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Generation and characterization of polyclonal antibodies specific to N-terminal extension of p85 isoform of ribosomal protein S6 kinase 1 (p85 S6K1)

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Aim. Generation of polyclonal antibodies specific to the ribosomal protein S6 kinase isoform – p85S6K1 and directed to the N-terminal (1-23 aa) extension of p85S6K1. **Methods**. Animal immunization with synthetic (1–23 aa) peptide, ELISA, western blot, immunoprecipitation, immunofluorescent analysis. **Results**. Polyclonal antibodies have been generated, which specifically recognize only p85 but not p70 isoform of S6K1 in western blot, immunoprecipitation and immunofluorescence analysis. **Conclusions**. The obtained antibodies can be recommended for studies on the p85S6K1 and other S6K1 isoforms possessing the N-terminal extension – the identification of binding protein partners, analysis of subcellular localization under different physiological conditions, elucidation of the signal transduction pathways involving different S6K1 isoforms.

Keywords: Ribosomal protein S6 kinase 1 (S6K1), S6K1 isoforms, p70 S6K1, p85 S6K1, polyclonal antibodies.

Introduction

The ribosomal protein S6 kinase1 (S6K1) is a serine/threonine kinase activated in response to the growth factors, cytokines and nutrients. S6K1 has been linked to the diverse cellular processes, including protein synthesis, mRNA processing, glucose homeostasis, cell growth and survival. Studies on model organisms have highlighted a role that the S6K activity plays in a number of pathologies, including obesity, diabetes, ageing and cancer [reviewed in 1]. S6K1 belongs to the PI3K/Akt/mTOR pathway that is one of the major signalling pathways hyperactivated in many cancers causing the uncontrolled proliferation, increased survival, motility, and invasiveness of the cancer cells [2–4].

The major known substrate of S6K1 is the 40 S ribosomal subunit protein S6 [5, 6]**,** however about twenty additional S6K1 substrates have already been identified [reviewed in 1].

Two isoforms of the enzyme, denoted as p70S6K1 and p85S6K1, are generated from a single gene by the use of alternative mRNA translational start sites, and differ only by the 23 aa N-terminal extension unique for the p85 isoform [7]. The N-terminal extension of p85S6K1 bears a putative nuclear localization signal that targets this isoform to the nucleus [7]. This was confirmed by immunofluorescence analysis of the rat embryo fibroblasts (REF-52) with p85S6K1 specific antibodies directed to the 8–23 aa sequence within the 23aa N-terminal extension of p85S6K1 [8]. The same authors demonstrated that microinjection of the affinity purified anti-p85S6K1 IgG into the nucleus, but not the cytoplasm, blocked the serum-induced initiation of DNA synthesis and the G1/S transition.

Despite the absence of a classical NLS, p70S6K1 can be also localized in the nucleus due to the shuttling from the cytoplasm to the nucleus upon the growth factor stimulation [9]. Moreover, phosphorylation of S17 by CK2 greatly enhances the nuclear export of p70S6K1 [10].

A number of nuclear substrates of S6K are identified so far, such as transcription factor CREM, MDM2 ubiquitine ligase, splicing complex protein SKAR, etc. [reviewed in 1] but it is not clear which of S6K1 isoforms is responsible for the phosphorylation and what are the precise functions of p70S6K1 and p85S6K1 in the nucleus.

In addition to the full-length forms of S6K1, novel S6K1 shorter splice variants in mice and human that can also possess the N-terminal NLS have been reported [11, 12]. Unlike the full-length S6K1, the identified splice isoforms, having no catalytical activity due to the truncation in the catalytical domain, possess the cell transforming activity.

The immunohistochemical analysis of S6K1 in different human breast, endometrial and thyroid tumours, revealed an increased level of S6K1 and alterations in the S6K1 subcellular localization [13–15]. In 25 % of breast adenocarcinoma cases and in 8 % of endometrial tumours the nuclear localization of S6K1 has been detected [16–18]. However, it was not clear which of two S6K1 isoforms was detected in the nucleus since the antibodies used were specific to the C-terminal sequence common for the p70S6K1 and p85S6K1 isoforms.

The aim of this work was to generate the polyclonal antibodies specific to the N-terminal (1-23 aa) extension unique for p85S6K1 that could distinguish the p70 and p85 isoforms of S6K1. This type of antibodies may be used for the analysis of the subcellular localization of the S6K1 isoforms in cells under different physiological conditions as well as in tumour cells of different types. In addition they may be very useful for the analysis of S6K1 dependent cell signalling in context of different functions of the p70 and p85 isoforms of S6K1 and the recently discovered oncogenic S6K1 splice isoforms [11, 12].

Materials and Methods

Antibodies. Mouse monoclonal (C3/10) and rabbit polyclonal (anti-S6K1/C) antibodies specific to the C-terminal (454–525 aa) sequence of p85S6K1 have been generated as described previously [19, 20]. For the generation of mouse polyclonal antibodies (a-S6K1/1–77) specific to the N-terminus of S6K1 as an antigen we used recombinant peptide corresponding to the 1–77 aa sequence of p85S6K1 (unpublished data). Rabbit monoclonal antibodies (clone 16) specific to the N-terminal sequence (1–122 aa) of p70S6K1 were from BD Bioscience (USA).

Generation of polyclonal antibodies specific to the N-terminal extension of p85S6K1. As an antigen for immunization we used a «spider» construct. For this purpose, the synthetic peptide CR50 (MRRRRRR DGFYPAPDFRHREAED) that corresponded to the N-terminal (1–23 aa) extension of p85S6K1 was immobilized on the surface of sepharose beads by chemical crosslinking (Provided by Alta Bioschience, Birmingham, UK).

For immunization we used one rabbit (New Zealand White Rabbits, weight – 3 kg, age – 6 months). The antigen/adjuvant mixture was inoculated into ten sites subcutaneously along the back. We used 0.1 ml of the mixture per site. 0.5 ml of «spider» suspension (equivalent to CR50 peptide concentration of 1 mg/ml) was mixed with 0.5 ml of Complete Freund's Adjuvant (for initial inoculation) and 0.5 ml of Incomplete Freund's Adjuvent (for the following injections). The immunizations were performed every four weeks. When the titer of antiserum reached 10^{-4} the blood was collected for the anti-serum preparation and further purification of the antibodies.

Purification of the anti-p85S6K1 antibodies was performed by affinity chromatography. The CR50 peptide was immobilised on NHS-activated Sepharose (Amersham) according to the recommendation of manufacturer. Then 5 ml of anti-sera diluted with PBS (1:1) were incubated overnight at +4 °C on the wheel in 10ml column with 1ml of the affinity matrix priory washed in PBS. After incubation the affinity matrix was washed five times with 10 ml of PBS. Elution of the anti-CR50 antibodies was per-

formed by 100mM Glycine (pH 2.5) with immediate neutralisation of the antibody eluates by 1M Tris (pH 8.8). After dialysis in PBS the purified antibodies were aliquoted and stored at – 70 °C with 50 % glycerol.

Western blot analysis – The 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). The cell lysates (25 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 corresponding antibodies dilutions for 1 h at RT. After washing with TBST, membrane was incubated with the HRP-conjugated goat anti-mouse IgG in 1:6000 dilution for 1 h at RT. (Jackson ImmunoResearch, USA). Finally, the membrane was developed using an ECL kit (GE Healthcare, USA) and then exposed to Agfa X-ray film.

Immunoprecipitation – 1μg of mAbs purified from ascites or 1μg of affinity purified polyclonal antibodies was incubated with 25 μl of 50 % protein G-Sepharose and 500 μg of cell extract in a total volume of 500 μl of lysis buffer and rotated at + 4°C for 3h. Immune complexes bound to Protein G Sepharose beads were recovered by brief centrifugation and washed three times by lysis buffer. Finally, samples were boiled in Laemmli sample buffer for 5 min, separated by SDS-PAGE and immunoblotted with the corresponding antibodies. As a negative control we used Protein G Sepharose beads incubated with cell lysates and mAbs 2B specific to S6K2 isoform.

Immunofluorescence analysis – MCF-7 cells cultured on glass slides were fixed with neutrally buffered 10 % formalin (Sigma, USA) for 15 min at room temperature. Thereafter, the cells were treated with 0.2 % Triton X-100 in PBS (50 μl) for 15 min. Then the samples were incubated for 30 min in 50 mM ammonium acetate (pH 5.0) with 10 mM cupric sulphate to eliminate autofluorescence. Nonspecific binding was blocked by the incubation with 10 % FCS for 30 min at room temperature. The rabbit polyclonal antibodies (1 μg/ml) were applied in PBS (50 μl) at + 4 °C in humidified atmosphere overnight. (In experiment for deprivation of the CR50 antibodies the synthetic peptide CR50 (10 μg/ml) was added to the dilution of the CR50 antibodies prior to incubation with the cell samples). The secondary FITS-conjugated anti-rabbit antibodies were applied in PBS in dilution 1:400 for 1 h at + 30 °C (Jackson ImmunoResearch, USA). All microscopy studies were performed using Leica DM1000 microscope (Leica, Wetzlar, Germany).

Results and Discussion

Since the discovery and molecular cloning of the S6K1 gene only two isoforms of the kinase have been known for a long time and referred to as p85S6K1 (525 aa) and p70S6K1(502 aa) (Fig. 1). It is commonly accepted that these isoforms originated as a consequence of realization of the alternative mRNA translational starts [7]. However, recently several splice isoforms in mice (S6K1 Iso-2, 316 aa) [11] and human (hS6K1-h6A, 209 aa and S6K1-h6C, 219 aa) [12] possessing cell transforming properties have been identified.

According to the literature data S6K1 implicated in numerous cellular processes, including protein synthesis, mRNA processing, glucose homeostasis, cell growth and survival targeting about twenty protein substrates with different subcellular localization including the nuclear compartment [1]. It is not clear so far, whether p85S6K1, that have exclusively nuclear localization according to the immunofluorescence data [8], and the cytoplasmic p70S6K1 form, that in fact may shuttle between cytoplasm and nucleus [10] in response to the extracellular stimuli, have similar or different functions and whether they have the common substrates. Additionally, the immunohistochemical analysis of different tumor tissues revealed an increased level of S6K1 in the nuclear compartment [13–15] but which of the S6K1 isoforms is overrepresented is not clear as well.

The antibodies specific to the 1–23 aa N-terminal extension of p85S6K1 that may discriminate the p85 and p70 kDa isoforms of S6K1 may be very useful for such analyses. Earlier it has been claimed the

generation of polyclonal antibodies directed to the 8–23 aa extension of p85S6K1 [8]. However, for some reason the authors excluded the 1–7 aa sequence (MRRRRRR) bearing the nuclear localization site from the synthetic peptide used for immunization that could increase the specificity of antibodies.

The aim of this study was to produce the polyclonal antibodies specific to the entire N-terminal 1-23 aa extension of the p85 isoform of S6K1 that differ it from a shorter p70 isoform. For this purpose, as an antigen for immunization we used the synthetic peptide CR50 (MRRRRRRDGFYPAPDFRHREAED) corresponding to the N-terminal extension of p85S6K1 immobilised on a sepharose carrier. The animal immunization was performed according to the regular protocol. The titer of anti-p85S6K1 before final bleeding was 10^{-4}. The specificity of affinity purified CR50 polyclonal antibodies has been analysed with several tests. According to the data of western blot analysis of the MCF7 and HEK293 cell lysates, the CR50 polyAbs efficiently detected a 85 kDa band corresponding to the p85 isoform of S6K1 (Fig. 2). The specificity of the p85 isoform recognition by the CR50 antibodies was confirmed by western blot analysis of the same cell lysate with the anti-S6K1 antibodies of other origin (Fig. 2). All antibodies used as a referent specifically recognised the same p85 kDa band. Since these antibodies were generated to the common for the p85 and p70 isoforms sequences (located in the N- or C-terminal regions of S6K1), all of them efficiently recognised the p70S6K1 isoform. The C3/10 mAbs were generated in our laboratory previously and they are specific to the C-terminal (454–525 aa) sequence of p85S6K1 [19]. Commercially available mAbs (clon 16) from BD Bioscience are directed to the N-terminal sequence (1–122 aa) of p70 S6K1 and the polyclonal antibodies a-S6K1/1–77 were generated to the N-terminal (1–77 aa) sequence of p85S6K1 (unpublished data). Interestingly, all types of antibodies recognised a unique spectrum of additional minor bands that may reflect some non-specific binding of antibodies or recognition of the novel isoforms of S6K1. For example mAbs 16 (BD Bioscience) directed to the N-terminal region, common for p85 and p70,

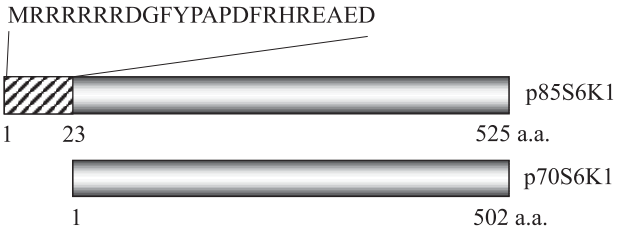


Fig. 1. Schematic representation of S6K1 isoforms – p85S6K1 and p70S6K1 that differs by N-terminal (1–23 aa) extension present in p85S6K1 and originated due to realization of alternative translation start

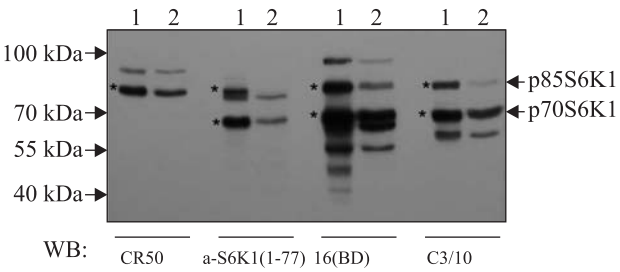


Fig. 2. Western blot analysis of cell lysates with anti-S6K1 antibodies of different origin: CR50, anti-S6K1 (1-77 aa), 16 (BD Bioscience) and C3/10. Cell lysates: 1 – MCF7 (25 μg); 2 – HEK293 (25 μg). p85S6K1 and p70S6K1 isoforms are indicated by asterisk

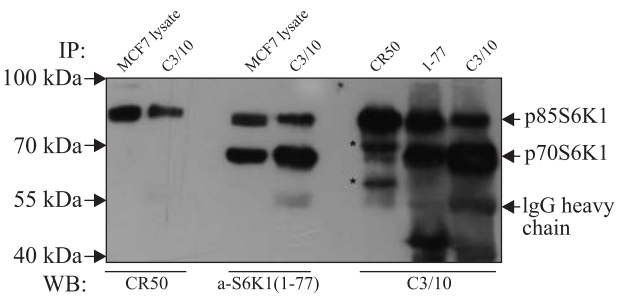


Fig. 3. Western blot detection of S6K1 isoforms immunoprecipitated from MCF7 cell lysates by anti-S6K1 antibodies (C3/10; CR50; a-S6K1(1–77)) and immunostained with different specificity anti-S6K1 antibodies. MCF7 cell lysate (25 μg)

could recognise a 65 kDa band that was not detected by the C3/10 mAbs directed to the C-terminal region common for both isoforms as well. On the other hand, C3/10 could recognise a 60 kDa band that is invisible for the mAbs 16. We cannot exclude as well that these bands represent proteolytically truncated either from N- or C-terminus forms of S6K1.

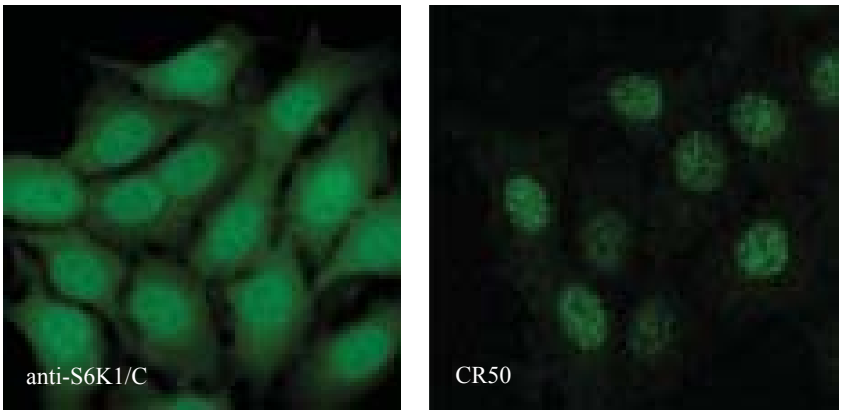
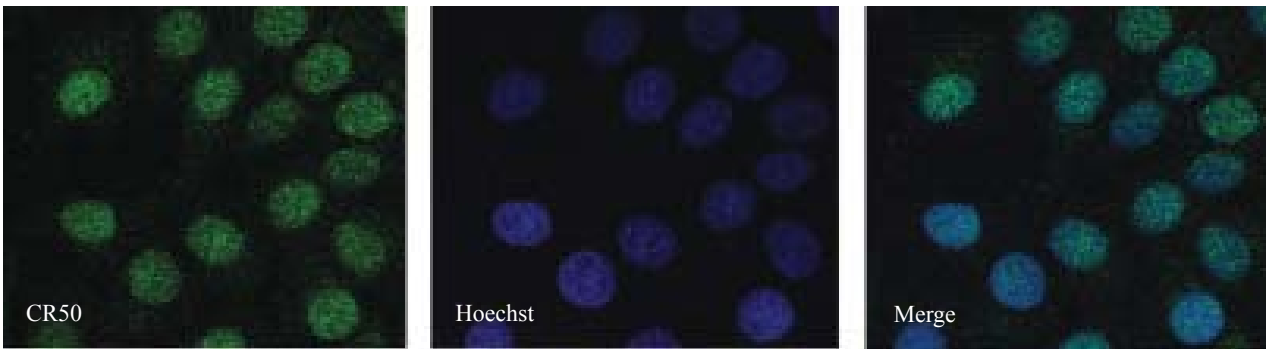
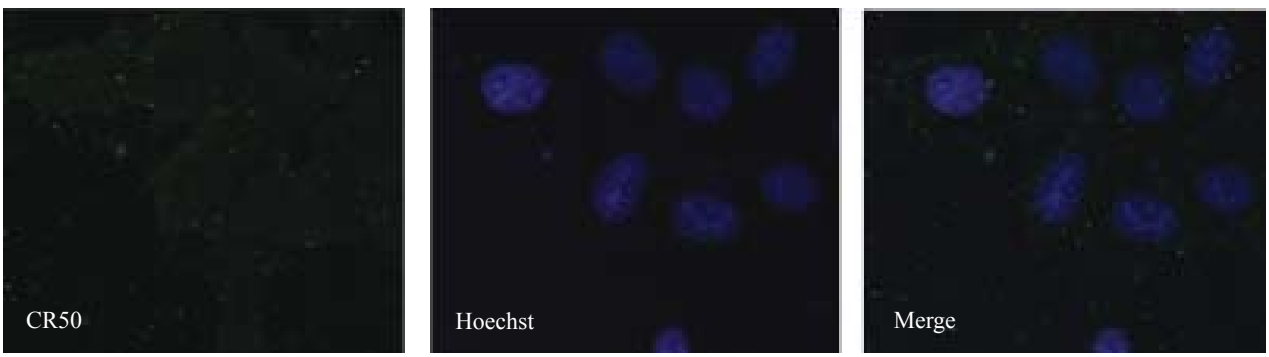


Fig. 4. Immunofluorescent localisation of endogenous p85S6K1 and p706SK1 in MCF7 cells. Exponentially growing MCF7 cells were cultured on coverslips as described in Materials and methods) and immunostained with the polyclonal antibdies specific to: C-terminus of S6K1 (anti-S6K1/C) and N-terminal extension of p85S6K1 (CR50)



A



B

Fig. 5. Immunofluorescent localisation of endogenous p85S6K1 in MCF7 cells. Exponentially growing MCF7 cells were cultured on coverslips as described Materials and methods) and immunostained with the polyclonal antibodies CR50 (*A*) and mixture of CR50 antibodies (*B*) with immunogenic synthetic peptide corresponding to the N-terminal (1–23 a.a.) extension of p85S6K1. The localization of nuclei in cells is depicted by Hoechst

For further validation of the CR50 polyAbs specificity we performed an immunoprecipitation of S6K1 from the cell lysates with a next analysis of the immunoprecipitates with different types of the anti-S6K1 antibodies. According to the data presented in Fig. 3 CR50 Abs precipitated p85 S6K1 but did not precipitate p70S6K1. As it was expected in western blot analysis of C3/10 mAbs immunoprecipitates that contain both p70kDa and p85 kDa S6K1 isoforms (since they are immunoblotted by C3/10) CR50 detected only p85S6K1. Several additional bands that were detected by the C3/10 mAbs in

CR50 immunoprecipitates (marked by asterisk) may reflect the proteolytical degradation of p85S6K1.

According to Thomas et al. [8] the antibodies to the 8–23 aa sequence of p85S6K1 detected this isoform exclusively in the nucleus. According to our data the CR50 polyAbs also detected S6K1 predominantly in the nucleus in immunocytochemical analysis of MCF7 cells in contrast to the anti-S6K1/C polyAbs specific for both S6K1 isoforms that detected S6K1 in the cytoplasm and in the nucleus to the similar extent (Fig. 4). The specificity of CR50 antibodies was further confirmed by the application of the synthetic CR50 peptide used as an antigen for immunization for the depletion of the CR50 antibodies in immunofluorescence analysis (Fig. 5). Immunostaining MCF7 cells by the mixture of CR50 antibodies and CR50 synthetic peptide completely abolished the nuclear staining.

As a conclusion, in this study we claim the generation of polyclonal antibodies specific to the N-terminal extension of the p85 isoform of S6K1 that could be also present in the recently identified S6K1 splice isoforms [11, 12] possessing cell transforming properties. A high specificity of the antibodies analysed in various assays such as western blot, immunoprecipitation and immunofluorescence analysis suggests the possibility of their application for the evaluation of the S6K1 isoforms functions by analysis of the S6K1 isoforms protein-protein interactions (identification of upstream regulators and downstream targets), subcellular localization under different physiological conditions or at different extracellular stimuli and alterations of the S6K1 isoforms expression and subcellular localisation in the malignant cells of different tumour types.

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Створення та характеристика поліклональних антитіл специфічних до p85 ізоформи кінази рибосомного білка S6 (p85 S6K1)

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Мета Отримати поліклональні антитіла специфічні до p85 ізоформи S6K1 и спрямовані до N-кінцевого (1–23 а.к.) подовження p85S6K1 **Методи.** Імунизація тварин синтетичним (1–23 а.к.) пептидом, ІФА, вестерн блот, імунопреципітація, іммуноцитохімічний аналіз. **Результати.** Отримано поліклональні антитіла, що розпізнають p85, але не p70 ізоформу S6K1 в вестерн блот анализі, іммунопреципітації і іммуноцитохімичному аналізі. **Висновки.** Отримані антитіла можуть бути рекомендованими для дослідження p85S6K1 та інших ізоформ S6K1, що мають N- кінцеве подовження – ідентифікації білків-партнерів, аналізу субклітинної локалізації за різних фізіологічних умов, аналізі ролі S6K1 ізоформ в сигнальній трансдукції.

Ключові слова: кіназа рибосомного білку S6 (S6K1), ізоформи S6K1, p70 S6K1, p85 S6K1, поліклональні антитіла.

Получение и характеристика поликлональных антител специфичных к p85 изоформе киназы рибосомного белка S6 (p85 S6K1)

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Цель. Получить полииклональные антитела специфичные к p85 изоформе S6K1 и направленные против N-концевого (1–23 а.к.) удлинения p85S6K1. **Методы.** Иммунизация животных синтетическим (1–23 а.к.) пептидом, ИФА, вестерн блот, иммунопреципитация, иммуноцитохимический анализ. **Результаты.** Получены поликлональные антитела распознающие p85, но не p70 изоформу S6K1 в вестерн блот анализе, иммунопреципитации и иммуноцитохимическом анализе. **Выводы.** Полученные антитела могут быть рекомендованы для изучения p85S6K1 и других изоформ S6K1 обладающих N-концевым удлинением – идентификации белков партнеров, анализе субклеточной локализации при разных физиологических условиях, анализе роли S6K1 изоформ в сигнальной трансдукции.

Ключевые слова: киназа рибосомального белка S6 (S6K1), изоформы S6K1, p70 S6K1, p85 S6K1, поликлональные антитела.

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