THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 24, Issue of June 11, pp. 16673–16676, 1999

Communication

Precursor of Pro-apoptotic Cytokine Modulates Aminoacylation Activity of tRNA Synthetase*

(Received for publication, January 21, 1999, and in revised form, April 13, 1999)

Sang Gyu Park‡, Keum Hee Jung‡, Jong Sang Lee‡, Yeong Joon Jo‡, Hiromi Motegi§, Sunghoon Kim‡¶, and Kiyotaka Shiba§

From the ‡National Creative Research Initiatives Center for ARS Network, Sung Kyun Kwan University, Suwon, Kyunggido, 440-746, Korea and the §Department of Cell Biology, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 170-8455, Japan

Endothelial monocyte activating polypeptide II (EMAPII) is a cytokine that is specifically induced by apoptosis. Its precursor (pro-EMAPII) has been suggested to be identical to p43, which is associated with the multi-tRNA synthetase complex. Herein, we have demonstrated that the N-terminal domain of pro-EMAPII interacts with the N-terminal extension of human cytoplasmic arginyl-tRNA synthetase (RRS) using genetic and immunoprecipitation analyses. Aminoacylation activity of RRS was enhanced about 2.5-fold by the interaction with pro-EMAPII but not with its N- or C-terminal domains alone. The N-terminal extension of RRS was not required for enzyme activity but did mediate activity stimulation by pro-EMAPII. Pro-EMAPII reduced the apparent K_m of RRS to tRNA, whereas the k_{cat} value remained unchanged. Therefore, the precursor of EMAPII is a multi-functional protein that assists aminoacylation in normal cells and releases the functional cytokine upon apoptosis.

Aminoacyl-tRNA synthetases (ARSs)[1] catalyze ligation of their cognate amino acids to specific tRNAs. Although basic architecture of the core domain is well conserved among ARSs, unique peptide extensions have been found in the N- or C-terminal ends of metazoan enzymes (1–3). Although these extensions have been thought to be involved in heterologous molecular interactions, their functional significance is not yet understood.

A macromolecular protein complex consisting of at least nine different ARSs has been found in higher eukaryotes (1–3). This multi-ARS complex also contains three nonsynthetase components, p18, p38, and p43 whose functions are not clear (4–7). Among these nonsynthetase components, p43 has been proposed to be a precursor of a tumor-specific cytokine, endothelial monocyte-activating polypeptide II (EMAPII) based on over 80% sequence identity between the two proteins (6). EMAPII was originally identified in the culture medium of murine fibrosarcoma cells induced by methylcholanthrene A (8). It triggers an acute inflammatory response (9, 10) and is involved in development-related apoptosis (11).

The precursor for EMAPII (pro-EMAPII) is processed at the Asp residue of ASTD/S sequence to release the C-terminal cytokine domain of 23 kDa (11). Its C-terminal domain shares homology with the C-terminal parts of methionyl-tRNA synthetases of prokaryotes, archaea and nematode, and also a yeast protein, Arc1p/G4p, which interacts with methionyl- and glutamyl-tRNA synthetases. The N-terminal domain of pro-EMAPII does not show homology to any known proteins, and its function has not been understood.

EMAPII is expressed in a wide range of cell lines and normal tissues (12) and its mRNA level is unchanged during apoptosis (11) although its production is induced by apoptosis. The present work was designed to address whether pro-EMAPII is identical to p43 and to understand its function in the normal cell. The results showed that pro-EMAPII is associated with the N-terminal extension of human arginyl-tRNA synthetase (RRS), facilitating aminoacylation reaction.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant tRNA Synthetases and Pro-EMAPII—Human pro-EMAPII is genetically separated into the N- and C-terminal domains by proteolytic cleavage at Asp^{147}. The cDNA encoding the full-length pro-EMAPII was isolated from pM338[2] by *Nde*I and *Xho*I digestion and then used as a template to separately amplify the DNA encoding its N- and C-terminal domains by PCR using the primer pairs of R1EF/S1ENB and R1ECF/S1EB (Table I). The PCR products were digested and cloned into pET28a using *Eco*RI and *Sal*I. The DNA encoding the 72-amino acid N-terminal extension of human RRS was also amplified by PCR using the primers of R1RNF and S1RNB (Table I) and cloned into the *Eco*RI and *Sal*I sites of pET28a. The resulting clones were transformed into *Escherichia coli* strain BL21-DE3, and the inserted genes were induced at 0.1 mM IPTG. The cells expressing the recombinant proteins were harvested, resuspended in 20 mM KH_2PO_4, 500 mM NaCl (pH 7.8), and 2 mM 2-mercaptoethanol, and then lysed by ultrasonication. After centrifugation of the lysate at 25,000 × *g*, the supernatants were recovered and the recombinant proteins containing a 6-histidine tag were isolated by nickel affinity chromatography according to the instructions of the manufacturer (Invitrogen).

The cDNAs encoding the full-length and N-terminal 72-amino acid truncated (ΔN72) human RRS proteins were also amplified by PCR with the primer pairs of R1RNF/S1RB and R1RTN/S1RB, respectively (Table I). The resulting PCR products were cloned into pGEX4T-1 using the *Eco*RI and *Sal*I sites to express as the glutathione *S*-transferase (GST) fusion proteins. Protein extracts were prepared as described above, and the GST fusion proteins were purified by glutathione affinity chromatography. The GST tag was then removed by thrombin cleavage and the RRS proteins were further purified according to the protocol of the manufacturer (Amersham Pharmacia Biotech). The plasmid pM109 containing the full-length human lysyl-tRNA synthetase (KRS) fused to a 6-histidine tag (13) was used to express the protein. The His-KRS

* This work was supported by a grant from National Creative Research Initiatives and Biotech 2000, 97-N1-06-01-A06 of the Ministry of Science and Technology, Korea (to S. K.) and by a grant from the Ministry of Education, Science and Culture of Japan (to K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biological Science, Sung Kyun Kwan University, 300 Chunchundong, Jangangu, Suwon, Kyunggido 440-746, Korea. Tel.: 82-331-290-5681; Fax: 82-331-290-5682; E-mail: shkim@yurim.skku.ac.kr.

[1] The abbreviations used are: ARS, aminoacyl-tRNA synthetase(s); RRS, arginyl-tRNA synthetase; EMAPII, endothelial monocyte activating polypeptide II; pro-EMAPII, EMAPII precursor; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; GST, glutathione *S*-transferase; KRS, lysyl-tRNA synthetase; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; BSA, bovine serum albumin.

[2] H. Motegi and K. Shiba, unpublished data.

TABLE I
Primers used for subcloning of human pro-EMAPII and RRS

Primers	Sequences
R1EF	5′-CCGGAATTCATGGCAAATAATGATGCT
R1ECF	5′-CCGGAATTCTCTAAGCCAATAGATGTT
S1EB	5′-CCGGTCGACTTATTTGATTCCACTGTT
S1ENB	5′-CTGGTCGACGTCGGCACTTCCAGC
R1RNF	5′-TGGAATTCATGGACGTACTGGTG
R1RTN	5′-CCGGAATTCATGATTAACATTATTAGC
S1RB	5′-ACGCGTCGACTTACATCCTTGGGCC
S1RNB	5′-GACGCGTCGACTTAATTTTTAGTTGG

fusion protein was purified using nickel affinity chromatography (CLONTECH).

Preparation of Polyclonal Rabbit Antibody Specific to Human Pro-EMAPII—The purified recombinant human pro-EMAPII (500 μg) was mixed with Freund's complete adjuvant at 1:1 volume ratio and then injected into two New Zealand White rabbits. Booster injections were conducted three times at 1-week intervals using the same amount of the protein mixed with the incomplete adjuvant at a 1:1 ratio. The rabbits were sacrificed by cardiac puncture, and the antiserum was obtained. The antibody was purified by protein A column chromatography. Specificity and titer were determined by Western blotting.

Immunoprecipitation—The purified N-terminal extension of human RRS (10 μg) was mixed with each of the full-length, N- or C-terminal domains of pro-EMAPII (10 μg each) at 4 °C overnight. The polyclonal rabbit (20 μg) antibody raised against human pro-EMAPII was then added to each of the mixtures and incubated on ice for 4 h. The protein A-agarose suspension in 20 μl of 50 mM Tris-HCl (pH 7.5) and 25 mM NaCl was also added, and incubation was continued at 4 °C for 5 h. The mixture was centrifuged, and the agarose pellet was washed three times with 400 μl of 50 mM Tris-HCl (pH 7.5) containing 25 mM NaCl and 0.01% Triton X-100. The agarose was treated with 50 mM Tris-HCl (pH 6.8) containing 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.2% bromphenol blue, and 10% glycerol, and the solution was then boiled for 5 min to elute the bound proteins. After centrifugation, the supernatant was loaded onto a 12% SDS-polyacrylamide gel. The proteins were separated by electrophoresis and detected by Coomassie Blue staining.

Two-hybrid Assay—Human proteins interacting with human pro-EMAPII were screened by a yeast two-hybrid system (14). The cDNA encoding the full-length pro-EMAPII was isolated by PCR using the primers R1EF and S1EB (Table I) and ligated next to the gene for LexA using the *Eco*RI and *Sal*I sites. The plasmid was transformed into yeast strain, EGY48 (*MAT, his3, trp1, ura3–52, leu2::pLeu2-LexAop6*/pSH 18–34 (*LexAop-lacZ*)). A human fetal brain cDNA library in which the proteins are expressed as fusion proteins with the B42 transcriptional activator (CLONTECH) was used to screen for proteins interacting with LexA-pro-EMAPII. The plasmids containing human cDNAs were transformed into EGY48 expressing LexA-pro-EMAPII. Interactions were detected by the induction of reporter genes, *LEU2* and *LacZ*, which resulted in cell growth on leucine-depleted yeast synthetic media containing 2% galactose and also formation of blue colonies on the yeast synthetic media containing 0.2 mM X-gal, 2% galactose, and 2% raffinose. The cDNAs encoding the N- and C-terminal domains of pro-EMAPII were cleaved from the histidine tag construction using *Eco*RI and *Sal*I and religated into the pLexA vector using the same sites.

Aminoacylation Assay—Aminoacylation activity of the purified human RRS was determined as described previously (15). The reaction mixture contained 125 mM Tris acetate (pH 7.4), 0.2 mg/ml bovine serum albumin, 5 mM ATP, 4 mM EDTA, 50 mM $MgCl_2$ and 0.1 μCi/μl [^{3}H]arginine. Aminoacylation of human KRS was carried out in a reaction mixture containing 50 mM HEPES (pH 7.5), 0.1 mg/ml BSA, 20 mM 2-mercaptoethanol, 4 mM ATP, and 0.12 μCi/μl [^{3}H]lysine. Human RRS and KRS were pre-incubated on ice with the full-length, N- or C-terminal domain of pro-EMAPII for 5 min and then added to their respective reaction mixtures at a concentration of 0.14 nM. The reaction was initiated by adding bovine liver total tRNA (0.34 μM). Reaction samples were taken at 1-min intervals and spotted on filter discs presoaked with 5% trichloroacetic acid. After 1 min, the filter discs were added to ice-cold 5% trichloroacetic acid and washed three times with fresh 5% trichloroacetic acid at 4 °C. The radioactivity adsorbed to the filters was quantitated by liquid scintillation counting. Reactions were also carried out at different concentrations of pro-EMAPII for kinetic analysis.

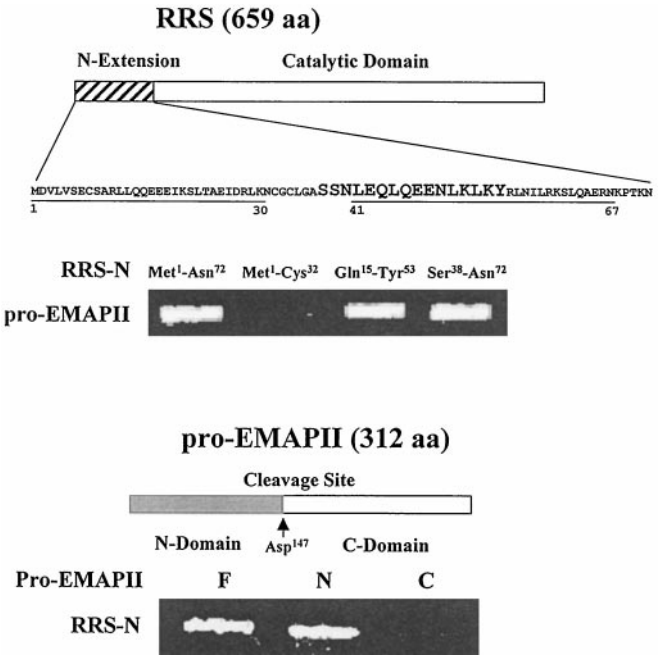

FIG. 1. **Interaction of pro-EMAPII with arginyl-tRNA synthetase.** The peptide regions of pro-EMAPII and RRS responsible for the interaction were mapped by two-hybrid analysis. The positive interactions were determined by cell growth on leucine-depleted yeast synthetic media (14). Three peptide fragments in the N-terminal extension of RRS (28) were tested for the interaction with pro-EMAPII. Amino acids commonly present in the two interacting peptides are shown in large letters. The peptides of Met^1-Lys^{30} and Leu^{41}-Asn^{67} were predicted to form α-helices (*underlined*). Human pro-EMAPII was divided into the N- and C-terminal domains at Asp^{147}. The N-terminal domain (*gray box*) showed the interaction with the N-terminal extension of RRS. *RRS-N* indicates the N-terminal 72-amino acid region. *F*, *N,* and *C* represent the full-length, N- and C-terminal domains of pro-EMAPII, respectively.

RESULTS

Screening of Proteins Interacting with Human Pro-EMAPII—To investigate the function of pro-EMAPII and its relationship to p43, we screened for protein(s) interacting with human pro-EMAPII using a yeast two-hybrid system (16, 17). The 312-amino acid polypeptide of human pro-EMAPII was fused to LexA (DNA-binding domain), and this fusion protein was used as a bait. Human proteins fused to B42 (transcriptional activator) were screened, and interaction between the two fusion proteins was detected by the induction of the reporter genes, *LEU2* and *LacZ*, in a yeast host strain (14).

Approximately 300,000 cDNA clones of human fetal brain were screened to identify proteins interacting with pro-EMAPII. The N-terminal 58-amino acid region of human RRS was selected as one of the six positive clones interacting with pro-EMAPII (data not shown). In the present work, we focused on the interaction between pro-EMAPII and RRS. The N-terminal 72-amino acid peptide region is only found in human (18) and hamster RRS proteins (19). We conducted deletion analysis to determine the peptide regions of pro-EMAPII and RRS responsible for the interaction. The peptides from Gln^{15} to Tyr^{53} and from Ser^{38} to Asn^{72} were able to interact with pro-EMAPII, suggesting that the residues from Gln^{15} to Ser^{38} are responsible for the interaction (Fig. 1). The N-terminal domain of pro-EMAPII showed the interaction with RRS but its C-terminal cytokine domain did not (Fig. 1).

Interaction between the N-terminal extension of RRS and pro-EMAPII was also tested by co-immunoprecipitation. The full-length, N- and C-terminal domains of pro-EMAPII and the 72-amino acid N-terminal extension of RRS were all expressed

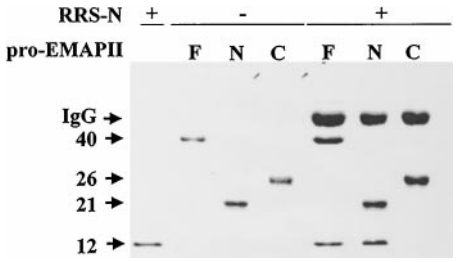

FIG. 2. **Immunoprecipitation of pro-EMAPII and RRS.** The 72-amino acid N-terminal extension of RRS and the full-length, N- and C-terminal domains of pro-EMAPII were expressed as His-tag fusion proteins and purified by nickel affinity chromatography. Each of the pro-EMAPII derivatives was mixed with the RRS peptide. Subsequently, anti-pro-EMAPII antibody was added to each mixture, and protein complexes were precipitated with protein A agarose. The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by Coomassie Blue staining. IgG (heavy chain) is shown as marked, and protein sizes are indicated in kDa.

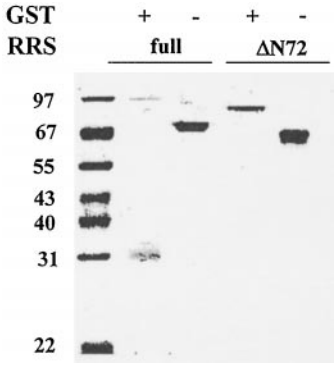

FIG. 3. **Purification of the full and N-terminal truncated RRS.** The full-length and 72-amino acid N-terminal truncated RRS (ΔN72) were expressed as GST fusion proteins. The GST tag was cleaved, and the two forms of RRS were purified. Marker sizes are shown in kDa.

as His-tag fusion proteins and were purified by nickel affinity chromatography (Fig. 2). The purified N-terminal peptide of RRS was mixed with each of the isolated full-length, N- and C-terminal pro-EMAPII in separate reactions. Polyclonal rabbit antibody raised against pro-EMAPII was then added to the mixture and precipitated with protein A-agarose. The proteins in the precipitate were dissolved and separated on an SDS-polyacrylamide gel. The N-terminal peptide of RRS was co-precipitated with the full-length or N-terminal domains of pro-EMAPII but not with its C-terminal domain (Fig. 2). These results further confirmed that the N-terminal domain of pro-EMAPII interacts with the N-terminal extension of RRS as initially identified by the two hybrid analysis (Fig. 1).

Pro-EMAPII Stimulates the Catalytic Activity of RRS—The functional significance of the interaction between RRS and pro-EMAPII was further investigated. We tested whether the aminoacylation activity of RRS was affected by interaction with pro-EMAPII. The full-length and N-terminal 72-amino acid truncated (ΔN72) RRS were expressed as GST-fusion proteins. The fused GST was removed by proteolytic cleavage, and the purified full-length and N-terminal truncated RRS proteins were used for the enzyme assay (Fig. 3).

The reaction catalyzed by tRNA synthetases proceeds in two steps. The first step is activation of the amino acid by reaction with ATP, and the second step involves transfer of the activated amino acid to the cognate tRNAs. Aminoacylation activity of the full-length RRS was enhanced approximately 2.5-fold in the presence of pro-EMAPII (Fig. 4, *left bars*). Since arginine-dependent [^{32}P]pyrophosphate-ATP exchange assay showed

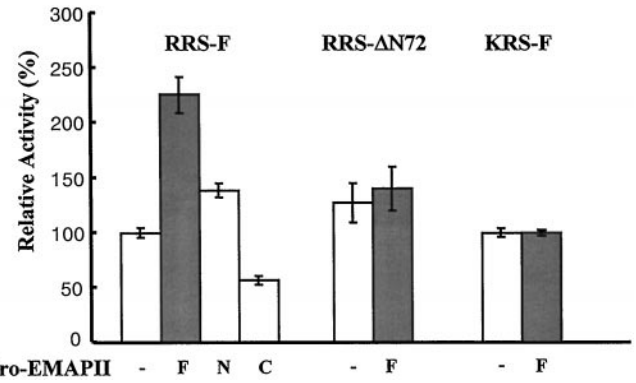

FIG. 4. **Stimulation of aminoacylation activity of RRS by interaction with pro-EMAPII.** Aminoacylation activities of the full-length and N-terminal truncated RRSs were determined in the absence and presence of the full-length, N- or C-terminal domains of pro-EMAPII. The activity of the full-length KRS was also determined in the absence and presence of the full-length pro-EMAPII. The activities of the full-length RRS without pro-EMAPII were normalized to 100%, and other activities were compared accordingly. The KRS activities with and without pro-EMAPII were also compared. The experiments were repeated three times. *F*, *N,* and *C* represent the full-length, N- and C-terminal domains of pro-EMAPII, respectively.

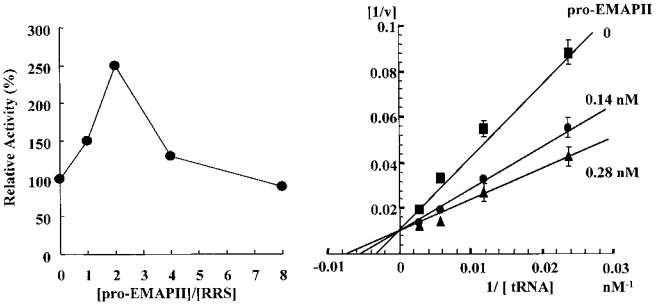

FIG. 5. **Kinetic analysis of the RRS aminoacylation reaction at different concentrations of pro-EMAPII.** Aminoacylation reactions of RRS were carried out at different concentrations of pro-EMAPII. *Left*, the relative reaction rates of RRS were plotted against the molar ratio of pro-EMAPII and RRS. *Right*, the effect of pro-EMAPII on the reaction was analyzed by a Lineweaver-Burk plot. Total bovine liver tRNA was added to the reaction from 42 to 336 nM. The reactions were repeated three times.

that the adenylation step of RRS was not affected by addition of pro-EMAPII (data not shown), the activity enhancement probably results from the second step of the reaction. Activity stimulation was not detected when the separated N- or C-terminal domain of pro-EMAPII was added, indicating that the full-length pro-EMAPII is necessary for the effect (Fig. 4, *left bars*). The truncated RRS retained aminoacylation activity comparable with the wild-type enzyme, suggesting that the N-terminal extension is not essential for the enzyme activity (Fig. 4, *middle bars*). However, the activity of this mutant was not increased by pro-EMAPII, indicating that interaction of pro-EMAPII with the N-terminal extension of RRS is essential for the stimulatory effect (Fig. 4, *middle bars*). To investigate whether the stimulatory effect of pro-EMAPII is specific for RRS, we employed human lysyl-tRNA synthetase (KRS) which does not appear to interact with p43 (7). The aminoacylation activities of KRS were measured in the absence and presence of pro-EMAPII. KRS activity was not affected by the addition of pro-EMAPII, suggesting that activity stimulation is specific to RRS (Fig. 4, *right bars*).

Kinetic analyses on the aminoacylation of RRS were carried out at different concentrations of pro-EMAPII to understand how pro-EMAPII enhances the RRS activity. The activity enhancement reached a maximum at a 2-fold molar excess of

pro-EMAPII to RRS and further addition of pro-EMAPII resulted in gradual decrease in the reaction rate (Fig. 5, *left panel*). A Lineweaver-Burk plot of the reaction showed that the apparent K_m of RRS with respect to tRNA was reduced by the addition of pro-EMAPII, whereas its k_{cat} value was not changed (Fig. 5, *right panel*). Excess pro-EMAPII probably binds to the tRNA substrate and lowers its effective concentration.

DISCUSSION

Pro-EMAPII (8) and p43 (6) have been independently isolated from different organisms. In this work, we found that pro-EMAPII interacts with RRS (Figs. 1 and 2). Previous cross-linking and genetic experiments showed the linkage of p43 and RRS (7, 20). Thus, all of these results support that p43 and pro-EMAPII are responsible for similar functions within the cell.

The full-length pro-EMAPII was required for the activity enhancement of RRS although the N-terminal domain of pro-EMAPII was sufficient for the direct interaction with pro-EMAPII (Fig. 4). It was previously shown that the C-terminal domain of pro-EMAPII contains tRNA binding activity (6). The kinetic analyses showed that pro-EMAPII affected only the apparent K_m value to tRNA and not k_{cat} of the enzyme (Fig. 5). Probably, tRNA recruited to the C-terminal domain of pro-EMAPII is delivered to the active site of RRS. Although the activity of RRS was enhanced about 2.5-fold by pro-EMAPII under our experimental conditions, its effect may be more significant *in vivo* because RRS present in the multi-protein complex would have limited accessibility to tRNA

Mammalian RRS exists in two forms differing by the N-terminal extension (15). The larger RRS containing the N-terminal extension is found in the multi-synthetase complex, whereas the smaller RRS exists in a free form (18, 19). The complex-associated larger RRS showed a 7-fold higher K_m for the tRNA substrate than the complex-free RRS, whereas other kinetic properties were similar (15). Perhaps, the higher K_m value of the complex-associated RRS for the tRNA substrate requires compensation by an active delivery of the tRNA substrate. In the case of RRS, the delivery of tRNA appears to be mediated by a *trans*-acting factor, pro-EMAPII. This mechanism is also reminiscent of yeast Arc1p, which forms a complex with methionyl-tRNA synthetase and stimulates its aminoacylation activity (21).

ARSs have developed different ways to modulate their catalytic activities and the efficiency of protein synthesis. For example, the N-terminal extension of rat aspartyl-tRNA synthetase facilitates the release of aminoacylated tRNA to elongation factor (22, 23), and the aminoacylation reaction of rabbit valyl-tRNA synthetase is enhanced by interaction with elongation factor EF-1H (24). The N-terminal extension of yeast glutaminyl-tRNA synthetase promotes specific recognition of its cognate tRNA (25), and the C-terminal appendix of *E. coli* methionyl-tRNA synthetase helps to dock its cognate tRNA to the active site (26). Whereas all of these functions are exerted by the peptide extensions connected *in cis* to the catalytic domains of ARSs, yeast Arc1p and mammalian pro-EMAPII are *trans*-acting factors. These factors may have more functional flexibility than the *cis*-acting peptide extensions because they can easily dissociate from the ARS and interact with cellular molecules for other physiological roles. Human tyrosyl-tRNA synthetase was recently shown to be secreted from apoptotic tumor cells and is cleaved to release the two distinct cytokine domains (27). Interestingly, the released C-terminal domain is homologous to EMAPII. These results along with our data suggest that protein synthesis and apoptosis are functionally coordinated via novel domains covalently or noncovalently linked to ARSs.

Acknowledgments—We thank Drs. Y. G. Ko, W. Seol, K. Zahn, and S. Martinis for critical comments on the manuscript.

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