Highly Differentiated Motifs Responsible for Two Cytokine Activities of a Split Human tRNA Synthetase*

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While native human tyrosyl-tRNA synthetase (TyrRS) is inactive as a cell-signaling molecule, it can be split into two distinct cytokines. The enzyme is secreted under apoptotic conditions in culture where it is cleaved into an N-terminal fragment that harbors the catalytic site and into a C-domain fragment found only in the mammalian enzymes. The N-terminal fragment is an interleukin-8 (IL-8)-like cytokine, whereas the released C-domain is an endothelial-monocyte-activating polypeptide II (EMAP II)-like cytokine. Although the IL-8-like activity of the N-fragment depends on an ELR motif found in c-chemokines and conserved among mammalian TyrRSs, here we show that a similar (NYR) motif in the context of a lower eukaryote TyrRS does not confer the IL8-like activity. We also show that a heptapeptide from the C-domain has EMAP II-like chemotaxis activity for mononuclear phagocytes and polymorphonuclear leukocytes. Eukaryote proteins other than human TyrRSs that have EMAP II-like domains have variants of the heptapeptide motif. Peptides based on these sequences are inactive as cytokines. Thus, the cytokine activities of split human TyrRSs depend on highly differentiated motifs that are idiosyncratic to the mammalian system.

Although aminoacyl-tRNA synthetases are key enzymes in protein biosynthesis that catalyze aminoacylation of their cognate tRNAs (1–4), we recently demonstrated that human tyrosyl-tRNA synthetase (TyrRS)1 has novel cytokine functions in addition to its role in protein synthesis (5). Under apoptotic conditions in culture, the full-length enzyme is secreted, where two distinct cytokines can be generated by an extracellular protease such as leukocyte elastase (5). While the full-length enzyme is inactive in assays for a variety of cytokine activities, the sequestered cytokines are released by splitting the native enzyme into N- and C-terminal halves (5). This finding established a link between protein synthesis and signal transduction.

The N-terminal fragment contains the Rossmann nucleotide binding fold characteristic of all class I aminoacyl-tRNA synthetases. It is almost the same molecular size as TyrRS from prokaryotes or lower eukaryotes (see Fig. 1A). This N-fragment of human TyrRS has the catalytic domain capable of aminoacylation of human tyrosine tRNA (5). Surprisingly, the N-fragment (human mini TyrRS) binds strongly to the interleukin-8 (IL-8) type A receptor and, like IL-8, functions as a chemotactant for polymorphonuclear leukocytes (PMNs) (5). The C-terminal fragment of human TyrRS has high sequence similarity to the mature form of a proinflammatory cytokine known as human endothelial-monocyte-activating polypeptide II (EMAP II) (6). The isolated C-domain of human TyrRS has potent chemotaxis activity for PMNs and mononuclear phagocytes (MPs), and stimulates production of myeloperoxidase, tumor necrosis factor-α, and tissue factor. These are the same activities found for mature EMAP II (5, 7, 8).

Aware that other proteins had some of the same sequences found in EMAP II and in human TyrRS, we wondered whether these proteins had any of the same cytokine activities. Alternatively, the sequences important for the cytokine activities of human TyrRS could have a different function in other proteins. In that case, the cytokine activities of human TyrRS might be viewed as highly differentiated adaptations of domains or motifs that are basic platforms for more than one kind of biological activity. For example, Saccharomyces cerevisiae TyrRS has an Asn-Tyr-Arg (NYR) motif similar to the Glu-Leu-Arg (ELR) sequence of human TyrRS and of α-chemokines like IL-8. The ELR motif is essential for the leukocyte chemoattractant activity of these proteins (5, 9, 10), and yet there is no obvious biological rationale why a yeast protein like S. cerevisiae TyrRS would have chemokine activity. Similarly, portions of S. cerevisiae Arc1p, bacterial, and Caenorhabditis elegans methionyl-tRNA synthetase (MetRS), and the β-subunit of prokaryotic phenylalanyl-tRNA synthetase (PhRS) have high sequence similarity to parts of mature EMAP II (11–13). Here again, there is no obvious rationale for EMAP II-like activity in the organisms that host these proteins. With these considerations in mind, we tested the functional significance of the IL-8- or EMAP II-like motifs in these proteins with respect to their potential chemokine activities. The results obtained strengthened the concept that, despite similarities of the motifs in human TyrRS with those in other proteins, the cytokine activities themselves are highly specific to human TyrRS.

**MATERIALS AND METHODS**

*Preparation of Peptides and Proteins—Custom syntheses of peptides, their high performance liquid chromatography purification, and their mass spectroscopic analyses were performed by Genosys Biotecnologies, Inc. (Woodlands, TX). Human mini TyrRS, S. cerevisiae TyrRS, and Escherichia coli TyrRS (with a C-terminal tag of six histidine residues) were overexpressed in E. coli strain BL 21 (DE 3) (Novagen, Madison, WI) by induction with isopropyl β-D-thiogalactopyranoside for 4 h. Using the procedures described by Novagen, the proteins were purified on a nickel affinity column (His-Bind™ resin; Novagen) from the supernatant of lysed cells. Endotoxin was removed from the protein.*
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**RESULTS**

Test for Cytokine Activity of Non-human TyrRS—Our previous results showed that the ELR motif of human mini TyrRS plays an important role in PMN receptor binding, as it does in α-chemokines (5). As shown in Fig. 1A, the critical ELR motif of α-chemokines like IL-8 is conserved among mammalian TyrRSs. The corresponding sequence element of *S. cerevisiae* or *P. carinii* TyrRSs is included for comparative purposes.

The maximal specific response represents 2000 cpm. The data represent mean values ± S.D. of three independent measurements. Previously reported (5) data of human mini and *E. coli* TyrRSs are included for comparative purposes. Panel C, each protein (1 nM) or medium alone was added to the lower compartments of chemotaxis chambers and PMNs (10^6 cells) were added to the upper compartments. Chambers were incubated for 45 min, and then migrating cells were counted in HPPs. Four measurements of PMN chemotaxis were done with each protein. Each determination was an average of nine HPF determinations. The data shown are representative of at least three independent experiments done in this way. Previously reported (5) data of human mini and *E. coli* TyrRSs are included for comparative purposes.

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**Fig. 1. Effect of some TyrRSs on PMN receptor binding and PMN migration. Panel A, schematic alignments of human mini TyrRSs with human full-length TyrRS (GenBank TM Data Bank accession number U89436), *S. cerevisiae* TyrRS (L12221), and *E. coli* TyrRS (J01719). The arrow shows the approximate PMN elastase cleavage site in human TyrRS (5). The location of CP1 (connective polypeptide 1 (48)) that splits the Rossmann nucleotide binding fold in TyrRS is indicated. Numbers on the right of the sequences correspond to the protein sizes. Partial alignments of bovine (AP087021), *S. cerevisiae* (L12221), *Schizosaccharomyces pombe* (AL031324), Pneumocystis carinii (20), *E. coli* (J01719), and *B. stearothermophilus* TyrRSs (J01546) corresponding to the ELR motif of human TyrRS are shown. Panel B, after incubation of PMNs with ^125^I-labeled human mini TyrRS (10 nM) in the absence or presence of a 100-fold molar excess of unlabeled TyrRSs for 2 h, cells were separated from bound radioactivity by centrifugation, and the cell sediment was resuspended and counted in a liquid scintillation counter. The latter procedure was repeated twice. Human PMNs were isolated from the blood by centrifugation (700 g) of human erythrocytes, washed twice with Hanks’ balanced salt solution, and incubated in RPMI 1640 medium containing heat-inactivated fetal bovine serum (10%), plated on tissue culture flasks, and incubated in a 5% CO2 incubator at 37 °C for 1–2 h (16). Nonadherent cells were removed by washing the flasks three times with Hanks’ balanced salt solution, and adherent cells were harvested by incubation with calcium-magnesium free phosphate-buffered saline containing EDTA (2 mM) for 15 min at 4 °C, followed by extensive washing.

**Chemotaxis Assays—**Cell migration of PMNs or MPs was performed in a microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) containing polycarbonate filters (5-µm pores) without or with polyvinylpyrrolidone, respectively (5, 17). PMNs or MPs were suspended in RPMI 1640 medium containing heat-inactivated fetal bovine serum (1%), and 10^4 cells were added/well to the upper chamber. The chemotactic stimulus was placed in the lower chamber, and cells were allowed to migrate for 45 min (for PMNs) or 3 h (for MPs) at 37 °C in a 5% CO2 incubator. After incubation, nonmigrating cells were removed, membranes were fixed in methanol, and migrating cells were visualized with the Hemacolor™ stain set (EM Diagnostic Systems, Gibbstown, NJ). Migrating cells were counted in high power fields (HPFs).

**Chemokine Receptor Binding Assays—**Custom radioiodination of human mini TyrRS with ^125^I was performed by Research & Diagnostic Antibody, Inc. (Richmond, CA). PMNs (2 × 10^6^ cells) in 120 μl of RPMI 1640 medium containing 20 mM Hepes (pH 7.4) and 10 mg/ml bovine serum albumin were incubated on ice for 2 h with 10 nM ^125^I-labeled human mini TyrRS (specific activity of ~60 Ci/mmol) in the absence or presence of a 100-fold molar excess of unlabeled TyrRSs. Cells were separated from bound radioactivity by centrifugation at approximately 8000 × g for 2 min through 500 μl of a 10% sucrose/phosphate-buffered saline cushion. The supernatant was aspirated, and the cell sediment was resuspended by using EcoLite (ICN Biomedicals, Irvine, CA) and counted in a liquid scintillation counter.

**Gel Filtration Chromatography—**Gel filtration was accomplished on a Superose 6 HR 10/30 FPLC column (Amersham Pharmacia Biotech). Gel filtration standard (Bio-Rad) that included thyroglobulin, bovine gamma globulin, chicken ovalbumin, equine myoglobin, and vitamin B-12 was used.
TyrRS inhibited binding of 125I-mini TyrRS to PMNs (Fig. 1C, schematic comparison of the domains homologous to the C-domain of human TyrRS (accession number Z73427), and S. cerevisiae Arc1p (X95481) are shown. Partial sequence alignments with the RIGRIIT motif of human EMAP II are listed. Peptides were synthesized based on the partial sequence alignments. A cysteine residue of the EMAP II peptide was replaced by an arginine to enhance peptide stability and solubility. (Previous experiments clarified that this substitution did not alter its biologic properties (21)).

Alignments of human TyrRS (accession number U10117), C. elegans MetRS (Z73427), and S. cerevisiae Arc1p (X95481) are shown. Partial sequence alignments with the RIGRIIT motif of human EMAP II are listed. Peptides were synthesized based on the partial sequence alignments. A cysteine residue of the EMAP II peptide was replaced by an arginine to enhance peptide stability and solubility. (Previous experiments clarified that this substitution did not alter its biologic properties (21)).

Panel A, schematic comparison of the domains homologous to the C-domain of human TyrRS. Alignments of human TyrRS (accession number U10117), C. elegans MetRS (Z73427), and S. cerevisiae Arc1p (X95481) are shown. Partial sequence alignments with the RIGRIIT motif of human EMAP II are listed. Peptides were synthesized based on the partial sequence alignments. A cysteine residue of the EMAP II peptide was replaced by an arginine to enhance peptide stability and solubility. (Previous experiments clarified that this substitution did not alter its biologic properties (21)).

Panel B, induction of PMN and MP migration (white and gray bars, respectively). Each peptide (1 nM) or medium alone was added to the lower compartments of chemotaxis chambers, and PMNs or MPs (10^4 cells) were added to the upper compartments. Chambers were incubated for 45 min (for PMNs) or 3 h (for MPs), and then migrating cells were counted in HPF. Results of chemotaxis assays were analyzed as in Fig. 1C.

E. coli TyrRS is NYR or ETV, respectively. Previously we showed that E. coli TyrRS neither activated nor bound to the IL-8 receptors on PMNs (5). A non-human eukaryotic TyrRS was not tested.

To delineate further the interaction of TyrRSs with PMNs, human mini TyrRS was radioiodinated to have a tracer for binding studies. Binding of human mini TyrRS for PMNs is not associated with cytokine activity, we also investigated the chemotaxis activities of human mini, S. cerevisiae, or E. coli TyrRS. As shown in Fig. 1C, incubation of PMNs with human mini TyrRS (1 nM) led to induction of PMN migration. In contrast, no chemotaxis was observed with either S. cerevisiae TyrRS or E. coli TyrRS (each at a concentration of 1 nM) (Fig. 1C). In addition to checking of the potential cytokine activity of S. cerevisiae TyrRS at a concentration of 1 nM, concentrations of 0.1, 10, 100, and 1000 nM were also tested. No significant chemotaxis was observed (data not shown). Thus, the binding of S. cerevisiae TyrRS to PMNs is not associated with a serendipitous cytokine function.

FIG. 2. Effect of heptapeptides on PMN and MP migration. Panel A, schematic comparison of the domains homologous to the C-domain of human TyrRS. Alignments of human TyrRS (accession number U89436) with human EMAP II (U10117), C. elegans MetRS (Z73427), and S. cerevisiae Arc1p (X95481) are shown. Partial sequence alignments with the RIGRIIT motif of human EMAP II are listed. Peptides were synthesized based on the partial sequence alignments. A cysteine residue of the EMAP II peptide was replaced by an arginine to enhance peptide stability and solubility. (Previous experiments clarified that this substitution did not alter its biologic properties (21)).

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human mini TyrRS α-chemokines

FIG. 3. Schematic comparison between human mini TyrRS and α-chemokines. Shown are schematic structures of α-chemokines (27, 28) and the subunit of homodimeric human mini TyrRS (predicted on the basis of the crystal structure of B. stearothermophilus TyrRS (26)). In this figure, C-terminal parts of human mini TyrRS and α-chemokines are omitted. The locations of β-strands or ELR motifs are delineated with solid arrows or circles, respectively. In the dimeric structure of TyrRS, the second subunit would appear below the plane of the one shown and is related by a 2-fold axis (26). Not a bacterial TyrRS can bind to the PMN receptor for human mini TyrRS.

To determine whether binding of S. cerevisiae TyrRS to PMNs was associated with cytokine activity, we also investigated the chemotaxis activities of human mini, S. cerevisiae, or E. coli TyrRS. As shown in Fig. 1C, incubation of PMNs with human mini TyrRS (1 nM) led to induction of PMN migration. In contrast, no chemotaxis was observed with either S. cerevisiae TyrRS or E. coli TyrRS (each at a concentration of 1 nM) (Fig. 1C). In addition to checking of the potential cytokine activity of S. cerevisiae TyrRS at a concentration of 1 nM, concentrations of 0.1, 10, 100, and 1000 nM were also tested. No significant chemotaxis was observed (data not shown). Thus, the binding of S. cerevisiae TyrRS to PMNs is not associated with a serendipitous cytokine function.

Dimeric Structure for Human Mini TyrRS—Prokaryotic and lower eukaryotic TyrRSs form stable dimers (18–20). We considered the possibility that the cytokine activities of human mini TyrRS were because of it having a different oligomeric state from the prokaryotic or lower eukaryotic homologs. For example, the region of the ELR motif might be in a distinct conformation in a monomeric versus a dimeric form of the protein. To investigate the oligomeric state of mini TyrRS, we determined the molecular weight of mini TyrRS by gel filtration chromatography on a Superose 6 HR 10/30 column. As controls, we performed gel filtration of E. coli and S. cerevisiae TyrRSs. Human mini TyrRS eluted at the same position as the E. coli or S. cerevisiae TyrRS, with an estimated molecular weight of 90 kDa (data not shown). This value corresponds closely to that expected for the dimeric form (84 kDa).

Cytokine Activities of Peptides Derived from Domains Similar to the C-Domain of Human TyrRS—A synthetic peptide comprising seven residues near the N terminus of human mature EMAP II (RIGRIIT) was shown previously to induce migration of PMNs and MPs (21). To gain further insight into the cytokine activity of the C-domain of human TyrRS, we used a sequence alignment to design the corresponding peptide (RVGIIT) of the C-domain (Fig. 2A). This C-domain peptide from TyrRS induced the same PMN and MP migration as did the mature EMAP II peptide (Fig. 2B). The effect of the C-domain peptide from TyrRS on PMN and MP migration showed a dose-dependence similar to that of the mature EMAP II.
peptide (data not shown). Thus, the C-domain of TyrRS and mature EMAP II share similar peptide motifs for chemotaxis at the same positions in their respective sequences.

Portions of S. cerevisiae Arc1p and C. elegans MetRS have high sequence homology with the C-domain of human TyrRS (6, 11, 13). Accordingly, we prepared the RVGKIIK-like peptide of S. cerevisiae Arc1p (RVGFIQK) and of C. elegans MetRS (RVGRIIK). Neither peptide induced PMN or MP migration (6, 11, 13). Accordingly, we prepared the RVGKIIT-like peptide high sequence homology with the C-domain of human TyrRS motif (where Xaa is any residue), mini TyrRS does not share the same positions in their respective sequences.

DISCUSSION

Because many residues and motifs are conserved among eukaryotic TyrRSs that are not found in their prokaryotic counterparts (22, 23), the binding of S. cerevisiae TyrRS to PMNs could reflect its structural similarity to human mini TyrRS. One reason why S. cerevisiae TyrRS does not have IL8-like cytokine activity might be attributed to the variation (NYR) of the sequence of critical ELR motif. We showed previously that a simple ELR → ELQ change inactivated the IL8-like activity of human mini TyrRS (5). In this connection, it is also noteworthy that prokaryotic and eukaryote cytoplasmic TyrRSs cannot cross-aminocylate their respective tyrosine tRNAs because of a sequence variation in a peptide motif that is needed for discrimination of a species-specific difference in their respective tRNA sequences (20, 24, 25). Thus, both cytokine and RNA-related activities of human TyrRS are sensitive to the most subtle sequence variations.

Human mini TyrRS differs in primary structure from more typical α-chemokines. For example, while mini TyrRS contains the ELR motif that is critical for receptor binding, this motif is at the middle of the Rossmann fold that forms the site for synthesis of tyrosyl-adenylate. In contrast, the ELR motif of α-chemokines is located near the N terminus. Also, whereas α-chemokines have conserved cysteines and a Cys-Xaa-Cys motif (where Xaa is any residue), mini TyrRS does not share the conserved residues. Despite these differences in primary structures, human mini TyrRS is predicted (based on the crystal structure of Bacillus stearothermophilus TyrRS (26)) to form the same six-stranded β-sheet as the α-chemokines (Fig. 3). Moreover, the predicted location of the ELR motif of human mini TyrRS is close to that of the α-chemokines (Fig. 3).

Under the experimental conditions of NMR or x-ray analyses, native IL-8 is a dimer (27, 28). However, at physiologically more relevant concentrations, monomeric and dimeric forms of IL-8 are in equilibrium, with the monomer being the prevalent form (29, 30). Dimerization-deficient IL-8 analogues were engineered by chemical modification or by mutations of residues at the dimer interface (31–33). Their structural analyses clarified that the IL-8 monomers have the same tertiary folding as the dimer (32, 34). In addition, their functional analyses showed that the monomers have full cytokine activity in vitro and in vivo (31–33). On the other hand, to mimic the dimeric form of IL-8, cross-linked single-chain dimers were designed (35). The results of chemotaxis and receptor binding assays showed that the dissociation of the dimer is not required for the biological activities (35). Thus, dimerization of IL-8 introduces no structural constraints for its tertiary folding or activities. Similarly, the ELR motif needed for the IL8-like activity is accessible in the dimeric structure found here for human mini TyrRS.

A long N-terminal segment precedes the ELR region of mini TyrRS (5). The same extension occurs in the yeast and bacterial proteins studied here so that the presence or absence of this extension cannot explain the unique cytokine activity of human mini TyrRS. Many of the ELR-containing chemokines have an N-terminal extension that is cleaved away to activate the chemokine (36, 37). These N-terminal extensions may block access to the ELR motif, and it is for that reason that activation occurs upon removal of the extensions. However, as explained above, the N-terminal region of the structural model of mini TyrRS does not physically block the ELR motif so that this region...
should be accessible for cytokine activation. Consistent with this conclusion, Hölzer et al. prepared a fusion protein in which the N terminus of IL-8 was fused to a Fab fragment (38). This chimeric protein showed specific binding to the IL-8 receptor, induced IL-8-mediated chemotactic activity, and stimulated release of myeloperoxidase.

Some amino acid residues in peptides corresponding to the RVGKIIT motif from the C-domain of human TyrRS are highly conserved among other proteins of both prokaryotes and eu-

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