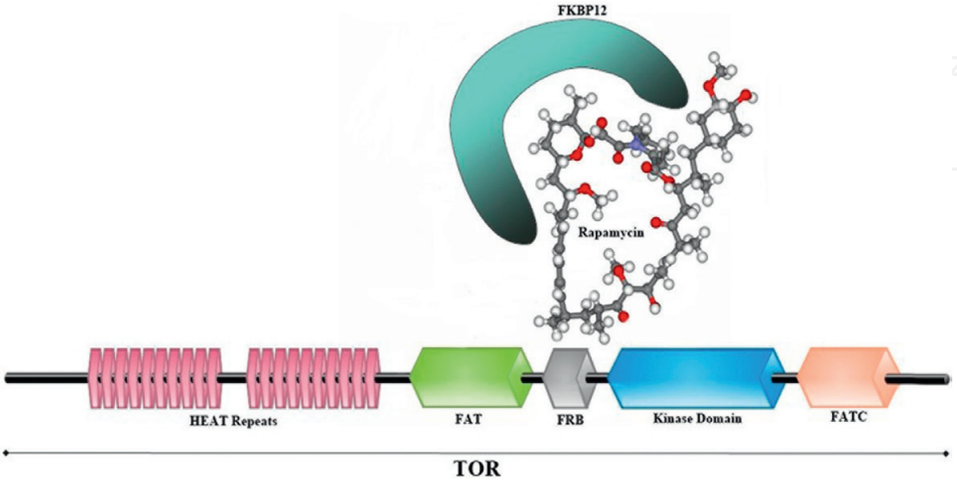

Figure 4. Association of rapamycin with TOR: schematic representation of structural organization of mTOR at domain level and its association with rapamycin–FKBP12 complex.

S6K1 being a downstream effector of mTOR, shows inhibitory response to rapamycin as is evidenced by loss of its ability to phosphorylate its substrate, ribosomal protein S6.

Early 1990s witnessed a rigorous search for identifying the rapamycin-sensitive regulatory phosphorylation sites in S6K1 and during 1995 three sites, T252 in the activation/T-loop, T412 in hydrophobic motif and S427 in the linker domain, which connects the auto-inhibitory domain to the catalytic domain were identified as principal rapamycin-sensitive sites [65]. These sites were shown to be dephosphorylated by rapamycin in hierarchical fashion T412 > S427 > T252 with T412 dephosphorylation most closely paralleling loss of kinase activity [65]. Besides, these sites were shown to be responsive to mitogenic stimulation as well [66]. Though all these three sites show rapamycin responsiveness, their conversions to either acidic or neutral amino acids reveal that T252 and T412 were critical regulatory sites where as T427 appeared to play a modulatory function [65]. Further T412 was shown to be principal site of rapamycin-induced S6K1 inactivation as T412E showed increased basal activity and was largely rapamycin resistant [65]. In 1995, Weng et al. reported the cooperativity among the two termini for exhibiting their regulatory effects on S6K1 activation by showing that amino- terminus truncated mutant, S6K1-ΔNT, was inactive. However, additional deletion of carboxy terminus, generating S6K1-ΔNT/ΔCT, rescued its phosphorylation and activation state. Surprisingly it was observed that the mutant was rapamycin resistant [45]. However, its responsiveness toward Wortmannin inhibition did not get compromised. These observations combined with the findings that the carboxy-terminal truncated S6K1 (S6K1-ΔCT) retains rapamycin sensitivity raised the speculations during latter half of 1990's that the inhibitory effect of the rapamycin–FKBP12 complex on S6K1 was either due to blockade of an upstream activator or activation of some phosphatase which mediate their influence through the two termini. The presence of Thr412 phosphorylation on S6K1-ΔNT/ΔCT isolated from rapamycin-treated cells questioned the idea that mTORC1 represents the sole S6K1 Thr412 kinase. In ΔNT/ΔCT, serum and insulin promote Thr412 phosphorylation and kinase activation in a completely rapamycin-resistant manner, suggesting that a rapamycin-insensitive kinase mediates Thr412 phosphorylation [38, 45]. This conundrum was soon resolved in 2005 by observing that rapamycin-insensitive mTORC2 mediates non-physiological S6K1 Thr412 phosphorylation in S6K1 mutants lacking a C-terminus [67]. It was also observed that unlike other AGC kinases S6Ks have atypically an extended C-terminus that imposes additional inhibitory influence on their state of activity [15]. Hence in the dead ΔNT allele, mTORC1 (mTOR/raptor) cannot dock to S6K1 and as such is incapacitated to phosphorylate Thr412, and mTORC2 (mTOR/rictor) cannot phosphorylate Thr412 due to steric hindrance imposed by the extended C-terminus [67]. In the partially rapamycin-resistant ΔCT mutant, both rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 cooperatively mediate Thr412 phosphorylation. In ΔNT/ΔCT (and F5A-ΔCT), only rapamycin-insensitive mTORC2 mediates Thr412 phosphorylation.

4. Other modes of S6K1 regulation

Studies of late propose the involvement of various post translational modifications (PTMs) in regulation of S6K1. Prominent among them are phosphatase-mediated dephosphorylation, acetylation and ubiquitination.

4.1. Dephosphorylation

S6K1 has been proposed to be regulated by co-ordinated action of phosphatases. This was observed while examining the action of rapamycin on phosphorylation state of S6K1.Whereas as rapamycin could dephosphorylate S6K1 through its inhibitory action on mTORC1 complex; the involvement of certain phosphatases to regulate this event was not ruled out. Indeed, S6Ks have been suspected to represent targets of PP2A (protein phosphatase 2A)-like phosphatases. This is supported by the evidence that regulation of several TOR substrates in *S. cerevisiae* occurs via suppression of PP2A-like phosphatases [68]. Further it has also been shown that PP2A interacts with wild type S6K1 and not ΔNT/ΔCT S6K1, a variant truncated of both the termini [69]. It is of late been observed that genetic ablation of PP2A regulatory subunit B (PP2A-B′) in *Drosophila melanogaster* leads to dS6K (Drosophila S6K) deregulation and a variety of metabolic defects [70]. Some further studies report that S6K1 phosphorylation (412) is enhanced in human cells upon knock down of PPP2R5C, the human PP2A-B′ orthologue [70]. All these studies support the role of phosphatases in regulating S6K1 activity. However, it is to be ascertained how mTOR influences these PP2A like phosphatase to modulate S6K1 Thr412 phosphorylation.

4.2. Acetylation and ubiquitination

Acetylation of S6K1 occurs reportedly by the action of two acetyltransferase enzymes, p300/CBP (cAMP-response-element-binding protein binding protein) and PCAF (p300/CBP-associated factor) [71].While as this PTM has been shown to occur on extreme C-terminus of S6K1 in response to the stimulation by mitogens, its dependence on phosphorylation has not been confirmed [72]. Acetylation has been primarily linked to the stabilization of S6K1 [71]. However, the concrete evidence to support this notion has remained largely elusive till date.

Polyubiquitination is another PTM, S6K1 is responsive to. S6K1 appears to experience the ubiquitination in response to mitogen stimulation [73, 74] through direct action of ubiquitin ligase, ROC1 [75]. RNA interference (RNAi) mediated knock down of ROC1 results in stabilization of S6K. These results are hence suggestive of the notion that polyubiquitination causes destabilization of S6K1 and a resultant attenuation of S6K1 downstream signals. Although regulation of S6K1 through ubiquitination still remains in its infancy; it however has opened a new window toward understanding detailed mechanistic inputs of proteasome mediated S6K1 regulation and function.

5. Functions associated with S6K1—a debatable discourse

S6K1, a downstream effector of mTOR is considered to be a multifaceted effector that regulates cell growth and proliferation by phosphorylating multiple ways. To add to its wide range of functions, S6K1 has much recently been shown to play a pivotal role in cellular senescence through its newly found substrate ZRF1 [76]. S6K1 gets activated in response to various signaling pathways including mTOR, PI3-kinase and MAPK, in a coordinated manner through sequential phosphorylation events directed at multiple sites [62]. While S6K1

extends its influence on various cellular functions, some of its associated functions still appear untenable, reason being the inadequate support data. Some of the S6K1 associated functions are briefly described below:

5.1. Cell growth and translational control—what is the connection?

S6K1 has been shown to control cell growth primarily by its ability to phosphorylate S6 protein of 40S ribosomal subunit (rpS6). These phosphorylations are shown to be localized at the C-terminus and happen in sequential order, namely S236 > S235 > S240 > S244 > S247. The dependence of S6 phosphorylation on S6K1 can traced by the observation that rpS6 deficient mice, rps6−/− show phenotypic resemblance to S6K1 deficient mice, S6K1−/−. Although these mice are viable and fertile, however both exhibit cell growth defects. It however is confusing to observe that $S6K1^{-/-}$ mice display minimal defects in rpS6 phosphorylation, while S6 K2, homolog of S6K1, deficient mice, that is, $S6K2^{-/-}$ grow to normal size despite exhibiting a significant reduction in rpS6 phosphorylation [77]. The conundrum can partly be resolved by the observation that claims selective recruitment of S6K1 and S6K2 to different cellular compartments and as such enables them to differentially phosphorylate rpS6 or alternatively by the preposition that rpS6 phosphorylation may ensue at a specific developmental stage where S6K1 but not S6K2 is active. Although a discordance seems to appear vis-a-vis S6K1 activation, rpS6 phosphorylation and cell growth, their complete understanding as far as therapeutics is concerned remains but pivotal. Although S6 phosphorylation was initially thought to be required for selectively translating mRNAs characterized by 5′-oligo Pyrimidine tract (5′-TOP). Later studies however confirmed that 5′ TOP mRNA translation remains unaffected in $S6K1^{-/-}/S6K2^{-/-}$ mice, which display minimal rpS6 phosphorylation [77], hence suggesting these two events to be independent [78].

S6K1 maintains its influence on cellular translation not only by the ability to phosphorylate ribosomal protein S6 (rpS6) but some studies provide evidences to establish S6K1 as a transcription factor kinase as well. One of the study shows S6K1 to phosphorylate eukaryotic initiation factor 4B (eIF4B), a component of cap-binding complex to control cap dependent translation. While as inactive S6K1 associates with eIF3 (a component of pre-initiation complex) and upon activation by mTORC1, via serum stimulation, dissociates to phosphorylate eIF4B, another component of pre-initiation complex [79, 80]. Besides it also controls translational initiation by phosphorylating PDCD4, a negative regulator of eIF4A (**Figure 5**). Phosphorylated PDCD4 gets ultimately degraded by ubiquitin ligase βTRCP [81]. The importance of S6K1 vis-a-vis translational control can further be demonstrated by the observation which implicates S6K1 in controlling translation elongation as well. The study carried shows S6K1 to phosphorylate and inactivate eEF2k (eukaryotic elongation factor-2 kinase, a negative regulator of translation [82].

5.2. Cell cycle progression-a reality check

S6K1 has been extensively discussed as a kinase that drives cells from G1 to S phase during cell cycle progress. However, the data in support does not seem to match this notion completely

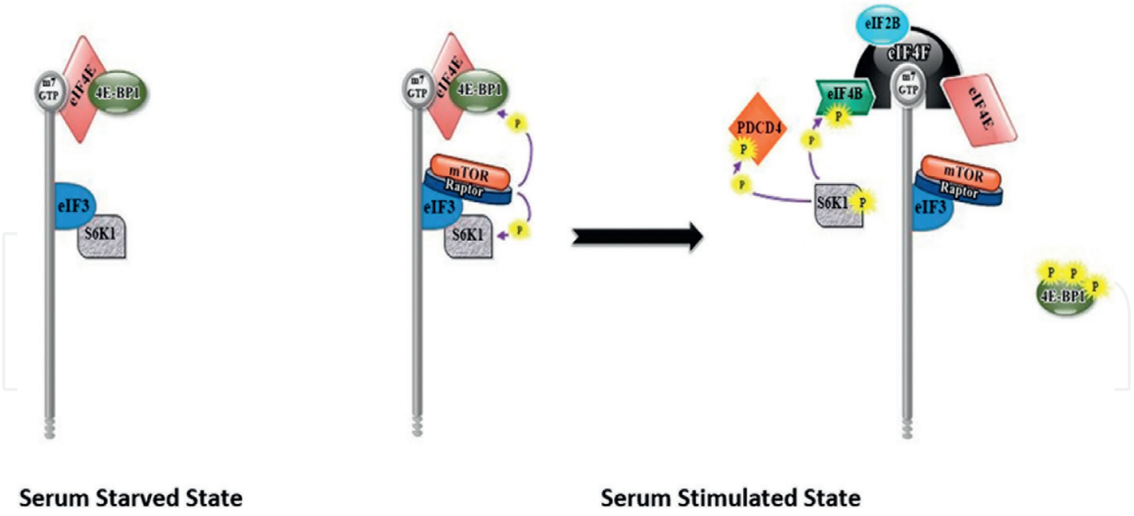

Figure 5. S6K1, a component of translation initiation complex: S6K1 is bound to eIF3 in basal (resting) state cells. Upon serum stimulation, mTORC1 is recruited to the complex, wherein it activates S6K1, which dissociates from eIF3 and phosphorylates several of its targets involved in the initiation of translation.

and in fact some of the data available even contests the veracity of these claims. Whereas Embryonic Stem (ES) cells devoid of S6K1 (S6K1−/−) and mouse embryonic fibroblasts (MEFs) obtained from S6K1 deficient mice do not show significant defects in cell proliferation [77], other studies implicate S6K1 in facilitating cell cycle progression through phosphorylation of estrogen receptor leading to the activation of its target genes that ultimately promotes cellular proliferation [83]. Additionally S6K1 exerts its control over cellular transcription through its ability to phosphorylate cAMP response element binding protein (CREB) isoform CREMr and transcription factor UBF-1 [84]. Phosphorylated UBF-1 in turn activates RNA Polymerase 1 driven transcription of genes encoding ribosomal RNAs and as such aid in ribosomal biogenesis. However, contrary to this belief McMullen et al. proposed no absolute requirement of S6K at either transcriptional or translational levels during ribosomal biogenesis. They showed that $S6K^{-/-}$ mice do not exhibit impaired cardiac hypertrophy, a response dependent on ribosomal biogenesis [85]. These observations put a question mark on the actual involvement of S6K1 in controlling cell cycle progression. Howsoever discordant the views with respect to direct involvement of S6K1 may be but the role of S6K1 in accelerating the cell cycle from G1 to S remains undisputed.

5.3. Cellular metastasis—handy option for therapeutic interventions

S6 kinase, being a downstream effector of mTOR is upregulated in various cancerous situations and as such regulates cellular metastasis. Its involvement in brain tumor pathogenesis was established by the fact that mTOR/S6K axis remains constitutively activated in glioma cells and upon S6K knockdown, the transformed phenotype of these cells is partially rescued [86]. Additionally constitutive activation of S6K1 has been reported in esophageal squamous cell carcinomas (ESCC) [87]. These and several other examples highlight the importance of S6K1 in various cancerous state and as such makes it a potential target for therapeutic interventions to treat cancer. A number of pharmaceutical companies have

already ventured in developing S6K specific inhibitors like ATP-competitive compounds that exhibit selective inhibition of S6K. This remains important in the backdrop of cancer relapses/resistance observed while treating them with mTOR inhibitor rapamycin or its rapalogues [88]. However, until recently it was not clear as to what contributes to the rapamycin resistance in these cells. This conundrum was to a greater extent resolved when MAPK interacting kinase (MNK) was found to be an active player in exerting rapamycin resistance in cancer cells. The study deliberated that cancer cell resistance to the mTORC1 inhibitor rapamycin involves MNK activation via a feedback signaling loop elicited by rapamycin [89, 90]. Much recently a study by Brown et al. reveals that post rapamycin treatment mTORC1 activity is sustained by MNK in cancer cells by way of promoting the association between MNK and mTORC1 to form a sub-complex. This way MNK facilitates binding of mTORC1 with its substrates and with phosphatidyl inositol 3′kinase-related kinase (PIKK) stabilizer, TELO2, while discouraging DEPTOR (endogenous mTOR inhibitor) binding [91]. In addition MNKs are also known for phosphorylating eukaryotic initiation factor 4E, eIF4e [92] and as such may play a role in cellular translation. Further the data generated in our lab hints out at the possible role of eIF4e in regulating S6K1 (unpublished data). We believe that prolonged exposure of cells to rapamycin results in activation of MNKs with the resultant activation of eIF4e. As a result S6K1 gets activated to counter the anti-proliferative/anti-cancerous effects of rapamycin (**Figure 6**). Thus therapeutic interventions envisaging S6K1 as a direct target would help overcome, to a larger extent, the resistance experienced toward mTORC1 inhibitors and as such emerge as more appropriate target for treating cancers.

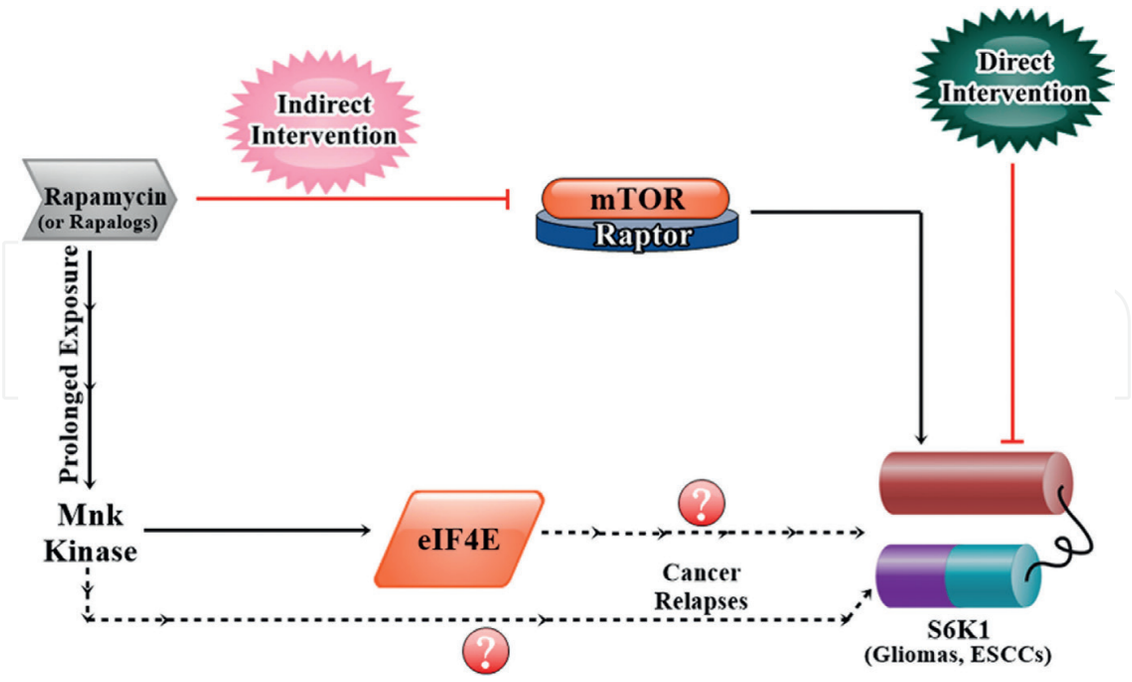

Figure 6. S6K1, a prudent target for treating cancers: an illustration representing S6K1 as a potential target for the specific inhibitors which could be used to overcome the resistance observed while using mTORC1 inhibitors during various cancerous situations.

6. Conclusion

Understanding S6K1 regulation and its associated functions over the last two decades has seen an upward trend. It has now been possible to establish S6K1 an important regulator of cell growth and proliferation. Further our understanding vis-à-vis S6K1 functions have also improved considerably to dissociate many of its functions from the ones related to phosphorylating rpS6 only. Instead its control over various cellular factors makes it more important candidate for regulating cellular physiology. Of late, S6K1 appears as a prudent target for treating various cancers and as such various trials have been initiated to use specific S6K1 inhibitors with the aim to treat different cancers where the routine practices of using generalized drugs appear less effective. However, achieving a complete success in this endeavor still seems difficult. A major challenge in this regard is to ensure that S6K1 s role is tumor specific regardless of its type or origin. Resolving such issues will of course be a task that would attract more research in the years to come.

Conflict of interest

The authors declare that there is no conflict of interest.

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