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# Human anti-asparaginyl-tRNA synthetase autoantibodies (anti-KS) increase the affinity of the enzyme for its tRNA substrate[1]

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**Abstract Autoantibodies directed against specific human aminoacyl-tRNA synthetases have been associated with a clinical picture including myositis, arthritis, interstitial lung disease and other features that has been referred to as the 'anti-synthetase syndrome'. Anti-asparaginyl-tRNA synthetase autoantibodies (anti-KS), the most recently described anti-synthetase autoantibodies, are directed against human cytosolic asparaginyl-tRNA synthetase and neutralize specifically its activity. Here we show that these antibodies recognize two epitopes on the human enzyme, an N-terminal epitope reactive in immunoblot experiments and a heat-labile epitope in the catalytic domain. In contrast to the well studied anti-Jo-1 autoantibodies anti-KS when bound to the synthetase increase the affinity of the synthetase for its tRNA substrate and prevent aminoacylation without interfering with the amino acid activation step. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Aminoacyl-tRNA synthetase; Anti-synthetase syndrome; Autoantibody; Autoimmune disease; Neutralization

## 1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are key components of the protein translation machinery that catalyze a two-step reaction leading to correct aminoacylation of isoacceptor tRNA. The 20 aaRSs present in most prokaryotic and eukaryotic species can be divided into two classes of 10 members each, distinguished by their catalytic domain topology [1,2]. A subset of the human autoimmune diseases, the idiopathic inflammatory myopathies, are characterized by the existence of high affinity neutralizing anti-cytoplasmic tRNA synthetase antibodies. To date, six different anti-synthetase antibodies have been described and each one inhibits a specific cytosolic aaRS – anti-PL12 (AlaRS), anti-PL7 (ThrRS), anti-OJ (IleRS), anti-Jo-1 (HisRS), anti-EJ (GlyRS), and anti-KS (AsnRS) [3].

The mechanism by which the six known anti-synthetase autoantibodies block aminoacylation is not fully understood. It is known, however, that some anti-synthetase antibodies can directly precipitate tRNA independent of the corresponding aaRS. However, other anti-synthetase antibodies, such as anti-asparaginyl-tRNA synthetase autoantibodies (anti-KS), react only with the enzyme:tRNA complex. Thus the mechanisms underlying aminoacylation inhibition are likely to be different among the different anti-synthetase antibodies. One hypothesis for the inhibitory effect of anti-synthetase antibodies on aaRS is that autoantibody might disrupt protein folding and/or conformational changes associated with substrate binding. Therefore, to understand the mechanism by which anti-KS inhibit aminoacylation, we examined the interaction of anti-KS on recombinant wild-type human cytosolic AsnRS (HsAsnRSc) and a series of HsAsnRSc deletion mutants corresponding to various enzyme domains. The specificity of anti-KS was compared to other known anti-synthetase antibodies, and the effect of anti-KS on HsAsnRSc was measured on the amino acid activation step and on the overall reaction.

## 2. Materials and methods

### 2.1. Autoimmune sera

Anti-KS sera were obtained from six different Japanese subjects (KS-prototype and KS1–KS5) for comparative immunological studies. Patients exhibiting rheumatological disease, characterized by a combination of inflammatory myositis with or without arthritis and interstitial lung disease, were screened for the presence of autoantibody as described previously by Hirakata and coworkers [4]. Control sera were collected from healthy normal subjects.

### 2.2. Construction of deletion mutants

HsAsnRSc mutants were constructed by site-directed mutagenesis (QuickChange™ Site-Directed Mutagenesis Kit, Stratagene) using polymerase chain reaction (PCR) and the plasmid containing wild-type HsAsnRSc cDNA as template. All cDNA sequences were cloned back into the pCal-n vector between the *Bam*HI and *Eco*RI restriction sites. Complementary anti-parallel primers (listed below) were extended during temperature cycling using *Pfu* DNA polymerase. Primers for $AsnRS_{1-221}$ (1) introduce two stop codons, primers for $AsnRS_{108-548}$ (2) a *Bam*HI restriction site. Mutated nucleotides are in bold:

1. 5′-GACAACCTGATCAAT**TAGTAG**TCTGACGTTGATGTCC-AGC-3′
2. 5′-GATTACCATTAAA AA**GGATCC**AGTCTCCCAGAGCCA-AAATGTGTG-3′

A *Bam*HI digestion for the $AsnRS_{108-548}$ construct eliminated a *Bam*HI–*Bam*HI fragment in order to obtain the final mutant constructs in phase with the calmodulin tag.

### 2.3. Expression and purification of recombinant asparaginyl-tRNA synthetases

The cDNA encoding human cytosolic AsnRS was amplified by PCR using a human liver 5′ RACE-Ready cDNA library from Clontech as template. Suitable primers were designed using EST sequence information of human ESTs encoding peptides showing high sequence

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[1] Enzyme: asparaginyl-tRNA synthetase (EC 6.1.1.22), EMBL accession number AJ000334.

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similarities with yeast AsnRS. The complete cDNA was cloned into the pCal-n expression vector [5]. BL21(DE3) pCal-n HsAsnRSc cells expressing the human enzyme were grown in LB-ampicillin at 37°C to an optical density of 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and incubation continued for 6 h at 23°C. Cells were then lysed by sonication. The bacterial extract was applied to a calmodulin affinity column for purification. The CBP tag was removed by proteolytic digestion with thrombin. *Brugia malayi* AsnRS [6] was purified similarly from an overproducing pCal-n clone. Purified yeast aspartyl-tRNA synthase (AspRS) was obtained from Dr. Gilbert Eriani, IBMC, Strasbourg, France.

*2.4. Immunoassays using autoimmune (anti-KS) serum*

The immunological reactivity of recombinant proteins was tested in two ways. For Western blot analysis, purified protein samples (wild-type and mutant AsnRS) were separated electrophoretically on a 12% SDS polyacrylamide gel and transferred to an Immobilon-P membrane [7]. Detection of bound protein was visualized using 5.0 μl of human anti-KS-type serum as a primary antibody. Anti-human IgG peroxidase conjugate with the SuperSignal® chemiluminescent substrate (Pierce) was used for detection of anti-KS.

ELISA plates were coated in duplicate with 1 μg of human recombinant antigen by overnight incubation at 4°C and were assayed as described elsewhere using mouse anti-human IgG conjugated to peroxidase [8].

*2.5. Aminoacylation and neutralization assays*

Aminoacylation assays measure incorporation of [$^{14}$C]Asn as previously described using unfractionated yeast tRNA and 2 μM purified HsAsnRSc [9].

Antibody-mediated neutralization assays using purified wild-type and mutant HsAsnRSc, *B. malayi* and *Thermus thermophilus* AsnRS [10] or suitable dilutions of bacterial extracts containing overexpressed yeast AsnRS [11] were performed as described elsewhere [5] using unfractionated tRNA.

*2.6. Gel retardation assays*

Gel retardation experiments were performed to examine the effect of anti-KS on tRNA binding to AsnRS. Yeast $tRNA^{Asn}$ was transcribed in vitro using [α-$^{32}$P]UTP. Labeled tRNA was purified by electrophoresis on an 8% polyacrylamide 8 M urea gel followed by overnight elution in 0.3 M sodium acetate (pH 7) and ethanol precipitated. The samples were redissolved in water to obtain 200 000 cpm/μl and renatured by heating for 3 min at 95°C and cooling for 5 min at 4°C. Renaturation buffer containing 10 mM Tris–HCl pH 8 and 10 mM $MgCl_2$, and 10 mM KCl was added. For each test, 200 000 cpm of $^{32}$P-radiolabeled yeast tRNA was incubated with 2 μM of recombinant protein for 5 min on ice in a total volume of 10 μl containing 25 mM HEPES buffer pH 7, 75 mM KCl, 10 mM $MgCl_2$, and 25% glycerol, and loaded on a native 6% (19:1) acrylamide–bisacrylamide gel. The electrophoresis was carried out in running buffer (50 mM sodium acetate and 110 mM magnesium acetate) at 4°C for 45 min. For experiments involving anti-KS, the protein was preincubated with 5 μl of serum (1:5 to 1:5000 dilution) on ice for 5 min.

*2.7. ATP–PPi exchange reaction*

An activity test was performed in the presence or absence of anti-KS serum using an ATP–$^{32}$PPi exchange reaction to verify if the first step of the aminoacylation reaction is inhibited by autoimmune serum. Since the first step of the aminoacylation reaction is reversible, i.e. ATP-mediated formation of asparagine adenylate, formation of radioactive ATP can be measured by providing $^{32}$PPi to a reaction mixture containing HsAsnRSc (2 μM) and asparagine [12].

*2.8. Competition of mutant HsAsnRScs for anti-KS*

The ability of various domains of HsAsnRSc to bind autoantibody was examined in competition tests. Both mutant proteins, $AsnRS_{108-548}$ (0–650 pmol) and $AsnRS_{1-221}$ (0–1780 pmol), which are devoid of aminoacylation activity, were tested for their capacity to compete with the wild-type enzyme (40 pmol) for the binding of neutralizing antibodies in order to localize the region of the human AsnRS responsible for aminoacylation inhibition. Evidence of competition exists if the wild-type enzyme regains its activity via the removal of neutralizing antibodies by the mutant proteins. Competitor protein was preincubated with anti-KS and control serum. After incubation, sera were used in aminoacylation inhibition assays as described above.

## 3. Results

*3.1. Anti-KS sera specifically interact with human AsnRS*

As previously described for the anti-KS prototype serum [5], five other anti-KS sera were tested for their neutralizing activity and reactivity in ELISA tests. All six anti-KS sera inhibited the aminoacylation reaction of HsAsnRSc. The inhibition level was reproducible with an average inhibition of 80%. Two others anti-synthetase sera tested, anti-AlaRS (anti-PL12) and anti-HisRS (anti-Jo-1), did not neutralize the human AsnRS activity. Also, polyclonal rabbit serum raised against the human protein did not inhibit human AsnRSc aminoacylation activity, suggesting that epitopes recognized by the rabbit serum are different from those recognized by anti-KS. In ELISA tests 50% of maximal reactivity was measured at KS serum dilutions of 1:5000 to 1:50 000. To investigate the specificity of the anti-KS sera, we used yeast AspRS as antigen in ELISA tests. AspRS belongs to the same subclass of synthetases (IIb) as AsnRS and both enzymes show significant sequence similarities. No immunoreactivity with anti-KS sera was observed. Furthermore, AsnRS from bacterial (*T. thermophilus*) or nematode (*B. malayi*) origin did not react with anti-KS sera. This demonstrates the high specificity of the KS autoantibodies.

*3.2. Localization of epitopes*

To localize the epitopes recognized by anti-KS sera, deletion mutants of the human synthetase were constructed by site-directed mutagenesis (Fig. 1A): $AsnRS_{108-548}$ lacking the first 107 amino acids (N-terminal eukaryote-specific domain) and $AsnRS_{1-221}$ corresponding to the first 221 amino

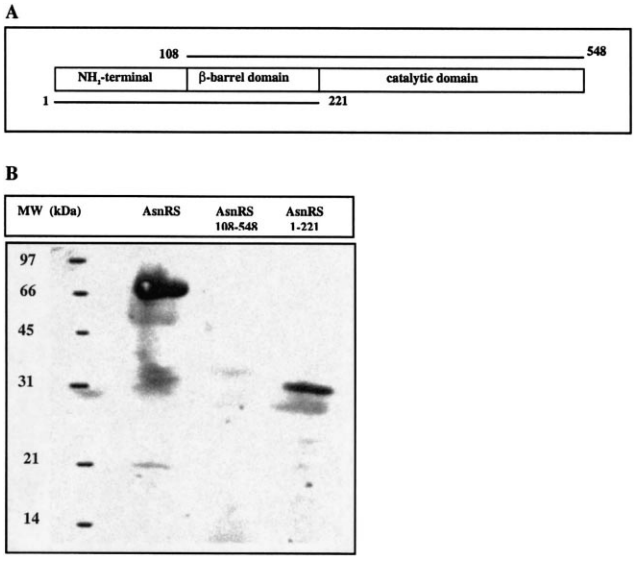


Fig. 1. Epitope localization. A: Schematic organization of HsAsnRSc according to the structural data from the thermophilic AsnRS enzyme [19]. Deletion mutants of the HsAsnRSc are designated by lines. B: Autoradiography of the Western blot showing the reactivity of two AsnRS mutants with anti-KS prototype serum.

acids (N-terminal eukaryote-specific domain and the β-barrel domain) of the human protein. Each mutant protein was expressed, purified and analyzed for its immunoreactivity in Western blot experiments, ELISA and a competition assay. For both mutant proteins, the β-barrel domain is required to obtain stability and proper folding.

Fig. 1B shows that the antibodies present in anti-KS serum specifically interact with the wild-type AsnRS, and the mutant protein corresponding to the N-terminal and the β-barrel domain of the protein ($AsnRS_{1-221}$). The mutant protein corresponding to the catalytic domain with the β-barrel domain of the protein ($AsnRS_{108-548}$) is not reactive in Western blots. The immunoreactivity of the human protein in Western blot experiments seems to be due to the reactivity of its N-terminal domain suggesting the presence of a linear epitope or an epitope with the capacity to refold on membrane in this region (residues 1–221). ELISA tests with anti-KS-type sera using $AsnRS_{1-221}$ and $AsnRS_{108-548}$ mutants in equimolar quantities as antigen show approximately a threefold higher reactivity with the catalytic domain mutant protein. These results suggest the presence of the immunodominant epitope in the catalytic domain of HsAsnRSc. To study the heat stability of the epitope(s), we preincubated wild-type enzyme, $AsnRS_{1-221}$ and $AsnRS_{108-548}$ at different temperatures and measured the residual ELISA reactivity with KS serum. Wild-type and the catalytic β-barrel domain mutant $AsnRS_{108-548}$ lost 50% reactivity after preincubation at 50°C. The N-terminal β-barrel mutant showed a stronger structural stability for its epitopes. Loss of 50% of immunoreactivity was observed for a temperature close to 65°C. This observation might explain the reactivity of the N-terminal epitope in Western blot experiments.

The mutant proteins $AsnRS_{1-221}$ and $AsnRS_{108-548}$ are devoid of aminoacylation activity. The correct folding of $AsnRS_{108-548}$ was tested by the ATP–PPi exchange reaction (data not shown) monitoring the first step of the aminoacylation reaction which requires a correct structural organization of the catalytic domain.

In competition tests, the mutant protein $AsnRS_{108-548}$ interacts with the neutralizing antibody allowing the wild-type enzyme in the presence of KS serum to recover its initial activity (Fig. 2). In contrast, the neutralizing antibodies do not recognize the mutant protein $AsnRS_{1-221}$ which is inactive

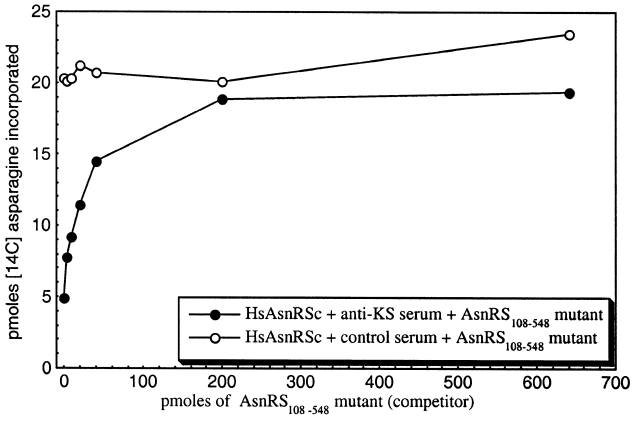


Fig. 2. Localization of the neutralizing autoepitope. Increasing concentrations of the competitor protein $AsnRS_{108-548}$ were added to wild-type HsAsnRSc (0.8 μM) in the presence of neutralizing KS autoantibody (1:10 dilution). The mutant protein $AsnRS_{108-548}$ is not active in the aminoacylation reaction.

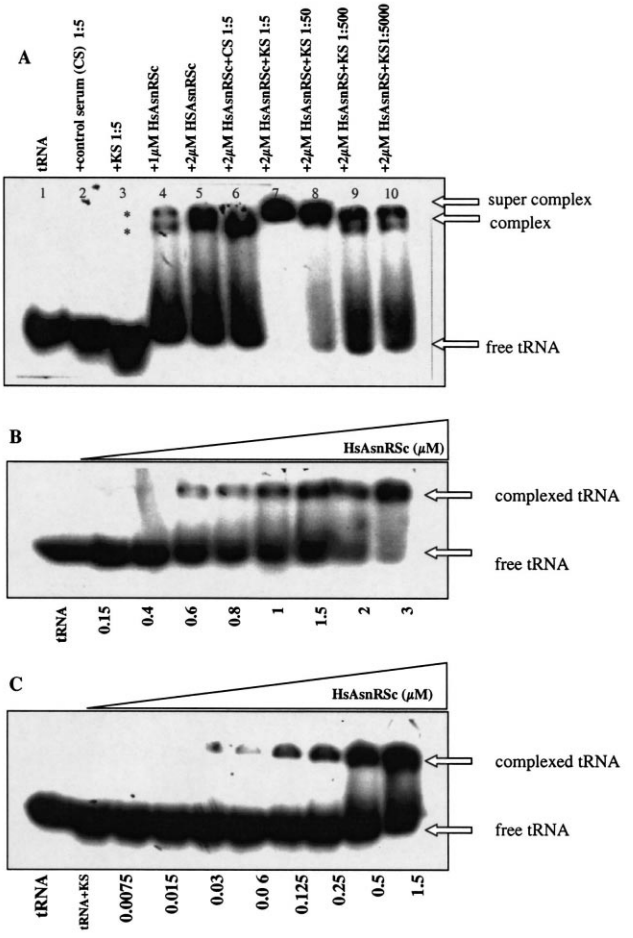


Fig. 3. Influence of KS serum on the affinity of HsAsnRSc for its tRNA substrate. A: Autoradiogram of a gel retardation experiment obtained with [α-$^{32}$P]UTP yeast tRNA. Radiolabeled tRNA is present in each lane. CS corresponds to control serum and KS to KS serum. Serum dilutions used are indicated. B, C: Autoradiograms of quantitative gel retardation experiments obtained with yeast $tRNA^{Asn}$ complexed to native HsAsnRSc in the absence (B) or presence (C) of KS autoantibody. Radiolabeled tRNA is present in each lane. Increasing concentrations of HsAsnRSc were used.

in the competition test. The absence of immunoreactivity of $AsnRS_{108-548}$ in Western blot analysis using anti-KS sera, together with its capacity to prevent neutralization by anti-KS serum, suggests that the neutralizing epitope is conformational and localized in the catalytic domain of the human enzyme.

### 3.3. Autoantibody alters the affinity of HsAsnRSc for tRNA

Analysis of the first step of the reaction (amino acid activation) by ATP–PPi exchange showed that there is no significant difference in radioactive ATP formation in the absence or presence of the anti-KS serum, indicating that antibody binding does not interfere with the amino acid activation step (data not shown). Therefore we studied the effect of the autoimmune serum on the affinity of the synthetase for its macromolecular substrate tRNA.

Gel retardation experiments demonstrated that KS antibodies do not recognize free tRNA (Fig. 3A, lane 3). Under non-saturating concentrations of synthetase (Fig. 3A, lanes 4 and 5) free tRNA and complexed tRNA can be observed. Two

forms of complexed tRNA (*) exist, most likely corresponding to one molecule of tRNA/dimer and two molecules of tRNAs/dimer of AsnRS. Control serum did not modify the pattern of free and complexed tRNA (Fig. 3A, lane 6). However, with the anti-KS serum (Fig. 3A, lane 7) the pattern is totally changed. No more free tRNA can be observed. A new band with a slightly slower migration compared to the complexed tRNA in the absence of KS appears, corresponding to a supercomplex composed of synthetase, tRNA and antibody. Anti-KS does not prevent the tRNA binding. When the antibody concentration decreases, the pattern corresponding to the synthetase–tRNA complex and free tRNA progressively reappears. According to the pattern obtained in the presence of anti-KS serum (Fig. 3A, lane 7), it seems that the antibody has a stabilizing effect on the tRNA–protein complex.

This effect was studied in more detail by gel retardation experiments in the absence (Fig. 3B) or presence (Fig. 3C) of anti-KS serum. Due to the shorter migration only the separation in uncomplexed or complexed tRNA is visible. In the absence of anti-KS, the shift corresponding to the tRNA–synthetase complex formation is obtained with a minimal concentration of 0.6 μM of human protein. When the KS serum is added (1:200 final dilution) this shift appears at about five times lower protein concentrations (60–125 nM). Therefore, the anti-synthetase serum with neutralizing activity increases the affinity of the synthetase for its substrate tRNA.

## 4. Discussion

Anti-KS represents the sixth in a series of human autoantibodies recognizing aminoacyl-tRNA synthetases. All such autoantibodies occur in persons with a variant of idiopathic inflammatory myopathies, characterized by immune-mediated lung and/or joint disease [3]. For unknown reasons, rarely does more than one anti-synthetase antibody occur in the same patient.

The results of our investigations with recombinant human AsnRS answer some questions underlying the inhibitory mechanisms of anti-KS. ATP–PPi exchange reactions showed no significant difference in radioactive ATP formation in the absence or presence of the anti-KS serum, indicating that antibody binding does not interfere with the amino acid activation step. Anti-KS, but not normal sera, leads to a higher affinity of the tRNA for the protein. The neutralizing autoepitope has been localized to the C-terminal part of human asparaginyl-tRNA synthetase based on binding studies with deletion derivatives. The tRNA affinity-increasing activity of the autoantibody was only observed with the entire enzyme. Therefore we cannot exclude the possibility that both activities involve different epitopes. If both activities involve the same epitope the increased affinity for tRNA might lead to aminoacylation inhibition by blocking the dissociation of the tRNA from its synthetase. Alternatively the autoantibody might induce a conformational change on the synthetase which interferes with the transfer of the activated amino acid to the acceptor stem of the tRNA. The heat-labile neutralizing epitope(s) recognized by anti-KS sera requires both the catalytic and β-barrel domains to adopt suitable structural conformations.

The N-terminal part of the human AsnRS, similar to the human HisRS, reactive with Jo-1, contains predicted helix structures [13]. The immunodominant epitope for Jo-1 is reportedly a coiled-coil structure localized in the first 60 amino acid of the human HisRS N-terminus. Jo-1 competes with the small substrates, ATP and histidine [14]. However, in contrast to that human HisRS/Jo-1 data, our results with AsnRS/anti-KS show three differences. First, anti-KS-type sera do react in an immunoblot experiments with residues 1–221 of the AsnRS N-terminus, but these epitopes are not involved in inhibition. This is not to say that the two N-terminal regions are totally distinct. Short regions of sequence similarity do exist in the N-terminal domains of many eukaryotic AsnRSs from different species. For example, a tRNA binding motif has been reported in the N-terminus of yeast aspartyl-tRNA synthetase and other eukaryotic class IIb aaRSs [15]. Secondly, anti-KS does not interfere with ATP and asparagine binding. Thirdly, HisRS inhibition by Jo-1 decreases with increasing phylogenetic distance from human. This was demonstrated by showing diminished inhibitory activity of Jo-1 using HisRSs isolated from distant prokaryotic and eukaryotic species [16]. In contrast, anti-KS does not significantly inhibit bacterial, yeast or nematode AsnRSs under the conditions tested. In the past, it has been suspected that anti-synthetase antibodies that do not precipitate tRNA alone might interfere with tRNA binding to aaRSs; however, to our knowledge, anti-KS is the first anti-synthetase antibody which has been shown to actually increase tRNA affinity.

Coiled-coil structures are frequently found in autoantigens and in other proteins and the concept of molecular mimicry has often been involved to explain the generation of autoantibodies [17]. The capacity of coiled-coils to bind to nucleic acids has been demonstrated and many intracellular autoantigens are nucleic acid-binding proteins. Binding of tRNA to synthetases may be an important factor leading to the development of these autoantibodies. Nucleic acid binding is a feature of several autoantigens in systemic autoimmune disease [18].

Ultimately, comparison of the three-dimensional atomic structures of eukaryotic AsnRS and aaRS:autoantibody complexes may help to define the basis for anti-synthetase antibody-mediated inhibition of aaRSs. These endeavors may prove technically challenging, given the flexibility of amino-terminal domains in eukaryotic aaRSs. The *T. thermophilus* AsnRS revealed that the motif 3 region of the catalytic domain interacts with a hinge region linking the N-terminal anticodon-binding domain to the catalytic core [19]. If this relationship also holds true for the human enzyme, anti-KS may interact with a conformation which includes the hinge region.

Our data do not directly address the underlying pathophysiological processes associated with the generation of anti-synthetase antibody in human autoimmune disease. These mechanisms are likely to be complex and may require in vivo confirmation of a hypothesis. Molecular mimicry between human and non-self antigens (e.g. pathogenic microbes) is often suggested as a mechanism for induction of autoimmunity. But this theory may be too simplistic to explain the etiology of anti-synthetase antibodies given the recent evidence from murine models of autoimmunity. Overexpression of the human MHCI complex in transgenic mice resulted in production of neutralizing Jo-1 antibody [20]. Therefore a complete understanding of mechanisms involved in the production of autoimmune phenomena may require detailed structure–function analysis across protein and non-protein families.

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