Stable accumulation of human ribosomal proteins SA, S2, S3, S4X, S6, S7 in rabbit reticulocyte cell-free expression system

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FIRE: THE FIRST-YEAR INNOVATION & RESEARCH EXPERIENCE

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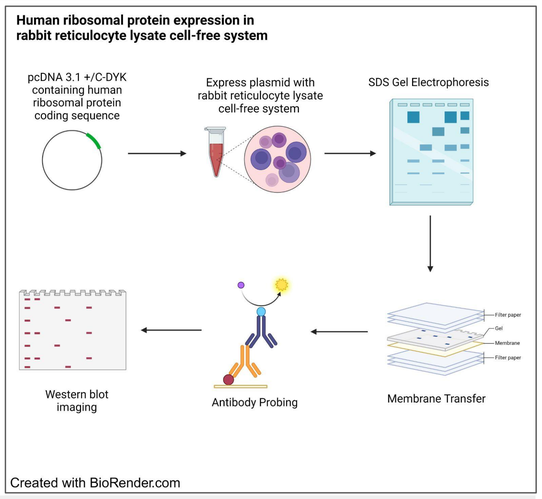
Introduction

- Ribosomal Proteins (RPs) are the main building blocks of the ribosome.
- While the structure and function of human RPs after ribosomal subunit assembly have been studied, the post-translational modifications (PTMs) that these individual RPs undergo before ribosomal subunit assembly have not been well characterized.
- Human RPs have functions in ribosome biogenesis, translation, and extra-ribosomal regulatory functions. Mutations in individual RPs have been shown to cause tissue-specific pathologies, which indicates that RPs may be involved in other roles outside the context of the ribosome[1].
- Using rabbit reticulocyte lysate (RRL) to express human RPs, allows for expression in a mammalian system, which gives a more accurate analysis of the PTMs of RPs. The RRL system also has implications in specifically studying Diamond Blackfan Anemia (DBA), which has been linked to several different mutations in different RPs[2].
- The rabbit reticulocyte lysate system is a cell-free system that is naturally enucleated during red blood cell maturation[3], but it still contains all of the components necessary for protein expression. Using this system, we are proposing that we can observe individual ribosomal proteins in the stage before they have been assembled the the nucleus. Without a nucleus present to facilitate ribosomal subunit assembly, we aim to determine the different PTMs and protein-protein interactions that allow for stability of individual human RPs in the cytoplasm of a cell-free system.
- Regarding the PTMs on RPs, previous site mapping has shown that some RPs are ubiquitinated and/or O-GlcNAcylated[4,5]. To determine the effect of ubiquitin and O-GlcNAc pathways on the expression and stability of these human RPs in the RRL system, we added MG132, a proteasome inhibitor, and TMG, an O-GlcNAcase inhibitor, to reactions with specific RPs to assess expression when these inhibitors were present.

Methods

- To express RPs in the RRL cell-free system, the pcDNA3.1/C-DYK plasmid was used with the ribosomal coding sequence already inserted. This cell-free system naturally expels its nucleus during red blood cell maturation[3], however the system maintains components of the nucleus that are necessary for protein synthesis, which allows for the observation of possible cytosolic PTMs influencing RP stability.
- After assembling the components of the cell-free system, Western blot analysis was performed via denaturing gel electrophoresis using 4-12% or 10% SDS-PAGE gels.
- Immunoprecipitations were also performed using anti-FLAG magnetic beads to amplify the signal of the ribosomal proteins, as well as to confirm the presence of the individual RPs and their PTMs.



Results

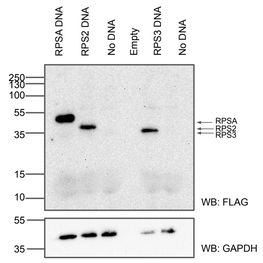


Figure 1: Coupled transcription/translation of pcDNA3.1/C-(K)DYK-RPSA, pcDNA3.1/C-(K)DYK-RPS2, and pcDNA3.1/C-(K)DYK-RPS3, and a no DNA control for RPSA and RPS2, and a no DNA control for RPS3 in rabbit reticulocyte lysate. Reactions were separated by denaturing electrophoresis and then subjected to Western blot with anti-FLAG antibody (upper panel). Arrows indicate the presence of the fusion proteins of ribosomal protein with FLAG. The predicted molecular weight of RPSA is 33.9 kDa, 32.3 kDa for RPS2, and 27.7kDa for RPS3.

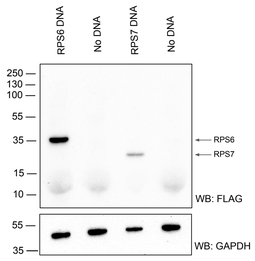


Figure 2: Coupled transcription/translation of pcDNA3.1+/C-(K)DYK-RPS6, pcDNA3.1+/C-(K)DYK-RPS7, or no DNA control in rabbit reticulocyte lysate. Reactions were separated by denaturing electrophoresis, and subjected to Western blot with anti-FLAG antibody (upper panel). Blots were stripped and reprobed with anti-GAPDH antibody for loading control (lower panel). Arrow indicates the presence of the fusion protein RPS6-FLAG with a predicted molecular weight of 29.7 kDa. Arrowhead indicates the presence of the fusion protein RPS7-FLAG with a predicted molecular weight of 23.1 kDa.

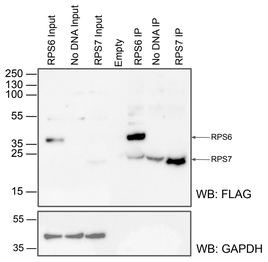


Figure 3: Coupled transcription/translation of pcDNA3.1+/C-(K)DYK-RPS6, pcDNA3.1+/C-(K)DYK-RPS7, or no DNA control in rabbit reticulocyte lysate, and performed immunoprecipitation for all 3 reactions. SDS-PAGE gel electrophoresis performed to separate reactions in lysate and IP samples, and western blot analysis using anti-FLAG antibody, seen in upper panel. Blots were stripped and reprobed with anti-GAPDH antibody for loading control, shown in lower panel. Arrows indicate presence of fusion protein RPS6-FLAG samples, with a predicted molecular weight of 29.7 kDa, and RPS7-FLAG in samples with a predicted molecular weight of 23.1 kDa.

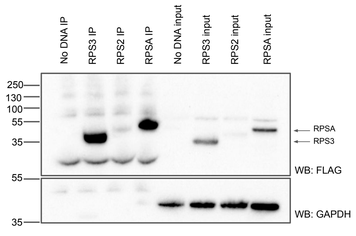


Figure 4: Coupled transcription/translation of pcDNA3.1/C-(K)DYK-RPSA, pcDNA3.1/C-(K)DYK-RPS2, pcDNA3.1/C-(K)DYK-RPS3, and a no DNA control in rabbit reticulocyte lysate with immunoprecipitation (IP) for each reaction completed in the lysate. Reactions were separated by denaturing electrophoresis and then subjected to Western Blot with anti-FLAG antibody (upper panel). Blots were then stripped and re-probed with anti-GAPDH antibody for loading control (lower panel). Arrows indicate the presence of the fusion proteins of RPSA and RPS3 with FLAG. Comparing IPs to inputs shows enrichment of the RPs.

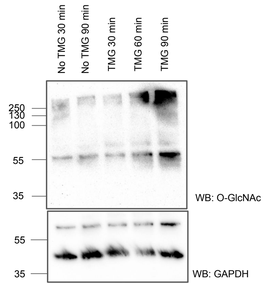


Figure 5: Expression of O-GlcNAcylated proteins in rabbit reticulocyte cell-free system with and without TMG inhibitor at different incubation times. Reactions were separated by denaturing electrophoresis, and subjected to Western blot with anti-O-GlcNAc antibody (upper panel). Blots were stripped and reprobed with anti-GAPDH antibody for loading control (lower panel).

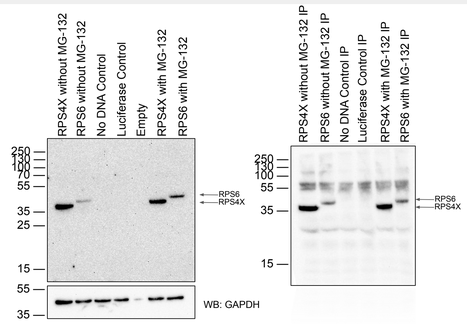


Figure 6: Coupled transcription/translation of pcDNA3.1+/C-(K)DYK-RPS4X, pcDNA3.1+/C-(K)DYK-RPS6, no DNA negative control, and Luciferase positive control in rabbit reticulocyte lysate. MG-132 proteasome inhibitor at 1X concentration was added to RPS4X and S6 samples in right two lanes. Immunoprecipitation was performed for all reactions and lysates (left) and IP (right) samples were run on separate gels. Reactions were separated by denaturing gel electrophoresis, and subjected to Western blot with anti-FLAG antibody (upper panels). Blot for lysates was stripped and reprobed with anti-GAPDH antibody for loading control (lower left panel). Arrows indicate the presence of the fusion protein RPS4X-FLAG with a predicted molecular weight of 30.6 kDa, and the presence of RPS6 with a predicted molecular weight of 29.7 kDa.

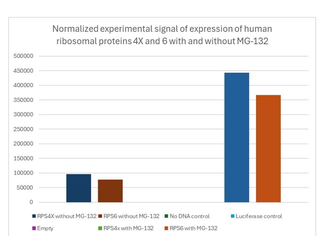


Figure 12: Normalized expression of RPS4X and S6 based on results from Figure 6 for coupled transcription/translation of human ribosomal proteins in rabbit reticulocyte system with and without addition of MG-132. Graph compares signal from each lane to the corresponding GAPDH loading control to normalize signal and compare levels of expression.

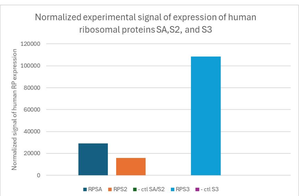


Figure 10: Normalized expression of RPSA,S2 and S3 based on results from Figure 1 above for coupled transcription/translation of human ribosomal proteins in rabbit reticulocyte system. Graph compares signal from each lane to corresponding GAPDH loading control to normalize signal and compare levels of expression.

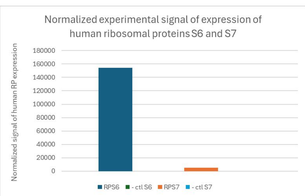


Figure 11: Normalized expression of RPS6 and S7 based on results from Figure 2 above for coupled transcription/translation of human ribosomal proteins in rabbit reticulocyte system. Graph compares signal from each lane to the corresponding GAPDH loading control to normalize signal and compare levels of expression.

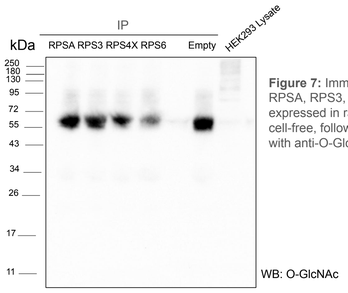


Figure 7: Immunoprecipitation of RPSA, RPS3, RPS4X, and RPS6 expressed in rabbit reticulocyte cell-free, followed by Western blot with anti-O-GlcNAc antibody.

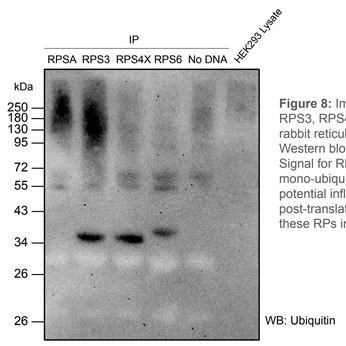


Figure 8: Immunoprecipitation of RPSA, RPS3, RPS4X, and RPS6 expressed in rabbit reticulocyte cell-free, followed by Western blot with anti-Ubiquitin antibody. Signal for RPS3, S4X, and S6 indicate a mono-ubiquitination event occurring, showing potential influence of ubiquitin post-translational modification on stability of these RPs in a cell-free system.

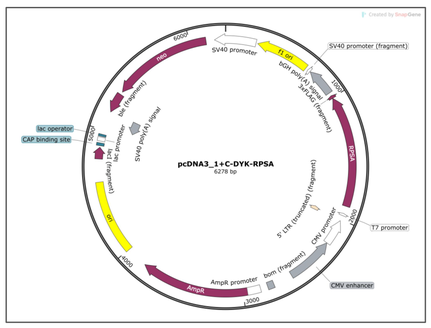


Figure 9: Sequence map of mammalian expression vector pcDNA3.1+C-DYK-RPSA, encoding the fusion protein of the ribosomal protein coding sequence and a 3xFLAG tag. The FLAG tag adds about 1 kDa to the molecular weight of the expressed ribosomal proteins. This map is identical for the rest of the ribosomal protein, S2,S3,S4x,S6,and S7, except for the individualized coding sequences for each ribosomal protein.

Discussion

- Based on our results, ribosomal proteins SA,S2,S3,S4X,and S6 have been shown to undergo a shift of about 8-10 kDa higher than their the predicted molecular weights. This shift is indicative of a possible monoubiquitination of the proteins since other post-translational modifications, even when coexisting at all potential target sites, are too small to cause such a large shift in observed mass.
- We propose that a single mono-ubiquitination (or ubiquitin-like) event promotes the stability of individual human RPs, consistent with recent findings[6]. The question of whether this ubiquitination occurs only in the cytoplasm of cells requires further investigation.
- We also see that each of the RPs tested have different levels of expression in this reticulocyte system. It was expected that if these proteins all had the same function in the ribosome, the proteins would all express at the same intensity. However, this is not the case; they are expressing at different levels, which is indicative of differential stability which might be related to the functions outside the context of the ribosome.
- In future directions, we will analyze the interplay between ubiquitin and other PTMs, such as O-GlcNAcylation, on each of the human RPs before their assembly into subunits, with an emphasis on blood cell lineage development.

Human RPs Expressed in RRL	Predicted Molecular Weight*	Observed Molecular Weight
RPSA	33.9 kDa	About 46 kDa
RPS2	32.3 kDa	About 40 kDa
RPS3	27.7 kDa	About 37 kDa
RPS4x	30.6 kDa	About 38 kDa
RPS6	29.7 kDa	About 36 kDa
RPS7	23.1 kDa	About 25 kDa

* Data from uniprot.org

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