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Regulation of catalytic activity of S6 kinase 2 during cell cycle

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Abstract

Ribosomal S6 kinase 2 (S6K2) is one of the kinases regulated by the mammalian target of rapamycin (mTOR) signaling pathway. Although it has been identified as a kinase homologous to S6K1, evidence suggests that the two kinases have non-overlapping functions, and the biological function of S6K2 still remains unknown. In order to identify the cell cycle stage(s) during which S6K2 plays a role, we assessed changes in the catalytic activity of S6K2 throughout the cell cycle. Our data show that S6K2 is active throughout the cell cycle with higher activity in G2 and M phases. We also show that S6K1 activity peaks sharply during M phase. Our data suggest that S6K1 and S6K2 likely play yet-unknown roles in G2 and M phases.

Keywords

Ribosomal S6 kinase 1 (S6K1); Ribosomal S6 kinase 2 (S6K2)

Introduction

S6K2 is a serine/threonine kinase to be identified first based on its homology to p70 S6 kinase, now known as S6K1 [1–5]. S6K1 and S6K2 act downstream of mTOR, a kinase that is a cellular nutrient- and mitogen-sensor and plays a role in translation as well as in cell growth and proliferation [6]. mTOR is the molecular target of the immunosuppressant drug rapamycin; binding of rapamycin to the immunophilin FKBP12 generates a complex that in turn binds and inhibits mTOR function, causing cell cycle delay or arrest [6,7]. Since rapamycin can inhibit cell proliferation in many different cell types, its use is widening beyond organ transplantation to include cardiac restenosis prevention and experimental cancer treatments. Understanding the mechanism by which S6K1 and S6K2 play a role in mTOR signaling pathways would allow us to better understand the pharmacological mechanism of rapamycin.

Regulation and function of S6K1 is better understood than that of S6K2, since it had been identified much earlier than S6K2. Both S6K1 and S6K2 are regulated by the same phosphoinositide-3-kinase (PI3K)/Akt/mTOR pathways [8]. S6K1 plays a role in cell size control; cells from the S6K1-null or S6K1/S6K2 double-null mice are smaller than those from the wildtype mice, and re-expression of S6K1, but not S6K2, in the double-null cells restores the normal size, indicating that S6K1 and S6K2 have non-overlapping cellular functions [1, 9,10]. Data also suggest that the two kinases share some functions; both S6K1 and S6K2 can phosphorylate the 40S ribosomal protein S6 [10,11]. S6 phosphorylation by S6K1 and S6K2 was thought to be responsible for upregulation of translation of a certain type of mRNA containing 5' terminus oligopyrimidine tract (5' TOP mRNA). However, cells from the S6K1/S6K2 double-null mice show inhibition of S6 phosphorylation, while maintaining 5' TOP

mRNA translation, thereby complicating our understanding of biological function of S6 phosphorylation by S6K1 or S6K2 [10]. Since both kinases phosphorylate S6 but the small cell size phenotype in S6K1-null background cannot be rescued by S6K2, it is likely that cell size regulation is mediated by a protein other than S6. The function of S6K2 aside from phosphorylating S6 is not currently known.

In order to better understand a more complete picture of S6K2 functions, we undertook detailed studies on changes in S6K2 activity during cell cycle progression. Most of the work on S6K2, to date has been mainly on its similarities to S6K1. Since rapamycin blocks cells from progressing beyond G1 phase, and since its target mTOR is one of the upstream regulators of S6K1, most of the work on S6K1, and by extension S6K2, has been focused on G0 and early G1 phases. However, since S6K1 and S6K2 likely have non-overlapping functions that are yet to be identified, we reasoned that studying how S6K2 activity changes throughout the cell cycle would help us understand the cell cycle stage during which S6K2 is likely to play a role. In this report we analyze catalytic activity of S6K2 in all phases of the cell cycle and compare it to that of S6K1. Our data show that S6K2 is active throughout the cell cycle, and that S6K2 and S6K1 may play a role in G2 and/or M phases.

Materials and methods

Cell culture, activation, and synchronization

HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, penicillin (250 units/ml), streptomycin (250 µg/ml), and L-Glutamine (292 µg/ml) at 37°C with 5.5% CO₂. 20 ng/ml of rapamycin (CalBiochem) was incubated with cells for 30 min prior to activation when indicated. Cell cycle synchronization using metabolic blocks such as nocodazole or thymidine has been previously described [12].

Immune complex kinase assay

Immune complex kinase assay was previously described in detail [11]. In brief, the assays were carried out by incubating the immunoprecipitated S6K2 with γ -³²P-ATP and GST-S6 (as exogenous substrate) at 30°C. Kinase reaction mixtures were separated via SDS-PAGE and the level of S6 phosphorylation was quantified using Phosphorimager (BioRad) or Typhoon 8600 (Amersham Biosciences). The protein levels of cell lysates were quantified via Bradford assay and lysates were adjusted to have equal protein concentrations before immunoprecipitation and kinase assay. Antibodies used for immunoprecipitation were; sc9381 anti-S6K2 Ab (Santa Cruz Biotechnology), 07–173 anti-S6K2 Ab (Upstate Biotechnology), anti-S6K2 antibody, or anti-S6K1 antibody (last two are kind gifts from Dr. J. Blenis, Harvard Medical School, Boston, MA) [2,13].

Immunoblot

Immunoblotting was carried out as previously described [11]. Protein concentrations in cell lysates were quantified and adjusted to have equal protein concentrations prior to loading. Membranes were immunoblotted with one of the following Ab; 07–173 anti-S6K2 (Upstate Biotechnology), anti-phospho histone H3, anti- β actin antibody (both from Cell Signaling Technology), and anti-S6K1 (kind gift from Dr. J. Blenis, Harvard Medical School, Boston, MA). These primary antibodies were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology) and visualized via enhanced chemiluminescence.

Cell cycle analysis

Cells were resuspended in PBS/EDTA (5 mM) solution and fixed by adding an equal volume of cold 100% ethanol. After 30 min cells were centrifuged, resuspended in PBS/EDTA, and incubated for 30 min with RNase A. Cells were then stained with propidium iodide (50 μ M) and analyzed via FACS. Analysis of the cell cycle phases was carried out using FlowJo software (TreeStar).

Results

In order to determine the levels of S6K2 catalytic activity throughout the cell cycle, we synchronized HeLa cells in S phase via double-thymidine block, released cells from the block, and collected cells at various time points as indicated. Cells were lysed and the lysates were adjusted to have equal protein concentrations. S6K2 was immunoprecipitated from cell lysates and its catalytic activity was assessed via the immune complex kinase assays using γ^{32} P-ATP and the exogenous substrate S6. As a negative control for catalytic activity we also collected cells that were treated with rapamycin, an indirect inhibitor of S6K2. Figure 1A shows that S6K2 is active throughout the cell cycle, although the activity seemed to be somewhat lower in S and G1 phases and higher in G2 and M phases. A duplicate set of plates was collected at the same time points and cells were stained with propidium iodide and analyzed via FACS in order to ascertain the cell cycle phase of each time point. Figure 1B shows that up to 4 h post release from double-thymidine block cells were in S phase, whereas after 6–8 h of release cells were in G2 phase. M phase corresponded to 9 and 10 h post-release, as indicated by an immunoblot showing that the phosphorylated form of histone H3, a marker of M phase, was only seen at hours 9 and 10. After 12 h post-release cells returned to G1 phase. The changes in the catalytic activity were not due to changes in the expression level of S6K2 in different cell cycle stages; the expression level of S6K2 stayed even throughout the cell cycle (Fig. 1C). Experiments in NIH3T3 cells showed similar results (data not shown).

There has been a study on cell cycle-dependent changes in S6K1 activity in Swiss mouse 3T3 cells, which showed that S6K1 catalytic activity is high during G0–G1 transition, decreases as it progressed through the cell cycle, but increases again at M–G1 transition [14]. In order to compare our results on S6K2 activity with that of S6K1 in the same system, we carried out similar experiments in Fig. 1 with S6K1 using HeLa cells. Figure 2A shows that S6K1 is catalytically active throughout the cell cycle in HeLa cells, with the highest level in M phase. This peak in activity in M phase was not due to an increase in S6K1 protein levels since immunoblot of S6K1 showed equivalent expression throughout the cell cycle (Fig. 2C). S6K1 undergoes a mobility shift as it becomes activated due to serine/threonine phosphorylation, whereas S6K2 does not undergo mobility shift in our hands [15,16]. Our data show that equivalent expression of S6K1 can be observed even with the shift (Fig. 2C). Mobility shift patterns showed that S6K1 is active throughout the cell cycle, although it was not sensitive enough to discern the peak activity phase. Our data confirms the previous results in Swiss mouse 3T3 cells that showed that S6K1 is active in M phase. There seems to be a slight cell line-dependent difference between HeLa and Swiss3T3 in that the peak activity in HeLa is during M phase rather than M–G1 transition as seen in Swiss3T3.

Since double-thymidine synchronization is less effective near the end of synchronization, namely after mid-G1 phase, we set out to analyze G1 and S phases of the cell cycle using a different synchronization method. We wanted to determine whether the relatively lower activity of S6K2 seen in G1 is real or due cells going out of synchrony, and we also wanted to validate that S6K2 in S phase is indeed lower in activity than in G2 or M phases. We used nocodazole to synchronize HeLa cells in M phase, and released cells from the block to analyze them during the transition between G1 and S phases, beginning at 7 h after release from nocodazole. Figure 3 shows that S6K2 catalytic activity stays even throughout the G1 to S

transition, but is much lower than that of M phase, confirming the data obtained in Fig. 1 showing that S6K2 activity is lower in G1 and S phases than in G2 and M phases. When we carry out the release for a longer period of time into G2/M phases, the data show similar results as in Fig. 1 (Fig. 3C).

Rapamycin has been shown to block cell cycle progression at G1 phase by inhibiting mTOR in G1. Since S6K2 is active throughout the cell cycle, we next assessed whether S6K2 catalytic activity past G1 phase is still regulated by mTOR and therefore rapamycin-sensitive. When cells in S/G2 phases of the cell cycle were treated with rapamycin, we observed that S6K2 activity is indeed inhibited, indicating that such activation of S6K2 past G1 phase is still rapamycin-sensitive.

In conclusion we find that S6K2 remains active throughout the cell cycle, with slightly higher activity in G2 and M phases. S6K1 in HeLa cells, which is also active throughout the cell cycle, shows its highest activity in M phase.

Discussion

Since S6K2 was first identified it has been assumed that it would behave in a similar manner to S6K1 since the two kinases have homologous catalytic domains. Therefore many of the studies on S6K2 were focused on the G0–G1 transition when quiescent cells are treated with various mitogens, as this is the cell cycle phase usually studied in the mTOR/S6K1 field. However, others and we have noticed that catalytic activity of S6K2 is weaker than that of S6K1 in this phase of the cell cycle [2]. One of the possible explanations for this is that G0–G1 transition is not the relevant cell cycle phase in which S6K2 plays a role. Since the full spectrum of S6K2 function is not known, we set out to identify the cell cycle phase in which S6K2 exerts its maximal catalytic activity. In this study we report that S6K2 catalytic activity remains high throughout the cell cycle in HeLa cells, with higher activity in G2 and M phases. S6K1, which also remains active throughout the cell cycle, shows its peak activity in M phase in HeLa cells.

Our data suggest that, since both kinases remain active throughout the cell cycle, it is not possible to pinpoint one particular cell cycle stage in which they play a role, and that these kinases may play a role that is broader and applicable to the entire cell cycle. Our data also suggest an intriguing possibility that there are as-yet-unknown functions of S6K1 in M phase and those of S6K2 in G2 and M phases, the cell cycle phases in which they show higher activities. Rapamycin blocks cells from progressing beyond G1 phase and therefore much focus has been rightly placed on the function of mTOR in G1 phase. However, it is becoming clear that mTOR has multiple functions that are not restricted only to G1 phase. Our data suggests the possibility that S6K1 and S6K2 may play a role in G2 and/or M phases, and that our efforts for identifying their substrate and functions should not solely be focused on G1 phase but rather throughout the cell cycle and especially in G2 and M phases. Further investigation is underway in order to identify G2- or M-phase-specific S6 kinase target(s).

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Abbreviations

S6K1

Ribosomal S6 kinase 1

S6K2

Ribosomal S6 kinase 2

Fig. 1.

The activity of S6K2 is regulated in a cell cycle-dependent manner. **(A)** HeLa cells were synchronized via double-thymidine block, released from the block, and lysed at various time points post release. S6K2 was immunoprecipitated from the lysates and its catalytic activity was assessed via immune complex kinase assay using $\gamma^{32}\text{P}$ -ATP and exogenous substrate S6. Lysates were adjusted to contain equivalent protein concentrations prior to assay. The graph, which is calculated as fold-activation over 2-h post-release activity, represents data from nine experiments ($P < 0.0001$). Rap: cells incubated with rapamycin (20 ng/ml) for 30 min prior to lysis. A representative immune complex kinase assay with a varying degree of ^{32}P -incorporation into exogenous substrate S6 is shown (top panel) as well as Coomassie staining of S6 substrate in the kinase assay, which shows equivalent use and loading of the substrate. **(B)** HeLa cells were collected at the same time points and incubated with propidium iodide and analyzed via FACS for cell cycle profile. Bottom panel: lysates from all time points were immunoblotted for phospho-H3, a marker for M phase. **(C)** Lysates from each time point were immunoblotted with anti-S6K2 antibody for S6K2 expression level (top panel) and with anti- β -actin antibody to show equivalent loading of lysates (bottom panel). Lysates for B and C were equilibrated to have equal protein concentrations prior to loading.

Fig. 2.

The activity of S6K1 during cell cycle progression in HeLa cells. **(A)** HeLa cells were synchronized and collected via double-thymidine block as described in Fig. 1. Lysates were adjusted to contain equivalent protein concentrations prior to assay. S6K1 immune complex kinase assays were carried out as described above. The graph, calculated as fold-activation over 2-h post-release activity, represents data from five experiments ($P < 0.0001$). Rap: cells treated with rapamycin in the manner described in Fig. 1. A representative immune complex kinase assay is shown; ³²P-incorporation into S6 (top panel) and Coomassie staining of S6 (bottom panel). **(B)** HeLa cells at each time point were incubated with propidium iodide and analyzed via FACS for cell cycle profile. **(C)** Lysates from all time points were immunoblotted for S6K1 expression level using anti-S6K1 antibody. β -actin immunoblot shows equivalent loading of lysates. Lysates were equilibrated to have equal protein concentrations before loading

Fig. 3. S6K2 activity in G1 and S phases. **(A)** HeLa cells were synchronized via nocodazole, released from the block, and lysed at the time points indicated. S6K2 immune complex kinase assays were carried out as described above. Lysates were adjusted to contain equivalent protein concentrations prior to assay. The graph is calculated as fold-activation over the 7-h post-release activity and represents data from three experiments ($P < 0.005$). Rap: cells incubated with rapamycin. Asyn: asynchronously-growing cells. A representative immune complex kinase assay is shown under the graph. **(B)** HeLa cells were incubated with propidium iodide and cell cycle profiles were analyzed by FACS. **(C)** HeLa cells were released from nocodazole block at 14 and 16 h and S6K2 catalytic activity and cell cycle phase were assessed as in Fig. 3A and B. **(D)** NIH3T3 cells were synchronized via serum withdrawal and allowed to enter cell cycle by addition of serum. After 13 h of cell cycle progression cells were treated with/without rapamycin for 30 min, harvested, and S6K2 catalytic activity and cell cycle phase were assessed as in Fig. 3A and B.