We previously identified a conserved A + U-rich element (ARE) in the 3'-untranslated region of bcl-2 mRNA. We have also recently demonstrated that the bcl-2 ARE interacts with a number of ARE-binding proteins (AUBPs) whose pattern changes during apoptosis in association with bcl-2 mRNA half-life reduction. Here we show that the AUBP AUF1 binds in vitro to bcl-2 mRNA. The results obtained in a yeast mRNA three-hybrid system have demonstrated that the 1–257-amino acid portion of p57 AUF1 (conserved in all isoforms), containing the two RNA recognition motifs, also binds to the bcl-2 ARE in vitro. UVC irradiation-induced apoptosis results in an increase of AUF1. Inhibition of apoptosis by a general caspase inhibitor reduces this increase by 2–3-fold. These results indicate involvement of AUF1 in the ARE/AUF1-mediated modulation of bcl-2 mRNA decay during apoptosis.

The bcl-2 gene encodes the multifunctional Bcl-2 protein known to be involved in cell growth, differentiation control, and prevention of apoptosis (1). Down-regulation of bcl-2 expression is a general response of the cell to apoptotic stimuli (2, 3). The mechanism by which Bcl-2 exerts a protective activity against apoptosis is still unclear, but the mechanism by which bcl-2 expression is regulated has recently been partially elucidated. A large amount of evidence indicates that up- and down-regulation of bcl-2 expression is modulated both at transcriptional and posttranscriptional levels, the latter of which includes mRNA stability and protein activity control. Expression of the bcl-2 gene was known to be regulated transcriptionally by a negative regulatory element (4). Two estrogen-responsive elements within the coding region involved in transcriptional regulation of bcl-2 have also been characterized recently in a breast cancer cell line (5). One of the posttranscriptional control mechanisms of bcl-2 expression has been described to be mediated by phosphorylation of Bcl-2 protein at different amino acid positions (6, 7). Another posttranscriptional mechanism of bcl-2 regulation has been identified in our laboratory. This mechanism modulates bcl-2 mRNA stability and involves a cis-acting A + U-rich element (ARE) located in the 3'-UTR of bcl-2 mRNA (8) that binds to a number of ARE-binding proteins (AUBPs) whose pattern undergoes modifications during apoptosis in association with bcl-2 mRNA decay (9).

AREs represent a class of cis-acting elements that modulate mRNA stability (10). They are present in a variety of mRNAs of genes required to be rapidly and finely modulated under particular conditions, such as response to growth factors, serum starvation, and apoptosis (8, 11–13). GM-CSF and c-fos mRNAs were the first to be studied in detail (14, 15). Compared with the AREs of these genes (16), the bcl-2 ARE possesses a moderate, constitutive, destabilizing activity that is dramatically enhanced upon application of apoptotic stimuli (8, 9).

The mechanism of action of ARE-mediated mRNA decay is under investigation by many laboratories. A number of AUBPs acting as trans-acting factors are known. In particular, some AUBPs belonging to the heterogeneous nuclear ribonucleoprotein family are involved in mRNA localization and stability control (17, 18). Among them, AUF1 and the embryonic lethal abnormal vision-like protein HuR are able to enhance or inhibit mRNA degradation, respectively. For example, there is evidence for binding and stabilization of c-fos, plasminogen activator inhibitor 2, and vascular endothelial growth factor mRNAs by HuR (19–23). This protein has also been implicated in the increase of p53-induced p21WAF1 mRNA after apoptotic stimuli (24). AUF1 was first identified as an mRNA-binding protein with selective affinity for AREs located within mRNAs such as c-myc, c-fos, and GM-CSF (25, 26). Although its destabilizing function is well documented, some studies suggest that AUF1 may have a role in the stabilizing complex on the a-globin mRNA (27) and, more recently, as a parathyroid hormone mRNA-binding protein that modulates mRNA stability (28). AUF1 is comprised of four isoforms of 37, 40, 42, and 45 kDa (29, 30). Although the role of each isoform has yet to be fully characterized, a direct correlation has been observed between each AUF1 isoform’s binding affinity and its RNA destabilizing activity.

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§§ Both authors contributed equally to this work.

†† To whom correspondence may be addressed: Dept. of Experimental Pathology and Oncology, School of Medicine, University of Florence, Viale G.B. Morgagni 50, 50134 Florence, Italy, and Robert Wood Johnson Medical School, Piscataway, New Jersey 08854.

From the 2Department of Experimental Pathology and Oncology, School of Medicine, University of Florence, 50124 Florence, Italy, 1Department of Pharmacology, School of Medicine, University of Milan, 20129 Milan, Italy, and **Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854.
activity toward different AREs, with isoforms p37 and p42 being the most effective (31).

Here, we demonstrate that AUF1 is a bcl-2 mRNA-binding protein and that potentially all its isoforms are able to form complexes with the bcl-2 ARE. At doses able to induce apoptosis, UVC irradiation induced an increase of cytoplasmic levels of the p45 AUF1 isoform, which was paralleled by enhancement of a bcl-2 mRNA-AUF1 complex and mediated by a mechanism that requires caspase activation. These results indicate that ARE-mediated bcl-2 mRNA down-regulation during apoptosis involves AUF1 and suggest different roles for its four isoforms.

**EXPERIMENTAL PROCEDURES**

**Plasmids Used for in Vitro Transcription and Transfection**—The schematic diagram of bcl-2 mRNA and sequences of ARE segments used for either in vitro transcription or cell transfection, bcl-2 ARE+ , a 396-bp segment of bcl-2 3'-UTR ranging from nucleotide 752–1147 of the bcl-2 cDNA sequence (GenBank accession number M14745) and containing the ARE, bcl-2 ARE, an 107 nt ARE that is a segment of bcl-2 evolutionary conserved cDNA sequence and harboring A+U-rich motifs. bcl-2 ARE, a 289 nt segment of bcl-2 3'-UTR ranging from nucleotide 752–1147 of the bcl-2 cDNA sequence (GenBank accession number M14745) carrying a deletion of 107 bp from nucleotide 944–1050 corresponding to the ARE core.

**FIG. 1.** Schematic diagram of bcl-2 mRNA and sequences of ARE segments used for either in vitro transcription or cell transfection. bcl-2 ARE+, a 396-bp segment of bcl-2 3'-UTR ranging from nucleotide 752–1147 of the bcl-2 cDNA sequence (GenBank accession number M14745) and containing the ARE, bcl-2 ARE, an 107 nt ARE that is a segment of bcl-2 evolutionary conserved cDNA sequence and harboring A+U-rich motifs. bcl-2 ARE, a 289 nt segment of bcl-2 3'-UTR ranging from nucleotide 752–1147 of the bcl-2 cDNA sequence (GenBank accession number M14745) carrying a deletion of 107 bp from nucleotide 944–1050 corresponding to the ARE core.

PCR product was amplified with FW1, as described above, and 5'-TTTCGACGTTTGGCGTTAAGACT-3' reverse (RV1) primers. The second PCR product was amplified with 5'-AAAAACTCTCAGACGGGACC-3' (FW2) and RV2 primers. The 12 overlapping nucleotides are underlined in RV1 and FW2 primers. The segment bcl-2 ARE was cloned in plasmid pCRII as described above, yielding pCRII/bcl2 ARE, and used for the synthesis of bcl-2 ARE riboprobe. A 107-bp segment of human bcl-2 mRNA located in the 3'-UTR from nucleotide 944–1050 (a short region with the highest evolutionary conservation containing AUUA pentamers and UUAUUUAU nonamer particularly rich in A+U motifs and therefore considered the ARE core), named bcl-2 ARE (32), was obtained by PCR amplification using the plasmid pBS-SK-H-Bcl-2 (33) as template, with 5'-TCAGAGAGGACGAGGGAG-3' forward and 5'-GATTTCAGCCAGTTACG-3' reverse primers. The bcl-2 ARE product was cloned in pCRII as described above, yielding pCRII/bcl-2 ARE to synthesize the bcl-2 ARE riboprobe. The remaining polylinkers of pCRII/bcl-2 ARE +, pCRII/bcl-2 ARE, and pCRII/bcl-2 ARE were removed by ApaI digestion and religation. Plasmids pBBB4 (obtained by Dr. Ann-Bin Shyu, University of Texas, Houston Health Science Center, Houston, TX) and pBBB-U1 have been described previously (8). Plasmid pBBB-U2 was obtained by cloning the BglII BamHI segment of pCRII/bcl2 ARE into the unique BglII site of plasmid pBBB4. Plasmids pBBB4, pBBB-U1, and pBBB-U2, used for RNase protection analysis of mRNA stability, contain the rabbit β-globin gene transcriptionally driven by the serum-inducible c-fos promoter (pBBB4) with inserted the bcl-2 ARE (pBBB-U1) or bcl-2 ARE (pBBB-U2).

**Cell Lines and Transformations**—The Jurkat T-cell leukemia line (clone E61; European Collection of Animal Cell Cultures) was cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humid-
AUF1 Is a bcl-2 AUBP Involved in Apoptosis

**FIG. 2.** Detection of AUF1 binding to the bcl-2 ARE by REMSA/supershift. Left panel, REMSA/supershifts with bcl-2 ARE+ riboprobe; lane 1, bcl-2 ARE+ riboprobe incubated with cytoplasmic extracts; lane 2, bcl-2 ARE+ riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; lane 4, bcl-2 ARE+ riboprobe incubated with cytoplasmic extracts and anti-TK Ab. Center panel, REMSA/supershifts with bcl-2 ARE. Lane 1, bcl-2 ARE riboprobe; lane 2, bcl-2 ARE riboprobe incubated with cytoplasmic extracts; lane 3, bcl-2 ARE riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; lane 4, bcl-2 ARE riboprobe incubated with cytoplasmic extracts and anti-TK Ab. Right panel, REMSA/supershifts with bcl-2 ΔARE. Lane 1, bcl-2 ΔARE riboprobe; lane 2, bcl-2 ΔARE riboprobe incubated with cytoplasmic extracts; lane 3, bcl-2 ΔARE riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; lane 4, bcl-2 ΔARE riboprobe incubated with cytoplasmic extracts and anti-TK Ab. Lane 1 indicates free RNA digested with RNase T1. Complexes and supershifts are indicated.

**FIG. 3.** Binding of all AUF1 isoforms to the bcl-2 ARE. All AUF1 isoforms synthesized in vitro were assayed in REMSA experiments for RNA binding activity with the three ARE riboprobes. Left panel, the bcl-2 ARE+ riboprobe incubated with each AUF1 isoform. Center panel, the bcl-2 ΔARE riboprobe incubated with each AUF1 isoform. Right panel, the bcl-2 ARE riboprobe incubated with each AUF1 isoform. FR represents free RNA digested with RNase T1. p37, p40, p42, and p45 indicate each AUF1 isoform used in shift experiments. Complexes are indicated.

RNA Electrophoretic Mobility Shift Assay (REMSA) and REMSA/Supershift—Cells (10^7) induced to apoptosis by irradiation with UVC (15 J/m^2, 254 nm), with or without a 2-h pretreatment with 100 μM Z-VAD-fmk (Bachem AG), were collected at various time points, washed with ice-cold phosphate-buffered saline, and lysed in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 40 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 0.2% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 rpm for 30 s in a microcentrifuge, and extracts were processed immediately or stored at −70 °C. REMSAs were performed by incubating the radiolabeled bcl-2 ARE+ bcl-2 ΔARE, and bcl-2 ARE riboprobes (5 × 10^5 cpm) with cytoplasmic protein extracts (30 μg, determined by BCA reagent; Pierce) or with in vitro synthesized AUF1 isoforms in a reaction mixture (20 μl) containing 10 mM Tris, pH 7.5, 0.1 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, 15 units of RNasin (Promega), and 50 μg of heparin for 20 min at room temperature, followed by digestion for 20 min at room temperature with 5 units of RNase T1 (La Roche Ltd.), which cuts RNA downstream to guanosine residues in a number of fragments. Samples were separated on a native polyacrylamide gel (5% polyacrylamide:bisacrylamide, 60:1). In REMSA/supershift experiments, RNase T1 was added after incubation of samples with 50 μg/ml polyclonal rabbit anti-AUF1 antibody (Ab) or with non-specific control anti-transketolase (TK) polyclonal Ab, a kind gift of
Fig. 4. Decay of $\beta$-globin mRNAs containing bcl-2 3'-UTR sequences.

NIH 3T3 cells were stably transfected with the chimeric constructs pBBB4 (rabbit $\beta$-globin gene), pBBB-U1 (bcl-2 ARE + in rabbit $\beta$-globin 3'-UTR), and pBBB-U2 (bcl-2 ARE in rabbit $\beta$-globin 3'-UTR). After serum starvation, 15% fetal calf serum was added to induce a pulse of transcription, and total RNA was extracted at the indicated time points. Autoradiograms show the representative PAGE analysis after RNase protection assays using two probes containing a 188-nt segment of the rabbit $\beta$-globin mRNA or a 120-nt segment of the murine glyceraldehyde-3-phosphate dehydrogenase mRNA, respectively. Phosphorimager-derived values of the rabbit $\beta$-globin protected segments have been normalized for the relevant values of the endogenous glyceraldehyde-3-phosphate dehydrogenase protected segment. Results shown on the graph are the means ± S.E. of three independent experiments.

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AUF1 Is a bcl-2 ARE-binding Protein Involved in Apoptosis

Prof. F. Paoletti (University of Florence), for 20 min at room temperature, electrophoresed as described above, and exposed to Hyperfilm MP (Amersham Biosciences).

Western Blot Analysis—Cytoplasmic proteins (50 μg, prepared as described above) were separated by 12.5% SDS-PAGE, electroblotted (Miniprotein apparatus; Bio-Rad) onto Hybond-C membrane (Amer)_descendants described above) were separated by 12.5% SDS-PAGE, electroblotted (Miniprotein apparatus; Bio-Rad) onto Hybond-C membrane (Ameresham Biosciences), and detected with ECL Western blotting analysis (Amersham Biosciences) with 300 ng/ml anti-AUF1 Ab. Each isofrom was identified on the basis of molecular mass markers (Bio-Rad).

UV-Cross-linking Assay and Immunoprecipitation—Aliquots of cytoplasmic proteins (50 μg, prepared as described above) were incubated with the 32P-labeled bcl-2 ARE riboprobe (106 cpm/μg; 5 × 104 cpm) in the presence of 0.5 mg/ml heparan sulfate and 2 μg of tRNA in a microplate (total volume, 10 μl) at room temperature for 10 min. RNA-protein complexes were cross-linked on ice by exposure to UVC for 5 min with 3000 μW/cm² in a Stratalinker 1800 (Stratagene). Samples were incubated with RNase A (1 μl, 1 mg/ml) for 30 min at 37 °C to digest unbound RNA. Proteins were immunoprecipitated by a 1-h incubation with polyclonal anti-AUF1 rabbit Ab (10 μg/ml), followed by overnight incubation with protein A-agarose (Sigma) at 4 °C. The immunoprecipitates were separated by 12.5% SDS-PAGE, autoradiographed, and analyzed by a Storm PhosphorImager (Molecular Dynamic). The molecular mass of each complex was evaluated on the basis of molecular mass markers (Bio-Rad).

The RNA Three-hybrid System (THS)—The yeast strain L40-coat and plasmid pIIIA/MS2-1, pIIIA/IRE-MS2, pAD-IRP1, and pACT2 (34) were gifts from Dr. M. Wickens (University of Wisconsin). The plasmid pRevR2 (35) was a gift from Dr. U. Putz (University of Hamburg, Germany). The hybrid RNA vector pIIIA/MS2-B2ARE harbors the 107-nucleotide sequence of the bcl-2 ARE region. The sequence was PCR-amplified from pBS-SK-H-bcl-2, with 5'-GACCCGGGT-CAGCTATTTACTGCAAAG-3' forward and 5'-GACCCGGGAGATTCCAAAGACAGGAGG-3' reverse primers, subcloned in pCR2 vector (Invitrogen), and inserted into the SmaI site of plasmid pIIIA/MS2-1. The resulting plasmid, pIIIA/MS2-bcl2, was transformed into L40-coat, and the chimeric RNA levels were assayed by Northern analysis (data not shown). To prevent transcription termination due to an RNA polymerase III termination signal in the poly-U stretch, a mutation at nucleotide 981 was introduced by PCR. The mispaired primers 5'-CATTATATTGTTACATTAAAGGAG-3' (forward) and 5'-CTTAAATGTGAAAAC-3' (reverse) were used to insert a single-base substitution (T→G, as indicated by lowercase letters), and the resulting segment was cloned into the pIIIA/MS2-1 plasmid as described previously. The AUF1 cDNA corresponding to the first 257 amino acids of p37 was amplified from pBAD/HISB-p37AUF1 (36) with the 5'-GAGGATCGGAATGTCGGAGGAGGCAG-3' forward and 5'-GACTCGAGTCTTCTCGCAAATCTCCTGCC-3' reverse primers to test the interaction between AUF1 and bcl-2 ARE. The PCR product was digested and inserted into the BamHI/XhoI sites of pACT2, in-frame with the GAL4 activation domain.

RESULTS

AUF1 Is a bcl-2 ARE-binding Protein Related to bcl-2 mRNA Half-life Regulation—In previous work (9), we demonstrated that the mRNA destabilizing ARE of bcl-2 binds specifically to cytoplasmic AUBPs, whose pattern undergoes modifications during apoptosis. The observation that proteins ranging from 30–50 kDa underwent the most noticeable increase led us to hypothesize that AUF1 could be a bcl-2 ARE-binding protein. To examine this possibility, REMSA supershift analysis of cytoplasmic protein complexes with bcl-2 ARE radiolabeled riboprobes (Fig. 1) has been carried out using an anti-AUF1 Ab and a nonspecific control anti-TK Ab (Fig. 2). When bcl-2 ARE+ or bcl-2 ARE riboprobes are used, the anti-AUF1 Ab (lane 3 of each panel), but not the nonspecific anti-TK Ab (lane 4 of each...
panel), is able to produce a supershifted complex, indicating AUF1 binding. By contrast, when the bcl-2 ΔARE riboprobe (in which the 107-nt ARE core was deleted) was used, supershift did not occur, indicating no AUF1 binding. Other REMSA experiments have been carried out with each AUF1 isoform (p37, p40, p42, and p45) synthesized in vitro. As shown in Fig. 3, all AUF1 isoforms shift bcl-2 ARE+ and bcl-2 ARE riboprobes but not the bcl-2 ΔARE riboprobe, indicating that binding of the bcl-2 mRNA to AUF1 requires the ARE core.

To evaluate the possibility that mRNA destabilizing activity and AUF1 binding of the bcl-2 ARE are two related events, analysis of mRNA half-life in cells expressing a reporter gene with or without the bcl-2 ARE was performed. For this purpose, three NIH 3T3 cell lines stably expressing a rabbit β-globin gene transcriptionally driven by the c-fos serum-inducible promoter with either bcl-2 ARE+ (pBBB-U1) or the ARE-deleted bcl-2 ΔARE (pBBB-U2) or without insert (pBBB4) were used. Following serum addition to induce a pulse of transcription of the serum-inducible pro-

moter with either bcl-2 ARE+ (pBBB-U1) or the ARE-deleted bcl-2 ΔARE (pBBB-U2) or without insert (pBBB4) were used. Following serum addition to induce a pulse of transcription of each reporter gene, total RNA was extracted at various times, and β-globin mRNA levels were quantitated by RNase protection using glyceraldehyde-3-phosphate dehydrogenase as an internal standard (Fig. 4). With respect to the β-globin transcript without insert (BBB4), the insertion of bcl-2 ARE+ (BBB-U1), which binds AUF1, reduces the half-life of reporter mRNA by 6-fold (from >12 h to 2 h). The insertion of bcl-2 ΔARE (BBB-U2), which does not bind AUF1, does not affect the half-life of β-globin mRNA.

All these results clearly indicate that the 107 nt of the ARE of bcl-2 (shared by bcl-2 ARE and bcl-2 ARE+), but not its flanking regions (bcl-2 ΔARE), contain the binding site for AUF1 and are also required for mRNA destabilizing activity. Thus, we have used the bcl-2 ARE as a riboprobe in further experiments.

We assumed that the smallest isoform, p37, was the prototype of AUF1 because its entire sequence, harboring two RNA
recognition motifs, is contained in the other three isoforms. The yeast RNA THS was used to demonstrate that recombinant p37 binds to the ARE of bcl-2 in vivo (Fig. 5). The reporter gene activation of the host yeast was tested in parallel with the indicated positive and negative controls. The 1–257-amino acid segment of p37 containing the two RNA recognition motifs of AUF1 elicited the activation of both LacZ and HIS3 genes, clearly demonstrating that binding of AUF1 to the bcl-2 ARE also occurs in vivo (Fig. 5).

An Increase of AUF1 and the bcl-2 mRNA-AUF1 Complex Are Associated with UVC-induced Apoptosis and Are Partially Prevented by the Caspase Inhibitor Z-VAD-fmk—The possibility that enhanced decay of bcl-2 mRNA during apoptosis is associated with changes of AUF1 levels was evaluated. Jurkat cells were irradiated with UVC at a dose previously established to commit cells to apoptosis (data not shown). REMSA experiments demonstrated an increase of the supershifted AUF1-bcl-2 ARE complex at 8 h compared with no UVC (Fig. 6, compare lane 3 with lane 4). To establish the involvement of the AUF1 isoforms in the increase of supershifted complex, we carried out time course Western blot analyses and immunoprecipitation experiments of UV-cross-linked complexes with anti-AUF1 Ab (Fig. 7, a and b, respectively). Each AUF1 isoform was identified by immunodetection and comparison with standard molecular mass markers. During the time course (0–10 h) after apoptotic stimulation (Fig. 7a), the cytoplasmic p45 isoform markedly increased within 2 h after UVC irradiation, peaking at 10 h. Pretreatment with Z-VAD-fmk attenuated this increase by 2–3-fold. The increase of p37 and p40/p42 isoforms after UVC irradiation was very low and was not affected by Z-VAD-fmk pretreatment. To determine whether the AUF1 variations observed during apoptosis are accompanied by an increase of some isoform-specific ARE binding activity, immunoprecipitation analyses of UV-cross-linked complexes were performed (Fig. 7b). Standard molecular mass markers and labeled AUF1 isoforms translated in vitro and run in the same gel were used for comparison. During the time course, the AUF1 isoform-bcl-2 ARE complex with an apparent molecular mass of about 45 kDa underwent a marked increase, which paralleled the increase of p45 AUF1 (Fig. 7a) in Western analysis and, analogously, is attenuated by pretreatment with Z-VAD-fmk. Another complex with lower molecular mass (about 37 kDa) was detected as a very faint band. No other complexes have been detected within the molecular mass range of the AUF1 isoforms. The additional bands with higher molecular masses observed in the immunoprecipitate (indicated by double-headed arrows) may represent AUF1 isoform dimers or multimers stabilized by UV-cross-linking. From all described results, we conclude that AUF1 is a bcl-2 AUBP and that an isoform-specific mechanism is involved in ARE-mediated bcl-2 mRNA decay during apoptosis. Furthermore, our results suggest that the p45 AUF1 isoform is the most likely candidate to play a pivotal role in this mechanism.
bcl-2 is one of the most studied apoptosis-related genes and is also implicated in cell cycle progression and cell differentiation. The expression of bcl-2 can be finely tuned by a variety of environmental or endogenous stimuli and regulated at both transcriptional (5, 33) and posttranscriptional levels (8). The diverse modes of regulation of bcl-2 expression probably reflect different requirements for down- or up-regulation in different physiological conditions or in pathological processes.

We described a posttranscriptional level of regulation of bcl-2 expression that is mediated by an ARE in the 3’-UTR of bcl-2 mRNA. Under normal conditions, this element has a moderate destabilizing activity toward the bcl-2 transcript. This activity increases following apoptotic stimuli, leading to enhanced bcl-2 mRNA degradation (8). The bcl-2 mRNA ARE binds to a number of AUBPs whose electrophoretic pattern changes after induction of apoptosis (9). A number of AUBPs that modulated mRNA stability have been described for other ARE-containing mRNAs such as VEGF (37, 38), GM-CSF (39, 40), and c-fos (15, 41, 42). We first demonstrated that AUF1 is a bcl-2 AUBP involved in bcl-2 down-regulation during apoptosis. AUF1 is a heterogeneous nuclear ribonucleoprotein protein implicated in mRNA stability regulation, and it shuttles dynamically between the nucleus and cytoplasm (43). Although the four isoforms of AUF1, namely, p45, p42, p40, and p37 (29, 30), are present in both the nucleus and cytoplasm of Jurkat cells (data not shown), all our experiments have been carried out using cytoplasmic extracts because the cytoplasm is the compartment of mRNA degradation. The binding of AUF1 to the ARE of bcl-2 was demonstrated in vitro by means of supershift REMSA experiments. The cytoplasmic extracts were incubated simultaneously with bcl-2 ARE radiolabeled riboprobes and either anti-AUF1 or anti-TK Ab. In these experiments, only the specific anti-AUF1 Ab was able to supershift the bcl-2 ARE-protein complex. All AUF1 isoforms (p37, p40, p42, and p45) have the potential to bind to the bcl-2 mRNA, as indicated in vitro by results obtained in REMSA experiments carried out with each single in vitro-synthesized isoform. Using p37, considered as the AUF1 prototype, in the yeast RNA THS we demonstrated that the AUF1 RNA binding motifs common to all four isoforms are also able to bind to the bcl-2 ARE in vivo.

Deletion of the evolutionary conserved ARE sequence particularly rich in AU motifs (and therefore considered as the ARE core) not only abolishes the ability of the bcl-2 ARE to bind AUF1 but also abolishes its mRNA destabilizing activity. This indicates that the deleted segment harbors the binding site for AUF1 and that AUF1 is required as a trans-acting factor for functional activity of the bcl-2 ARE.

The implication of AUF1 as a trans-acting factor involved in ARE-mediated, bcl-2 mRNA degradation during apoptosis was further demonstrated by results obtained in supershift REMSA experiments carried out after UVC irradiation of cultured cells. Furthermore, we found that the level of the p45 isoform, as evaluated by Western blot, increased markedly until 10 h after UVC irradiation. In the same extracts, the level of one AUF1/bcl-2 ARE complex paralleled the increased level of p45 AUF1, and its molecular mass was also about 45 kDa. This complex was also the only one to undergo a clear decrease in Z-VAD-fmk-treated cells compared with untreated cells. This decrease also parallels the decrease in the 45-kDa isoform observed by Western blot of extracts from cells pretreated with Z-VAD-fmk with respect to untreated cells. Whereas these data are suggestive, the specific AUF1 isoform(s) involved in bcl-2 ARE binding and mRNA degradation during apoptosis could not be precisely identified on the basis of its molecular mass. Nevertheless, our results indicate that at least one AUF1 isoform is a candidate to play a pivotal role in this regulatory mechanism.

Arao et al. (43) found that p45 and p42 are predominantly nuclear proteins and that, consequently, the observed increase, if actually attributable to one of these isoforms, could be explained by nuclear to cytoplasm shuttling. This possibility is supported by our previous observation that the apoptotic stimulus by cycloheximide (50 μM), utilized to block protein synthesis, resulted in an analogous increase in bcl-2 ARE-bound, cytoplasmic AUBPs (9). In our model, the relative balance of the AUF1 isoforms seems to determine the fate of the bcl-2 mRNA in response to apoptotic stimuli. We speculate that this balance could be affected in response to other types of endogenous and environmental stimuli. Results obtained from Western blot as well as immunoprecipitation assays suggest that binding of AUF1 isoforms to bcl-2 mRNA in cellular extracts could also depend on the other amino acid sequence motifs by which the isoforms differ.

We also speculate that the scarce signal of the relatively low molecular mass complex (about 37 kDa), the absence of other complexes in the range of the AUF1 molecular masses, and the presence of higher molecular mass complexes in immunoprecipitates could result from the AUF1 isoform ability to multimerize as reported by Wilson et al. (44). Our results also strengthen the hypothesis that the various isoforms of AUF1 may reflect the need of cells to differentially modulate ARE-containing mRNAs during apoptosis. Furthermore, mRNA turnover of ARE-containing genes has already been demonstrated to be a finely tuned process. For example, Wang et al. (24) demonstrated that the ARE-containing mRNA of p21<sup>WAF1</sup>, a p53-inducible gene responsible for cell cycle inhibition upon UVC irradiation, was stabilized by the AUBP HuR. This would account for the arrest of cell growth that occurs at the first stages of apoptosis. Other conditions, such as hemin-induced erythropoietic differentiation, are able to impair ARE activity by sequestration of AUF1 into a complex of proteins (45).

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AUF1 Is a bcl-2 A + U-rich Element-binding Protein Involved in bcl-2 mRNA Destabilization during Apoptosis

Andrea Lapucci, Martino Donnini, Laura Papucci, Ewa Witort, Alessio Tempestini, Anna Bevilacqua, Angelo Nicolin, Gary Brewer, Nicola Schiavone and Sergio Capaccioli

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