The EMAP II Cytokine Is Released from the Mammalian Multisynthetase Complex after Cleavage of Its p43/proEMAP II Component*

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Endothelial-monocyte-activating polypeptide II (EMAP II) is an inflammatory cytokine released under apoptotic conditions. Its proEMAP II precursor proved to be identical to the auxiliary p43 component of the aminoacyl-tRNA synthetase complex. We show here that the EMAP II domain of p43 is released readily from the complex after in vitro digestion with caspase 7 and is able to induce migration of human mononuclear phagocytes. The N terminus of in vitro-processed EMAP II coincides exactly with that of the mature cytokine isolated from conditioned medium of fibrosarcoma cells. We also show that p43/proEMAP II has a strong tRNA binding capacity (K_D = 0.2 μM) as compared with its isolated N or C domains (7.5 μM and 40 μM, respectively). The potent general RNA binding capacity ascribed to p43/proEMAP II is lost upon the release of the EMAP II domain. This suggests that after onset of apoptosis, the first consequence of the cleavage of p43 is to limit the availability of tRNA for aminoacyl-tRNA synthetases associated within the complex. Translation arrest is accompanied by the release of the EMAP II cytokine that plays a role in the engulfment of apoptotic cells by attracting phagocytes. As a consequence, p43 compares well with a molecular fuse that triggers the irreversible cell growth/cell death transition induced under apoptotic conditions.

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 are associated within a multienzyme complex containing three auxiliary proteins, as well (1). The p38 auxiliary component contributes a scaffold protein for the assembly of the complex (2, 3). The p18 subunit of the complex might be an anchor for auxiliary proteins, as well (1). The p38 auxiliary component contributes a scaffold protein for the assembly of the complex (2, 3). The p18 subunit of the complex might be an anchor for auxiliary proteins, as well (1). The p38 auxiliary component contributes a scaffold protein for the assembly of the complex (2, 3). The p18 subunit of the complex might be an anchor for auxiliary proteins, as well (1). The p38 auxiliary component contributes a scaffold protein for the assembly of the complex (2, 3). The p18 subunit of the complex might be an anchor for auxiliary proteins, as well (1). The p38 auxiliary component contributes a scaffold protein for the assembly of the complex (2, 3). The p18 subunit of the complex might be an anchor for auxiliary proteins, as well (1).
Purification of the Multisynthetase Complex from Mouse Liver—Liver (160 g) from 120 mice were homogenized in a Waring Blendor (2 × 15 s) after addition (1 ml per g) of extraction buffer (50 mM Tris- HCl, pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol) containing protease inhibitors (1 mM diisopropyl phosphofluoridate, 2 mM phenylmethylsulfonyl fluoride). Extract was cleared by centrifugation at 30,000 × g for 30 min and subjected further to high speed centrifugation at 260,000 × g for 2 h. The supernatant was applied on a 1,700-ml (5 × 85 cm) Sephacryl S-400 HR column (Amersham Pharmacia Biotech) equilibrated in Buffer A (75 mM potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol, and 10% glycerol) and developed at a flow rate of 4 ml/min. Fractions with LysRS activity were combined and applied on a 30-ml (1.6 × 15 cm) tRNA-Sepharose column (21) developed at a flow rate of 1 ml/min. The complex was eluted with a linear gradient of potassium phosphate buffer (75 to 350 mM). After a 4-fold dilution with a solution containing 2 mM DTT and 10% glycerol, fractions were applied on a 1-ml Resource Q column (Amersham Pharmacia Biotech) equilibrated in Buffer B (25 mM Tris- HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 10% glycerol) and developed at 1 ml/min with a linear gradient of KCl from 50 to 500 mM. Fractions were dialyzed against 25 mM potassium phosphate buffer, pH 7.5, 2 mM DTT, and 5% glycerol and stored at −20 °C.

Expression and Purification of p43 and of Derivatives Thereof—Human p43 (h-p43), as well as its N-terminal (h-p43N) and C-terminal (h-p43C; EMAPII), were expressed in Escherichia coli with the PET-28b expression system (Novagen). The h-p43 cDNA was produced by polymerase chain reaction between the two oligonucleotides p43–3 (-ccctcgagtaataaatgatgctgttctgaagagac) and p43–2 (-ccctcgagtttattcaattggccat) and p43-m2 (-ccctcgagtttttttcatttgctga), which introduced an NcoI site. The h-p43N cDNA was amplified between oligonucleotides p43–3 and p43–160 (-ccctcgagtttaattccactattggccat) and p43-m2 (-ccctcgagtttttttcatttgctga), which introduced a stop codon. The nucleotide sequence of the constructs was checked by DNA sequencing.

For purification of h-p43 without His tag, cell extract obtained as described above in 20 ml of Tris- HCl, pH 7.0, 50 mM NaCl, 2 mM DTT, 55% glycerol. For purification of h-p43C without His tag, cell extract obtained from citrated venous blood (buffy coats) from healthy donors in a two-step procedure by density-gradient centrifugation. Peripheral blood mononuclear cells were recovered from citrated venous blood (buffy coats) from healthy donors according to the method of Boyum (26). Blood was diluted 1:3 in Hanks' buffered saline, and 25 ml of this cell suspension was layered over 15 ml of Ficoll-Paque (Amersham Pharmacia Biotech). After centrifugation at 600 × g for 20 min at 2°C, mononuclear cells were collected, and cell suspension was washed twice with Hanks' buffered saline to remove platelets. For further monocyte isolation cells were resuspended in 15 ml of Hanks' buffered saline and placed into the sample tube of a JE-6B elutriator rotor (Beckman). At a constant rotor speed of 2300 rpm flow rate was increased successively from 7 ml/min to 18 ml/min. Monocytes were reproducibly eluted in fractions 5 to 9, containing more than 90% monocytes (CD14⁺ cells) as determined by fluorescence-activated cell sorter analysis with an anti-CD14 monoclonal antibody. After isolation cells were centrifuged (1000 rpm; 5 min), and monocytes were cultured not longer than 24 h in serum-free macrophase medium, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at a concentration of 2 × 10⁶ cells/ml. To avoid cell adhesion to the plastic surface hydrophobic culture plates (In Vitro Systems) were used.

Preparation of Antibodies and Western Blotting—Polyclonal antibodies were raised in rabbit against purified h-p43N and h-p43C following repeated injections at 2-week intervals of 0.5 mg of homogenous protein emulsified with complete (first injection) or incomplete Freund's adjuvant. Other antibodies directed to murine EMAPI (28) or to components of the multisynthetase complex (23) were as described. After SDS-PAGE (24), Western blotting was conducted essentially as described (25), using polyvinylidene difluoride transfer membranes (Hybond-P; Amersham Pharmacia Biotech), goat anti-rabbit IgG conjugated with peroxidase, and the ECL detection reagents.

Monocyte Chemotactic Assay—Monocytes were isolated from buffy coats of healthy donors in a two-step procedure by density-gradient centrifugation. Peripheral blood mononuclear cells were recovered from citrated venous blood (buffy coats) from healthy donors according to the method of Boyum (26). Blood was diluted 1:3 in Hanks' buffered saline, and 25 ml of this cell suspension was layered over 15 ml of Ficoll-Paque (Amersham Pharmacia Biotech). After centrifugation at 600 × g for 20 min at 2°C, mononuclear cells were collected, and cell suspension was washed twice with Hanks' buffered saline to remove platelets. For further monocyte isolation cells were resuspended in 15 ml of Hanks' buffered saline and placed into the sample tube of a JE-6B elutriator rotor (Beckman). At a constant rotor speed of 2300 rpm flow rate was increased successively from 7 ml/min to 18 ml/min. Monocytes were reproducibly eluted in fractions 5 to 9, containing more than 90% monocytes (CD14⁺ cells) as determined by fluorescence-activated cell sorter analysis with an anti-CD14 monoclonal antibody. After isolation cells were centrifuged (1000 rpm; 5 min), and monocytes were cultured not longer than 24 h in serum-free macrophase medium, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at a concentration of 2 × 10⁶ cells/ml. To avoid cell adhesion to the plastic surface hydrophobic culture plates (In Vitro Systems) were used.
Dual Function of p43/EMAPII

RESULTS

The p43 Component of the Multisynthetase Complex Is a Substrate of Caspase 7—The p43 component of the multisynthetase complex is the homolog of proEMAPII, the precursor of the EMAPII cytokine (5). Previous studies showed that [35S]labeled mouse proEMAPII produced in an in vitro transcription/translation system is a substrate of caspase 7 and, to a lesser extent, of caspase 3 (20). Because the only known cellular species of p43 is that associated within the multisynthetase complex (5), we surmised that if p43 is proEMAPII, the precursor of the cytokine, the caspase-7 cleavage site on p43 should be readily accessible in the complex. On the contrary, if the p43 component of the complex would be protected against caspase digestion, it could not be the direct precursor of EMAPII.

Homogeneous mouse multisynthetase complex was incubated in the presence of mouse caspase 7, and time course of p43 digestion was monitored by Western blotting with anti-EMAPII antibodies directed to the C-terminal moiety of p43 (Fig. 1). After 90 min of incubation, the p43 polypeptide (80 ng of protein taking into account that 2 molecules of p43 (2 × 40 kDa) are associated per molecule of complex (1.5 MDa)) was almost entirely converted into EMAPII, an 18-kDa polypeptide. In contrast, after a 90-min digestion with caspase 7 of recombinant mouse p43 (60 ng of the dimeric protein) expressed in E. coli (see below), only ~50% of the p43 polypeptide was converted into EMAPII. We concluded that the p43 component of the complex is a substrate of caspase 7 and that its association within the complex facilitates its cleavage. Caspase 3 or caspase 8 did not efficiently cleave the complex-associated form of p43 or the isolated subunit (not shown).

To test the behavior of the other components of the complex toward caspase 7 treatment, the multisynthetase complex subjected to controlled proteolysis was analyzed by SDS-PAGE followed by Coomassie staining (Fig. 2) and Western blotting (see Fig. 2 and below) using antibodies directed to individual components. Complete analysis revealed that IleRS, LeuRS, MetRS, ArgRS, p38, and probably GlnRS were not affected by the addition of caspase 7. In contrast, GluProRS (163 kDa), LysRS (68 kDa), and AspRS (57 kDa), in addition to p43, were cleaved by caspase 7 and converted to polypeptides of 105 + 60 kDa for GluProRS, 66 and 61 kDa for LysRS, and 55 kDa for AspRS. As shown in Fig. 2, when Z-DEVD-CMK, a potent and specific inhibitor of caspase 7, was added in the incubation mixture, cleavage of p43, GluProRS, LysRS, and AspRS was completely abolished.

Caspase 7 Treatment Releases EMAPII from the Complex—If EMAPII is a cleavage product of the complex-associated form of p43, then it should be readily released from the complex after caspase 7 treatment. The mouse complex was incubated with caspase 7 as described above and subjected to size fractionation on a Superose 12 column (Fig. 3). A major peak was eluted with an apparent mass corresponding to the native complex (fraction C). Two additional minor peaks were observed corresponding to the elution volumes of proteins of ~100 kDa (fraction C) and of small proteins eluting near the inclusion volume of the column (fraction E). Fraction F corresponded to the elution of components of the incubation buffer in the total bed volume of
A purified multienzyme complex from mouse (75 μg) was subjected to caspase 7 treatment (2200 units) followed by size fractionation on a Superose 12 HR 10/30 column equilibrated in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM DTT. Elution was monitored at 220 nm. According to the elution profile, fractions were combined to give samples A and B, corresponding to complex-associated proteins, and C–F, corresponding to free species. B, samples A–F were concentrated by ultrafiltration on Centricon-C4 (10,000 molecular weight cutoff; Sartorius) and analyzed by Western blotting using antibodies directed to the p43C or p43N region of p43, to the repeated units (GluRS) or ProRS domain (ProRS) of GluProRS, and to LysRS, AspRS, MetRS, ArgRS, or p38. Lanes T and T' corresponded to control samples of the multisynthetase complex before (T) or after (T') caspase treatment, recovered before size exclusion chromatography.

The consequences of caspase 7 treatment of the complex on the activity of its enzymatic components were appraised by measuring their tRNA aminoacylation capacity in the presence of saturating amounts of crude yeast or beef tRNA. Using these assay conditions, only ProRS activity was shown to be affected, with a 40% reduced initial velocity. This partial loss of activity could be related to the instability of the dimeric enzyme, which proved to readily dissociate into inactive monomers when released from the bifunctional GluProRS (30).

The EMAPII Domain Released by Caspase 7 Has Cytokine Activities—To establish that the EMAPII polypeptide initially isolated by Stern and co-workers (14) from conditioned medium of murine methylcholanthrene A fibrosarcoma cells does indeed correspond to the C-terminal domain of p43, substantial amounts of this polypeptide were isolated to perform its structural and functional characterization. Preparative digestion of the murine multisynthetase complex (500 μg of complex) was conducted with 10,000 units of caspase 7. Because two monomers of EMAPII (mass of the monomer = 18 kDa) could be isolated by molecules of complex (1500 kDa), a maximum of 12 μg of EMAPII were expected. To prevent absorption of this polypeptide on glassware, 0.01% Tween 20 was included in all buffers. EMAPII was isolated to homogeneity following two chromatographic steps on Resource Q and Mini-S columns and concentrated by ultrafiltration. Analysis by SDS-PAGE revealed a single polypeptide of 18 kDa (Fig. 4B), and a single N-terminal amino acid sequence was obtained by Edman degradation, KXPIDA... (the identity of the first amino acid residue was not determined). It precisely matches the sequence of the mouse p43 protein starting from residue 145, following the Asp144 residue from the ASTD sequence, corresponding to the caspase 7 cleavage site. It also coincides with the N-terminal amino acid sequence of the EMAPII cytokine initially isolated from conditioned medium of fibrosarcoma cells (14).

EMAPII has been shown to induce the migration of mononuclear phagocytes (MPs) and polymorphonuclear leukocytes...
and to stimulate the production of tissue factor by MPs and the release of myeloperoxidase from polymorphonuclear leukocytes. The cytokine activity of the EMAPII product derived from the multisynthetase complex by in vitro processing was assessed by its capacity to induce migration of human MPs (Fig. 5). EMAPII derived from the multisynthetase complex induced migration of MPs in a biphasic dose-response manner typical for chemokines. The chemotactic response was already significant at a concentration as low as 10 pg/ml, reached a maximum at about 1 ng/ml, and decreased to less significant values at an EMAPII concentration of 100 ng/ml. This dose response is very similar to that originally observed with EMAPII purified from tumor cell supernatants (14). When used at a protein concentration of 1 ng/ml, the recombinant cytokine (p43Ct) displayed a similar chemotactic activity (Fig. 5). The recombinant native human proEMAPII produced in E. coli (h-p43) also induced migration of MPs (results not shown).

The N and C Domains of p43 Contribute a Bipartite tRNA Binding Site—We previously reported that the C-terminal domain of p43 is a monomer and displays a weak nonspecific tRNA binding capacity ($K_D \approx 40 \mu M$; see Ref. 5). To determine whether cleavage of p43 into EMAPII could modify the tRNA binding property of p43, we expressed and purified to homogeneity different forms of p43 from different origins. Murine and human p43 (m-p43 and h-p43) (Fig. 4A), as well as the human p43N and p43C moieties corresponding to the two polypeptides
generated by caspase 7 treatment (Fig. 4B), were expressed in E. coli with a C-terminal His tag. A C-terminal fragment deprived of a His tag was also isolated (p43C-H).

A double-hybrid screen of interactions between components of the complex showed that p43 associated with itself (2). In addition, multisynthetase complexes purified to homogeneity contain two copies of the p43 polypeptide per molecule of complex. These results suggested that p43 could be a dimer, even though p43C is a monomer when analyzed by gel filtration (5).

The oligomeric structure of p43 was determined by sedimentation equilibrium (Fig. 6). When p43 was subjected to centrifugation equilibrium at an initial protein concentration of 38 μM, experimental data were fitted to a single species with a dimer-tetramer equilibrium, with a dissociation constant of 7.5 and 40 μM, respectively. As a control, we observed that p43C and p43C-H (a derivative without His tag) have indistinguishable tRNA binding capacities. Therefore, the two domains of p43 are likely to synergistically contribute a potent tRNA binding site on p43.

DISCUSSION

Here we showed that the p43 component of the mammalian multisynthetase complex is a substrate for caspase 7, an apoptotic protease. The free recombinant, as well as the natural complex-associated p43 species, are substrates of caspase 7. However, we found that the p43 entity associated within the complex was more efficiently processed into EMAPII than its soluble form. This result suggests that the site of cleavage is made more accessible to the caspase when p43 is forced into a conformation suited for its association with the other components of the complex. Although the EEVD sequence at position 175 to 178 of murine p43 would be an ideal site for caspase 7, according to its known preferred peptide substrates (31) the 141ASTD/S145 sequence is used in vitro by caspase 7 (this study), and the N terminus of the in vivo-generated EMAPII product also starts at residue Ser145 (14).

We analyzed the ability of p43 to form stable complexes with various tRNAs. Radiolabeled in vitro-transcribed tRNAs (yeast tRNA<sup>Asp</sup> or tRNA<sup>Lys</sup>, human tRNA<sup>Asp</sup> or tRNA<sup>Lys</sup>) were incubated with human p43 or with its isolated p43N or p43C polypeptides at the concentrations indicated. After electrophoresis at 4 °C on a 6% native polyacrylamide gel, the mobility shift of tRNA was visualized by autoradiography.

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Following cleavage at the 141ASTD/S145 site, the EMAPII cytokine lost its propensity to associate with the complex and is released as a monomer. The N terminus of p43 remains bound to the complex. These results are in full agreement with our
previous analysis of protein-protein interactions involved in the assembly of the complex. We showed by a two-hybrid analysis that p43 is able to interact with p38, the scaffold component of the complex, but also with GluRS and ArgRS (2). Furthermore, in vitro studies revealed that the isolated N-terminal moiety of p43 associates with ArgRS, but its C-terminal region corresponding to EMAPII does not (3). Similarly, p43 binds to p38 via its N-terminal domain. Thus, we anticipated that the release of EMAPII would not destabilize the quaternary structure of the complex.

Examination of each of the components of the complex after caspase treatment revealed that, with the exception of ProRS, aminocyl-tRNA synthetases remain associated. ProRS is carried by a multifunctional polypeptide (32) containing an N-terminal domain corresponding to GluRS, a linker region made of repeated units with nonspecific RNA binding properties (33), and a C-terminal ProRS domain. Cleavage of the GluProRS polypeptide by uncontrolled proteolysis led to the release of ProRS from the complex (30). Because of the size of the GluRS (105 kDa) and ProRS (60 kDa) polypeptides observed after cleavage by caspase, the finding that antibodies raised against (105 kDa) and ProRS (60 kDa) polypeptides observed after inhibition of translation in apoptotic cells, their proteolytic products do not possess cytokine activity. Secretion of EMAPII, the C-terminal domain of p43, results in the recruitment of macrophages (14, 18, 38) that engulf apoptotic cells, thus preventing inflammation caused by the release of their cellular content because of secondary necrosis of apoptotic cells. In this regard, p43 may compare with a molecular fuse. In its native pro-EMAPII form, it is an important cofactor for aminocytosine activity. After the fuse has blown, translation is irreversibly switched off, and EMAPII enters the cell death signaling pathway. In vitro assays showed that full-length recombinant p43 is also a potent cytokine (17, 19). This suggests that association of p43 within the multisynthetase complex inhibits its cytokine activity and/or sequesters proEMAPII in a cellular compartment and prevents its entry into apoptotic pathways until it is cleaved off the complex. Only a processed form of p43 is recovered in the supernatant of apoptotic cells. The pathway of EMAPII secretion remains to be deciphered.

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