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Expression of the cytoplasmic nucleolin for post-transcriptional regulation of macrophage colony-stimulating factor mRNA in ovarian and breast cancer cells

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ABSTRACT

The formation of the mRNP complex is a critical component of translational regulation and mRNA decay. Both the 5′ and 3′UTRs of CSF-1 mRNA are involved in post-transcriptional regulation. In CSF-1 mRNA, a small hairpin loop structure is predicted to form at the extreme 5′ end (2–21 nt) of the 5′UTR. Nucleolin binds the hairpin loop structure in the 5′UTR of CSF-1 mRNA and enhances translation, while removal of this hairpin loop nucleolin binding element dramatically represses translation. Thus in CSF-1 mRNA, the hairpin loop nucleolin binding element is critical for translational regulation. In addition, nucleolin interacts with the 3′UTR of CSF-1 mRNA and facilitates the miRISC formation which results in poly (A) tail shortening. The overexpression of nucleolin increases the association of CSF-1 mRNA containing short poly $(A)_{n \leq 26}$, with polyribosomes. Nucleolin both forms an mRNP complex with the eIF4G and CSF-1 mRNA, and is co-localized with the eIF4G in the cytoplasm further supporting nucleolin's role in translational regulation. The distinct foci formation of nucleolin in the cytoplasm of ovarian and breast cancer cells implicates the translational promoting role of nucleolin in these cancers.

1. Introduction

The untranslated regions (UTRs) in mRNA have a significant role in post-transcriptional regulation. Both the 5′ and 3′UTRs are involved in mRNA stability and translation efficiency [1]. The regulatory elements found in the 5′UTR includes the 5′cap, secondary structure, and internal ribosome entry site (IRES). The 5′cap is a binding site for eIF4F, which is composed of the 5′cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A1. The formation of the eIF4F complex recruits the 43S preinitiation complex, a major rate-limiting step in translation initiation [2]. The 5′cap also protects the mRNA from decay process [36].

The secondary structure in the 5′UTR modulates translation by binding proteins or other factors. The stem-loop structures in the 5′UTR can activate or inhibit translation depending on mRNA species and cellular circumstances [3,4]. A hairpin loop located near the 5′cap with a $dG = -25 \sim -35$ kcal/mol has been described using the GFP reporter mRNA to inhibit translation initiation by blocking the access of the 43S preinitiation complex [5]. Dmitriev et al. reported that the translation of mRNAs with long and highly structured 5′UTRs depends strongly on the 5′cap [3]. In contrast, highly structured IRES facilitates a cap-independent translation initiation [4,6].

The 3′UTR contains miRNA target sites, AU-rich elements (AREs), and poly $(A)_n$ tail, and is involved in the determination of mRNA fate and translational regulation. Binding of miRNA forms miRISC in the 3′UTR, inducing mRNA deadenylation followed by degradation [7,8]. The poly $(A)_n$ tail is also involved in mRNA stability and translation. Poly (A) binding protein (PABP) acts as a scaffold for many other proteins and regulates translation [9]. PABP interacts with eIF4F and mediates 5′-3′ interaction, resulting in mRNP closed-loop formation and facilitating the transfer of ribosomal subunits from the 3′- to the 5′-end for recycling [10,11,36,37].

Following translation, mRNA is assembled by the deadenylation complex, which leads to the decay process [7,12]. In humans, deadenylation is a major step in the decay process, which is followed by decapping and the exonuclease digestion of mRNA. Deadenylation is a biphasic process with consecutive actions of the Pan2-Pan3 and Ccr4-NOT deadenylation complexes. Pan2-Pan3 triggers the first phase of poly$(A)_n$ tail shortening to ~110 nt, and Ccr4-NOT then follows by shortening the poly$(A)_{110}$ tail to 10–110 nt [13]. In miRNA-directed mRNA decay, the formation of the miRISC on the 3′UTR induces rapid mRNA decay by accelerating biphasic deadenylation [14]. Similarly, AU-rich elements (AREs) in the 3′UTR are destabilizing elements [15] which are involved in mRNA decay by triggering biphasic deadenylation [16].

Our recent findings, however, indicate that nucleolin mediates the miRNA-directed deadenylation of CSF-1 mRNA but increases translation without affecting the total mRNA level or half-life of the deadenylated

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form of CSF-1 mRNA [17]. This suggested that nucleolin-induced mRNA deadenylation may have a previously unidentified function in regulating translation. Nucleolin up-regulates the translation of a group of mRNAs by binding the structured (folded) region, *i.e.*, intra RNA-RNA hybrid structure [18], G-quadruplex [17], and AU-rich elements [19,20,21]. The CSF-1 mRNA, which encodes macrophage colony stimulating factor [22], a cytokine with roles in progression of several cancers including ovarian and breast cancers, interacts with nucleolin through the G-quadruplex and AREs in the 3′UTR [17,23]. The stability and translation of CSF-1 mRNA is also regulated by miRNAs [17,24]. Our findings indicated that the binding of nucleolin to the G-quadruplex and AREs in the CSF-1 mRNA 3′UTR not only mediates miRNA-directed CSF-1 mRNA deadenylation, but also blocks further miRISC-directed mRNA decay and increases the translation of CSF-1 mRNA [17].

In this paper, we find that nucleolin foci can be visualized in the cytoplasm of ovarian and breast cancer cells, and co-localizes with eIF4G. Overexpression of nucleolin in the cytoplasm increases the polyribosome-associated CSF-1 mRNA containing shortened poly $(A)_{n\leq 26}$ tails. A hairpin loop structure is predicted at the extreme 5′ end (2-21 nt) of the 5′UTR. Nucleolin interacts with this hairpin loop structure in the 5′UTR, increasing translation only in the presence of this hairpin loop nucleolin binding element. Removal of this element encoding the hairpin loop structure in the 5′UTR dramatically represses translation of mRNA. Collectively, our data suggests that the interaction of nucleolin with eIF4G and with the hairpin loop structure in the 5′UTR of CSF-1 mRNA are largely responsible for enhanced CSF-1 mRNA translation and stability.

2. Materials and methods

2.1. Cell lines

Hey and SKOV3 ovarian cancer cell lines were cultured in DMEM/F12 (Mediatech) supplemented with 10% fetal bovine serum. BT20 breast cancer cell line was cultured in MEM supplemented with 10% fetal bovine serum. MDA-MB-231 breast cancer cell line was cultured in DMEM supplemented with 10% fetal bovine serum. Hey ovarian cancer cell lines with chromosomally-integrated nucleolin (NCL)-GFP or GFP were generated.

2.2. Preparation of cytoplasmic and nuclear protein

Cytoplasmic and nuclear protein fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce).

2.3. Indirect immunofluorescence microscopy

The procedure described by Kedersha and Anderson [27] was followed. Cells were fixed in 4% paraformaldehyde for 5 min, and permeabilized in cold methanol for 15–20 min before incubation with the nucleolin antibody for 1 h at 37 °C. The N-terminal or C-terminal immunogen-raised rabbit monoclonal nucleolin antibodies (Abcam) and middle domain raised mouse monoclonal nucleolin antibody (Santa Cruz Biotechnology) were used. The Alexa-conjugated secondary antibodies (Life Technologies) were incubated for 1 h at 37 °C, and the resulting image was photographed under the fluorescence microscope.

2.4. Nucleolin shRNA transfection assay

Transfection and sequence information of nucleolin shRNA was described previously [17].

2.5. Luciferase reporter constructs

CSF-1 mRNA 5′ and 3′UTRs were ligated with Firefly luciferase gene and cloned into the pTRE3G-BI plasmid (Clontech). Triple mutation was described previously [17].

For constitutive expression of reporter constructs, CSF-1 mRNA 5′ and 3′UTRs were ligated with Firefly luciferase gene and cloned into the pcDNA3.1 plasmid.

2.6. Translation induction assay and ribosome profile

The 'Tet-ON' system provides better control of transcription as well as translation, since there is no accumulation of translation products (*i.e.,* luciferase) in the cell before the addition of tetracycline. Chromosomally-integrated SKOV3 and Hey cell lines with a Tet-ON 3G inducible system (Clontech) were generated with the plasmid constructs indicated in Fig. 2A.

In translation induction assay, cells were treated after 24 h incubation for 4 h with 20 ng/ml doxycycline which was then removed by PBS wash, 5 times. After doxycycline removal, cells were harvested and lysed every hour for the assay of luciferase activity. A ribosome profile was generated as described previously [17]. $N = 10$.

2.7. RNA electrophoretic mobility shift assay

REMSA was described previously [26]. The sequences at extreme 5′ end of the 5′UTR (2-21 nt), 5′UTR without hairpin loop sequence (22-105 nt), and G-quadruplex from the 3′UTR (2855-2892 nt) were used to generate ^{32}P–labeled RNA probe for REMSA. ^{32}P–labeled RNA probe was solubilized in 50 mM Tris-$HCl_{pH7.4}$ and 100 mM KCl to maintain secondary structure. Linear 5′hairpin RNA sequence is induced by solubilizing in 50 mM Tris-$HCl_{pH7.4}$ without 100 mM KCl and heat denatured for 5 min before REMSA. Myc-DDK-tagged nucleolin (Origene) was purchased.

2.8. Analysis of luciferase RNA half-life

To determine luciferase reporter mRNA half-life in Hey ovarian cancer cells, actinomycin-D (Act-D) chase experiments were performed with 3 μg/ml of Act D (Sigma) added to inhibit new transcription. For cells undergoing transfection of various constructs, the experiments were performed 2 days after transfection. Cells were harvested at 0 h, 1 h, 2 h, and 4 h after Act D treatment, total cellular RNA extracted using Trizol (Invitrogen), and luciferase RNA, GAPDH, or *Renilla* mRNA levels were analyzed by qRT-PCR. Luciferase mRNA half-lives were calculated after qRT-PCR, normalized to GAPDH mRNA, values were plotted, and the time period required for a given transcript to decrease to one-half of the initial abundance was 0063alculated. GAPDH mRNA is not affected by nucleolin, and has a long half-life (>18 h). Three independent experiments were performed.

2.9. Native mRNP immunoprecipitation

Native co-immunoprecipitation (Co-IP) of the endogenous mRNP complex was done by the protocol described previously with modifications [17,26]. Cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). To maintain the intact native state of the mRNP complex, cytoplasmic lysates were not fixed or sonicated. To partially disrupt the mRNP complex, cytoplasmic lysates were treated with RNase A (10 ng/ul) and T1 (1 U/ul) for 15 min at 37 °C before immunoprecipitation. For nucleolin IP, 5 μg mouse monoclonal anti-human nucleolin antibody raised against middle domain (Santa Cruz Biotechnology), or rabbit monoclonal anti-human nucleolin antibodies raised against N- or C-terminal residues (Abcam), were used. For eIF4G IP, 5 μg rabbit polyclonal anti-human eIF4G antibody (Santa