New Concepts

Activation of Angiogenic Signaling Pathways by Two Human tRNA Synthetases†

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ABSTRACT: Aminoacyl-tRNA synthetases establish the rules of the genetic code by joining amino acids to tRNAs that bear the anticodon triplets corresponding to the attached amino acids. The enzymes are thought to be among the earliest proteins to appear, in the transition from a putative RNA world to the theater of proteins. Over their long evolution, the enzymes have acquired additional functions that typically require specialized insertions or domain fusions. Recently, fragments of the closely related human tyrosyl- and tryptophanyl-tRNA synthetases were discovered to be active in angiogenesis signaling pathways. One synthetase fragment has proangiogenic activity, while the other is antiangiogenic. Activity was demonstrated in cell-based assays in vitro and in vivo in the chick embryo, and in the neonatal and adult mouse. The full-length, native enzymes are inactive in these same assays. Activation of angiogenesis activity requires fragment production from the native enzymes by protease cleavage or by translation of alternatively spliced pre-mRNA. Thus, these tRNA synthetases link translation to a major cell-signaling pathway in mammalian cells. The results with animals suggest that therapeutic applications are possible with these tRNA synthetases.

The past decade heralded a new era of discovery for aminoacyl-tRNA synthetase research as several independent studies demonstrated that the enzymes are involved in activities beyond translation. This family of enzymes is conserved throughout all organisms to carry out ligation of amino acids to their cognate tRNAs. However, idiosyncratic variations found in particular synthetases gave rise to new biological roles.

In the conventional role, tRNA synthetases are responsible for establishing the genetic code through selection and pairing of amino acids with their cognate tRNAs. This canonical family of enzymes also has a broad repertoire of other functions such as RNA splicing, RNA trafficking, transcriptional and translational regulation, and cell signaling (*1*). Additionally, several synthetase-like proteins were reported to participate in a wide range of specialized activities such as DNA synthesis, cell signaling, and regulation of amino acid biosynthesis (*2*). These activities illustrate the versatility of the tRNA synthetase scaffold and its capacity for modifications and additions of domains that expand the functions.

Recent studies on two mammalian synthetases, tyrosyl-tRNA synthetase (TyrRS)[1] and tryptophanyl-tRNA synthetase (TrpRS), showed that these proteins participate in angiogenic signal transduction pathways (*3*−*7*). This discovery was stimulated in part by the finding that the antiangiogenic factor endothelial monocyte activating polypeptide II (EMAP II) was a processed form of the tRNA synthetase-associated protein p43 (*8*, *9*), and in part by the discovery of an interleukin 8 (IL-8)-like activity for one of these enzymes (*3*, *4*). The latter cytokine is known to be involved in angiogenic signaling pathways (*10*).

Translation Proteins and Cell Signaling: A New Paradigm

EMAP II Is a Tumor-Derived Inhibitor of Angiogenesis. A new antiangiogenic factor was isolated from methylcholanthrene A-induced fibrosarcomas in 1993 (*11*). Treatment of this tumor in mice with tumor necrosis factor resulted in thrombosis and vessel hemorrhage, increased vascular permeability, and tumor regression (*12*−*14*). A search for tumor factors that altered endothelial functions led to EMAP II (*11*), a 23 kDa protein that activates polymorphonuclear (PMN) cells and mononuclear phagocytes (MPs) (*15*).

Surprisingly, EMAP II is a fragment of the intracellular protein p43, which combines with at least nine tRNA synthetases in a multi-synthetase complex (*8*, *9*). Within the complex, p43 occupies a central position (*16*) where it

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[1] Abbreviations: TyrRS, tyrosyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; EMAP II, endothelial monocyte activating polypeptide II; PMN, polymorphonuclear; MP, mononuclear phagocyte; IL-8, interleukin 8; CAM, chick chorioallantoic membrane; IFN-γ, interferon γ; HUVEC, human umbilical vein endothelial cell; IDO, indoleamine 2,3-dioxygenase.

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modulates the activity of arginyl-tRNA synthetase (*9*). Despite the failure to identify a conventional secretion signal peptide for either p43 or EMAP II, normal and apoptotic cells actively secrete p43 (*17*) and EMAP II (*11*, *18*, *19*).

In addition to being an activator of PMN cells and MPs, EMAP II has antiangiogenic activity. In two experimental models (mouse matrigel and rabbit cornea models), EMAP II potently inhibited basic fibroblast growth factor-stimulated angiogenesis (*20*). In a model of primary and metastatic tumor growth, EMAP II had significant antitumor activity (*20*). The connection of EMAP II/p43 to translation and to regulation of tumor angiogenesis was unexpected. Although EMAP II was reported to bind to cell surface α-ATP synthase on serum-starved cells (*21*), the binding has not been demonstrated to be required for mediation of the antiangiogenic response of EMAP II. Still, this work was the first attempt to study potential signaling interactions between EMAP II and cell surface molecules.

A Human TyrRS Fragment Is Angiogenic and Stimulates Immune Cells. The sequence of human TyrRS revealed an EMAP II-like protein fused to the C-terminal side of the catalytic domain (*22*). The amino acid sequence of this C-domain is 48% identical with that of human EMAP II (*22*). This level of identity was unexpected because none of the sequenced prokaryote (*Escherichia coli*) or basal eukaryote (*Saccharomyces cerevisiae*) TyrRSs contained this fusion. However, "stand alone" proteins homologous to EMAP II, such as Arc1p in the yeast *S. cerevisiae* (*23*) and Trbp 111 in the extreme thermophile *Aquifex aeolicus*, are found throughout evolution (*24*). Segmented animals, such as mammals and insects, adopted the EMAP II module as a C-terminal domain fused to TyrRS. This observation stimulated examination of a role for the C-domain of human TyrRS.

Throughout evolution, a number of insertions and domain additions were fixed into aminoacyl-tRNA synthetases. In the case of the EMAP II-like C-domain of human TyrRS, initial studies showed the domain was dispensable for aminoacylation in vitro and in vivo (*25*). Although not required for aminoacylation, the C-domain was conserved among all mammalian and insect TyrRSs. Given that it was not necessary for activity and that it appeared late, we examined whether it had any of the cytokine activities of the homologue EMAP II.

The isolated C-domain had cytokine activities similar to those of EMAP II. In response to the C-domain, MPs migrated and produced tumor necrosis factor-α and tissue factor, and PMN cells migrated and produced myeloperoxidase. These responses are those of EMAP II (*3*). EMAP II is also an antiangiogenic factor (*20*, *26*), although the related C-domain of mammalian TyrRS was not tested for that activity. Significantly, these activities were found only after the C-domain was released from TyrRS. In the context of the full-length protein, the cytokine activities were quenched (*3*).

The N-terminal catalytic domain (designated mini TyrRS, Figure 1) was serendipitously discovered to also have cytokine activity (*3*, *4*). Mini TyrRS specifically stimulated migration of PMN cells with a bell-shaped dose dependence [as seen with CXC-chemokines (*27*, *28*)] and maximum activity between 1 and 10 nM. Here again, full-length, unsplit TyrRS was inactive. The surprising cytokine activity of mini

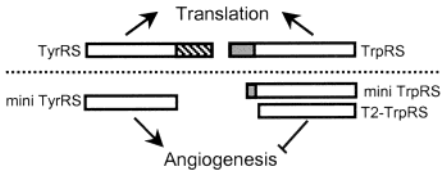

FIGURE 1: Extra domains found in cytoplasmic human TyrRS and TrpRS (shaded regions) modulate the activities of these proteins. The full-length proteins participate in translation, while truncated variants stimulate or inhibit angiogenesis.

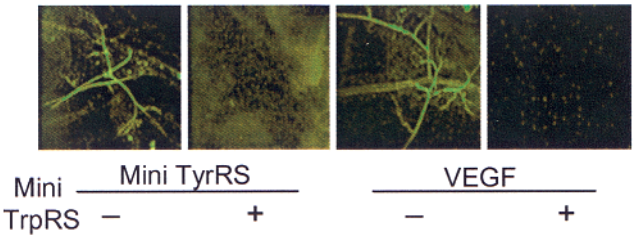

FIGURE 2: Angiogenesis is activated by mini TyrRS and inhibited by mini TrpRS in a murine matrigel model of angiogenesis. Matrigel implants containing mini TyrRS, VEGF, or mini TrpRS were injected subcutaneously. Five days later, the mice were injected intravenously with the fluorescein-labeled endothelial binding lectin *Grifonia* (*Bandeiraea*) *simplicifolia* I, isolectin B4 (*5*, *6*). The matrigel plugs were resected and fluorescent images taken by confocal microscopy. The third and fourth panels are adapted from Wakasugi et al. (*6*). Copyright 2002 National Academy of Sciences, U.S.A.

TyrRS was unique to the mammalian enzyme and mimicked that of IL-8 (*3*, *4*). The CXC-chemokine IL-8 stimulated PMN cell migration in this assay with a maximum at 100 nM.

IL-8 belongs to a class of cytokines the activity of which is dependent on the three-amino acid motif Glu-Leu-Arg (ELR). In CXC-chemokines, ELR predicts neutrophil activation (*29*, *30*) and stimulation of angiogenesis (*27*, *31*). Human mini TyrRS contains a single ELR motif that is absent from the yeast and *E. coli* enzymes. The ELR motif in mini TyrRS correlated with activation of PMN cells in chemotaxis (*3*, *4*). For example, a point mutation within the ELR motif (R93Q) abolished PMN migration. Similarly, an ELR to DLQ mutation inactivates IL-8 (*27*). Further work demonstrated binding of mini TyrRS to CXCR1, a cell surface IL-8 receptor (*3*). Thus, the ELR element was essential for cytokine activity of mini TyrRS as well as of IL-8. Secondary structural modeling supported the concept that the ELR tripeptide was similarly disposed in a loop in each of the respective proteins (*4*).

As noted above, CXC-chemokines that contain the ELR motif have proangiogenic activity. Indeed, mini TyrRS was angiogenic in mouse matrigel and chick chorioallantoic membrane (CAM) assays for angiogenesis (Figure 2) (*5*). Activity was dependent on the ELR motif; mutation from ELR to ELQ abolished activity (*5*). Full-length TyrRS was inactive in these same assays. Thus, removal of the C-domain by proteolytic cleavage is required for activation of the cytokine functions of mini TyrRS.

That full-length human TyrRS was inactive in the cytokine assays suggested that cellular export and proteolytic processing could regulate the signal transduction activities of mini TyrRS and the C-domain. In a preliminary screen of TyrRS

export, apoptotic U937 cells released TyrRS and not several other synthetases that were tested. Release occurred under conditions similar to those that lead to release EMAP II (*32*). Because EMAP II is known to be secreted under certain conditions (*11*, *18*, *19*), perhaps the EMAP II-like domain of TyrRS is what mediates export of the synthetase. A second level of regulation of cytokine activity would be proteolytic processing. In this connection, the extracellular protease leukocyte elastase released mini TyrRS from the full-length enzyme (*3*). This protease is found at the tumor−host interface and at sites of inflammation (*33*).

TrpRS Variants Are Potent Inhibitors of Angiogenesis. TrpRS is a close homologue of TyrRS. The active site Cα atoms of the two enzymes are superimposable with an rms deviation of only 1.1 Å (*34*). Like human TyrRS, human TrpRS contains an appended domain that is found only in mammalian TrpRSs (Figure 1). In this instance, the extra domain is appended to the N-terminus and is similar to a domain found in eukaryote tRNA synthetases for histidine, glycine, and methionine (*35*−*37*). It is also repeated as three tandem domains that join human glutamyl- with prolyl-tRNA synthetase to give the Glu-Pro tRNA synthetase that is found in higher eukaryotes (*38*). As isolated domains, the peptides have helix−turn−helix structures that bind tRNA (*39*, *40*). Thus, tRNA synthetases may have adopted this module to facilitate tRNA binding. However, the N-terminal domain in human TrpRS is not required for aminoacylation (*6*), and its function is unknown.

Prior to the discovery that fragments of human TyrRS had cytokine activities, TrpRS gene and protein expression were demonstrated to be upregulated by the antiproliferative cytokine interferon-γ (IFN-γ) (*41*−*45*). Among tRNA synthetases, the IFN-γ induction of TrpRS was unique. Interestingly, an alternatively spliced variant of TrpRS mRNA was also upregulated by IFN-γ (*46*, *47*). The protein encoded by the alternatively spliced mRNA was predicted to be an N-terminally truncated TrpRS variant that began at Met^{48} (designated mini TrpRS). In a proteome analysis of IFN-γ-treated cells, a protein spot consistent with the theoretical mass and pI for mini TrpRS was identified (*45*). Additionally, this spot reacts with anti-TrpRS antiserum in a Western blot and was suggested to be the product of alternatively spliced TrpRS mRNA (*45*). Supporting that finding, we re-examined the two-dimensional (2D) proteome analyses of other IFN-γ-treated cells and found a protein strongly upregulated by IFN-γ with a mass and isoelectric point similar to those predicted for mini TrpRS (*41*, *42*, *44*, *48*).

Given the cytokine activities of human TyrRS fragments, human TrpRS and the truncated variant mini TrpRS were investigated for activities on human umbilical vein endothelial cells (HUVECs) and for angiogenesis in vivo. Mini TrpRS blocked VEGF-induced migration of HUVEC cells, while full-length TrpRS had no effect (*6*). Further verification of the activity of mini TrpRS was observed in two different angiogenesis assays in vivo. In both chick CAM and mouse matrigel assays, mini TrpRS inhibited VEGF-stimulated angiogenesis (Figure 2) (*6*). The cell signaling activity was specific to the truncated mini TrpRS variant that lacked most of the N-terminal helix−turn−helix domain. Similar N-terminally truncated variants produced by leukocyte elastase cleavage (T1-TrpRS and T2-TrpRS) had antiangiogenic activity. The smallest variant T2-TrpRS exhibited the most potent activity against VEGF-stimulated angiogenesis (*6*, *7*), with dose-dependent inhibition of angiogenesis (in the mouse matrigel assay) having an IC_{50} of 1.7 nM (*7*).

In a mouse model of angiogenesis in which VEGF is believed to play an important role (*49*, *50*), both mini TrpRS and T2-TrpRS inhibited the natural process of retinal angiogenesis. T2-TrpRS was particularly potent in this system. It completely inhibited angiogenesis in 70% of the eyes that were tested (*7*). As found in the other studies, full-length human TrpRS was inactive in the mouse retina model (*7*). In a separate experiment, Alexafluor 488-labeled T2-TrpRS was injected as a biologic to examine the mechanism of inhibition of retinal angiogenesis. The fluorescent T2-TrpRS bound to pre-existing blood vessels in the retina primary layers and effectively inhibited further angiogenesis. In contrast, Alexafluor 488-labeled TrpRS did not bind specifically to any site within the retina (*7*). The binding results were consistent with the activity of T2-TrpRS and the inactivity of full-length TrpRS in other angiogenesis assays. Thus, T2-TrpRS may act directly on existing blood vessels to inhibit further development.

Because the cell signaling activity of human TrpRS like that of TyrRS is found exclusively in truncated variants, we imagine mini TrpRS must be generated by a regulated mechanism. For example, proteolysis of full-length TrpRS could activate the protein for inhibition of angiogenesis.

To gain more insight into the biological significance of the cytokine activities of tRNA synthetases, we examined a database of human tissues and cell lines for the gene expression profile of human TrpRSs (*51*). The profile showed a striking overexpression (greater than 3-fold median expression calculated for all tissues and cells lines that were examined) in three tissues, i.e., placenta, lung, and spleen (Figure 3). In addition, HUVEC cells and whole blood had slightly elevated expression levels. Compared to those of 15 other cytoplasmic tRNA synthetases included in the database, the overexpression and tissue specificity were unique. Among normal tissues, no other synthetase showed significant overexpression. Because TrpRS expression is upregulated by IFN-γ (*41*−*45*), the unique tissue-specific TrpRS expression, relative to other synthetases, was perhaps not surprising.

We examined whether TrpRS overexpression in placenta, lung, and spleen correlated with the expression of another IFN-γ inducible gene, i.e., indoleamine 2,3-dioxygenase (IDO). IDO catalyzes the rate-limiting step in the catabolism of tryptophan along the mammalian kynurenine pathway (*52*). One hypothesis for the IFN-γ regulation of TrpRS is that IDO upregulation leads to tryptophan depletion. Clear evidence for coordinated expression of TrpRS and IDO has been demonstrated in a few systems (*53*, *54*). Thus, the coordinated upregulation of TrpRS and IDO could serve as a mechanism for maintaining protein synthesis during tryptophan depletion (*43*, *55*). A comparison of the relative expression levels of TrpRS and IDO (*51*) shows that the two enzymes have strikingly different expression profiles in the three human tissues where TrpRS is expressed at high levels. None of the tissues or tumor cells that overexpressed either TrpRS or IDO also overexpressed the other protein (Pearson's $r = 0.102$). IDO mRNA was ubiquitous in all normal tissue, but it was only overexpressed in thymus. These results suggest in these tissues that TrpRS upregulation is not linked to IDO expression. Thus, at least in many circumstances,

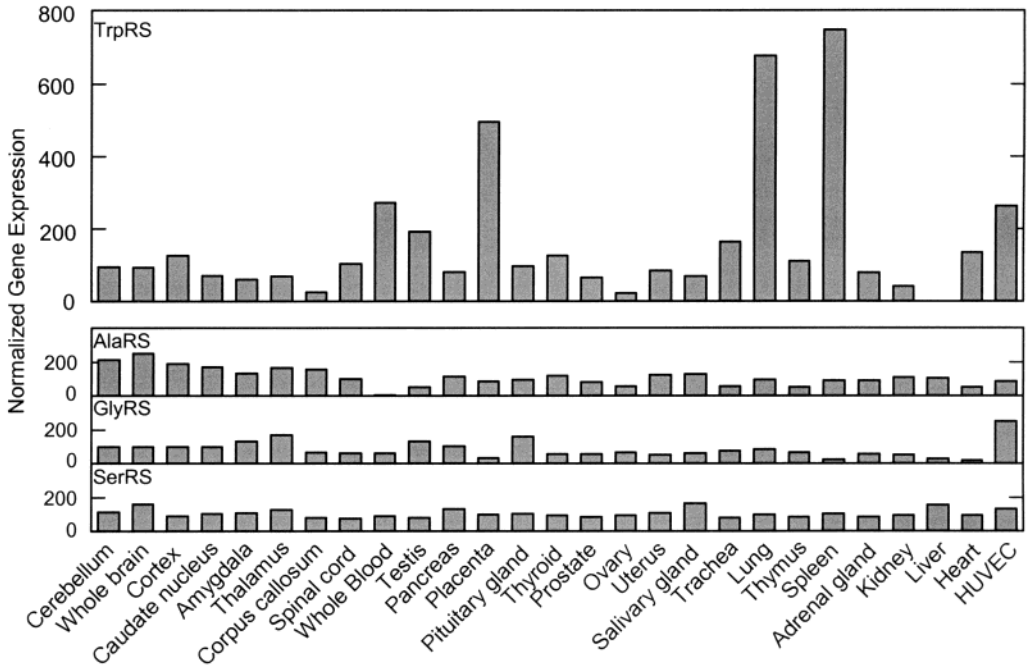

FIGURE 3: Tissue-specific gene expression profiles for TrpRS, AlaRS, GlyRS, and SerRS were obtained from a human transcriptome database (*51*). The gene expression level (vertical axis) was normalized (relative to 100) to its respective median level of expression measured in 46 different tissues or cells. The data from selected tissues and cells are displayed.

upregulation of TrpRS contributes to functions distinct from protein synthesis maintenance during IDO expression.

Profiling other genes with tissue-specific overexpression in placenta, lung, spleen, and HUVEC cells is also of interest. We identified 27 genes that were overexpressed in parallel with high-level production of TrpRS. Fifteen of these were involved in signal transduction or cell adhesion pathways. The coincidence between TrpRS expression and the expression of a number of other genes involved in cell signaling or angiogenesis (such as endoglin, PECAM1, Gro α, granulin, and furin) is consistent with the idea that TrpRS contributes to biological processes involving cell signaling such as angiogenesis regulation.

Anecdotal reports disclosed the overexpression of TrpRS in a variety of other conditions. For example, TrpRS is overexpressed in guinea pigs during delayed-type hypersensitivity reactions (*56*), in *Drosophila* during development of salivary gland (*57*), and in IFN-γ-treated bladder transitional cells (*44*). While the canonical function of TrpRS is aminoacylation of $tRNA^{Trp}$ for protein synthesis, the increase in TrpRS levels during specific developmental events and in specific diseases may be a response related to its role in cell signaling.

Cell Signaling Regulated by tRNA Synthetases. The appended domains of TyrRS and TrpRS are similar to the pro domains of many cytokines and growth factors. These domains maintain an inactive cell signaling status for the tRNA synthetases. Following the appropriate cellular induction, the bioactive fragments can be produced by removing the pro domain through specialized cellular mechanisms, including protease cleavage and alternative splicing of mRNA. After processing, the fragments are active as regulators of angiogenesis: mini TyrRS as a stimulator of blood vessel development and mini TrpRS or T2-TrpRS as a potent inhibitor of blood vessel development. Thus, these two synthetases acquired opposing signal transduction activity, possibly as a coordinated mechanism for regulating angiogenesis.

The mechanistic basis for the action of tRNA synthetase fragments has yet to be established. T2-TrpRS localizes to blood vessels and inhibits VEGF- and mini TyrRS-stimulated blood vessel development. Thus, T2-TrpRS may intervene at a step common to both of these angiogenic factors. Similarly, mini TyrRS-stimulated angiogenesis is inhibited by the antiangiogenic factor IP-10 and also by mini TrpRS, suggesting that mini TyrRS acts through a signaling mechanism that can be downregulated by various antiangiogenic factors. The regulation of angiogenesis by mini TyrRS and T2-TrpRS suggests a unique opportunity for therapeutic intervention.

While the biological rationale for cell signaling activities of TyrRS and TrpRS may not yet be evident, these enzymes have features that are particularly meaningful in the context of cell signaling. For example, the proangiogenic ELR motif of mini TyrRS and the EMAP II-like cytokine domain is a feature unique to higher eukaryotic TyrRSs. It is absent in lower eukaryotes such as yeast and the lower animal *Caenorhabditis elegans* which lacks a vascular system. Rational mutation of yeast TyrRS gives rise to a functional TyrRS with acquired cell signaling activity similar to that of human mini TyrRS (*68*). Thus, specific mutations of TyrRS during evolution allowed the development of new functional activities.

Interestingly, angiogenic human mini TyrRS is opposed by T2-TrpRS, a related enzyme with antiangiogenic activity. The activity of these related angiogenesis mediators is correlated to the presence of the ELR motif within the synthetases. Angiogenic mini TyrRS contains an ELR motif, while it is lacking in antiangiogenic T2-TrpRS. The activity of another class of angiogenic mediators, CXC-chemokines, is also determined by the presence or absence of the ELR motif, where ELR-containing chemokines are angiogenic and

those lacking ELR are antiangiogenic (*29*, *58*). In addition to the relationship between TyrRS and TrpRS, another biological mechanism relates TrpRS to cell signaling pathways. The antiproliferative cytokine IFN-γ, which is known to upregulate a number of antiangiogenic CXC-chemokines (*28*, *31*, *59*−*61*), also upregulates the expression of TrpRS.

Fragments of plasminogen and collagen with antiangiogenic activity and a ribonuclease A-like protein with angiogenic activity have been described (*62*−*64*). Their biological significance in natural angiogenesis and their therapeutic utility remain to be determined. Some of the same questions pertain to the fragments of TyrRS and TrpRS discussed here. However, the strong links between these enzymes and known cell signaling factors and regulators, and the lack of activities of TyrRS and TrpRS orthologs from organisms lacking a vasculature, are consistent with mini TyrRS and mini TrpRS having a natural role in angiogenesis.

Certain tRNA synthetases have been identified as autoantigens in the serum of patients with polymyositis, a wasting muscle disease. In most patients, the antibodies are directed against histidyl-tRNA synthetase (15−25%), and a few patients have antibodies to alanyl-, asparaginyl-, glycyl-, isoleucyl-, or threonyl-tRNA synthetases (*65*, *66*). Human histidyl-tRNA synthetase was recently reported to stimulate chemotaxis of activated monocytes and immature dendritic cells (*67*). That this synthetase interacts directly with immune cells suggests an active role for the synthetase in the etiology of anti-synthetase antibodies in polymyositis patients (*67*). Thus, while the cytokine and cell signaling activities of TyrRS and TrpRS have been demonstrated in some detail, it is likely that other synthetases, including histidyl-tRNA synthetase, have activities in signal transduction pathways. The elucidation and understanding of these activities is a major goal of future research.

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