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The processed isoform of the translation termination factor eRF3 localizes to the nucleus to interact with the ARF tumor suppressor

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ABSTRACT

The eukaryotic releasing factor eRF3 is a multifunctional protein that plays pivotal roles in translation termination as well as the initiation of mRNA decay. eRF3 also functions in the regulation of apoptosis; eRF3 is cleaved at Ala73 by an as yet unidentified protease into processed isoform of eRF3 (p-eRF3), which interacts with the inhibitors of apoptosis proteins (IAPs). The binding of p-eRF3 with IAPs leads to the release of active caspases from IAPs, which promotes apoptosis. Although full-length eRF3 is localized exclusively in the cytoplasm, p-eRF3 localizes in the nucleus as well as the cytoplasm. We here focused on the role of p-eRF3 in the nucleus. We identified leptomycin-sensitive nuclear export signal (NES) at amino acid residues 61–71 immediately upstream of the cleavage site Ala73. Thus, the proteolytic cleavage of eRF3 into p-eRF3 leads to release an amino-terminal fragment containing NES to allow the relocalization of eRF3 into the nucleus. Consistent with this, p-eRF3 more strongly interacted with the nuclear ARF tumor suppressor than full-length eRF3. These results suggest that while p-eRF3 interacts with IAPs to promote apoptosis in the cytoplasm, p-eRF3 also has some roles in regulating cell death in the nucleus.

1. Introduction

eRF3 is an evolutionarily conserved polypeptide chain releasing factor that functions in translation termination and termination-coupled events. The yeast eRF3 gene *GST1* was initially identified as essential gene for G1-to-S phase transition in the cell cycle [1]. The gene was also cloned as the omnipotent suppressor *SUP35* [2]. Two eRF3 genes, *GSPT1* and *GSPT2*, have been identified to date in mammals [3,4], the products of which were later renamed as eRF3a and eRF3b, respectively [5]. eRF3 consists of two regions, an amino-terminal unstructured domain (N-domain) and translation elongation factor eEF1A-like GTP-binding domain (C-domain). The role of eRF3 is well-established in translation termination, in which eRF3 interacts with another releasing factor, eRF1 in its C-domain to accelerate the polypeptide chain releasing reaction catalyzed by the ribosome [6–9].

On the other hand, the N-domain of eRF3 is not necessary for the termination reaction, but interacts with the poly(A)-binding protein PABP to play a pivotal role in the initiation of mRNA decay [10]. eRF3, via an interaction with PABP, also functions in the translation cycle by efficiently recycling the terminating ribosome to the initiation complex [11]. Furthermore, eRF3 was shown to be involved in the initiation of nonsense-mediated mRNA decay (NMD), which rapidly degrades aberrant mRNAs containing premature termination codons [12]. Thus, eRF3 is a multifunctional regulator of gene expression.

The proteolytically processed isoform of eRF3 (p-eRF3) acts as a regulator of apoptosis. eRF3 has been shown to be cleaved at the 73A residue by an unknown protease, leading to the exposure of a conserved inhibitor of apoptosis protein (IAP)-binding motif (IBM) at its N-terminus [13]. Smac/DIABLO and Omi/HtrA2 are IBM-containing proteins that have been extensively examined in humans. Both proteins are localized in the intermembrane space of mitochondria and are released into the cytosol during apoptosis to interact with IAPs, which leads to the activation of caspase due to their liberation from IAP inhibition [14–20]. In a similar manner, the N-terminally processed p-eRF3 also promotes apoptosis by binding with IAPs [13]. This study defined eRF3 as a novel regulator of cell death. The above-described functions of eRF3 are thought to occur in the cytoplasm; however, this study suggested that p-eRF3 may also function in the nucleus; p-eRF3 is localized not only in

Abbreviations: IAP, inhibitor of apoptosis protein; NES, nuclear export signal; ORF, open reading frame.

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the cytoplasm, but also in the nucleus, whereas full-length eRF3 is localized exclusively in the cytoplasm. This prompted us to investigate the mechanism responsible for the nucleocytoplasmic shuttling of eRF3 and the role of p-eRF3 in the nucleus. In the present study, we demonstrate that the amino acid sequence (60–71) of eRF3, which is localized immediately upstream of the cleavage site, acts as a functional NES, and the removal of NES by an unknown protease allows p-eRF3 to localize in the nucleus and interact with the nuclear tumor suppressor ARF, which is known to inhibit cell growth in a p53-dependent or -independent manner [21]. The role of p-eRF3 in the nucleus has been discussed herein.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 5% fetal bovine serum (Sigma) and maintained at 37 °C in 5% CO_2. NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma). HeLa cells were transfected with plasmid using Polyethyleneimine MAX (Polysciences). NIH3T3 cells were transfected with plasmids using LipofectAMINE 2000 (Invitrogen).

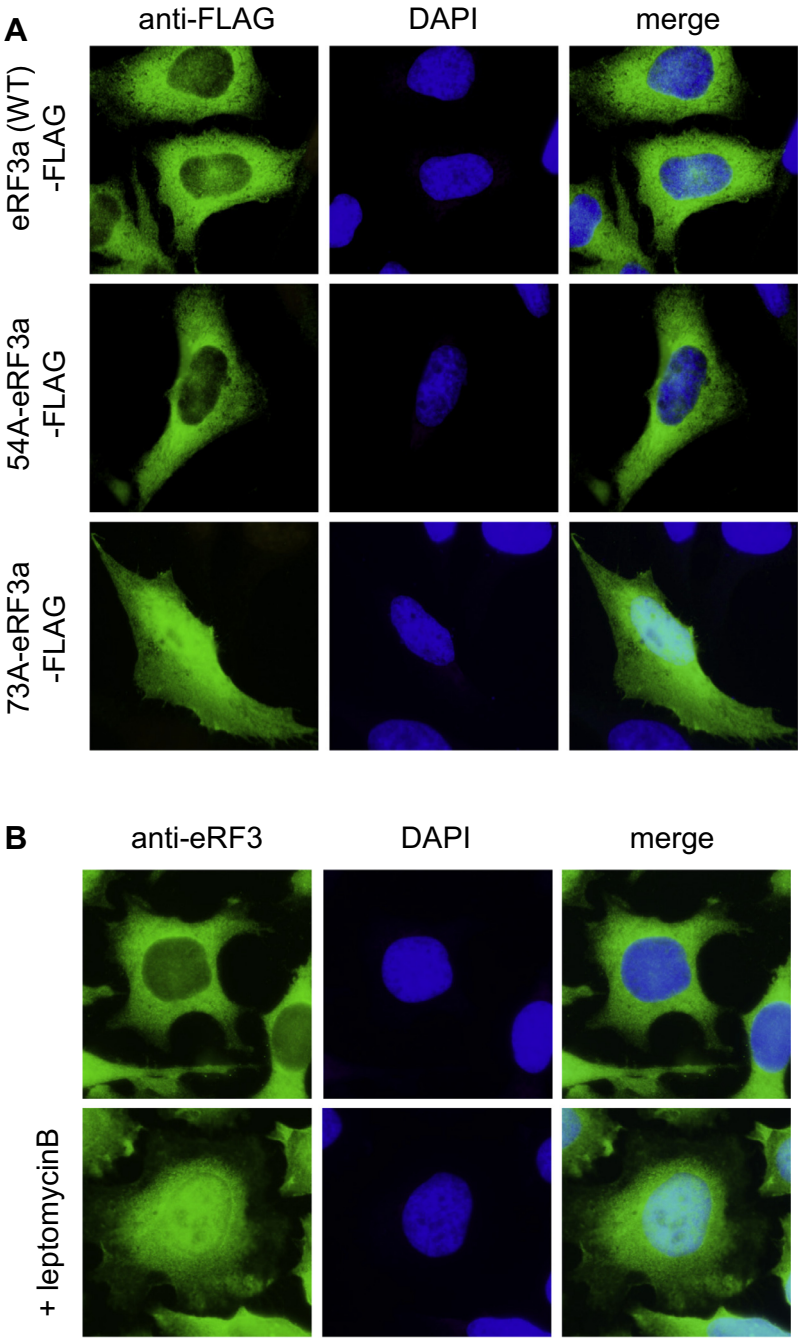

Fig. 1. p-eRF3 localizes in the nucleus as well as the cytoplasm, and is exported from the nucleus in a CRM1-dependent manner. (A) HeLa cells were transfected with pCMV-MycFLAG-Ub-hGSPT1, pCMV-MycFLAG-Ub-(54A)-hGSPT1, and pCMV-MycFLAG-Ub-(73A)-hGSPT1 expressing Ub-(wt)-eRF3a-FLAG, Ub-(54A)-eRF3a-FLAG, and Ub-(73A)-eRF3a-FLAG proteins, respectively, and cells were stained using an anti-FLAG antibody combined with anti-mouse Alexa 488. Nuclei were stained with DAPI. The localization of the FLAG-tagged eRF3 was observed using fluorescence microscopy. (B) HeLa cells treated with or without leptomycin B for 4 h were stained by an anti-eRF3 antibody combined with anti-rabbit Alexa 488. Nuclei were stained with DAPI. The localization of endogenous eRF3 was observed using fluorescence microscopy.

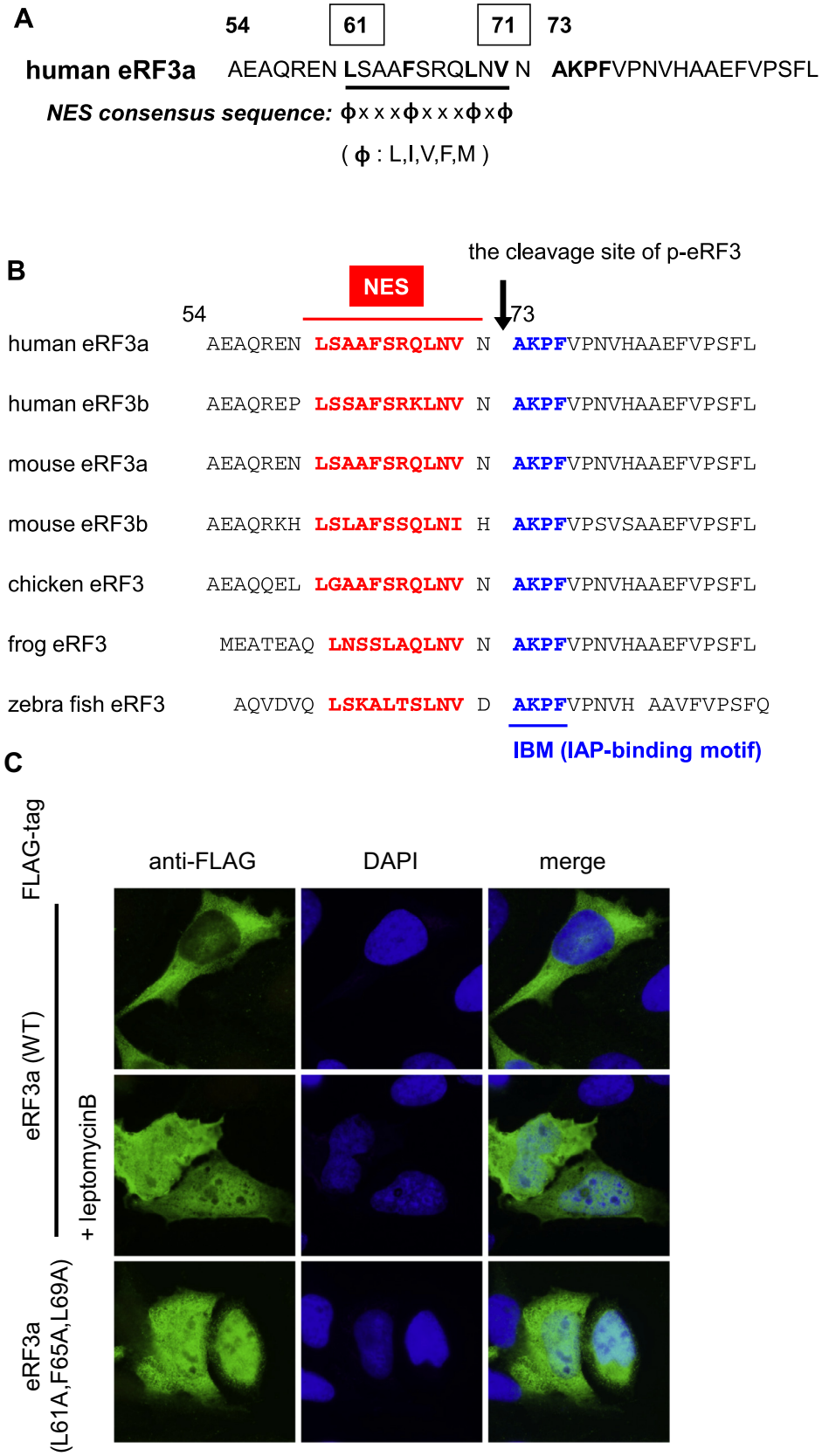

Fig. 2. Identification of the nuclear export signal (NES) motif in eRF3. (A) The leptomycin-sensitive nuclear export signal (NES) in human eRF3a was identified at amino acid residues 61–71 immediately upstream of the cleavage site Ala73. (B) The alignment of amino acid sequences around NES and the cleavage site of p-eRF3. The amino acid sequences of human eRF3a, human eRF3b, mouse eRF3a, mouse eRF3b, chicken eRF3, frog eRF3, and zebra fish eRF3 are shown. (C) HeLa cells were transfected with pFLAG-CMV2-hGSPT1 and pFLAG-CMV2-hGSPT1 (L61A,F65A,L69A), expressing FLAG-eRF3a and FLAG-eRF3a (L61A,F65A,L69A), respectively, and the cells were stained by an anti-FLAG antibody combined with anti-mouse Alexa 488. Nuclei were stained with DAPI. The localization of FLAG-tagged eRF3a was observed using fluorescence microscopy. HeLa cells expressing FLAG-eRF3a, which were treated with leptomycin B, were also shown (+leptomycin B).

2.2. Plasmid

To construct pFLAG-CMV2-hGSPT1 (L61A,F65A,L69A), the corresponding region of hGSPT1 cDNA was amplified by inverse PCR using primer pairs NH149 (5′-AAC GTC AAC GCC AAG CCC TTC-3′)/NH150 (5′-GGC TTG CCG GCT GGC GGC CGC GCT GGC GTT CTC CCG CTG-3′), and pFLAG-CMV2-hGSPT1 [4] as a template. To construct pCMV-5xMyc-p14ARF, ORF of p14ARF was amplified by PCR using primer pairs NH174 (5′-GGC GAA TTC ATG GTG CGC AGG TTC TTG G-3′)/NH175 (5′-TCC TCA GCC AGG TCC ACG GGC-3′), and random primer cDNA library obtained from HeLa cells as a template. The resulting cDNA fragment was digested with EcoRI and inserted into the EcoRI and EcoRV sites of pCMV-5xMyc [22].

2.3. Immunostaining

HeLa cells grown on cover glass were fixed by 4% paraformaldehyde (PFA) in PBS(-) for 15 min. After washing 2 times with PBS(-) containing 10 mM glycine, the cells were permeabilized by PBS(-) containing 1% goat serum and 0.1% Triton X-100 for 15 min. The cells were then washed once with PBS(-) and incubated with an anti-FLAG antibody (Sigma) (1/400 dilution) or anti-eRF3 antibody [23] in PBS(-) containing 1% goat serum in a moisture box overnight. After washing 3 times with PBS(-), cells were incubated with Alexa 488 (Invitrogen) (1/400) and 4′, 6-diamidino-2-phenylindole (DAPI) (Dojindo) (1/1000) in PBS(-) containing 1% goat serum for 90 min, and were then washed 3 times with PBS(-). The cover glass was mounted on a glass slide using Prolong Gold (Invitrogen).

2.4. Immunoprecipitation

NIH3T3 cells were lysed in buffer A (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A) and cells were placed on ice for 15 min after sonication (10 pulses). The supernatant was prepared by centrifugation at 15,000 rpm for 15 min at 4 °C and incubated with anti-Flag IgG agarose (Sigma) for 30 min in the cold room. The resin was washed three times with buffer A. Bound protein was eluted with SDS–PAGE sample buffer and analyzed by Western blotting.

3. Results

3.1. Removal of the amino acid residues (54–72) of eRF3 is responsible for the nuclear localization of p-eRF3

A previous study demonstrated that p-eRF3 interacted with IAPs to promote apoptosis [13]. This study also reported that while full-length eRF3 localized exclusively in the cytoplasm and associated with ER, p-eRF3 was released from the ER and localized both in the cytoplasm and nucleus. These results suggest that eRF3 may be a nucleocytoplasmic shuttling protein. Thus, we attempted to elucidate the mechanism responsible for regulating the subcellular localization of eRF3/p-eRF3. Because p-eRF3 is generated by proteolytic cleavage at Ala73, we hypothesized that the N-terminal region (1–72) released from eRF3 may contain a signal that defines cytoplasmic localization. To examine this possibility, we analyzed the subcellular localization of the N-terminal deletion mutants of eRF3. HeLa cells were transfected with plasmids expressing ubiquitin (Ub)-tagged eRF3a-fusion proteins, in which ubiquitin was fused with eRF3a deletion mutants [23]. According to the Ub fusion protein approach, the Ub moiety of the expressed fusion proteins is cleaved by multiple ATP-dependent proteases, and eRF3a fragments without Ub can be produced [13]. We confirmed that p-eRF3 (73A-eRF3) localized both in the cytoplasm and nucleus, whereas full-length eRF3 localized exclusively in the cytoplasm (Fig. 1A). As we already demonstrated in our previous study that the caspase-cleaved form of eRF3 (33Q-eRF3) localized in the cytoplasm [23], we examined 54A-eRF3 to further narrow the region of eRF3 required for its cytoplasmic localization. 54A-eRF3 exhibited cytoplasmic localization (Fig. 1A). These results indicate that the 19-amino-acid region (54–72) contains a signal that defines the cytoplasmic localization of eRF3.

3.2. eRF3 is exported from the nucleus in a CRM1-dependent manner

Since the CRM1-dependent nuclear export system has already established in detail, we next examined the effects of Leptomycin B, a specific inhibitor of CRM1, on the subcellular localization of eRF3. As shown in Fig. 1B, eRF3 localized not only in the cytoplasm, but also in the nucleus following the Leptomycin B treatment, which indicates that eRF3 is a nucleocytoplasmic shuttling protein that is exported from the nucleus in a manner dependent on CRM1.

3.3. Identification of a functional nuclear export signal (NES) in eRF3

The above results identified eRF3 as a dynamic protein, the cellular localization of which was regulated through the CRM1-dependent nuclear export pathway and the 19-amino-acid region (54–72) contained a cytoplasmic localization signal. We noticed a stretch of hydrophobic residues with a characteristic spacing that resembled leucine-rich NES in the 19-amino-acid region (54–72) (Fig. 2A) [24]. This sequence was highly conserved from zebra fish to humans (Fig. 2B). To confirm that the NES-like sequence identified in eRF3a acts as a functional NES, we introduced alanine mutations into the consensus hydrophobic residues (61LSAAFSRQLNV71). eRF3a (L61A/F65A/L69A) localized both in the cytoplasm and nucleus, as observed for wild type eRF3a in cells treated with LMB (Fig. 2C). We also obtained similar results by using GFP-fused eRF3a (data not shown). These results indicate that 61LSAAFSRQLNV71 of eRF3a is a functional NES. Moreover, p-eRF3 that fused with the amino acid sequence (60–71) of eRF3a at its C-terminus again re-localized exclusively in the cytoplasm, which further supported our conclusion (data not shown). Thus, the proteolytic cleavage of eRF3 by an unknown protease leads to the removal of the NES located immediately upstream of the cleavage site to produce p-eRF3, which in turn allows p-eRF3 to localize in the nucleus as well as the cytoplasm.

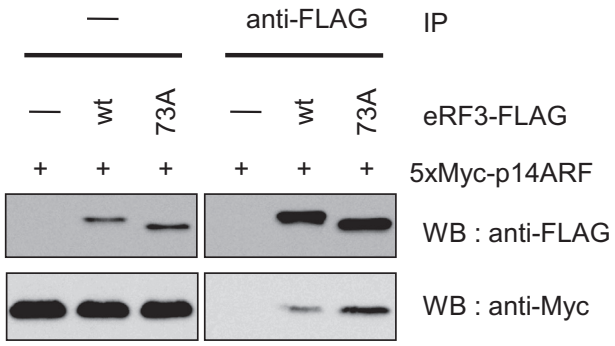

Fig. 3. p-eRF3 interacts with the ARF tumor suppressor in living cells. NIH3T3 cells were transfected with pCMV-5xMyc-p14ARF and either pCMV-MycFLAG, pCMV-MycFLAG-Ub-hGSPT1/eRF3a or pCMV-MycFLAG-Ub-(73A)-hGSPT1/eRF3a. Whole cell extracts were immunoprecipitated (IP) using an anti-FLAG antibody. The immunoprecipitates and inputs were analyzed by Western blotting using anti-FLAG and anti-Myc antibodies.

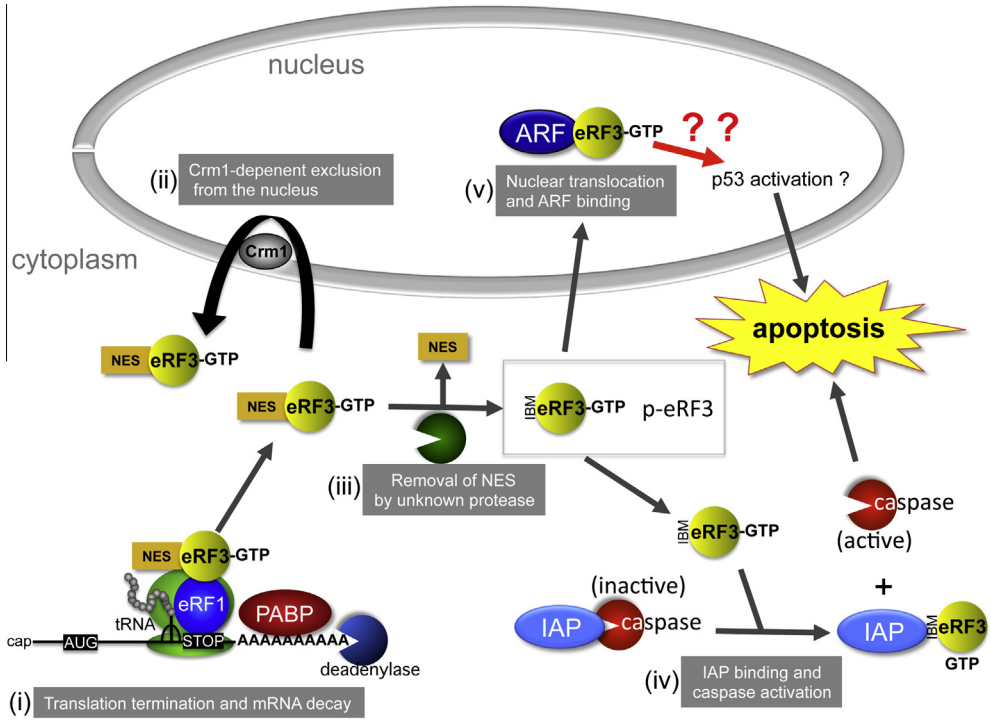

Fig. 4. A hypothetical model describing the possible role of nucleocytoplasmic shuttling of eRF3. (i) In the cytoplasm, eRF3 binds with eRF1 to regulate the termination of translation and termination-coupled initiation of mRNA decay. (ii) NES located immediately upstream of the IAP-binding motif (IBM) of eRF3 acts as a signal to exclude eRF3 from the nucleus. (iii) The removal of NES by an unknown protease produces p-eRF3, which exposes IBM at the N-terminus. (iv) The binding of p-eRF3 IBM with IAPs disrupts their interaction with the initiator caspase-9 and effector caspases-3 and -7, which allows the activation of caspases. (v) The removal of NES allows the nuclear translocation of p-eRF3 and its interaction with the nuclear tumor suppressor ARF.

3.4. p-eRF3 interacts with p14ARF

To gain further insight into the role of nuclear translocated p-eRF3, we next examined the interaction between p-eRF3 and the tumor suppressor p14ARF. A previous study identified p14ARF as a binding partner of eRF3 by yeast two-hybrid screening. eRF3 was shown to bind to p14ARF in an *in vitro* binding assay [25]. However, the interaction between full-length eRF3 and p14ARF was not demonstrated in living cells because eRF3 was localized exclusively in the cytoplasm, whereas p14ARF was in the nucleus. Thus, we expressed 5xMyc-tagged p14ARF and either Flag-eRF3a or Flag-p-eRF3a (73A-eRF3) in NIH3T3 cells, and performed immunoprecipitation experiments with the anti-Flag antibody. As shown in Fig. 3, p-eRF3a (73A-eRF3a) bound more strongly with p14ARF than full-length eRF3a.

4. Discussion

A previous study demonstrated that eRF3 is proteolytically processed into an IAP-binding protein to promote apoptosis [13]. The binding of p-eRF3 with IAPs disrupts their interaction with the initiator caspase-9 and effector caspases-3 and -7, which allows the activation of caspases. In the present study, we have newly demonstrated the following: (i) eRF3 is a nucleocytoplasmic shuttling protein and is exported from the nucleus in a manner dependent on CRM1; (ii) 61LSAAFSRQLNV71 located immediately upstream of the proteolytic cleavage site of eRF3 acts as a functional NES; (iii) the removal of NES allows p-eRF3 to localize to the nucleus as well as cytoplasm; (iv) p-eRF3 more strongly interacts with the nuclear ARF tumor suppressor than full-length eRF3 (see Fig. 4). Thus, the proteolytic cleavage of eRF3 at Ala73 leads to the removal of NES located immediately upstream of the cleavage site to produce p-eRF3, which in turn allows p-eRF3 to translocate to the nucleus and interact with p14ARF. p14ARF is best known for its induction of p53-dependent cell death or growth arrest; p14ARF binds to inactivate Mdm2, an E3 ubiquitin ligase for p53, and stabilize p53 to stimulate the transcriptional activity of p53. Therefore, besides promoting apoptosis by binding to IAPs, p-eRF3 may translocate to the nucleus to further promote apoptosis through the p14ARF-p53 pathway. This possibility is now under investigation in our laboratory.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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