Abstract. Upf2 protein predominantly localizes to the cytoplasmic fraction, and binds to the exon junction complex (EJC) on spliced mRNA. The present study aimed to determine the cellular site where the interaction between Upf2 and EJC occurs. First, the cell lysate was fractionated into the cytoplasm and nucleoplasm, and western blotting to detect levels of Upf2 protein was performed. Upf2 was clearly detected in the cytoplasm and in the nucleoplasm. Secondly, immunostaining was performed, and the majority of Upf2 was detected in the cytoplasmic perinuclear region; a small quantity of Upf2 was detected in the intranuclear region. RNase treatment of the cells reduced the Upf2 immunostained signal. The immunopurified fractions containing nuclear and cytoplasmic Upf2 also contained one of the EJC core factors, RBM8A. These results implied the existence of Upf2 in the nucleoplasm and the cytoplasm, and it appeared to be involved in the construction of the mRNA complex. In order to verify the construction of Upf2-binding EJC in the nucleoplasm, an in situ proximity ligation assay was performed with anti-Upf2 and anti-RBM8A antibodies. These results demonstrated that their interaction occurred not only in the cytoplasmic region, but also in the intranuclear region. Taken together, these results suggested that Upf2 combines with EJC in both the cytoplasmic and the intranuclear fractions, and that it is involved in mRNA metabolism in human cells.

Introduction

Previously, it has been reported that the aberrant copy number variation (CNV) of the human Upf2 gene locus is associated with neural development disorders, based on a search for CNVs among patients with intellectual disabilities (1). Upf3b, the binding partner of Upf2, is also associated with similar developmental disorders, and this protein is one component of the exon junction complex (EJC) formed on mRNA (2). The EJC, and its associated proteins, are essential factors of the nonsense-mediated mRNA decay (NMD) pathway, and NMD has various effects on cellular function. Therefore, it is possible that a deficiency of them may lead to neuro-developmental disorders.

mRNA forms messenger ribonucleoprotein particles (mRNPs) with various factors and is exported from the nucleus to the cytoplasm following transcription. During these processes, various quality control systems are responsible for maintaining the integrity of the genetic information. One of these processes is NMD, which eliminates mRNA that contains premature termination codons (PTCs) (3-6). PTCs may arise from a variety of transcription errors, including nonsense mutations in the genome, and they often cause recessive inherited disorders. Regardless of their source, PTCs in one allele have the potential to produce deleterious C-terminal truncated proteins, and NMD is considered to suppress the expression of truncated proteins that possibly have dominant negative toxicity caused by competitive inhibition of wild-type-allele-derived proteins. During the processes of mRNA metabolism, almost all the mRNAs that are exported from the nucleus to the cytoplasm pass through a quality control process, so that those containing PTCs may be eliminated to effectively maintain the integrity of the genetic information by NMD.

The conventional NMD mechanism requires the presence of various factors, including the SMG-1-Upf1-eRF1-eRF3 (SURF) complex (formed with SMG1, Upf1 and eukaryotic release factors), Upf2, Upf3b, EJC, and others. The EJC comprises RBM8A (Y14), Magoh, eIF4A3 and other proteins, and this is formed on the mRNA via splicing, serving as a platform for the assembly of the NMD-associated complex (7-10) or other functional complexes. The complex formed comprising Upf1, Upf2 and Upf3b is well established (4,11). Upf3b and Upf2 are reported to localize at the EJC and receive SURF when the ribosome encounters PTCs, as determined from biochemical experiments (12).

Immunostaining experiments have revealed that EJC and Upf3b predominantly localize in the nucleus, whereas Upf1
predominantly localizes in the cytoplasm. On the other hand, although Upf2 has a nuclear localization signal, Upf2 is located predominantly in the cytoplasmic perinuclear region (11,13). The interaction of NMD factors is well established (14-16), and NMD is considered to progress according to the following three steps: First, Upf3b and EJC bind to mRNA molecules via RNA splicing in the nucleus; subsequently, perinuclear Upf2 binds to Upf3b on the exporting mRNA; and finally, following the formation of the EJC-Upf2-mRNA complex, it interacts with the SURF complex through Upf1 on the ribosomes arrested at a PTC, resulting in the selective degradation of mRNAs with PTCs (4,12). It has been reported that NMD occurs either in the cytoplasm or in a nuclear-associated manner when newly synthesized mRNA undergoes the first round of translation, a process known as pioneer round translation (3,5,6,17-19). On the other hand, the inhibitory effect of endogenous expression of dominant-negative Upf proteins on NMD activity was assessed, and it was proposed that the interaction between the Upf complex and EJC occurs in the cytoplasm, rather than in a nucleus-associated manner (20). Therefore, although NMD occurs in the cytoplasm, the possibility of nuclear-associated NMD during the export of mRNPs still remains.

According to previously proposed models (10,15,16), Upf2 accumulates at the perinuclear region and forms a complex with EJC on the exporting mRNA outside of the nuclei. However, almost all models have been established solely on spatial observations, and therefore have not provided any information concerning the mechanism for Upf2 localization. If only perinuclear Upf2 proteins are involved in forming a complex with EJC, nuclear-associated NMD should take place close to the nucleus, perhaps immediately following export from the nuclear pore complex. Alternatively, if intranuclear Upf2 protein forms complexes with EJC in the nuclei, the mRNA-EJC-Upf2 complex would appear in time for an approach by SURF and the ribosome, even in nuclei. Indeed, at present, no published studies have identified the location of endogenous Upf2-EJC complex formation.

In the present study, fractionation of cell lysates and series of immunostaining experiments were performed. Taken together, our study using a proximity ligation in situ assay has demonstrated that endogenous Upf2 interacts with one of the EJC core factors, RBM8A, in the inner nucleus prior to mRNA export through the nuclear pore, and constructs the mRNA-protein complex.

Materials and methods

Cell culture. HeLa and A549 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics (final concentration, 10,000 U/ml penicillin and 10 mg/ml streptomycin; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cells were allowed to adhere and proliferate for 224 h at 37°C in 5% CO₂ prior to the following experiments.

Cellular fractionation. The preparation of nucleoplasmic and cytoplasmic fractions was performed as previously described (21). NE-PER nuclear and cytoplasmic extraction reagent (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA) was used according to the manufacturer's protocol, and prepared fractions were denatured with 2X Laemmli Sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for western blot analysis.

Western blotting. The procedures for whole lysate preparation and western blot analysis have been described (22). Protein concentration of the lysates was measured by the Bradford method. In brief, denatured samples (25 μg) were applied to 10% acrylamide gels. Following SDS-PAGE, gels were transferred to polyvinylidene difluoride membranes and blocked with 5% skim milk for 1 h. Then, blocked membranes were subjected to antibody binding. The first antibodies were rabbit anti-human Upf2 polypeptide antisera (prepared in our laboratory), monoclonal mouse anti-human lamin A/C antibody (1:1,000; cat. no. sc-7292; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and monoclonal rabbit anti-human caspase 3 (1:1,000; cat. no. 9665; Cell Signaling Technology, Inc., Danvers, MA, USA). In addition, monoclonal mouse anti-RBM8A antibody was purchased from Sigma-Aldrich (1:500; AC4 clone; cat. no. Y1253). β-actin (ACTB) was used as the reference protein, and was detected using mouse monoclonal anti-β-actin antibody purchased from Sigma-Aldrich (1:5,000; AC15 clone; cat. no. A5441). Incubation with primary antibody were performed at 4°C, overnight. Primary antibodies were detected with horseradish peroxidase-conjugated polyclonal goat anti-mouse (1:5,000; cat. no. P0447) or anti-rabbit (1:5,000; cat. no. P0448) secondary antibodies (Dako, Glostrup, Denmark).

Gene expression/knockdown. One day prior to the small interfering RNA (siRNA) transfection experiments, HeLa cells were seeded on to culture plates or dishes. The depletion of Upf2 was performed using stealth RNA interference (RNAi) molecules (HSS178005), and the knockdown of RBM8A was achieved using HSS115052; the two siRNAs were obtained from Invitrogen™; Thermo Fisher Scientific, Waltham, MA, USA). Two double-stranded molecules of the Stealth RNAi™ negative control kit (Thermo Fisher Scientific) and medium GC duplex were used as the negative control. Transfections were performed using Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific), according to the manufacturer's protocol.

Immunostaining and observation of the cells. The full details of the procedure followed for immunostaining were previously described (23). Briefly, HeLa cells were washed three times with phosphate-buffered saline (PBS) and fixed at room temperature with 4% paraformaldehyde solution (Taab Laboratory Equipment Ltd, Aldermaston, Reading, UK) in PBS for 10 min. After washing three times with PBS, the cells were treated with 0.2% Triton X-100 (Sigma-Aldrich)/PBS for 10 min at room temperature. Samples were washed three times with PBS. For the ribonuclease (RNase) treatment experiments, cells were fixed with cold ethanol for 10 min and treated with 10 ng/ml RNaseA solution (Qiagen GmbH, Hilden, Germany) at 37°C for 30 min, followed by three further washes with PBS. Blocking was performed with 1% bovine serum albumin (BSA; Wako Pure Chemical Industries,
Laser Ltd.) with gentle agitation for 30 min at room temperature. Following this procedure, the cells were incubated with the first antibodies, rabbit anti-human Upf2 polyclonal antiserum prepared in our laboratory, anti-human lamin A/C antibody (Santa Cruz Biotechnology, Inc.), mouse anti-RBM8A antibody purchased from Sigma-Aldrich (4C4 clone) or rabbit anti-RBM8A antibody (prepared in our laboratory) with gentle agitation for 2 h at room temperature. Binding of the first antibody was detected using Alexa Fluor 488- or 594-conjugated secondary antibodies (Molecular Probes Life Technologies, Carlsbad, CA, USA) with gentle agitation at room temperature for 60 min, and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). After washing the cells with PBS three times, ProLong® Gold Antifade reagent (Invitrogen™, Thermo Fisher Scientific) was used to prevent fading. Images were captured with either an Axiovert 200 M inverted fluorescence microscope or an LSM 710 confocal point-scanning microscope (Carl Zeiss, München, Germany). ZEN 2008 SP1.1 software (Carl Zeiss) was used to analyze signal intensity.

**Immunoprecipitation of Upf2.** To confirm the association between mRNA and Upf2, HeLa cells were collected using a cell scraper and suspended in cold NET-2 buffer [50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.05% Igepal-CA630; Sigma-Aldrich]. For nuclear fractions, cells were treated with cNE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific, Inc.) and nuclear fractions were collected. Subsequently, nuclear fractions were suspended in cold NET-2 buffer. The samples were sonicated using the Vibra cell sonicator (Sonics & Materials, Inc., Newtown, CT, USA), and the sonicated samples were centrifuged for 15 min at 15,000 x g. The supernatant was treated with anti-Upf2 antiserum and normal rabbit serum as the control. Following an incubation with agitation in a cold room, Protein G-conjugated agarose beads (Invitrogen™; Thermo Fischer Scientific) were added to the samples, and the samples thus obtained were again incubated in a cold room. Following sufficient washing with NET-2 buffer, the beads were resuspended in Laemmlı sample buffer (BioRad Laboratories, Inc.) with 2-mercaptoethanol (Sigma-Aldrich). The sample was boiled, and the resultant sample buffer solution was processed for western blotting, which was performed as described above.

**Detection of complex formation with a proximity ligation in situ assay.** The direct observation of Upf2-EJC complex formation was performed using a Duolink® kit (Olink Bioscience, Uppsala, Sweden; product now owned by Sigma-Aldrich) following the manufacturer’s protocol (23). This method enables the visualization of complex formation in cells by proximity ligation of single-stranded DNA conjugated with a secondary antibody (24), and is available for the complex in nuclei and cytoplasm (25). Signal can be observed when the distance between the secondary antibodies is <40 nm. Therefore, this method can detect not only direct protein-protein interactions, but also complex formation, assuming that the bound first antibodies are proximal enough. The first antibodies used in the present study were as described in the above Immunostaining and observation section. Images were captured with either an Axiovert 200 M inverted fluorescence microscope or an LSM 710 confocal point-scanning microscope (Carl Zeiss, München, Germany).

**Results**

**Detection of Upf2 by western blotting in the nuclear fraction.** In order to assess whether Upf2 was resident in the nuclei, HeLa cells were fractionated into their respective nuclear and cytoplasmic fractions. These fractions were analyzed using western blotting. The purity of each fraction was evaluated by blotting with anti-lamin A/C (nuclear marker) and anti-caspase 3 (cytoplasmic marker) antibodies. Almost all the lamin A/C was detected in the nuclear fraction, and caspase 3 was predominantly detected in the cytoplasm. Therefore, it was confirmed that the fractionation process had been successful, and Upf2 was clearly detected in the two fractions (Fig. 1).

**Detection of Upf2 and RBM8A by immunostaining.** Subsequently, to confirm the results of western blotting, immunostaining experiments were performed. Previous reports have revealed the cytoplasmic and perinuclear localization of endogenous or exogenous Upf2 (11,13). In accordance with these reports, the results in the present study also demonstrated that Upf2 is predominantly localized to the cytoplasmic perinuclear region, although a limited quantity was observed in the nucleus (Fig. 2A). Subsequently, knockdown experiments were performed to confirm the specificity of the obtained signals. The Upf2-specific siRNA, prepared in our own laboratory, successfully depleted the Upf2 signals in the perinuclear and intranuclear regions (Fig. 2A). The knockdown efficiency was confirmed by western blotting of the siRNA-treated and control siRNA-treated cells (Fig. 2B). These findings suggested that the signals from immunostaining observed in the present study had originated from the Upf2 molecules. Thus, it was deduced that Upf2 proteins exist not only in the perinuclear region, but also in the nucleoplasmic region.
Upf2 binds to RNA complex. To analyze which factors bind to the RNA molecule, RNase treatments are available and have been used in a number of previous studies (26-28). To examine whether Upf2 protein binds to RNA molecules in cells, methanol-fixed cells were treated with RNaseA solution. Following washing of the cells, cells were stained with anti-lamin A/C and anti-Upf2 antibodies, as described in the Materials and methods section. In addition, RBM8A was also stained as a control for the RNase treatment. As shown in Fig. 3, the majority of Upf2 in the cytoplasm and the nucleoplasm
disappeared on RNase treatment. This suggested that appreciable quantities of Upf2 bind to the mRNA molecules in the nucleoplasm, as found for the cytoplasm and the perinuclear region. The signal from lamin A/C remained, and this clearly demonstrated that the structural integrity of the nucleus was not affected by the RNase treatment.

The presence of Upf2-binding RBM8A in the nuclear fraction was subsequently investigated. Following fractionation, Upf2 protein was immunopurified using a specific antibody. RBM8A was detected to a limited extent in the immunoprecipitate of the nuclear fraction, similarly to the cytoplasmic fraction (Fig. 4).

Detection of complex formation between Upf2 and RBM8A using a proximity ligation in situ assay. Finally, to verify the interaction between Upf2 and EJC in the nucleoplasm, a proximity ligation in situ assay with rabbit anti-Upf2 and mouse anti-RBM8A antibodies (a component of EJC) was performed (7-10,29). Signals from the proximity ligation in situ assay were detected under a fluorescence microscope from the nuclei and the cytoplasm. In addition, knockdown of either the Upf2 or the RBM8A gene resulted in a reduction in signal intensity (Fig. 5). Under a confocal laser scanning microscope, sliced images were obtained, and the images revealed the nuclear-localized signals in addition to cytoplasmic signals (data not shown). These findings were not cell-type-specific, since similar results were obtained with human A549 cells under identical conditions. These results suggested that the Upf2 protein resides proximally to RBM8A in the nuclei and cytoplasm, and is included in the EJC.

Discussion

Previous reports have demonstrated that Upf2 binding at the perinuclear region efficiently promotes NMD before translation. Although Upf2 has a putative nuclear localization signal (NLS) sequence and is localized to the perinuclear region, whether Upf2 is present in the nucleus remains unclear. Thus, the current study investigated the presence of Upf2 in the nucleus. The data suggested that nuclear Upf2 co-localizes with mRNPs in the nucleus. Thus, the previously proposed model (10,15,16), which included cytoplasmic binding, requires the addition of a nucleoplasmic fraction.

Taken together, our results suggest that nuclear Upf2 co-localizes with mRNPs in the nucleus. The previously
proposed model (10,15,16), which included cytoplasmic binding, therefore requires the addition of a nucleoplasmic fraction. Since Upf2-associated NMD occurs in the cytoplasm (20), nuclear complex formation may not be associated with the cytoplasmic NMD reaction. In addition, the distribution of the Duolink signal did not perfectly correlate with the localization of Upf2, and complex formation and cytoplasmic Upf2 are able to exist without complex formation with EJC. Therefore, the mechanism that would account for the binding of Upf2 to the EJC in the nucleus has yet to be elucidated. Essentially, the molecular function of Upf2 has not been firmly established, other than the requirement for NMD activity. Since NMD occurs in the cytoplasm (20), given its nuclear function, additional functions for Upf2 may be assumed. In a transcriptome analysis, depletion of Upf2 was demonstrated to cause physiological changes in various genes without PTC (30). Therefore, Upf2 may have additional functions that would be required for the proper development of the human neural system. Additionally, the quantity of Upf2 that resides in the nucleus has yet to be fully established, since there is a possibility that the western blots and immunoprecipitation data also contained nuclear-flanked Upf2. Therefore, further investigations are revealed to reveal additional functions of Upf2, including its role in the nucleus.

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