Secreted Histidyl-tRNA Synthetase Splice Variants Elaborate Major Epitopes for Autoantibodies in Inflammatory Myositis

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Background: Autoantibodies (anti-Jo-1) to cytoplasmic histidyl-tRNA synthetase (HisRS) are associated with inflammatory myositis. Results: HisRS and two splice variants (SVs) cross-react with anti-Jo-1 antibodies and are secreted; at least one SV transcript is up-regulated in dermatomyositis. Conclusion: Secreted HisRS SVs contain major epitopes of anti-Jo-1 autoantibodies. Significance: Secreted HisRS and its SVs share epitopes for potential extracellular anti-Jo-1 antibody binding.

Inflammatory and debilitating myositis and interstitial lung disease are commonly associated with autoantibodies (anti-Jo-1 antibodies) to cytoplasmic histidyl-tRNA synthetase (HisRS). Anti-Jo-1 antibodies from different disease-afflicted patients react mostly with spatially separated epitopes in the three-dimensional structure of human HisRS. We noted that two HisRS splice variants (SVs) include these spatially separated regions, but each SV lacks the HisRS catalytic domain. Despite the large deletions, the two SVs cross-react with a substantial population of anti-Jo-1 antibodies from myositis patients. Moreover, expression of at least one of the SVs is up-regulated in dermatomyositis patients, and cell-based experiments show that both SVs and HisRS can be secreted. We suggest that, in patients with inflammatory myositis, anti-Jo-1 antibodies may have extracellular activity.

Idiopathic inflammatory myositis (IIM)2 is an autoimmune disease that is strongly associated with autoantibodies and is frequently associated with interstitial lung disease (ILD) (1). Myositis-specific antibodies (MSAs) and myositis-associated antibodies define two distinct groups (2). MSAs are directed against histidyl-, threonyl-, alanyl-, glycyl-, isoleucyl-, and asparaginyl-tRNA synthetases. Interestingly, in any single patient, these MSAs are mutually exclusive (1).

Among the myositis-specific anti-aaRS Abs, those directed against cytoplasmic histidyl-tRNA synthetase (HisRS) are the most common (3) and were first described >30 years ago (4). Approximately 25–30% of patients with dermatomyositis (DM) or polymyositis have anti-HisRS Abs (3). In contrast, autoantibodies directed against the other five aaRSs collectively constitute a much smaller percentage (3–5). Anti-HisRS Abs, which were historically designated as anti-Jo-1 Abs, bind to sites that are spread across the entire protein and include both linear and conformational epitopes (6, 7).

Among the various epitopes, the N-terminal portion of HisRS is especially prominent (6–8). In ELISA, recombinant HisRS(1–60) (constituting the first 60 amino acids (aa)) reacted with anti-Jo-1 Abs, whereas a truncated HisRS lacking the first 60 aa failed to react (7). Interestingly, the first 60 aa of HisRS are encoded by the first two exons of the mRNA of HARS and are absent from HisRSs of prokaryotes and lower eukaryotes. As expected, anti-Jo-1 Abs do not react with Escherichia coli HisRS (9). According to our structural analysis, this small domain (designated as a WHEP domain) forms a helical coiled-coil structure (9). Other work showed that HisRS(1–48) induced migration of CD4+ and CD8+ lymphocytes, IL-2-activated monocytes, and immature dendritic cells. In contrast, HisRS(61–509), which lacks the first 60 aa, failed to stimulate these inflammation-related cell migration events (8). Other in vivo studies in mice suggest that HisRS has an etiological relationship to the disease (10).

Despite the wealth of data on the association of HisRS with anti-Jo-1 Ab in IIM/ILD, the cross-reactivity of splice variants (SVs) with anti-Jo-1 Abs is undefined. In this in mind, we previously identified HisRSCD, a natural HisRS SV that has an internal deletion that ablates the entire catalytic domain (CD) and joins the N-terminal WHEP domain (1–60 residues) to the C-terminal anticodon-binding domain (ABD) (9). The result is a change in both quaternary and tertiary structures. Thus, HisRSCD is a monomer (HisRS is a homodimer) shaped like a dumbbell-like structure, where a flexible linker joins its two ends.

* This work was supported, in whole or in part, by National Institutes of Health Grant CA92577 from NCI and Grant GM88278. This work was also supported by Innovation and Technology Fund of Hong Kong Grants UIM181, UIM192, and UIM199; aTyr Pharma; and an NFCR Fellowship (to Mingjie Zhang and Paul Schimmel) in the form of compensation, stock ownership, or both.

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domains and the ABD has an altered conformation. Although the epitopes were not mapped, HisRS\(\Delta\)CD reacted with anti-Jo-1 Abs from patient sera (9).

Interestingly, we identified another novel HisRS SV in muscle tissue, which we designated as HisRS\(^{\text{WHEP}}\). This SV is composed solely of the first 60 aa of HisRS, which constitute the WHEP domain. It results from a splice event that introduces a stop codon from intron 2. With this discovery, we then set out to investigate whether transcripts for HisRS\(\Delta\)CD and HisRS\(^{\text{WHEP}}\) are up-regulated in patients with IIM/ILD. In addition, we investigated recombinant forms of these variants and their constituent domains for their reaction with anti-Jo-1 Abs from patients. Our results demonstrate that both the expression and cross-reactivity of HisRS\(\Delta\)CD and of HisRS\(^{\text{WHEP}}\) are associated with IIM and therefore support the possibility of extracellular anti-Jo-1 antibody binding to HisRS and its SVs.

**EXPERIMENTAL PROCEDURES**

**PCR Identification of HisRS\(^{\text{WHEP}}\)—** A human skeletal muscle cDNA library was used as a template (Clontech, Palo Alto, CA). PCR was performed with a pair of primers (FP1 (AGTGGACAAGCGGGATGCGAGAGC)/RP1 (GCTTGGAGTCTTCCTGATAG)), and the PCR product was validated by direct sequencing. A color-coded trace from sequencing is presented in supplemental Fig. S1.

**Sample Preparation for Gene Expression Analysis—** All human tissue poly(A)\(^{+}\) RNAs were purchased from Clontech (catalog nos. 636170, 636591, 636128, 636105, 636113, 636119, 636121, 636101, 636118, 636146, 636125, 636162, and 636120). Muscle biopsies from DM patients were kindly provided by the Telethon Network of Genetic Biobanks (Milan, Italy). These samples consisted of 10 muscle biopsies from Caucasian DM patients (including five males and five females). The diagnosis was based on clinical manifestation and histology. Total RNA was isolated from muscle using a PARIS kit (Invitrogen) and was pooled together as the DM group. The control group was pooled total RNA from two healthy Caucasian subjects (including one male and one female; Clontech catalog no. 636534). First-strand cDNAs were synthesized as described previously (9).

**Quantitative PCR and Data Analysis—** Quantitative PCRs (qPCRs) were performed as described previously (9, 11). The qPCR primer sequences were as follows: qFP1, CACGGTGCA- GAAGTCTTGAGT; qRP1, TCCCCATATCTTCCCCATCATG; qFP2, GTGCTCAAAAAACCCCCCAAGTAGAG; qRP2, C- ACAGTGCTACAGCCTCTG; qFP3, ACCCCCAATGTA- AGAGCGA; qRP3, TCTCGCAAGCTGCAATCGT; qFP\(_{\text{MxA}}\), ACTGTAGGCCTATCCAG; and qRP\(_{\text{MxA}}\), TTCAGGAGGACGCTGAGT.

**Detection of HisRS Proteins by Western Blot Analysis—** Total cell lysates (TCLs) of monocytic THP-1 cells and human primary skeletal muscle cells (Cell Application, San Diego, CA) were prepared in 50 mM Tris buffer (pH 8.0) containing 1% Triton X-100 and 5 mM EDTA. TCLs (50 \(\mu\)g) were applied to electrophoresis and subsequent Western blot analysis with anti-HisRS mAb (Abnova, Walnut, CA).

**Quantification of HisRS Levels in Monocytic THP-1 Cells—** The cellular HisRS concentration was determined by standard sandwich ELISA (capture Ab, home-made anti-human HisRS mouse mAb; detection Ab, anti-human HisRS mAb (Abnova), biotinylated in-house). Recombinant human HisRS protein was used as the quantification standard (see below).

**Protein Expression and Purification—** The cDNAs encoding native human HisRS (aa 1–506), HisRS\(\Delta\)CD (aa 1–60 plus aa 405–506), HisRS\(^{\text{WHEP}}\) (aa 1–60), CD (aa 54–398), and ABD (aa 406–506) were cloned into the pET21a vector with a C-terminal His\(_{6}\) tag. From our experience, the C-terminal 3 aa (CIC, aa 507–509) reduce protein homogeneity; thus, these residues were removed in all constructs. The constructs were transformed into E. coli BL21(DE3) cells, and expressed proteins were purified by nickel-nitrioltriacetic acid affinity chromatography and further separated by size-exclusion chromatography in 1× PBS buffer with 1 mM DTT. The purity and homogeneity of each protein were checked by analytical size-exclusion chromatography and SDS-PAGE.

**Depletion ELISA—** Anti-Jo-1 autoantibody-positive patient sera were obtained from RDLS Inc. (Los Angeles, CA). A 96-well enzyme immunoassay/radioimmunoassay plate (Corning, Corning, NY) was coated with 50 \(\mu\)l (2 \(\mu\)g/ml) of one of the recombinant proteins (see above) or BSA (as a control) in PBS buffer. After washing and blocking, patient sera containing anti-Jo-1 autoantibodies (in a dilution giving 25% of the maximum effect when applied to a HisRS-coated plate) were added and incubated overnight at 4 °C. After incubation, supernatant was applied to another plate (precoated with the respective recombinant protein) to check the depletion efficiency. The samples with a pre-depletion efficiency of >95% were applied to another plate coated with HisRS for indirect ELISA. The detection Ab was HRP-conjugated goat anti-human IgG (10 ng/well IgG; AbD Serotec, Raleigh, NC). The results were obtained with a FLUOstar OPTIMA instrument (BMG Labtech, Offenburg, Germany).

**Secretion Assay—** Coding sequences for HisRS, HisRS\(\Delta\)CD, and HisRS\(^{\text{WHEP}}\) were cloned into the pCI-neo-2×myc vector (Promega, Madison, WI) through the NheI/NotI restriction sites. These constructs were transfected into HEK293T cells or C2C12 myoblasts using Lipofectamine LTX with PlusTM reagents (Invitrogen) following the manufacturer's instructions. To achieve similar overexpression levels, the DNA constructs of HisRS\(\Delta\)CD or HisRS\(^{\text{WHEP}}\) were transfected at 1 \(\mu\)g for 28 × 10\(^{6}\) cells, whereas that of HisRS was transfected at 0.1 \(\mu\)g. Empty vector was transfected as a control. The transfected cells were split when confluent and plated at 2 \(\times\) 10\(^{5}\) cells/cm\(^2\) in a 60-mm dish. Media were refreshed after 3 h, and both media and TCLs were harvested after another 24 h of incubation. The media were precloned with 5 \(\mu\)l of Dynabeads-protein G (Invitrogen) for 1 h at 4 °C. Anti-Myc polyclonal Ab (1.5 \(\mu\)g; Sigma) was mixed with 5 \(\mu\)l of Dynabeads-protein G in PBS for 1 h at room temperature. The Ab/bead mixture was added to the precloned media and further incubated for 2 h at 4 °C. The protein-Ab-bead complex was washed with radioimmune precipitation assay buffer (12) and eluted with 0.1 M glycine buffer (pH 2.0). The eluent was neutralized by adding 1 M Tris-HCl (pH 8.0; v/v, 10:1). TCLs were prepared in radioimmune pre-
We noted an expressed sequence tag (EST) BP267368 annotation in the University of California Santa Cruz EST database (13). This transcript has a 122-bp insertion of nucleotides from intron 2, located between exons 2 and 3 (supplemental Fig. S1A). Because the intron insertion introduces a stop codon immediately at the end of exon 2, it could, in principle, encode just the WHEP domain of HisRS. To verify this variant, we designed primers that targeted the exon 1 and exon 4 regions of human HARS (Fig. 1A). PCR with a muscle cDNA template and the aforementioned pair of primers yielded a product of 473 nucleotides, which is larger than that expected for the 351-nucleotide transcript that would encode the same region of full-length HARS (Fig. 1B). This product confirmed features of the EST BP267368 annotation. However, in contrast to EST BP267368, our SV had neither a T-to-C substitution in exon 2, which would yield a L56P substitution in HARS, nor a synonymous A-to-G substitution in exon 3 (supplemental Fig. S1, B and C). In addition, our analysis differed in having a synonymous T-to-A substitution in the sequence of the insertion into intron 2. The inserted sequence was flanked by consensus GT-AG splice junctions (Fig. 1C) and created a new exon cassette. We designated this cassette as exon 2B. The transcript
that results from this splice event harbors a canonical start codon, so translation would start at the typical initiator ATG and terminate after exon 2 (Fig. 1C). The consequence is a protein composed of solely the first 60 aa of human HisRS. Because this protein is made up of only the WHEP domain, we named it HisRSWHEP (Fig. 1, C and D).

Expression of Transcripts for HisRSWHEP in 13 Human Tissue Types—We next compared the transcript levels of HisRSWHEP and HARS in 13 human tissue types, which were total leukocytes, bone marrow, spleen, lung, heart, kidney, liver, pancreas, small intestine, colon, thyroid, adipose, and skeletal muscle. The SYBR Green qPCR method was employed. The transcript for HARS was somewhat evenly distributed across all 13 tissue types, deviating no more than 3 times from the median value (Fig. 1E). (Because the transcript for the housekeeping gene (HKG) RPL9 (60 S ribosomal protein L9) is the most evenly distributed among ~20 HKGs, the levels of the HARS transcripts were normalized to that for RPL9.) In comparison, the transcript level of HisRSWHEP was highest in lung (3.5 times above the median level) (Fig. 1E). The transcript levels of HisRSWHEP were below 0.1% of those of HARS. Interestingly, the expression level of HisRSWHEP was low in normal skeletal muscle tissue in comparison with other tissues.

Detection of HisRSWHEP Protein—We used a standard Western blot method to search for the translation product of the HisRSWHEP transcript. For this purpose, a mAb raised against the N-terminal region (aa 1–97) of human HisRS was used. Considering the relatively small amounts of HisRSWHEP mRNA and the difficulty in obtaining adequate amounts of human tissues, human cell lines cultured in vitro were employed. Although not detected in human skeletal muscle cells, the 6.8-kDa HisRSWHEP protein was readily observed in human monocytic THP-1 cells (Fig. 1F, red arrow). Consistent with the relatively low amount of its mRNA, HisRSWHEP was present at a level estimated close to 1% of that of HisRS, which was detected with the same Ab (Fig. 1F, black arrow). We also determined the cellular HisRS level in monocytic THP-1 cells by standard sandwich ELISA (see “Experimental Procedures”). Our results show that the intracellular HisRS level was 0.94 ± 0.17 μM (mean ± S.E., n = 4).

HisRSWHEP Transcript Is Up-regulated in Pool of Muscle Biopsies from DM Patients—Anti-Jo-1 Abs are present in 15–30% of polymyositis patients and 5–10% of DM patients (14). To evaluate the possibility of HisRSWHEP being an antigen in muscle biopsies of patients with IIM/ILD, we examined its mRNA transcript in pooled muscle biopsies from 10 DM patients. (Because of difficulties in defining primers that were sufficiently specific, the transcript for HisRSΔCD was not measured.) Because MXA (myxovirus resistance gene, a type 1 interferon (α/β)) was reported to be up-regulated in myositis (15), its transcript was included as a positive control. Two HKGs, RPL9 and RPS11 (40 S ribosomal protein S11), were also included in our analysis. As a control, we used total RNA from two healthy Caucasian subjects. Relative to the control, the transcript for HisRSWHEP was significantly up-regulated in RNA samples from DM muscle biopsies (2.7 ± 0.2-fold, p < 0.0001) (Fig. 1G). The transcript for native HARS was also up-regulated (2.1 ± 0.3-fold, p < 0.0001) (Fig. 1G).
DISCUSSION

Several previous studies suggest that low-abundant transcripts, which were previously considered as unimportant, are biologically significant in differentiation, metabolism, and phenotypic alternation (21–25). To better understand the expression of HisRSWHEP, we measured the concentration of human HisRS in monocytic THP-1 cells and showed that intracellular HisRS has a concentration of 0.94 \( \pm \) 0.17 M (see above), which is roughly comparable with the reported concentration of methionyl-tRNA synthetase in rabbit reticulocytes (26). On the basis of our estimation that the HisRSWHEP protein is close to 1% of full-length HisRS, we estimate that the cellular content of HisRSWHEP is \( \sim 10 \) nM. Even if only a fraction is secreted, this concentration is well within the range of known dissociation constants (\( K_d \)) for aARS in cell signaling events. For example, the aARS complex-interacting multifunctional protein 1 is reported to bind to CD23 with a \( K_d \) of 4.3 nM (27); glycyl-tRNA synthetase binds to CDH6 with a \( K_d \) of 3.4 nM (16); and a fragment of tyrosyl-tRNA synthetase, known as minitryptosyl-tRNA synthetase, stimulates polymorphonuclear cell migration at 1 nM (28). In addition, these concentrations are higher than the effective concentrations of many cytokines, which are in the picomolar to lower nanomolar range. Thus, in healthy young people (\(<45\) years of age), the serum TNF-\( \alpha \) level is estimated to be \( \sim 0.19 \) pm, IL-6 is estimated to be \( \sim 0.16 \) pm, and MCP-1 is estimated to be \( \sim 16.4 \) pm (29). From this perspective, our results harmonize well with what is known about many other systems.

Novel functions for the WHEP domains in tryptophanyl-tRNA synthetase, glutamylprolyl-tRNA synthetase, and glycyl-tRNA synthetase have been reported previously (30–34). Interestingly, the WHEP domain-containing N-terminal 48-aa
A fragment of HisRS was previously associated with a novel inflammatory function, whereas the residual protein lacking this fragment was inactive (8). Here, we established that two HisRS SVs, unknown at the time of the work of Howard et al. (8) and which each harbor the WHEP domain, are expressed in cultured cells and cross-react with a substantial portion of the anti-Jo-1 Abs from the tested patient population. Both SVs and HisRS can also be secreted. In addition, in a DM patient population undiagnosed as to anti-Jo-1 Ab status, expression of HisRS and at least one of these SVs appears to be up-regulated (Fig. 1G).

Non-translational functions for SVs, natural proteolytic fragments, and even a truncated bipartite synthetase (from the recruitment of a novel stop codon) have been reported for various human tRNA synthetases or synthetase-associated proteins (31, 35–43). These non-translational functions reach into many parts of cell biology and homeostatic mechanisms, including angiogenesis, hematopoiesis, and control of tumor growth. In addition, some of these functions are extracellular and are enabled by the capacity of at least some aARSs to be secreted, as evidenced by their detection in human and mouse sera (16, 17, 44–47). With this in mind, there are suggestions of immunomodulation-related functions, such that aARS fragments have activities that can act to resolve inflammation (48, 49). Thus, in light of the many examples of non-catalytic fragments of aARSs having extracellular functions and given the data presented here showing the reactivity of SVs of HisRS for anti-Jo-1 Abs, the up-regulation of at least one of them in a DM patient population, and their secretion from cultured cells, we propose that these SVs deserve further investigations related to muscle health and the etiology of inflammatory muscle diseases.

Possibly, HisRS and its two WHEP domain-containing SVs are involved in maintaining immune homeostasis in muscle. When immune surveillance or clearance is needed, HisRS proteins attract immune cells to muscle tissue. In support of this hypothesis, the N-terminal WHEP domain of HisRS may be chemotactic for lymphocytes and activated monocytes (8). Possibly because of their persistent presence, in DM patients, the HisRS proteins are eventually seen as “foreigners,” and autoantibodies against HisRS, especially the WHEP domain, are generated. These autoantibodies may antagonize the immune homeostatic role of HisRS proteins and gradually lead to myositis.

Acknowledgment—We thank the Telethon Network of Genetic Biobanks for kindly providing human DM muscle biopsies.

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