CHAPTER 1

The Physiological Role of Lysyl tRNA Synthetase in the Immune System

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Contents

Abstract

Lysyl tRNA synthetase (LysRS) is an aminoacyl-tRNA synthetase (AaRS). This group of ancient proteins, known for their critical role in translation, was found in recent years to function in a variety of other roles.

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Besides its enzymatic activity in aminoacylation of tRNA, LysRS can produce dinucleotide diadenosine tetraphosphate (Ap_4A). Intracellularly, it is found mainly in the cytoplasm as a part of a multisynthetase complex where it interacts with several proteins, most notably AIMP2.

Besides its role in translation it has been demonstrated that LysRS can act as a cytokine-like molecule, secreted by cells and having distinct effects on macrophages. Moreover, LysRS can bind to the transcription factors USF2 and MITF and can influence their transcriptional activities following immunological stimulation of mast cells.

In this review, we focus on the nontranslational functions of LysRS related to the immune system. We begin with a short discussion of "gene sharing," proceed to a description of its structural and enzymatic function and then describe some of the *in vivo* functions of this enzyme.

Lysyl tRNA synthetase (LysRS) is an aminoacyl-tRNA synthetase (AaRS). This ancient family of enzymes has diverged to take on alternate and very diverse functions in the cell that extend well beyond their primary role in protein synthesis. Modules of synthetase ancestors or paralogs have also been adapted for other cellular purposes. One of these alternate roles for an AaRS was discovered when it was shown that AlaRS could bind to an upstream region of its own gene and autoregulate transcription (Putney and Schimmel, 1981). Another example is that human TyrRS is specifically cleaved to produce two distinct cytokines (Wakasugi and Schimmel, 1999). The cleavage itself unmasks the cytokine activities that are imbedded in the native protein, but not elucidated until cleavage occurs. A further example has been described whereby a secreted alternative splice fragment of TrpRS is a potent regulator of angiogenesis (Wakasugi *et al.*, 2002). Such observations have powerful implications on a link between protein synthesis, the immune system, and apoptosis.

In this review, we focus on the nontranslational functions of LysRS that are important to the immune system. We will first briefly discuss ''gene sharing,'' then describe the structural and enzymatic functions of LysRS and finally examine the *in vivo* functions of this enzyme.

1. GENE SHARING AND NONCANONICAL PROTEIN ROLES

Although the initial paradigm relating genes and proteins was that one gene encodes for one protein with one specific function, it has become clear that through alternative splicing one gene can be the source of

numerous, sometimes radically different, proteins. Piatigorsky was a pioneer in the study of alternative functions of similar proteins. For many years, he studied the crystallins, proteins with a critical role in the lens and cornea. His group (Wistow *et al.*, 1987) discovered that duck ε-crystallin was essentially identical to lactate dehydrogenase B4 (LDH64), an ancient, highly conserved glycolytic enzyme. Since ε-crystallin seemed to be confined to birds and crocodiles, it was possible that this represented a recent, atypical event in evolution. These researchers later revealed, however, this was not the case and sequence relationships have been found between all crystallins and specific enzymes (Piatigorsky and Wistow, 1989). The basic reason for the evolution of these enzymes into an alternative role is still not obvious since no common catalytic function is evident among these enzymes/crystallins. It seems that properties such as the ability to accumulate to high intracellular concentrations without precipitation, and thermodynamic stability, a characteristic of many enzymes, are probably the basis for their selection as crystallins. Thus this secondary protein function, which is not ''stable'' during evolution, might seem unrelated to what we perceive as the main attributes of the double function protein but nevertheless may play a critical role in certain biological systems.

This phenomenon was termed ''gene sharing,'' which is defined as a situation whereby a gene may acquire and maintain a second function without duplication or loss of the primary function (Piatigorsky, 2007). While the term gene sharing has been used to define the use of a single protein for two different functions, other names for a similar phenomena are also used such as ''moonlighting'' or noncanonical roles. Most people describing the alternative roles of AaRSs tend to use these terms perhaps due to the critical role of these proteins in translation.

It is important to note that the use of ''off the shelf'' proteins as components of new complexes with novel functions has possible important evolutionary advantages. It allows relatively rapid adaptation for new roles without the need for the creation of totally new proteins. Thus, it is not surprising that gene sharing or noncanonical roles of proteins are widespread.

2. AMINOACYL-tRNA SYNTHETASES (AaRSs) AND THE MULTISYNTHETASE COMPLEX

2.1. General description

The first function described for AaRSs was aminoacylation of their specific tRNAs. AaRSs are essential for decoding the genetic code during protein translation. Researchers trying to obtain insights as to the

beginning of life have studied in great detail these ancient proteins in a range of different organisms as probes for understanding basic aspects relating to the evolution of the genetic code (Ataide and Ibba, 2004; Pezo *et al.*, 2004; Ribas de Pouplana and Schimmel, 2001; Schimmel and Ribas de Pouplana, 2001; Stathopoulos *et al.*, 2001). There are two classes of tRNA synthetases, I and II, that are distinguished by the architecture of their active-site catalytic cores.

The AaRSs are essential housekeeping genes and thus critical for cellular survival. As mentioned earlier, many of these proteins have additional role/roles. These are related either to their catalytic activity or to an additional functional activity.

Decades ago, it was reported that during evolution many of the AaRSs incorporated additional domains (Schimmel and Ribas de Pouplana, 1995). The initially recognized roles of those domains were tRNA recognition and modification functions. However, later it was revealed that these appended domains can help in the regulation of important biological processes, including cell cycle control, tissue differentiation, cellular cemotaxis, and inflammation (Park *et al.*, 2005b, 2008; Ribas de Pouplana and Geslain, 2008).

As described earlier for crystallins, the reason for the evolution of a specific additional role of an ancient protein is not always obvious to us. In many cases, it is not linked to the known enzymatic activity of that specific protein. One suggestion has been that the critical role that AaRSs play in translational regulation evolved to provide a link between translation and a regulatory process such as cell cycle progression. This might be the case in some modes of regulation, but as will be described in the following section, the relationship between LysRS's noncanonical roles and translation is not straightforward. The alternative functions of this protein are probably related also to some other attributes of this protein and not to its role in translation *per se*.

Already in the 1970s, it was reported that most AaRSs reside in large protein complexes in the cytoplasm (Bandyopadhyay and Deutscher, 1971). A stable cytosolic complex of nine AaRSs (leucyl-, lysyl-, prolyl-, isoleucyl-, methionyl-, glutamyl-, glutaminyl-, arginyl-, and aspartyl-tRNA synthetases) was found in mammals (Mirande *et al.*, 1985). Moreover, it was suggested that the AaRSs responsible for coupling to those tRNA molecules that are charged hydrophobic and nonaromatic amino acids, are all present within the complex, while those aminoacylating the smallest and largest amino acids are absent (Wolfson and Knight, 2005). In addition to the nine AaRSs, the complex contains three nonsynthetase proteins: AIMP1, AIMP2, and AIMP3 (until recently, these proteins were known as P43, P38, and P18, respectively).

A variety of tRNA synthetase complexes have been identified in organisms varying from prokaryotes to archea and to eukaryotes, yet it

seems that the larger multisynthetase complex appeared only later in evolution and thus is found only in mammalians (Hausmann and Ibba, 2008a). It was postulated that the existence of the multisynthetase complex is important for efficient translation. Since depletion of any of the synthetases can cause a significant decrease in translation and cellular viability assessing the physiological importance of the multisynthetase complex for translation is difficult.

Only in 2008, there was more direct evidence for the importance of the multisynthetase complex in translational regulation finally provided (Kyriacou and Deutscher, 2008). Already in the 1980s, it was shown that in addition to its role in translation, ArgRS has a role in posttranslational modification, as it arginylates the NH_2 terminus of some proteins, a modification that can serve as a signal for ubiquitin-dependent protein degradation (Ferber and Ciechanover, 1987). Interestingly, there are two forms of ArgRS: one of around 72 Kd, which is found inside the multisynthetase complex, and the other of around 60 Kd found in the cytoplasm (Deutscher and Ni, 1982; Vellekamp *et al.*, 1985). Both forms have similar *in vitro* in catalytic activities (Vellekamp *et al.*, 1985). Kyriacou and Deutscher hypothesized that the shorter cytoplasmatic form is probably important for protein arginylation, while the longer form found in the multisynthetase complex is responsible for translation and the fact that it is part of this complex is critical for this role. A Chinese hamster ovary (Cho) cell line encoding temperature sensitive full-length arginine tRNA synthetase was used in this study. ArgRS is nearly deleted at higher temperatures in these cells. The cells were complemented with recombinant DNA encoding either the short 60 Kd or the longer 72 Kd form of ArgRS. They found that the shorter ArgRS, though just as active *in vitro* as the longer form, was significantly less efficient in complementing the translational activities of ArgRs *in vivo*. Although these results were obtained only for one type of AaRS, they certainly support the notion that the existence of the multisynthetase complex allows much more efficient translational efficacy.

It is interesting to note here the two aspects of the study described earlier. Firstly, in order to obtain insights about the specific function of ArgRS, it was essential to use an approach of reconstitution of the endogenously depleted AaRS with transfected mutated forms of the same plasmid. In recent experiments, we have used a similar approach to try to delineate noncanonical roles of LysRS. Secondly, the protein modifier role of ArgRS needs mention. In the same way that this enzyme can arginylate certain proteins at their NH_2 terminus, it has been postulated that LysRS adenylates NH_2 residues (Chou *et al.*, 2007).

Whatever be the initial important evolutionary advantage of allowing the formation of this multimeric complex, it is clear now that such a complex has advantages for the cells besides its importance in translation.

While the main role of the multisynthetase complex may have been initially to enable efficient protein translation, perhaps through the ''tRNA channeling,'' there are other attributes to proteins found in a complex such as this, for example, their stability might be totally different (Han *et al.*, 2008). The multisynthetase complex not only allows a more efficient functioning of its enzyme components in translation, but can also be viewed as a depot of 11 proteins.

2.2. The multisynthetase complex as a protein "depot"

The depot hypothesis postulates that there are some common features of macromolecular depots and their released daughter proteins (Lee *et al.*, 2004a; Ray *et al.*, 2007). While the macromolecular depots may allow efficient processes that demand close proximity of related proteins to take place, the same proteins released from the multimeric complexes (daughter proteins) commonly acquire a totally different role outside the complex either as monomers or as parts of newly formed complexes. The best studied example of a new complex which includes ''daughter proteins'' released from a multimeric complex is that of the large protein assembly called ''interferon-γ-activated inhibitor of translation'' (the GAIT complex) (Ray *et al.*, 2007). This is an assembly of four proteins that can bind specific structures at the 3′ end of at least several scores of messenger RNAs. The complex was initially identified through the study of proteins bound to the 30-nucleotide untranslated region of the ceruloplasmin mRNA in human monocytic cells. This sequence was demonstrated to be involved in the inactivation of translation following IFN-γ stimulation of those cells. The components of the GAIT complex assemble in two steps: initially, GluProRS is released from the multisynthetase complex and together with a known RNA binding protein: NS1-associated protein-1 (NSAP1) form a pre-GAIT complex after around 2 h of IFN-γ treatment. The complex is inactive until approximately 14 h later in human monocytes, when ribosomal protein L13a, which is released from the large ribosome units, together with another protein which is known mainly for its enzymatic roles, glyceraldehydes 3-phosphate dehydrogenase (GAPDH), join to form the active GAIT complex. Therefore, the GAIT complex contains two daughter proteins each released from a different depot of large multicomplex proteins that are involved in protein translation to ultimately regulate translational activity of several mRNAs. Interestingly, the trigger for release of both GluProRS and ribosomal protein 13a from the large complexes seems to be specific phosphorylation of these proteins (Mazumder *et al.*, 2003; Sampath *et al.*, 2004).

Mammalian GluProRS is unusual because it is a large protein containing two different AaRSs. These AaRSs are linked by a linker containing three homologous protein domains, called WHEP domains (Ribas de Pouplana

and Geslain, 2008). The complex mechanism involved in the binding of GluProRS to the GAIT element in mRNA and the translational inactivation of the related mRNA was recently described in detail (Jia *et al.*, 2008). It seems that all the RNA binding of the GAIT complex to GAIT element RNA is directly mediated by the WHEP domains. The first two WHEP domains are expressed in isolation and can compete with the GAIT complex for binding to the GAIT element, however, these two domains are insufficient for silencing mRNA translation since for that the whole complex is needed. The second and third WHEP domains were found to be used for GluProRS–NSAP1 binding, an association that later undergoes a conformational switch with the addition of phospho-L13a and GAPDH. Thus, a complex picture emerges in which WHEP domains change their interactions and conformation over the period of time following IFN-γ stimulation.

It seems that the important role in the GAIT complex is carried out only by the AaRS appended domains and not by the AaRSs themselves. In this regard, it is interesting to note that, so far, the WHEP domains have only been found to AaRS and not to other proteins. Thus, the large GluProRS released from the multisynthetase complex is used for cellular regulation due to its appended domains and not for any enzymatic activity. A further study identified the single site of L13a phosphorylation responsible for its release from ribosomes and for activation of the GAIT system in IFN-γ-treated monocytic cells (Mukhopadhyay *et al.*, 2008). This site is phosphorylated by the death-associated protein kinase-1 (DAPK) and zipper-interacting protein kinase (ZIPK), both of which contain a functional 30UTR GAIT element. This study revealed the existence of an RNA-based negative-feedback module.

It is interesting to note that the GAIT complex seems to have evolved only later in evolution as it is not found in rodents. While the enzymatic domains of tRNA synthetase are highly conserved through evolution, it would seem that the acquisition of additional, more specific function occurred later in evolution (Ribas de Pouplana and Geslain, 2008).

Several of the multisynthetase complex proteins have been demonstrated to have additional roles besides those related to translation in the multisynthetase complex. Thus, GlutRS and MetRS were shown to be involved in antiapoptotic regulation and rRNA biogenesis (Kim *et al.*, 2000; Ko *et al.*, 2001). Various roles have been described for the AaRS-interacting factors AIMP1–3. AIMP1 acts have several roles as an extracellular cytokine/hormone-like molecule (Kim *et al.*, 2008a; Lee *et al.*, 2008; Park *et al.*, 2006b). AIMP2 has important intracellular roles as a downregulator of c-Myc during lung cell differentiation (Kim *et al.*, 2003) and also as a positive regulator of p53 (Han *et al.*, 2008), while AIMP3 is a tumor suppressor that activates ATM/ATR, which is required for repair of DNA damage (Kim *et al.*, 2008b; Park *et al.*, 2005a, 2006a). Although it would seem that the multisynthetase complex may be the

source of these functional proteins, this has not yet been verified experimentally for proteins other than GluProRS and LysRS. Of all the multisynthetase complex proteins, the enzyme with the most diverse activities besides translational regulation is LysRS on which the rest of this review will focus.

3. LysRS

3.1. Structural and molecular characteristics

As mentioned earlier, the two classes of AaRSs are distinguished by the architectures of their active-site catalytic cores. LysRS in mammals is a class II synthetase, though there are some organisms whose LysRS is a class I synthetase. Like all AaRSs, LysRS is also organized as a modular arrangement of functional domains. The class II LysRS (and the closely related AspRS and AsnRS) have an arrangement of functional domains in which the order of the functional domains is the opposite of that found in most AaRSs; that is, the catalytic domain is at the C-terminal end of the polypeptide, whereas the anticodon-binding site is encoded by the N-terminal part of the protein (Cusack, 1995; Guo *et al.*, 2008). Class II LysRS is one of the most conserved AaRSs (Guo *et al.*, 2008). When human and *Escherichia coli* enzymes are compared, the highest similarity exists for the C-terminal aminoacylation domain (50%), while there is still 26% similarity for the N-terminal anticodon-binding domain (Guo *et al.*, 2008).

In eukaryotes, LysRS contains an N-terminal appendage which is lysine-rich and has been shown to enable nonspecific tRNA binding and thus allow increased catalytic efficiency of the enzyme, especially at the low concentration of deacylated tRNA prevailing *in vivo* (Francin and Mirande, 2003; Francin *et al.*, 2002). Recently, the crystal structure of a tetrameric form of LysRS has been described (Guo *et al.*, 2008). The crystals revealed an unusual tetramer, a structure not seen with any of the other class II AaRS. This crystal structure has already revealed important insights regarding possible interaction mechanisms between LysLysRS and proteins such as AIMP2—one of the three nonenzymatic components of the MSC complex (Guo *et al.*, 2008). There are two eukaryote-specific insertions, one of which is embedded in the tetramer interface from the side of the catalytic domain. This seems to be a hotspot for variations during evolution, and is different even from structures in the related AspRS. This region could thus be involved in the building of new protein interfaces during evolution (Guo *et al.*, 2008).

Unlike several other AaRSs, both mitochondrial and cytoplasmatic LysRS are encoded by the same gene, with the difference being the inclusion of exon 2 between exons 1 and 3 for the mitochondrial isoform encoding. In humans, it has been shown that the mature LysLysRS mRNA

consists of 70% of the cytoplasmic isoform and 30% of the mitochondrial isoform (Tolkunova *et al.*, 2000).

In summary, LysRS in mammalians is a class II AaRS, which has an N-terminal appendage (lacking in LysRS of lower eukaryotes) allowing higher catalytic efficiency due to nonspecific binding of tRNA, and several other nonconserved regions, which allow new protein–protein interactions.

3.2. Interacting proteins

LysRS is found in mammalian cells mainly as a part of the multisynthetase complex. Numerous studies have been performed to map this complex's localization and binding partners (see, e.g., Han *et al.*, 2006; Quevillon *et al.*, 1999) and it may also be found in a tetrameric form (Guo *et al.*, 2008) (Fig. 1.1).

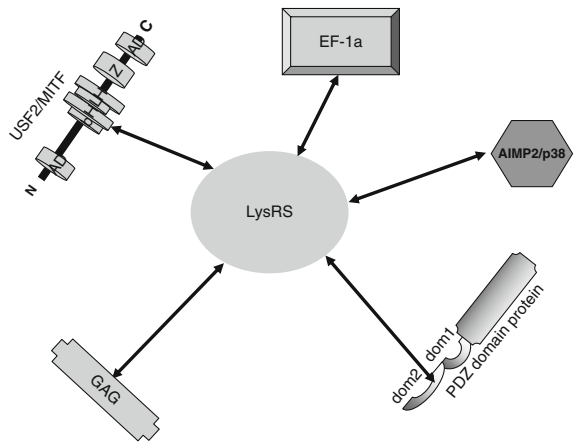

FIGURE 1.1 LysRS interactions. LysRS has been demonstrated to have direct interactions with several proteins. In the multisynthetase complex it has direct interaction with AIMP2/p38, and has a critical role in the maintenance of stability of the complex. A role for EF-1a in channeling tRNA–aminoacids complexes to the ribosome has been suggested by several researchers, direct interactions with mammalian truncated LysRS has only been recently demonstrated *in vitro*. We have demonstrated that LysRS can bind to two bHLH-Leucine zipper transcription factors-MITF and USF2. The HIV virions (and some other retroviruses) contain LysRS. Critical for this incorporation is the interaction with the GAG protein. LysRS is the only AaRS for which interactions with PDZ domain containing proteins has been described. Two independent groups have described interactions with PDZ domain proteins—one with PSD-15 and TIP-15 (two proteins derived by alternative splicing of the same gene), and the other one with syntegenin/mda-9. It seems that the binding to the PDZ domains is mediated at least in part by a PDZ domain binding element found in the C-terminus of LysRS.

Besides these interactions, which are probably critical to the canonical roles of LysRS, several other protein–protein interactions of LysLysRS have been described over the years by different research groups. The protein interactions of LysRS whose functional role has not yet been studied in detail will be described now. Later in this review, we will detail our own work regarding the function of the interactions of LysRS with the transcription factor MITF (Carmi-Levy *et al.*, 2008; Lee *et al.*, 2004b) and with components of the HIV virus in which it is packaged.

3.2.1. PDZ domain interactions

Proteins harboring the postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain function as scaffolds in organizing multiprotein complexes (Nourry *et al.*, 2003). The PDZ domain-containing proteins have a critical role in the connections of various membrane proteins such as cell adhesion molecules, receptors, and ion channels with downstream signaling molecules (Nourry *et al.*, 2003; Zimmermann, 2006). Interestingly, PDZ-containing proteins usually contain more than just PDZ domains and include other protein interaction domains, such as SH3, PTB, and WW (Nourry *et al.*, 2003). In most cases, the PDZ domains recognize the C-terminus of their protein binding partners, though by now it is known that some PDZ domains can recognize internal peptide sequences and sometimes even lipids (Nourry *et al.*, 2003). Specific roles for PDZ domain-containing proteins have been described in various biological systems. Of note is the particular role of some PDZ domain proteins in cancer.

Two independent groups, who have tried to isolate proteins interacting with the PDZ domain proteins, reported the specific binding of LysRS to those proteins. We will now describe their findings in some detail as they reveal a possible important connection between LysRS and signal-transduction pathways.

The first group performed a yeast two-hybrid screen with HTLV-1 TAX as a bait and isolated a PDZ domain protein called TIP-15, which harbors the first two PDZ domains of a larger protein known as PSD-95 (Rousset *et al.*, 1998). TIP-15 was then used as bait itself in another two-hybrid screen, resulting in the isolation of 12 proteins with high affinity to this protein, including LysRS (Fabre *et al.*, 2000). Coimmunoprecipitation experiments in mammalian cells confirmed the interactions of four of these proteins, one of them being LysRS. TIP-1, a truncated protein containing only one PDZ domain, did not bind to LysRS. A canonical PDZ domain binding motif at the C-terminus of LysRS was identified in this study for the first time (Fabre *et al.*, 2000).

Recently, another group (Meerschaert *et al.*, 2008) independently described an interaction of LysRS with a PDZ domain protein known as both syntenin-1 (isolated as a protein that binds syndecan

(Grootjans *et al.*, 1997)) and as mda-9 (isolated as a melanoma differentiation associated gene (Lin *et al.*, 1998). This specific PDZ domain protein has been shown to be overexpressed in breast and gastric cancer cells and in melanoma, where it promotes the migration and metastasis of cancer cells (Boukerche *et al.*, 2005; Koo *et al.*, 2002). Numerous proteins have been found to be able to bind syntenin-1/mda-9 including various glutamate receptors, the serine/threonine kinase Unc51.1 and rab5,19 (Meerschaert *et al.*, 2008). Meerschaert's group used mass spectrometry to discover genes interacting *in vivo* with syntenin-1/mda-9 in mammalian cells (Meerschaert *et al.*, 2008). They identified several AaRSs derived from the multisynthetase complex as binding partners of this protein. Subsequent experiments identified LysRS as the direct syntenin-1/mda-9 binding protein on which the binding of other multisynthetase complex proteins is dependent. They also identified the PDZ domain binding motif in the C-terminus of LysRS (Meerschaert *et al.*, 2008).

As an initial functional assay, this group studied the effect of syntenin-1/mda-9 binding on LysRS aminoacylation activity. They found that recombinant syntenin-1/mda-9, when used at an equimolar ratio to LysRS, inhibited LysRS activity by approximately 40%. This assay, however, was performed *in vitro* without any further *in vivo* studies (Meerschaert *et al.*, 2008).

The findings that two different PDZ domain proteins bind specifically to LysRS and not to other AaRSs, strongly suggest that LysRS–PDZ interactions have a role in signal transduction. Syntenin-1/mda-9 binding to LysRS seems to result in decreased translation rates (Meerschaert *et al.*, 2008), yet there might be other roles for this interaction, such as facilitating the interaction of LysRS with other signal-transduction molecules. In this regard, it is interesting to note that syntenin-1/mda-9 was originally isolated as a melanoma differentiation gene and that LysRS binds to the transcription factor MITF and is involved in its regulation. This transcription factor has been shown to have an important role both as a melanocyte differentiation regulation and as a melanoma oncogene. All this suggests that the any connection between LysRS, MITF, and syntenin-1/mda-9 in melanoma should be investigated.

3.2.2. Elongation factor 1A (EF-1A) and LysRS

A role for elongation factor 1A (EF-1A) in channeling the tRNA–aminoacyl complex from the tRNA to the ribosome has been proposed (Hausmann and Ibba, 2008b). The archeal multisynthetase complex, which is much smaller than its mammalian counterpart and contains only three synthetases, LeuRS, LysRS, and ProRS, was recently investigated (Hausmann and Ibba, 2008b). EF-1A was found to bind to LeuRS in a stable complex which increased Leu-tRNA synthesis to several folds. The interaction of EF-1A with the archaeal multisynthetase complex

contributes to the translational process by enhancing the aminoacylation rates of the AaRSs in the complex and by subsequent channeling of the cognate tRNA–aminoacid complexes to the ribosome (Hausmann *et al.*, 2007). Interactions between EF-1A and various AaRSs have been described in eukaryotes. Already in 1994, the importance of the N-terminal appendage of human AspRS EF-1A was described (Reed and Yang, 1994). It is important to mention that both LysRS and AspRS are similar class II AaRSs with a terminal appendage to the original structure which has a role in the binding of tRNA (Francin *et al.*, 2002). Yang and his colleagues in 2008 published their research regarding the effect of EF-1A and AspRS on stimulated lysylation *in vitro*. They noted that only upon the removal of the amino-terminal appendage from human LysRS could it bind to EF-1A and its lysylation activity was stimulated by this binding (Guzzo and Yang, 2008). The observed stimulation of lysylation activity was unlikely due to stabilization of synthetase by EF-1A since the half-life of LysRS under the assay conditions was several hours while the aminoacylation assays were completed in minutes. Since it was the truncated LysRS which was influenced by EF-1A binding, the physiological relevance of EF-1A binding to LysRS are still not clear. Furthermore, this group did not study any other activity of LysRS such as its ability to synthesize Ap_4A.

It is interesting to note that similar to LysRS, EF-1A has been implicated in several critical biological processes. For example, a truncated form of this enzyme has been found to be an important oncogene in prostate cancer (Mansilla *et al.*, 2005), and it has been claimed that the effect of G-rich GT oligonucleotides on cellular cytotoxicity correlated with their binding to nuclear EF-1A. We found strong binding of EF-1A to MITF in a yeast two-hybrid screen (Razin, unpublished data). We have not yet studied this *in vitro* interaction further, but it could be postulated that under certain conditions not only LysRS but also an associated elongation factor might have a role in transcriptional regulation.

3.2.3. Mutated superoxide dismutase

Amyotrophic lateral sclerosis or as it is popularly known, Lou Gehrig's disease, is a progressive motor neuron degenerative disease which invariably results in death. The majority of the familial cases of this disease were found to be related to mutations in the gene encoding Cu, Zn superoxide dismutase. In 1997, mouse models of ALS were used to try and locate the downstream effectors of mutated superoxide dismutase. It was demonstrated that mutated superoxide dismutase can specifically bind to mitochondrial LysRS (Kunst *et al.*, 1997). More than 10 years later, it was found that LysRS that is bound to mutated superoxide dismutase displays a high propensity to misfold and aggregate prior to its import into mitochondria (Kawamata *et al.*, 2008). This misfolded LysRS can then

undergo proteasomal degradation. Ultimately, the interactions between mutated superoxide dismutase and mitochondrial LysRS result in mitochondrial morphological abnormalities and cellular toxicity. Thus, it seems that mitochondrial LysRS is the first protein whose abnormal binding to mutated superoxide dismutase leads to mitochondrial toxicity and might play a significant role in the development of mutated superoxide dismutase-related ALS.

3.3. LysRS and the autoimmune response

Autoantibodies can cause a variety of diseases. A surprising finding regarding autoantibodies is that their repertoire is rather limited and has been assessed as less than 2% of total human proteins (Plotz, 2003). Interestingly, mouse models of autoimmunity have displayed similar autoantibodies to those found in humans, again emphasizing the nonrandomness of this repertoire. However, it seems that for most autoantigens, the reason that specific proteins turn into antigens is far from obvious since there is no clear cut functional relationship between the different proteins. Several AaRSs have been found to be autoantigens. The most studied are antibodies to HistRS (known as anti-Jo-1 antibodies), which are characteristic of autoimmune inflammatory myopathy (myositis) in humans (Nagaraju *et al.*, 2000), and anti-IleRS (known as anti-OJ antibodies). Patients with anti-OJ antibodies have been shown to have antibodies to other constituents of the multisynthetase complex (Gelpi *et al.*, 1996; Targoff *et al.*, 1993). The reason for the common occurrence of AaRSs as autoantigens was postulated by Plotz and colleagues to be linked to their ability to act as chemoattractants to immune cells (Plotz, 2003). Unknown to Plotz at the time of writing of his review was the possible activity of LysRS as a chemokine-like molecule. Autoantibodies were isolated from the sera of patients who had developed transplant-associated coronary artery disease (TxCAD) following cardiac transplantation (Linke *et al.*, 2001). They isolated a total of six positive clones out of 40,000 clones from a HUVEC cDNA library, one of which was LysRS, and two were ribosomal proteins. So, LysRS would also seem to be an autoantigens, though it is not as prominent as several other AaRSs.

3.4. Functional roles

A variety of roles for LysRS have been described. We will initially describe some alternate biochemical roles and later its physiological functions.

3.4.1. Lysine residue adenylation

Wagner and colleagues recently described the ability of LysRS to adenylate lysine residues (Chou and Wagner, 2007). They used both human and *E. coli* LysRS to study adenylation of Hint. Hint-1 is a member of the histidine triad (HiT) superfamily of proteins, all of which can bind and hydrolyze nucleotides or their derivatives. It has great structural similarity to the tumor suppressor FHIT, which can bind both Ap_4A and Ap_3A, though it is much more efficient in the hydrolysis of Ap_3A. All the reactions were carried out *in vitro* with purified recombinant proteins. The adenylated proteins are found for relatively short times and that complicates any efforts to identify them *in vivo*. Radiolabled nucleotides were added to the purified proteins to prove that the Hint is labeled by the addition of AMP, in a manner that is dependent upon the formation of lysyl-AMP. They found that Hint labeling was dependent on Mg^{2+}, known to be required for aminoacyl–adenylate formation, whereas addition of Zn^{2+}, which favors Ap_4A formation, was inhibitory. Ap_4A inhibited the formation of lysyl-AMP with an IC_{50} value in the low micromolar range. This *in vitro* study also revealed that the lysyl-AMP intermediate formed by LysRS could be a natural substrate for the Hint proteins. Therefore, the same protein that can be adenylated by LysRS can also hydrolyze adenylated lysine residues.

The physiological importance of the adenylation of proteins at lysine residues is still not clear. As mentioned, this adenylation is short lived and hence hard to detect *in vivo.* Further studies may reveal a very important role for LysRS in this regard once *in vivo* studies can provide the hard evidence.

3.4.2. Synthesis of dinucleotides

Already in the 1960s, Zamecnik demonstrated that under certain conditions LysRS in purified *E. coli* could produce dinucleotides *in vitro*, and characterized the important product of this reaction as adenosine tetraphosphate, a compound which had been synthesized chemically just a short time before (Randerath *et al.*, 1966; Zamecnik *et al.*, 1966). Much later in the 1980s, other groups studied the role of LysRS from mammalian sources as a producer of Ap_4A. At this time, they were limited to *in vitro* studies due to their inability to efficiently knockdown LysRS. Zinc was shown to be critical for the activation of the production of Ap_4A via LysRS and PhenylRS in sheep's liver (Brevet *et al.*, 1982). Zinc greatly stimulates the initial rate of *in vitro* synthesis of Ap_4A. Later, however, it was shown that AMP can be omitted from the reaction and that zinc levels can be markedly reduced provided catalytic amounts of LysRS are added to the reaction mixture (Hilderman and Ortwerth, 1987). Therefore, the *in vivo*

role of zinc in the production of Ap_4A might be much less critical than initially thought. As mentioned earlier, other AaRSs apart from LysRS were able to produce Ap_4A *in vitro.* Hilderman and colleagues demonstrated that one type of tRNAlys (tRNALys) was much more efficient in inducing Ap_4A production by the ArgRS/LysRS complex than the more common tNAlys and tRNALys (Hilderman and Ortwerth, 1987). These experiments suggested a role for LysRS in efficient Ap_4A synthesis. Furthermore, two studies demonstrated the differential efficacy of LysRS in the production of Ap_4A within the complex and external to it (Wahab and Yang, 1985a,b). According to their calculations, based on *in vitro* experiments with purified multisynthetase complexes, LysRS was six times more efficient in the production of Ap_4A once unbound from the complex. One must, of course, note that these are the results of *in vitro* studies where different concentrations of reaction reagents, such as zinc, might significantly alter the final results; but still one might expect an increase in cellular Ap_4A levels if free LysRS levels are increased intracellularly.

3.4.3. LysRS as a cytokine-like molecule

Several AaRSs have been implicated as having a possible role as secreted extracellular modulators of cellular function (Kleeman *et al.*, 1997; Tolstrup *et al.*, 1995; Wakasugi and Schimmel, 1999; Wakasugi *et al.*, 2002). Media was obtained from HEK293 cells overexpressing one of 11 different synthetases, and screened to determine which synthetases were secreted (Park *et al.*, 2005b). Only AlaRS and LysRS were detected in the culture media. Various other cell lines were used to investigate whether endogenous LysRS was secreted in a signal-dependent manner. HCT116 colon cancer cells, DU145, SKN-SH, and MCF-7 cells had substantially increased LysRS secretion rates in response to treatment with TNF-α but not TGF-β. Recombinant LysRS was shown to bind to the membranes of two macrophage-like cell lines (RAW264.7 and THP-1). Following LysRS stimulation, increased secretion of TNF by these two cell lines was detected. Thus, it seems that LysRS and TNF-α form a positive feedback loop in these cells. This study further demonstrated that RAW264.7 macrophage-like cells had increased migrational capacity following LysRS stimulation. The activity of MMP-9, but not MMP-2, was significantly induced by LysRS in these cells. LysRS also induced the migration of peripheral blood mononuclear cells. Selective inhibitors were used to determine that the MAPK pathway has a pivotal role in this LysRS-cytokine signal-transduction pathway. Altogether, the results of this study suggest that LysRS can act as a cytokine-like molecule under certain circumstances. This activity might be related to LysRS's role as an autoantigen (as mentioned earlier).

3.4.4. Physiological roles of LysRS

LysRS is probably the AaRS to which the largest number of physiological roles has been attributed in addition to aminoacylation (Freist and Gauss, 1995). One example is the unique heat shock inducible LysRS gene (lysU) in bacteria (Charlier and Sanchez, 1987) and another is the existence of a specific iron inducible LysRS in tomato roots with a role in plant adaptation (Giritch *et al.*, 1997). Here, we will focus on LysRS's functions in higher eukaryotes and then discuss in some detail the proposed role of LysRS in transcriptional regulation.

3.4.4.1. LysRS and transcription factors We have been studying transcription factor networks in mast cells for more than a decade (Nechushtan and Razin, 1998). We began our studies by assessing the fate of c-Fos and c-Jun proteins following IgE-Ag stimulation of mast cells. These proteins are well known as both oncogenes and early response transcription factors. Surprisingly, at the time we found out that IgE-Ag stimulation leads to the binding of c-Fos not to Jun but to what was then a newly discovered transcription factor known at that time as Fos interacting protein (FIP) and now known by the name of USF2 (Lewin *et al.*, 1993). This transcription factor is a bHLH-leucine zipper transcription factor found in most cells that has an important role in many biological processes. The transcription factor MITF was cloned around this time (Hodgkinson *et al.*, 1993; Tachibana *et al.*, 1994). This is also a bHLH-leucine zipper transcription factor, which is structurally most similar to the TFE transcription factors. TFE transcription factors and MITF are now considered part of a transcription factor family known as MiT (Hemesath *et al.*, 1994). The transcription factors with the highest similarity to this family are USF1 and USF2. Our special interest in MITF was due to the finding that mast cells are depleted in MITF mutated mast cells and because of its similar structure to USF2. Indeed, we found that IgE-Ag induction of mast cells results in USF2 binding to MITF (Nechushtan *et al.*, 1997). Since MITF was demonstrated to have a critical role in mast cell physiology (Kitamura *et al.*, 2002; Nechushtan and Razin, 2002), we decided to try and isolate the MITF binding proteins. We used the yeast two-hybrid assay as a screening method, utilizing a truncated MITF lacking both transactivation domains to probe a library from a mast cell line (Razin *et al.*, 1999) (Fig. 1.2).

Several proteins were found to bind to MITF with high affinity. We initially concentrated our efforts on the study of a protein known at that time as PKCI (PKC interacting protein), but now known as Hint-1 (Razin *et al.*, 1999). We demonstrated that this HIT family protein can bind and inhibit transcriptional activation by MITF. Interestingly, we also demonstrated that Hint-1 could be released from MITF following IgE-Ag stimulation of mast cells and that Hint-1 can bind to MITF in melanoma cells.

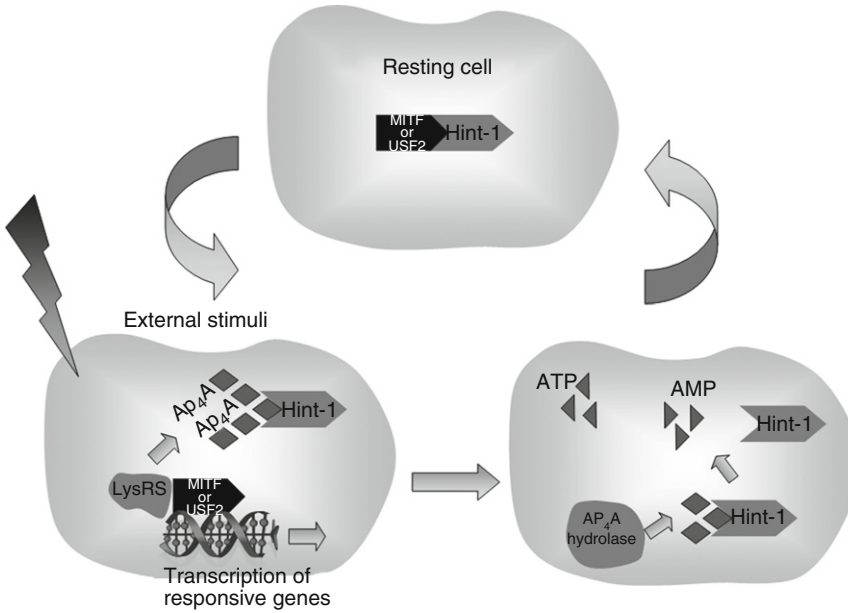

FIGURE 1.2 A model of LysRS/Ap_4A transcriptional regulation. We and others demonstrated that in resting cells, MITF is inhibited by Hint-1. In activated cells, LysRS starts to produce Ap_4A which can bind to Hint-1 and release it from MITF (the best studied example being IgE-Ag stimulated mast cells; similar findings in cardiomyocytes have been described). The initial increase in Ap_4A levels and activation of MITF is followed by degradation of Ap_4A by Ap_4A hydrolase, and decreased production of Ap_4A by LysRS, which leads to the unassociated HINT being available to bind to MITF again and so inhibit its transcriptional activity. This figure summarizes three articles on the role of Ap_4A in transcriptional regulation.

Weinstein and colleagues have recently presented preliminary data at the 2008 AACR meeting whereby they corroborate our results regarding the binding of Hint-1 to MITF and its ability to inhibit MITF (Genovese *et al.*, 2008). Their results were obtained with several types of human melanocytes and human melanoma cell lines and they seem to suggest that Hint-1 could modulate MITF activity in melanoma and might have a role in the progression of this kind of tumor (Genovese *et al.*, 2008).

One of the proteins found to be highly bound to MITF was LysRS (Razin *et al.*, 1999). We verified our observation from the yeast two-hybrid assay with coimmunoprecipitation utilizing extracts from mast cells (Lee *et al.*, 2004b). We noted that several earlier studies from the 1980s previously demonstrated that LysRS can produce the unique dinucleotide

Ap_4A. We found using Biacore that Hint-1 binds specifically to Ap_4A (Lee *et al.*, 2004b). We then demonstrated *in vitro* that MITF could be dissociated from Hint-1 by the application of Ap_4A but not by other dinucleotides, such as Ap_3A, and Ap_5A (Lee *et al.*, 2004b). We therefore hypothesized that the association of LysRS and MITF is not related to LysRS's role as a tRNA synthetase, but to one of its ''moonlight'' functions as a producer of Ap_4A (Lee *et al.*, 2004b). We proposed that Ap_4A produced by LysRS played a critical role in the regulation of several transcription factors through its ability to control gene expression. As an initial *in vivo* assay to test this hypothesis, we introduced Ap_4A into cultured mast cells using the rather crude ''cold shock'' method, and demonstrated that this introduction increased the expression of some MITF-regulated genes (Lee *et al.*, 2004b).

We later demonstrated that LysRS associates with USF2, which unlike MITF, is ubiquitously expressed in eukaryotic cells. In mast cells, we have found that similarly to MITF, USF2 is negatively regulated by Hint-1 and Ap_4A acts as a positive regulator of USF2 by a molecular mechanism similar to that described for MITF (Lee and Razin, 2005). This finding lent support to the notion that LysRS and Ap_4A may be involved in regulation of gene transcription in many cell types and not limited to those where MITF is expressed (Lee and Razin, 2005).

To elucidate the mechanisms involved in this transcriptional regulation pathway, we decided to study the regulation of both the synthesis of Ap_4A by LysRS and its degradation. Interestingly, we found that following IgE-Ag stimulation of mast cells, there is a transient two- to threefold increase of intracellular Ap_4A (Carmi-Levy *et al.*, 2008).

Several enzymes that can degrade Ap_4A have been described (Hankin *et al.*, 1997; Swarbrick *et al.*, 2005; Vollmayer *et al.*, 2003). However, only one of these, NUDT2, which is a member of the Nudix family, has been shown to reside intracellularly and has been proposed as the intracellular enzyme responsible for the degradation of Ap_4A (Abdelghany *et al.*, 2001; Hankin *et al.*, 1997). We downregulated rat NUDT2 by electroporating cultured rat mast cells with NUDT2 siRNA. This downregulation caused significantly prolonged elevation of Ap_4A following IgE-Ag stimulation of mast cells (Carmi-Levy *et al.*, 2008). This result was the first demonstration of a physiological role of NUDT2 in the regulation of the increase in intracellular Ap_4A levels in response to an immunological stimulus.

Following the initial characterization of Ap_4A, various studies demonstrated impressive increases in intracellular Ap_4A levels following a variety of external stimuli. However, most of these observations were performed before modern techniques were available, such as siRNA silencing of specific genes, and thus the enzymes responsible for the production and degradation of Ap_4A under various conditions have not yet been delineated.

As mentioned, we hypothesized that increased Ap_4A levels should allow the release of Hint-1 from MITF and so increase the transcriptional activity of MITF. By blocking NUDT2, we managed to increase intracellular Ap_4A levels in a more physiological manner than with the cold shock method (Carmi-Levy *et al.*, 2008). This increase in Ap_4A levels resulted in an increase in the expression of some, but not all, USF2- and MITF-regulated genes. The effect was not dramatic, with increases of up to two-fold in most genes (Carmi-Levy *et al.*, 2008). A limitation of this approach is that the mast cells were stimulated in a way that was chosen to achieve the maximal IgE-Ag response of the cells. Thus, inhibiting the endogenous hydrolase, which basically prolongs the time that endogenous Ap_4A levels are high, does not result in very large differences in IgE-Ag-stimulated Ap_4A peak levels. Such conditions might not be typical of endogenous situations since IgE-Ag stimulation probably happens at lower than optimal conditions *in vivo*.

It is important to note here that we did witness an increased release of MITF from Hint-1 and increases in the expression of luciferase driven by MITF responsive genes following inhibition of Ap_4A hydrolase expression (Carmi-Levy *et al.*, 2008). Our study lends further support to the notion that intracellularly produced Ap_4A has a role in the regulation of gene transcription.

3.4.4.2. LysRS and HIV tRNA is used as a primer to initiate the reverse transcriptase (RT)-catalyzed synthesis of the minus-strand in retroviruses. Different retroviruses use different tRNAs as a primer. Kleiman and colleagues defined the roles of lys tRNA and LysRS in retroviruses and more specifically in HIV. In lentiviruses, including human immunodeficiency virus type 1 (HIV-1), tRNA3Lys serves as the primer tRNA (Jiang *et al.*, 1993; Mak and Kleiman, 1997). In avian retroviruses and HIV-1, the primer tRNAs are selectively packaged, and the percentage of the tRNA population of the primer tRNA type increases in the virus. In HIV-1, the relative concentration of tRNALys increases from 5% to 6% in the cytoplasm to 50–60% in virus (Huang *et al.*, 1994). Importantly in HIV-1, increases in the concentration of primer tRNA3Lys in the viral population is correlated with an increase in tRNA3Lys annealing and viral infectivity (Gabor *et al.*, 2002). In addition to tRNA3Lys it was found out that LysRS is also selectively packaged into HIV-1 (Cen *et al.*, 2001). It seems that the main role of LysRS in HIV is the targeting of tRNALys for virion incorporation (Cen *et al.*, 2001; Gabor *et al.*, 2002; Kovaleski *et al.*, 2006). Gag alone is sufficient for the incorporation of LysRS into Gag virus-like particles (Cen *et al.*, 2001), whereas GagPol is required in addition for the incorporation of tRNALys (Huang *et al.*, 1994) (Fig. 1.3).

Overexpression of LysRS in the cell results in a near doubling of the incorporation of both tRNALys and LysRS into HIV-1 (Cen *et al.*, 2004).

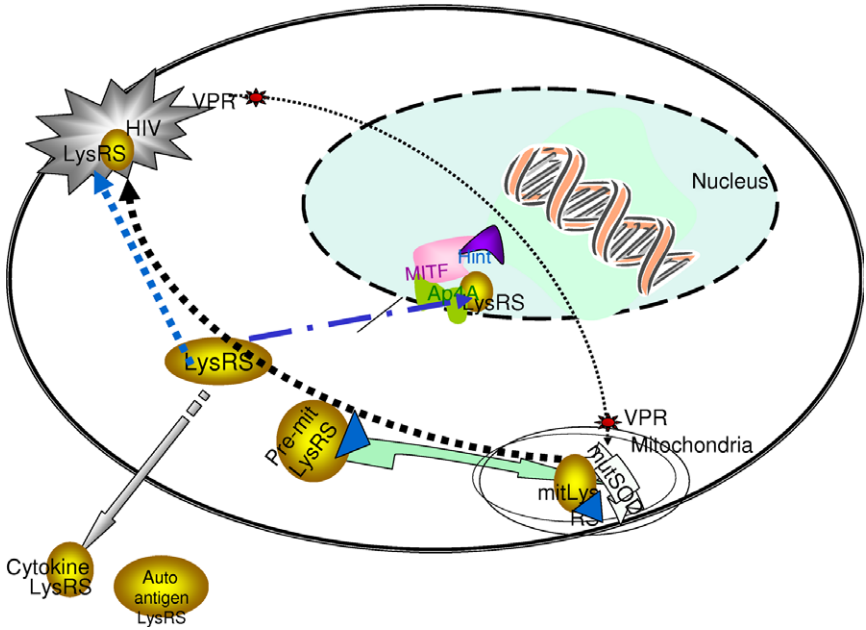

FIGURE 1.3 Roles of LysRS. LysRS is produced in two forms from the same gene—a mitochondrial form, harboring a mitochondrial localization sequence, and a regular form. The premitochondrial form of LysRS is cleaved once it enters the mitochondria and 17 amino acids are lost and mitochondrial LysRS (mitoLysRS) is obtained. Besides its role in translation, mitochondrial LysRS can specifically bind mutated superoxide dismutase (mutSOD), an interaction which is proposed to have a role in the pathogenesis of amyotropic lateral sclerosis. Mitochondrial LysRS has also been proposed as the LysRS used by the HIV virion. It has proposed that a protein released from the HIV virion, VPR leads to increased release of mitochondrial LysRS which can be incorporated into HIV. Alternatively, it has been proposed that newly synthesized LysRS can be incorporated into the HIV virions before incorporation into the multisynthetase complex. LysRS is also secreted and has cytokine-like properties. In addition, LysRS has been found to be an autoantigen in certain autoimmune disorders. LysRS has also been found in the nucleus. LysRS is a producer of Ap_4A, and can influence the dissociation of the transcriptional inhibitor Hint-1 from transcription factors such as MITF.

Thus, considering the huge clinical importance of HIV and the important role of LysRS in the biology of HIV, it was interesting to try and locate the source of LysRS in the virions. Regarding this question, there is currently a heated debate in the literature. Kleiman's group claim that the source for viral LysRS is newly synthesized cytoplasmatic LysRS that binds to the GAG molecule before it binds to the multisynthetase complex (Cen *et al.*, 2004). This claim is based on the use of truncated and tagged exogenous LysRS. In contrast, Mirande and colleagues claim that the source for HIV

LysRS is mitochondrial LysRS that is released in higher amounts from the mitochondria following HIV infection and specific mitochondrial degradation as a result of the action of HIV-derived VPR protein (Francin *et al.*, 2002). Their conclusions were based on the use of specific antibodies to the mitochondrial LysRS. Neither of these research groups studied activated immune cells, in which there might be substantial release of LysRS from the multisynthetase complex, and only used model cell lines *in vitro* for their studies. Therefore, it seems that while the critical role of LysRS in the life cycle of HIV is now supported by evidence from several laboratories, the source of LysRS in HIV and its specific form in the virus is still not clear.

4. CONCLUDING REMARKS

LysRS has several roles besides its function as a key enzyme involved in translation. In the immune system, it may function both as an extracellular cytokine-like molecule and a signal-transduction protein in a signal-transduction pathway ultimately regulating gene expression. It has a critical role in HIV viral genesis. LysRS has been implicated as having a role both in autoimmune disease and in amyotrophic lateral sclerosis. It is found in cells in the cytoplasm mainly as part of the multisynthetase complex, in mitochondria and in some cases has been found to be nuclear or membrane bound. Studies of the regulation of LysRS function inside the cells should provide us with critical insights as to the basic mechanisms of LysRS function in its various noncanonical roles. It seems that much more work is needed to understand the complex regulation of LysRS function and the involvement of LysRS in such seemingly unrelated processes such as HIV genesis and the response to external stimuli.

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