The Cytokine Portion of p43 Occupies a Central Position within the Eukaryotic Multisynthetase Complex*

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Multicellular eukaryotes contain a macromolecular assembly of nine aminoacyl-tRNA synthetase activities and three auxiliary proteins. One of these, p43, is the precursor of endothelial monocyte-activating polypeptide II (EMAP II), an inflammatory cytokine involved in apoptotic processes. As a step toward understanding this paradoxical association, the EMAP II portion of p43 has been localized within the rabbit reticulocyte multisynthetase complex. Immunoblot analysis demonstrates strong reaction of anti-EMAP II antiserum with p43, as well as cross-reactivity with isoleucyl-tRNA synthetase. Electron microscopic images of immunocomplexes show two antibody binding sites. The primary site is near the midpoint of the multisynthetase complex at the intersection of the arms with the base. This site near the lower edge of the central cleft is assigned to the C-terminal cytokine portion of p43. The secondary site of antibody binding is in the base of the particle and maps the location of isoleucyl-tRNA synthetase. These data allow refinement of the three-domain model of polypeptide distribution within the multisynthetase complex. Moreover, the central location of p43/EMAP II suggests a role for this polypeptide in optimizing normal function and in rapid disruption of essential cellular machinery when apoptosis is signaled.

The aminoacyl-tRNA synthetase family is characterized by the common enzymatic activity of covalent coupling of amino acids to their appropriate transfer RNAs. Recently, interest in aminoacyl-tRNA synthetases has been stimulated by their potential as targets for new antibiotics and by their association with cytokines. The remarkable structural diversity of these enzymes, as well as their myriad roles in biology and disease, have recently been reviewed (1, 2).

A unique feature of aminoacyl-tRNA synthetases from multicellular eukaryotes is their assembly into an ∼1 × 10^6-Da multiprotein complex (for reviews see Refs. 3 and 4). Electron microscopic studies (5, 6) show it to be cup shaped with a characteristic Y-like view in which a deep central cleft is visible. The aminoacyl-tRNA synthetase components of this particle are the arginyl-tRNA synthetase dimer, the aspartyl-tRNA synthetase dimer, the bifunctional glutamyl-/prolyl-tRNA synthetase, glutaminyl-tRNA synthetase, isoleucyl-tRNA synthetase, leucyl-tRNA synthetase, the lysyl-tRNA synthetase dimer, and methionyl-tRNA synthetase. The complex also contains three auxiliary proteins. It has been suggested that p18 and p38 are used for interactions with protein translation factors (7) or protein–protein interactions within the multisynthetase particle (8), respectively. However, p43 appears to have multiple functions. One role for p43 may be in tRNA trafficking (see Ref. 9 and references therein), but a variety of other biological functions are possible, because it is a precursor form (10) of endothelial monocyte-activating polypeptide II (EMAP II). Release of EMAP II leads to acute inflammation and plays a role in apoptotic processes (reviewed in Ref. 11). Processing of p43 into EMAP II by caspase 7 may be part of a coordinated scheme that attracts macrophages to sites of apoptosis (12). This cytokine is also found in autoimmune lesions in the nervous system (13). It sensitizes tumors to tumor necrosis factor α (14) and inhibits neovascularization of metastatic carcinomas (15). Thus, the presence of the precursor form of EMAP II in an assembly of enzymes necessary for protein biosynthesis appears incongruous.

As an initial step toward understanding this intriguing association, this study localizes the cytokine portion of p43 within the multisynthetase complex using antibodies directed against EMAP II. The information obtained is used to refine the three-domain model of the particle and to propose a role for p43 within the multisynthetase complex.

EXPERIMENTAL PROCEDURES

Protein Purification—Isolation of the multisynthetase complex from rabbit reticulocyte lysate (Green Hectares) was carried out as described previously (16).

Immunoblot Analysis—Samples of 0.8-μg multisynthetase complex were subjected to denaturing electrophoresis (17) on a 10% polyacrylamide gel and then transblotted onto nitrocellulose membrane using standard methods (18). Reaction of component polypeptides with comparable dilutions of either normal rabbit serum (Sigma) or of rabbit anti-rabbit generated against recombinant mature EMAP II (19) were detected with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (Sigma) using bromochloroindoyl phosphate and nitroblue tetrazolium colorimetric substrates (Schleicher and Schuell).

Immunoelectron Microscopy—Samples (8 μg) of two separate preparations of multisynthetase complex were placed into an equal volume of high performance liquid chromatography buffer (25 mM Hepes, pH 7.2, 100 mM NaCl) containing a 1:5 or 1:10 dilution of anti-EMAP II antiserum. After 1.5 or 3 h, the entire mixture was applied to a 30-cm × 4.6-mm BIOSEP-SEC-S4000 column (Phenomenex) in the same buffer for isolation of immunocomplexes (20). Samples were prepared for electron microscopy by staining with 1% uranyl acetate as described previously (20). Electron micrographs of the negatively stained samples were obtained with a LEO 912AB microscope operated at 100 kV using an absolute magnification of 31,500.

Image Analysis—Micrographs were digitized using a flatbed scanner at an optical resolution corresponding to 6.4 Å per pixel on the image scale. Orientation of the multisynthetase complex and position of antibody binding site or sites were recorded for each immunocomplex iden-

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Localization of p43/EMAP II within the Multisynthetase Complex

RESULTS

Specificity of Anti-EMAP II for p43—The specificity of the antiserum directed against EMAP II for the cytokine was previously demonstrated (19). However, an immunoblot analysis (Fig. 1) was carried out to determine its reactivity within the context of the multiple components of the multisynthetase complex. Anti-EMAP II antibodies are strongly reactive with p43. Much weaker, but clearly detectable, cross-reactivity is observed with isoleucyl-tRNA synthetase. Thus, two locations of antibody binding are expected when this polyclonal antiserum is used in immunochemistry experiments.

Mapping of Antibody Binding Sites within the Multisynthetase Complex—Fig. 2 contains examples of electron microscopic images of individual multisynthetase complex particles to which anti-EMAP II antibodies are bound. Schematic drawings are included to illustrate each type of immunocomplex. Images in rows 1 and 2 show the most common antibody binding site. As described in terms of characteristic orientations and the three-domain model of the multisynthetase particle (5, 16), this site is slightly above the midpoint of the particle but close to the intersection of one arm and the base. Row 1 depicts immunocomplexes with full views of the front or back of the multisynthetase complex. Bound antibodies are seen lying to the side of the particle (panels 1A to 1C) or protruding from the top of the cleft (panels 1D to 1F). These images demonstrate the proximity of the antibody binding site to the lower edge of the central cleft. In row 2, the multisynthetase complex is in intermediate orientations, where the particle is rotated or tilted so that the cleft is no longer seen. The clear visibility of bound antibody molecules indicates binding to the surface of the particle near its midpoint. That this anti-EMAP II antiserum contains antibodies directed against more than one epitope within p43 is demonstrated by panels 2D to 2F, in which two closely spaced antibodies are bound. Consistent relative stoichiometry measurements (data not shown and Ref. 3) indicate position of the particle components as determined by reversible staining with Ponceau S. Identity of each polypeptide is given to the right of the figure. Aminocarboxyl-tRNA synthetases are identified with the one-letter amino acid abbreviation.

Images in row 3 are examples of antibody binding to the secondary site. Panels 3A to 3C again show front or back views of the multisynthetase complex. The bound antibodies are near the edge of the base furthest from the upper arms. Panels 3D to 3F show immunocomplexes with one antibody in each of the primary and secondary binding sites.

Immunocomplexes were also observed in which one antibody is bound to the multisynthetase complex in a third characteristic orientation. This is the approximately square view that is interpreted to be of the top or bottom of the particle. These images do not provide exact placement of the antibody binding sites. However, when multiple antibodies are bound (row 4), one can determine the spatial orientation of the two binding sites. As shown in panels 4A to 4C, the bound antibodies are on opposite sides of the particle. Using the interpretation that these views are down the long axis of the particle, the two binding sites must be on opposite faces of the multisynthetase complex. The two sites can be distinguished from one another based on images seen in panels 4D to 4F. These show two closely spaced bound antibodies on one side of the particle and a third on the opposite side. The first is likely the primary site near the center of the molecule because of the common observation of two closely spaced antibodies at this site in immunocomplexes where the multisynthetase particle is seen from the side. The binding site with one antibody is then defined as the secondary site in the particle base. It is noteworthy that no immunocomplexes with the multisynthetase complex in the triangular or intermediate views showed more than one antibody bound to the base of the particle.

Fig. 3 contains images of immunocomplexes in which individual anti-EMAP II antibodies are bound to two separate multisynthetase complex particles. All of the orientations of bound antibodies and multisynthetase particles are represented in such dimers. In the images in row 1, antibodies are bound to the primary binding site near the midpoint of the

![Image](https://example.com/image1.png)

**Fig. 1.** Immunoblots indicating reactivity of normal rabbit serum (left panel) and anti-EMAP II antiserum (right panel) with p43 and isoleucyl-tRNA synthetase. In each panel, lane 1 contains pre-stained markers whose mass is given in kDa at the left of the figure; lane 2 contains multisynthetase complex. Dots to the right of lane 2 in each panel indicate position of the particle components as determined by reversible staining with Ponceau S. Identity of each polypeptide is given to the right of the figure. Aminocarboxyl-tRNA synthetases are identified with the one-letter amino acid abbreviation. Composite figures were prepared for presentation using Adobe Photoshop.

**Fig. 2.** Schematics and negatively stained electron microscopic images of immunocomplexes in which antibodies directed against EMAP II are bound to individual multisynthetase particles. Scale bar equals 25 nm. Columns 1 and 4 contain schematics showing the orientation of the multisynthetase complex and antibody binding site(s) that correspond to the images in the adjacent panels. Row 1, side views showing the primary antibody binding in a central position near or within the cleft between two domains of the particle. Antibodies lie either to the side (A to C) or top (D to F) of the multisynthetase particle. Row 2, intermediate side views with one (A to C) or two (D to F) antibodies bound to the central site. Row 3, views depicting the secondary binding site in the base of the particle. Immunocomplexes are shown with only the secondary site (A to C) or both sites (D to F) occupied. Row 4, approximately square end views demonstrating that the primary and secondary antibody binding sites are on opposite faces of the particle.
multisynthetase complex. Panels 1A and 1B show the most common views in which the antibodies lie to the side of each multisynthetase particle. The closeness of the two particles and limited antibody visibility strongly emphasize that the binding site is in a central position within the particle. That is, bound antibody extends from the top of the particle. Again such images place the antibody binding site deep in the central cleft. Row 2 shows immunocomplexes with antibodies bound to the secondary site in the base of the multisynthetase complex. This is especially clear in the particle on the right side of panel 2A. In panels 2B and 2C, two bound antibodies are visible. In each case, one is at the primary site, and the other is at the secondary site. The images in row 3 reiterate that the two binding sites are on opposite faces of the multisynthetase complex. In panel 3A, two closely spaced bound antibodies connect the two multisynthetase particles. A third bound antibody is on the opposite side of the particle on the right. The central stain-filled hole indicates that this is in the top orientation. Both particles in panel 3B are viewed from the bottom. The two antibodies bound to the particle in the upper right are distinctly seen on opposite edges. Panel 3C is a similar example, except that a third antibody is lying very near the upper edge of the particle on the right.

**Location and Spatial Relationship of Anti-EMAP II Binding Sites within the Multisynthetase Complex**—Fig. 4 is a cartoon depicting the locations of anti-EMAP II antibody binding to the multisynthetase complex. In this schematic, when viewed from the front, the three domains and central cleft between the arms of the particle are visible. The primary binding site is located at the midpoint of the particle at the lower edge of the cleft. This site was defined from the 280 (85%) images showing side views of the multisynthetase complex and bound antibodies near the midpoint of the particle where the arms meet the base. As the multisynthetase complex is rotated around its long axis into intermediate views, the secondary binding site in the base becomes partially visible. This was designated the minor site because only 50 (15%) of the side views of the multisynthetase particle showed antibody in this site. These assignments were emphasized by the 169 immunocomplexes with multiple antibodies bound to side orientations of the multisynthetase complex. Of these, 135 (80%) had two closely spaced bound antibodies near the center of the particle. In the remaining 34 (20%), one antibody was bound to the secondary site in the base of the particle, and either one or two antibodies were bound to the primary site.

When the multisynthetase complex is fully rotated to the back orientation, then the face containing the secondary binding site is uppermost, whereas the primary site is on the hidden face. Placement of the binding sites on opposite faces of the multisynthetase complex is required for consistency with images of the top or bottom of the particle. That is, 37 (59%) of the immunocomplexes with top or bottom views of the multisynthetase particle and more than one bound antibody showed two closely spaced antibodies bound on one side of the particle, that is, in the primary binding site. However, the remaining images showed antibodies bound to opposite sides of the particles and
so indicated that the binding sites are on opposite faces of the multisynthetase complex. Overall 418 immunocomplexes were analyzed in which the positions of 697 bound antibodies were mapped. Only 27 (4%) were inconsistent with either the primary or secondary site as depicted in Fig. 4.

**DISCUSSION**

**Assignment of the Primary Antibody Binding Site to p43 and the Secondary Site to Isoleucyl-tRNA Synthetase—**Immunoblot analysis shows that the strongest reaction of antibodies within the anti-EMAP II antiserum is with p43. Regardless of whether this is because of higher titer of this population, stronger affinity, or both, this result suggests that the majority of bound antibodies observed in electron microscopic images of immunocomplexes will react with this polypeptide. In each of the types of immunocomplex analyzed in this study, a marked majority of antibody binding was to an area at the midpoint of the multisynthetase complex near the lowest portion of the central cleft. These data support the assignment of the primary binding site to the cytokine portion of p43. Similarly, the weak reaction of anti-EMAP II antiserum with the isoleucyl-tRNA synthetase polypeptide, coupled with the observation of much smaller percentages of antibodies bound to the base of the multisynthetase particle, suggest that the secondary binding site is within this enzyme.

Additional evidence in support of these assignments comes from the three-domain model of the multisynthetase complex (16), in which isoleucyl-, leucyl-, and glutamyl-/prolyl-tRNA synthetases were placed together in the base of the particle. The association of these enzymes was confirmed by an extensive two-hybrid analysis (21). Moreover, the glutamyl-/prolyl-tRNA synthetase has been located in or near the base using immunoelectron microscopy (20). Cross-linking, (16) genetic analysis (8), and immunoprecipitation experiments (22) have documented that p43 is associated with arginyl- and glutamyl-tRNA synthetases. Both of these enzymes have been assigned to the domains making up the upper arms of the model of the multisynthetase complex (16). Thus, it is logical to assign the primary anti-EMAP II binding site in the central portion of the particle to p43.

**Refinement of the Three-domain Model of the Multisynthetase Complex—**Based on the location of the primary binding site, p43 can be reliably placed in a central position in the model of the multisynthetase complex (Fig. 5). To satisfy the constraints of previous studies (8, 16), the polypeptide is depicted as an elongated molecule extending the width of the complex. That p43 likely consists of at least two distinct domains connected by a linker region accessible to proteases is suggested by in vivo processing of pro-EMAPII into the 22-kDa cytokine by caspases (12). The directionality of p43 within the multisynthetase complex can also be established. That is, based on the spatial relationship of the anti-EMAP II binding sites, the C-terminal cytokine portion of the polypeptide is located on the opposite side of the particle from isoleucyl-tRNA synthetase.

The new data and results from recent genetic analyses (8, 21) allow further refinement of the three-domain model of the multisynthetase complex. The polypeptide positions within domain III have been rearranged to a clockwise progression from glutamyl-/prolyl-tRNA synthetase to leucyl-tRNA synthetase to isoleucyl-tRNA synthetase when viewed from the bottom of the model. The base domain has also been rotated slightly to allow more overlap with components in the arms.

**Suggested Role of p43 / EMAP II in the Multisynthetase Complex—**A biological utility of the seemingly paradoxical association of a proinflammatory and apoptosis-inducing cytokine with essential enzymes of protein biosynthesis has been suggested in discussions of the presence of EMAP II and EMAP II-like domains within tyrosyl-tRNA synthetase and methionyl-tRNA synthetase and of p43/EMAP II within the multisynthetase complex (reviewed in Ref. 23). Specifically, association of EMAP II with aminoacyl-tRNA synthetases may poise cells for rapid acceleration of cell death by disruption of the protein translation machinery after generation of an apoptotic signal. The chemotactic effects of the cytokine then promote inflammatory processes for removal of cellular debris.

This study has shown that p43/EMAP II is located at or very near the junction of the three major domains of the multisynthetase complex. Thus, it is well positioned to affect the overall structure and stability of the particle and so may promote rapid breakdown of the multisynthetase complex upon release of the cytokine. Under normal cellular conditions, the central location of p43/EMAP II and its ability to modulate the activity of arginyl-tRNA synthetase (22) suggest that p43/EMAP II may play a role in optimizing aminoacylation events within the multisynthetase complex. This may involve direct effects on individual enzymes or general effects on the structure or stability of the particle.

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