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Ribosomal protein S3 is secreted as a homodimer in cancer cells

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ABSTRACT

Protein secretion is a general phenomenon by which cells communicate with the extracellular environment. Secretory proteins, including hormones, enzymes, toxins, and antimicrobial peptides have various functions in extracellular environments. Here, we determined that ribosomal protein S3 (rpS3) is homodimerized and secreted in several cancer cell lines such as HT1080 (human fibrosarcoma) and MPC11 (mouse plasmacytoma). Moreover, we found that the secreted rpS3 protein increased in doxorubicin-resistant MPC11 cells compared to that in MPC11 cells. In addition, we also detected that the level of secreted rpS3 increased in more malignant cells, which were established with continuous exposure of cigarette smoke condensate. These findings suggest that the secreted rpS3 protein is an indicator of malignant tumors.

1. Introduction

Most secreted proteins are made on ribosomes attached to the rough endoplasmic reticulum (ER). These secretory proteins generally contain signal peptides in their N-terminus that are recognized and bound by a signal recognition particle (SRP). The secretory protein-SRP complex binds to the SRP receptor on the ER membrane to be transferred into the ER lumen. Then, proteins destined for secretion are exported outside of the cell using the ER/Golgi-dependent secretory pathway. However, it has been recently documented that some proteins can be exported outside of cells without using the ER/Golgi-dependent secretory pathway.

It has been rarely reported that ribosomal proteins are secreted into the extracellular environment. For example, the rpS27 protein, which is called metallopanstimulin-1, increases in the sera of patients with several cancers. Additionally, Yamamoto et al. [1–3] showed that homodimerized rpS19 increases monocyte migration through an interaction with the C5a receptor (the complement C5-derived leukocyte chemotactic factor) on monocytes. Robert et al. [4] also showed that *Escherichia coli* 50S ribosomal protein L7/L12 is present as four copies organized as two dimers in the ribosome, and that dimers of these proteins support protein synthesis.

Ribosomal protein S3 (rpS3) plays a key role in protein translation as a ribosomal component. rpS3 possesses various extraribosomal functions such as DNA repair [5–10], cell signaling [11–14], apoptosis/survival [15], host-pathogen interactions [16,17] and transcriptional regulation [18–20]. In our previous study [12], we discovered that rpS3 interacts with nm23-H1, which acts as a suppressor of metastasis in certain human tumors and as a poor prognostic indicator against various hematologic malignancies. Additionally, we confirmed that rpS3 inhibits secretion of matrix metalloproteinase-9 (MMP-9), and that the interaction between rpS3 and nm23-H1 is critical for the invasiveness of HT1080 cells. Additionally, leucine 190 of rpS3 and histidine 118 and serine 120 of nm23-H1 are key residues for interaction of these proteins.

In this study, we determined that rpS3 is secreted into the extracellular environment, and that the secreted rpS3 is homodimerized. We also demonstrated that secreted rpS3 is intimately associated with tumor malignancy in several cancer cell lines [21].

2. Materials and methods

2.1. Cell culture

Human fibrosarcoma HT1080 or mouse plasmacytoma (MPC11) cells were maintained in DMEM (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (Thermo Scientific) and grown at 37 °C in a humidified atmosphere of 5% CO_2. MPC11 cells were cultured in serial increasing concentrations of doxorubicin for 45 days to establish the doxorubicin-resistant

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MPC11 subline. The doxorubicin-resistant MPC11 subline was cultured in a doxorubicin-free medium before experiments.

2.2. Protein precipitation

The cells were incubated with serum free media for 24 h to confirm secretion and dimerization of rpS3. After the incubation, media were collected and concentrated by ultrafiltration in an Amicon Ultra-15 Centrifugal Filter Device (Millipore, Billerica, MA, USA). Concentrated media were subjected to Western blotting to detect appropriate proteins.

2.3. In vitro binding assay

The complete coding region of rpS3 was inserted into pGEX5X-1 (Amersham Bioscience, Piscataway, NJ, USA) or pET21a as described previously [22,23]. Glutathione-S-transferase (GST), GST-fused rpS3 (GST-rpS3), and His-tagged rpS3 (His-rpS3) were expressed in *E. coli* BL21 for the GST pull-down assay. The expressed proteins were then purified using with glutathione–Sepharose (GSH)-4B beads (Amersham Pharmacia, Uppsala, Sweden) and Ni–NTA-agarose resin (Qiagen, Valencia, CA, USA). GSH-4B bead-immobilized GST or GST-rpS3 proteins were incubated for 12 h at 4 °C with purified His-tagged rpS3 proteins for the *in vitro* binding assay. Then, the co-precipitates were washed three times in lysis buffer. The co-precipitates were boiled in 2× sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose (PVDF) membrane, and immunoblotted using anti-His antibody.

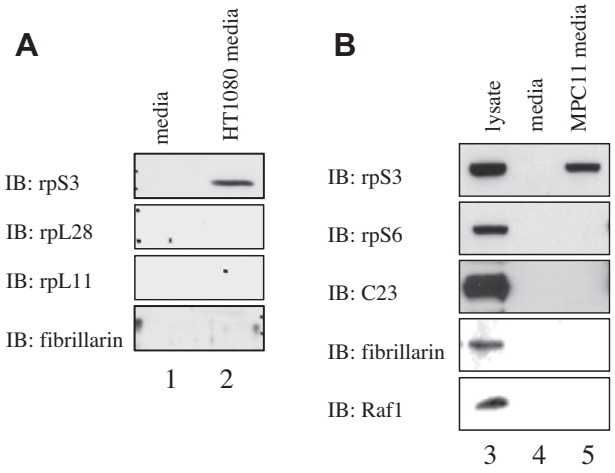

Fig. 1. The ribosomal protein S3 (rpS3) protein is secreted into cell culture medium. HT1080 (A) or MPC11 (B) cells were incubated in serum-free media for 24 h and then the cell culture media were collected and concentrated with an Amicon filter. Cell lysates (lanes 1 and 3), non-cell culture media (lane 4), and the concentrated cell culture media (lanes 2 and 5) were immunoblotted with specific antibodies. C23, fibrillarin, and Raf1 antibodies were used to confirm cell death (C23 as a nucleolar protein, fibrillarin as a nuclear protein, Raf-1 as a cytoplasmic protein).

2.4. Acidic-native gel electrophoresis

To verify rpS3 dimerization, purified His-tagged rpS3 from the medium was subjected to electrophoresis with an acidic-native gel (1.5 M acetate–KOH pH 4.3, 50% glycerol, 30% acrylamide, 0.8% methylene bis-acrylamide, 10% ammonium persurfate, TEMED as separating gel; 0.25 M acetate–KOH [pH 6.8], 30%

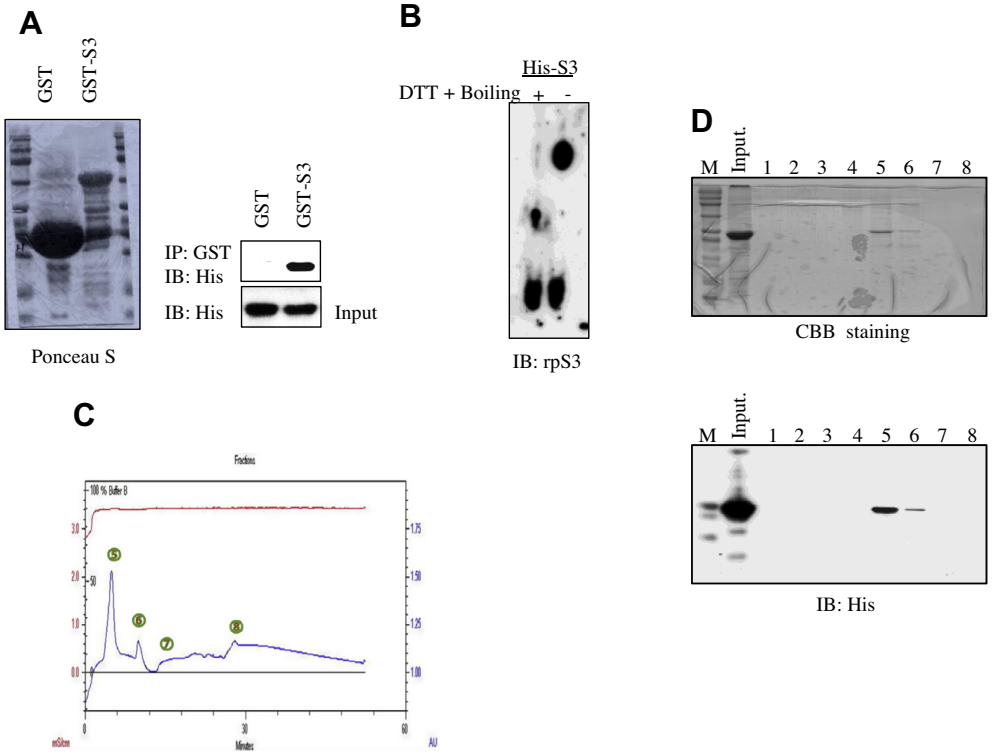

Fig. 2. Ribosomal protein S3 (rpS3) proteins are homodimerized *in vitro*. (A) The GST and GST-fused rpS3 proteins were bound in glutathione–Sepharose 4B beads, and the immobilized proteins were incubated with purified His-tagged rpS3 protein. After extensive washing in PBS, the co-precipitants were prepared for Ponceau-S staining (left panel) and immunoblotting (right panel). (B) The purified His-tagged rpS3 was subjected to acidic native gel electrophoresis with or without DTT and boiling. (C and D) The His-tagged rpS3 was subjected to size exclusion column chromatography. Two peaks (numbers 5 and 6) are indicated by the UV-detector (Nos. 1–4 are not shown), and both peaks were collected for immunoblot analysis. M; protein marker, Input; 1 μg of the purified His-tagged rpS3.

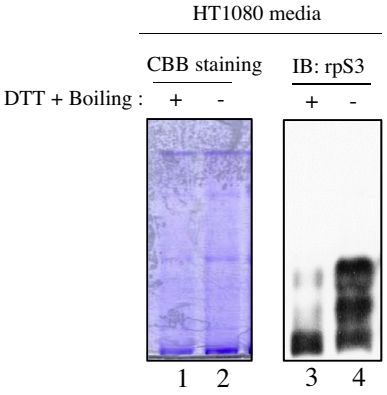

Fig. 3. The secreted ribosomal protein S3 (rpS3) protein is homodimerized. HT1080 cells were incubated in serum-free media for 24 h, and the cell culture media were collected and concentrated. The concentrated media were subjected to acidic native gel electrophoresis with (lanes 1 and 3) or without (lanes 2 and 4) DTT and boiling. Then the separated proteins underwent Coomassie Brilliant Blue (CBB) staining (left panel) and an immunoblot analysis (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acrylamide, 0.8% methylene bis-acrylamide, 10% ammonium persurfate, TEMED as a stacking gel; 0.35 M β-alanine, 0.14 M acetic acid, 600 ml H_2O as running buffer [pH 4.3]; 50% glycerol, 0.25 M acetate-KOH [pH 6.8] as dissolving buffer). The separated samples were transferred to a PVDF membrane and immunoblotted or stained with Coomassie Brilliant Blue (CBB).

2.5. Size-exclusion column chromatography

The purified His-tagged rpS3 protein (25 μg) was analyzed by liquid chromatography. The chromatography system used for the analysis of proteins consisted of a Superdex 200 10/300 GL column (GE Healthcare). First, chromatography was performed by adding dH_2O to remove the equilibration buffer (20 mM Tris–HCl, 50 mM NaCl, pH 8.0) to equilibrate the column and adding the elution buffer for His-purification (20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4) at room temperature. The purified His-rpS3 protein was loaded on the column, and the eluted protein was measured at 260 nm with a UV spectrophotometer equipped with Bio-Rad DuoFlow (Hercules, CA, USA). Each fraction was collected and underwent CBB staining or immunoblotting.

3. Results and discussion

3.1. RpS3 protein is secreted into cell culture media

Both HT1080 and MPC11 (mouse plasmacytoma) cells were cultured in serum-depleted media for 24 h, and then the culture media were collected and precipitated to investigate whether the rpS3 protein was secreted into the cell culture media. The level of rpS3 protein with other ribosomal proteins was quantified (Fig. 1A and B). Interestingly, the rpS3 protein was only detected in the culture media of HT1080 cells or MPC11 cells. Additionally, fibrillarin (Fig. 1A and B) and C23 (Fig. 1B) as nuclear markers and Raf-1 as a cytoplasmic marker were not detected in the cell culture media. Therefore, secretion of rpS3 was not due to cell death.

3.2. RpS3 protein is homodimerized in vitro

Secreted proteins maintain stability in various ways including dimerization. To investigate whether the rpS3 protein was homodimerized, the recombinant His-tagged and GST-fused rpS3 proteins were used in the GST pull-down assay (Fig. 2A). As shown in Fig. 2A, the immobilized GST-fused rpS3 was co-precipitated with His-tagged rpS3. Next, the purified His-tagged rpS3 protein was separated using native gel electrophoresis (Fig. 2B). His-tagged rpS3 boiled with dithiothreitol was located at the site corresponding to their monomeric size, whereas the untreated sample generated a newly sized protein that was presumably a dimeric form of His-tagged rpS3. Size exclusion chromatography was performed to determine whether the newly sized His-tagged rpS3 was homodimeric, (Fig. 2C and D). Here, the His-tagged rpS3 protein was separated as two peaks from a size exclusion chromatography column (Fig. 2C). Subsequently, each peak was collected and subjected to SDS–PAGE. CBB and Western blot analysis revealed that the rpS3 protein could be another size besides a monomer.

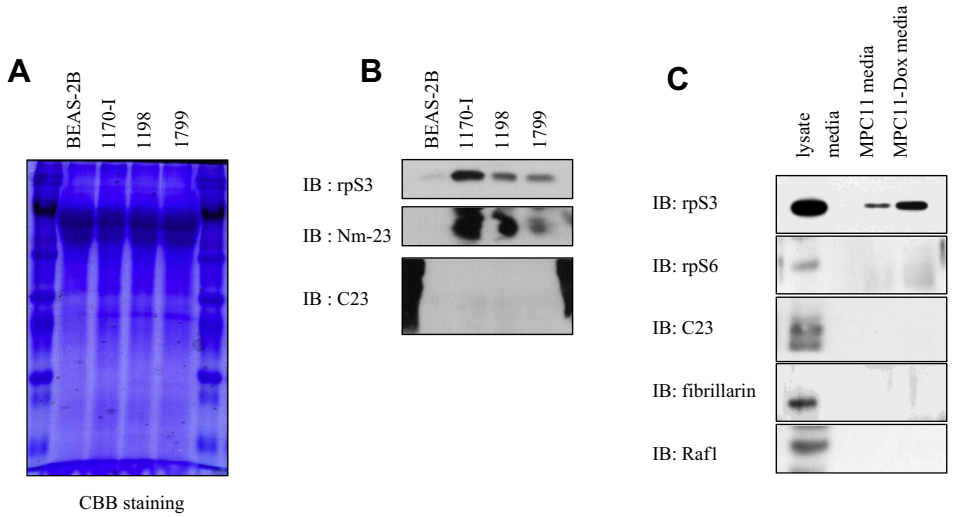

Fig. 4. The level of secreted ribosomal protein S3 (rpS3) protein increases in malignant cells. (A and B) Cell culture media of each cell line derived from BEAS-2B cells were collected and concentrated, and subjected to Coomassie Brilliant Blue (CBB) staining (A) and an immunoblot analysis (B) (BEAS-2B: immortalized normal, 1170-I: tumorigenic, 1198: transformed but non-tumorigenic, 1799: non-transformed). Nm23-H1 was a representative secreted protein, and C23 was used as a cell death marker. (C) The cell culture media of MPC11 and MPC11-Dox cells were collected and concentrated, and were subjected to immunoblot analysis using the indicated antibodies. Non-cell culture media were used as a negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. The secreted rpS3 protein is homodimerized

Acidic-native gel electrophoresis was performed with concentrated cell culture media to further examine whether the secreted rpS3 from HT1080 cells is dimerized. Subsequently, an immunoblot analysis showed that multiple bands appeared in the non-denatuing sample differently from the denatured sample (Fig. 3). Combined with the previous data, this finding suggests that rpS3 is homodimerized and secreted into the extracellular fluid.

3.4. The level of secreted rpS3 protein increases in malignant cells

HT1080 cells are a well known malignant fibrosarcoma cell line that secretes a number of proteins including MMP-2, MMP-9, and NM23-H1. To clarify whether the secretion of rpS3 is implicated in tumor malignancy, we used a series of special tumor cell lines whose malignancy is well established with continuous exposure to cigarette smoke condensates. The cell lines are derived from a single immortalized human normal bronchial epithelial cell line, BEAS-2B (Fig. 4A and B), and their malignancy can be classified as follows: non-transformed (1799), transformed but non-tumorigenic (1198), and tumorigenic (1170-I) cell lines. As shown in Fig. 4B, secretion of rpS3 increased dramatically in 1170-I cells compared to that of other cells similar to NM23-H1. Additionally, 1198 or 1799 cells showed moderately increased secretion of rpS3 than that of BEAS-2B. A doxorubicin-resistant MPC-11 cell line was generated to examine the relationship between secreted rpS3 and tumor malignancy. The level of secreted rpS3 in doxorubicin-resistant MPC-11 cells increased significantly compared to that in MPC-11 parent cells (Fig. 4C). Taken together, these results suggest that rpS3 secretion is closely related with tumor malignancy.

Collectively, we demonstrated that malignant cells release rpS3 protein and that the secreted rpS3 is homodimerized in the extracellular environment. Based on our findings, the level of secreted rpS3 may serve as a blood marker for tumor malignancy. Additionally, further investigation of the rpS3 secretion pathway may contribute to the development of a diagnostic marker for malignant tumors.

Disclosure of potential conflicts of interest

No potential conflict of interest are disclosed.

Acknowledgements

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References

[1] Y. Shibuya, M. Shiokawa, H. Nishiura, T. Nishimura, N. Nishino, H. Okabe, K. Takagi, T. Yamamoto, Identification of receptor-binding sites of monocyte chemotactic S19 ribosomal protein dimer, Am. J. Pathol. 159 (2001) 2293–2301.

[2] H. Nishiura, Y. Shibuya, T. Yamamoto, S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a, Laboratory investigation, J. Tech. Methods Pathol. 78 (1998) 1615–1623.

[3] K. Horino, H. Nishiura, T. Ohsako, Y. Shibuya, T. Hiraoka, N. Kitamura, T. Yamamoto, A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells, Laboratory investigation, J. Tech. Methods Pathol. 78 (1998) 603–617.

[4] A.V. Oleinikov, G.G. Jokhadze, R.R. Traut, A single-headed dimer of *Escherichia coli* ribosomal protein L7/L12 supports protein synthesis, Proc. Natl. Acad. Sci. USA 95 (1998) 4215–4218.

[5] T.S. Kim, H.D. Kim, J. Kim, PKCdelta-dependent functional switch of rpS3 between translation and DNA repair, Biochim. Biophys. Acta 1793 (2009) 395–405.

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

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

[8] V. Hegde, M. Wang, W.A. Deutsch, Human ribosomal protein S3 interacts with DNA base excision repair proteins hAPE/Ref-1 and hOGG1, Biochemistry 43 (2004) 14211–14217.

[9] K.M. Seong, S.O. Jung, H.D. Kim, H.J. Kim, Y.J. Jung, S.Y. Choi, J. Kim, Yeast ribosomal protein S3 possesses a beta-lyase activity on damaged DNA, FEBS Lett. 586 (2012) 356–361.

[10] Y. Kim, H.D. Kim, J. Kim, Cytoplasmic ribosomal protein S3 (rpS3) plays a pivotal role in mitochondrial DNA damage surveillance, Biochim. Biophys. Acta 1833 (2013) 2943–2952, http://dx.doi.org/10.1016/j.bbamcr.2013.07.015 [Epub ahead of print].

[11] H.D. Kim, T.S. Kim, J. Kim, Aberrant ribosome biogenesis activates c-Myc and ASK1 pathways resulting in p53-dependent G1 arrest, Oncogene 30 (2011) 3317–3327.

[12] S.H. Kim, J. Kim, Reduction of invasion in human fibrosarcoma cells by ribosomal protein S3 in conjunction with Nm23-H1 and ERK, Biochim. Biophys. Acta 1763 (2006) 823–832.

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

[14] Y.J. Joo, J.H. Kim, U.B. Kang, M.H. Yu, J. Kim, Gcn4p-mediated transcriptional repression of ribosomal protein genes under amino-acid starvation, EMBO J. 30 (2011) 859–872.

[15] N. Sen, B.D. Paul, M.M. Gadalla, A.K. Mustafa, T. Sen, R. Xu, S. Kim, S.H. Snyder, Hydrogen sulfide-linked sulfhydration of NF-kappaB mediates its antiapoptotic actions, Mol. Cell 45 (2012) 13–24.

[16] F. Wan, A. Weaver, X. Gao, M. Bern, P.R. Hardwidge, M.J. Lenardo, IKKbeta phosphorylation regulates RPS3 nuclear translocation and NF-kappaB function during infection with *Escherichia coli* strain O157:H7, Nat. Immunol. 12 (2011) 335–343.

[17] X. Gao, F. Wan, K. Mateo, E. Callegari, D. Wang, W. Deng, J. Puente, F. Li, M.S. Chaussee, B.B. Finlay, M.J. Lenardo, P.R. Hardwidge, Bacterial effector binding to ribosomal protein s3 subverts NF-kappaB function, PLoS Pathog. 5 (2009) e1000708.

[18] F. Wan, D.E. Anderson, R.A. Barnitz, A. Snow, N. Bidere, L. Zheng, V. Hegde, L.T. Lam, L.M. Staudt, D. Levens, W.A. Deutsch, M.J. Lenardo, Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation, Cell 131 (2007) 927–939.

[19] S. Yadavilli, L.D. Mayo, M. Higgins, S. Lain, V. Hegde, W.A. Deutsch, Ribosomal protein S3: a multi-functional protein that interacts with both p53 and MDM2 through its KH domain, DNA Repair 8 (2009) 1215–1224.

[20] E.M. Wier, J. Neighoff, X. Sun, K. Fu, F. Wan, Identification of an N-terminal truncation of the NF-kappaB p65 subunit that specifically modulates ribosomal protein S3-dependent NF-kappaB gene expression, J. Biol. Chem. 287 (2012) 43019–43029.

[21] J.E. Kim, K.H. Koo, Y.H. Kim, J. Sohn, Y.G. Park, Identification of potential lung cancer biomarkers using an *in vitro* carcinogenesis model, Exp. Mol. Med. 40 (2008) 709–720.

[22] H.D. Kim, T.S. Kim, Y.J. Joo, H.S. Shin, S.H. Kim, C.Y. Jang, C.E. Lee, J. Kim, RpS3 translation is repressed by interaction with its own mRNA, J. Cell. Biochem. 110 (2010) 294–303.

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