J. Biochem. **143,** 325–332 (2008)
doi:10.1093/jb/mvm229

Anti-Peptide Antibodies for Examining the Conformation, Molecular Assembly and Localization of an Intracellular Protein, Ribosomal Protein S6, In vivo

Masatoshi Nakagawa[1], Nobuko Ohmido[2], Katsumi Ishikawa[1], Susumu Uchiyama[3], Kiichi Fukui[3] and Takachika Azuma[1,*]

[1]Division of Biosignaling, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan; [2]Faculty of Human Development, Kobe University, Tsurukabuto 3-11, Nada, Kobe, Hyogo 657-8501, Japan; and [3]Laboratory of Dynamic Cell Biology, Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received November 4, 2007; accepted November 9, 2007; published online November 26, 2007

Ribosomal protein S6 (rpS6) is known to relate to cell proliferation. Our recent proteome analysis of human metaphase chromosomes revealed the enrichment of rpS6 during mitosis. Here, structure, localization and molecular assembly *in vitro* and *in vivo* of a human rpS6, were examined using antibodies (Abs) prepared by immunizing rabbits with synthetic peptides. Five peptides, Ser6-Asp20 (S6-1), Ile52-Gly66 (S6-2), Asp103-Gly117 (S6-3), Asn146-Lys160 (S6-4) and Arg178-Ile192 (S6-5) were chosen as epitopes of human rpS6. These peptides except for S6-3 induced strong Ab production, and with an enzyme-linked immunosorbent assay, anti-S6-2, anti-S6-4 and anti-S6-5, showed high reactivity to recombinant rpS6 (r-rpS6), while anti-S6-1 did not, suggesting that S6-2, S6-4 and S6-5 were exposed on the r-rpS6 surface, while S6-1 was less exposed or possessed a different conformation. The immunostaining of HeLa cells as well as isolated chromosomes suggested that rpS6 occurs in endoplasmic reticulum (ER) but less possible on chromosomes since no Abs showed localization of rpS6 to chromosomes. In addition, the immunostaining suggested that only S6-4 is exposed on the protein surface, while S6-2 and S6-5 are buried by the interaction with other macromolecules in HeLa cells. Present our result shows new possibility of antibodies as tools for structure-oriented cell biology.

Key words: anti-peptide antibody, conformation, peptides, ribosomal protein S6, epitope.

Abbreviations: Ab, antibody; Ag, antigen; HPLC, high-pressure liquid chromatography; *H. sapiens, Homo sapiens*; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; rpS6, ribosomal protein S6; r-rpS6, recombinant ribosomal protein S6; *S. cerevisiae, Saccharomyces cerevisiae*; S6K, ribosomal protein S6 kinase; *T. thermophilus, Thermus thermophilus*.

Antibodies (Abs) have long been used for studying protein structure in solution because of their specificity in recognition of structure and conformation (*1*). Abs recognize the antigenic determinant (epitope) that has structural complementarity to the Ab-combining site (paratope). Epitopes have been classified into two types, 'linear or segmental' and 'conformational or topographic' (*2–6*). Linear epitopes are determined by their linear amino acid sequences, while conformational epitopes are related to the three-dimensional protein structure and are considered to be an assemblage of several amino acid segments. Abs prepared by immunization of animals with native proteins seem to largely recognize conformational epitopes and sometimes fail to react with denatured proteins or peptides (*2*). These Abs, therefore, cannot be used for detection of proteins by western blotting analysis after SDS–PAGE. On the other hand, Abs prepared by immunization with synthetic peptides are able to bind to both native and denatured proteins, and can be used for isolating and characterizing gene products.

Due to advances in the methodology of peptide synthesis, in addition to progress in the theoretical understanding of the immunochemistry of Ag–Ab recognition (*7*), anti-peptide Abs have been widely used for identification of proteins in cell extracts or living cells that contain corresponding peptide segments (*8*). Ab reactivity is considered to depend on two factors; the degree of epitope exposure on the protein surface and the secondary structure of the peptide segments in the target proteins. Although most peptides used as immunogens possess no obvious secondary structure, the rigid conformation of the corresponding segments in a folded protein was expected to affect Ab reactivity. However, Stanfield *et al*. (*9*) showed that the conformation of a peptide in complex with an anti-peptide Ab was different from that in which the peptide was a portion of the intact protein, forming a type II β-turn in the former, and an α-helix in the latter. It was also shown that anti-peptide Abs were able to create combining sites for peptide accommodation by an induced fit mechanism (*10*). Therefore, it is likely that the secondary structure

*To whom correspondence should be addressed. Tel: +81-4-7121-4082, Fax: +81-4-7121-4089, E-mail: tazuma@rs.noda.tus.ac.jp

of a peptide segment is less important in determining Ab reactivity than its degree of exposure on the surface. Peptide segments folded into the interior of a protein or buried in the protein interface would not be accessible to Abs. This suggests that the reactivity of an anti-peptide Ab is a measure of the folding state of the recognized peptide sequence, in the case of purified protein, as well as of interactions with other macromolecules in the case of proteins present in cells.

In the present experiments, we prepared Abs by immunizing rabbits with synthetic peptides possessing amino acid sequences corresponding to human rpS6 as a model for the analysis of the protein–macromolecule interactions in living cells using Abs. Recently, it has been reported that rpS6 phospholylation as well as the activation of several protein kinases is essential for regulating the protein synthesis and the cell size (*11*). In mammals, phosphatidylinositol 3-kinase (PI3K) is a component of a growth-regulation pathway, one of whose downstream targets is rpS6 that is the major phosphoprotein among the components in 40 S ribosomal subunit. The phosphorylation of rpS6 at multiple sites was induced by several kinds of stimuli like serum, growth factors, insulin and tumour promoting agents (*12–15*) and is caused by rpS6 kinase (S6K1 and S6K2) including PI3K, at early stage after stimuli and regulates the translational activation of terminal oligopyrimidine tract (TOP) mRNAs, which encode the important components of the protein synthesis, led to the expression of proteins and the progression of cell cycle (*16*). Although rpS6 phospholyration is thus important for cell cycles in eukaryotes, biophysical and biochemical information such as 3D structure or localization of rpS6 in cells has not been provided yet. Since recent proteome analysis of human metaphase chromosomes suggested the possibility of rpS6 to be a chromosomal protein (*17*), although it is generally known to occur in ribosomes, its localization in cells throughout cell cycle was required to confirm by the other methods such as immunostaining. In order to choose epitopes of Abs for immunostaining, we utilized the information on 3D structure, assuming the analogy of 3D structure between human rpS6 and that of *Thermus thermophilus*. We chose five epitopes: four of them (S6-2, S6-3, S6-4 and S6-5) were expected to locate on the protein surface and another (S6-1) was from eukaryote-specific N-terminal region. Since rpS6 is abundant in cells, the binding of Abs to this protein was expected to be easily visualized by immunostaining and behave as a positive control for determining the localization. Before the analysis by immunostaining, we examined the reactivity of the Abs with peptide or recombinant rpS6 protein (r-rpS6) by the enzyme-linked immunosorbent assay (ELISA), in which peptide or r-rpS6 was each coated onto plastic plates, respectively. This assay was expected to provide information on the extent of exposure of the peptide segments on the monomeric rpS6. In addition, we compared the reactivity of Abs with r-rpS6 on plates by ELISA and rpS6 on isolated chromosomes or in HeLa cells by immunostaining and attempted to explain the observed differences of Abs, if any, in terms of accessibility of the epitopes. In other words, we showed that only an Ab reacted with the epitope exposed on the surface of the target protein can be used for the analysis of its localization *in vivo*.

MATERIALS AND METHODS

Two-Dimensional Gel Electrophoresis—Chromosomes and nucleic fractions were isolated from HeLa cells by sucrose density gradient centrifugation and then, the proteins extracted by acetic acid extraction method were separated by radical-free and highly reducing two-dimensional gel electrophoresis (RFHR 2-DE) as described previously (*17*).

Preparation of Antigens—The peptides corresponding to the amino acid segments of rpS6, Ser6-Asp20 (S6-1), Ile52-Gly66 (S6-2), Asp103-Gly117 (S6-3), Asn146-Lys160 (S6-4) and Arg178-Ile192 (S6-5), were prepared. A Cys residue was added at the N- or C-terminus of these peptides for conjugation with carrier proteins. We used a solid phase Fmoc strategy for synthesizing the peptides. The synthesis was performed on NovaSyn-TGR resin (Novabiochem, Darmstadt, Germany) and the peptides were purified by high-performance liquid chromatography (HPLC).

The synthetic peptides were conjugated to bovine serum albumin (BSA) using m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, Pierce). MBS was first reacted with BSA at pH 8.5 (HEPES buffer containing 150 mM NaCl) for 2 h, followed by gel filtration with a PD-10 column equilibrated with HEPES buffer containing 1 mM EDTA. Peptides with a free Cys residue at either the N- or C-terminus were added to the MBS–BSA solution at a peptide to BSA ratio of 50:1 and reacted for 1 h. The unreacted peptides were removed on a PD-10 column. The amount of peptides conjugated to the BSA was estimated by mass spectroscopy.

Mass Spectrometry—MS analyses were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to identify the number of peptides conjugated to BSA. BSA and peptide-conjugated BSA were dissolved in water and mixed with matrix solution [2:1 (v/v) mixture of 0.1% trifluoroacetic acid/acetonitrile] saturated with sinapinic acid (Sigma) on respective sample plates and then dried at room temperature. All MS experiments were carried out using a REFLEX III mass spectrometer (Bruker Daltonics) in linear-positive ion mode. Sample ionization was performed by irradiation using a nitrogen laser with emission set at 337 nm.

Preparation of Recombinant rpS6 Protein—The rpS6 cDNA was prepared by reverse transcriptase PCR (RT–PCR) from mRNA of HeLa cells and cloned using the GATEWAY system (Invitrogen). The protein fused with a His Tag at N-terminus was expressed in *Escherichia coli* strain BL (DE3) (Stratagene). Cells were grown in LB medium containing 100 μg/ml ampicillin and induced with isopropyl-β-D(−)-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After incubation at 4°C for 4 h, cells were harvested by centrifugation. The cell pellet was re-suspended in the sonication buffer (20 mM Tris–HCl pH 7.4, 130 mM NaCl, 1 mM PMSF) and cells were lysed by sonication. The precipitate was collected by centrifugation and dissolved

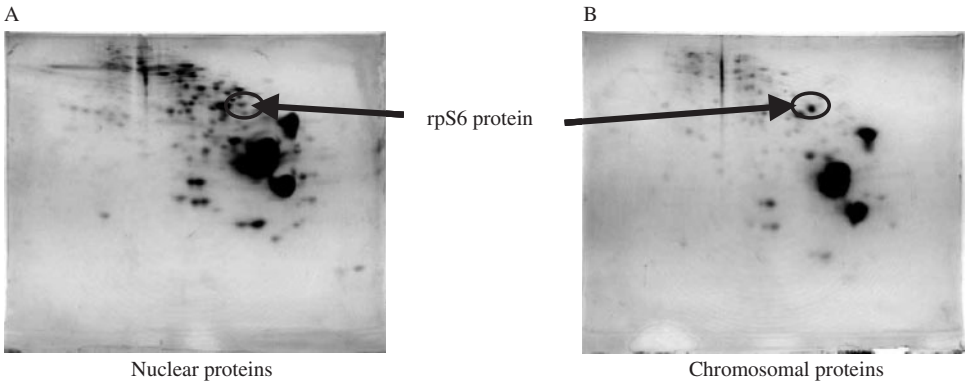

Fig. 1. **Two-dimensional gel electrophoresis.** (A) The proteins extracted from nucleic fraction. (B) The proteins extracted from isolated chromosome fraction.

in refolding buffer (20 mM Tris–HCl pH 7.4, 130 mM NaCl, 8 M urea). This protein solution was dialysed twice against a Ni column buffer (20 mM Na_2HPO_4 pH 7.4, 500 mM NaCl, 6 M Urea) containing 50 mM imidazole, and applied to a Ni column. After washing, the target protein was eluted with Ni column buffer containing 500 mM imidazole. The urea-denatured purified protein was then folded by a stepwise dilution of urea concentration by dialysis against 20 mM Tris–HCl (pH 8.0), 1 mM EDTA solution containing respective concentrations of 4, 2, 1 and 0 M urea. The folded protein was purified using an anion exchange column, and the target protein was then concentrated and dialysed against phosphate-buffered saline (PBS).

Immunization and Ab Purification—Rabbits were purchased from Oriental (Japan). The peptide-BSA conjugates were emulsified with complete Freund's adjuvant (CFA) and rabbits were immunized subcutaneously followed by booster immunizations on Days 14, 28 and 56. They were bled 9 weeks post-immunization. Anti-BSA Abs were removed by passing through a column of BSA–Sepharose, and flow-through fractions were used for further analysis.

ELISA—In order to estimate the amount of Abs capable of binding to the peptides or r-rpS6 protein, micro titre plates were coated with either peptides or r-rpS6 protein at 0.33 μM (50 μl/well) at 4°C, followed by incubation with 5% skimmed milk (250 μl/well). Sera from the immunized rabbits were then applied to the plates and IgG bound to Ags was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (MP Biomedicals, Inc.).

Immunostaining of rpS6 in HeLa Cells—The immunostaining was carried out according to the procedure of Maeshima and Laemmli (*18*) with a small modification. HeLa S3 cells were grown on 18 × 18 mm poly-L-lysine-coated glass coverslips in RPMI1640 (Nacalai Tesque) supplemented with 10% fetal calf serum (GIBCO BRL), 1% penicillin and streptomycin, under conditions of 37°C in a 5% CO_2 incubator for 3 days. After washing with PBS, the cells were incubated in 0.5 mg/ml $NaBH_2$ in XBE2 (10 mM HEPES pH 7.6, 2 mM $MgCl_2$, 100 mM KCl, 5 mM EGTA) for 5 min. Permeabilization was carried out with 0.5% Triton X-100 in XBE2 at room temperature, and cells were then fixed with 2% paraformaldehyde (PFA)/XBE2 for 15 min and incubated with 10 μg/μl of the purified anti-peptide Abs (diluted 1:100 in XBE2 containing 1% BSA, 0.05% Tween-20) for 60 min. The bound anti-peptide Abs were detected by FITC-labelled goat anti-rabbit IgG Abs (Sigma) (1:200 in XBE2 containing 1% BSA, 0.05% Tween-20). The slides were embedded in Vectashield containing 1 μg/ml DAPI. Images were obtained using a fluorescence microscope, BX60 (OLYMPUS), equipped with a sensitive-cooled CCD camera (PXL 1400, Photometrics). The B- or UV-light excitation filters are used for the excitation of FITC and DAPI, respectively. Captured images were digitally stored and analysed using imaging software (Photoshop 7.0, Adobe). Chromosomes isolated from HeLa S3 cells (*17*) were diluted with XBE2 and then fixed with 1/10 vol of 8% (PFA)/XBE2 for 15 min. Equal volume of 60% sucrose/XBE2 was added into chromosome solution and centrifuged at 2000 r.p.m. for 10 min. Immunostaining was performed with same manner as described above.

RESULTS

Two-Dimensional Gel Electrophoresis (2-DE)—The proteins isolated from nuclear fraction or chromosome fraction were analysed by RFHR 2-DE (Fig. 1). The spots obtained were subjected to proteome analysis and the protein consisting of each spot was deduced on the basis of amino acid sequences (*17*). Since the rpS6 spot occurred in both chromosome and nuclear fractions and since its amount in chromosome fraction varied with preparation methods employed, it was considered that rpS6 interacted rather weakly with the surface of chromosome and can be classified as 'chromosome peripheral proteins' or 'chromosome coating proteins' (*17*). Since rpS6 has been known to exist only in ribosome in general, further analysis of localization by different methodology was required.

Ag Preparation and Immunization—Amino acid sequence of *Homo sapiens* (*H. sapiens*) rpS6 (*19*) and its comparison to those of other species (*20*, *21*) are shown in Fig. 2A. Compared to prokaryotic rpS6, the eukaryotic rpS6 has an additional about 44 amino acid residues in N-terminus and more than 40 amino acid residues in C-terminus, respectively. The homology of these additional regions is quite high among eukaryotes.

A

```
                          S6-1                                      S6-2
H.sapiens        1:MKLNISFPATGCQKLIEVDDERKLRTFYEKRMATEVAADALGEEWKGYVVRISGGNDKQG 60
melanogaster     1:MKLNVSYPATGCQKLFEVVDEHKLRVFYEKRMGQVVEADILGDEWKGYQLRIAGGNDKQG 60
S.cerevisiae     1:MKLNISYPVNGSQKTFEIDDEHRIRVFFDKRIGQEVDGEAVGDEFKGYVFKISGGNDKQG 60
thermophilus     1:--------------------------------------------MRRYEVNIVLNPN--- 13
E.coli           1:--------------------------------------------MRHYEIVFMVHPD--- 13
                                                                  S6-3
H.sapiens       61:FPMKQGVLTHGRVRLLLSKGHSCYR--PRRTGERKRKSVRGCIVDANLSVLNLVIVKKGE 118
melanogaster    61:FPMKQGVLTHGRVRLLLKKGHSCYR--PRRTGERKRKSVRGCIVDANMSVLALVVLKKGE 118
S.cerevisiae    61:FPMKQGVLLPTRIKLLLTKNVSCYR--PRRDGERKRKSVRGAIVGPDLAVLALVIVKKGE 118
Thermophilus    14:LDQSQLALEKEIIQRALEN-YGARVEKVEELGLRR---------------LAYPIAKDPQ 57
E.coli          14:-QSEQVPGMIERYTAAITG-AEGKIHRLEDWGRRQ---------------LAYPINKLHK 56
                                               S6-4
H.sapiens      119:KDIPGLTDTTVPRRLGPKRASRIRKLFNLSKEDDVRQYV--VRKPLN--KEGKKPRTKAP 174
melanogaster   119:KDIPGLTDTTIPRRLGPKRASKIRKLYNLSKEDDVRRFV--VRRPLPA-KDNKKATSKAP 175
S.cerevisiae   119:QELEGLTDTTVPKRLGPKRANNIRKFFGLSKEDDVRDFV--IRREVT---KGEKTYTKAP 173
Thermophilus    58:----GYFLWYQVEMPE-DRVNDLARELRIR--DNVRRVMV-VKS---------------- 93
E.coli          57:----AHYVLMNVEAPQ-EVIDELETTFRFN--DAVIRSMVMRTKHAVT------------ 97
                      S6-5
H.sapiens      175:KIQRLVTPRVLQHKRRRIALKKQRTKKNKEEAAEYAKLLAKRMKEAKEKRQEQIAKRRRL 234
melanogaster   176:KIQRLITPVVLQRKHRRIALKKKRQIASKEASADYAKLLVQRKKESKAKREE--AKRRRS 233
S.cerevisiae   174:KIQRLVTPQRLQRKRHQRALKVRNAQAQREAAAEYAQLLAKRLSERKAEKAE--IRKRRA 231
Thermophilus    94:-------QEPFLANA--------------------------------------------- 101
E.coli          98:-EASPMVKAKDERRERRDDFANETADDAEAGDSEE------------------------- 131

H.sapiens      235:SSLRASTSKSESSQK                                              249
melanogaster   234:ASIRESKSSVSSDKK                                              248
S.cerevisiae   232:SSLKA----------                                              236
Thermophilus   102:---------------                                              102
E.coli         132:---------------                                              132
```

B

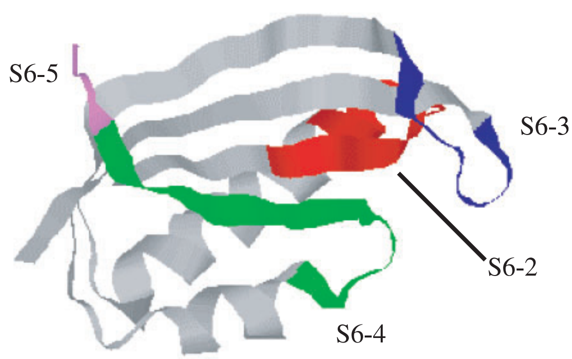

Fig. 2. **Comparizon of amino acid sequences and structural assignment of epitopes at rpS6.** (A) Amino acid sequence of *H. sapiens* (human) rpS6 and comparison to other species are shown. Missing amino acid residues are indicated by dashes (–). Numbers show the amino acid positions and coloured letters are epitopes immunized to the rabbit (brown: S6-1, red: S6-2, blue: S6-3, green: S6-4, violet: S6-5). (B) Three-dimensional structure of *T. thermophilus* rpS6 (PDB code, 1RIS) are shown in ribbon model with the program RASMOL (*22*). The S6-2 (red), S6-3 (blue), S6-4 (green) and S6-5 (violet) epitopes are indicated on the S6.

We chose five peptide segments as epitopes on the basis of three-dimensional structure of *T. thermophilus* rpS6 (Fig. 2B): S6-1, eukaryote-specific region; S6-2, signature motif; S6-3, loop region in *T. thermophilus* rpS6; S6-4, high homology region among species; S6-5, nuclear localization signal sequence. Table 1 shows the amino acid sequences of the peptides used in the present experiment. The number of peptides conjugated to one BSA molecule was estimated by mass spectroscopy (data not shown). The spectra displayed single broad peak and the average number of BSA-conjugated peptides was ~10.

The generation of anti-peptide Abs at various dilutions of antisera to their respective peptides was examined by ELISA using peptide-coated plates and is shown in Fig. 3. Each peptide–BSA conjugate provided a high Ab production. No significant difference was observed in Ab production between peptides conjugated through Cys at the N- *versus* the C-terminus (data not shown). However, a peptide corresponding to the amino acid segment S6-3 provided little production of Abs capable of binding to this peptide.

Preparation of r-rpS6 Protein—The r-rpS6 was prepared with a His tag sequence at the N-terminus for the rapid and high purity purification. The purity of r-rpS6 was examined by SDS–PAGE (Fig. 4). Two bands with different MWs are evident in the purified material. Since both components were capable of binding to Ni

Table 1. **Amino acid sequences of synthesized peptides and location in human rpS6 protein.**

Peptides	Position	Amino acid sequences
S6-1	S6-D20	SFPATGCQKLIEVDD
S6-2	I52-Q66	ISGGNDKQGFPMKQ
S6-3	D103-G117	DANLSVLNLVIVKKG
S6-4	N146-K160	NLSKEDDVRQYVVRK
S6-5	R178-I192	RLVTPRVLQHKRRRI

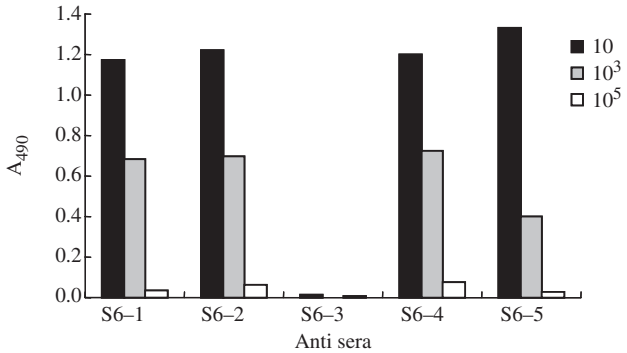

Fig. 3. **Response of rabbit sera immunized with each peptide.** The immune responses were checked by an ELISA using the plate coated with each antigen peptide. Absorbances at 490 nm (A_{490}) were plotted against dilutions of antisera.

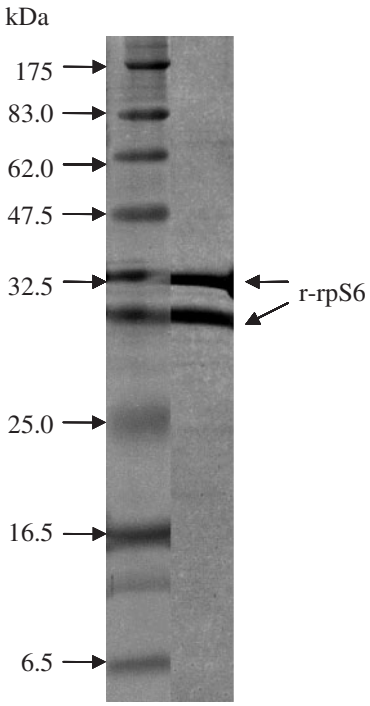

Fig. 4. **Analysis of r-rpS6 purification by SDS–PAGE.** 1, Molecular weight marker; 2, r-rpS6 protein purified on a Ni column and an anion exchange column.

column, which indicated the existence of the His tag at the N-terminus of these proteins, it was considered that the molecular heterogeneity had not arisen from proteolysis at the N-terminal segment and that either post-translational modification at the C-terminus or digestion by proteases during preparation have occurred. Since no significant differences were observed in Ab reactivity with these components by western blotting using anti-S6-2, anti-S6-4 and anti-S6-5 Abs, we utilized this mixture as the source of r-rpS6 for further experiments.

Binding of Abs to r-rpS6—The binding of anti-peptide Abs was examined by ELISA using plates coated with the respective peptides or r-rpS6, and the results shown in Fig. 5 indicated that reactivity varied greatly among Abs, in spite of the fact that they showed a similar capability of binding to the corresponding free peptides, except for S6-3. Anti-S6-5 Abs showed the most efficient binding to r-rpS6. On the other hand, the weak binding of anti-S6-1 to r-rpS6 suggested that the segment at the N-terminal region was buried in the protein interior and was less exposed, or that the His-tag introduced at the N-terminus hindered Ab binding to the S6-1 epitope. High reactivity of anti-S6-2, anti-S6-4 and anti-S6-5 Abs to r-rpS6 indicates the exposure of the corresponding epitopes on the r-rpS6 surface.

Binding of Abs to rpS6 Protein in HeLa Cells and Isolated Chromosomes—The binding of Abs to the proteins in HeLa cells was examined by immunostaining using anti-S6-1, S6-2, S6-4 and S6-5. Anti-S6-4 Abs showed strong reactivity to rpS6 proteins in the cytoplasm where they have been shown to exist in as a ribosomal protein but not in the nucleus, as shown in Fig. 6A. In contrast to the reactivity of anti-S6-4, the anti-S6-5 Abs bound only at very low levels to rpS6 in HeLa cells (Fig. 6A), in spite of their strong reactivity with r-rpS6. This suggests that rpS6 interacts with other macromolecules such as S18 or mRNA in the cell, and that anti-S6-5 could not therefore bind to the epitope. On the other hand, anti-S6-1 did show evidence of binding to rpS6 in HeLa cells (although rather weak), despite a minimal level of binding to r-rpS6. When the isolated chromosomes were examined by immunostaining, rpS6 was not detected using any Abs including anti-S6-4 Abs (Fig. 6B). The results suggest that rpS6 is not localized on the chromosome isolated by the method employed in the present study, although rpS6 was detected from both nucleic and chromosomes fraction by 2-DE (Fig. 1). Judging from the observations of immunostaining of HeLa cells and isolated chromosomes, it was considered that rpS6 might have been washed out from the surface because the interaction between rpS6 and chromosomes is very weak.

DISCUSSION

One of the goals in clarifying the structural biology of proteins is to understand the role of their quaternary structure in living cells. However, the structural aspects of protein function and interactions with various intracellular components in living cells is not yet well understood, although information is available on the three-dimensional structures of proteins as provided by X-ray crystallography and nuclear magnetic resonance. In this study, we attempted to deduce the configuration of peptide segments within the protein structure, *i.e.* whether they exist on the surface, or in the interior

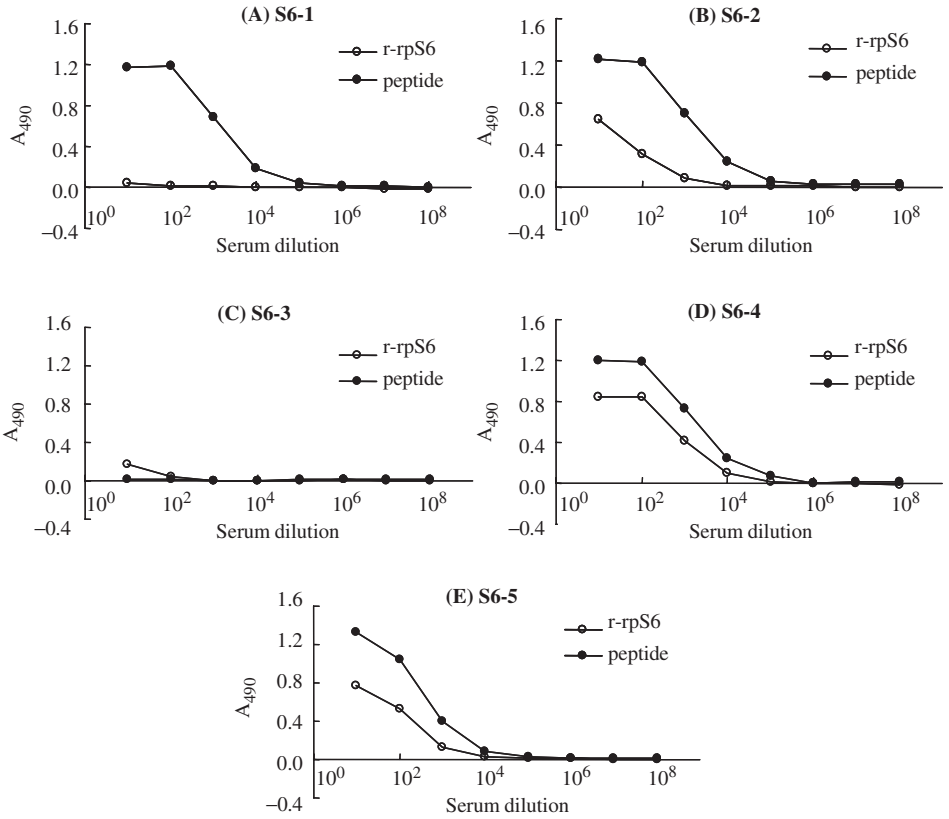

Fig. 5. **Binding of anti-peptide Abs to respective peptides or rS6 (0.33 μM) coated on the ELISA plates.** Absorbances at 490 nm (A_{490}) were plotted against dilutions of antisera.

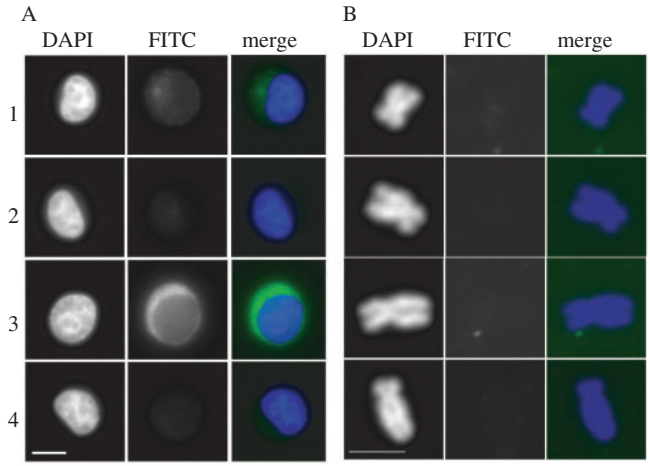

Fig. 6. **Immunostaining of HeLa S3 cells (A) and isolated chromosomes from HeLa S3 cells (B) for anti-peptide Abs.** 1, anti-S6-1; 2, anti-S6-2; 3, anti-S6-4; 4, anti-S6-5. Nuclei were stained with DAPI. The bound anti-peptide Abs were detected by FITC-labelled goat anti-rabbit IgG Abs.

due to protein folding, or are located at the interface of protein–macromolecule cellular interactions. For this purpose, we prepared anti-peptide Abs by immunizing rabbits with peptides of 15 amino acid residues and examined the relative Ab reactivity with the peptides *versus* the intact protein.

We assumed that Ab reactivity is determined by accessibility to the epitopes. We considered that the peptides, whether free in solution or coated on the plates, were fully exposed and reactive with the anti-peptide Abs. Since the peptide segments located on the surface of rpS6 might not be exposed completely, unlike the free peptide, it was expected that reactivity with rpS6 would be less than that with the peptide. In fact, in ELISA, r-rpS6 was ~100-fold less reactive than the peptides (data not shown). However, it is worth considering that the relative binding of Abs is a measure of the exposure of the segment on the surface. In this context, we surmised that the secondary structure of the segments did not significantly affect the reactivity of anti-peptide Ab, since Stanfield *et al*. (*9*) showed that the conformation of a peptide in complex with anti-peptide Ab was different from that in the intact protein; the former forming a type II β-turn, while the latter an α-helix.

We chose rpS6 as a model for the analysis of the protein–macromolecule interactions in living cells using Abs because it exists as a complex with other proteins and RNA as well as a major component of ribosome small subunit. An X-ray crystallographic study of rpS6 from *T. thermophilus* (*23*, *24*, *25*) showed that this protein contains four β-strands and two α-helices (Fig. 2B). The small (30S) subunit of the *E. coli* bacterial ribosome has been studied as a model for the ordered assembly of a large ribonucleoprotein complex (*26*). According to this model, rpS6 forms a heterodimer

with another ribosomal protein, S18, with a dissociation constant of <10 nM (*27*) and the S6-S18 heterodimer interacts with 16S RNA (*28*). It was expected that a large surface area was involved in these interactions (*23*).

S6-1 comprises the N-terminal region of rpS6. Since this region does not exist in rpS6 from bacteria such as *T. thermophilus* (Fig. 2), for which the tertiary structure was determined by X-ray crystallography (*23*), structural information was not yet available. We predicted that this region would be exposed on the protein surface *in vivo* on the basis of the reactivity of anti-S6-1 Abs using immunostaining of HeLa cells. However, anti-S6-1 Abs did not bind to r-rpS6 at substantial levels, suggesting that the S6-1 epitope is not exposed on the surface of r-rpS6. Such a disagreement between r-rpS6 and rpS6 in HeLa cells in terms of reactivity with anti-S6-1 Ab can be explained in terms of a conformational change. We expect that the N-terminal region of rpS6 is less exposed when free, but is exposed in HeLa cells due to an allosteric effect from interaction with other macromolecules. It is also possible that the His-tag introduced at the N-terminus causes steric hindrance in the reaction with anti-S6-1 Abs.

In *T. thermophilus*, S6-2 consists of a β-strand (β1) and an α-helix (α1). We expected that rpS6 in HeLa cells would bind to the anti-S6-2 Abs because the region containing the S6-2 epitope did not interact with other molecules such as RNA or S18 in *T. thermophilus* (*28*). However, the finding that anti-S6-2 Ab bound to r-rpS6 but not to rpS6 in HeLa cells suggested that human rpS6 interacts with other macromolecules *via* the segment containing the S6-2 epitope, or that the conformation of this portion changed after interaction with other macromolecules, even if the interaction occurred through segments other than S6-2.

The region containing the S6-4 epitope is homologous with respect to the amino acid sequences of various species (*24*, *25*). The corresponding region of *T. thermophilus* formed a β-strand (β4). The fact that the anti-S6-4 Abs reacted with both r-rpS6 and rpS6 in HeLa cells suggested that this epitope is exposed on the protein surface without taking part in interactions with other macromolecules. Among the anti-peptide Abs capable of reacting with r-rpS6, only anti-S6-4 reacted strongly with HeLa cell rpS6. It is of interest that no reaction with cytoplasmic proteins was observed with anti-S6-5 Abs, in spite of their strong reactivity to r-rpS6 (Fig. 6A). This was explicable in terms of steric hindrance resulting from interaction with macromolecules. We prepared Abs to S6-4 and S6-5 by immunization of three rabbits with the respective peptides and found that anti-S6-4 Abs reacted with r-rpS6 as well as with rpS6 in HeLa cells, while anti-S6-5 Abs failed to react with HeLa cell rpS6 (data not shown). Therefore, the specificity of these Abs did not depend on differences among individual animals. Abs such as anti-S6-5 cannot be used for cytostaining or immunoprecipitation experiments.

As described above, we were able to prepare highly reactive anti-S6-4 Ab capable of binding to human rpS6. Although all Abs possessed a similar level of reactivity to the respective peptides, they behaved differently with rpS6 protein in HeLa cells. Such variation in reactivity to proteins in living cells could not be explained in terms of affinity for the peptide or for the purified proteins, and seemed to depend on the extent of epitope exposure on the protein surface. Steric hindrance caused by the other components would affect interactions between anti-peptide Ab and epitope, suggesting that anti-peptide Abs can be used as tools for predicting sites of protein–macromolecule interactions in living cells. Even using anti-S6-4 Ab capable of reacting with rpS6 in cytoplasm, we could not detect it on the isolated chromosomes or in nucleus. The results suggest that rpS6 exists largely on the ER *in vivo* and attaches to chromosomes during the isolation process. This conclusion is consistent with our comparative proteome analysis of metaphase chromosomes isolated by three different methods, where rpS6 is largely dissociated from chromosomes upon thoroughly purification (*17*).

This study was supported by Special Coordination Funds of the Ministry of Education, Culture Sports, Sciences and Technology of the Japanese Government (to N.O., T.A. and K.F.).

REFERENCES

1. Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E., and Wilson, A.C. (1984) The antigenic structure of proteins: a reappraisal. *Ann. Rev. Immunol.* **2**, 67–101
2. Sachs, D.H., Schechter, A.N., Eastake, A., and Anfinsen, C.B. (1972) An immunological approach to the conformational equilibria of polypeptides. *Proc. Natl. Acad. Sci. USA* **69**, 3790–3794
3. Berzofsky, J.A., Buckenmeyer, G.K., Hicks, G., Gurd, F.R.N., Feldman, R.J., and Minna, J. (1982) Topographic antigenic determinants recognized by monoclonal antibodies to sperm whale myoglobin. *J. Biol. Chem.* **257**, 3189–3198
4. Geysen, H.M., Barteling, S.J., and Meloen, R.H. (1985) Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. *Proc. Natl. Acad. Sci. USA* **82**, 178–182
5. Tainer, J.A., Getzoff, E.D., Paterson, Y., Olson, A.J., and Lerner, R.A. (1985) The atomic mobility component of protein antigenicity. *Annu. Rev. Immunol.* **3**, 501–535
6. Roda, S.J., Geysen, H.M., Mason, T.J., and Schoofs, P.J. (1986) The antibody response to myoglobin. I. Systematic synthesis of myoglobin peptides reveals location and substructure of species-dependent continuous antigenic determinants. *Mol. Immunol.* **23**, 603–610
7. Berzofsky, I.A., Berkower, I.J., and Epstein, S.L. (1993) Antigen-antibody interaction and monoclonal antibodies. in *Fundamental Immunology* (Paul, W.E., ed.) pp. 421–425, Raven Press Ltd., New York
8. Getzoff, E.D., Tainer, J.A., Lerner, R.A., and Geysen, H.M. (1988) The chemistry and mechanism of antibody binding to protein antigens. *Adv. Immunol.* **43**, 1–98
9. Stanfield, R.L., Fieser, T.M., Lerner, R.A., and Wilson, I.A. (1990) Crystal structures of an antibody to a peptide and its complex with peptide antigen at 2.8 A. *Science* **248**, 712–719
10. Rini, J.M., Schulze-Gahmen, U., and Wilson, I.A. (1992) Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Science* **255**, 959–965

11. Ruvinsky, I. and Meyuhas, O. (2006) Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem. Sci.* **31**, 342–348
12. Thomas, G., Martin-Pérez, J., Siegmann, M., and Otto, A.M. (1982) The effect of serum, EGF, PGF2 alpha and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. *Cell* **30**, 235–242
13. Blenis, J., Spivack, J.G., and Erikson, R.L. (1984) Phorbol ester, serum, and rous sarcoma virus transforming gene product induce similar phosphorylations of ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **81**, 6408–6412
14. Rosen, O.M., Rubin, C.S., Cobb, M.H., and Smith, C.J. (1981) Insulin stimulates the phosphorylation of ribosomal protein S6 in a cell-free system derived from 3T3-L1 adipocytes. *J. Biol. Chem.* **256**, 3630–3633
15. Blenis, J. and Erikson, R.L. (1984) Phosphorylation of the ribosomal protein S6 is elevated in cells transformed by a variety of tumor viruses. *J. Virol.* **50**, 966–969
16. Thomas, G. (2002) The S6 kinase signaling pathway in the control of development and growth. *Biol. Res.* **35**, 305–313
17. Uchiyama, S., Kobayashu, S., Takata, H., Ishihara, T., Nori, N., Higashi, T., Hayashihara, K., Sone, T., Higo, D., Nirasawa, T., Takao, T., Matsunaga, S., and Fkui, K. (2005) Proteome analysis of human metaphase chromosomes. *J. Biol. Chem.* **280**, 16994–17004
18. Maeshima, K. and Laemmli, U.K. (2003) A two-step scaffolding model for mitotic chromosome assembly. *Dev. Cell* **4**, 467–480
19. Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S., Wagner, L., Shenmen, C.M., Schuler, G.D., Altschul, S.F., Zeeberg, B., Buetow, K.H., Schaefer, C.F., Bhat, N.K., Hopkins, R.F., Jordan, H., Moore, T., Max, S.I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A.A., Rubin, G.M., Hong, L., Stapleton, M., Soares, M.B., Bonaldo, M.F., Casavant, T.L., Scheetz, T.E., Brownstein, M.J., Usdin, T.B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S.S., Loquellano, N.A., Peters, G.J., Abramson, R.D., Mullahy, S.J., Bosak, S.A., McEwan, P.J., McKernan, K.J., Malek, J.A., Gunaratne, P.H., Richards, S., Worley, K.C., Hale, S., Garcia, A.M., Gay, L.J., Hulyk, S.W., Villalon, D.K., Muzny, D.M., Sodergren, E.J., Lu, X., Gibbs, R.A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A.C., Shevchenko, Y., Bouffard, G.G., Blakesley, R.W., Touchman, J.W., Green, E.D., Dickson, M.C., Rodriguez, A.C., Grimwood, J., Schmutz, J., Myers, R.M., Butterfield, Y.S.N., Krzywinski, M.I., Skalska, U., Smailus, D.E., Schnerch, A., Schein, J.E., Jones, S.J.M., and Marra, M.A. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. USA* **99**, 16899–16903
20. Watson, K.L., Konrad, K.D., Woods, D.F., and Bryant, P.J. (1992) Drosophila homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11302–11306
21. Leer, R.J., van Raamsdonk-Duin, M.M.C., Molenaar, C.M.T., Cohen, L.H., Mager, W.H., and Planta, R.J. (1982) The structure of the gene coding for the phosphorylated ribosomal protein S10 in yeast. *Nucleic Acids Res.* **10**, 5869–5878
22. Sayle, A. and Milner-White, E.J. (1995) RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **20**, 374–376
23. Brodersen, D.E., Clemons, W.M., Carter, A.P., Wimberly, B.T., and Ramakrishnan, V. (2002) Crystal structure of the 30S ribosomal subunit from *Thermus thermophilus*: structure of the proteins and their interactions with 16SRNA. *J. Mol. Biol.* **316**, 725–768
24. Sedelnikova, S.E., Agalarov, S.C., and Eliseikina, I.A. (1991) Crystals of protein S6 from the 30 S ribosomal subunit of *Thermus thermophilus*. *J. Mol. Biol.* **220**, 549–550
25. Lindahl, M., Svensson, L.A., Liljas, A., Sedelnikova, S.E., Eliseikina, I.A., Fomenkova, N.P., Nevskaya, N., Nikonov, S.V., Garber, M.B., Muranova, T.A., Rykonova, A.I., and Amons, R. (1994) Crystal structure of the ribosomal protein S6 from *Thermus thermophilus*. *EMBO J.* **13**, 1249–1254
26. Held, W.A., Ballou, B., Mizushima, S., and Nomura, M. (1974) Assembly mapping of 30S ribosomal proteins from *Escherichia coli*. Further studies. *J. Biol. Chem.* **249**, 3103–3111
27. Recht, M.I. and Williamson, J.R. (2001) Central domain assembly: thermodynamics and kinetics of S6 and S18 binding to an S15-RNA complex. *J. Mol. Biol.* **313**, 35–48
28. Agalarov, S.C., Sridhar Prasad, G., Funke, P.M., Stout, C.D., and Williamson, J.R. (2000) Structure of the S15,S6,S18-rRNA complex: assembly of the 30S ribosome central domain. *Science* **288**, 107–113