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Immunohistochemical Studies of Human Ribosomal Protein S3 (rpS3)

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The human ribosomal protein S3 (rpS3) was expressed in *E. coli* using the pET-15b vector and the monoclonal antibodies (mAbs) were produced and characterized. A total of five hybridoma cell lines were established and the antibodies recognized a single band of molecular weight of 33 kDa on immunoblot with purified rpS3. When the purified rpS3 was incubated with the mAbs, the UV endonuclease activity of rpS3 was inhibited up to a maximum of 49%. The binding affinity of mAbs to rpS3 determined by using a biosensor technology showed that they have similar binding affinities. Using the anti-rpS3 antibodies as probes, we investigated the cross-reactivities of various other mammalian brain tissues and cell lines, including human. The immunoreactive bands on Western blots appeared to be the same molecular mass of 33 kDa in all animal species tested. They also appear to be extensively cross-reactive among different organs in rat. These results demonstrated that only one type of immunologically similar rpS3 protein is present in all of the mammalian brain tissues including human. Furthermore, these antibodies were successfully applied in immunohistochemistry in order to detect rpS3 in the gerbil brain tissues. Among the various regions in the brain tissues, the rpS3 positive neurons were predominantly observed in the ependymal cells, hippocampus and substantia nigra pars compacta. The different distributions of rpS3 in brain tissues reply that rpS3 protein may play an important second function in the neuronal cells.

Keywords: Cross-reactivity, Human ribosomal protein S3 (rpS3), Immunohistochemistry, Monoclonal Antibodies (mAbs)

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Introduction

There is an increasing awareness of the importance of oxidative damage to DNA, which may be mediated primarily by highly reactive oxygen species (ROS) such as the free radical produced by ionizing radiation. These ROS are highly reactive and capable of producing a large array of base modifications of DNA. These modifications could have a role in mutagenesis, carcinogenesis, and aging (Hollstein *et al.*, 1984; Deutsch *et al.*, 1997). The attack that free radicals have on DNA is varied and profound; single- and double-strand breaks and the production of baseless sites in DNA are a common result of free radical damage to DNA (Lindahl, 1993). DNA repair represents a major pathway in any effort against free radical attack on DNA. The formation of damage to DNA is subject to repair by the base excision repair pathway, which is initiated by *N*-glycosylase that cleaves to the glycosylic bond between the sugar and the damaged base, resulting in an apurinic/apyrimidinic (AP) site. Most of the N-glycosylases that act on oxidatively damaged purines and pyrimidines also possess AP lyase activity (Doetsch and Cunningham, 1990; Hedge *et al.*, 2001).

Ribosomal protein S3 (rpS3) is one of the components of the 40S ribosomal small subunit of eukaryotic ribosomes that are cross-linked to the initiation factor eIF2 (Westermann *et al.*, 1979) and eIF3 (Tolan *et al.*, 1983). As rpS3 has a nuclear localization of signal in the N-terminal region, it is believed that the translation functions of rpS3 operate in the cytosol, and the repair function operates in the nucleus (Lee *et al.*, 2002). In addition to their role in ribosomal functions, many ribosomal proteins have secondary functions in replication, transcription, RNA processing, DNA repair, and malignant transformation (Wool, 1996). Human rpS3 gene is localized on the chromosome 11q13.3-q13.5, whose gene product is known to be involved in the initiation of translation (Polakiewicz *et al.*, 1995; Lee *et al.*, 2002; Lim *et al.*, 2002).

It has been reported that human, *Drosophila*, and rat rpS3

possess an AP lyase activity that cleave to abasic sites. Also, ribosomal proteins are in general highly conserved between human and rat (99%), compared to rat and yeast (60%), rat and eubacteria (27%), and rat and archaebacteria (34%) (Kim *et al.*, 1995; Wool *et al.*, 1995). Human rpS3 has an endonuclease activity included in the UV-damaged DNA repair. UV damaged DNA contains cyclobutane pyrimidine dimers, AP sites and other lesions such as thymine glycols functioning as a general base-damage endonuclease (Kim *et al.*, 1995; Kim *et al.*, 2005). Furthermore, human rpS3 has been shown to induce apoptosis in some cell lines (Jang *et al.*, 2004). Recently, the interaction with Hsp90 protects from ubiquitination and proteasome-dependent degradation of the protein (Kim *et al.*, 2006).

Recombinant rat rpS3 protein that is expressed in *E. coli* has an AP endonuclease activity as well. Both human and murine rpS3 have an associated AP endonuclease activity, which cleaves to the 3' side of the AP site via a β-lyase mechanism (Kim *et al.*, 1995). The yeast rpS3 proteins were reported to have an endonuclease activity on AP DNA. Also, *E. coli* endonuclease III has an AP lyase activity that is associated with DNA N-glycosylase activity for the damaged pyrimidine bases (Demple and Linn, 1980; Kim and Linn, 1988; Jung *et al.*, 2001). Skin is continuously exposed to many hazardous environmental agents including UV, ozone, and ionizing radiation. These hazardous agents are highly expressed in the development of skin cancer (Rhie *et al.*, 2001; Lim *et al.*, 2002). In addition, Pogue-Geile *et al.* (1991) demonstrated that in colorectal cancer, rpS3 mRNA were present at levels 4-10 fold higher than those found in the normal colon. However, it is not clear whether the higher mRNA levels are the result of increased transcription or decreased mRNA degradation.

In this report, we expressed human rpS3 protein and injected it as an immunogen into BALB/c mice to obtain monoclonal antibodies (mAbs). Several mAbs were produced from the fusion experiments. The mAbs, which specifically recognized rpS3 on Western blots, were characterized and used as probes for a cross-reactivity study. In addition, the expression of the rpS3 in the various regions of the brain was demonstrated by immunohistochemical analysis.

Materials and Methods

Materials. Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen. Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was purchased from Sigma. Fetal bovine serum (FBS), Basal medium Eagle, Dulbecco's Modified Eagles medium (DME), hypoxanthine-aminopterin-thymidine (HAT), and penicillin/streptomycin antibiotics were purchased from Gibco BRL. All other chemicals and reagents were of the highest analytical grade available.

Construction and purification of recombinant human rpS3. For pET-rpS3 an expression vector was constructed to express human rpS3. On the basis of the cDNA sequence of human rpS3, two oligonucleotides were synthesized. The forward primers, 5'-CTC GAGATGGCAGTGCAAATATCCAAGAAG-3', contained *Xho*I restriction sites and the reverse primers, 5'-GGATCCTTATGCTG-TGGGGACTGGCTGGGG-3', contained *Bam*HI restriction sites. The PCR reaction was performed in a thermal cycler (Perkin-Elmer, model 9600). The reaction mixture was made up in a 50 ml siliconized reaction tube and heated at 94°C for 5 min. The program for PCR consisted of 30 cycles of extension at 94°C for 1 min, denaturation at 55°C for 1 min, annealing at 72°C for 3 min, and the final extension at 72°C for 10 min. The PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into a TA cloning vector and then transformed into a DH5a competent cell. The plasmids of selected colonies were purified by using an alkaline lysis method (Sambrook *et al.*, 1989). The purified TA vector containing human rpS3 cDNA was digested with *Xho*I and *Bam*HI. The purified insert was ligated into a pET-15b expression vector, which vector had been digested with the same restriction enzymes. The host *E. coli* BL21 (DE3) was transformed with pET-rpS3 and then the transformants were selected on a LB plate containing ampicillin. The selected colonies were cultured in LB medium containing ampicillin. IPTG was added to the final concentration of 1 mM, and the incubation was continued for 3 h. The cells were harvested and a 5 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) was added and sonicated. After centrifugation (15,000 × *g*, 30 min), supernatants containing pET-rpS3 were immediately loaded onto a 2.5 ml Ni^{2+}-nitrilotriacetic acid sepharose column. After the column was washed with 10 volumes of a binding buffer and six volumes of a washing buffer (35 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), the proteins were eluted with an elution buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Purified proteins were combined and the salts were removed using PD-10 column chromatography. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

Production and purification of anti-human rpS3 mAbs. Production and purification of mAbs to the enzyme was performed as previously described (Choi *et al.*, 1996; Jang *et al.*, 2003; Eum *et al.*, 2005; Kim *et al.*, 2005). Briefly, the mAbs were produced by cell fusion after immunization of BALB/c mice with purified human rpS3. Also, Western blotting was performed according to the method of Towbin *et al.* (1979) using a produced human rpS3 mAbs.

Epitope mapping. One-dimensional epitope mapping was carried out according to a procedure previously described (Choi *et al.*, 1995). Ten micrograms of purified enzyme was mixed with an equal volume of *Staphyloccus aureus* V-8 protease solution (50 μg/ml in SDS sample buffer) and immediately applied onto a SDS-PAGE, and the separated peptides were transferred and immunoblotted as described above.

Immobilization and analysis of enzyme-rpS3 mAb interaction on BIAcore. Protein-protein interaction between monoclonal antibodies and rpS3 was performed using a Pharmacia Biosensor BIAcore instrument. CM5 research-grade sensor chips were used for all experiments. The indirectly oriented immobilization of antibodies on the CM5 sensor chip was carried out as follows. First, rabbit anti-mouse IgG Fc (ramfc) was coupled to the chip by injecting 100 ng of ramfc in 10 mM sodium acetate, pH 4.5, at a flow rate of 5 μl/min at 20°C. The carboxyl-methyl dextran matrix of the sensor chip was activated using a 30 μl (6 min) injection of a mixture of 0.2 M 1-ethyl-3-[(dimethylamino)propyl]carbodiimide and 0.05 M N-hydroxysuccinimide in water to convert the carboxyl group of the sensor chip matrix to an N-hydroxy- succinimide ester. This ester is susceptible to nucleophilic attack by amino groups of proteins, resulting in an amide linkage of the protein to the sensor chip. Under these conditions, typically 3,700 resonance units of ramfc were immobilized on the CM5 chip. The interactions of rpS3 with monoclonal antibodies were measured by two subsequent injections; the monoclonal antibodies were captured by ramfc, and followed by rpS3. Protein-protein interaction studies were carried out in HEPES-buffered saline (10 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20). Kinetic rate constants (k_{off} and k_{on}) and the equilibrium dissociation constants ($K_D = k_{off}/k_{on}$) were determined using the BIAlogue Kinetics Evaluation Software.

UV Endounclease assay. Plasmid pBluescript was used as a substrate for endonuclease activity. UV-irradiation, or non UV-irradiated plasmids were incubated with purified ribosomal protein. The whole reaction mixture (total 20 μl) contained 2 μl of the UV irradiated-, or unirradiated plasmid (0.1 mg) and recombinant ribosomal protein S3. The reaction mixture was incubated at 37°C for 1 h, and 3 μl of a loading dye that contained 1% SDS was added. This whole loading mixture was electrophoresed on a 1% agarose gel that contained 0.1 μg/ml of ethidium bromide, and the band pattern was observed under UV light.

Cell culture. Rat pheochromocytoma cell line PC12 and mouse macrophage cell line J774A1, human hepatoblastoma cell line HepG2, squamous cell carcinoma SiHa, and cervical adenocarcinoma cell line HeLa cells were obtained from Korea Cell Line Bank. All cell lines were grown according to the supplier's instruction.

Cross reactivities among mammalian brains including human and cell lines. Several animal brains from cow, pig, dog, cat, rat, and chicken were removed and homogenized in 10 mM potassium phosphate buffer that contained 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF. In the case of human brain, a small fragment of cerebral cortex was removed from 45-year old male who required surgery after an accident. The individual 25% (w/v) homogenates were centrifuged at 10,000 × *g* for 1 h. Five microliters of each supernatant were mixed with SDS sample buffer and boiled for 10 min. And then the samples were applied to SDS-PAGE and western blotting. Also, several cultured cells (PC12, J774A1, HepG2, SiHa, HeLa) were harvested for Western blotting.

Immunohistochemistry. This study used the progeny of Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experiment Animal Center, Hallym University. The animals were housed at constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light/dark cycle and free access to food and water. Procedures involving animals and their care conformed to the institutional guidelines, which are in compliance with current international laws and policies (NIH *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-23, 1985, revised 1996) and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee. Animals were anesthetized with pentobarbital sodium, and perfused via the ascending aorta with 200 ml of 4% paraformaldehyde in a phosphate buffer (PB). The brain were removed, postfixed in the same fixative for 4 h, and rinsed in PB that contained 30% sucrose at 4°C for 2 days. Thereafter the tissues were frozen and sectioned with a cryostat at 30 μm. Consective sections were collected in six-well plates that contained phosphate buffer saline (PBS). These free-floating sections were first incubated with 10% normal horse serum for 30 min at room temperature. They were then incubated in the mouse anti-rpS3 mAbs overnight at room temperature. After washing three times for 10 min with PBS, the sections were incubated sequentially in horse anti-mouse IgG and streptavidin. Between the incubations, the tissues were washed with PBS three times for 10 min each. The sections were visualized with 3,3'-diaminobenzidine (DAB) in a 0.1 M Tris buffer and mounted on gelatin-coated slides. The immunoreactions were observed under the Axioscope microscope.

Other methods. Discontinuous SDS-PAGE was carried out as described by Laemmli (1970). Spectrophotometric measurements were carried out with a Kontron model UVIKON 930 double-beam spectrophotometer.

Results and Discussion

It is well known that ribosomal proteins are the major component of ribosome. These proteins are synthesized in the cytoplasm and transferred to the nucleus. In the nucleus, the ribosomal proteins assemble with processed rRNA and form two ribosomal subunits, 40S or 60S. These are exported to cytoplasm and participate in protein synthesis (Warner, 1989). In *Drosophila*, rpS3 revealed an endonuclease activity that cleaves to AP sites (Wilson *et al*., 1994), glycosylase activity (Deutsch *et al*., 1997), and dRpase (deoxyribo phosphodiesterase) activity (Sandigursky *et al*., 1997). These activities imply that rpS3 may be involved in the DNA repair process. Since the multifunctional activity of rpS3 protein, various human diseases including skin cancer, colorectal cancer, etc are related to this protein. In the present study, we produced five hybridoma cell lines to the human rpS3 protein and characterized it by peptide mapping, immuno-reactivity test and immunohistochemical analysis.

Functional expression of human rpS3 protein. In order to produce the human rpS3 mAbs, we have the cloned and constructed recombinant human rpS3. To develop an expression system to overexpress and simply purify the

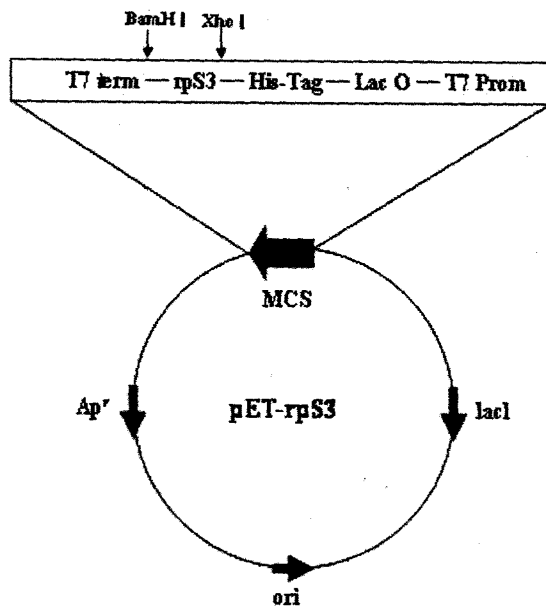

Fig. 1. Construction of recombinant human rpS3 expression vector system based on the vector pET-15b. Synthetic oligomers were cloned into *Xho*I, *Bam*HI sites and human rpS3 cDNA was cloned into *Xho*I and *Bam*HI sites of pET-15b.

human rpS3 protein, we constructed the pET-human rpS3 expression vector (pET-rpS3), which contains consecutive cDNA sequence encoding the human rpS3 and six histidine residues at the amino-terminus (Fig. 1).

Expression and purification of the recombinant human rpS3 was performed as described in the **Materials and Methods** section. In brief, following the induction of expression, the human rpS3 protein was purified by Ni^{2+}-affinity chromatography. The purification result is shown in Fig. 2A. The purified proteins were found to be nearly homogeneous as determined by a SDS-PAGE analysis with Coomassie Brilliant blue staining. The recombinant human rpS3 proteins have an estimated molecular mass of 33 kDa. Several reports showed that the ribosomal proteins from various eukaryotes are expressed at very low levels in *E. coli*. This event was probably caused by the codon bias because of the ribosomal proteins high content of arginine codons in eukaryotic ribosomal proteins, which are inadequately utilized by bacterial translational machinery (Dieci *et al*., 2000; Jung *et al*., 2001). In our experimental system, human rpS3 was highly expressed when the pET-15b expression vector was used and the resulting purified recombinant human rpS3 protein molecular weight is about 33 kDa (Fig. 2A). A similar observation reported that the recombinant human rpS3 protein molecular weight is 33 kDa (Lee *et al*., 2002).

Production of mAbs. We previously identified the recombinant human rpS3 as a 33 kDa protein and exhibited a single protein band on a SDS-PAGE. To enhance the immunogenicity of the protein and obtain antibodies with a better reactivity on

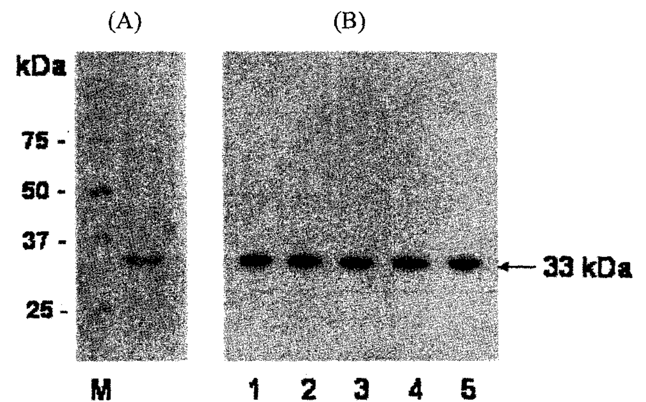

Fig. 2. SDS-PAGE of purified rpS3 (A) and corresponding immunoblots (B) of purified human rpS3 probed with representative human rpS3 mAbs: lane 1, rpS3-1; lane 2, rpS3-2; lane 3, rpS3-3; lane 4, rpS3-4; lane 5, rpS3-5. All of them recognized the purified human rpS3.

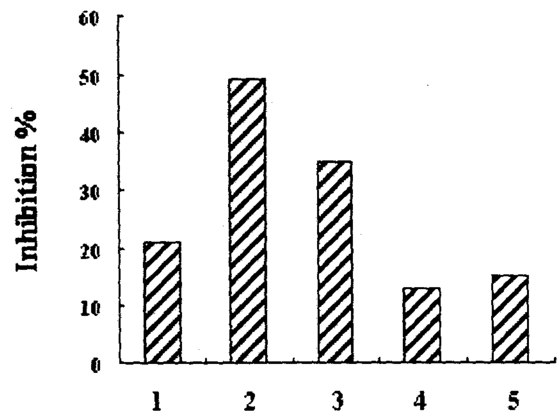

Fig. 3. Inhibition of UV endonuclease activity by the purified mAbs. Lane 1, rpS3-1; lane 2, rpS3-2; lane 3, rpS3-3; lane 4, rpS3-4; lane 5, rpS3-5. One μg of purified human rpS3 was incubated with purified mAbs (10 μg in 90 μl PBS) for 1 h at room temperature and enzyme activity determined as described in **Materials and Methods**.

Western blot, the purified protein was denatured by the addition of SDS (final concentration 0.1%) and heating. Then the denatured immunogen was injected into 6~8 weeks old BALB/c mice intraperitoneally. From two fusion experiments, 25 positive clones were initially screened by immunodot-blot analysis. Because goat anti-mouse IgG antibody was used as a second antibody, all mAbs screened by the procedure are IgG classes. Among the hybridoma, some of the hybridoma clones either lost the ability to produce mAbs continuously or produced mAbs reacting with the antigen weakly on the immunoblot. Thus we selected the 5 mAbs showing strong reactivity for further study. The immunoreactivities of 5 mAbs with purified recombinant human rpS3 are shown in Fig. 2B.

Inhibition of mAb on UV endonuclease activity of rpS3 protein. Human rpS3 has an identical amino acid sequence to

Table 1. Interaction of anti-rpS3 monoclonal antibody with rpS3

mAbs	Association rate constant (k_{on}) (M^{-1} s^{-1})	Dissociation rate constant (k_{off}) (s^{-1})	Equilibrium dissociation constant (K_D) (nM)
rpS3-1	5.1×10^4	5.3×10^{-5}	1.0 ± 0.12
rpS3-2	5.0×10^4	5.2×10^{-5}	1.0 ± 0.15
rpS3-3	4.8×10^4	5.3×10^{-5}	1.1 ± 0.18
rpS3-4	4.9×10^4	5.2×10^{-5}	1.1 ± 0.22
rpS3-5	3.9×10^4	5.1×10^{-5}	1.3 ± 0.18

Results are the averages of two separate experiments, with the error expressed as the range of the two data sets.

UV endonuclease III, which activity cleaves DNA lesions caused by UV irradiation. Recombinant murine rpS3 protein that is expressed in *E. coli* has an AP endonuclease activity as well. Both human and murine rpS3 have an associated apurinic/apyrimidine (AP) endonuclease activity, which cleaves to the 3' side of the AP site via a β-lyase mechanism (Kim *et al.*, 1995). The incubation of samples of purified rpS3 protein clones of mAb inhibited UV endonuclease activity and the extent of this inhibition reached a maximum of approximately 49% (Fig. 3). This inhibitory effect of mAb on rpS3 protein indicates that the antibody may bind at or near the active site of the protein and presumably inhibit the UV endonuclease reaction. Alternatively, the inhibition may be a result of an immoblilization of the protein brought about by the binding of the antibodies to epitopes remote from the active site.

Epitope mapping. The different immunoreactivities of the anti-rpS3 mAbs with the rpS3 protein were further examined by an epitope mapping analysis with V-8 protease. The rpS3 was digested with V-8 protease and immunoblotted with anti-rpS3 mAbs. The five mAbs can be assigned to two different groups according to their reactivities in the epitope mapping. RpS3-1, rpS3-2, and rpS3-3 displayed a similar band patterns, indicating that these antibodies recognize the same or very similar epitopes. However, the mAbs rpS3-4 and rpS3-5 showed somewhat different band pattern (data not shown).

Protein-protein interactions of anti-rpS3 monoclonal antibodies with rpS3. To compare further the antigenic reactivities of anti-rpS3 monoclonal antibodies with rpS3 quantitatively, we examined the interactions directly in the Pharmacia BIAcore. The equilibrium binding constant between antigen and antibody can be measured in a variety of ways, as long as the complex can be separated from free ligand once the reaction has reached equilibrium. However, few methods allow the analysis of the interaction in real time and thus the determination of kinetic rate constants. The biosensor technology uses the optimal phenomenon of surface plasmon resonance to monitor the interaction of an immobilized ligand to a protein in the flow solution that is passed over it (Fagerstam *et al.*, 1992; Malmqvist, 1993). By using methods described above, k_{on} and k_{off} values were calculated for rpS3 mAbs. Each measurement was done at least twice and up to four times on different surfaces. The results of kinetic experiments are summarized in Table 1. The binding affinities of anti-rpS3 mAbs for rpS3 were similar each other. All mAbs showed a very slow dissociation, almost close to the limit of the machine (~1×10^{-5} s^{-1}), and there were no differences in dissociation rate constant (k_{off}) among the rpS3 mAbs. These results indicate that the molecular recognition processes of each mAbs to rpS3 showed almost same kinetic parameters.

Cross-reactivity of mAbs among mammalian including human and avian species. In order to confirm the cross-reactivity of the mAbs with the mammalian and avian rpS3 proteins, brain tissues from a human, cow, pig, dog, cat, rat, and chicken were removed and the total proteins of the brain homogenates were separated on SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the mAbs. The immunoreactive bands on western blot appeared as protein bands of the same molecular mass, 33 kDa, in all animals tested even though the band intensities were somewhat different each other (Fig. 4A). These data indicated that only one type of rpS3 protein is present in all tissues tested, and that all of the reactive proteins are immunologically similar even though the expression level is different.

It is well known that the rpS3 is an extremely well conserved ribosomal protein. Human rpS3 gene is composed six introns and seven exons like *Xenoppus* and *Fugu* homologus (Pellizzoni *et al.*, 1994; Crosio *et al.*, 1996). Several studies investigated the homology of amino acid sequence between vertebrate, invertebrate, plant, bacteria and that of the human rpS3 protein. In vertebrate, the 99% and 94% homology were observed between mouse, pufferfish and human, respectively. While the invertebrate homologs show a somewhat lower conservation, with identity percentage from 85% (*D. virilis*) to 65% (*S. cerevisiae*) and the identity between the bacterium (*H. halobium*) and human rpS3 is the lowest with 32% identity. The less conserved part of rpS3 is the C-terminal end of the protein, and the sequences of this part is determined the rate of identity between various species and human (Chan *et al.*, 1996; Lim *et al.*, 2002; Lyamouri *et al.*, 2002).

Cross-reactivity of mAbs among various mammalian cell lines and rat organs. To examine the cross-reactivity of

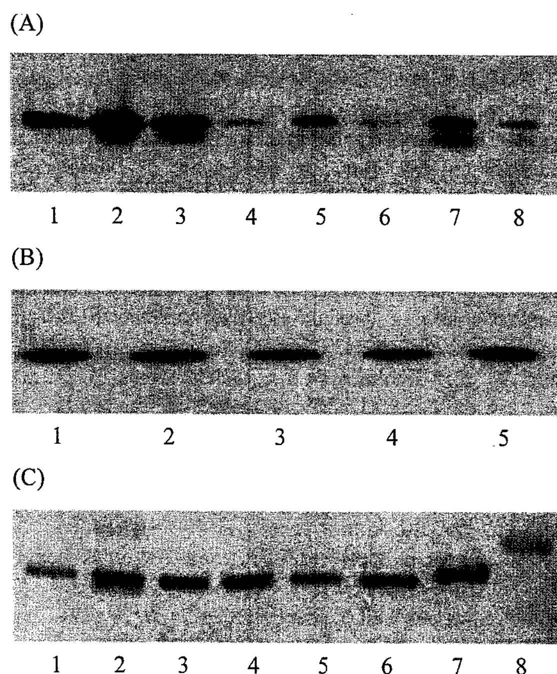

Fig. 4. Cross-reactivities of rpS3 from mammalian and an avian species (A), several cell lines (B), and various rat tissues (C) with mAbs made against human rpS3: A. lane 1, human; lane 2, cow; lane 3, pig; lane 4, dog; lane 5, cat; lane 6 rat; lane 7, chicken lane 8, rabbit. Animal brains were removed, and total proteins of the brain homogenates were immunoblotted with human rpS3 mAbs. B. lane 1, HeLa cells; lane 2, PC 12 cells; lane 3, J774A1 cells; lane 4, SiHa cells; lane 5, HepG2 cells. C. lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, stomach; lane 6, lung; lane 7, testes; lane 8, skeletal muscle. Total proteins were extracted and immunoblotted with human rpS3 mAbs.

mAbs among different organs, we prepared several mammalian cell lines and rat organs. The various cultured cell lines (HeLa, J774A1, PC12, SiHa, HepG2) were harvested and total proteins were extracted and immunoblotted with the mAbs, respectively. As shown in Fig. 4B, the mAbs all recognized a protein band of the same molecular mass of 33 kDa on the immunoblot.

In addition, in order to examine the cross-reactivity of the mAbs with various rat organs, we prepared several rat tissues (brain, liver, kidney, heart, stomach, lung, testes and skeletal muscle). Total proteins from the several tissues of the rat were extracted and immunoblotted with mAbs. As shown in Fig. 4C, the mAbs also recognized the same protein band on the immunoblot, except for skeletal muscle. The recognition of a protein band with a molecular mass of about 33 kDa in skeletal muscle indicates that it may be the premature form or isoprotein type. The results we obtained are consistent with other observations that the human rpS3 protein is highly conserved among several tissues including human.

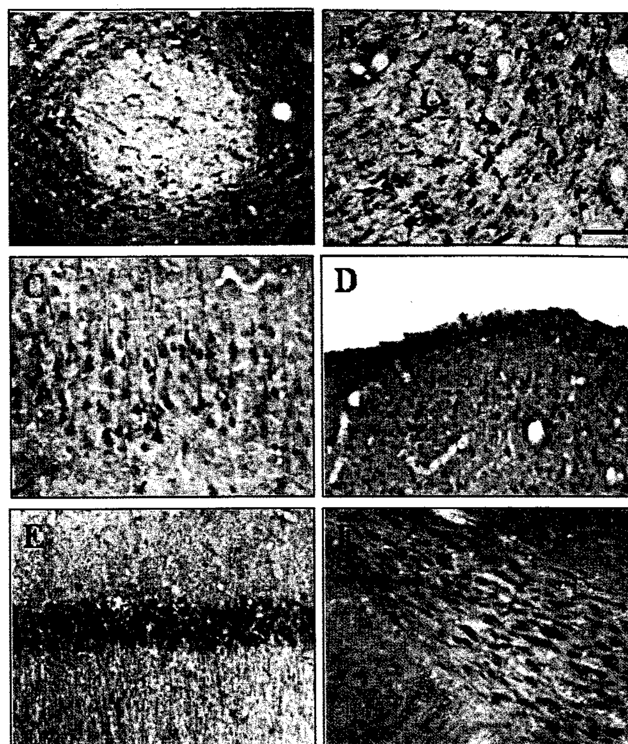

Fig. 5. The distribution of rpS3 in various regions of gerbil brains. (A) Amygdala; (B) Diagonal band; (C) Cerebral cortex; (D) Ependymal cell; (E) Hippocampus CA1 area; (F) Substantia nigra pars compacta. Bar = 50 μm.

Immunohistochemistry. The mAbs were applied in immunohistochemical studies on gerbil-brain slices. The rpS3 immunoreactive neurons were widespread in various regions of the brain; amygdala, diagonal band, cerebral cortex, ependyma, hippocampus, and substantia nigra pars compacta (Fig. 5). Among the various regions in the brain tissues, the rpS3 positive neurons were predominantly observed in the ependymal cells, hippocampus and substantia nigra pars compacta. Recently, several studies showed that environmental risk factors including ROS increased risk for Parkinson's diseases in substantia nigra pars compacta (Liou *et al.*, 1997; Gorell *et al.*, 1998; McCormack *et al.*, 2002). Particularly, our previous studies provided evidence for the occurrence of oxidative stress and oxidative DNA damage is related to cerebral ischemia in hippocampus (Won *et al.*, 1999; 2001). Thus, the results we obtained implied that rpS3 might have secondary function in the brain and further study is needed to confirm the role of this protein relative to various neurological diseases.

In summary, we described the production and characterization of the first complete set of high-affinity monoclonal antibodies against human rpS3. Since antibodies enable the specific and sensitive detection of rpS3 protein in tissues, thus they could be used for the quantitative evaluation of the expression level

of the protein, Furthermore, the availability of an inexhaustible supply of homogenous antibodies could greatly benefit studies on the physiological role of the rpS3 protein in brain metabolism. This supply of homogenous antibodies would also benefit other biochemical studies, such as studies regarding immuno-affinity purification as well as monitoring diagnosis kit for rpS3-related diseases. Furthermore the immunohistochemical analysis in the brain tissues will give the valuable informations to identify secondary function of rpS3 related to the neuronal diseases such as brain ischemia.

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References

Bradford, M. A. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Chan, Y. L., Olivera, J., Paz, V. and Wool, I. G (1996) The primary structure of rat ribosomal protein S3a and of S3b. *Biochem. Biophys. Res. Commun.* **228**, 141-147.

Choi, E. Y., Jang, S. H., and Choi, S. Y. (1996) Human brain GABA transaminase is immunologically distinct from those of other mammalian brain. *Neurochem. Int.* **28**, 597-600.

Choi, E. Y., Park, S. Y., Jang, S. H., Song, M. S., Cho, S. W. and Choi, S. Y. (1995) Production and characterization of monoclonal antibodies to bovine brain succinic semialdehyde reductase. *J. Neurochem.* **64**, 371-377.

Crosio, C., Cecconi, F., Mariottini, P., Cesareni, G., Brenner, S. and Amaldi, F. (1996) *Fugu* intron oversize reveals the presence of U15 snoRNA coding sequences in some introns of the ribosomal protein S3 gene. *Genome Res.* **6**, 1227-1231.

Demple, B. and Linn, S. (1980) DNA N-glycosylase and UV repair. *Nature* **287**, 203-208.

Deutsch, W. A., Yacoub, A., Jaruga, P., Zastawny, T. H. and Dizdaroglu, M. (1997) Characterization and mechanism of action of *Drosophila* ribosomal protein S3 DNA glycosylase activity for the removal oxidatively damaged DNA base. *J. Biol. Chem.* **272**, 32857-32860.

Dieci, G, Bottarelli, L., Ballabeni, A. and Ottonello, S. (2000) t-RNA assisted overproduction of eukaryotic ribosomal proteins. *Prot. Exp. Pur.* **18**, 346-354.

Doetsch, P. W. and Cunningham, R. P. (1990) The enzymology of apurinic/apyrimidinic endonucleases. *Mutat. Res.* **236**, 173-201.

Eum, W. S., Choi, H. S., Kim, D. W., Jang, S. H., Choi, S. H., Kim, S. Y., Park, J., Kang, J. H., Cho, S. W., Kwon, O. S., Hwang, I. K., Yoo, K. Y., Kang, T. C., Won, M. H. and Choi, S. Y. (2005) Production and characterization of monoclonal antibodies against human ceruloplasmin. *J. Biochem. Mol. Biol.* **38**, 71-76.

Fagerstam, L. G, Karlsson, A. F., Karlsson, R., Persson, B. and Ronnberg, I. (1992) Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis. *J. Chromatogr.* **597**, 397-410.

Gorell, J. M., Johnson, C. C., Rybicki, B. A., Peterson, E. L. and Richardson, R. J. (1998) The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurol.* **50**, 1346-1350.

Hedge, V., Kelley, M. R., Xu, Y., Mian, S. and Deutsch, W. A. (2001) Conversion of the bifunctional 8-oxoguanine/b-d apurine/apyrimidine DNA repair activaties of Drosophila ribosomal protein S3 into the human S3 monofunctional β-elimination catalyst through a single amino acid change. *J. Biol. Chem.* **276**, 27591-27596.

Hollstein, M. C., Brooks, P., Linn, S. and Ames, B. R. (1984) Hydroxymethyluracil DNA glycosylase in mammalian cells. *Biochemistry* **81**, 4003-4007.

Jang, C. Y., Lee, J. Y. and Kim, J. (2004) RpS3, a DNA repair endonuclease and ribosomal protein, is involved in apoptosis. *FEBS Lett.* **560**, 81-85.

Jang, S. H., Kim, A. Y., Bahn, J. H., Eum, W. S., Kim, D. W., Park, J., Lee, K. S., Kang, T. C., Won, M. H., Kang, J. H., Kwon, O. S., Yoon, H. Y., Lee, E. Y., Cho, S. W. and Choi, S. Y. (2003) Human glutamate dehydrogenase is immunologically distinct from other mammalian orthologus. *Exp. Mol. Med.* **35**, 249-256.

Jung, S., Lee, J. and Kim, J. (2001) Yeast ribosomal protein S3 has an endonuclease activity on AP DNA. *Mol. Cells* **12**, 84-90.

Kim, D. W., Eum, W. S., Choi, H. S., Kim, S. Y., An, J. J., Lee, S. H., Sohn, E. J., Hwang, S. I., Kwon, O. S., Kang, T. C., Won, M. H., Cho, S. W., Lee, K. S., Park, J. and Choi, S. Y. (2005) Human brain pyridoxal-5'-phosphate phosphatase: production and characterization of monoclonal antibodies. *J. Biochem. Mol. Biol.* **38**, 703-708.

Kim, J., Chubatsu, L. S., Admon, A., Stahl, J., Fellous, R. and Linn, S. (1995) Implication of mammalian ribosomal protein S3 in the processing of DNA damage. *J. Biol. Chem.* **270**, 13620-13629.

Kim, J. and Linn, S. (1988) The mechanisms of action of *E. coli* endonuclease and T4 UV endonuclease (endonuclease) at AP sites. *Nucleic Acids Res.* **16**, 1135-1141.

Kim, S. H., Lee, J. Y. and Kim, J. (2005) Characterization of a wide range base-damage-endonuclease activity of mammalian rpS3. Biochem. *Biophys. Res. Commun.* **328**, 962-967.

Kim, T. S., Jang, C. Y., Kim, H. D., Lee, J. Y., Ahy, B. Y. and Kim, J. (2006) Interaction of Hsp90 with ribosomal proteins protects from ubiquitination and proteasome-dependent degradation. *Mol. Biol. Cell.* **17**, 824-833.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, C. H., Kim, S. H., Choi, J. I., Choi, J. Y., Lee, C. E. and Kim, J. (2002) Electron paramagnetic resonance study reveals a putative iron-sulfur cluster in human rpS3 protein. *Mol. Cells* **13**, 154-156.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* **362**, 709-715.

Lim, Y., Lee, S. M., Kim, M., Lee, J. Y., Moon, E. P., Lee, B. J. and Kim, J. (2002) Complete genomic structure of human rpS3: identification of functional U15b snoRNA in the fifth intron. *Gene* **286**, 291-297.

Liou, H. H., Tsai, M. C., Chen, C. J., Jeng, J. S., Chang, Y. C., Chen, S. Y. and Chen, R. C. (1997) Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. *Neurol.* **48**, 1583-1588.

Lyamouri, M., Enerly, E., Kress, H. and Lambertsson, A. (2002) Conservation of gene order, structure and sequence between three closely linked genes in *Drosophila melanogaster* and *Drosophilar virilis*. *Gene* **282**, 199-206.

Malmqvist, M. (1993) Biospecific interaction analysis using biosensor technology. *Nature* **361**, 186-187.

McCormack, A. L., Thiruchelvam, M., Manning-Bog, A. B., Thiffault, C., Langston, J. W., Cory-Slechta, D. A. and Di Monte, D. A. (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurol. Dis.* **10**, 119-127.

Pellizzoni, L., Crosio, C., Campioni, N., Loreni, F. and Pierandrei-Amaldi, P. (1994) Different forms of U15 snoRNA are encoded in the introns of the ribosomal protein S1 gene of Xenopus laevis. *Nucleic Acids Res.* **22**, 4607-4613.

Pogue-Geile, K., Geiser, J. R., Shu, M., Miller, C., Wool, I. R., Meisler, A. I. and Pipas, J. M. (1991) Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. *Mol. Cell. Biol.* **11**, 3842-3849 .

Polakiewicz, R. D., Munroe, D. J., Sait, S. N., Tycowski, K. T., Nowak, N. J., Shows, T. B., Housman, D. E. and Page, D. C. (1995) Mapping of ribosomal protein S3 and internally nested snoRNA U15A gene to human chromosome 11q13.3-q13.5. *Genomics* **25**, 577-580.

Rhie, G. E., Seo, J. Y. and Chung, J. H. (2001) Modulation of catalase in human skin in vivo by acute and chronic UV radiation. *Mol. Cells* **11**, 399-404.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Labolatory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbar, New York, USA.

Sandigursky, M., Yacoub, A., Kelly, M. R., Deutsch, W. A. and Franklin, W. A. (1997) The *Drosophila* ribosomal protein S3 contains a DNA deoxyribophosphodiesterase (dRpase) activity. *J. Biol. Chem.* **272**, 17480-17484.

Tolan, D. R., Hershey, J. W. and Traut, R. T. (1983) Crosslinking of eukaryotic initiation factor eIF-3 to the 40S ribosomal subunit from rabbit reticulocytes. *Biochimie* **65**, 427-436.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Warner, J. R. (1989) Synthesis of ribosomes in *Sacchromyces cerevisiae. Microbiol. Rev.* **53**, 256-271.

Westermann, P., Heumann, W., Bommer, U. A., Bielka, H., Nygard, O. and Hultin, T. (1979) Crosslinking of initiation factor eIF-2 to proteins of the small subunit of rat liver ribosomes. *FEBS Lett.* **97**, 101-104.

Willson, D. M., Deutsch, W. A. and Kelly, M. R. (1994) Drosophila ribosomal protein S3 contains an activity that cleaves DNA at apurinic/apyrimidine sites. *J. Biol. Chem.* **269**, 25359-25364.

Won, M. H., Kang, T. C., Jeon, G. S., Lee, J. C., Kim, D. Y., Choi, E. M., Lee, K. H., Choi, C. D., Chung, M. H. and Cho, S. S. (1999) Immunohistochemical detection of oxidant DNA damage induced by ischemia-reperfusion insults in gerbil hippocampus *in vivo*. *Brain Res.* **836**, 70-78.

Won, M. H., Kang, T. C., Park, S. K., Jeon, G. S., Kim, Y. W., Seo, J. H., Choi, E. M., Chung, M. H. and Cho, S. S. (2001) The alteration of N-methyl-D-aspartate receptor expression and oxidative DNA damage in the CA1 area at the early time after ischemia-repurfusion insult. *Neurosci. Lett.* **301**, 139-142.

Wool, I. G., Chan, Y. L. and Gluck, A. (1995) Structure and evolution of mammalian ribosomal proteins. *Biochem. Cell Biol.* **73**, 933-947.

Wool, I. G. (1996) Extraribosomal functions of ribosomal proteins. *Trends Biochem. Sci.* **21**, 164-165.