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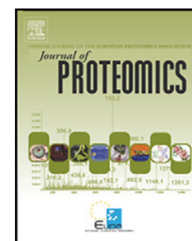

Identification of human, rat and chicken ribosomal proteins by a combination of two-dimensional polyacrylamide gel electrophoresis and mass spectrometry

Anh Thu Nguyen-Lefebvre[a], *Sandrine Gonin-Giraud*[a], *Alexander Scherl*[b], *Patrizia Arboit*[b], *Laure Granger*[a], *Jean-Charles Sanchez*[b], *Jean-Jacques Diaz*[a], *Olivier Gandrillon*[a,*], *Jean-Jacques Madjar*[a]

[a]*Université de Lyon, F-69622, Lyon, France, Université Lyon 1, Villeurbanne, CNRS, UMR5534, Centre de Génétique Moléculaire et Cellulaire, France*
[b]*Biomedical Proteomics Research Group, Department of Structural Biology and Bioinformatics, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland*

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ABSTRACT

To identify the exact spot position of human, rat and chicken ribosomal proteins (RP) separated by two-dimensional polyacrylamide gel electrophoresis (2-DE), a 2-DE system was designed to separate RP with a pI > 8.6 according to their charge in the first dimension and to their molecular mass in the second dimension. Individual proteins were excised from the gels and identified by mass spectrometry after digestion by trypsin. In addition, a mixture of purified RP from these three species was also analyzed by tandem mass tag spectrometry. By combining those two methods 74 RP from human, 76 from rat and 67 from chicken were identified according to the nomenclature initially defined for rat liver RP and by using the Swiss-Prot/trEMBL databases. Whereas human and rat RP were well described, most of RP from chicken were not characterized in databases, since 35 out of 67 chicken RP identified in this study were not listed yet. We propose here the first comprehensive description of chicken RP and their comparison to those from human and rat.

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1. Introduction

Ribosomes are large ribonucleoprotein complexes that provide an accurate structure for mRNA translation and protein synthesis. They were first described in 1955 as dense particles or granules in the cytoplasm of eukaryotic cells observed by electron microscopy [1]. The eukaryotic ribosome contains four ribosomal RNA (rRNA) and about 80 ribosomal proteins (RP) [2]. This complex exhibits a molecular mass of about 4 MDa and a sedimentation coefficient of 80S. The ribosome is composed of two subunits that are evolutionary conserved throughout the living world, a small subunit which is involved in decoding of the mRNA and a large subunit that harbours the peptidyl transferase center buried in the rRNA [3]. In eukaryotes, the 40S small subunit is composed of only one 18S rRNA of 1,900 nucleotides and about 30 RP, whereas the 60S large subunit contains three rRNA (5S, 5.8S and 28S) of 120, 160 and 4,700 nucleotides respectively, and about 50 RP. Eukaryotic RP, like prokaryotic RP, exhibit small molecular masses (from 3.4 kDa to 47.7 kDa) and most of them are extremely basic [4]. Their relationship to each other as well as to the rRNA was unambiguously described when the atomic architecture of

* *Corresponding author.*
E-mail address: olivier.gandrillon@univ-lyon1.fr (O. Gandrillon).

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the prokaryotic ribosome was revealed by X-ray crystallography [5 and original references herein].

In the seventies, eukaryotic RP have been extensively analyzed using different kinds of two-dimensional polyacrylamide gel electrophoresis systems (2-DE). In these systems, RP were numbered according to their electrophoretic mobility in both dimensions and different nomenclatures were thus proposed rendering these analyses difficult to exploit between different laboratories. Following a protocol initially designed for prokaryotic ribosomal proteins [6], Sherton and Wool proposed the first nomenclature for rat liver RP after separation of the small and large ribosomal subunits. Briefly after extraction, RP were separated according to their electric charge in the first dimension at pH 8.6 in an 8% polyacrylamide gel, then in the second dimension at pH 4.5 in an 18% polyacrylamide gel, both gels containing urea. Based on the nomenclature initially defined for rat liver RP in this electrophoretic system [7], a uniform nomenclature for mammalian RP was then proposed [8] according to the similarities in 2-DE migration of RP originating from different species. In addition, after cloning and sequencing of the first cDNA of mouse RP mRNA [9], databases were filled up with a considerable amount of new RP cDNA sequences of a large number of species. Due to the highly conserved sequences of RP, the nomenclature originally adopted for mammalian RP was thus extended to other eukaryotic species. However, at present the validation by mass spectrometry of the identity of RP associated to their names deposited in databases and separated in previously published 2-DE gels is still missing. Furthermore the fact that there is no 2-DE gels available that could be easily set up in many laboratories to separate RP remains a strong limitation for the analyses of ribosomal protein composition.

We report here the validated spot position of human, rat and chicken RP by 2-DE, according to the name of RP deposited in the databases. For this, we designed a 2-DE system in which basic RP were separated according to their charge at pH 8.6 in the first dimension and to their molecular mass in the second dimension in a slab gel, the composition of which is derived from that originally described by Laemmli [10] that is widely used in most laboratories. Proteins extracted from the gels were identified by mass spectrometry for all three species. To complete this identification, purified RP were also analyzed in a quantitative manner using the isobaric tagging technology [11] (TMT) allowing to identify a few more RP, mainly the so called acidic ones, *i.e.* those with a $pI<8.6$.

2. Materials and methods

2.1. Cell cultures

HeLa cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM from Gibco/Life Technology, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS from Biowest, Nuaillé, France), 50 U/mL penicillin, 50 μg/mL streptomycin and 1 μg/mL amphotericin B (both from Gibco/Life Technology) as already described [12].

Human and rat dermal fibroblasts (HDF and RDF respectively) were obtained from CELLnTEC Advanced Cell System (Bern, Switzerland) and were grown according to the manufacturer's protocol at 37 °C with 5% CO_2 in the CnT-05 fibroblast medium (CELLnTEC Advanced Cell System).

Transforming growth factor alpha and beta (TGF-α and TGF-β)-induced erythrocytic cells (T2EC) were generated from SPAFAS white leghorn chickens (PA12 line from INRA, Tours, France) as described [13]. Cells were expanded at 37 °C with 5% CO_2 in culture medium (minimum essential medium alpha (α-MEM from Gibco/Life Technology), 10% FBS, 10^{-3} M HEPES (2-(4-(2-hydroxyethyl)piperazin-1-yl) ethanesulfonic acid), 10^{-4} M 2-mercaptoethanol, 10^{-6} M dexamethasone (all from Sigma-Aldrich Co., Saint Louis, MO, USA), 5 ng/mL human TGF-α (Active Bioscience, Hamburg, Germany), 1 ng/mL human TGF-β-1 (PeproTech INC, Rocky Hill, NJ, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (both from Gibco/Life Technology)).

CEF were generated from SPAFAS white leghorn chickens (LD1 line from INRA, Tours, France). Cells were grown at 37 °C with 5% CO_2, as previously described [14], in medium 199 with Earl's salts, supplemented with 10% tryptose phosphate broth (TPB from Difco, Detroit, MI, USA), 5% FBS, 1% normal chicken serum (NCS), 0.1% sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B (all from Gibco/Life Technology).

2.2. Cell fractionation and ribosome purification

10^7 HeLa cells were washed twice with cold Dulbecco's phosphate buffered saline (D-PBS from Gibco/Life Technology) kept at 4 °C. Cells were scraped off the culture dishes in preheated D-PBS, trypsin (0.25% w/v) at 37 °C. Cells in suspension were washed once with D-PBS kept at 4 °C and then centrifuged at $370\times g$ for 5 min at 4 °C. The supernatant was removed and cells were resuspended and kept for 10 min at 4 °C in 300 μL of lysis buffer made of 0.5% IGEPAL® CA-630 (Sigma-Aldrich), 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl pH 7.4, 2 mM 1,4-dithioerythritol (DTE from Sigma-Aldrich), protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail II (Sigma-Aldrich). Cell lysate was centrifuged at $800\times g$ for 10 min at 4 °C to spin down the nuclei. Post-nuclear supernatant was centrifuged at $12{,}000\times g$ for 10 min at 4 °C to spin down the mitochondria. Post-mitochondrial supernatant was layered on 1 mL of a 1 M sucrose cushion made in 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris–HCl pH 7.4 in a thick-wall polycarbonate centrifuge tube, and centrifuged for 2 h at $305{,}000\times g$ at 4 °C in a Beckman Coulter-Optima™MAX-XP Ultracentrifuge (Brea, CA, USA). Ribosome pellet was rinsed twice with distilled water at 4 °C and resuspended in 300 μL of 50 mM Tris–HCl pH 7.4 containing 25 mM KCl, 5 mM $MgCl_2$ and 2 mM DTE. Concentration of ribosomes was determined by measuring the absorbance at 260 nm with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Fibroblast ribosomes from human, rat and chicken were obtained as described previously for HeLa cells but with lysis buffer made in 125 mM sucrose instead of 250 mM.

Ribosomes from T2EC grown in suspension were obtained as described for fibroblasts without the trypsinization step. By those cell fractionation procedures, ribosomes from cultured cells were purified as a mixture of cytoplasmic free and membrane-bound ribosomes.

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