Characterization of p43(ARF), a Derivative of the p43 Component of Multiaminoacyl-tRNA Synthetase Complex Released during Apoptosis*

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In human, nine aminoacyl tRNA synthetases are associated with the three auxiliary proteins, p18, p38, and p43, to form a stable multiprotein complex. The p43 component, which has a potent tRNA binding capacity, is associated to the complex via its N-terminal moiety. This protein is also the precursor of the endothelial monocyte-activating polypeptide II (p43(EMAPII)), corresponding to the C-terminal moiety of p43, a cytokine generated during apoptosis. Here we examined the cellular pathway that, starting from the p43 subunit of the complex, leads to this extracellular cytokine. We identified a new intermediate in this pathway, named p43(ARF) for Apoptosis-released Factor. This intermediate is produced in cellulo by proteolytic cleavage of endogenous p43 and is rapidly recovered in the culture medium. This p43 derivative was purified from the medium of human U937 cells subjected to serum starvation. It contains 40 additional N-terminal amino acid residues as compared with the cytokine p43(EMAPII) and may be generated by a member of the matrix metalloproteinase family. Recombinant p43(ARF) is a monomer in solution and binds tRNA with a $K_d$ of $-6 \times 30$-fold lower than that of p43. Highly purified p43(ARF) or p43(EMAPII) do not stimulate the expression of E-selectin by human umbilical vein endothelial cells. Our results suggest that the cleavage of p43 and its cellular delocalization, and thus the release of this tRNA binding subunit from the complex, is one of the molecular mechanisms leading to the shut down of protein synthesis in apoptosis.

Protein synthesis is essential for cell growth. In cells undergoing apoptosis, the translation process is rapidly inhibited and correlates with caspase-dependent cleavage of the eukaryotic translation initiation factors eIF3, eIF4B, and eIF4G (1) and with phosphorylation of eIF2a (2). Inhibition of protein synthesis by addition of cycloheximide to culture cells also promotes apoptosis. Translation is not completely inhibited at early stages of apoptosis. For instance, the mRNA encoding antia apoptotic proteins XIAP or c-Myc may be produced, allowing cell recovery during the process of programmed cell death. By contrast, translation is not inhibited during the process of necrotic cell death (3). Although modification of translation initiation factors seems to be one of the primary steps of regulation of translation in apoptotic cells, other components of the protein synthesis machinery, including aminoacyl-tRNA synthetases, are also the target of apoptotic proteases (4, 5).

In higher eukaryotic organisms, from Drosophila to mammals, the nine aminoacyl-tRNA synthetases specific for amino acids Glu, Pro, Ile, Leu, Met, Gln, Lys, Arg, and Asp form a Multiaminoacyl-tRNA Synthetase complex (MARS)5 with the three auxiliary proteins p18, p38, and p43 (6). The p43 subunit is an RNA-binding protein (5) organized around a pseudo-dimeric OB-fold-based domain (7). It occupies a central position within the multisynthetase complex (8). It has been proposed that p43 might play a role of a cofactor for aminoacylation (9), but this function remains a subject of controversy (10). The presence of p43, or of p43-related proteins, in the cytoplasm but also in the nucleus of rabbit kidney cells has been observed by immunoelectron microscopy (11).

The p43 component of MARS is also the precursor of the Endothelial-monocyte-activating polypeptide II (p43(EMAPII)) isolated from methylcholanthrene A-induced fibrosarcoma cells, a cytokine generated during apoptosis (12–14). The mature p43(EMAPII) has been ascribed to a proinflammatory cytokine that stimulates chemotactic migration of polymorphonuclear granulocytes and mononuclear phagocytes and induces tissue factor activity on endothelial cells. Whether p43(EMAPII) or its precursor, the p43 component of MARS, is the real cytokine remains controversial (13, 15). p43 as well as p43(EMAPII) have also been reported to have antiangiogenic properties that

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5 The abbreviations used are: MARS, multiaminoacyl-tRNA synthetase; EMAPII, endothelial monocyte-activating polypeptide II; ARF, apoptosis-released factor; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; GFP, green fluorescent protein; DTT, dithiothreitol; 7-AAD, 7-amino-actinomycin D; FACS, fluorescence-activated cell sorter; IL-3, interleukin 3.
**p43(ARF), an Apoptosis-released Factor**

would limit establishment of neovasculature and thus would suppress tumor growth (16, 17).

We previously reported that the p43 component of MARS is a substrate for caspase 7 (5), but other data also indicated that p43 may not be primarily cleaved by this apoptotic protease (18), suggesting that the maturation of p43 into p43(EMAPII) may involve intermediate maturation stages. In this report, we investigated the pathway of p43 cleavage during apoptosis, we identified and characterized a new intermediate referred to as p43(ARF) for Apoptosis-released Factor, and analyzed the subcellular localization and the putative functions of p43, p43(ARF), and p43(EMAPII) during development of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Induction of Apoptosis**—The 32D murine myeloid precursor cell line (gift from Matthias Clauss, Max-Planck-Institut, Bad Nauheim, Germany) is dependent on an exogenous supply of IL-3 for growth (19). 32D cells were grown in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml of penicillin and streptomycin, and induced to undergo apoptosis by removal of tetracycline from the culture medium. Stably transfected cells were cultured in the absence of FCS to induce apoptosis and stained with the vital dye 7-amino-actinomycin D (7-AAD) and with Annexin V-PE (BD Biosciences) and subjected to FACS analysis (PAS-III; Partec) to identify early apoptotic cells (Annexin V-PE-positive and 7-AAD-negative).

**Purification of p43(ARF) from the Supernatant of U937 Apoptotic Cells**—U937 cells (10⁸ cells) growing in RPMI medium containing 10% FCS were washed three times in RPMI without FCS and incubated in serum-free RPMI medium for 5 days at 37 °C. Cell culture medium was centrifuged at 40,000 × g for 20 min at 4 °C, diluted 1.5-fold by addition of 60 mM Tris-HCl, pH 7.0, and 0.03% Tween 20, and directly applied to a Mono S HR 5/5 column. Elution was achieved by a linear gradient (60-column volume) of NaCl from 0 to 300 mM in 20 mM Tris-HCl, pH 7.0, 2 mM DTT, and 0.01% Tween 20. Fractions containing p43(ARF), detected by Western blotting, were dialyzed (20 mM NaCl) and eluted by a linear gradient (40-column volume) of NaCl from 0 to 250 mM. Fractions containing p43(ARF) were dialed (20 mM Tris-HCl, pH 8.5, 2 mM DTT, and 0.01% Tween 20), applied to a Mono Q HR 5/5 column equilibrated in the same buffer, and eluted by a linear gradient (50-column volume) of NaCl from 0 to 300 mM. Fractions containing p43(ARF) were concentrated by ultrafiltration (Vivaspin 500, 5000 MWCO) and subjected to N-terminal amino acid sequence analysis by automated Edman degradation.

**Protein Overexpression and Purification**—The cDNA fragment encoding p43(ARF) was produced by PCR with oligonucleotides 5'-cccctcagtgtctgagagcataaa and 5'-cccctcagtgtctgagagcataaa and inserted into the NcoI-XhoI sites of the bacterial expression vector pET-28b (Novagen) to give pET/p43(ARF). The construct was verified by DNA sequencing.

The protein encoded by pET/p43(ARF) was expressed in *Escherichia coli* BL21(DE3) grown in Luria Bertani medium supplemented with kanamycin (50 µg/ml). Culture (1.5 liters) was grown at 37 °C to an _A₆₀₀_ of 0.5, transferred at 28 °C, and expression was induced by addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside for 4 h. Cells were washed twice with ice-cold extraction buffer (30 mM Tris-HCl, pH 7.0, 30 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM DTT), resuspended in 23 ml of the same buffer containing 1 mM diisopropyl-fluorophosphate, and sonicated. All subsequent steps were conducted at 4 °C. After centrifugation at 18,000 × _g_ for 30 min, the clear
supernatant was applied to a 33-ml S-Sepharose FF column (Amersham Biosciences). p43(ARF) was eluted by a linear gradient (25-column volume) of NaCl from 50 to 350 mM in 20 mM Tris-HCl, pH 7.0, containing 1 mM DTT. After extensive dialysis against 20 mM Tris-HCl, pH 7.0, and 1 mM DTT, fractions were applied to a 25-ml Q-Sepharose FF column (Amersham Biosciences) equilibrated in the same buffer. The flowthrough fraction was recovered and immediately applied to a 30-ml Source 15S column equilibrated in 20 mM Tris-HCl, pH 7.0, 50 mM NaCl, 1 mM DTT and eluted by a linear gradient (23-column volume) of NaCl from 50 to 300 mM. Fractions containing p43(ARF) were concentrated by ultrafiltration, dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 mM sodium bis, 29:1, containing 5% glycerol, and stored at −20 °C at a protein concentration of ~30 mg/ml. Protein concentration was determined by using a calculated absorption coefficient of 0.35 A₄₅₀ units-mg⁻¹·cm⁻².

**Sedimentation Equilibrium**—Ultra-centrifugation experiments were conducted as described previously (23) in a Beckman Optima XL-A analytical ultracentrifuge, using a 60 Ti rotor and a double sector cell of 12-mm path length. Equilibrium was verified from the superimposition of duplicate scans recorded at 4-h intervals. The experimental sedimentation equilibrium data were fitted to a model for a single homogeneous species following Equation 1

\[
c(r) = c(r_{ref}) \exp[(M_s(1 - \nu)\omega^2/2RT)(r^2 - r_{ref}^2)] \quad (\text{Eq. 1})
\]

where \( c(r) \) is the protein concentration at radial position \( r \), \( c(r_{ref}) \) is the concentration of the protein at an arbitrary reference radial distance \( r_{ref} \), \( M_s \) is the molecular mass, and \( \nu \) the partial specific volume (0.733 at 4 °C for p43(ARF)) of the solvent, \( \rho \) is the density of the solvent, \( \omega \) is the angular velocity of the rotor, and \( R \) and \( T \) are the molar gas constant and the absolute temperature, respectively.

**Gel Retardation Assay**—Protein-tRNA interactions were assayed using a band shift assay as previously described (5). The genes for human tRNA₂Met, for rabbit elongator tRNA₂Met, and for minihelices corresponding to the acceptor-TΨC stem-loop domain of these two tRNAs, placed under the control of the T7 polymerase promoter, were subjected to in vitro transcription as described (10, 24). Briefly, ³²P-labeled tRNAs were synthesized in a reaction mixture (50 μl) containing 1 μg of linearized template DNA, 40 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol, 0.01% Triton X-100, 1 mM each CTP, UTP, and GTP, 10 μM [α-³²P]ATP (200 Ci/mmol), 1000 units/ml T7 RNA polymerase. After incubation at 37 °C for 1 h, the transcripts were purified by electrophoresis on a denaturing 12% polyacrylamide gel (mono:bis, 19:1), recovered from the gel by soaking in H₂O, precipitated with ethanol, and resuspended in 5 mM MgCl₂. Transcripts were renatured by heating at 90 °C and slow cooling (90 to 30 °C in 2 h).

Homogeneous protein was incubated at increasing concentrations with radiolabeled tRNA in an 11-μl volume containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% glycerol, and bovine serum albumin at 0.1 mg/ml. After incubation at 25 °C for 30 min, the mixture was placed on ice and loaded on a 6% polyacrylamide gel (mono:bis, 29:1) containing 5% glycerol in 0.5× Tris borate-EDTA at 4 °C. After electrophoresis, the gel was fixed, dried, and subjected to autoradiography. Free and bound tRNA were quantified by densitometry analysis. Because the amount of labeled transcripts added in the assays was negligible compared with the amount of protein added, concentration of protein at which half of the tRNA formed a complex corresponded to the apparent Kᵰ value of the complex.

**Confocal Imaging**—The cDNAs encoding human p43 and its derivatives were introduced between the EcoRI and BamHI sites of pEGFP-N1 (BD Biosciences). HeLa-ST cells were grown in F12 medium supplemented with 10% FCS. Cells were transfected with Effectene (Qiagen). Cells were grown in 8-well Lab-Tek II chambers (Nalge Nunc International) and observed by confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope.

**RESULTS**

**Release of p43(ARF) during Apoptosis**—When murine 32D cells were subjected to apoptosis by IL-3 deprivation, p43(EMAPII) was recovered in the culture medium (Fig. 1) as reported previously (4). The release of mature p43(EMAPII) is observed after 16–20 h of IL-3 withdrawal. However, several other p43-related polypeptides, the native p43 polypeptide and a proteolytic intermediate of ~26–28 kDa, were also observed in the culture medium (Fig. 1). We also detected the presence of other proteins in the culture medium, including the 76-kDa species of ArgRS, an integral component of MARS, 12 h after onset of apoptosis (results not shown). The finding that the matured form of p43, p43(EMAPII), but also the p43 and ArgRS proteins that are integral components of the MARS complex, were found in the culture medium but were not subjected to proteolysis suggested that cytolysis also occurred in the population of apoptotic 32D cells. Accordingly, ~30–40% of the cells were permeable to trypan blue after 12 h of IL-3 starvation, as observed previously (19). Thus, the presence of p43(EMAPII) in the culture medium could be attributed either to secretion from apoptotic cells or to leakage from permeable necrotic cells. Therefore, we looked for a cell type where cytolysis would not rapidly occur after onset of apoptosis.
were counted (a 800-ml culture containing 10^9 U937 cells was subjected to apoptosis. To characterize the p43 derivative generated during apoptosis, the degradation product of p43 was named p43(ARF), for Apoptosis-released Factor. No p43(EMAPII) degradation product could be detected in the culture medium of U937 cells subjected to apoptosis. The human promonocytic U937 cell line undergoes apoptosis before cytolysis occurs (25). When U937 cells were subjected to serum starvation, DNA fragmentation and cleavage of poly(ADP-ribose) polymerase, two landmarks of apoptosis, occurred after 52–68 h of serum withdrawal (Fig. 2). The viability of the cells, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, decreased after 52 h of starvation but the extent of dead cells that did not exclude trypan blue remained constant after more than 100 h of incubation (Fig. 2). In the meantime, antibodies directed to p43(EMAPII) were used to monitor the level of p43 in cell extracts prepared at different times of starvation. The p43 content of U937 cells drastically decreased after 92 h of serum deprivation, and a single polypeptide of ~26 kDa could be detected in the culture medium after 52–68 h of onset of apoptosis (Fig. 2). This polypeptide was distinct from p43(EMAPII) used as a marker. Only trace amounts of this polypeptide could be observed in the cellular fraction. By contrast with 32D cells, no polypeptide related to ArgRS, a marker of MARS, was detected in the culture medium fraction (results not shown). These results clearly showed that the p43 component of MARS is subjected to proteolysis during progression of apoptosis and that a degradation product is released in the culture supernatant. This degradation product of p43 was named p43(ARF), for Apoptosis-released Factor. No p43(EMAPII) degradation product could be detected in the culture medium of U937 cells subjected to apoptosis.

Purification and Structural Characterization of p43(ARF)— To characterize the p43 derivative generated during apoptosis, a 800-ml culture containing 10^9 U937 cells was subjected to serum withdrawal for 5 days and p43(ARF) was purified from the cell culture supernatant following three chromatographic steps on MonoS, MonoQ, and MiniS PE columns. The elution of p43(ARF) was monitored by Western blotting with antibodies directed to p43(EMAPII). About 0.2 μg of p43(ARF) was isolated and its N-terminal sequence was determined by automatic Edman degradation. The sequence SGTEQIKGG was obtained and exactly matched the sequence of human p43 from residues 107 to 116. The calculated molecular mass of p43(ARF) is 22.5 kDa, in reasonably good agreement with the apparent mass of 26 kDa estimated by SDS-PAGE. The recombinant p43(ARF) protein produced in E. coli displayed an electrophoretic mobility similar to p43(ARF) isolated from the supernatant of U937 cells. The discrepancy between the observed and calculated masses is certainly related to the aberrant migration of p43, which also displays an apparent molecular mass of 43 kDa for a calculated mass of 34 kDa.

We showed previously that caspase 7, an apoptotic protease, is able to convert in vitro p43 into p43(EMAPII) (5). The sequence of cleavage of p43 into p43(ARF) (103TTVS SGTK110) does not correspond to any consensus cleavage site for a protease of the caspase family. Matrix metalloproteinases, including macrophage elastase, correspond to a family of proteases that possess a broad capacity to cleave components of the extracellular matrix but also non-matrix proteins that regulate a variety of biological processes (26). Recombinant p43 and the MARS complex were subjected to controlled proteolytic digestion by elastase (Fig. 3). In both cases, the free and MARS-associated forms of p43 were cleaved by elastase to give a polypeptide of ~26 kDa, comigrating with recombinant p43(ARF), which was resistant to further proteolysis. The N-terminal amino acid sequence, SXGTKE, of the polypeptide recovered after elastase digestion of recombinant p43 was determined by Edman degradation. It is located 1 amino acid residue upstream of the N-terminal sequence of p43(ARF) isolated from apoptotic U937 cells. This suggests that, in vivo, elastase could be involved in processing p43 into p43(ARF), with the additional release of the N-terminal Ser residue by an aminopeptidase. Alternatively, another member of the large matrix metalloproteinase family with a slightly different specificity might be involved in the processing of p43 into p43(ARF). Whatever the protease involved in the cleavage, our data show that this segment of p43 is freely accessible.

Recombinant p43(ARF) was expressed in E. coli from the pET28b plasmid but without a His tag. Indeed, because the
calculated isoelectric point of p43(ARF) is very basic (pI of 8.72), the protein eluted from the sulfopropyl cation exchanger at a NaCl concentration of 260 mM was already more than 80% homogeneous. The purified recombinant p43(ARF) is readily soluble at a protein concentration up to 30 mg/ml. It displayed an apparent molecular mass of 26 kDa by SDS-PAGE (Fig. 3). The oligomeric structure of p43(ARF) was determined by sedimentation equilibrium (Fig. 4). We previously determined that p43 is a dimer in solution, whereas p43(EMAPII) is a monomer (5). The p43 derivative p43(EMAPII) is a compact domain based on an OB-fold structure (7). When p43(ARF) was subjected to centrifugation equilibrium at an initial protein concentration of 33 μM, experimental data could be fitted to a single species with a molecular mass of 22,641 ± 812 Da. The monodispersity of p43(ARF) in solution, bearing a 40-amino acid residue extension as compared with p43(EMAPII), containing 12 Lys residues, suggests that p43(ARF) is a discrete entity with a well defined structural organization. Taking into account a calculated molecular mass of 22,504 Da for the monomer, we concluded that p43(ARF) is a monomer in solution. Thus, the site of dimerization of p43 is comprised within the first 106 amino acid residues of the protein.

**Test of the Cytokine Properties of p43(ARF)**—The matured form of p43, p43(EMAPII), is thought to be an active cytokine that activates endothelial cells (13), causing a rise in Ca²⁺ concentration, the release of von Willebrand factor antigen, cell surface expression of P-selectin, the induction of tissue factor activity, and an enhanced expression of E-selectin. However, other reports have suggested that p43 itself, the precursor of p43(EMAPII), could be a real cytokine (15). To analyze the cytokine function of p43, p43(ARF), and p43(EMAPII), we examined the expression of E-selectin, one of the major landmarks of vascular inflammation induced by various cytokines such as IL-1β and tumor necrosis factor α (27) by HUVEC cells subjected to various stimuli. Addition of p43 at high concentration produced an increase in E-selectin expression (Fig. 6). When p43 was heat-treated (100 °C, 10 min) prior to incubation on a native gel (Fig. 5). The apparent $K_d$ determined for rabbit elongator tRNA^Met or for human tRNA^Arg was ~6 nm. Using the same tRNA substrates, $K_d$ of ~40 and ~0.2 μM were observed for p43(EMAPII) and p43, respectively (not shown). Stable complexes were also observed with p43(ARF) in the presence of minihelices representing the acceptor-TΨC stem-loop domains of these two tRNAs, with $K_d$ of ~11 nm (Fig. 5).

Thus, the strong interaction observed between p43(ARF) and these RNA substrates did not require the L-shaped structure of the tRNA molecules.

**FIGURE 3. Cleavage of p43 by elastase.** The rabbit MARS complex (a) or recombinant human p43 (b) were subjected to controlled elastase digestion at a protein:elastase ratio of 1:1000 or 1:10, respectively. At the times indicated, proteolysis was stopped by heating with SDS. The samples were analyzed by SDS-PAGE, and p43-related polypeptides were visualized by Western blotting with anti-p43(EMAPII) antibodies (a) or by Coomassie blue staining (b). Recombinant p43(ARF) and p43(EMAPII) were used as markers.

**FIGURE 4. Human p43(ARF) is a monomer in solution.** Recombinant p43(ARF) (initial concentration of 33 μM) was analyzed by equilibrium sedimentation at 15,000 rpm in 20 mM Tris-HCl, pH 7.5, 100 μM NaCl, 10% glycerol, and 1 mM DTT at 4 °C. Experimental values (open circles) were fitted (curves) to a monodisperse solute of 22,641 ± 812 Da. The residuals are indicated.

**p43(ARF), an Apoptosis-released Factor**
tion, the stimulation was decreased. Preincubation of p43 with anti-p43 antibodies had only a limited effect. By contrast, incubation of HUVEC with p43(ARF) or p43(EMAPII) at high concentration did not stimulate E-selectin expression.

To further understand the possible role of p43, p43(ARF), and p43(EMAPII) during the development of programmed cell death, these three proteins were expressed in human cells under the control of an inducible promoter. In a tetracycline-free medium, the three recombinant proteins were expressed to a level close to that of endogenous p43, as assessed by Western blot analysis with anti-p43(EMAPII) antibodies (results not shown). The aim of this experiment was to determine whether expression of p43 or of the matured products p43(ARF) or p43(EMAPII) may trigger apoptosis or may modify its time course of appearance and development. Alternatively, because all p43 is processed into p43(ARF) under apoptotic growth conditions (Fig. 2d), inactivation of this RNA binding cofactor associated with the nine aminoacyl-tRNA synthetases of MARS could be the main defect generated by the cleavage of the p43 component of MARS. The time course of apoptosis development was assessed by FACS after staining cells with 7-AAD, a nucleic acid dye excluded from viable cells, and with Annexin V-PE, which binds to phosphatidylserine that is translocated to the outer side of the plasma membrane at the early stages of apoptosis (Fig. 7). Expression of plasmid-borne p43, p43(ARF), or p43(EMAPII) in addition to endogenous p43 was not lethal for the cells and did not cause any noticeable phenotype. In particular, expression of p43 in excess neither accelerated nor impaired the cellular program of apoptosis (Fig. 7). Expression of p43(ARF) or of p43(EMAPII), the two p43 derivatives produced during apoptosis, did not induce the cells to enter apoptosis in the absence of other stimuli. When apoptosis was induced by serum starvation, expression of p43(ARF) had only a moderate effect on the time course of apoptosis development (Fig. 7). The percentage of apoptotic cells (annexin V-positive and 7-AAD-negative) was slightly increased in the presence of p43(ARF): 9.8, 14.7, 31.3, and 49.4% of apoptotic cells after 72, 80, 96, and 104 h of serum starvation, as compared with 7.2, 7.3, 16.7, and 28.8% in the absence of plasmid-borne p43(ARF). These results do not support the idea that the release of p43(ARF) or of p43(EMAPII) from MARS would be sufficient to induce apoptosis but leave open the possibility that the main cellular consequence of the cleavage of p43 might be related to the general breakdown of protein synthesis in apoptotic cells.

Subcellular Localization of p43 and of p43 Derivatives—To investigate the consequence of p43 cleavage on its subcellular localization, p43, p43(ARF), and p43(EMAPII) were fused with the green fluorescent protein (GFP) appended to their C-terminal ends. The fusion proteins were expressed in human cells for transient expression studies, and their stability and in vivo localization were analyzed 24 h after transfection. The various p43-GFP fusion proteins were not degraded in vivo as assessed by Western blot analysis with anti-GFP antibodies (results not shown). Their subcellular localization was analyzed by confocal microscopy on living cells. The recombinant p43 component of MARS was only found in the cytoplasmic compartment (Fig. 8). It should be noticed that the p43 protein, even when overexpressed after transient transfection in human cells, was com-
pletely excluded from the nuclear compartment. As a control, GFP alone showed a diffuse pattern throughout the cell, with the exception of the nucleoli that displayed a limited GFP fluorescence. By contrast with p43-GFP, the p43(ARF)-GFP and p43(EMAPII)-GFP fusion proteins were found to be present in the cell cytoplasm but also in the nucleus (with the exception of the nucleoli). Thus, the release of the C-terminal domain of p43 upon apoptosis is accompanied by a delocalization of p43(ARF) or p43(EMAPII) into the whole cell and the OB-folded RNA-binding domain of p43(ARF) is no more excluded from the nuclear compartment. A GFP fusion protein containing the N-terminal domain of p43 upstream from the cleavage site of p43(ARF), from residues 1 to 106, was exclusively found in the cytoplasm (results not shown).

**DISCUSSION**

The p43 subunit of MARS, a 312- amino acid polypeptide, is involved in apoptosis. It was initially isolated as p43(EMAPII), a cytokine generated in apoptosis (13). This derivative of p43 corresponds to its C-terminal domain starting from residue Ser147. It can be generated by cleavage with caspase 7, an apoptotic protease, both in cellulo (4) and in vitro (5). Recombinant p43 is also the substrate of other proteases, but they generate proteolytic products distinct from p43(EMAPII) (18). Whether the precursor (p43) or its maturation product (p43(EMAPII)) is secreted from apoptotic cells is not well established (4, 15). Here we characterized a new maturation product of p43 in human U937 cells induced in apoptosis.

As compared with other cell types used in previous studies, this promonocytic cell line can be induced in apoptosis independently of cytolysis. Induction of apoptosis in murine 32D cells or in human adenocarcinoma cell lines resulted in the recovery of p43, p43(EMAPII), and of uncharacterized intermediates in the culture medium (4, 15, 28). The appearance of a maturation product of p43 in the cytoplasm of U937 cells induced to undergo apoptosis is concomitant with DNA fragmentation, but it does not accumulate within the cell. Indeed, it is rapidly and specifically released from apoptotic cells and was therefore referred to as an Apoptosis-released Factor, p43(ARF). Neither its precursor (p43 from MARS) nor any other component of MARS was detected in the culture medium of apoptotic cells.

Concerning the putative function of p43 as a cytokine, the following observations are puzzling. Native recombinant p43, the precursor of p43(ARF), and p43(EMAPII) have been reported to have the capacity of inducing migration of the endothelial cells (17). The full-length recombinant p43 showed the highest activity (17), but the p43 subunit of MARS was exclusively found associated within the complex in extracts of exponentially growing human cells (14). This suggests that association of p43 within the MARS complex is a means to regulate the balance between the two functions of p43 and to sequester this protein in the cytoplasm of growing cells, as shown by the subcellular localization of a p43-GFP fusion protein. Although p43 could be _per se_ a bona fide cytokine, its association with the synthetases would withdraw it from this cellular process. Thus, complex formation would be a means to regulate the spatio-temporal activity of p43. It has also been
reported that the cytokine activity of p43(EMAPII) could be mimicked by a synthetic peptide, RIGRIVTVLE (29), but the crystal structure of p43(EMAPII) showed that only the side chains of Val163 and Thr164 are accessible to protein interactions (7), suggesting that the effects of the peptide are serendipitous. We also show in this work that highly purified preparations of p43, p43(ARF), or p43(EMAPII) do not induce the expression of E-selectin, a marker of the induction of endothelial cells, when used at concentrations similar to well known cytokines. This suggests that at least some of the effects previously ascribed to p43 or to some of its derivatives should be carefully reexamined. The finding that p43(ARF) is produced intracellularly in U937 but does not accumulate within the cell suggests that it is secreted and fulfills another function, as p43(ARF) or as a polypeptide subjected to an additional maturation step, p43(EMAPII).

Even if the amount of p43(ARF) recovered within the cell is low, a small but significant amount may localize to the nucleus, as exemplified by the finding that a p43(ARF)-GFP fusion protein could be translocated to the nucleus. This observation may explain some of the findings previously reported. For instance, p43-related polypeptides were identified within the nucleus by immuno-gold labeling on ultrathin sections of kidney cells, suggesting that p43 may also play a role in this cellular compartment (11). However, we show here that the p43-GFP fusion is efficiently excluded from the nucleus, whereas the p43(ARF)-GFP and p43(EMAPII)-GFP fusion proteins are no more excluded from the nuclear compartment. Thus, p43(ARF) may have in the nucleus a non-canonical function in addition to the role of its precursor in cytoplasmic protein synthesis. This function may be related to its high propensity to associate with nucleic acids.

Interestingly, monomeric p43(ARF) binds nucleic acids with a more than three orders of magnitude increase in affinity as compared with p43(EMAPII). The N-terminal polypeptide sequence of p43(ARF), made of 40 additional amino acid residues as compared with p43(EMAPII), is very rich in charged residues (2 Asp, 6 Glu, and 12 Lys) and displays a calculated isoelectric point of 9.79. This highly charged polypeptide may be responsible for the aberrant mobility of p43(ARF) observed by SDS-PAGE. The consensus sequence EKKXEKKXEKK-GEKK, containing four EKK motifs, is extensively conserved from Xenopus to human (supplemental Fig. S1). The precursor p43 also contains this signature sequence but binds nucleic acids with a 30-fold lower affinity. Thus, the conversion of p43 into p43(ARF) is accompanied by the exposure of this RNA binding site or by a conformational change of this domain that builds a high affinity binding site for nucleic acids. The possibility that the domain carrying this consensus sequence is not freely accessible in the native p43 protein is also supported by the observation that complete elastase digestion of the p43 component of MARS requires 100-fold higher protease concentrations than that required for the cleavage of recombinant p43. The accessibility of the highly charged segment of p43, also carrying the cleavage sites leading to p43(ARF) and p43(EMAPII), depends on the structural state of the protein.

The involvement of the components of MARS in pathways other than tRNA aminoacylation is not unusual. For instance, the bifunctional GluProRS of MARS is able to translocate to another complex that causes translational silencing of specific mRNAs (30), and LysRS may form an alternative complex with MITF and Hint and play a role in the activation of the microphthalmia transcription factor (31). In both cases, these are full-length components of MARS that are recruited in alternative complexes, which should cause profound structural rearrangement of MARS and may impair its activity in global translation. Concerning p43, after proteolytic cleavage, p43(ARF) loses its ability to associate with other components of MARS and is released in the intracellular space, but the N terminus polypeptide of p43, from residues 1 to 106, retains the capacity to associate within MARS and the structural integrity of the particle is not affected.6 In contrast to necrotic cells that retain the capacity to synthesize proteins (3), translation inhibition occurs in apoptosis (1). Modification of initiation factors by phosphorylation or caspase-dependent cleavage is one of the major control points of translation regulation in apoptosis. The cleavage of p43 that supports translation when associated within MARS could be an additional control to regulate translation at the elongation step. An efficient shut down of protein synthesis at early stages of apoptosis may contribute to avoidance of production of abnormal or inflammatory proteins that would trigger an inflammatory response that is generally not observed in apoptosis.

The two cleavage sites on p43, after Ser106 and after Asp146, are well conserved among species. The two consensus sequences TTSøS106↓, where ø is a hydrophobic amino acid residue, and SAD146↓SK, for a putative matrix metalloproteinase and for caspase 7, respectively, are conserved from hen to human (supplemental Fig. S1). This suggests that the same series of events leads to the release of the p43 component of MARS in these cell types. However, MARS is ubiquitous to all metazoan species, from Drosophila to human, studied so far (32). Thus, because the two consensus cleavage sites are not recovered from the p43 component of MARS from fish or Xenope (supplemental Fig. S1), the possibility that p43 may have a role as a switch in translation and as a putative cytokine seems to be a recent advance in evolution.

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