Identification of the 49-kDa Autoantigen Associated with Lymphocytic Hypophysitis as α-Enolase

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Lymphocytic hypophysitis is part of the spectrum of organ-specific autoimmune diseases, and although its histopathology is well documented, its pathogenesis is unclear. Serum autoantibodies directed against a 49-kDa cytosolic protein are detected by immunoblotting in 70% of patients with biopsy-proven lymphocytic hypophysitis. Here we report the purification and identification of this first target autoantigen in lymphocytic hypophysitis. The autoantigen has a molecular mass of 49 kDa, a cytosolic localization, and a ubiquitous tissue distribution. The 49-kDa protein was purified from monkey brain and human placental cytosol. Limited amino acid sequencing after proteolytic digestion of the human placental protein showed identity with α-enolase. The identification was confirmed using sera from patients with pituitary autoimmunity, which strongly reacted with recombinant human α-enolase and yeast enolase, but not with rabbit muscle β-enolase. This indicates that the immunoreactive epitopes are largely conserved from yeast to human, but are not present in β-enolase. α-Enolase autoantibodies are not specific to pituitary autoimmune disease and have been reported in other autoimmune diseases. However, this study is the first to indicate a role for α-enolase as an autoantigen in lymphocytic hypophysitis. (J Clin Endocrinol Metab 87: 752–757, 2002)

LYMPHOCYTIC HYPOPHYSITIS is the term used for autoimmune disease of the pituitary, and it is part of the spectrum of organ-specific autoimmune endocrinopathies (1). The disease is characterized by infiltration of the pituitary by immune cells (2, 3) and is often associated with hypopituitarism (4). Approximately 85% of patients are women (5, 6). Over half of the female cases in the literature presented in late pregnancy or within 18 months postpartum (7).

One of the hallmarks of autoimmunity is the presence of autoantibodies that target organ-specific proteins (called autoantigens). Traditional pituitary autoantibody assays using immunofluorescence (8) were problematic (9). Serum pituitary autoantibodies were demonstrated by immunofluorescence in only a few cases of hypophysitis (10, 11). The development of an immunoblotting assay (12) has now enabled the detection of a number of pituitary target autoantigens (1) and their characterization by mol wt. Immunoblotting has detected pituitary autoantibodies in lymphocytic hypophysitis (1), isolated ACTH deficiency (13), and other autoimmune endocrine disorders (14). However, the identity, function, and possible role of these autoantigens are unknown.

Serum autoantibodies directed against a 49-kDa pituitary cytosolic protein were detected in 70% of patients with biopsy-proven lymphocytic hypophysitis and in 55% of patients with the clinical picture of hypophysitis, compared with 9.8% of normal subjects (1). However, a significant percentage of patients with other autoimmune diseases also had antibodies to this protein (1). In addition, 28% of Swedish patients with idiopathic hypopituitarism and 28% of their relatives were found to have the same autoantibody activity compared with 6.8% of controls (15).

We have shown in previous studies that the 49-kDa cytosolic protein is conserved across species. It is present in pituitary tissue from monkeys, sheep, and rats and appears enriched in the mouse AtT20 corticotroph cell line (1). Most major target autoantigens have been shown to be conserved through evolution. In endocrine autoimmunity, they are often tissue-specific enzymes, such as 21-hydroxylase in Addison’s disease (16) and thyroid peroxidase in Hashimoto’s thyroiditis (17). An ideal situation for diagnostic purposes would be if the 49-kDa autoantigen were pituitary specific, enabling the development of a disease-specific pituitary autoantibody assay. However, this is not the case for the 49-kDa protein; it is distributed in all tissues tested with the exception of skeletal muscle (1).

The aim of this study was to purify and identify the 49-kDa cytosolic target autoantigen associated with lymphocytic hypophysitis as a starting point to determine why pituitary autoimmune disease may occur. Monkey brain and human placenta were chosen as tissue sources because of the tissue volume required and their ready availability.

Materials and Methods

Preparation of cytosolic proteins

Cynomolgous monkey brain was obtained in accordance with animal ethics guidelines of the Commonwealth Serum Laboratories (Melbourne, Australia). Human placenta was collected soon after delivery with the informed consent of the donor in accordance with the guidelines of the human research ethics committees of the Hunter Area Health Service and University of Newcastle. Monkey brain and human placental tissues were homogenized in PBS, 250 mM sucrose, and a cocktail...
of protease inhibitors [Roche Molecular Biochemicals (Indianapolis, IN)]. Complete protease inhibitor tablets (two per 50 ml), 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride] using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), followed by a Teflon-glass tissue grinder. The cytosolic fraction was obtained by centrifugation at 100,000 × g at 4°C for 30 min.

**Purification of the 49-kDa target protein**

A carboxymethyl (CM)-Sepharose column ( XK-26, with 150 ml resin, Pharmacia Biotech, Uppsala, Sweden) was linked in tandem to a diethylaminoethyl (DEAE)-Sepharose column (XK-26, with 150 ml resin). Both columns were equilibrated with buffer A (20 mM Tris-Cl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.4). Monkey brain cytosol or human placental cytosol was loaded onto the tandem columns with buffer A at 5 ml/min. The columns were disconnected, and proteins bound to the DEAE column were eluted with a linear gradient of 0–400 mM NaCl (in buffer A) at a flow rate of 5 ml/min. Forty 5-ml fractions were collected. All fractions were assayed for the 49-kDa target protein by immunoblotting, and positive fractions were pooled for further purification.

After this point, monkey brain and human placenta were processed slightly differently, as outlined below.

For the placental preparation the DEAE-Sepharose fraction was next applied to a Poros-Q column equilibrated with buffer A, and the 49-kDa protein was found in the unbound fraction. This fraction was then loaded onto phenyl-Sepharose.

Partially purified fractions of the 49-kDa target protein from DEAE-Sepharose (for monkey brain) or from the Poros-Q column (for human placenta) were applied to a phenyl-Sepharose CL-4B column (5 ml; Pharmacia Biotech) in 20 mM Tris-Cl, pH 7.4, containing 2 mM NaCl. The column was eluted in batches with 150, 100, 50, and 0 mM NaCl in buffer A. A final elution was performed with 1% Triton X-100 in buffer A to strip the column. All batch-eluted fractions were immunoscreened for the 49-kDa target protein by immunoblot.

The Triton-eluted fraction from monkey brain was concentrated by vacuum centrifugation to about 2 ml and applied to a Mono-Q column ( XK5, 5-ml column, Pharmacia Biotech) equilibrated with 10 mM phosphate buffer, pH 7.4. Bound proteins were eluted with a NaCl gradient from 0–200 mM at 1 ml/min. Forty 1.5-ml fractions were collected, and immunoreactive fractions were concentrated to 200 μl using a centrifugal filter concentrator with a 30-kDa molecular mass cut-off (Millipore Corp., Bedford, MA). A silver-stained gel (18) showed a single protein in Mono-Q fractions. Direct N-terminal amino acid sequencing of the purified target antigen was attempted on an automatic amino acid sequencer (model ABS-470A Protein Sequencer with an on-line model 120A PTH analyzer, PE Applied Biosystems, Foster City, CA).

**Microsequencing**

The 49-kDa protein was excised from a Coomassie-stained 15% polyacrylamide gel containing the human placental phenyl-Sepharose fractions (Fig. 3B, lane 7). The protein was digested with trypsin, and the peptides were extracted into trifluoroacetic acid and acetonitrile and separated on a 100-mm column containing the human placental phenyl-Sepharose. The immunoreactive 49-kDa target protein did not bind to CM-Sepharose, or to Poros-Q columns (Fig. 3). Therefore, unlike the monkey brain protein, the human placental protein did not bind to DEAE-resin under these conditions. The unbound fraction as previously described (12).

Western blots of purified enolase preparations

Bakers' yeast enolase and rabbit skeletal muscle enolase were purchased from Sigma (St. Louis, MO). Histone-tagged recombinant human α-enolase (r-α-enolase) expressed from the ENO1 gene product in JM109 (Escherichia coli) cells, was partially purified on a histone-Ni²⁺ affinity column (19), resulting in a 46-kDa protein (Fig. 5A, lane 3). The purified enolase enzymes and human pituitary cytosol were run on SDS-PAGE, transferred to polyvinylidene difluoride, and probed with goat polyclonal antienolase antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The second antibody was rabbit antigoat IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The polyclonal antienolase antibodies are known to recognize α-, β-, and γ-enolase subunits. The different forms of enolase were also probed with normal control sera and sera from patients with lymphocytic hypophysitis known to have pituitary autoantibodies directed against the 49-kDa cytosolic protein.

**Results**

**Purification of the 49-kDa protein from monkey brain cytosol**

The main autoantigen detected by serum autoantibodies in patients with lymphocytic hypophysitis is a 49-kDa cytosolic protein that is not pituitary specific (1). Our initial aim was to purify it from monkey brain cytosol. The 49-kDa protein did not bind to CM-Sepharose (data not shown), but bound to DEAE-Sepharose (Fig. 1). The immunoreactive protein eluted from DEAE-Sepharose between 210 and 270 mM NaCl (Fig. 1A). Normal control serum did not react specifically with this protein (Fig. 1B). The corresponding Coomassie blue stain revealed a 49-kDa protein of comparatively low abundance that correlated with the immunoreactivity (Fig. 1C).

To further purify the antigen, the DEAE fractions were applied to hydrophobic interaction chromatography on phenyl-Sepharose. The immunoreactive 49-kDa target protein did not elute until the column was stripped with 1% Triton X-100 (results not shown). This fraction was applied to a Mono-Q column and was only detected in fractions that passed directly through (Fig. 2A). Silver staining of a 10% polyacrylamide gel run under the same conditions revealed that the target protein was purified to apparent homogeneity, but the total amount of protein in these fractions was very low (Fig. 2B). The concentrated 49-kDa protein sample was loaded directly onto the amino acid sequencer, but no sequence data were obtained, suggesting that it was N-terminally blocked.

**Partial purification of the 49-kDa protein from human placental cytosol**

In parallel experiments, the 49-kDa immunoreactive protein was partially purified from human placenta (Fig. 3). It did not bind to CM- or DEAE-Sepharose, or to Poros-Q columns (Fig. 3, lanes 3–5). Therefore, unlike the monkey brain protein, the human placental protein did not bind to DEAE-resin under these conditions. The unbound fraction...
was then applied to phenyl-Sepharose and bound well. The 49-kDa protein was eluted from phenyl-Sepharose with 500 mM NaCl (Fig. 3A, lane 6).

Despite the fact that the 49-kDa protein purified from monkey brain or human placenta both react with autoantibodies in the same patient's serum, these proteins behaved differently on chromatography. It was important to determine whether they are indeed the same protein. Peptide mapping was used to illustrate the degree of homology between these proteins. Partial proteolysis with V8 protease (also called endoproteinase Glu-C) gave the same distinctive digestion patterns for the 49-kDa protein purified from monkey brain or human placenta (Fig. 4), indicating that they are related, if not identical.
Microsequencing of the 49-kDa placental protein

As the monkey brain and human placental 49-kDa proteins were shown to be similar in size, immunoblot, and peptide mapping, the placental protein was chosen for internal microsequencing. The 49-kDa band in the phenyl-Sepharose fractions enriched for the target protein was excised from a polyacrylamide gel (Fig. 3B, lane 7). Trypsindigested fragments were separated by microbore HPLC, and five peptides were sequenced. Peptide 1 fully matched human α-enolase (residues 16–18, Table 1). Note that it did not match the closely related β- or γ-enolases, both of which differ by two amino acids in this region. Peptides 2–5 contained more than one sequence. To ensure an unbiased database search, the entire sequence data obtained for these peptides was used in a regular expression format to search the nonredundant protein database (using ProteinInfo version 2.0.1 at Proteometrics: www.proteometrics.com). Peptide 2 contained two sequences that matched 100% to human enolase (α-enolase 249–260 and α- or β-enolase 279–290; Table 1) and not to any other protein. Note that the former sequence also matched with a region of shared sequence between these two enolases, which is a form of α-enolase that is truncated after Ile95. This demonstrates that the 49-kDa target protein is α-enolase.

Patient serum reacts to enolase

Finally, we confirmed the identification of enolase and investigated which enolase isoforms were recognized by sera from patients with lymphocytic hypophysitis. Enolase was obtained from three sources: rabbit muscle β-enolase, recombinant human α-enolase, and bakers’ yeast enolase (Fig. 5A). These proteins were probed with antibodies in human sera. Patient serum autoantibodies strongly recognized recombinant human α-enolase and weakly recognized yeast enolase (Fig. 5B). Normal control serum did not strongly recognize any of the three enolase sources or any 49-kDa protein in pituitary cytosol (Fig. 5C). An immunoblot with an antienolase polyclonal antibody that detects α-, β-, and γ-enolase isoforms confirmed the presence of enolase in all lanes (Fig. 5D). These data support the sequence data and suggest that patient sera recognize evolutionarily conserved sequences within α-enolase.

Discussion

This report is the first to identify a target autoantigen in pituitary autoimmune disease. We have identified the 49-kDa cytosolic autoantigen in lymphocytic hypophysitis as the ubiquitous glycolytic enzyme, α-enolase. Initially using monkey brain cytosol, an enriched source of the 49-kDa protein (1), we purified the 49-kDa autoantigen to homogeneity, but direct N-terminal amino acid sequencing failed. In parallel, the autoantigen was purified from human placenta, which expresses many proteins found in the brain and pituitary. It proved to be a particularly abundant source. The proteins from monkey brain or human placenta were shown to be highly related through digestion of the 49-kDa proteins with the same protease, producing an almost identical peptide map. The human placental form of the 49-kDa autoantigen was identified as α-enolase by amino acid microsequencing of two tryptic peptides.

Enolase is a ubiquitous enzyme whose amino acid sequence is highly conserved through evolution (22). Its main recognized role is to catalyze the conversion of phosphoglycerate to phosphoenolpyruvate in glycolysis (23), but it has other functions, which will be discussed below. Mammalian enolase exists as homodimeric or heterodimeric isoenzymes of a combination of three subunits: α-, β-, and γ-enolase. α-Enolase is expressed ubiquitously, β-enolase is predominantly expressed in muscle tissue, and γ-enolase is largely restricted to neuronal and neuroendocrine tissue (24).

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Sequence</th>
<th>Match (human database)</th>
<th>% Identity</th>
<th>Predicted M₀ (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GNPTVEVDLFTSK</td>
<td>GNPTVEVDLFTSK (α-enolase residues 16–28)</td>
<td>100</td>
<td>46–49</td>
</tr>
<tr>
<td>2</td>
<td>[LVY][VGI][ESI][GHP] [MDN][DQI][VLG]A [LDA][LEPS][YEG][FNK]</td>
<td>1) (R)-YISPDQLADLYK (α-enolase residues 279–290)</td>
<td>100</td>
<td>46–49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) (K)-VVIGMDVAASEF (α- or β-enolase residues 249–260)</td>
<td>100</td>
<td>46–49</td>
</tr>
<tr>
<td>3</td>
<td>[AS]GEHNI</td>
<td>(K)-AVEHII (α-enolase residues 70–75)</td>
<td>67</td>
<td>46–49</td>
</tr>
<tr>
<td>4</td>
<td>[VS][AL][TS][ILN][PS] [GKR]</td>
<td>(K)-KDATNVG (α-, β-, or γ-enolase 212–217)</td>
<td>67</td>
<td>46–49</td>
</tr>
<tr>
<td>5</td>
<td>[AS][TV][YP][WF][L][AY][P [LT][LY]</td>
<td>(R)-AAVPSGASTGIY (α- or γ-enolase residues 33–44)</td>
<td>58</td>
<td>46–49</td>
</tr>
</tbody>
</table>

Note that peptide 2 contained three amino acid sequences, represented by the three amino acids (single letter code) enclosed in square brackets. Using regular expression searching of peptide 2 against the non-redundant database the sequence matched two different enolase tryptic peptides. Similarly, peptides 3–5 contained 2 sequences. All the residue numbers listed in brackets refer to the human α-enolase sequence.
FIG. 5. Antibodies in patient sera recognize α-enolase. Enolase from three sources was run on SDS-PAGE and stained with Coomassie blue (A), or immunoblotted with patient sera (B), normal control sera (C), or a polyclonal antibody recognizing all forms of enolase (D). Human pituitary cytosol was used in lane 1, whereas the other lanes contained rabbit muscle β-enolase (lane 2), recombinant human α-enolase (lane 3), or bakers’ yeast enolase (lane 4).

Each subunit consists of 434 amino acids, of which 336 (77%) are identical. As the 49-kDa protein from monkey brain behaved slightly differently on column chromatography compared with human placental α-enolase, this may suggest that the protein in brain could be neuron-specific enolase. Neuron-specific enolase is made up of two γ-enolase subunits (25) and is known to be abundant in brain (24).

Commercial enolase preparations were immunoblotted with sera from patients with lymphocytic hypophysitis to confirm anti-enolase antibody reactivity. Patient sera recognized recombinant α-enolase and yeast enolase, but not rabbit muscle β-enolase. This suggests that the epitope recognized by sera from patients with hypophysitis is not present in β-enolase. Other researchers reported similar findings. Serum from a patient with discoid lupus erythematosus recognized yeast enolase, but not rabbit muscle β-enolase (26). The exclusion of β-enolase as a target autoantigen is further supported by previous immunoblotting assays by our group showing that patient sera did not recognize the 49-kDa target autoantigen in skeletal muscle cytosolic preparations (1). In the current study we were unable to directly test γ-enolase, but subsequently we have shown by two-dimensional gel electrophoresis that sera from patients with peripartum hypophysitis recognize both α- and γ-enolase in pituitary tissue and placenta (27). Yeast enolase shares a high degree of homology with human enolase (22). As a result, antienolase antibodies in our study recognized evolutionarily conserved epitopes in yeast enolase.

Usually, organ-specific autoimmune diseases have tissue-specific enzymes as target autoantigens (28). Classic examples include thyroid peroxidase in thyroid autoimmunity (17) and 21-hydroxylase in Addison’s adrenalitis (16). However, like enolase, other ubiquitous enzymes have been associated with organ-specific autoimmunity. The multimeric enzyme complex, dihydrolipoamide acetyl-transferase, is an autoantigen in primary biliary cirrhosis (29).

Patient serum reactivity to α-enolase is not specific for lymphocytic hypophysitis. The ever-growing list of organ-specific and nonorgan-specific autoimmune conditions associated with autoantibodies to α-enolase includes systemic rheumatic diseases (30), anti-neutrophil cytoplasmic antibody-positive vasculitis and systemic lupus erythematosus with renal disease (31), endometriosis (32), cancer-associated retinopathy (33), discoid lupus erythematosus (26), mixed cryoglobulinemia or arthritis with kidney involvement (34), primary biliary cirrhosis and autoimmune hepatitis (35), primary sclerosing cholangitis (36), inflammatory bowel disease (37), primary membranous nephropathy (38), and, finally, systemic sclerosis and rheumatoid arthritis (39). We have demonstrated anti-49-kDa autoantibodies, which we now recognize as enolase, in patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (40). The presence of anti-α-enolase antibodies in lymphocytic hypophysitis is therefore consistent with the findings of other studies of autoimmunity, but renders their detection nonspecific. There has been no explanation for why α-enolase is an autoantigen in so many autoimmune diseases. It may relate to its other properties. In addition to its role in glycolysis, α-enolase functions as a plasminogen receptor (19, 41), as a controller of cell growth and differentiation by down-regulation of c-myc protooncogene expression (21), as a structural protein in the lens of some species (42), and possibly as a suppressive lymphokine (43).

The plasminogen-binding property of streptococci is implicated in the bacteria’s tissue invasion processes. Studies of pathogenic streptococcal bacteria have shown expression of α-enolase on the bacteria surface, displaying strong plasminogen binding activity (44). Streptococcal antibodies that cross-react with human α-enolase could be part of the molecular mimicry that contributes to acute rheumatic fever and other autoimmunity related to streptococcal infection (45). In lymphocytic hypophysitis, some patients have presented with a clinical picture of meningoencephalitis before the development of a pituitary mass (4). It is not unreasonable to suggest enolase as the link between the infective trigger and the development of autoimmunity.

Although studies have shown that some normal control subjects have circulating tissue-specific autoantibodies [e.g. against thyroid peroxidase (46)], they usually target different epitopes on the autoantigen compared with patient sera. In one study of cancer-associated retinopathy, one epitope of α-enolase recognized by patient sera caused more in vitro cytotoxicity and apoptosis of retinal cells than the sera of control subjects (47). Similarly, epitope mapping could determine specific regions of the enzyme that lead to pathogenic antienolase reactivity against the pituitary in lymphocytic hypophysitis patients.

In summary, this study identifies the 49-kDa autoantigen in lymphocytic hypophysitis as α-enolase. Although not disease specific, it is now clear that these autoantibodies are
common markers of autoimmune disease. They may be helpful in the diagnosis of patients with a clinical picture of lymphocytic hypophysitis who would otherwise not need pituitary biopsy.

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