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Journal of Autoimmunity 26 (2006) 215–222

journal of autoimmunity

www.elsevier.com/locate/issn/08968411

Monoclonal antibody 76F distinguishes IA-2 from IA-2β and overlaps an autoantibody epitope

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Received 23 September 2005; revised 22 December 2005; accepted 23 December 2005

Abstract

IA-2 and IA-2β are highly related proteins that are autoantigens in type 1 diabetes, and provide a model for developing reagents and assays that distinguish similar proteins with unique autoantibody epitopes.

Monoclonal antibodies (mAb) to IA-2 and IA-2β were prepared and tested for their ability to bind to the related proteins and their ability to compete for specific autoantibody epitope binding by sera from patients with type 1 diabetes.

Monoclonal antibodies that specifically bound IA-2 (76F) or bound both IA-2 and IA-2β (A9) were isolated and characterized. 76F mAb recognized IA-2 of human, rat and mouse origin in native and denatured forms and had an epitope specificity for residues 626–630 (FEYQD) which are found in the juxtamembrane (JM) region of human and mouse IA-2, but not IA-2β. This region overlaps with the autoantibody epitope JM2. Binding to the 76F monoclonal antibody was specifically inhibited by sera with antibodies to the JM2 epitope but not with antibodies to the adjacent JM1 epitope, indicating that unique epitopes can be distinguished by this approach.

76F mAb has the unique property to distinguish between the two closely related autoantigens IA-2 and IA-2β by targeting an IA-2 specific epitope of the juxtamembrane region. The findings define an approach to develop assays for specific antibody epitope measurements which may be relevant for disease prognosis and monitoring intervention therapies.

Keywords: IA-2; Type 1 diabetes mellitus; Monoclonal antibody; Autoantibodies

1. Introduction

The protein tyrosine phosphatase (PTP)-like insulinoma antigen 2 (IA-2), also known as ICA512 (islet cell autoantigen 512), PTPN and PTP35, is a 979 amino acid transmembrane protein expressed within secretory granules of brain and neuroendocrine cells including pancreatic islet β cells [1]. The full length protein is cleaved to yield a 65 kDa mature protein form that contains a lumenal domain, and an intracellular cytoplasmic domain characterized by a juxtamembrane (JM) region of around 100 amino acids prior to a single PTP-like domain of around 300 amino acids [2–4]. IA-2 is thought to be involved in the regulation of secretory granule exocytosis. It links secretory granules to the cytoskeleton via the PDZ

Abbreviations: mAb, monoclonal antibody; JM, juxtamembrane; T1DM, type 1 diabetes mellitus; TBS, Tris-buffered saline.

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doi:10.1016/j.jaut.2005.12.001

(protein domain named for PSD-95, Disc large and Zo-1) domain of β2-syntrophin [5] and upon stimulation of insulin secretion is released through cleavage of its cytoplasmic domain, thereby allowing the mobilization of secretory granules to the cell surface [6]. Further supporting a role for IA-2 in insulin secretion, IA-2-deficient mice that were generated by targeted disruption of the mouse IA-2 gene showed alterations in glucose tolerance tests and insulin secretion [7].

IA-2 has a homologue known as IA-2β or phogrin [8]. Targeted disruption of IA-2β also results in alterations in glucose tolerance [9]. Both IA-2 and IA-2β are also major targets of autoantibodies in type 1 diabetes mellitus (T1DM) [2, 10–13]. Autoantibodies bind to the intracellular domain of the proteins and predominantly to epitopes that are shared between the two molecules, or unique to IA-2. Autoantibody cross-reactivity between the two molecules is likely to be due to the high degree of homology within their cytoplasmic domains [14–20]. Despite this, it is uncertain whether IA-2β shares all of the functional characteristics reported for IA-2 [1,5,6,21] and there is a need to distinguish IA-2 from IA-2β in order to correctly identify their functions and roles in the pathogenesis of T1DM. Here we report the generation of a mouse monoclonal antibody that distinguishes expression of the two proteins. The antibody also has a unique property of binding to amino acids also recognized by IA-2 specific autoantibodies and was therefore used in a proof of principle autoantibody epitope specific assay.

2. Materials and methods

2.1. Generation of monoclonal antibodies

Six-week-old BALB/c mice received four weekly intraperitoneal injections with incomplete Freund's adjuvant plus 100 μg of recombinant human IA-$2_{389-979}$ produced in *Escherichia coli* and purified by histidine trapping [12]. IA-$2_{389-979}$ includes all residues expressed in the mature IA-2 protein. Four days after the last injection, mice were killed, and splenocytes isolated and fused. Hybridomas were screened with a radiobinding assay [15] and positive hybridomas cloned by limiting dilution. Nine IA-2 reactive clones were obtained. Five of these bound the intracellular domain of IA-2 and 4 bound the extracellular domain of IA-2. The five intracellular domain-binding clones were able to compete with each other for IA-2 binding, indicating that they bound a similar epitope. Similarly, the extracellular domain binding antibodies also competed with each other for IA-2 binding. The intracellular domain binding antibody 76F was used for characterization in this study. A second set of hybridomas were generated by injecting 6-week-old non-obese diabetic mice with incomplete Freund's adjuvant plus recombinant IA-$2\beta_{741-1033}$ following the same protocol. IA-$2\beta_{741-1033}$ includes most residues found in the intracellular IA-2β protein and all known epitopes of IA-2β autoantibodies. Two IA-2β reactive hybridomas were cloned. A9 was used for characterization in this study.

2.2. Western blotting

Tissues or cultured cells were gently homogenized in lysis buffer (50 mM Tris, pH 7.5, 10 mM sodium phosphate, 150 mM sodium chloride, 1% Triton X-100, 5 mM EDTA, 10 mM sodium chloride, 5 mM iodoacetate, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and incubated for 30 min on ice. The lysate was centrifuged at $10{,}000 \times g$ for 20 min to remove insoluble material and the protein concentration determined. Aliquots of tissue lysates representing 10 μg of protein or 10 ng of recombinant protein representing the juxtamembrane, cytoplasmic and the PTP-like domain of IA-2 were separated by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS) containing 0.05% Tween-20 and 2% skimmed milk powder, then incubated with tissue culture supernatants containing 76F mAb at 1/10 dilution. After washing in TBS–0.05% Tween-20, antibody binding was visualized with a horseradish peroxidase-conjugated anti-mouse IgG (Sigma, Poole, UK) using a chemiluminescent substrate (ECL, Amersham Pharmacia Biotech, Amersham, UK).

2.3. Immunohistochemistry

Sensitive detection of IA-2 was achieved by immunoperoxidase labelling with signal amplification using streptavidin-biotin complexes as previously described [22]. Paraffin sections 5–6 μm thick of rat pancreas were dewaxed and rehydrated for immunohistochemistry. Endogenous peroxidase was blocked by treatment with 0.3% hydrogen peroxide in methanol and sections treated with normal swine serum (20% in TBS). Sections were then incubated overnight at 4 °C with 76F monoclonal (mAb), or polyclonal antibodies to insulin (ICN, Thame, UK) or glucagon (Sigma, Poole, UK). Binding of 76F was visualized by incubation with the appropriate second antibody and the peroxidase-labelled streptavidin method with 3,3′-diaminobenzidine substrate using a commercial kit (LSAB Plus, Dako, Cambridge, UK). Sections were then counterstained with haematoxylin, dehydrated, and mounted for microscopic inspection. Consecutive sections labelled with antibodies to insulin and glucagon were incubated with the second primary antibody (to insulin or glucagon) for 1 h at room temperature, washed, and antibody binding detected with FITC-conjugated anti-guinea pig or anti-rabbit immunoglobulin (Stratech, Luton, UK).

For intracellular staining of cell lines, βTC3 cells were plated on 24-mm poly-L-lysine-coated glass coverslips, grown for 2 days and fixed for 20 min with 3% paraformaldehyde in PBS. After quenching of autofluorescence in 15 mM glycine the cells were permeabilized for 4 min in 0.1% (w/v) Triton X-100 in PBS and then incubated for 10 min in 0.2% (w/v) gelatin in PBS to prevent non-specific antibody binding. Primary antibodies (guinea pig anti-Ins 1:600 (DAKO); 76F or A9 hybridoma supernatant diluted 1/2 in 0.2% (w/v) gelatin, 16.5% (v/v) goat serum, 0.3% (Triton X-100, 40 mM phosphate buffer, 0.45 M NaCl) were applied for the appropriate