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Development of Monoclonal Antibodies Specific to Ribosomal Protein S6 Kinase 2

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Abstract

Go to:

Ribosomal protein S6 kinase 2 (S6K2) is a serine/threonine kinase that belongs to the family of AGC kinases, which includes PKB/Akt, PKC, PDK1, and SGK1. Mammalian cells express two isoforms of S6K, termed S6K1 and S6K2. Each of these has nuclear and cytoplasmic spicing variants, which originate from different initiation start codons. Nuclear isoforms of S6K1 and S6K2 are slightly longer, as they possess additional sequences at the N-terminus with nuclear localization signals. Biochemical and genetic studies implicated S6Ks in the regulation of cell size, growth, and energy metabolism. Deregulation of S6K signaling has been linked to various human pathologies, making them excellent targets for drug discovery. The aim of this study was to produce monoclonal antibodies directed at the N-terminal regulatory region of S6K2, which shows very low homology to S6K1 or other members of the AGC family. To achieve this goal, two S6K2 fragments covering 1–64aa and 14–64aa N-terminal sequences were expressed in bacteria as GST/6His fusion proteins. Affinity purified recombinant proteins were used as antigens for immunization, hybridoma screening, and analysis of generated clones. We produced a panel of S6K2-specific antibodies, which recognized recombinant S6K2 proteins in ELISA and Western blot analysis. Further analysis of selected clones revealed that three clones, termed B1, B2, and B4, specifically recognized not only recombinant, but also endogenous S6K2 in Western blot analysis of HEK293 cell lysates. Specificity of B2 clone has been confirmed in additional commonly used immunoassays, including immunoprecipitation and immunocytochemistry. These properties make B2 MAb particularly valuable for elucidating signal transduction pathways involving S6K2 signaling under physiological conditions and in human pathologies.

Introduction

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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 isoforms of S6K in mammalian cells, termed S6K1 and S6K2.[1,2] Both isoforms have cytoplasmic and nuclear splicing variants (S6K1/I and S6K2/I), which originate from alternative translation initiation sites. The 23- and 13-amino acid extensions at the N-termini of S6K1 and 2 correspondently possess nuclear localization signals (NLS) that target longer isoforms to the nucleus. In contrast to S6K1, S6K2 contains an additional NLS at the C-terminus, which determines its predominantly nuclear localization.[3,4] S6K1 and 2 share a similar modular organization in which kinase and kinase extension domains are flanked by regulatory regions with different protein-protein interaction sequences and signaling 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 low level of homology (38% and 12%, respectively).[5,6] A unique feature of C-terminal regulatory regions in S6Ks is the presence of autoinhibitory pseudosubstrate sequences, which resemble the carboxyl-terminal segment of ribosomal protein S6 (rpS6) that contains all five S6K-directed sites of phosphorylation. 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 implicates this isoform in regulating cytoskeletal rearrangements through specific interaction with the PDZ domain of the F-actin binding protein neurabin.[7] S6K2 meanwhile, contains a proline-rich region in its C-terminus that 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. Deregulation of S6K function has been closely associated with a number of pathologies, including obesity, diabetes, and cancer. Therefore, S6Ks are considered good targets for the development of novel diagnostic and therapeutic approaches.

S6Ks are activated in response to growth factors, hormones and nutrients, and signaling via the phosphatidylinositol 3-kinase (PI3K), and mammalian target of rapamycin (mTOR) pathways is critical in the process of activation. It has been proposed that multiple phosphorylations within the autoinhibitory sequences in response to mitogenic stimulation release the inhibitory interaction between the N- and C-terminal regulatory regions and open the kinase domain for subsequent phosphorylations by mTOR (in the hydrophobic motif) and PDK1 (in the activation loop). In an activated state, S6K1 and 2 interact with and phosphorylate a diverse range of proteins that link S6K signaling to various cellular processes. Both isoforms have been shown to associate with and phosphorylate a diverse range of cellular proteins, including 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), and mRNA binding proteins (hnRNPF/H, SKAR, CBP80).[9] Distinct roles of S6K1 and 2 in controlling cellular functions are emphasized by the formation of discrete multienzyme complexes, involving each kinase. For example, S6K1, but not S6K2, was shown to form a regulatory complex with initiation factor 3 (eIF3), implicating this isoform in controlling the initiation of protein synthesis.[10] Furthermore, the recruitment of activated S6K1 to the exon junction complex (EJC) on newly spliced mRNA was shown to be implicated in the regulation of mRNA processing via specific interaction with mRNA binding protein, Aly/REF-like target (SKAR).[11] The interaction of S6K1 with another mRNA binding protein, cap-binding protein 80 (CBP80), has been linked to the control of the pioneer round of translation in the nucleus.[12,13]

S6K2 has also been found in complexes with mRNA binding proteins, which belong to the family of heterogenous ribonucleoproteins (hnRNPs). The interaction of S6K2 with hnRNP-F was shown to be required for the induction of cell proliferation.[14] It is well established that both S6K1 and 2 possess a pro-survival function that is mediated by different signaling mechanisms. In the case of S6K1, pro-survival signaling is linked to binding and phosphorylating MDM2 and BAD1.[15–17] In contrast, the anti-apoptotic function of S6K2 was dependent upon formation of a signaling complex with B-Raf and PKCε.[18] Importantly the S6K2/B-Raf/PKCε complex has been linked to mediating chemoresistance in cancer cells.

The above studies clearly indicate that S6K1 and 2 can be found in distinct regulatory complexes located in different cellular compartments in the cytoplasm and nucleus, including endoplasmic reticulum, cytoskeleton, mitochondria and nucleolus. To date, subcellular localization and the expression of S6K isoforms in cell lines and tissues have not been adequately studied. The lack of antibodies specific to S6K1 and 2 is possibly an important contributing factor for this gap of knowledge. Taking this into account, we present in this study the production and characterization of monoclonal antibodies specific to S6K2.

Materials and Methods

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Expression and affinity purification of GST-S6K2 fusion proteins

cDNA fragments of human S6K2, corresponding to N-terminal sequences 1–64aa and 14–64aa, 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 corresponding were expressed in BL21(DE3) *Escherichia coli* cells. The expression of F1 and F2 fusion proteins was induced by 1 mM isopropyl-b-D(2)-thiogalactopyranoside (IPTG) for 3 h at 30°C. Affinity purification of recombinant proteins from the insoluble fractions was carried out on Ni-NTA-agarose (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The purity of fusion proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Production of hybridomas

Hybridoma production was performed according to the standard protocol.[16] In brief, female BALB/c mice (8 weeks old) were immunized with 20 µg of recombinant F1 protein in 50% complete Freund's adjuvant by intraperitoneal injection (i.p.). Subsequent immunizations (four in total) were carried with 20 µg of recombinant protein with incomplete Freund's adjuvant by i.p. injection at 2-week intervals. The titer of anti-F1 antibodies in blood sera was monitored by ELISA (it was 10^{-6} after 45 days). Finally immunized mice were boosted with 15 µg of antigen by i.p. injection in PBS without adjuvant. Three days later, splenocytes from immunized mouse and SP2/0 myeloma cells cultured in RPMI 1640 medium containing 20% fetal calf serum (FCS) were fused in the presence of PEG (MW2000, Merck, Darmstadt, Germany). Positive hybrids were selected by ELISA for F1 GST-His fusion peptide and then counter-screened against GST-His alone to eliminate anti-GST clones. Selected hybridoma clones were subcloned using limiting dilution method. For further characterization of generated S6K2-specific antibodies, hybridoma medium or IgGs purified from ascetic fluid were used.

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 µg/well). To block non-specific binding, 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, plates were loaded with 100 µL aliquots of hybridoma culture media and were 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, Madison, WI) were added to each well and incubated at 37°C for 1 h. Plates were washed again four times, then substrate solution (0.02% H_2O_2), 0.5 mg/mL 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) sodium salt (Sigma-Aldrich, St. Louis, MO), and 0.1 M citrate-phosphate buffer (pH 5.8) were 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, Piscataway, NJ) 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 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, Meylan, France). Cell lysates (30 µg) were separated by SDS-PAGE and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). 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 media of positive clones for 1 h at RT. After washing three times with TBST, HRP-conjugated goat anti-mouse IgG in 1:6000 dilution (Promega) were incubated with the membrane for 1 h at RT. Finally, the membrane was developed using an ECL kit (GE Healthcare) and then exposed to Agfa X-ray film.

Immunoprecipitation

One µg of MAbs purified from ascites was incubated with 15 µL of 50% slurry of protein G-Sepharose (GE Healthcare) 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 with 1 mL of lysis buffer. Finally samples were boiled for 5 min in Laemmli sample buffer, separated by SDS-PAGE and immunoblotted with corresponding antibodies. As a control, we used Protein G Sepharose beads incubated with monoclonal antibodies or cell lysates alone.

Immunocytochemistry

Immunocytochemical staining was performed according to the basic protocol as described previously.[17] In brief, human embryonic kidney (HEK) 293 cells were grown on a 6-well plate in complete DMEM medium with 10% FCS until they reached 80–90% confluence. Then, cells were fixed with methanol (1 mL of ice cold methanol/well) at −20°C for 10 min. After washing three times with 0.5% BSA in PBS (pH 7.4), fixed cells were incubated with purified anti-S6K2

monoclonal antibody (clone B2) at a final concentration of 2 μg/mL at 4°C for 12 h. After washing three times with 0.5% BSA in PBS (pH 7.4), secondary HRP-conjugated goat anti-mouse IgG (1:500, Promega) was added and incubated with cells for 2 h at RT. The staining was performed by DAB. Light microscopy was carried out with the use of an Axioplan microscope (Carl Zeiss, Jena, Germany).

Results and Discussion

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The aim of this study was to produce monoclonal antibodies directed at the N-terminal regulatory region of S6K2/I and S6K2/II, which shows very low homology to S6K1 or other members of the AGC family.

S6K2 MAb production

cDNA fragments of S6K2 coding for N-terminal 1–64 aa and 14–64aa sequences were cloned into pET42a vector, expressed in *E. coli*, and recombinant proteins (F1 and F2 correspondently) were purified as recommended by the manufacturer using Ni-NTA-agarose (Fig. 1A, B). Peptide F1 covering NLS located within the 1–13 aa sequence of S6K2/I was used as an antigen for immunization. We maintained the standard scheme of immunization, boosting animals every 2 weeks. The titer of anti-S6K2 antibodies in the serum of immunized mice was nearly 10^{-6} on the 45th day of immunization.

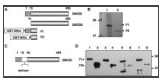

FIG. 1.
Generation of monoclonal anti-S6K2-specific antibodies. (**A**) Schematic of S6K2 peptides (F1 and F2) cloned in pET42a vectors, purified as GST-His-tag fusions from bacteria cells, and used for mice immunization (F1) and positive clones screening (F1, F2). **...**

Spleen cells from one of the immunized mice were then fused with SP2/0 myeloma cells. The resulting hybridoma cells were selected in HAT medium and tested for the production of anti-S6K2 antibodies on the tenth day after fusion. Screening of ten 96-well plates with growing hybrids by ELISA using S6K2 recombinant peptide F1 as antigens allowed us to identify 15 positive clones. We used recombinant GST-His protein as an antigen in the second round of ELISA screening in order to detect clones producing antibodies specific to GST-His. Only four from 15 selected primary clones were negative towards GST/His. ELISA screening of these clones with S6K2 recombinant peptides F1 and F2 demonstrated positive response for both peptides, indicating that none of the clones was specific to NLS region (1–13 aa) of S6K2 and the epitopes for all four clones were located within the 14–64 aa sequence of S6K2 (Fig. 1C). The summary of ELISA screening of hybrid cells is presented in Table 1.

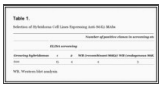

Table 1.
Selection of Hybridoma Cell Lines Expressing Anti-S6K2 MAbs

Next we examined the specificity of selected hybridoma clones in Western blotting using recombinant S6K2 proteins (F1 and F2). The obtained data show that MAbs from four ELISA selected clones specifically recognized both recombinant peptides (Fig. 1D).

Application of anti-S6K2 MAbs in Western blot analysis with total cell lysates

To further analyze antibodies, we tested all selected clones in Western blotting with HEK293 cell lysates (Fig. 2A). Only MAbs from three selected clones (B1, B2, and B4) could recognize endogenous S6K2 with similar specificity. As a positive control, we used anti-S6K2/C-term rabbit polyclonal antibodies that were characterized and applied in previous studies.(18–20) These results clearly show that antibodies produced by B1, B2, and B4 clones specifically recognize endogenous S6K2, albeit with different specificity. B4 MAbs could recognize an additional 60 kDa band that may represent non-specific binding.

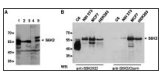

FIG. 2.
Monoclonal antibodies against human S6K2 specifically recognize S6K2 in cell of different origin. (**A**) Western blot analysis of HEK293 cell lysates with anti-S6K2/C-term polyclonal antibodies (lane 1) and anti-S6K2 MAbs B1 (lane 2), B2 (lane 3), B3 (lane **...**

Taking into account that as antigen for immunization and hybridoma screening we used human S6K2 peptides, in the next stage we analyzed the cross reactivity of generated MAbs with S6K2 from other mammals. For further experiments we chose the B2 clone. Western blot analysis of cell lysates from cell lines of different origin showed the cross-reactivity of MAbs produced by B2 clone with S6K2 from mouse, rat, and human (Fig. 2B). B2 MAb detected endogenous S6K2 in all cell lysates analyzed. However, in contrast to anti-S6K2/C-term polyclonal antibodies, B2 MAbs detected additional protein bands in rat and mouse cells (Fig. 2B) that may represent isoforms of S6K2 lacking of C-terminal sequence.

Application of anti-S6K2 (2B) MAbs for immunoprecipitation

To broaden the application of generated antibody, we tested B2 MAbs in immunoprecipitation of endogenous S6K2. For this purpose we used HEK 293 cell lysates. The data (Fig. 3A) clearly indicated that B2 MAbs precipitated endogenous S6K2 as it was further detected in Western blotting by anti-S6K2/C-term rabbit polyclonal antibodies. Taking into account that molecular weight of S6K2 forms (54 and 56 kDa) are very close to IgG heavy chains we used anti-S6K2 rabbit antibodies for Western blot analysis.

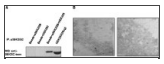

FIG. 3.

Monoclonal antibodies against S6K2 (B2) specifically immunoprecipitate endogenous S6K2 from HEK293 cell lysate and detects S6K2 in immunocytochemistry. (**A**) Western blot analysis of S6K2 kinase precipitated from HEK 293 cell lysates by MAbs B2 and detected **...**

Application of anti-S6K2 MAbs for cytochemistry

Finally MAbs produced by clone B2 were used in immunocytochemistry. HEK293 cells were grown and fixed as described in the section on Materials and Methods. Anti-S6K2 (B2) MAbs produced strong DAB staining (right panel) in comparison to the control (left panel) of secondary antibodies (Fig. 3B). Our data suggest that in HEK293, S6K2 was predominantly detected in cell cytoplasm. These data correlate with our previous observation in which S6K2 was detected predominantly in the cytoplasm of thyroid follicle cells; however, in the monolayer culture of thyrocytes in the course of follicles outspreading, S6K2 have been observed in nuclei as well. (18)

Taken together, this study describes the production of anti-S6K2 monoclonal antibodies (clone B2), which are suitable for various immunoassays, including ELISA, Western blot analysis, immunprecipitation, and immunocytochemistry. The produced antibodies provide a very useful tool to study the function of S6K2 and its possible isoforms in different macromolecular complexes.

Acknowledgments

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Author Disclosure Statement

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The authors have no financial interests to disclose.

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