Effect of Modulation of hnRNP L Levels on the Decay of bcl-2 mRNA in MCF-7 Cells

Mi-Hyun Lim¹, Dong-Hyoung Lee¹, Seung Eun Jung¹, Dong-Ye Youn¹, Chan Sun Park², and Jeong-Hwa Lee¹

¹Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul 137-701, ²Bioindustry Research Center, Korea Research Institute Bioscience and Biotechnology, Daejeon 305-806, Korea

It has been shown that CA repeats in the 3'-untranslated region (UTR) of bcl-2 mRNA contribute the constitutive decay of bcl-2 mRNA and that hnRNP L (heterogenous nuclear ribonucleoprotein L) interacts with CA repeats in the 3'-UTR of bcl-2 mRNA, both *in vitro* and *in vivo*. The aim of this study was to determine whether the alteration of hnRNP L affects the stability of bcl-2 mRNA *in vivo*. Human breast carcinoma MCF-7 cells were transfected with hnRNP L-specific shRNA or hnRNP L-expressing vector to decrease or increase hnRNP L levels, respectively, followed by an actinomycin D chase. An RT-PCR analysis showed that the rate of degradation of endogenous bcl-2 mRNA was not affected by the decrease or increase in the hnRNP L levels. Furthermore, during apoptosis or autophagy, in which bcl-2 expression has been reported to decrease, no difference in the degradation of bcl-2 mRNA was observed between control and hnRNP L-knock down MCF-7 Cells. On the other hand, the levels of AUF-1 and nucleolin, transacting factors for ARE in the 3'UTR of bcl-2 mRNA, were not significantly affected by the decrease in hnRNP L, suggesting that a disturbance in the quantitative balance between these transacting factors is not likely to interfere with the effect of hnRNP L. Collectively, the findings indicate that the decay of bcl-2 mRNA does not appear to be directly controlled by hnRNP L *in vivo*.

Key Words: hnRNP L; bcl-2 mRNA stability

INTRODUCTION

Apoptosis is essential for the normal development and maintenance of homeostasis, and a disruption of apoptotic pathways is associated with development of multiple disease states, including cancer [1]. The bcl-2 family of genes encodes specific proteins that regulate programmed cell death in different physiological and pathological conditions [2-4]. Among those genes, Bcl-2 is a representative anti-apoptotic protein, the levels of expression of which determine cellular fates, survival or death, under a variety of conditions. The expression of bcl-2 can be modulated at different levels by modulation of the rate of bcl-2 transcription [5,6]. It has been also shown that the destabilization of bcl-2 mRNA precedes the decreased bcl-2 protein levels, followed by the induction of apoptosis in several types of cells, suggesting that the stability of bcl-2 mRNA is an important determinant of cellular Bcl-2 protein levels [7,8]. mRNA stability is generally determined by interactions of stabilizing or destabilizing proteins with cis-elements that are located either in the coding or noncoding

regions [9]. Several reports have described that a conserved AU-rich element (ARE) is present in the 3'-untranslated region (UTR) of bcl-2 mRNA and that interactions of ARE with a number of ARE-binding proteins (AUBP), including AUF-1, is associated with the decay of bcl-2 mRNA during apoptosis [10-12]. Other transacting proteins such as nucleolin or Tino have been identified to target ARE in the 3'UTR of bcl-2 as a stabilizing or destabilizing protein, respectively [13,14].

On the other hand, in a previous study, we demonstrated that the CA repeats in the 3'UTR of bcl-2, upstream to ARE, exert destabilizing effects on the constitutive decay of bcl-2 mRNA [15]. A recent study reported that heterogeneous nuclear ribonucleoprotein L (hnRNP L) interacts with the CA repeats of bcl-2 mRNA *in vitro* as well as *in vivo*. However, cytosolic extracts from MCF-7 cells, in which hnRNP L levels were down-regulated, have only a partial effect potential on the recovery of the degradation of rates of CA repeats of bcl-2 mRNA, as evidenced by *in vitro* degradation assays [16]. These results suggest that hnRNP L may not be the actual transacting factor for the CA repeats of bcl-2 mRNA. However, *in vitro* decay systems have limitations, in that a short riboprobe is used as a target. The latter is derived from the 3'UTR of bcl-2 mRNA flanking CA repeats, thereby excluding ARE. Moreover, hnRNP L has the ability to

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Corresponding to: Jeong-Hwa Lee, Department of Biochemistry, College of Medicine, The Catholic University of Korea, 505, Banpo-dong, Seocho-gu, Seoul 137-701, Korea. (Tel) 82-2-2258-7293, (Fax) 82-2-596-4435, (E-mail) leejh@catholic.ac.kr

ABBREVIATIONS: hnRNP L, heterogeneous nuclear ribonucleoprotein L; UTR, untranslated region; ARE, AU-rich element.

interact with AUF-1, an ARE-binding protein [17]. It is possible that hnRNP L affects the interaction of ARE and AUBP thereby modulating the stability of bcl-2 mRNA. Therefore, we determined the rate of decay of endogenous bcl-2 mRNA, which includes CA repeats as well as ARE, as a function of the quantity of hnRNP L *in vivo*.

METHODS

Cell culture and transfection

MCF-7 cells, a human breast adenocarcinoma cell line, were maintained in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics solutions (penicillin G 100 unit/ml and streptomycin 100 μ g/ml; Hyclone) at 37°C in a humidified incubator under a 5% CO₂ and 95% humidified atmosphere.

Down-modulation of hnRNP L expression was performed using short-hairpin RNA (shRNA) targeting hnRNP L. Briefly, the annealed double stranded nucleotides (sense: 5'-GATCCCCGTCATACCCCTTACACTCTTTCAAGAGAAGAGTGTAAGGGTATGGACTTTTGGAAA-3' and antisense: 5'-AGCTTTTCCAAAAAGTCCATACCCCTTACACTCTTCTCTTGAAAGAGTGTAAGGGTATGGACGGG-3') were ligated into the BamHI and HindIII sites of the pSUPER.puro vector (OligoEngine, Seattle, WA). MCF-7 cells were trans-

fected with the hnRNP L shRNA -expressing plasmid (sh-hnRNP L) or with the pSUPER.puro vector (vector) alone using a microporator (MP-10; iNCYTO, Suwon, Korea) at 990 V and a pulse width of 50 msec. The over-expression of hnRNP L was accomplished by transfection of one μ g of hnRNP L/pCR3.1 using a microporator, followed by incubation for 48 h in a 6-well plate.

Isolation of total RNA and quantitative real-time RT-PCR

Cells were treated with actinomycin D (Calbiochem, San Diego, CA) to block transcription or to induce apoptosis or autophagy by exposure to staurosporin (200 nM; Sigma, St. Louis, MO) or Hank's balanced salt solution (HBSS, Invitrogen, Grand Island, NY), respectively, for the indicated times. Total RNA was extracted from cells using RNA-Bee reagent (Tel-Test, INC., Friendswood, TX) according to the manufacturer's protocol. For each sample, one μ g of RNA was reverse-transcribed using M-MuLV reverse transcriptase, RNase inhibitor and random hexamers (Fermentas INC., Ontario, Canada). To validate the expression level of each gene, quantitative real-time RT-PCR was performed using an Mx3000P[®] QPCR System (Stratagene, La Jolla, CA) using SYBR[®]Premix Ex TaqTM (TaKaRa BIO INC., Shiga, JAPAN) including ROX dye. The relative expression level was calculated by normalizing the amount of target mRNA to that of 18s rRNA. The primers used in this study

Fig. 1. Down-regulation of hnRNP L expression using shRNA does not affect the degradation of bcl-2 mRNA. (A) MCF-7 cells were transfected with pSUPER.puro vector (vector) or shRNA-hnRNP L (sh-hnRNP L) for 72 h and hnRNP L expression as well as Bcl-2 expression was then examined by western blot. The expression of β -actin was used as a loading control. (B, C) Real-time RT-PCR analysis of hnRNP L mRNA expression or bcl-2 mRNA expression after transfection of sh-hnRNP L for 72 h. The relative levels were normalized to 18s rRNA. The value of vector-transfected MCF-7 cells was arbitrarily designated as 1.0. (D) The decay of endogenous bcl-2 mRNA was measured by Real-time RT-PCR after treatment of actinomycin D. At the indicated time points (0, 2, 4, 6, and 8 h) after addition of actinomycin D (5 μ g/ml), total RNA was extracted from MCF-7 cells transfected with vector (■) or with sh-hnRNP L (▲) and processed for real time PCR as described in Methods. Data were normalized to 18s rRNA and the remaining bcl-2 mRNA is presented as % of initial levels. Values are the mean of three independent experiments $\pm$ SD.

were as follows: hnRNP L (5'-TTGTGGCCCTGTCCAGAGAATT-3' and 5'-GTTTGTGTAGTCCCAAGTATCCTG-3', 18s rRNA (5'-CGCCGCTAGAGGTGATTA-3' and 5'-TTGGCAATGCTTTCGCT), bcl-2 (5'-CTTCCATGTGTGTTGCCGATCA-3' and 5'-CCCAGGGCAAAGAAATGCAAGTGA-3').

Immunoblot assay

Cells were scraped off the plates and transferred to microcentrifuge tubes. The cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.5) with protease inhibitors and immediately sonicated twice for 15 sec each on ice followed by centrifugation at 13,200 rpm at 4°C for 10 min to remove insoluble materials. Equal amounts of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 0.1% TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) containing 5% non-fat dried milk for 1 h and incubated with the following primary antibodies: anti-hnRNP L (1 : 2,000; Santa Cruz, CA), anti- β -actin (1 : 10,000; Sigma), anti-AUF-1 (1 : 2,000; Upstate, Lake Placid, NY), anti-nucleolin (1 : 200; Santa Cruz). Blots were detected using Pierce ECL western blotting substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

RESULTS

Effect of down-regulation of hnRNP L on the decay of bcl-2 mRNA in MCF-7 cells

We previously reported that the degradation of bcl-2 mRNA was related to CA repeats in the 3'UTR of bcl-2 mRNA which was shown to interact with hnRNP L [15, 16]. To determine the contribution of hnRNP L to bcl-2 mRNA decay, MCF-7 cells were transfected with shRNA specific for hnRNP L, followed by actinomycin D chase experiments. The hnRNP L levels in the MCF-7 cells transfected with sh-hnRNP L were decreased to 70% and 78% that of vector-transfected MCF-7 cells in protein levels and mRNA levels, as determined by a western blot and real-time RT-PCR, respectively (Fig. 1A, B). The Bcl-2 protein levels and mRNA levels did not appear to be affected by the down-regulation of hnRNP L (Fig. 1A, C). The decay of endogenous bcl-2 mRNA was then examined by treating cells with actinomycin D, a transcription blocker, and real-time RT-PCR. As shown in Fig. 1D, the rate of degradation of endogenous bcl-2 mRNA in MCF-7 cells that had been transfected with sh-hnRNP L was similar with that in vector-transfected MCF-7 cells. The bcl-2 mRNA remaining at 8 h after three actinomycin D treatment was about 50% both in MCF-7 cells that had been transfected with the vector and sh-hnRNP L. These results suggest that low levels of hnRNP L had no effect on the stability of bcl-2 mRNA.

Effect of the overexpression of hnRNP L on the decay of bcl-2 mRNA

To explore the effect of the overexpression of hnRNP L on the stability of bcl-2 mRNA, MCF-7 cells were tran-

siently transfected with a pCR3.1 vector or hnRNP L/pCR3.1 vector. A western blotting assay indicated that hnRNP L levels were increased by 2.8 fold as the result of the transfection of the hnRNP L-expressing vector (Fig. 2A) compared to control MCF-7 cells that had been transfected with the pCR3.1 vector. We then compared the rates of degradation of bcl-2 mRNA from MCF-7 cells overexpressing hnRNP L and from control MCF-7 cells by chase experiments using actinomycin D. Fig. 2B shows that the half life of the bcl-2 mRNA from MCF-7 cells overexpressing hnRNP L was 4.5 h and the corresponding value for control MCF-7 cells was 4.8 h.

Down-regulation of hnRNP L does not affect bcl-2 mRNA levels during apoptosis or autophagy

Since the down-regulation of hnRNP L had no effect on the constitutive decay of bcl-2 mRNA, we examined bcl-2 mRNA expression during apoptosis in MCF-7 cells in which hnRNP L expression is decreased. The expression of bcl-2 was shown to be downregulated to activate the apoptotic process [18,19]. Thus, we determined bcl-2 mRNA levels in MCF-7 cells treated apoptotic stimulus such as staurosporin up to 12 h after suppression of hnRNP L levels. As shown in Fig. 3A the degree of decrease of bcl-2 mRNA after exposure to staurosporin was not significantly different between MCF-7 cells that had been transfected with the vector and sh-hnRNP L.

Fig. 2. Effect of overexpression of hnRNP L on the stability of bcl-2 mRNA in MCF-7 cells. (A) Western blot for hnRNP L expression after transfection of hnRNP L-expressing vector for 48 h. The expression of β -actin was used as a loading control. (B) bcl-2 mRNA half-life was calculated from decay curves by linear regression between 0 and 8 h as in Fig. 1. Values, shown as mean $\pm$ SD, are based on two independent experiments.

Fig. 3. Decay of bcl-2 mRNA in vector and sh-hnRNP L-transfected MCF-7 cells during apoptosis or autophagy. After the down-regulation of hnRNP L, MCF-7 cells were exposed to 200 nM of staurosporine (A) or incubated with HBSS (B). Total RNA was isolated at the indicated time points and real-time RT-PCR was performed with bcl-2 and 18s rRNA primers as in Fig. 1. The remaining bcl-2 mRNA at each time point was plotted against time as a percentage of its initial value. Results are shown as means $\pm$ SD of three independent experiments.

Bcl-2 has also been shown to be suppressed in starvation-induced autophagy in MCF-7 cancer cells [20]. To induce autophagy, MCF-7 cells transfected with the vector or sh-hnRNP L were incubated in HBSS. The decrease in bcl-2 mRNA levels during autophagy in sh-hnRNP L-transfected MCF-7 cells was not notably different with that in vector-transfected MCF-7 cells. The levels of bcl-2 mRNA remaining 30 min after the induction of autophagy was 25% and 29% of the initial levels in MCF-7 cells that had been transfected with vector and sh-hnRNP L, respectively (Fig. 3B).

Effect of alteration of hnRNP L expression on the levels of other bcl-2 ARE binding proteins

The ARE in the 3'UTR of bcl-2 has been shown to mediate bcl-2 mRNA decay during apoptosis, either by increasing the interaction of a destabilizing factor such as AUF-1 or by reducing interactions with a stabilizing factor such as nucleolin [8,11,12,14]. To investigate whether the down-regulation of hnRNP L expression could affect other proteins that bind to bcl-2 mRNA, we examined the levels of AUF-1 and nucleolin, both of which are binding factors of ARE of bcl-2, after the suppression or induction of hnRNP L levels. A western blotting analysis demonstrated that the expression of nucleolin was not influenced by the down-regulation of hnRNP L or by the induction of hnRNP L in

Fig. 4. Effect of hnRNP L expression on AUF-1 and nucleolin levels. Total protein lysates were prepared from MCF-7 cells transfected with sh-hnRNP L or hnRNP L-expressing vector. A western blot was performed for hnRNP L, AUF-1 and nucleolin levels. The expression of β -actin was included as a loading control.

MCF-7 cells (Fig. 4). The expression of AUF-1, however, was a little bit decreased by suppression of hnRNP L but not apparently affected by increase of hnRNP L (Fig. 4).

DISCUSSION

HnRNP L, an abundant nucleoplasmic protein, is known to function as a splicing regulator which promotes or represses the removal of introns or skipping exons *via* binding to CA repeats in the intron [21-23]. In addition to CA repeats in the intron, hnRNP L has been reported to interact with CA repeats or CA clusters in the 3'UTR of several genes, although the effect of their interaction on the stability of mRNA varies and depends on the target genes. In the case of eNOS pre-mRNA, hnRNP L plays a protective role from RNA cleavage which is specified by CA repeats [21]. Furthermore, an association of hnRNP L with the CA-rich cluster of the 3'UTR of VEGF confers stability to VEGF mRNA upon exposure to hypoxia [24]. However, deletion of the binding sites for hnRNP L in the 3'UTR of iNOS mRNA resulted in destabilization, indicating that hnRNP L promotes the degradation of iNOS mRNA [25]. We previously demonstrated that hnRNP L binds to CA repeats in 3'UTR of bcl-2 mRNA *in vitro* and *in vivo* [16]. However, the consequences of their interaction on the stability of bcl-2 mRNA was not obvious in the previous *in vitro* degradation assay, which is based on the down-modulation of hnRNP L did not provide for a prominent recovery in the decay of the bcl-2 riboprobe, including CA repeats [16].

In the present study, we investigated the issue of whether the stability of endogenous bcl-2 mRNA is influenced by hnRNP L levels in MCF-7 cells. Our results show that the down or up-regulation of hnRNP L has no effect on the constitutive decay of bcl-2 mRNA, as evidenced by actinomycin D chase experiments (Fig. 1, 2). In addition to the constitutive decay, the decrease in bcl-2 mRNA during apoptosis or autophagy was not significantly changed by the down-regulation of hnRNP L in our experiments (Fig. 3). These findings suggest that, even though hnRNP L specifically binds to CA repeats of bcl-2 mRNA, their interaction is not an essential or sufficient requirement for CA-repeats

to mediate the decay of bcl-2 mRNA, which is consistent with the previous *in vitro* degradation results. Thus, in addition to hnRNP L, the participation of an additional protein or proteins, could be required to initiate the CA repeat-mediated degradation of bcl-2 mRNA. In support of this conclusion, we previously observed that a larger complex with less mobility, in addition to the main complex, was formed with the riboprobe of CA repeats bcl-2 in REMSA (RNA electrophoretic mobility shift assays), both of which were supershifted with hnRNP L antibodies, indicating that the larger complex includes hnRNP L, CA repeats in bcl-2 mRNA and probably an unidentified protein [16]. Furthermore, hnRNP L has been shown to associate with several proteins such as AUF-1, hnRNP A2, hnRNP I and hnRNP L itself [17,25,26]. Thus, it is probable that interactions of hnRNP L with another protein *via* CA repeats of bcl-2 mRNA is an important prerequisite for the degradation of bcl-2 mRNA, which is not adequately disturbed by modulation of hnRNP L levels.

ARE is another cis element that is involved in the stability of bcl-2 mRNA, which is located in the 3'UTR of bcl-2 mRNA, 35 bp downstream from the CA repeats. Since the distance between the two elements is not great, it is possible that the decrease in a transacting factor for CA repeats may alter the accessibility of the transacting factor to another cis-element, namely, ARE. Thus, although their expression levels were not significantly affected by the down-regulation of hnRNP L, it can be postulated that the affinity of ARE with AUF-1 or nucleolin may be altered by the decrease in hnRNP L levels, resulting in the destabilizing or stabilizing of bcl-2 mRNA. In either case, the primary effect of hnRNP L on bcl-2 mRNA decay is not likely to be exposed. And it should be also noted that expression of AUF-1 was slightly decreased by reduction in hnRNP L levels, suggesting the possibility of involvement of hnRNP L on the stability of AUF-1 mRNA or protein.

In summary, the findings presented herein indicate that the modulation of hnRNP L expression does not significantly affect the degradation of bcl-2 mRNA in MCF-7 cells, suggesting the complex regulatory mechanism for bcl-2 mRNA stability involving CA repeats and ARE.

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