Identification of Nucleolin as an AU-rich Element Binding Protein Inolved in bcl-2 mRNA Stabilization*  

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bcl-2 mRNA contains an AU-rich element (ARE) that functions in regulating bcl-2 stability. Our earlier studies indicated that taxol- or okadaic acid-induced bcl-2 mRNA destabilization in HL-60 cells is associated with decreased binding of trans-acting factors to the ARE. To identify factors that play a role in the regulation of bcl-2 mRNA stability, bcl-2 ARE-binding proteins were purified from HL-60 cells. Three polypeptides of 100, 70, and 32 kDa were isolated from a bcl-2 ARE affinity matrix. Matrix-assisted laser desorption ionization mass spectrometry analysis identified these proteins as full-length nucleolin and proteolytic fragments of nucleolin. RNA gel shifts assays indicated that recombinant nucleolin and proteolytic fragments of nucleolin, RNA gel shifts assays indicated that recombinant nucleolin (residues 284–707) binds specifically to bcl-2 ARE RNA. In addition, recombinant nucleolin decreases the rate of decapping of mRNA in HL-60 cell extracts in an ARE-dependent manner. Taxol or okadaic acid treatment of HL-60 cells results in proteolysis of nucleolin in a similar time frame as drug-induced bcl-2 mRNA down-regulation. These findings suggest that nucleolin functions as a bcl-2-stabilizing factor and that taxol and okadaic acid treatment induces apoptosis in HL-60 cells through a process that involves down-regulation of nucleolin and destabilization of bcl-2 mRNA.

The mammalian bcl-2 gene encodes a 29-kDa protein that functions as an inhibitor of programmed cell death or apoptosis. Overexpression of bcl-2 is thought to be an important component in the development of B cell lymphomas and certain leukemias (1, 2). In addition to its importance in cancer development, high bcl-2 expression in hematological tumors is frequently an obstacle to cancer chemotherapy (3, 4). Numerous reports describe the effects of anti-cancer and apoptotic agents on the steady state levels of bcl-2 mRNA and Bcl-2 protein (3, 5–7). For example, taxol induces apoptosis in ovarian cancer cells through a process that involves bcl-2 mRNA destabilization (5). Similarly, okadaic acid (OA)1-induced apoptosis of human HL-60 leukemia cells is preceded by destabilization of bcl-2 mRNA as well as down-regulation of Bcl-2 protein levels (6, 8).

Many of the elements that regulate mRNA stability are located in the 3′-untranslated region (3′-UTR) of the mRNA (for review, see Ref. 9). Prominent among these elements are the AU-rich elements (AREs), which are found in numerous short-lived cytokine and oncogene mRNAs. AREs increase the rate of poly(A) shortening as well as the rate of subsequent decay of the mRNA body (10, 11). AREs also appear to increase the rate of decapping of mRNA (12). Cellular factors that interact with AREs have been found to modulate the stability of ARE-containing mRNAs in vivo in both positive and negative fashions. For example, HuR has been found to stabilize ARE-containing mRNAs (13, 14), whereas AUF1 (15) and tristetraproline (16–18) contribute to specific ARE mRNA destabilization. Although the mechanisms by which these proteins modulate the decay of ARE-RNAs is not fully understood, it has been reported that ARE-binding proteins recruit the exosome to ARE-mRNAs (19), thereby promoting rapid 3′-5′ exonuclease-lytic decay (19, 20).

Schiavone et al. (7) reported that there is an ARE in the 3′-UTR of bcl-2 mRNA that is a functional destabilizing element. Subsequently, Donnini et al. (21) found that UVC-induced apoptosis of Jurkat cells leads to decreased bcl-2 mRNA levels accompanied by increased binding of a number of cytoplasmic proteins to a bcl-2 ARE riboprobe. Recently it was found that UVC treatment of Jurkat cells leads to increased binding of the p45 isoform of AUF1 to ARE RNA (22), suggesting that AUF1 plays a role in bcl-2 mRNA destabilization in those cells.

In other cells, less is known about the cis-acting elements or trans-acting factors that regulate bcl-2 mRNA stability in response to apoptotic agents. We have recently reported that HL-60 cell extracts contain proteins that bind to the first ARE in bcl-2 mRNA (ARE 1) (nucleotides 921–1057 of bcl-2 cDNA (1)) and that these proteins are inactive or decreased in HL-60 cells treated with taxol or okadaic acid for 32 h (8). UV-induced RNA cross-linking assays revealed that HL-60 cell extracts contain ~8 proteins ranging in size from 32 to 100 kDa that bind to ARE 1 RNA in vitro (8). Interestingly, RNA cross-linking to ~70- and ~38-kDa proteins was dramatically reduced after 20 h of taxol or OA treatment, and cross-linking to 4 proteins of 45–60 kDa was progressively reduced with 10–34 h of OA or taxol treatment. Taken together these studies suggested that taxol- or OA-induced bcl-2 mRNA destabilization in HL-60 cells involves inactivation of trans-acting factors, which stabilize bcl-2 mRNA through interaction with at least one of the four AU-rich elements present in the 3′-UTR of bcl-2 mRNA. Although these earlier studies ruled out the participation of the mRNA-stabilizing protein HuR in the formation of HL-60 protein-ARE complexes, the identity of the proteins that bind to bcl-2 ARE 1 remained unknown. Accordingly, to shed

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light on the mechanisms of taxol- and OA-induced apoptosis, we identified bcl-2 ARE 1-binding proteins from HL-60 cells that are responsive to taxol and OA treatment. We also examined the effect of one of the ARE-binding proteins on ARE mRNA stability in cell extracts to determine its potential as a modulator of bcl-2 mRNA stability in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture—Human HL-60 leukemia cells (ATCC) were grown in RPMI 1640 medium (In Vitro) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in 95% air, 5% CO2 in a fully humidified incubator. Cells were treated with taxol (200 nM) or okadaic acid (20 nM) as previously described (8).

Preparation of Cell Extracts—For protein purification HL-60 cells (2 × 106) were harvested by centrifugation at 100 × g for 5 min at 4 °C. The cells were washed twice with phosphate-buffered saline and suspended in 50 ml of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% protease inhibitor mixture (Sigma) containing 104 mM (2-aminoethyl)-benzenesulfonyl fluoride, 0.08 mM aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin, and 1.5 mM E-64. Cells were lysed with three 10-s bursts using a Virsonic Sonicator (Virtis) and subjected to low speed centrifugation (10,000 × g) for 10 min followed by centrifugation at 100,000 × g for 1 h at 4 °C to produce a S100 extract.

HL-60 RNA extracts for RNA decay analyses were prepared from 2 × 106 cells. Untreated cells or cells treated with 20 μM OA for 24 h were harvested and washed with PBS and lysed with buffer containing with phosphate-buffered saline, suspended in 200 μl of buffer D (10 mM Hepes, pH 8.0, 3.0 mM MgCl2, 40 mM KCl, 0.1% protease inhibitor mixture, 0.2% Nonidet P-40, 10 mM glycerol, 1 mM dithiothreitol), and incubated on ice for 10 min. The lysate was centrifuged at 10,000 × g for 2 min followed by centrifugation at 100,000 × g for 1 h at 4 °C. Samples were stored in aliquots at −80 °C after flash-freezing on dry ice.

Heparin-Sepharose Column Chromatography—A 5-ml Hi-Trap Heparin-Sepharose column (Amersham Biosciences) was equilibrated with 50 ml of start buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% protease inhibitor mixture) and 750 μl of 40 mM heparin-Sepharose (Amersham Biosciences) was added to the column. The column was washed with 50 ml of wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% protease inhibitor mixture). Before mixing with the biotin-RNA, heparin-Sepharose column fractions were dialyzed against buffer B (100 mM NaCl, 0.1% protease inhibitor mixture) and 10 μl of this was spotted on a nitrocellulose filter. The biotin-RNA was added and the filter washed with 1 ml of buffer B (100 mM NaCl). Bound RNA was eluted with 200 μl of elution buffer (200 mM NaCl, 0.1% protease inhibitor mixture) and concentrated using a Microcon 10 concentrator (Millipore).

RNA Gel Mobility Shift Assays—RNA gel mobility shift assays were performed as described previously (8). Briefly, column fractions (5 μl) or purified protein (2–7.5 μg) were mixed with [32P]RNA transcripts (20,000 cpm) in 20 μl of RNA binding buffer (8) and incubated on ice for 10 min. Samples were analyzed on 15% agarose, Tris-acetate-EDTA gel, which was electrophoresed at 4 °C, dried on nitrocellulose paper, and analyzed by phosphorimaging using a STORM 860 (Molecular Dynamics). For antibody supershift assays, column fractions (10 μl) were mixed with 50 μg/ml monoclonal anti-human nucleolin antibody (Santa Cruz) for 10 min at room temperature before incubation with [32P]RNA. For competition assays, 10× (90 nM) or 25× (225 nM) concentrations of homologous RNA (unlabeled ARE 1) or non-homologous RNA were added to samples containing 20,000 cpm [32P]ARE 1 RNA before the addition of purified recombinant nucleolin. Non-homologous homologous β-globin transcript, a 284-nucleotide CAT transcript, and a 112-nucleotide α-opener transcript. RNA concentrations were determined by absorbance at 260 nm.

Western Blot—Protein concentrations in cell extracts were estimated by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. Equal amounts of protein (40 μg) were boiled in denaturation buffer for 5 min and separated on a 12% polyacrylamide, SDS gel. The proteins were electroblotted to Immobilon-P membrane (Millipore). To confirm equal loading, a Ponceau S stain was included in the gel. The membrane was incubated with 5% (w/v) nonfat dry milk in TBS-T buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) at 4 °C. The membrane was then incubated at room temper-
After washing with buffer C, the SPRINT perfusion chromatography system was equilibrated with buffer C. The loaded onto a 1.7-ml metal chelate (POROS MC-M) column (BioCAD) of 0 mouse secondary antibody (0.2 g/ml) (Santa-Cruz) in fresh blocking buffer. Unbound antibody was removed by four 10-min washes in TBS-T buffer. The membrane was dried, and analyzed by phosphorimaging.

Expression and Purification of Recombinant Nucleolin—A recombinant pET21a plasmid carrying a truncated nucleolin gene encoding residues 284–707 and six histidines (25) was a gift from Dr. France Carrier (University of Maryland). Escherichia coli BL21 cells transformed with pET-ΔNuc-His were grown until the A600 ∼ 0.6 and then induced overnight with 0.4 mM isopropyl-1-thio-D-galactopyranoside at 30 °C. After induction cell pellets were resuspended in buffer C (20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonl fluoride) and lysed by sonication. The lysate was centrifuged at 12,000 g/ml) (Santa-Cruz) for 1 hour at room temperature followed by four 10-min washes with TBS-T. The blot was then developed with lumino reagent (Santa-Cruz). Cruz markers (Santa-Cruz) were used as internal molecular weight standards.

RESULTS

Purification of Proteins That Bind to bcl-2 ARE 1—To purify bcl-2 ARE-binding proteins, HL-60 cell extracts were subjected to heparin-Sepharose chromatography followed by ARE RNA affinity chromatography. In the first purification step, HL-60 S100 extracts were applied to a heparin-Sepharose column equilibrated with start buffer. Proteins were eluted from the column with a linear gradient of 0–1.0 M NaCl. As shown in Fig. 1, proteins were eluted across the entire salt gradient. Fractions eluted from the column were assayed for protein content (Fig 2A) and tested for ARE 1 binding by UV-induced RNA cross-linking (Fig. 2B) and RNA gel mobility shift assays (Fig. 2C). As shown in Fig. 2, fractions 51, 53, 55, and 58 contained proteins that bind to ARE 1 in both assays. In the UV-cross-linking assays the predominant cross-linked proteins were in the 40–60-kDa molecular mass range in the total protein extracts, whereas in heparin column fraction 58, the most efficiently cross-linked proteins were 70 and 100 kDa. This presumably reflects differences in the abundance of these proteins in the different samples. Fractions 57–59 were pooled and selected for further purification because these fractions contained an ~70-kDa protein that potentially corresponds to the 70-kDa protein observed to be responsive to taxol and OA treatment (8). Additionally, these fractions contained proteins that exhibited efficient RNA cross-linking and consisted of the least complex mixture of proteins (Fig. 2A).

To further purify the ARE-binding proteins, fractions 57–59 were subjected to ARE RNA affinity chromatography. An ARE RNA affinity matrix was prepared by coupling ARE 1 RNA synthesized by in vitro transcription to biotin using Photoprobe™ biotin. The biotinylated ARE RNA was incubated with pooled fractions 57–59 in RNA binding buffer and then incubated with avidin D-Vectrex matrix. After extensive washing of the 70-kDa protein observed to be responsive to taxol and OA treatment (8). Additionally, these fractions contained proteins that bind to ARE 1 in both assays. In the UV-cross-linking assays the predominant cross-linked proteins were in the 40–60-kDa molecular mass range in the total protein extracts, whereas in heparin column fraction 58, the most efficiently cross-linked proteins were 70 and 100 kDa. This presumably reflects differences in the abundance of these proteins in the different samples. Fractions 57–59 were pooled and selected for further purification because these fractions contained an ~70-kDa protein that potentially corresponds to the 70-kDa protein observed to be responsive to taxol and OA treatment (8). Additionally, these fractions contained proteins that exhibited efficient RNA cross-linking and consisted of the least complex mixture of proteins (Fig. 2A).

To further purify the ARE-binding proteins, fractions 57–59 were subjected to ARE RNA affinity chromatography. An ARE RNA affinity matrix was prepared by coupling ARE 1 RNA synthesized by in vitro transcription to biotin using Photoprobe™ biotin. The biotinylated ARE RNA was incubated with pooled fractions 57–59 in RNA binding buffer and then incubated with avidin D-Vectrex matrix. After extensive washing of the RNA-biotin-avidin matrix, bound proteins were eluted with a 0.2 M step gradient of 0.2–1.2 M NaCl in buffer B. SDS-PAGE analysis of the eluted proteins (Fig. 3A) indicated that the majority of the proteins in the pooled heparin fractions did not bind to the ARE RNA matrix (compare lanes S and FT). However, the first three salt-eluted fractions contained a small number of proteins including three prominent bands of ~100, ~70, and 32 kDa. The 100-kDa protein was significantly enriched relative to its concentration in the sample applied to the column (Fig. 3A).

Fractions eluted from the RNA matrix were desalted and analyzed by UV cross-linking and gel shift assays. As shown in Fig. 3B, the 0.2, 0.4, and 0.6 M NaCl fractions contained proteins of ~100, 70, and 50 kDa that UV-cross-link to ARE-RNA.
All four salt-eluted fractions also produced a shift in the electrophoretic mobility of ARE 1 RNA (Fig. 4A). Proteins in these fractions exhibited specificity for ARE 1 RNA, as they did not bind to a 40-nucleotide RNA transcript containing a repeat of the AUUUA pentamer ((AUUU)₅A) (Fig. 4B). The different sizes of the ribonucleoprotein complexes (Fig. 4A) presumably are due to binding of more than 1 protein to the 220-nucleotide probe RNA (e.g., different combinations of the 50-, 70-, and 100-kDa proteins may be present in the ribonucleoprotein complexes).

Identification of the 100-, 70-, and 32-kDa Proteins—The 100-, 70-, and 32-kDa bands were selected for further analysis because SDS-PAGE indicated they were enriched in the affinity matrix fractions and they were among the most abundant proteins in the eluted fractions (Fig. 3). Additionally, the 100- and 70-kDa proteins were efficiently cross-linked to ARE RNA (Figs. 2 and 3). The 50-kDa band was not examined further because it was present in lower abundance and was not well separated from other protein bands on the SDS gel. To determine the identity of the selected proteins, the corresponding bands in the 0.4 and 0.6 M NaCl fractions were excised from the Coomassie Blue-stained SDS-polyacrylamide gel (Fig. 3A) and subjected to MALDI-mass spectrometry (MS) analysis. This analysis included in-gel digestion with trypsin followed by MALDI-MS analysis of peptides eluted from each gel slice. Two peptides from the 100-kDa protein were selected for sequencing by tandem mass spectrometry. Comparison of the sequences of the two peptides with the sequence of peptides in the NCBI protein data base using the program BLAST (28) indicated that the two peptides match amino acids 611–624 and 349–352, respectively, of human nucleolin. The mass/charge ratio (m/z) of seven additional peptides in the MALDI mass spectrum matched those of nucleolin. Other peaks in the spectrum were identified as peptides from trypsin or a MALDI mass standard, but no other non-nucleolin peptides were identified. Similar MALDI-MS analysis of the 70- and 32-kDa proteins revealed the presence of peptides that match the mass of nucleolin peptides, indicating they both are proteolytic fragments of nucleolin.

Fig. 2. UV cross-linking and RNA binding assays of heparin-Sepharose column fractions. Selected column fractions were desalted and subjected to UV cross-linking and gel shift assays. A and B, 10-μl aliquots of the indicated fractions were incubated with ³²P-ARE 1 RNA in RNA binding buffer and exposed to UV light (254 nm) for 30 min. Complexes were digested with RNase A and T₁, and proteins were separated by SDS-PAGE. MW, molecular weight markers. A, image of the Coomassie Blue-stained gel. B, phosphorimage of the SDS gel detected on a STORM imager. S, sample. C, gel shift assay. Indicated fractions were incubated with ³²P-ARE 1 RNA in RNA binding buffer. Free and complexed RNAs were separated by electrophoresis on a 1% agarose gel, which was dried and analyzed by phosphorimaging, as in B.

Fig. 3. RNA affinity purification of heparin column fractions. Heparin-Sepharose fractions 57–59 were pooled and incubated with biotinylated ARE 1 RNA. The sample was then incubated with avidin D-Vectrex, and bound proteins were eluted with a step gradient of 0.2–1.0 M NaCl in buffer B. Aliquots of the eluted fractions were assayed for binding to bcl-2 ARE 1 by UV cross-linking as in Fig. 2. After UV treatment, proteins were separated by SDS-PAGE, and the gel was stained with Coomassie Blue (A) and exposed to a phosphorimage plate (B). In panel A, M indicates molecular weight markers. Panels A and B, lane 1, sample (S) applied to column; lane 2, flow-through (FT); lanes 3 and 4, wash (W) fractions; lanes 5–8, fractions eluted with 0.2, 0.4, 0.6, and 0.8 M NaCl, respectively. The asterisks indicate bands excised from the gel for MALDI-MS analysis.
Binding of Nucleolin to ARE 1 in Vitro—Gel shift assays indicated that proteins eluted from the ARE affinity matrix form complexes with ARE RNA but do not bind to (AUUU)_5A RNA. However, these column fractions contained a number of proteins, only some of which may bind to ARE RNA. To confirm that nucleolin is one of the proteins present in the observed ARE-protein complexes (Fig. 4A), a gel mobility supershift assay was performed using anti-nucleolin antibody. As shown in Fig. 5, incubation of 32P-ARE RNA with pooled aliquots of the 0.4 and 0.6 M NaCl fractions from the ARE 1 affinity column produced a shift in the RNA mobility (Fig. 5, lane 2). When ARE RNA and the pooled fractions were incubated in the presence of anti-human-nucleolin monoclonal antibody, a supershifted protein-RNA-antibody complex was observed (Fig. 5, lane 3). Thus, nucleolin is present in the protein-RNA complexes formed with the affinity column fractions. Whether the 100- and/or 70-kDa forms of nucleolin are present in the protein-RNA complexes remains unknown. Also, it is not known if the 60-kDa protein detected in the UV-cross-linking assay (Fig. 3) is a fragment of nucleolin. The partial supershift seen with anti-nucleolin antibody suggests additional proteins may be present in the protein-RNA complexes (Fig. 5).

The UV cross-linking assays suggest that nucleolin binds directly to ARE RNA rather than binding to another protein that is bound to ARE RNA. To confirm this, the ARE RNA binding activity of purified recombinant nucleolin was tested. A fragment of human nucleolin containing a His tag encoded on pET21a-Nuc (25) was expressed in E. coli and purified by Ni²⁺ column chromatography. This recombinant nucleolin (∆Nuc-His), which contains amino acids 284–707, contains the RNA binding domain of nucleolin (residues 284–629) and retains its RNA binding activity (29). As shown in Fig. 6, incubation of ∆Nuc-His with ARE RNA resulted in formation of a protein-RNA complex in a gel shift assay. Thus, recombinant nucleolin can bind ARE RNA in the absence of other HL-60 proteins.

To examine the specificity of nucleolin binding to RNA, homologous or heterologous RNAs were added as competitors in RNA gel shift assays. In the presence of a 10-fold molar excess of unlabeled ARE RNA, the apparent size of nucleolin 32P-ARE RNA complexes was reduced, and no probe RNA was bound in the presence of a 25-fold excess of ARE RNA (Fig. 6). In contrast, the addition of the same concentration of β-globin, CAT, or α-operon RNA transcripts did not inhibit the formation of nucleolin-ARE RNA complexes, as indicated by the absence of free 32P-ARE RNA in the gel shift assay (Fig. 6). Thus, recombinant nucleolin exhibits a ≥25-fold preference for ARE RNA over other mRNAs. The intermediate mobility of the protein-RNA complex observed in the presence of a 10-fold excess of unlabeled ARE RNA (Fig. 6) further suggests that nucleolin-ARE RNA complexes formed in the presence of excess nucleolin contain 2 or more nucleolin molecules.

To examine the effect of taxol and OA on nucleolin in HL-60 cells, Western blotting was performed. As previously reported, treatment of HL-60 cells with taxol or OA reduces the bcl-2 ARE RNA binding activity of cell extracts and leads to bcl-2 mRNA destabilization (8). Results reported here indicate that nucleolin binds specifically to bcl-2 ARE 1, suggesting it may be one of the proteins that plays a role in regulating bcl-2 mRNA stability in HL-60 cells. This raises the question of whether taxol or OA treatment down-regulates or inactivates nucleolin and subsequently decreases binding to bcl-2 mRNA. To address this question nucleolin levels were examined in drug-treated cells by Western blotting. As shown in Fig. 6, untreated HL-60 cells contain a major band corresponding to full-length nucleolin (apparent mass of 100 kDa) and a less intense nucleolin band of ~70 kDa. In cells treated with taxol or OA, the full-length nucleolin band progressively

**Fig. 4.** RNA binding activity of affinity matrix fractions. Aliquots of fractions eluted from the ARE 1 affinity column (Fig. 3) were incubated with 32P-RNA transcripts in binding buffer. RNA-protein complexes were electrophoresed on a 1% agarose-Tris acetate-EDTA gel. A, fractions eluted with 0.2, 0.4, 0.6, and 0.8 M NaCl, respectively, were incubated with a 200-nucleotide 32P-labeled ARE 1 transcript. B, fractions eluted with 0.4 and 0.6 M NaCl, respectively, were incubated with a 32P-labeled transcript containing the sequence (AUUU)_5A.

**Fig. 5.** Nucleolin antibody supershift assay of affinity column fractions. Aliquots of affinity column fractions eluted with 0.4 and 0.6 M NaCl were pooled and incubated with 32P-labeled bcl-2 ARE 1 RNA in binding buffer for 10 min at 4 °C. Anti-nucleolin antibody (lane 3) or buffer (lane 2) was then added to the reaction mixture, and the samples were incubated for 25 min at room temperature. Free (RNA) and complexed RNAs were separated on a 1% agarose-Tris acetate-EDTA gel, which was analyzed by phosphorimaging.

**Fig. 6.** RNA binding specificity of recombinant nucleolin. Recombinant nucleolin (∆Nuc-His) was incubated with 32P-labeled bcl-2 ARE 1 RNA in the presence or absence of a 10× or 25× molar excess of unlabeled competitor RNAs as indicated. After incubation in RNA binding buffer, samples were separated on a 1% agarose-Tris acetate-EDTA gel, which was dried and analyzed by phosphorimaging. RNAf, free RNA.
decreased and proteolytic fragments of 55–85 kDa increased during 10–34 h of drug treatment. Thus, nucleolin proteolysis occurred in a similar time frame as the changes in ARE RNA binding activity that have been observed upon taxol or OA treatment (8). In both untreated and treated cells the amount of nucleolin increased after 10–34 h of growth; however, in untreated cells there was no increase in proteolytic fragments of nucleolin (Fig. 7B).

Nucleolin Stabilization of ARE mRNA in Vitro—To investigate the potential of nucleolin to stabilize bcl-2 mRNA, RNA decay assays were performed using the in vitro assay system developed by Ford and Wilusz (27). Soluble cytoplasmic extracts (S100) were prepared from untreated and OA-treated HL-60 cells. Capped and polyadenylated mRNAs were used in these assays to mimic in vivo decay, which likely involves cap-stimulated deadenylation (30) by poly(A)-specific ribonuclease (31) followed by rapid decay of the mRNA body (27). 32P-Labeled β-globin-, β-globin-ARE, bcl-2-coding region, and bcl-2-coding region-ARE transcripts were synthesized by in vitro transcription reactions in which the 7mGpppG cap was added co-transcriptionally. Poly(A) tails of ~150 nucleotides were subsequently added, and the mRNAs were incubated with S100 extracts in the presence of poly(A) (to activate deadenylation). At various times the reactions were stopped, RNA was isolated, and its recovery was analyzed by gel electrophoresis. As shown in Fig. 8, β-globin-ARE RNA decayed faster than β-globin RNA in untreated (control) HL-60 extracts. This is consistent with the more rapid decay of

![Fig. 7. Western blot analysis of nucleolin in HL60 cells treated with taxol or okadaic acid. A, extracts from cells treated for 0, 10, 20, or 34 h with 20 nM OA or with 200 nM taxol for 10, 20, 24, or 34 h (as indicated) were separated on a 12% polyacrylamide-SDS gel. After electrophoresis, proteins were transferred to Immobilon P paper and probed with monoclonal anti-human nucleolin antibody. B, untreated cells were harvested at 0, 10, 20, 24, and 34 h after dilution into fresh media, and extracts were probed with anti-nucleolin and anti-β-actin antibodies, as indicated.]

![Fig. 8. Effect of nucleolin on decay of β-globin ARE mRNA in HL-60 extracts. A, decay of β-globin-ARE RNA in untreated (Control) or OA-treated HL-60 cell extracts. Nuc, nucleolin. B, decay of β-globin RNA in extracts of OA-treated or untreated HL-60 cells. The effect of nucleolin on decay of each mRNA was assessed by the addition of ∆Nuc-His to control and OA-treated cell extracts before incubation with mRNAs. 5' capped, polyadenylated 32P-labeled RNAs were incubated with S100 extracts and isolated at the indicated times. Extracts were prepared from untreated cells or cells treated for 24 h with OA (20 nM). RNA recovery was assessed by polyacrylamide gel electrophoresis and phosphorimaging. % RNA remaining indicates the amount of full-length mRNA recovered as a function of time of incubation in cell extracts. Data points are the average of two independent analyses; the maximum error range was ± 6%.]

β-globin-ARE RNA than β-globin RNA that was observed in vivo in transfected NIH 3T3 cells (8). Incubation of β-globin-ARE RNA in OA-treated S100 extracts resulted in more rapid decay than in extracts of untreated HL-60 cells (Fig. 8A). This decrease in stability is similar to the destabilization of bcl-2 mRNA observed in vivo after OA treatment of HL-60 cells. In contrast, β-globin mRNA was equally stable in extracts of untreated and drug-treated cells (Fig. 8B). Similar results were observed with transcripts containing a portion of the bcl-2-coding region (bcl-CR) versus a transcript containing the bcl-2-coding region and the ARE (bcl-CR-ARE). Bcl-CR-ARE decayed faster in untreated or OA-treated extracts (Fig. 9A) than bcl-CR transcripts (Fig. 9B). When purified recombinant nucleolin (ΔNuc-His) was added to extracts of OA-treated cells or to control extracts, both β-globin-ARE (Fig. 8A) and bcl-CR-ARE RNA (Fig. 9A) decayed at considerably slower rates. However, decay of β-globin and bcl-CR mRNAs (lacking ARE-1) in drug-treated extracts were not affected by the addition of nucleolin (Figs. 8B and 9B). Similar assays with capped, polyadenylated CAT mRNA (284 nucleotides) showed no effect of nucleolin on mRNA decay rates (data not shown). Thus, nucleolin increased the stability of mRNA in HL-60 cell extracts in an ARE-dependent manner.

**FIG. 9.** Effect of nucleolin on decay of bcl-2 ARE mRNA in HL-60 extracts. A, decay of bcl-CR-ARE RNA in untreated (Control) or OA-treated HL-60 cell extracts. Nuc, nucleolin. B, decay of bcl-CR RNA in extracts of OA-treated or untreated HL-60 cells. The effect of nucleolin on decay of each mRNA was assessed by the addition of ΔNuc-His to control and OA-treated cell extracts before incubation with mRNAs. Decay assays were performed as in Fig. 8. % RNA remaining indicates the amount of full-length mRNA recovered as a function of time of incubation in cell extracts. Data points are the average of two independent analyses; the maximum error range was ± 5%.

**DISCUSSION**

Our previous studies indicated that HL-60 extracts contain ~8 proteins or polypeptides that bind to bcl-2 ARE 1 RNA (8). Prominent among these was a 70-kDa protein that exhibited sharply reduced cross-linking after 10 h of taxol or OA treatment of HL-60 cells. These studies suggested that the 70-kDa protein and possibly others binds and stabilizes bcl-2 mRNA in HL-60 cells. Furthermore, treatment with taxol or OA reduces this binding and leads to bcl-2 mRNA destabilization. In this study we have purified three polypeptides of 100, 70, and 32 kDa by ARE RNA affinity chromatography. MALDI-MS analysis indicated that the polypeptide with an apparent mass of 100 kDa is nucleolin (which has a calculated mass of 76 kDa) and that the 70 and 32 kDa polypeptides are proteolytic fragments of nucleolin. Both the 100 and 70 kDa forms of nucleolin cross-link to ARE 1 RNA, whereas the 32-kDa fragment did not cross-link. The presence of nucleolin in protein-ARE complexes formed with affinity column fractions was confirmed by RNA supershift assays with anti-nucleolin antibody. Gel shift assays further demonstrated that recombinant nucleolin (ΔNuc-His) binds ARE 1 RNA specifically. Western blots confirmed that nucleolin is highly expressed in HL-60 cells. Collectively, these results suggest that nucleolin plays a positive role in the reg-
ulation of bcl-2 mRNA stability. Consistent with this we observed that taxol or OA treatment of HL-60 cells leads to proteolysis of nucleolin in a similar time frame as bcl-2 mRNA down-regulation. Strong support for the concept that nucleolin stabilizes bcl-2 mRNA in HL-60 cells comes from our finding that recombinant nucleolin decreases the decay rate of ARE-containing mRNAs in OA-treated HL-60 cell extracts.

Nucleolin is an abundant, multifunctional protein that exhibits an unusual range of activities, including DNA binding, RNA binding, and possibly DNA-RNA helix unwinding (for review, see Refs. 32 and 33). Nucleolin contains four RNA binding domains that are involved in recognition of a variety of RNAs. In addition to playing an essential role in pre-rRNA processing (34), nucleolin is thought to regulate ribosomal DNA transcription (35–37) and is a component of the transcription factor LR1 (38). Interestingly, nucleolin has also been found to play a role in regulation of the stability of interleukin 2 mRNA (39) and amyloid precursor protein mRNA (40). Nucleolin has also been observed to bind to human preprorenin mRNA (41) and to be a component of a fragile X mental retardation protein (FMRP)-associated messenger ribonucleoprotein (mRNP) particle (42). Stabilization of interleukin 2 mRNA in response to T-cell activation involves binding of nucleolin to a response element in the 5′-UTR of interleukin 2 mRNA. Nucleolin also has been shown to bind to a 29-nucleotide element in the 3′-UTR of amyloid precursor protein mRNA (43) and contribute to the stabilization of the mRNA (44). Interestingly, the predicted secondary structure of bcl-2 ARE 1 contains a stem-loop with a sequence in the loop (UCCCA) that is similar to the loop sequence (UCCCCGA) in the nucleolin recognition element in pre-rRNA (45). However, there is little sequence similarity between bcl-2 ARE 1 and the 29-nucleotide element in amyloid precursor protein mRNA or the 22-nucleotide response element in interleukin 2 mRNA which are bound by nucleolin.

Previous observations that bcl-2 mRNA is destabilized by treatment of HL-60 cells with OA, an inhibitor of protein phosphatases 1 and 2A (46), or taxol, which induces phosphorylation of c-Raf-1 and Bcl-2 proteins (47), suggested that bcl-2 mRNA down-regulation may involve phosphorylation of a stabilizing trans-factor (8). Interestingly, nucleolin is a phosphoprotein that is highly expressed in proliferating cells (48). Nucleolin is a substrate for several kinases including protein kinase C (49), and there is evidence suggesting that nucleolin is a substrate for protein phosphatases (50, 51). Importantly, nucleolin is threonine-phosphorylated by p34cdc2 kinase during mitosis (32, 33). Because taxol is a mitotic inhibitor (52), the accumulation of taxol-treated cells in M phase would be expected to increase the extent of nucleolin phosphorylation. Phosphorylation of nucleolin appears to enhance its auto-degradation and to regulate some of its disparate activities (for review, see Ref. 32).

Based on these reports we hypothesize that in proliferating cells nucleolin binds to and stabilizes bcl-2 mRNA, leading to overexpression of Bcl-2 protein. When cells are treated with okadaic acid or taxol, nucleolin is phosphorylated and subsequently cleaved into fragments that no longer bind bcl-2 mRNA. In the absence of nucleolin and possibly other stabilizing proteins, bcl-2 mRNA is then rapidly degraded via an ARE-dependent decay mechanism. This hypothesis is further supported by the observation that treatment of human fibroblasts with okadaic acid leads to hyperphosphorylation of N60, a proteolytic fragment of nucleolin (53). A similar process likely occurs with taxol treatment, since taxol-induced bcl-2 mRNA decay is blocked by the tyrosine kinase inhibitor genistein (5).

The genetic hallmark of follicular lymphoma is represented by a (t14:18) chromosomal translocation that moves the bcl-2 gene into close proximity to the IgH transcription enhancer (1). Although it is clear that transcription of the bcl-2 gene is enhanced in follicular lymphoma, increased levels of Bcl-2 protein are often observed in other cancers where the (t14:18) translocation has not occurred, such as chronic lymphocytic leukemia, acute myeloid leukemia, multiple myeloma, and metastatic breast cancer (2, 3, 54). This raises the intriguing possibility that in these tumors increased bcl-2 mRNA stability contributes more to the elevated levels of Bcl-2 protein than transcriptional alterations. Thus, deciphering the role of nucleolin in up-regulating the expression of the bcl-2 gene may provide important leads into the molecular mechanisms of cancer development and progression.

In summary, this study has identified a novel mechanism by which a chemotherapeutic drug can induce apoptosis in cancer cells. This mechanism entails the drug-induced down-regulation of nucleolin, a protein involved in stabilization of bcl-2 mRNA. It is possible that other anticancer drugs which inhibit cell proliferation or promote cellular differentiation may also induce apoptosis via nucleolin down-regulation. For example, induction of apoptosis in U937 leukemia cells is accompanied by notable decreases in the levels of nuclear and cytoplasmic nucleolin (55). Under these conditions less nucleolin would be available in the cytoplasm to stabilize bcl-2 mRNA. The potential of nucleolin in having a broader role in cancer development and drug-induced apoptosis is currently under investigation.

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REFERENCES

1. Cleary, M. L., Smith, S. D., and Sklar, J. (1986) Cell 47, 19–28
2. Stehbe, K. G., Jadaz, A., Tepee, D., and Drexl, H. G. (1995) Leukemia (Baltimore) 9, 1841–1845
3. Reed, J. C. (1997) Adv. Pharmacol. 41, 501–532
4. Gao, G., and Dou, Q.-P. (2000) Mol. Pharmacol. 58, 1001–1010
5. Liu, Y., and Priest, D. G. (1996) Cell. Pharmacol. 3, 405–408
6. Riordan, F. S., Foroni, L., Hoffbrand V., Mehta A. B., and Wickremasinghe R. G. (1998) PDES Lett. 435, 195–198
7. Schiavone, N., Rosini, P., Quattrone, A., Dinnini, L., Lupacci, A., Citti, L., Bevilacqua, L., Nicolin, A., and Capaccioli, S. (2000) PASES J 14, 174–184
8. Bandyopadhyay, S., Sengupta, T., Fernandez, D., and Spicer, E. K. (2003) Biochem. Pharmacol. 65, 1101–1102
9. Jacobson, A., and Peltz, S. W. (1996) Annu. Rev. Biochem. 65, 693–739
10. Wilson, T., and Treisman, R. (1988) Nature 336, 396–399
11. Shyu, A-B., Belasco, J. G., and Greenberg, M. E. (1991) Genes Dev. 5, 221–231
12. Gao, G., Wilusz, C. J., Pelz, S. W., and Wilusz, J. (2001) EMBO J. 20, 1134–1143
13. Fan, X. C., and Steitz, J. A. (1998) EMBO J. 17, 3448–3460
14. Peng, S. S., Chen, C. Y., Xu, N., and Shyu, A. B. (1998) EMBO J. 17, 3461–3470
15. Zhang, W., Wanger, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993) Mol. Cell. Biol. 13, 7652–7665
16. Carballo, E., Lai, W. S., and Blackshear, P. J. (1996) J. Biol. Chem. 271, 1001–1005
17. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1996) Mol. Cell. Biol. 16, 4311–4323
18. Raghavan, A., Robison, R. L., McNabb, J., Miller, C. R., Williams, D. A., and Bohanban, P. J. (2001) J. Biol. Chem. 276, 47588–47596
19. Chen, C.Y., Gherzi, R., Ong, E., Chen, E., Rainer, J., Ruijin, G. J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001) Cell 107, 451–464
20. Mukherjee, D., Gao, M., O’Connor, J. P., Rainer, J., Ruijin, G., Luta, C. S., and Wilusz, J. (2002) EMBO J. 21, 165–174
21. Dinnini, L., Lapucci, A., Pagucci, L., Witzert, E., Tempestini, A., Brewer, G., Bevilacqua, M., Nicolin, A., Capaccioli, S., and Schiavone, N. (2001) Biochem. Biophys. Res. Commun. 297, 1063–1069
22. Lapucci, A., Dinnini, M., Pagucci, L., Witzert, E., Tempestini, A., Bevilacqua, M., Nicolin, A., Brewer, G., Schiavone, N., and Capaccioli, S. (2002) J. Biol. Chem. 277, 16139–16146
23. Guise, T. C., and Draper, R. E. (1994) J. Mol. Biol. 241, 246–262
24. Tholankunnel, B. G., Raymond, J. R., and Melbon, C. C. (1999) Biochemistry 35, 15564–15572
25. Yang, C., Maguire, D. A., and Carrier, F. (2002) Nucleic Acids Res. 30, 2251–2260
26. Shyu, A-B., Greenberg, M. E., and Belasco, J. G. (1989) Genes Dev. 3, 60–72
27. Ford, L. P., and Wilusz, J. (1999) Methods Companion Methods Enzymol. 17, 21–27
28. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
Nucleolin Binds to a bcl-2 AU-rich Element
Identification of Nucleolin as an AU-rich Element Binding Protein Involved in \textit{bcl-2} mRNA Stabilization
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