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Inhibition of the catalytic activity of human transaldolase by antibodies and site-directed mutagenesis

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Abstract Transaldolase is a key enzyme of the pentose phosphate pathway. While antibody (Ab) 169, directed against the N-terminal 139 residues of human transaldolase (TAL-H), had no effect on enzyme activity, Ab 12484 raised against full length and functional recombinant TAL-H inhibited catalytic activity. This tentatively mapped the catalytic site to the C-terminal 140–336 amino acid portion of TAL-H. Dihydroxyacetone transfer reactions catalyzed by transaldolase depend on Schiff base formation by a lysine residue. Replacement of lysine-142 by glutamine using site-directed mutagenesis resulted in a complete loss of enzyme activity, suggesting that lysine-142 is essential for the catalytic activity of TAL-H.

Key words: Transaldolase; Antibody-mediated inhibition; Site-directed mutagenesis

1. Introduction

The pentose phosphate pathway (PPP) enzyme transaldolase catalyzes the transfer of a C_3 fragment corresponding to dihydroxyacetone from sedoheptulose 7-phosphate to glyceraldehyde 3-phosphate forming erythrose 4-phosphate and fructose 6-phosphate. PPP fulfills two essential functions, formation of ribose 5-phosphate for synthesis of nucleotides and generation of NADPH for biosynthetic reactions and to maintain glutathione at a reduced state, thus, to protect sulfhydryl groups and cellular integrity from oxygen radicals [1]. Importance of PPP has been documented in host defense mechanisms in all tissues against oxidative stress [1], in embryogenesis [2], neurulation [3], myelination [4], inflammation [5–8], lymphocyte activation [9,10], phagocytosis [6,8], cardiac arrhythmias [11], resistance to radiation of malignant tumors [12]. We recently demonstrated that transaldolase is expressed selectively in oligodendrocytes at high levels, possibly linked to production of large amounts of lipids as a major component of myelin and to the protection of myelin sheaths from oxygen radicals and that high-affinity autoantibodies to human transaldolase (TAL-H) are present in serum and cerebrospinal fluid of patients with multiple sclerosis (MS) [13].

The active site of transaldolase of *Candida utilis* includes seven amino acids around a lysine residue that mediates Schiff base formation during the dihydroxyacetone-transfer reaction [14,15]. In the yeast *Saccharomyces cerevisiae* replacement of a single lysine residue at position 144 of yeast transaldolase (TAL-Y) resulted in a complete loss of enzyme activity [16]. Other residues in the active site did not appear essential for function of TAL-Y [16]. In the present study, we demonstrate that antibody 169, raised against a 139 amino acid long N-terminal domain of TAL-H [17], had no effect on enzyme activity, while Ab 12484, raised against a full length and functional recombinant TAL-H molecule, suppressed enzyme activity. Inhibition by Ab 12484 was reduced in the presence of excess substrate concentrations, suggesting that an interaction with the catalytic site of TAL-H may be responsible, at least in part, for antibody-mediated inhibition of enzyme activity. Using amino acid replacement by site-directed mutagenesis, we show that lysine-142 is critical for catalytic activity of human transaldolase (TAL-H).

2. Materials and methods

2.1. Prokaryotic expression of recombinant protein

Full-length TAL-H protein was expressed as a fusion protein with glutathione *S*-transferase (GST) encoded by pGEX-2T plasmid vector [18]. A *Bgl*II site was generated by polymerase chain reaction mediated mutagenesis immediately 5′ of the first methionine codon of TAL-H cDNA [17]. The open reading frame of TAL-H was amplified by PCR using a set of 5′ (4/2*Bgl*II, 5′-GTA-CTT-AGA-TCT-ATG-TCG-AGC-TCA-CCC-GTG-3′; first methionine codon of TAL-H is underlined) and 3′ (oligodT-18) primers and cloned directly into the pCR-1000 vector (InVitrogen, San Diego, CA). Subsequently, a 1033 nucleotide long *Bgl*II fragment of cDNA clone 4/2–4/1, between nucleotide positions 57 and 1090, respectively, was transferred into the *Bam*HI site of pGEX-2T, immediately downstream of the thrombin cleavage site. Optimum stimulation of expression of the recombinant fusion protein was obtained with 1 mM isopropylthio-β-galactoside (IPTG) after 2 h. TAL-H/GST fusion protein was affinity-purified through binding of GST to glutathione-coated agarose beads as specified by the supplier (Pharmacia). TAL-H protein was cleaved from GST by 1 NIH unit of thrombin (Sigma, St. Louis, MO) in 1 ml of PBS containing 600 μg fusion protein. TAL-H protein was separated from the agarose bead-bound GST by centrifugation.

2.2. Antibodies

Highly specific polyclonal antibodies 169 and 170 directed to the 139 amino acid long N-terminal segment of TAL-H were developed earlier [17]. Antibody 12484 to the functional 336 amino acid long full length recombinant TAL-H was raised in rabbits as earlier described [17]. For enzyme inhibition assays, immunoglobulins were precipitated from sera with 50% ammonium sulfate [19], dialyzed against phosphate buffered saline pH 7.4, and quantified by the Bradford assay [20].

2.3. Testing of transaldolase enzyme activity

Transaldolase enzyme activity was measured by the transfer of the dihydroxyacetone three-carbon unit from the donor D-fructose-6-phosphate, to the acceptor D-erythrose-4-phosphate [21]. Enzyme activity was assayed in the presence of 3.2 mM D-fructose 6-phosphate, 0.2 mM

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Abbreviations: PPP, pentose phosphate pathway; TAL-H, human transaldolase; rTAL-H, recombinant TAL-H; mTAL-H, mutant TAL-H; GST, glutathione *S*-transferase.

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D-erythrose-4-phosphate, 0.1 mM NADH, 10 μg of α-glycerophosphate dehydrogenase/triosephosphate isomerase at a 1:6 ratio in 40 mM triethanolamine, 10 mM EDTA pH 7.6 at room temperature by continuous absorbance reading at 340 nm for 10 min. The assay was conducted in the activity range of 0.001–0.01 units/ml using yeast transaldolase (TAL-Y, Sigma, St. Louis, MO) and, subsequently, rTAL-H as positive controls. For antibody-mediated inhibition assays, 50 ng of rTAL-H in PBS was pretreated with purified immunoglobulins for 30 min at 4°C and tested for enzyme activity.

2.4. Site-directed mutagenesis

A single mutagenic oligonucleotide, in addition to two outer amplification primers, in a single amplification/mutagenesis reaction was utilized. Prior to amplification, the mutagenic oligonucleotide was phosphorylated with T4 polynucleotide kinase (GIBCO-BRL) and, subsequently, incorporated into the amplification product by including a thermostable ligase during PCR [22]. Thus, mutagenic PCR reaction was performed by including a phosphorylated mutagenic oligonucleotide (4/1m, corresponding to positions 467–498 of TAL-H cDNA, 5′-CCG-AAT-TCT-TAT-ACA-GCT-GTC-ATC-AAC-CTG-GG-3′ to achieve an A→C mutation at the underlined nucleotide position 480) with the previously utilized two outer 5′ (4/2*Bgl*II) and 3′ (oligodT-18) amplification primers. PCR reactions included 200 pmol of each of the three primers, 200 μM of each dNTP, thermostable ligase buffer (25 mM potassium acetate, 20 mM Tris pH 7.6, 10 mM magnesium acetate, 0.1% Triton X-100, 10 mM dithiothreitol, 1 mM NAD^+), 5 U of *Taq* DNA polymerase (Perkin-Elmer/Cetus), 40 U of *Taq* ligase (New England Biolabs.), and 1 μg of p4/2–4/1 template DNA [17]. The amplified products were cloned into the pCRII vector (Invitrogen, San Diego, CA). Resultant plasmids were sequenced by the chain termination method [23] and analyzed for the presence of the planned point mutation (at position 480 for mutagenic oligonucleotide 4/1m) and absence of unwanted mutations. Clone $4/1m_2$ with the desired mutation was digested with *Bgl*II restriction enzyme, utilizing recognition sites in the 5′ outer primer and downstream of the polyadenylation signal, and cloned into the *Bam*HI site of the pGEX-2T vector [18]. The mutated TAL-H (mTAL-H) was expressed as a fusion protein with GST as described for wild-type human transaldolase (rTAL-H).

2.5. Western blot analysis

500 ng of recombinant TAL-H protein in 10 μl per well was separated by SDS-PAGE and electroblotted to nitrocellulose [24]. Nitrocellulose strips were incubated in 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20, and 5% skim milk, with antibodies (at a 1000-fold dilution unless indicated otherwise) for 1 h at room temperature. For detection of rabbit antibodies, after washing, the strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN). In between the incubations the strips were vigorously washed in 0.1% Tween-20, 100 mM Tris pH 7.5, and 0.9% NaCl. The blots were developed with a substrate comprised of 1 mg/ml 4-chloronaphthol and 0.003% hydrogen peroxide.

2.6. ELISA

96-well plates were precoated at 4°C with 100 ng of antigen per well in 0.01 M $NaHCO_3$ (pH 9.55). Uncoated sites were blocked with 10% goat serum/0.1% Tween-20 in PBS pH 7.4 at room temperature for 1 h. Subsequently, sera were added to the wells in 10% goat serum/0.1% Tween-20 in PBS. After incubation for 1 h, the plates were washed with 0.1% Tween-20 in PBS. The plates were then further incubated with horseradish peroxidase-conjugated secondary antibodies, washed with 0.1% Tween-20 in PBS, and developed with 2,2′-azino-bis(2-ethylbenzthiazoline-6-sulfonic acid) as earlier described [25].

3. Results and discussion

3.1. Production of fully functional human recombinant transaldolase (rTAL-H)

The full-length TAL-H protein was expressed as a fusion protein with glutathione *S*-transferase (GST) encoded by pGEX-2T plasmid vector. By Western blot analysis of protein lysates, a 66 kDa fusion protein (38 kDa TAL-H+28 kDa GST) was detected using antibody 169 [17]. TAL-H/GST fusion protein was affinity-purified through binding of GST to glutathion-coated agarose beads, cleaved from GST with thrombin, and the TAL-H protein was separated from the agarose bead-bound GST by centrifugation (Fig. 1). The purified full length recombinant TAL-H (rTAL-H) was found to be highly functional in the transaldolase enzyme assay by showing a specific activity of ≥15 U/mg protein. As references, purified yeast transaldolase (TAL-Y) had an enzyme activity of ~12 U/mg protein, while normal human lymphocytes contain 0.015 U transaldolase activity/mg of total protein [17].

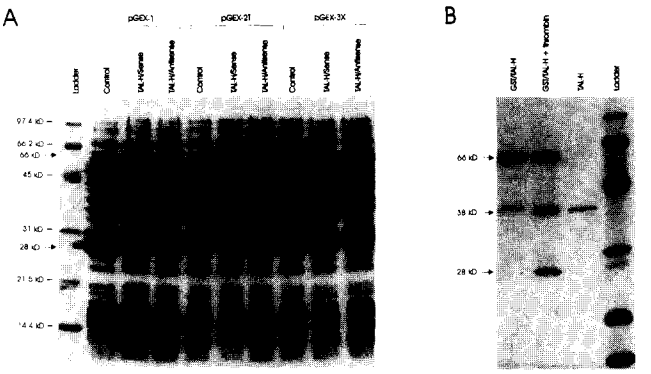

Fig. 1. Expression and affinity-purification of full-length TAL-H protein. (A) SDS-PAGE of protein lysates. A 66 kDa fusion protein containing the 38 kDa TAL-H protein fused to the 28 kDa GST protein was expressed in the pGEX-2T/TAL-H/Sense construct. (B) SDS-PAGE analysis of products after successive steps of purification. The 66 kDa fusion protein was affinity-purified from the total bacterial lysate through binding to glutathion-coated agarose beads (lane GST/TAL-H). The fusion protein was cleaved with thrombin to separate TAL-H from GST. Lane 'GST/TAL-H + thrombin' shows result of proteolytic cleavage. TAL-H protein was separated from the agarose bead-bound GST by centrifugation (lane TAL-H).

3.2. Inhibition of enzymatic activity by Ab 12484 raised against the full length TAL-H protein – lack of inhibition by Ab 169 directed against residues 1–139 of TAL-H

TAL-H-specific rabbit Ab 169 directed to the N-terminal 139 amino acid long segment of TAL-H [17] and Ab 12484, raised against the full length TAL-H protein, were utilized to map functionally relevant domains of TAL-H. Abs 169 and 12484 displayed similar levels of binding affinity to rTAL-H based on a quantitative ELISA assay (Fig. 2A). Pretreatment of rTAL-H with Ab 169 up to a concentration of 1 mg/ml had no effect on enzymatic activity of rTAL-H (Fig. 2B). By contrast, Ab 12484 dose-dependently suppressed enzymatic activity of rTAL-H. Maximal suppression by Ab 12484 was obtained at a concentration of 300 μg/ml. To ensure that the inhibitory effects were due to the presence of antibodies and not other factors, immunoglobulins precipitated from inhibitory sera with 50% ammonium sulfate, were utilized. Preimmune rabbit sera had no effect on enzyme activity under similar conditions.

3.3. Enzyme-inhibitory activity of Ab 12484 is partially reversible by substrate excess

TAL-H activity was routinely measured using fructose 6-phosphate as the dihydroxyacetone donor substrate. Assays were done in the presence of 3.2 mM fructose 6-phosphate which allowed determinations of enzymatic activity at maximal

velocity (Fig. 3). Under these conditions activity of TAL-H was suppressed in the presence of Ab 12484 by as much as 74%. To determine whether antibody-mediated suppression of enzymatic activity involved an interaction with the catalytic site of TAL-H, concentration of fructose 6-phosphate was increased during the enzyme assay. The inhibitory effect of Ab 12484 on transaldolase activity could be partially overcome by addition of excess of fructose 6-phosphate (Fig. 3). Increasing the concentration of fructose 6-phosphate from 3.2 mM to 320 mM augmented activity of Ab 12484-treated TAL-H from 1.9 ± 0.1 to 4.9 ± 0.4 U/mg enzyme protein. These findings indicate that Ab 12484 decreases both affinity of the enzyme for fructose 6-phosphate and the V_{max} (K_{cat}) of the reaction. Based on a lack of inhibition by Ab 169, the catalytic site of TAL-H was tentatively allocated to the C-terminal portion (residues 140–336) of the molecule.

3.4. Lysine 142 mediates catalytic activity of TAL-H

The active site of TAL in *C. utilis* has been mapped to a heptapeptide around a lysine residue which directly mediates Schiff base formation in the dihydroxyacetone transfer reaction

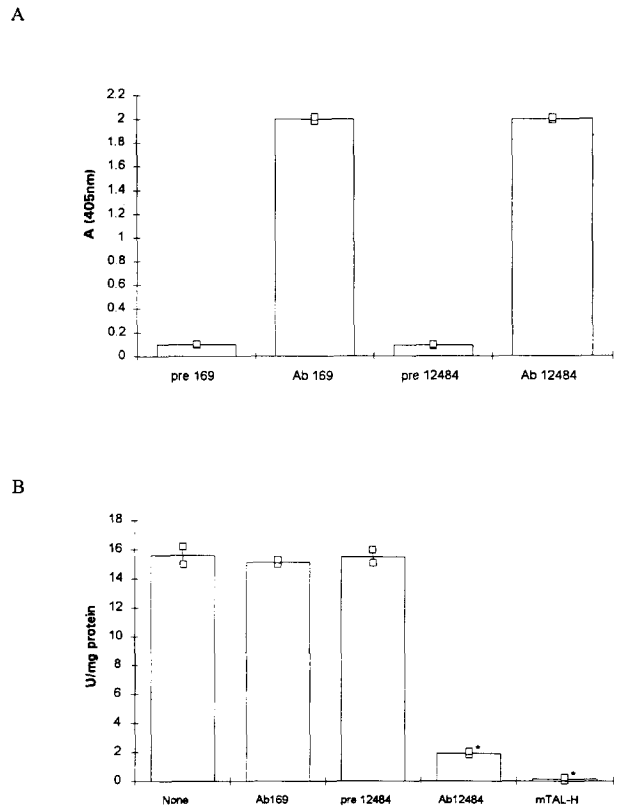

Fig. 2. (A) Binding of preimmune rabbit sera (pre) and TAL-H specific Abs 169 and 12484 to 100 ng of affinity-purified human recombinant transaldolase (rTAL-H) in ELISA. Antibodies were added at a dilution of 1 : 1,000. Values indicate mean ± S.D. of four parallel optical density measurements at 405 nm. (B) Inhibition of enzymatic activity of rTAL-H by Ab 12484. 50 ng of rTAL-H was preincubated with 300 μg of purified immunoglobulins from the indicated sera for 30 min at 4°C in 500 μl of PBS pH 7.4 and assayed for enzyme activity. Values indicate mean ± S.D. of four independent measurements. Enzyme activity of mutant TAL-H with Lys → Gln substitution at position 142 (mTAL-H) was < 1% of that of wild-type rTAL-H. *significant suppression: $P < 0.01$.

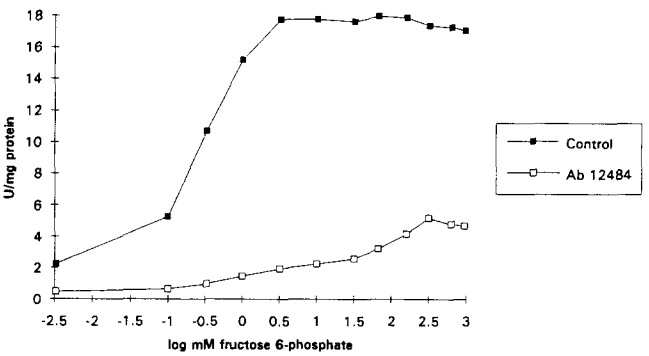

Fig. 3. Effect of excess fructose 6-phosphate substrate on inhibition of transaldolase activity by Ab 12484. Following preincubation without (Control) or with 300 μg of Ab 12484 for 30 min (Ab 12484), enzymatic activity of 50 ng of rTAL-H was measured in the presence of fructose 6-phosphate at the indicated concentrations. Values represent mean of three experiments.

[14,26]. Sequence alignment of active site heptapeptides of TAL from *C. utilis* [14] and *S. cerevisiae* [16] with a corresponding heptapeptide of TAL-H [17] revealed that in addition to the essential lysine residues mediating Schiff base formation with the substrate, the remaining residues are identical or functionally homologous except for substitution of an alanine in both TAL-C and TAL-Y with a serine at position 144 in TAL-H (Fig. 4). Both TAL-Y and TAL-H are rich in lysines. While TAL-H contains 31 lysines, TAL-Y has 36 lysine residues. The overall homology between TAL-H and TAL-Y is 58% [17]. Lysine-144 in TAL-Y is critical for its activity [16] and it corresponds to lysine-142 in TAL-H. To determine the significance of lysine-142 in the catalytic activity of TAL-H, it was replaced by glutamine creating an A→C mutation at nucleotide position 480 in the TAL-H cDNA clone 4/2–4/1 [17]. Absence of unwanted mutations was verified by sequencing the expression vector carrying the mutant cDNA $4/1m_2$ (Fig. 5A). The mutant TAL-H protein (mTAL-H) was expressed in *E. coli* JM101 bacteria as a 66 kDa fusion protein with the 28 kDa GST protein as described for wild-type TAL-H. The fusion protein was affinity-purified from the total bacterial lysate through

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TAL-C IKIASTY
      ||||||:
TAL-Y IKIASTW
      ||:x|||
TAL-H IKLSSTW
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Fig. 4. (A) Sequence homologies between the translated amino acid sequence of cDNA 4/2–4/1 (TAL-H) and the yeast transaldolase protein (TAL-Y). Potential phosphorylation sites are indicated in TAL-H. Calmodulin-dependent protein kinase (Calkin) and cAMP-dependent protein kinase (cAMP-P) sites are underlined with solid lines. Casein kinase II recognition motifs are in italic and underlined. Serine and threonine residues in potential protein kinase C phosphorylation sites are marked with asterisks. A lysine (K) residue at position 144 that has been shown to be essential for function of TAL-Y, corresponding to position 142 in TAL-H, is bold-faced and underlined. (B) Sequence alignment of the active sites in TAL from *C. utilis* (TAL-C), *S. cerevisiae* (TAL-Y), and man (TAL-H). Lysine residues (K) potentially involved in Schiff base formation with the substrate are underlined. Identical (|), functionally homologous (:), and functionally dissimilar (x: A, neutral/hydrophobic versus S, neutral/polar) residues are indicated.

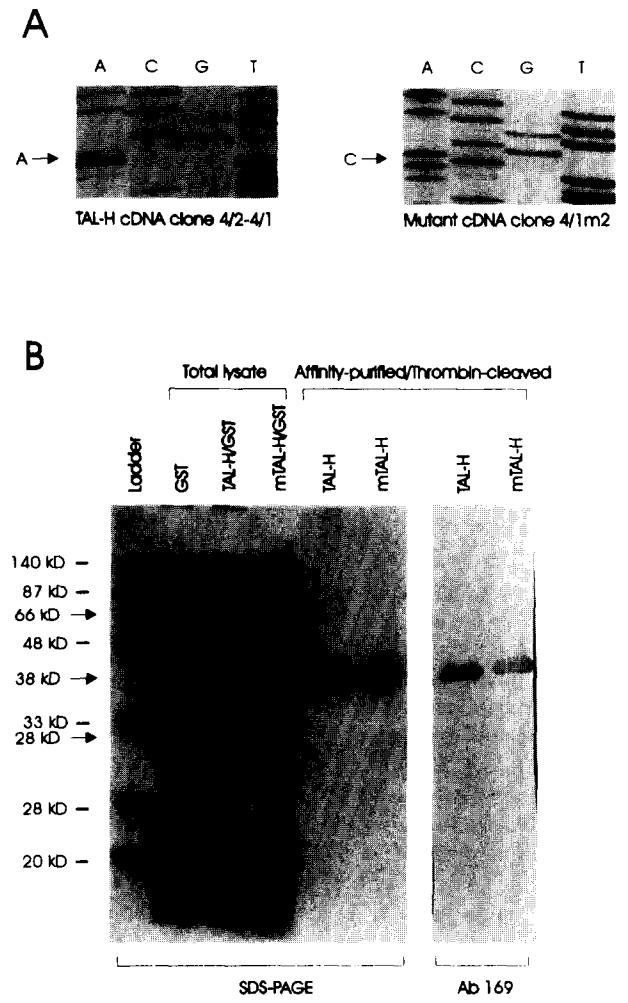

Fig. 5. Site-directed mutagenesis of TAL-H. (A) Nucleotide sequence of wild-type TAL-H cDNA clone 4/2–4/1 and of mutant clone $4/1m_2$. Lysine-142 of TAL-H (AAG triplet) was replaced by glutamine (CAG triplet) by creating an A → C mutation at nucleotide position 480 of TAL-H cDNA clone 4/2–4/1. (B) SDS-PAGE and Western blot analysis of mutant TAL-H protein (mTAL-H). mTAL was expressed and affinity-purified as described for wild-type TAL-H. Lanes for SDS-PAGE contained 40 μg of total lysate or 500 ng of purified protein. Lanes for Western blot contained 100 ng of purified protein which was detected by Ab 169 at a dilution of 1:5,000.

binding to glutathione-coated agarose beads and mTAL-H was cleaved from GST with thrombin. Subsequently, mTAL-H was separated from the agarose bead-bound GST by centrifugation. Authenticity of mTAL-H was verified based on its molecular weight and immunoreactivity with TAL-H specific Ab 169 (Fig. 5B). Replacement of the lysine residue at position 142 in TAL-H resulted in a complete loss (> 99% suppression) of enzyme activity (Fig. 2B). No difference was noted between the wild-type or mutant TAL-H in reactivity with Ab 12484, suggesting that multiple epitopes outside the catalytic domain of the molecule are recognized by these antibodies.

High-affinity antibodies to TAL-H are present in patients with MS [13]. Catalytic activity of wild-type rTAL-H (50 ng) was also inhibited in the presence of 300 μg/ml purified immunoglobulins from MS patients ALV and LAK, by 37% and 26%, respectively (not shown). These data demonstrated that autoantibodies from patients with MS recognized not only the immunoblotted full-length TAL-H protein and its N-terminal fragment [13] but functionally relevant C-terminal epitopes as well. The pathological significance of inhibition of transaldolase activity by autoantibodies is unknown at this time, but capture of TAL-H by immunoglobulins on the surface of B lymphocytes may play an important role in presenting TAL-H to T cells. In fact, activated B cells, by virtue of antigen capture on their surface immunoglobulins, are particularly efficient presenters of low abundance antigens [27]. Proliferation of T cells was stimulated by TAL-H in both patients (LAK, stimulation index, SI = 3.1 [13]; ALV, SI = 4.6, not shown). The identification of enzyme inhibitory autoantibodies capable of binding to native TAL-H may provide an example of how specific autoantibodies can influence autoimmune T cell responses in patients with MS.

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