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Generation of monoclonal antibodies specific to ribosomal protein S6 kinase 1

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*The **aim** of this study was to produce monoclonal antibodies directed to the N-terminal regulatory region of S6K1, which shows very low homology to S6K2. **Methods**. Hybridoma technology, ELISA, Western blot, Immunoprecipitation. **Results**. Three hybridoma clones (A1, A2 and A3) producing mAbs specific to ribosomal protein S6 kinase 1 have been generated. Specificity of mAbs has been confirmed by Western blot and immunoprecipitation technique. **Conclusions**. The obtained antibodies are suitable for elucidating signal transduction pathways involving S6K1.*

Keywords: ribosomal protein S6 kinase, mAbs.

Introduction. Ribosomal protein S6 kinases (S6Ks) belong to the AGC family of Ser/Thr kinases, which includes PKA, PKCs, PKB/Akt, RSK, SGK and PDK1. There are two highly homologous isoforms of S6K in mammalian cells, termed S6K1 and S6K2 [1–4]. Both isoforms have nuclear (S6K1/I and S6K2/I) and cytoplasmic (S6K1/II and S6K2/II) variants originated from different initiation start codons. The 23- and 13-amino acid extensions at the N-termini of S6K1 and S6K2 correspondently possess nuclear localization signals (NLS). S6K1 and S6K2 have a similar modular organization, in which kinase and kinase extension domains are flanked by the regulatory N- and C-terminal regions with different protein-protein interaction sequences and signalling motifs. A high level of homology is shared between S6K1 and 2 kinase and kinase extension domains (83 % and 76 % respectively). However, the N- and C-terminal regulatory regions of S6K1 and 2 exhibit a low level of homology (38 % and 12 % respectively) [5, 6]. The structure of S6Ks has not been solved so far, but deletion/mutational studies suggest that the acidic N-terminal sequences of S6K1/2 interact with positively charged residues in the C-terminal autoinhibitory regions, thereby keeping their kinase domains in inactive conformations.

The presence of a PDZ domain-binding motif at the C-terminus of S6K1 that is absent in S6K2 implicates this isoform in regulating cytoskeletal rearrangements through specific interaction with the PDZ domain of the F-actin binding protein – neurabin [7, 8]. S6K2 meanwhile, contains a proline-rich region in its C-terminus, which may facilitate interaction with SH3 domain – or WW domain-containing molecules [2, 8].

Genetic and biochemical studies have linked S6Ks to diverse cellular processes, including mRNA processing, translation, glucose homeostasis, cell size and growth, cellular metabolism and survival. The deregulation of S6Ks function has been closely associated with a number of diseases, including obesity, diabetes and cancer. Therefore, S6Ks are considered as good targets for the development of novel diagnostic and therapeutic approaches.

S6Ks are activated in response to the growth factors, hormones and nutrients signalling via the phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways. In activated state both isoforms have been shown to associate with and phosphorylate a diverse range of cellular proteins, including the regulators of translation (rpS6, eIF4B, eEF2K and PDCD4), protein kinases (PDK1, mTOR and PKCs), small GTPases (Rac and cdc42), ubiquitin ligases (MDM2 and Roc1), pro-survival factors (Bad1) (reviewed in [9]). At the same time S6K1 and 2 can form distinct multienzyme complexes suggesting different roles of S6K1 and 2 in controlling diverse cellular functions [10–17]. For example, S6K1, but not S6K2, has been shown to form a regulatory complex with initiation factor 3 (eIF3), implicating this isoform in controlling the initiation of protein synthesis [10]. S6K2, but not S6K1, has been found in complexes with mRNA binding proteins, which belong to the family of heterogenous ribonucleoproteins (hnRNPs) and this interaction is required for the induction of cell proliferation [14].

Taking this into account, we present in this study the production and characterization of monoclonal antibodies specific to S6K1 that would be valuable for elucidating signal transduction pathways involving S6Ks.

Materials and methods. *Expression and affinity purification of GST-S6K2 fusion proteins.* A cDNA fragments of rat S6K1, corresponding to N-terminal sequences (1–74 aa and 24–74 aa) were amplified by PCR using specific oligonucleotide primers. All fragments were cloned into *pET42a* vector («Novagen», Madison, WI) in frame with the N-terminal GST and 6His-tag sequences. Recombinant proteins designated as F1 and F2 correspondently were expressed in BL21(DE3) *Escherichia coli* cells. Affinity purification of recombinant proteins from the insoluble fractions of bacterial lysate was carried out by Ni-NTA-agarose («QIAGEN», Germany) chromatography at denatured conditions according to manufactures recommendations. The purity of fusion proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell lines. HEK293 (human embryonal kidney), MCF-7 (human breast adenocarcinoma), NIH/3T3 (mouse embryonic fibroblast), and C6 (rat glial cells) were propagated in DMEM medium supplemented with 10 % (v/v) fetal calf serum (FCS) and 2 mM L-glutamine. Sp 2/0 (mouse myeloma) cells were cultured in RPMI-1640 medium supplemented with 10 % (v/v) FCS and 2 mM L-glutamine.

Development of hybridoma. Hybridoma development has been performed according to the protocol published in [18, 19]. As an antigen for immunization S6K1 N-terminal recombinant peptide F1 has been used. Positive hybridoma clones were selected by ELISA for F1 GST-His fusion peptide and then counter screened against GST-His alone to eliminate anti-GST clones. The selected hybridomas were subcloned using limiting dilution method. For further characterization of the generated S6K1 specific antibodies the hybridoma culture supernatant or IgGs purified from ascitic fluid were used [18].

ELISA assays – F1, F2 recombinant proteins or GST alone were diluted in PBS (pH 7.4) and incubated for 1 h at 37 °C in 96-well polystyrene plates (0.5 g/well). To block non-specific binding, the plates were washed once with PBS containing 0.1 % Tween-20, and incubated with 200 μl of 2 % bovine serum albumin (BSA) in PBS (pH 7.4) for 1 h at 37 °C. Subsequently, the plates were loaded with 100 μl aliquots of hybridoma culture supernatant and incubated for 1 h at 37 °C. After extensive washing, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:5000 v/v, «Promega», USA) were added to each well and incubated at 37 °C for 1 h. The plates were washed again four times, then a substrate solution, which contains 0.02 % H_2O_2, 0.5 mg/ml 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) sodium salt («Sigma-Aldrich», USA) and 0.1 M citrate-phosphate buffer (pH 5.8) was added to each well. After 15-min incubation at 37 °C, the light absorbance of the each well was determined at 490 nm.

Production and purification of monoclonal antibodies from mouse ascites – BALB/c mice were injected with 0.5 ml of Pristane and 7–10 days later inoculated with $5 10^6$ hybridoma cells. The ascitic fluid was collected after 10–14 days. The fraction of immunoglobulins was precipitated from ascitic fluid with 50 % ammonium sulphate and used for affinity purification by Protein G-Sepharose CL-4B («GE Healthcare», USA) chromatography. The IgG fractions were pulled together and dialyzed in a phosphate-buffered saline (PBS), pH 7.4. The aliquots of purified antibodies were stored at –70 °C.

Western blot analysis. Cell lysates were prepared using a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 50 mM NaF, 5 mM EDTA, and a mixture of protease inhibitors («Roche Molecular Diagnostics», France). Cell lysates (30 µg) were separated by SDS-PAGE and transferred onto Immobilon-P membrane («Millipore», USA). The membrane was blocked with 5 % skim milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05 % Tween-20) for 1 h at RT, and then incubated with culture supernatants of positive clones for 1 h at RT. After washing with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG in 1:6000 dilution for 1 h at RT. Finally, the membrane was developed using ECL kit («GE Healthcare») and then exposed to Agfa X-ray film.

Immunoprecipitation. 1µg of mAbs purified from ascites was incubated with 15 µl of 50 % protein G-Sepharose and 500 µg of cell extract in a total volume of 500 µl lysis buffer and rotated at 4 °C for 3 h. Immune complexes bound to Protein G-Sepharose beads were recovered by brief centrifugation and washed three times. Finally, samples were boiled in Laemmli sample buffer for 5 min, separated by SDS-PAGE and immunoblotted with corresponding antibodies. As a negative control we used Protein G Sepharose beads incubated with cell lysates and mAbs 2B specific to S6K2 isoform.

Results and discussion. The aim of this study was to produce monoclonal antibodies specific to the N-terminal regulatory region of S6K1, which shows very low homology to S6K2 or other members of the AGC family.

S6K1 mAbs production. A cDNA fragments of S6K1 coding for N-terminal 1–74 aa and 24–74 aa sequences were cloned into *pET42a* vector, expressed in *E. coli* and recombinant proteins (F1 and F2 correspondently) were purified as recommended by manufacturer using Ni-NTA-agarose (Fig. 1, *A, B*). Peptide F1 covering NLS located within 1–23 aa sequence of S6K1/I was used as an antigen for immunization. Following presented scheme of immunization the titre of anti-S6K1 antibodies in the serum of immunized mice was nearly (10^{-6}) on 45th day of immunisation.

Spleen cells from one of the immunized mice were then fused with SP2/0 myeloma cells. The resulting hybridoma cells were selected by growing in HAT medium, and tested for the production of anti-S6K1 antibodies on 10th day after fusion. Screening of ten 96 well plates with growing hybridomas by ELISA using S6K1 recombinant peptide F1 as antigens, allowed us to identify 12 positive clones. Next, we used the recombinant GST-His protein as an antigen in second round of ELISA screening in order to detect the clones producing antibodies specific to GST-His. Only three from twelve selected primary clones did not react with GST/His (data not shown). ELISA screening of these clones with S6K1 recombinant peptides F1 and F2 demonstrated positive response to both peptides (data not shown), indicating that none of the clones was specific to NLS region (1–23 aa) of S6K1 and the epitops for all three clones were located within the 24–74 aa sequence of S6K1 common for both peptides (Fig. 2).

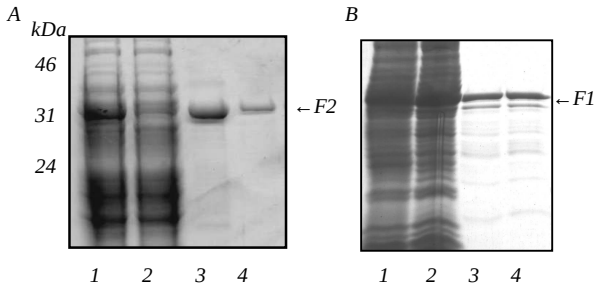

Fig. 1. Expression and afffinity purification of S6K1 recombinant peptides F1 and F2. SDS-PAGE analysis of purified S6K1-GST-His-taged fusion peptides F1 (*A*) and F2 (*B*): *1, 2* – lysates of bacterial cells before and after chromatography; *3, 4* – fractions of eluted purified peptides

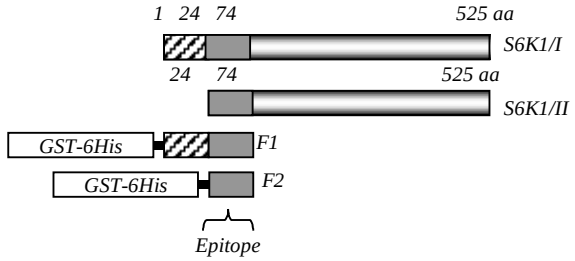

Fig. 2. Schematic representation of S6K1 peptides (F1 and F2) cloned in *pET42a* vectors, purified as GST-His-tag fusions from bacteria cells and representation of S6K1 epitope localization recognized by monoclonal antibodies

Application of anti-S6K1 mAbs for Western blotting. To analyse antibodies further we tested all selected clones in Western blotting of cell lysates. Taking into account that for immunization we used the rat S6K1 N-terminal recombinant fragment we used for the analysis rat, mouse and human cell lines. According to the data of Western blot, mAbs of all three clones (A1, A2 and

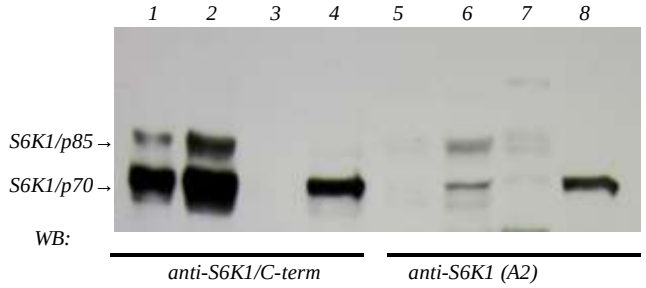

Fig. 3. Representative Western blot analysis of MCF7 (*1*, *5*), HEK293 (*2*, *6*), NIH3T3 (*3*, *7*) and C6 (*4*, *8*) cell lysates, with monoclonal antibodies against S6K1 (clone A2) and rabbit polyclonal anti-S6K1/C-term

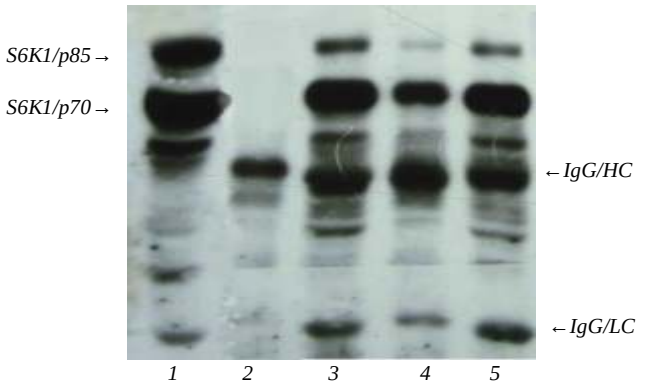

Fig. 4. Monoclonal antibodies against S6K1 (A1, A2, A3) specifically immunoprecipitate endogenous S6K1 from HEK293 cell lysate. Western blot analysis of S6K1 kinase precipitated from HEK 293 cell lysates by mAbs A1 (*3*), A2 (*4*), A3 (*5*) and detected by anti-S6K1/C-term polyclonal antibody: *1* – total cell lysate; *2* – immunoprecipitate by anti-S6K2 mAb B2 [22] as a control

A3) efficiently recognized the endogenous S6K1/p70 in lysate of rat cell line C6 and to a less extent the S6K1 variants (p85 and p70) in human and mouse cell lines. Western blot analysis of cell lysates with A2 mAb is presented in Fig. 3. Data for A1 and A3 mAbs are not presented. As a positive control we used the anti-S6K1/C-term rabbit polyclonal antibodies that were characterised and applied in the previous studies [20–22]. These results clearly show that the antibodies produced by A1, A2 and A3 clones specifically recognize the endogenous S6K1.

Application of anti-S6K1 MAbs for immunoprecipitation. To widen a range of applications for the antibodies generated, we tested them in immunoprecipitation of the endogenous S6K1 from cell lysates. For this purpose we use HEK293 cell lysates. The data presented in Fig. 4 clearly indicate that all three mAbs precipitated the endogenous S6K1 (S6K1/p85 and S6K/p70) as it was further detected in WB by the anti-S6K1/C-term rabbit polyclonal antibodies.

Taken together, this study describes the generation of three hybridoma clones, which produce the anti-S6K1 monoclonal antibodies specific to the kinase N-terminal regulatory region. These antibodies are suitable for various immunoassays, including ELISA, Western blotting and immunprecipitation. The produced antibodies provide a very useful tool to study the function of S6K1, its possible isoforms and different macromolecular complexes they are involved in.

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Створення моноклональних антитіл, специфічних до кінази 1 рибосомного білка S6

Резюме

Мета. *Отримати моноклональні антитіла проти N-кінцевої регуляторної ділянки кінази рибосомного білка S6-S6K1, який має низький рівень гомології з ізоформою S6K2.* ***Методи***. *Гібридомна технологія, ІФА, Вестерн-блот, імунопреципітація.* ***Результати***. *Створено три гібридомних клони (A1, A2 і A3), які продукують моноклональні антитіла, специфічні до кінази S6K1.* ***Висновки***. *Одержані моноклональні антитіла за своїми характеристикам можна використовувати для дослідження S6K1-залежних сигнальних шляхів.*

Ключові слова: кіназа рибосомного білка S6, МкАТ.

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Получение моноклональных антител, специфичных к киназе 1 рибосомного белка S6

Резюме

Цель. *Получить моноклональные антитела против N-концевого регуляторного участка киназы рибосомного белка S6 – S6K1, имеющего низкий уровень гомологии с S6K2 изоформой.* ***Методы***. *Гибридомная технология, ИФА, Вестерн-блот, иммунопреципитация* ***Результаты***. *Созданы три гибридомных клона (A1, A2 и A3), продуцирующих моноклональные антитела, специфичные к киназе S6K1. Специфичность антител подтверждена методами Вестерн блота и иммунопреципитации.* ***Выводы***. *Полученные моноклональные антитела по своим характеристикам можно использовать для изучения S6K1-зависимых сигнальных путей.*

Ключевые слова: киназа рибосомного белка S6, МкАТ.

REFERENCES

1. *Jeno P., Ballou L. M., Novak-Hofer I., Thomas G.* Identification and characterization of a mitogenicactivated S6 kinase // Proc. Natl Acad. Sci. USA.–1988.–**85**, N 2.–P. 406–410.
2. *Gout I., Minami T., Hara K., Tsujishita Y., Filonenko V., Waterfield M. D., Yonezawa K.* Molecular cloning and characterization of a novel p70 S6 kinase, p70 S6 kinase beta containing a proline-rich region // J. Biol. Chem.–1998.–**273**, N 46.–P. 30061–30064.
3. *Martin K. A., Schalm S. S., Romanelli A., Keon K. L., Blenis J.* Ribosomal S6 kinase 2 inhibition by a potent C-terminal repressor domain is relieved by mitogen-activated protein-extracellular signal-regulated kinase kinase-regulated phosphorylation // J. Biol. Chem.–2001.–**276**, N 11.–P. 7892–7898.
4. *Koh H., Jee K., Lee B., Kim J., Kim D., Yun Y. H., Kim J. W., Choi H. S., Chung J.* Cloning and characterization of a nuclear S6 kinase, S6 kinase-related kinase (SRK); a novel nuclear target of Akt // Oncogene.–1999.–**18**, N 36.–P. 5115–5119.
5. *Pende M., Kozma S. C., Jaquet M., Oorschot V., Burcelin R., Le Marchand-Brustel Y., Klumperman J., Thorens B., Thomas G.* Hypoinsulinaemia, glucose intolerance and diminished b-cell size in S6K1-deficient mice // Nature.–2000.–**408**, N 6815.–P. 994–997.
6. *Ohanna M., Sobering A. K., Lapointe T., Lorenzo L., Praud C., Petroulakis E., Sonenberg N., Kelly P. A., Sotiropoulos A., Pende M.* Atrophy of S6K1(-/-) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control // Nat. Cell. Biol.–2005.–**7**, N 3.–P. 286–294.
7. *Burnett P. E., Blackshaw S., Lai M. M., Qureshi I. A., Burnett A. F., Sabatini D. M., Snyder S. H.* Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton // Proc. Natl Acad. Sci. USA.–1998.–**95**, N 14.–P. 8351–8356.
8. *Lee-Fruman K. K., Kuo C. J., Lippincott J., Terada N., Blenis J.* Characterization of S6K2, a novel kinase homologous to S6K1 // Oncogene.–1999.–**18**, N 36.–P. 5108–5114.
9. *Fenton T. R., Gout I. T.* Functions and regulation of the 70 kDa ribosomal S6 kinases // Int. J. Biochem. Cell Biol.–2011.–**43**, N 1.–P. 47–59.
10. *Wang X., Li W., Williams M., Terada N., Alessi D. R., Proud C. G.* Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase // EMBO J.–2001.–**20**, N 16.–P. 4370–4379.
11. *Richardson C. J., Broenstrup M., Fingar D. C., Julich K., Ballif B. A., Gygi S., Blenis J.* SKAR is a specific target of S6 kinase 1 in cell growth control // Curr. Biol.–2004.–**14**, N 17.–P. 1540–1549.
12. *Ma X. M., Yoon S. O., Richardson C. J., Julich K., Blenis J.* SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs // Cell.–2008.–**133**, N 2.–P. 303–313.
13. *Wilson K. F., Wu W. J., Cerione R. A.* Cdc42 stimulates RNA splicing via the S6 kinase and a novel S6 kinase target, the nuclear cap-binding complex // J. Biol. Chem.–2000.–**275**, N 48.–P. 37307–37310.
14. *Goh E. T,. Pardo O. E., Michael N., Niewiarowski A., Totty N., Volkova D., Tsaneva I. R., Seckl M. J., Gout I.* Involvement of heterogeneous ribonucleoprotein F in the regulation of cell proliferation via the mammalian target of rapamycin/S6 kinase 2 pathway // J. Biol. Chem.–2010.–**285**, N 22.–P. 17065–17076.
15. *Fang J., Meng Q., Vogt P. K., Zhang R., Jiang B. H.* A downstream kinase of the mammalian target of rapamycin, p70S6K1, regulates human double minute 2 protein phosphorylation and stability // J. Cell. Physiol.–2006.–**209**, N 2.–P. 261–265.
16. *Harada H., Andersen J. S., Mann M., Terada N., Korsmeyer S. J.* p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD // Proc. Natl Acad. Sci. USA.–2001.–**98**, N 17.–P. 9666–96670.
17. *Harada H., Becknell B., Wilm M., Mann M., Huang L. J., Taylor S. S., Scott J. D., Korsmeyer S. J.* Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A // Mol. Cell.–1999.–**3**, N 4.–P. 413–422.
18. *Savinska L. O., Kijamova R. G., Pogrebnoy P. V., Ovcharenko G. V., Gout I. T., Filonenko V. V.* Comparative characterization of S6 kinase α and β isoforms expression in mammalian tissues // Biopolym. Cell.–2001.–**17**, N 5.–P. 374–379.
19. *Harlow E., Lane D.* Antibodies: a laboratory manual.–New York: Cold Spring Harbor Lab. press, 1988.
20. *Panasyuk G., Nemazanyy I., Ovcharenko G., Lyzogubov V., Gout I., Filonenko V.* Generation and characterization of monoclonal antibodies to protein kinase 2 (CK2) beta subunit // Hybridoma (Larchmt).–2005.–**24**, N 4.–P. 206–210.
21. *Khoruzhenko A. I., Cherednyk O. V., Filonenko V. V.* Subcellular localization of S6K1 and S6K2 forms of ribosomal protein S6 kinase in primary monolayer culture of rat thyrocytes // Biopolym. Cell.–2008.–**24**, N 1.–P. 35–40.
22. *Savinska L. A., Usenko V. S., Lyzogubov V. V., Pogrebnoy P. V., Patchevsky S. S., Ovsharenko G. V., Cheshuk V. S., Gout I. T., Matsuka G. Kh., Filonenko V. V.* P70 S6 kinase α and β isoforms expression in cell lines and breast tumors // Biopolym. Cell.–2003.–**19**, N 1.–P. 64–70.

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