Lysyl-tRNA Synthetase-generated Lysyl-Adenylate Is a Substrate for Histidine Triad Nucleotide Binding Proteins*§

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Histidine triad nucleotide binding proteins (Hints) are the most ancient members of the histidine triad protein superfamily of nucleotidyltransferases and hydrolyases. Protein-protein interaction studies have found that complexes of the transcription factors MITF or USF2 and lysyl-tRNA synthetase (LysRS) are associated with human Hint1. Therefore, we hypothesized that lysyl-AMP or the LysRS-lysyl-AMP may be a native substrate for Hints. To explore the biochemical relationship between Hint1 and LysRS, a series of catalytic radiolabeling, mutagenesis, and kinetic experiments was conducted with purified LysRSs and Hints from human and Escherichia coli. After incubation of the E. coli or human LysRS with Hints and [α-32P]ATP, but not [α-32P]GTP, 32P-labeled Hints were observed. By varying time and the concentrations of lysine, Mg2+ or LysRS, the adenylation of Hint was found to be dependent on the formation of lysyl-AMP. Site-directed mutagenesis studies of the active site histidine triad revealed that Hint labeling could be abolished by substitution of either His-101 of E. coli hint or His-112 of human Hint1 by either alanine or glycine. Ap3A, believed to be synthesized by LysRS in vivo, and Zn2+ were shown to inhibit the formation of Hint-AMP with an IC50 value in the low micromolar range. Consistent with pyrophosphatase being an inhibitor for aminoacyl-tRNA synthetase, incubations in the presence of pyrophosphatase resulted in enhanced formation of Hint-AMP. These results demonstrate that the lysyl-AMP intermediate formed by LysRS is a natural substrate for Hints and suggests a potential highly conserved regulatory role for Hints on LysRS and possibly other aminoacyl-tRNA synthetases.

Histidine triad nucleotide binding protein (Hint)2 belongs to a histidine triad (HIT) superfamily that has a characteristic C-terminal active site motif, HXXHXX, where X is a hydrophobic residue (1). HIT enzymes are a ubiquitous superfamily consisting primarily of nucleoside phosphoramidases, dinucleotide hydrolyases, and nucleotidylyltransferases (1). Five distinct branches of HIT superfamily have been recently classified by a phylogenetic study of HIT proteins (2, 3). Recently, Hint1 homologs isolated from rabbit (4), human (5), chicken (6), yeast (4), and Escherichia coli (5) have been shown to be purine nucleoside phosphoramidases. Human Hint1 has been shown to associate with, and possibly regulate several transcription factors such as TFIIH (7), MITF (8, 9), and USF2 (10). Additionally, Hint1 knock out mice have been shown to have an increased susceptibility to the induction of ovarian and mammary tumors by the carcinogen dibenzanthracene and to spontaneous tumors (11). Up-regulation of Hint1 and the significantly reduced in vivo tumorigenicity of 5-aza-2C-treated non-small-cell lung cancer cell line NCI-H522 suggested that hHint1 might be a tumor suppressor (12). Recently, Weisie and Huber (13) reported that Hint1 triggers apoptosis independent of its phosphoramidase activity (13). Human Hint2, which is 61% identical to Hint1, has recently been shown to be a mitochondrial apoptotic sensitizer that is down-regulated in hepatocellular carcinomas (14). Although Hints are efficient hydrolyses of purine nucleoside phosphoramidates, cellular function and biochemical relevance of the enzymatic phosphoramidase activity has not been determined.

The Fhit (fragile histidine triad) branch of the HIT superfamily is only found in eukaryotes. Like Hint, Fhit is also a homodimer with tumor suppressor activity (15). Human Fhit is a diadenosine P1,P3-triphosphate (Ap3A) hydrolase as well as phosphoramidase (16, 17). The precise mechanism of action by which it affects tumor development is not well understood, although site-directed mutagenesis studies have indicated that the Fhit tumor suppressor function is likely not dependent on its Ap3A hydrolase activity (18). In contrast to the first two branches, the galactose-1-phosphate uridylyltransferase branch has been shown to be the second enzyme in the Leloir pathway necessary for galactose utilization (19, 20). Although, galactose-1-phosphate uridylyltransferase is a homodimer with little overall sequence homology with Hint1 or Fhit, it does share some tertiary structure similarity (21). The fourth family is aprataxin, which is mutated in ataxia-oculomotor apraxia1 (22, 23) and possesses phosphoramidase and Ap3A hydrolase activity as well as DNA/RNA binding properties (2); recently, the physiological substrate for aprataxin has been shown to be abotive adenylated DNA ligation intermediates (3). The last family is the scavenger mRNA decapping enzyme, DcpS/Dcs-1, a 7-methyl-GpppG hydrolase (24, 25).

Recently, hHint1 has been isolated from complexes with lysyl-tRNA synthetase (LysRS) and MITF or USF2 transcription factors (9, 10). To elucidate the biochemical connection...
between LysRS and Hint1, both E. coli LysRS (ecLysU) and human LysRS (hLysRS) were purified and shown to label echinT and hHint1 with [α-32P]ATP but not with [γ-32P]ATP, [α-32P]GTP, or [γ-32P]GTP. This was found to be consistent with chemical degradation and mutagenic experiments revealing that the Hint-AMP intermediate formed upon interaction with LysRS contained a phosphoramidate linkage between AMP and a strictly conserved and catalytically essential active site histidine (His-112 of hHint1, Fig. 1). Furthermore, by varying individual components in the aminoacylation reaction, the formation of the Hint-AMP intermediate was found to be dependent on the formation of the lysyl-AMP intermediate. Hence, Hints may function in part to regulate the catalytic activity of LysRS by providing a possible new mechanism of pre-transfer editing or by scavenging highly reactive inappropriately released aminoacyl-adenylates. Moreover, the interaction of Hint1 with transcription factors such as USF2 and MITF may be mediated by the adenylation of Hint1 by LysRS.

EXPERIMENTAL PROCEDURES

Protein Purification—The E. coli BS68 strain harboring the pBAS39 (derived from pET3a) plasmid for expressing ecLysU as a C-terminal His6-tag fusion protein was a gift from Dr. Paul Schimmel (The Scripps Research Institute) (26). The plasmid pM368 encoding hLysRS expresses a fusion protein containing the N-terminal MRGSHHHHHHSSGWVD sequence appended to full-length hLysRS was a gift from Dr. Karin Musier-Forsyth (University of Minnesota) (27). Both LysRS enzymes were purified by using Ni2+/agarose binding according to a previously published procedure (27). Wild type and mutants of hHint1 and echinT were purified by an AMP-agarose column from ec hinT knock out strain (BB2) as described previously (5).

Labeling of Hints by Purified E. coli LysU—E. coli LysU (6.25 μM) was incubated with [α-32P]ATP or [α-32P]GTP (0.33 μM, 800 Ci/mmol, MP Biomedicals) in buffer A (10 μl, 25 mM Tris HCl, pH 7.8, 100 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, protease inhibitor tablet (Roche Applied Science)) at 23 °C for 10 min followed by the addition of either buffer A (5 μl), echinT (5 μl, 1.25, 3, or 7.5 μM), hHint1 (5 μl, 1.25, 3, or 7.5 μM), or the chimera mutant (5 μl, 1.25, 3, or 7.5 μM) and incubated for 10 min. The reaction was terminated by the addition of SDS sample buffer (4×, 5 μl, Invitrogen). The reaction mixture was boiled for 10 min, and the proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. Labeled proteins were visualized by subjecting dried polyvinylidene difluoride membranes to autoradiography with a storage phosphor screen for 12 h followed by scanning with a Storm 840 PhosphorImager.

Lysyl-AMP-dependent Adenylation of Hints by E. coli LysU and Human LysRS—The concentration of ecLysU, hLysRS, lysine, [α-32P]ATP, Mg2+, Zn2+, nucleotides, yeast inorganic pyrophosphatase (Sigma), Hint proteins, and incubation time period for each experiment is described in the legends to Figs. 3–9. The reactions were terminated by the addition of SDS sample buffer (4×), and samples were analyzed as described above. Quantitation of the intensity of the 32P signal was carried out with ImageQuant software (GE Healthcare).

Measurement of AMP Production by TLC Assay—The TLC assay was employed to measure the formation of AMP by aminoacyl-tRNA synthetase (28, 29). E. coli LysU (1 μM) was added to the reaction mixture containing ATP (250 μM), lysine (200 μM), and the reaction was allowed to proceed for 10 min at 37 °C. The reaction was terminated by the addition of SDS sample buffer (4×) and analyzed as described above.

FIGURE 1. X-ray crystallographic structure of hHint1. A, overall dimer structure with bound AMPCP (Protein Data Bank code 1AV5). Shown is the active site region in one monomer with bound AMPCP (Protein Data Bank code 1AV5) (8) and with bound AMP (C); histidine 112 of hHint1 is the nucleophilic residue that forms a covalent P-N bond with its substrate (Protein Data Bank code 1KPF) (48).
experiments were carried out with buffer A containing 2.5 mM acetate (100 mM), 5% acetic acid solution. The 32P signal was observed after the addition of Hints for 10 min. Control experiments were carried out with buffer A containing 2.5 μM Hints but not ecLysU (lanes 1, 5, and 8). Incubation of ecLysU (6.25 μM) was carried out at 23 °C for 10 min with [α-32P]GTP (0.33 μM) only weak labeling of ecLysU (59 kDa) was observed by the addition of hHint1 (2.5 μM, lane 3) and the chimera (2.5 μM lane 4) but not with buffer A (lane 2) and buffer A (Fig. 2A).

μM), [α-32P]ATP (7.5 μM) in buffer B (100 mM Tris HCl, pH 7.0, 10 mM NaCl, 10 mM MgCl2, 5 mM dithiothreitol, 0.03 unit/μl inorganic pyrophosphatase) at 23 °C. Aliquots (2 μl) were withdrawn and quenched in ice-cold sodium acetate solution (200 mM, pH 5.0). After 10 or 30 min, echintT (2.5 μM) was added to the remaining reaction mixture, and AMP formation was monitored for an additional 10 min. Aliquots of quenched reactions (1 μl) were spotted in duplicate on polyethyleneimine-cellulose plates (20 × 10 cm, Selecto Scientific) pre-developed with water. The air-dried TLC plate was developed in ammonium hydroxide solution. The [32P]signal was detected and quantitated as described above.

RESULTS

The Chimeric Mutant of hHint1 and echintT—Human Hint1 and echintT shared 50% sequence identity over 107 alignable amino acids, but their C termini are distinct. The C terminus may play an important role in determining substrate specificity. Homology modeling studies revealed that the C termini of echintT are likely to reside in a similar location to that of the hHint1.3 To address this possibility, a human/E. coli chimera was created by replacing the C terminus of hHint1 (Gln-120 to Gly-126) with the C terminus of echintT (Pro-109 to Leu-119).

FIGURE 2. Nucleotidylation of Hint by ecLysU. A, incubation of ecLysU (6.25 μM) was carried out at 23 °C for 10 min with [α-32P]ATP (0.33 μM). Labeling of echintT (0.5 μM, lane 1), hHint1 (lanes 3 and 4), and the chimera (lanes 9 and 10) was observed after the addition of Hints for 10 min. Control experiments were carried out with buffer A containing 2.5 μM Hints but not ecLysU (lanes 1, 5, and 8). B, incubation of ecLysU (6.25 μM) was carried out at 23 °C for 10 min with [α-32P]GTP (0.33 μM). Only weak labeling of ecLysU (59 kDa) was observed by the addition of hHint1 (2.5 μM, lane 3) and the chimera (2.5 μM lane 4) but not with buffer A (lane 2).

Nucleotidylation of Hints by ecLysU—Adenylation but not guanylation of Hints was observed after incubation with ecLysU (Fig. 2A), whereas the control experiments without ecLysU (Fig. 2A, lanes 1, 5, and 8) revealed negligible labeling of Hints. Adenylation of echintT (Fig. 2A, lanes 3 and 4, 13 kDa), hHint1 (Fig. 2A, lanes 6 and 7, 14 kDa), and the chimera mutant (Fig. 2A, lanes 9 and 10, 14 kDa) was compared which demonstrate that labeling of echintT and the chimera was more intense than the labeling of hHint1, indicating the potential role of the C-terminal loop in facilitating labeling. In contrast to adenylation, incubation of ecLysU with [α-32P]-GTP revealed weak labeling of ecLysU (59 kDa) after the addition of hHint1 (Fig. 2B, lane 3) and the chimera (Fig. 2B, lane 4) but no labeling with only buffer A (Fig. 2B, lane 1) or echintT (Fig. 2B, lane 2).

Enhanced Adenylation of Hint by ecLysU by the Addition of Lysine—To first investigate whether the lysyl-tRNA synthetase activity associated with ecLysU was responsible for the adenylation of Hints, the effect of lysine on Hint-AMP formation by 11-fold, when the amount of lysine enhanced the amount of Hint-AMP by factors of 11 or 5.

3 T.-F. Chou, Y. Y. Sham, and C. R. Wagner, unpublished data.
observed. Because the small amount of Hint-AMP was observed in the absence of added lysine (Fig. 3, lanes 2 and 4), it is highly likely that a small amount of contaminating lysine or lysyl-AMP bound with ecLysU is present with the purified enzyme. In addition, at the higher echinT concentration, we do observe small amounts of labeled ecLysU (Fig. 3, lanes 4 and 5, 59 kDa), possibly indicating that at high enough concentrations of Hint, adenylation of ecLysU can be observed.

Time Dependence of Hint Adenylation by ecLysU—To evaluate the rate of adenylyl-Hint formation, after preincubation of ecLysU (0.022 μM), lysine (2.2 μM), and [α-32P]ATP (0.38 μM) for 1 min, excess echinT (1.4 μM) was added to initiate the reaction; the reaction was carried out for an additional 8 min with aliquots removed and quenched at 1-min intervals. As shown in Fig. 4, maximal Hint-AMP formation was observed between 1 and 3 min after initiation. Consistent with rapid consumption of ATP by ecLysU and turnover of the Hint-AMP intermediate, over the course of the next 5 min, a 6-fold decrease in the intensity of echinT labeling was observed. In subsequent experiments, the labeling of echinT was carried out for maximum of 1 min.

Adenylation of ecLysU by echinT—Incubation of ecLysU (0.022 μM) with lysine and [α-32P]ATP for 1 min followed by the addition of various concentrations of echinT (0.056–14 μM) revealed that the intensity of the Hint-AMP was proportional to the added amount of echinT, reaching a plateau between 1.4 and 2.8 μM. Moreover, labeling of ecLysU was noticeable when incubations were carried out with at least a 56-fold excess of echinT. The prevalence of an additional M, band, consistent in size with covalently linked ecLysU and echinT, was observed at the highest echinT concentration (14 μM) (Fig. 5). The possibility that this band may result from the labeling of LysRS at multiple sites cannot be ruled out. Labeling of the high M, band and ecLysU is likely to suppress the amount of observed echinT-AMP.

Adenylation of Hint Is Dependent on the Concentration of Lysine and Mg2+—Because lysine is one of the substrates and Mg2+ is absolutely required for lysyl-AMP formation (30), it is of interest to determine the effect of both molecules on adenylation of echinT in more detail. Hence, various concentrations of lysine (0.025–2500 μM) were examined to reveal a saturation-type behavior with respect to the amount of lysine; under these conditions maximal intensity was observed at 25 μM lysine (Fig. 6A). In addition, labeling was observed only when Mg2+ was present in the reaction mixture, with maximal intensity reached at a concentration of 1500 μM (Fig. 6B). Taken together, the observed reliance of echinT adenylation on lysine and Mg2+ is consistent with a labeling reaction dependent on the formation of lysyl-AMP by ecLysU.

Inhibition of Hint Adenylation by Zn2+, Ap4A, and Ap3A—Divalent Zn2+ has been shown to stimulate Ap4A formation by ecLysU. The mechanism has been shown to rely on the ability of Zn2+ to stimulate nucleophilic attack by the γ-phosphate of ATP on lysyl-AMP (31). In addition, Zn2+, but not Ap3A, has been shown to inhibit Hint activity with an IC50 of (9.5 ± 2.1) μM. Therefore, we chose to test the effect of Zn2+, Ap4A, and other nucleotides on the formation of Hint-AMP. As shown in Fig. 7, Zn2+ (15 μM) inhibited Hint-AMP formation by 80%, and Ap4A inhibited Hint-AMP formation with an IC50 of 15 μM. Moreover, about 70–90% inhibition was observed for incubations with 150 μM Ap4A, Ap3A, or ATP. Consistent with the preference of LysRS for ATP and not GTP, incubations with 150 μM GTP decreased Hint-AMP by only 50%, whereas inhibition by either AMP or GMP was not observed (supplemental

4 T.-F. Chou and C. R. Wagner, unpublished data.
Fig. 1. These results provided additional evidence that lysyl-AMP is required for adenylation of echinT.

Dependence of Hint Adenylation on the Concentration of echinT and ecLysU—To examine the concentration effect of echinT and ecLysU on Hint-AMP intermediate formation, we first fixed the ecLysU concentration (0.025 μM) and varied the concentration of echinT from 0.05 to 12.5 μM. A plateau in the amount of echinT labeling was observed over the concentration range 2.5–12.5 μM echinT (Fig. 8A). When the ecLysU concentration was varied from 0.025 to 14 μM with a fixed echinT concentration (6.25 μM), the intensity of Hint-AMP was found to increase with increasing amounts of ecLysU (Fig. 8B).

Effect of Inorganic Pyrophosphate (PPi) on Hint-AMP Formation—Yeast inorganic pyrophosphatase, which hydrolyzes PPI, has been used to enhance the catalytic activity of LysRS by reducing product inhibition by PPI (32). The addition of inorganic pyrophosphatase to aminoacyl-tRNA synthetase reaction mixtures has been suggested to more closely mimic the in vivo conditions for aminoacylation (33, 34). Therefore, adenylation of echinT was also carried out with and without inorganic pyrophosphatase to evaluate the effect of PPI on Hint-AMP formation. As shown in supplemental Fig. 2, in the presence of inorganic pyrophosphatase-enhanced Hint-AMP formation was observed (3-fold).

Effect of Hint on AMP Formation by ecLysU—To further establish the effect of Hint on LysRS-mediated reaction, a direct measurement of AMP production by ecLysU with or without echinT was compared using the described TLC assay (28, 29). The observed rate constant for AMP formation by ecLysU is 0.08 ± 0.01 min⁻¹ and 0.16 ± 0.02 min⁻¹ in the presence of echinT. Variants are represented by S.D.

Adenylation of Hint Mutants by ecLysU and hLysRS—Mutation of His-101 of echinT and His-112 of hHint1 to Ala or Gly completely abolished formation of Hint-AMP (Fig. 9), indicating that the adenylation site was the nucleophilic active site histidine. As shown in Fig. 9A, incubation of ecLysU with echinT, the chimera, and hHint1 revealed similar labeling...
intensities for echinT and the chimera but about 100-fold lower intensity for hHint1. Labeling by hLysRS demonstrated a 10-fold higher intensity for echinT than that for hHint1 and a 47-fold higher intensity for the chimera (Fig. 8B), suggesting that the Hint C terminus is responsible for mediating the differential labeling observed by the echLysU and hLysRS.

**DISCUSSION**

Aminoacyl-tRNA synthetases are essential enzymes that charge amino acids with their corresponding cognate tRNAs to form aminoacyl-tRNAs, which are a prerequisite for protein biosynthesis. Aminoacyl-tRNA synthetases synthesize aminoacyl-tRNA in a two-step reaction (35). In the first step, a carboxylate of an amino acid reacts with ATP to form an activated aminoacyl-AMP intermediate. In the second step, a tRNA reacts with the intermediate to form the aminoacyl-tRNA. In addition to the aminoacylation reaction, lysyl-tRNA synthetase and several other aminoacyl-tRNA synthetases are known to catalyze the formation of Ap₄A (36–38).

In addition to their well recognized aminoacylation activity, several aminoacyl-tRNA synthetases appear to have alternative cellular and possibly physiological roles (39). For example, the association of human LysRS with the human immunodeficiency virus proteins Vpr (40) and Gag (41, 42) and the involvement of histidyl-tRNA synthetase and aspartyl-tRNA synthetase with pro-inflammatory cytokines (43) suggest that aminoacyl-tRNA synthetases have multiple physiological functions. Recently, hLysRS has been shown to suppress gene transcription in complexes with hHint1 and MITF (9) or USF2 (10). Dissociation of hHint1 from the complex by Ap₄A leads to an observable increase of the transcription of specific genes. Nevertheless, the ubiquitous presence of Hints suggests that an underlining and highly conserved physiological function is likely to exist. Although protein phosphoramidate adenylates

**FIGURE 8.** Dependence of Hint adenylation on the concentration of echinT and echLysU. Adenylation was carried out in buffer A containing lysine (2.5 μM) with [α-³²P]ATP (0.32 μM) at 23 °C. A, incubations with echLysU (0.025 μM) for 1 min followed by the addition of echinT (0.05–12.5 μM) for 1 min. Steady-state levels of Hint-AMP were observed when the concentration of echinT reached 2.5 μM. B, incubations with echLysU (0.025–14 μM) for 1 min followed by the addition of echinT (6.25 μM) for 1 min. The intensity of Hint-AMP is proportional to the increased concentration of echLysU.

**FIGURE 9.** Adenylation of Hints by echLysU and hLysRS. Labeling was carried out in buffer A containing lysine (35 μM) and inorganic pyrophosphatase (0.02 unit/ml) with [α-³²P]ATP (0.9 μM) at 23 °C for 1 min followed by the addition of Hint proteins (3.6 μM) for 1 min with echLysU (0.1 μM) (A) and hLysRS (1 μM) (B). WT, wild type.
such as adenylated DNA ligase are well known, thus far they have been shown not to be substrates for Hints (4). Because no small molecule nucleoside phosphoramidates are known to exist, we hypothesized that, based on the association of hHinT with hLysRS, aminoacyl-adenylates may be natural substrates for Hints.

To investigate the relationship between LysRS and Hint-AMP formation, a series of experiments was carried out under various conditions to verify the necessity for lysyl-AMP formation. Consistent with the preference of aminoacyl-tRNA synthetase for ATP over GTP, labeling of Hints was only observed for incubations with [α-32P]ATP (Fig. 2). In addition, GTP was found to be a significantly poorer inhibitor of Hint-AMP formation than ATP. In support of our findings, recent modeling studies attempting to develop a molecular rationale for the substrate specificity of eLysRS have concluded that the enzyme has a greater propensity to bind ATP in the necessary productive conformation than GTP (44).

Given the catalytic processes involving LysRS (Fig. 11), we proposed that formation of Hint-AMP is dependent on the formation of lysyl-AMP. As can be seen in Figs. 2 and 5, increasing the amount of lysine in the reaction solution resulted in enhanced and saturable Hint labeling, as expected from an enzymatic process. Hint labeling was found to be dependent on Mg2+, which is required for aminoacyl-adenylate formation, whereas the addition of Zn2+, which favors Ap4A formation, was found to be inhibitory. In addition, our observation that Ap4A and Ap3A, which are neither substrates nor inhibitors of Hints, are potent inhibitors of Hint-AMP formation is consistent with the inhibitory effect of Zn2+ and the necessity of lysyl-AMP for labeling. That we can observe a plateau in the amount of labeling as we increase the amount of echinT is indicative of a substrate-saturable enzymatic reaction, with an apparent K_{m} of (0.85 ± 0.34) μM, which is about 25-fold lower than that observed for the cognate tRNA_{Lys} (45). The removal of PPi, a byproduct of aminoacyl-tRNA synthetase aminoacyl-adenylate formation, by pyrophosphatase has been shown to significantly enhance tRNA aminoacylation (34). Because the binding site for both PPi and the incoming tRNA are overlapping, inorganic pyrophosphatase has been shown to aid the reaction by reducing product inhibition and the rate of the reverse reaction. Similarly, a greater amount of Hint adenylation was observed with reaction mixtures containing inorganic pyrophosphatase.

Previously, it has been proposed that Hints, like other members of the HIT family, carry out substrate hydrolysis by employing nucleophilic catalysis (1, 46). Of the four conserved active site histidines, one has been shown to be catalytically essential and presumably required for intermediate formation. Adenylated enzymes were not observed when the putative nucleophilic histidine in either echinT (His-101) or hHinT (His-112, Fig. 1) was mutated to alanine or glycine. Chemical degradation studies and pulse-chase experiments with cold ATP have demonstrated that the Hint-adenylate is sensitive to acid, but not base, and that the intermediate has a half-life of <1 min. This result is consistent with kinetic experiments with model adenosine phosphoramidate substrate that have revealed the half-life of the echinT-AMP, hHinT1-AMP, chima-ra-AMP to be 0.15 ± 0.05, 0.30 ± 0.02, and 7.2 ± 2.0 s, respectively (47). In addition, the rate of AMP production by eLysU increases by ~2-fold in the presence of echinT. Taken together, these results are consistent with formation of a covalent P-N bond between the active site histidine and AMP. As observed for other HIT proteins, such as Fhit and galactose-1-phosphate uridylyltransferase (19, 48, 49), which also proceed through active site histidine-AMP intermediates, the catalytic mechanism used by Hints proceeds through formation of an adenylated enzyme intermediate (Fig. 10). The extent to which Hint hydrolysis of the aminoacyl-adenylate is dependent on protein-protein transfer or the release of lysyl-AMP from the active site of LysRS remains to be determined (Fig. 11).

Although E. coli and human Hints are nearly 50% sequence similar and their active sites consist of a conserved set of side chains, the C termini of each enzyme is quite unique. X-ray crystal structure analysis of the human and rabbit structures has clearly demonstrated that the C termini of one monomer is in close proximity to the active site of the opposite monomer (21, 48, 50). Consequently, we hypothesized that a chimeric Hint in which the C terminus of the human enzyme was replaced by that of the C terminus of the E. coli enzyme may be responsible for substrate specificity differences. Indeed, regard-
less of the LysRS, the chimera was more effectively labeled than either of the two parental Hints. In particular, the bacterial C terminus improved adenylation of hHint1 by ecLysU by nearly 100-fold, with the chimeric protein appearing to be as effectively labeled as the echinT by ecLysU. Surprisingly, hLysRS more effectively labeled echinT than hHint1. As can be seen in Fig. 9B, the E. coli C terminus appears to be at least partially responsible for enhanced adenylation formation, since chimera labeling is enhanced 47-fold. The additional 4-fold increase in chimera labeling relative to echinT may reflect additional hHint1 specific determinants of adenylation or difference in the half-life of the Hint-AMP intermediate.

Taken together, these results demonstrate that the lysyl-AMP is a physiological substrate for Hints. In retrospect, the ability of Hints to hydrolyze aminoacyl-adenylates should not be surprising given the similar structure and the greater reactivity when compared with nucleoside phosphoramidates. Given the broad conservation of Hints in all three kingdoms of life and their wide spread tissue distribution, a common evolutionary conserved function appears likely. Although it is possible that Hints are cellular scavengers of inadvertently released aminoacyl-adenylates, the results of recent E. coli protein-protein interaction studies demonstrating that elongation factor (EF-Tu) binds not only to lysyl, alanyl-, and isoleucyl-tRNA synthetase but also echinT suggests that the ability of Hints to hydrolyze aminoacyl-adenylates may possibly have a role to play as regulators of protein translation (51). Whether this role may be to assist in pre-transfer editing or the regulation of adenylate formation remains to be determined. In addition, in mammalian cells, the adenylation of Hint by LysRS might be responsible for the regulation of complex formation with the transcription factors, USF2 and MITF. Ongoing enzyme- and cell-based studies should clarify their apparently necessary cellular role.

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