Primary Structure and Functional Expression of Human Glycyl-tRNA Synthetase, an Autoantigen in Myositis*

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Two identical cDNAs encoding human glycyl-tRNA synthetase were isolated from K562 and HEL cell act11 libraries by screening with “anti-EJ” autoantibodies from a patient with dermatomyositis, previously shown to specifically inhibit this enzyme. The sequenced cDNA had 2,985 base pairs, with a predicted protein of 685 amino acids and M, 77,500. The antigen reactive with anti-EJ was immunoaffinity-purified from HeLa cells, and two proteolytic peptides matched areas of the predicted amino acid sequence. The sequence was 61.6% identical with that of silk moth glycyl-tRNA synthetase. Several very highly conserved regions were identified, including the single class II synthetase motif region and an N-terminal region similar to one found in at least three other synthetases. Near identity in other regions suggested that they also had important functions. Expression of the cDNA in Escherichia coli yielded a protein of expected size that reacted with the autoantibodies and was active as glycyl-tRNA synthetase.

Aminoacyl-tRNA synthetases perform an essential function in protein synthesis by catalyzing the esterification of an amino acid to its cognate tRNA (1). These enzymes are necessarily present in each cell and must properly recognize the tRNA and the amino acid in order to maintain fidelity of translation. Recent progress in the analysis of the structure of these enzymes has provided significant insights into their classification and evolutionary development, their mechanisms of substrate recognition and catalytic activity, as well as a more general understanding of nucleic acid-protein interactions (1–8).

The primary structures for most bacterial and yeast forms of these enzymes have been determined (1), and for some, three-dimensional structures are known (3, 8–10). Two distinct classes of synthetases have been recognized based on consensus sequences, with similar certainty of structural features, amino acid attachment sites, and other properties between members of a class (2, 4, 9, 11, 12). There are major differences between yeast and corresponding bacterial forms, although significant areas of homology are commonly seen, with conservation of consensus sequences and certain structural features (1, 3, 11).

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Nucleic acid and amino acid sequences for only a minority of higher eukaryotic synthetases are available, including those for the human forms of histidyl-, threonyl-, tryptophanyl-, aspartyl-, valyl-tRNA synthetases (13–17), and a 170-kDa synthetase ini-
**Human Glycyl-tRNA Synthetase**

2. **fig. 3. A.** Sequencing strategy for EJ2 cDNA. *A,* thin line represents full EJ2 cDNA; heavy line shows the coding region. EcoRI sites are indicated. 2. position of cDNA fragments of clone EJ2 when aligned 5'→3'. 2A, 1.0 kb; 2B, 0.8 kb; 2C, 0.6 kb. Thin arrows show directions and extent of individual sequencing reactions from fragments. Thick arrows show sequencing across EcoRI sites from Gly-A cDNA.

**fig. 3. B.** Nucleotide and deduced amino acid sequence of human glycyl-tRNA synthetase. 1) EcoRI sites between fragments = bold nucleotide sequence, with fragment position indicated (E, fA, fB, E, = 1.0-, 0.8-, and 0.6-kb cDNA fragments); 2) polyadenylation signal = underlined nucleotide sequence; 3) the consensus pattern of class II synthetase motif 2 = (F,Y,H)-R-X-(E,D)-(4,12)-(R,H)-X-X-X-F (D,E) (4). The region that satisfied this pattern = underlined amino acid sequence; 4) areas of sequenced peptides = shaded. The directly determined 20 and 25 amino acid sequences matched the predicted sequences exactly; 5) area of N-terminal consensus sequence (found in HisRS, TrpRS, and GluProRS = double underline. GenBank™ accession no.: U09587.
B

VallysSerProIleThrGlyAsnAspLeuSerSerProProValSerPheAsnAsnMetPhe

VallysSerProIleThrGlyAsnAspLeuSerSerProProValSerPheAsnAsnMetPhe

ValArgGlyPheLeuGlyHisPheValAspProSerGluProSerGluAspHis

ValArgGlyPheLeuGlyHisPheValAspProSerGluProSerGluAspHis

ProLysPheGluAsnValAlaAspGluPheProArgGluAspAlaValGluValIleAsp

ProLysPheGluAsnValAlaAspGluPheProArgGluAspAlaValGluValIleAsp

SerGlyGluSerAlaArgAspLeuGluAspLeuValGluValIleAsn

SerGlyGluSerAlaArgAspLeuGluAspLeuValGluValIleAsn

GluLysAspLeuArgPheArgGlnHiaMetGlueanLeuGluProPheProLeuAla

GluLysAspLeuArgPheArgGlnHiaMetGlueanLeuGluProPheProLeuAla

ValAspProGluCysPheLeuTyrAlaCya

ValAspProGluCysPheLeuTyrAlaCya

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GluLysProLeuGlyHisPheValAlaSerProGluProGluSerGluGly

GluLysProLeuGlyHisPheValAlaSerProGluProGluSerGluGly

AlaIleGlyLysLeuAlaGluAspLeuValMetGluLeuValLeuIleCys

AlaIleGlyLysLeuAlaGluAspLeuValMetGluLeuValLeuIleCys

Materials and Methods

Sera—Sera with autoantibodies were obtained from patients with myositis (34). "Anti-EJ" antibody (code letters based on the prototype patient) was defined by immunoprecipitation of a specific set of tRNAs and a 75-kDa protein from HeLa cell extract (33). Anti-EJ was presumed to react with GlyRS because IgG with anti-EJ (but not anti-EJ-negative IgG) inhibits GlyRS specifically and completely (80–100% inhibition compared to activity with control IgG) (33, 34), with no significant, specific effects on other synthetases. The restricted set of tRNAs precipitated by anti-EJ are assumed to be forms of tRNA^Glu.

1 The abbreviations used are: GluProRS, glutamyl-prolyl-tRNA synthetase; GlyRS, glycyl-tRNA synthetase; HisRS, histidyl-tRNA synthetase; ThrRS, threonyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; EJ, code letters from prototype patient designating 75-kDa antigen; IPTG, isopropyl-1-thio-p-galactopyranoside; Jo-1, HisRS (as autoantigen); PCR, polymerase chain reaction; kb, kilo base; bp, base pair.

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Zrnmunoblot—Immunoblot was performed as described previously (35, 36). 10% SDS-PAGE, nitrocellulose membrane, 5% non-fat milk for blocking and serum dilution, and a goat anti-human IgG/alkaline-phosphatase conjugate (Sigma) were used. Either whole HeLa cell extract, HeLa EJ protein, or Escherichia coli lysate with or without recombinant protein were used as antigen preparations. The HeLa extract was a sonicate of \(1 \times 10^4\) cells, centrifuged at 12,000 xg for 30 min (35). HeLa EJ protein was prepared from HeLa extract by immunoprecipitation with anti-EJ serum, performed as described previously (35, 36). Isolation of cDNA and Confirmation—Two high GC content cDNA expression libraries were screened using anti-EJ serum, one from K562 cells (purchased from Clontech, Palo Alto, CA) and the other from HEL cells (a gift from Dr. Mortimer Poncz). Screening and subcloning were performed as described previously (36). The identity of proteins encoded by cDNA clones was confirmed by using affinity-purified antibodies prepared by incubating nitrocellulose with plaque proteins from clone EJ2 (or wild-type hgt1 as control) with anti-EJ serum and elution with glycine buffer at pH 2.3 (35). Further confirmation was obtained from the amino acid sequence of peptides of HeLa EJ protein. EJ was purified by immunoaffinity chromatography, from extract of \(=10^7\) HeLa cells per application (10 150-cm² flasks) using a gel prepared from 1 ml of anti-EJ serum, with IgG cross-linked to protein A-Sepharose with dimethyl suberimidate (Amersham Antibody Orientation Kit, Schliecher & Schuell), and eluted with glycine buffer, pH 2.3. Eight such eluates were pooled, separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The 75-kDa protein was subjected to in situ limited proteolysis, and the amino acid sequence of two peptides was determined by Edman degradation (37, by Harvard microchemistry. Nucleotide Sequencing—Nucleotide sequencing was performed by standard methods (38). The nucleotide sequence of the 5' and 3' termini of all cDNA fragments from both identified clones were sequenced from pUC18. Remaining nucleotide sequence was determined for both strands of all three fragments of clone EJ2 from M13mp18. Northern Blot—Total HeLa RNA was electrophoresed in a denaturing 1% agarose gel containing 1 M formaldehyde and transferred onto nylon membrane, as done previously (36). The RNA was probed separately with each cDNA fragment labeled with \(^{32}P\). Expression of Recombinant Protein—The Gly-A cDNA, encoding the full protein, was derived by PCR from reverse-transcribed HeLa mRNA.
as previously performed (36), subcloned into pQE expression plasmid, and transformed into E. coli M15/pREP4 (Qiagen expression kit, Chatsworth, CA).

Aminoacylation—Aminoacylation was performed as described (33, 39), using whole yeast tRNA, \([^{1}H]glycine\), and as enzyme source either lysates of E. coli with plasmid with insert induced by IPTG, diluted 1:20 with Tris buffer or control lysates at an equal protein concentration (5.7 mg/ml). The ability of antibody to inhibit aminoacylation was determined by preincubation of the enzyme source with half-volume of 5 mg/ml IgG from either anti-EJ serum or normal serum (33, 39).

Sequence Analysis—The Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group was used for computer analysis of the nucleotide and amino acid sequences, performed on a VAX 8240 computer (40), and modifications were used for analysis of short sequence matches (41).

RESULTS

Isolation of cDNA Clones—Two clones were specifically reactive with the screening anti-EJ serum and not normal serum, labeled EJ1 (from K562 library) and EJ2 (from HEL). Among anti-EJ sera from eight other patients, three were strongly and specifically reactive, and five showed weaker, equivocal reaction. Control sera did not react. Supporting the impression that these cDNAs encoded the autoantigen, affinity-purified antibodies (eluates from proteins of clone EJ2 after incubation with anti-EJ serum), but not controls (eluates from wild-type) reacted by immunoblot with a 75-kDa protein of HeLa extract that was identical in size to that recognized by whole anti-EJ sera (Fig. 1). They did not recognize other proteins bound by whole anti-EJ sera.

Characterization of cDNAs—Both clones, EJ1 and EJ2, revealed a similar set of three insert fragments, labeled fA (1.0 kb), fB (0.8 kb), and fC (0.6 kb). Sequencing of the 5’ and 3’ termini revealed that EJ1 and EJ2 inserts were identical, and only EJ2 was further studied. By Northern blot, an identical hybridization pattern was seen when total HeLa cell RNA was probed with each fragment, including a major band at 2.4 kb and 2 minor larger bands (Fig. 2).

The full nucleotide sequence of both strands of each of the three fragments was determined according to the scheme in Fig. 3A. Analysis of the possible predicted proteins that could be derived from the three fragments indicated that the alignment shown in Fig. 3A (5’fA-fC-fB3’) could produce a protein in the expected 75-kDa range (Fig. 1 and Ref. 33). PCR with primers derived from the ends of the predicted coding sequence, using reverse-transcribed HeLa mRNA as template, produced a product of expected size (labeled Gly-A). Sequencing across the proposed EcoRI restriction sites (Fig. 3A), showing that the first site was flanked by sequence of fA and fC, and the second by fC and fB, confirmed the alignment and that no sequence was lost from these sites. 20- and 25-residue sequences from proteolytic peptides of EJ protein were identical to regions of the sequence predicted by fB cDNA (Fig. 3B), confirming that EJ2 cDNA encoded EJ protein.

Expression of Gly-A—After expression of the PCR-derived Gly-A cDNA in E. coli, the recombinant protein migrated similarly in SDS-polyacrylamide gel electrophoresis to HeLa EJ protein (Fig. 4). This protein reacted strongly by immunoblot with the three sera that reacted strongly with plaques (two examples shown in Fig. 4), and weakly but detectably with the other anti-EJ sera.

E. coli lysate containing the expressed protein had very high GlyRS activity (incorporation of \([^{1}H]glycine\) into tRNA), but no significant HisRS activity. A representative experiment is shown in Table I. Lysate of E. coli with vector without Gly-A insert had very low activity, representing endogenous E. coli GlyRS. Activity with Gly-A but without IPTG induction was higher, representing low level expression without induction. With IPTG, a 39-fold increase in GlyRS activity was seen compared to uninduced lysate. No activity above base line was seen with expressed original fragment cDNAs. Normal IgG partially inhibited GlyRS activity of induced lysate (average 18.2%), as expected from previous studies and felt to be nonspecific (31, 35). Inhibition by IgG with anti-EJ was much stronger (95.6%), supporting the conclusion that the induced activity was due to recombinant human GlyRS.

![Figure 4. Immunoblot against recombinant and natural EJ protein (glycyl-tRNA synthetase).](image-url)
**Human Glycyl-tRNA Synthetase**

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**DISCUSSION**

The evidence presented confirmed the nature of the proteins encoded by the cDNAs. The identification as EJ protein was supported by: (a) reaction of anti-EJ autoantibodies with the Agt11 fusion protein and with the bacterially expressed product of Gly-A cDNA and (b) the exact match between sequences of peptides derived from EJ antigen with regions of the predicted amino acid sequence from EJ protein. This finding supported its identification as GlyRS, since previous data showed that anti-EJ autoantibodies strongly inhibit GlyRS activity (33, 34). This was directly demonstrated by evidence of GlyRS functional activity. The evidence is independent of the strong homology with the silk moth form or the presence of a class II synthetase signature motif.
The demonstration that the cDNA encodes the EJ antigen helped confirm that it was the cytoplasmic, rather than mitochondrial, form. Anti-EJ autoantibodies can completely inhibit GlyRS activity of cell extracts and react with the 75-kDa antigen after sedimentation of mitochondria. Anti-EJ would not be expected to react with the mitochondrial form. Anti-synthetases are broadly cross-reactive between mammalian species, but anti-HisRS reacted less well with reptile, amphibian, avian, and fish species, and not at all with yeast, bacterial, or other lower forms (44, 45).

The major band seen by Northern blot at approximately 2.4 kb is consistent in size with the cDNAs, indicating that most of the large 5'-untranslated region of B. mori GlyRS is absent. The significance of the larger hybridizing mRNAs is unclear. If they represent specific hybridization, the similarity between insect and human forms is less than 70% (201, 202). The human and B. mori GlyRS further highlights its potential importance.

Comparison to silk moth GlyRS—The degree of amino acid identity between human and B. mori GlyRS (61.6%) was less than that between the catalytic domains of Drosophila and human GluProRS (about 79%) (20), the only other synthetase for which both insect and human forms are known. There is less identity between yeast and human forms (56% for aspartyl-129, 53% for threonyl-141, and 47.5% for histidyl-tRNA synthetases (13)).

The human and B. mori forms show extended areas of almost complete identity, and other areas of lesser homology. The longest extended conserved region, amino acids 242–294, includes a class II synthetase motif. Three motifs conserved between members of class II have been identified, and this region fulfills precisely the signature pattern of motif 2 (4) (also seen in B. mori GlyRS), thought to interact with the tRNA acceptor stem (11). The near-identity between human and B. mori GlyRS extends proximally from motif 2 to include at least 28 additional amino acids. This may represent a region referred to as "signature region 1," that is conserved for a given enzyme across species but not between other class II synthetases (13) and thus a candidate region for tRNA or amino acid recognition (13).

Class II motif I has not been found in this or other forms of GlyRS. The motif I region is at the dimer interface (11); its absence in bacterial GlyRS may relate to its different quaternary structure (heterotetramer), but eukaryotic GlyRS is a dimer (46). Thus, a region analogous to motif 1 might be present in the N-terminal portion. Between amino acids 66 and 82, and amino acids 129 and 141, are regions of near-identity between B. mori and human, suggesting that one may serve the function of motif 1 for GlyRS.

Similarly, a region analogous to motif 3 that may bind the ATP would be expected toward the C-terminal portion, and a very highly conserved section of this region, between 589 and 610, might possibly be involved. Another highly conserved area, between amino acids 373 and 394, and the adjacent 398–415, could possibly, based on its position, represent "signature region 2," another vertically conserved region (11, 13).

Evolutionary Significance—Like the eukaryotic forms, and unlike other class II synthetases, bacterial GlyRS has only one class II motif (3, 4). Surprisingly, however, it is motif 3. The bacterial form also differs, as noted, in being a tetramer, and shows no significant overall or regional homology to the human or B. mori forms. It was suggested that this may mean that eukaryotic GlyRS evolved from another class II synthetase, ThrRS, since the motif 2 region of B. mori GlyRS had several identities and similarities with those of all four available forms of ThrRS (24). The 9-amino acid region just adjacent to GlyRS motif 2 showed some similarity to a region of Neurospora leucyl-tRNA synthetase (Fig. 6) (47), a class I synthetase, and it is noteworthy that another region of human GlyRS had a 7 consecutive amino acid match with that enzyme. Sequences of additional forms of these synthetases may help clarify their evolutionary relationships.

A relatively conserved region of interest was seen between amino acids 20 and 56 that is similar to N-terminal regions of TrpRS (15) and HisRS (13), and the connecting region of GluProRS (18, 20). Its possible significance has been noted previously (13, 18, 20, 24). It was suggested that in GluProRS, it serves as a core for formation of the multi-enzyme synthetase complex (20). Helical regions in the N-terminal portion of class II synthetases have been suggested as tRNA-binding regions (11), and previous comparison of this consensus sequence to similar structures in other proteins suggested that it may be a nucleic acid binding motif (13). Its strong conservation between human and silk moth GlyRS further highlights its potential importance.

GlyRS as Autoantigen—This recombinant protein had both EJ antigen and GlyRS functional activity, providing stronger evidence than previously available that EJ antigen is GlyRS. Similar evidence has been found for the Jo-1 antigen and HisRS (48, 49).

The ability of the autoantibodies to inhibit the functional activity of GlyRS suggests that they react with a functional site, but it is unlikely to be the tRNA-binding site since the autoantibody immunoprecipitates tRNA without binding to it directly, implying that tRNA is bound to the enzyme. The location of the reactive epitopes cannot be determined from these studies, but the availability of this cDNA clone will help in localizing them.

The reason for production of these autoantibodies is not known, but the possibility of molecular mimicry between antigens and infectious agents is frequently suggested. The demonstration of short areas of shared sequences between epitope regions and proteins of infectious agents could support such hypotheses (41). Examples of short amino acid matches between human GlyRS and proteins of human viruses are shown in Fig. 6. One is in the motif 2 region with similarities to ThrRS (also a synthetase autoantigen). However, it is not yet known if
these represent autoantibody binding sites on GlyRS. The signifi-
cance of such observations remains to be determined.

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