Human 1A6/DRIM, the homolog of yeast Utp20, functions in the 18S rRNA processing

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Abstract

1A6/DRIM is a nucleolar protein with a nucleolar targeting sequence in its 3′-terminus. Bioinformatic analysis indicated that human 1A6/DRIM shares 23% identity and 43% similarity with yeast Utp20, which has been reported as a component of U3 snoRNA protein complex and has been implicated in 18S rRNA processing. In the present study, we found, by utilizing RT-PCR with RNA extracted from anti-1A6/DRIM immunoprecipitates and Northern blotting, that 1A6/DRIM is associated with U3 snoRNA. Pulse-chase labeling assays showed that silencing of 1A6/DRIM expression in HeLa cells resulted in a delayed 18S rRNA processing. Furthermore, immunoprecipitations revealed that 1A6/DRIM was also associated with fibrillarin, another U3 RNP component in HeLa cells. These results indicate that 1A6/DRIM is involved in 18S rRNA processing and is the bona fide mammalian Utp20.

Keywords: 1A6/DRIM; UTP20; 18S rRNA processing; U3 snoRNA

1. Introduction

1A6/DRIM was originally identified from the malignant derivative of an immortalized gastric epithelial cell line [1] and was found to be down-regulated in metastatic breast cancer cells [2]. However, its role in tumorigenesis is still largely unknown. 1A6/DRIM is a nucleolar protein, suggesting a biological function of 1A6/DRIM in nucleolus. 1A6/DRIM is closely related to yeast Utp20 [2–7], a component of the small-subunit (SSU) processome that is essential for 18 rRNA processing, suggesting that 1A6/DRIM might also be implicated in 18S rRNA processing.

Nucleolus in eukaryotes is a compartment for biosynthesis of ribosomes including processing of pre-ribosomal RNA (pre-rRNA) and sequential assembly of a large number of ribosomal proteins on the rRNAs. A large polycistronic precursor RNA containing the sequences for the matured rRNAs (18S, 5.8S, and 28S rRNA), two external transcribed spacers (ETS), and two internal transcribed spacers (ITS) is transcribed by RNA polymerase I. After being chemically modified at numerous sites, the pre-rRNA is processed to produce 18S, 5.8S, and 28S rRNAs; 5S rRNA is independently transcribed by RNA polymerase III.

Many chemical modification steps in the pre-rRNA processing are guided by small nucleolar RNAs (snoRNAs). These snoRNAs correctly position the modification enzymes on the pre-rRNA by base pairing with target sites. To fulfill this task, snoRNAs interact with a plethora of nucleolar proteins to form snoRNA protein complexes (snoRNPs). The snoRNAs can be divided into two major classes: the box C/D and box H/ACA snoRNAs. Box C/D snoRNAs such as U3 and U14 are involved...
in 18S rRNA processing [8, 9]. U14 snoRNA is responsible for 2’-O-methylation of the pre-rRNA and consequently promotes the base-pairing reaction [10], whereas U3 snoRNA functions in base pairing the flanking regions of 18S rRNA to facilitate 18S rRNA processing [11, 12, 13].

Pre-rRNA processing and snoRNPs components have been extensively studied in the yeast [3, 14–18], whereas the data about human snoRNPs has been scarce. Recently, Andersen and colleagues have performed proteomic analysis of the human nucleolus and identified 271 nucleolar proteins [19], and Scherl et al. [20] identified 213 proteins from the nucleolus of HeLa cells. Collectively, this work yields a total of about 350 nucleolar proteins in human. However, the specific function of these proteins in rRNA biosynthesis is yet to be defined.

We report here that 1A6/DRIM is a component of human snoRNPs and is involved in 18S rRNA processing.

2. Materials and methods

2.1. Cell culture and 1A6/DRIM RNAi

HeLa cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C. For silencing 1A6/DRIM expression, a specific sequence to 1A6/DRIM (5’-GCCAUAGCCUGAAAGAUUU-3’) was chemically synthesized together with the synthesis of an unrelated siRNA (siNC, 5’-ACUACC-GUUGUUUAAGGUG-3’) as the control (Shanghai GenePharma Co., Ltd). The synthesized siRNAs were transfected into cells at a concentration of 100 nM with LipofectAMINE 2000™ (Invitrogen) according to the manufacturer’s instruction.

2.2. Metabolic labeling and analysis of RNA transcripts

Pulse-chase labeling was performed as described previously [21]. In brief, 72 h after transfection of chemically synthesized siRNA, HeLa cells were pre-incubated in methionine-free medium for 15 min and then switched to medium containing 50 μCi/ml L-[methyl-³H] methionine (PerkinElmer Life Sciences) for 30 min. Cells were then chased in medium containing 15 μg/ml of non-radioactive methionine. Total RNA was isolated with Trizol reagent (Invitrogen). The [methyl-³H] methionine labeled RNAs were resolved on a 1% agarose–formaldehyde gel and detected by fluorography.

2.3. Preparation of cellular extracts and immunoprecipitation

Immunoprecipitation was performed as described previously [22]. Briefly, HeLa cell lysates were prepared in buffer A (25 mM Tris–Cl [pH 7.5], 100 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.05% NP-40) and used directly for immunoprecipitation. Monoclonal anti-1A6/DRIM antibody 6D9 or mouse IgG were coupled with 50% suspension of protein A sepharose beads (Amersham Biosciences) in IPP500 (500 mM NaCl, 10 mM Tris–Cl [pH 8.0], 0.05% NP-40). Beads were incubated with cellular extracts for 2 h at 4 °C. After extensive washes, precipitated proteins were analyzed by Western blotting and co-precipitated RNAs were isolated with Trizol reagent (Invitrogen).

2.4. RT-PCR for amplification of U3 snoRNA

Reverse transcription was performed with the co-precipitated RNAs using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instruction. PCR was performed using following U3-specific primers [23]: Forward primer: 5’-AACGCTT(HindIII) ACCACTCA-GACCGCGTCTC-3’, and reverse primer: 5’-GAATTC(HindIII) ACCACTCA-TCAGGGATC-3’. Aliquots of PCR products were loaded onto 2.5% agarose gel and visualized under UV spectrometer (Alpha Innotech Corporation). The PCR products were cloned into pGEM-T-easy vector (Promega) and the sequence was verified by DNA sequencing.

2.5. Northern blotting

Northern blotting was performed as described previously [23]. RNA sample was heated to 70 °C for 3 min in loading buffer (80% [v/v] formamide, 90 mM Tris–borate, 2 mM EDTA, pH 8.0, 0.025% [w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF) before loaded onto a 7% polyacrylamide–8.3 M urea gel. RNA was blotted onto Hybond membrane (Amersham) by electroblotting (Bio-Rad) for 30 min at 200 mA. Prehybridization was performed for 2 h at 65 °C in 6×SSC, 5×Denhardt’s solution (0.1% [w/v) Ficoll 400, 0.1% [w/v) BSA, 0.1% [w/v) polyvinylpyrrolidone-40), 0.1% (w/v) SDS, 100 μg/ml denatured herring sperm DNA, and 125 μg/ml total yeast RNA. The [³²P]-CTP-labeled U3-RNA probe was added at an amount of 2–6×10⁶ cpm/ml in prehybridization solution. Hybridization was carried out for 16 h at 65 °C. After washing three times with 2×SSC, 0.1% (w/v) SDS for 30 min at 65 °C, the blot was dried and analyzed autoradiographically.

![Fig. 1](image-url) 1A6/DRIM was associated with U3 snoRNA in HeLa cells. Immunoprecipitations were performed with anti-1A6/DRIM antibody 6D9 or pre-immune mouse IgG (mIgG) in HeLa cells. (A) Proteins from the precipitates were separated on 6% SDS-PAGE and transferred onto PVDF membrane followed by Immunoprecipitations were performed with 6D9. 1A6/DRIM is detected as a 310-kDa band and indicated with an arrow head. (B) RNA was extracted from the precipitates described above for RT-PCR analysis with U3 snoRNA-specific primers. Ten percent of RNAs were used as inputs. (C) Northern blotting analysis with [³²P]-CTP labeled U3 RNA probe of the 6D9-precipitated RNA. Thirty percent of RNAs were used as inputs.
2.6. Immunoblotting

Precipitated proteins were separated on SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences). The membranes were then probed with 1A6/DRIM-specific antibody 6D9 at 1:500 dilution or anti-fibrillarin antibody (Santa Cruz) at 1:1000 dilution after being blocked with 5% milk in PBS/T (0.5% Tween-20 in PBS buffer). After extensive washing with PBS/T, the membranes were incubated with HRP-conjugated secondary antibodies. The immuno-complex was detected with the ECL-Kit (Amersham Biosciences) before exposed to X-ray film.

3. Results

In order to understand the biological function of 1A6/DRIM protein, we first analyzed human 1A6/DRIM protein by bioinformatics. A BLAST search of the translated GenBank database with the human 1A6/DRIM protein sequence indicated that human 1A6/DRIM protein is an Utp20 homolog in Saccharomyces cerevisiae. The yeast Utp20 (YBL004W, YBA4, YBL0101) is a protein of 287 kDa that shares a 23% identity
Northern blotting was performed with [32P]-CTP labeled U3 length U3 snoRNA. To further verify the RT-PCR results, sequenced. The results revealed that they were identical to full-

3. Immunoprecipitation was performed with 6D9 on HeLa cellular lysates. Precipitates were separated on SDS-PAGE and transferred onto PVDF membrane. The blot was detected by anti-fibrillarin antibody. IgG LC: IgG light chain.

and 43% similarity with human 1A6/DRIM [NP055318.1] [2,3]. In addition, bioinformatic analysis indicated that the structure and charge distribution of the human 1A6/DRIM and yeast Utp20 are also very similar, both human 1A6/DRIM and yeast Utp20 harbor HEAT motifs and have a similar distribution of basic amino acids.

Yeast Utp20 has been implicated in the function of U3 snoRNA. In order to investigate whether human 1A6/DRIM is also functionally connected to U3 snoRNA, immunoprecipitation was performed with a monoclonal anti-1A6/DRIM antibody 6D9 in HeLa cells. RNAs were extracted from the immunoprecipitation pellets and the co-precipitated U3 snoRNA was analyzed by RT-PCR. Immunoprecipitation with pre-immune mouse IgG was also included as a control. As shown in Fig. 1 while immunoprecipitation with pre-immune mouse IgG did not yield any PCR product, 6D9 was able to immunoprecipitate a unique 220-bp fragment (Fig. 1B). Next, the 220-bp PCR product was cloned into pGEM-T-easy vector and five pGEM-T-easy-U3 clones were sequenced. The results showed that fibrillarin was indeed immunoprecipitated by the anti-1A6/DRIM antibody (Fig. 3).

4. Discussion

In a previous study, we showed that 1A6/DRIM is a nucleolar protein and identified its nuclear leading and nucleolar targeting sequences in the C-terminus [24]. Our results also indicated that the 1A6/DRIM’s nucleolar targeting sequence containing basic amino acids could be a functional motif in the nucleolus. To understand the function of 1A6/DRIM in the nucleolus, we investigated whether 1A6/DRIM is associated with another U3 snoRNA component, Fibrillarin. Immunoprecipitation was performed in HeLa cells using anti-1A6/DRIM 6D9. Precipitates were loaded onto SDS-PAGE and transferred onto PVDF membrane. The blot was then probed with an anti-fibrillarin antibody. The results showed that fibrillarin was indeed immunoprecipitated by the anti-1A6/DRIM antibody (Fig. 3).

Recent studies have utilized mass spectrometry combined with tandem affinity-purification (TAP) to identify the protein complexes involved in the U3 snoRNPs. In yeast, Utp1-17, Utp18, Utp20, Utp21, and Utp22 have been identified as U3 snoRNP components [15]. The yeast system is widely used as a model system for studying ribosome biogenesis because it is relatively easy to manipulate. However, in mammalian, only a few proteins have been identified to be U3 snoRNP components, albeit proteomic studies have indicated 350 proteins in the nucleolus [19,20].

For rRNA biosynthesis in mammalian cell, a 47S pre-rRNA is first transcribed, which contains 18S, 5.8S, and 28S rRNA as well as 5′ETS, ITS1, ITS2, and 3′ETS. The pre-rRNA is then cleaved at specific sites to produce a series of intermediates and consequently matured rRNAs. Several cleavage pathways have been described for processing of the pre-rRNA to form the matured rRNAs [25,26]. At least two cleavage pathways have been described in mammalian cells (Fig. 2A). For the formation of 18S rRNA, the major pre-rRNA processing pathway (pathway A) is to cleave 47S rRNA to form a 41S rRNA intermediate followed by an additional cleavage to form matured 18S rRNA. U3 snoRNPs mainly function in this pathway. Alternatively, 47S rRNA is cleaved through pathway B to produce a 34S rRNA, then a 20S rRNA and finally the matured 18S rRNA. In the present study, when 1A6/DRIM was silenced by siRNA, the level of 41S rRNA intermediate was reduced, suggesting that the pathway A was partially inhibited by 1A6/DRIM silencing, whereas pathway B was not affected.
Consequently, 1A6/DRIM deficiency resulted in a delayed 18S rRNA processing. The observation that U3 snoRNP-mediated 18S rRNA processing (pathway A) was inhibited by 1A6/DRIM knockdown suggests that 1A6/DRIM is required in U3 snoRNPs-mediated 18S rRNA processing. In the meantime, Northern blotting showed that U3 snoRNA levels were not affected by 1A6/DRIM silencing, indicating that the delayed 18S rRNA processing was not due to a general defect in U3 snoRNA biogenesis but a specific effect of 1A6/DRIM protein deficiency.

In this study, we showed that 1A6/DRIM is associated with U3 snoRNA in HeLa cells. We also detected fibrillarin in 1A6/DRIM-specific immunoprecipitates. Together with the requirement of 1A6/DRIM for the maturation of 18S RNA, these results support that 1A6/DRIM is a U3 snoRNP component.

In yeast, in addition to function as U3 snoRNP components, HEAT proteins Upf10 and Up20 are also capable of mediating protein–protein interaction in the nucleolus [15,19,27,28]. Structural analysis revealed that 1A6/DRIM harbors 11 HEAT motifs [2], suggesting that 1A6/DRIM might also mediate protein–protein interactions in the nucleolus. Whether the HEAT motifs are essential for 1A6/DRIM nucleolar function has to be defined in the future studies.

In mammalian cells, 18S rRNA processing is a highly complicated process in which many snoRNAs including box C/D snoRNAs such as U3 and U14 and box H/ACA snoRNAs such as snR10 and snR30 are involved [29,30]. Up to date, only a few nucleolar proteins such as MPP10 [31–33], Imp3p and Imp4p [34–37], and hU3-55K [22,38,39] have been identified to be specifically associated with U3 snoRNA and the rest pre-rRNA processing proteins are associated with multiple snoRNPs. For example, mammalian protein 15.5K, which mediates U3 binding of hU3-55K, is involved in assembly of three RNP complexes, the U4/U6 snRNP, the box C/D snoRNP, and the U3 box B/C motif-associated RNP complex [40]. These complexes are compositely and functionally distinct. In the present study, we demonstrated that 1A6/DRIM functions in 18S rRNA processing as a component of U3 snoRNPs. However, we cannot exclude the possibility of 1A6/DRIM involvement in other snoRNPs such as U14 snoRNPs. Further studies are warranted to investigate whether 1A6/DRIM is also involved in the function of other snoRNPs and whether the association of 1A6/DRIM and U3 snoRNA is by mechanism of a direct interaction.

DRIM was so named for its down-regulation in the metastatic derivative cell line MDA-MB 435 (4A4) compared with the non-metastatic variant of the cell line MDA-MB 435 (2C5) by differential display [2]. However, whether the DRIM mRNA level correlates with metastatic propensity of breast cancer cells is still not clear [41], and how DRIM might function in cell proliferation and migration is currently unknown. In light of our current findings, it will be interesting in the future studies to investigate whether the pathophysiological activities of 1A6/DRIM in cell proliferation and cancer metastasis is connected to its role in rRNA biogenesis.

In conclusion, our results demonstrated that human 1A6/DRIM, by association with U3 snoRNA, functions in the processing of 18S rRNA and is the bona fide mammalian UTP20.

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