NIH Public Access

Author Manuscript

Clin Immunol. Author manuscript; available in PMC 2012 July 24.

Published in final edited form as:
Clin Immunol. 2008 June ; 127(3): 366–374. doi:10.1016/j.clim.2008.01.018.

Selective Screening of Secretory Vesicle-Associated Proteins for Autoantigens in Type 1 Diabetes: VAMP2 and NPY are New Minor Autoantigens

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Abstract

The four major autoantigens (IA-2, I-2β, GAD65 and insulin) of type 1 diabetes are all associated with dense core or synaptic vesicles. This raised the possibility that other secretory vesicle-associated proteins might be targets of the autoimmune response in type 1 diabetes. To test this hypothesis 56 proteins, two-thirds of which are associated with secretory vesicles, were prepared by in vitro transcription/translation and screened for autoantibodies by liquid phase radioimmunoprecipitation. Two secretory vesicle-associated proteins, VAMP2 and NPY, were identified as new minor autoantigens with 21% and 9%, respectively, of 200 type 1 diabetes sera reacting positively. These findings add support to the hypothesis that secretory vesicle-associated proteins are particularly important, but not the exclusive, targets of the autoimmune response in type 1 diabetes. Selective screening of the human proteome offers a useful approach for identifying new autoantigens in autoimmune diseases.

Keywords

autoantibodies; autoantigens; GAD65; IA-2; protein tyrosine phosphatase; proteome; secretory vesicles; type 1 diabetes

INTRODUCTION

In the past, screening methods used to identify autoantigens varied widely and in many cases autoantigens were discovered by chance [1]. The human genome makes it possible to

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prepare thousands of proteins and to screen them for autoantigens by their reactivity with sera from patients with autoimmune diseases. Massive high throughput screening, however, is still at a very early stage [2]. An alternative approach is to select a limited number of candidate proteins and screen them with a sensitive liquid phase radioimmunoassay. In the case of type 1 diabetes, proteins associated with secretory vesicles are of particular interest since the major type 1 diabetes autoantigens (i.e., IA-2, IA-2β, GAD and insulin) are associated with secretory vesicles or their pathways [3–9].

In the past, new autoantigens have been difficult to identify because of insensitive methods and denatured antigens, especially when evaluated by methods such as Western blot or solid phase assays (e.g., ELISA). This is a particularly important issue since most autoantibodies react with conformational epitopes [10,11]. These earlier methods are now being replaced by liquid phase radioimmunoprecipitation assays using recombinant proteins [3,4] which avoid some of the earlier problems. In these latter assays the proteins are radiolabeled making sensitive quantitation possible, prepared as recombinant molecules by in vitro transcription/translation thereby decreasing the presence of irrelevant molecules found in many antigen preparations, and assayed in liquid phase to decrease the likelihood of denaturation.

In the present study, 56 recombinant proteins including 37 associated with secretory vesicles or their pathways, were screened for autoantigens using a liquid phase radioimmunoprecipitation assay with a panel of sera from newly diagnosed patients with type 1 diabetes and normal controls.

RESEARCH DESIGN AND METHODS

Preparation of radiolabeled recombinant proteins

For the screening assays, DNA sequences of selected proteins were obtained from the GenBank (http://www.ncbi.nih.gov/Genbank/). Coding regions of the proteins were amplified by PCR from a brain cDNA library or from expressed sequence tags with sequence-specific forward primers containing both ATG and T7 promoter and sequence-specific reverse primers containing a stop codon sequence and a poly-A tail. In some cases, large molecules were divided into two overlapping fragments (e.g., TOP2, (5′), TOP2, (3′)). Each PCR product was confirmed by sequence analysis. PCR-generated cDNA then was used to prepare ^{35}S-methionine-labeled proteins (Amersham, Arlington Heights, IL) by an in vitro transcription/translation system (TNT T7 Quick for PCR DNA; Promega, Madison, WI). Each translated protein was evaluated for expected molecular mass by SDS-PAGE and then used directly in a liquid phase radioimmunoprecipitation assay. In the screening procedure used here, the time consuming step involved in inserting each of the cDNAs into a vector was avoided. For the validation assays, the coding regions of VAMP2 (vesicle-associated membrane protein 2) and NPY (neuropeptide Y) were amplified by PCR from a brain cDNA library with sequence-specific primers containing restriction endonuclease recognition sites. Each PCR product was cloned into pGBKT7 vectors (Clontech; Mountain View, CA). The constructs then were verified by DNA sequencing and used to prepare ^{35}S-methionine-labeled proteins by an in vitro transcription/translation system. Radioimmunoprecipitation assays were performed as described.

Serum samples

Sera from newly diagnosed patients with type 1 diabetes that were assayed in one of our laboratories (S.A.I) as part of an earlier unrelated protocol for autoantibodies to IA-2 and GAD65 were used in the present study. For the screening study, fifty sera that were single or double autoantibody-positive (31 males, 19 females: age range, 4–19) were selected and divided randomly into two panels, each containing 25 sera. Because there were insufficient sera from any one subject to test all 56 recombinant proteins, approximately one-half of the proteins were tested with sera from each of the panels. Some proteins were screened with sera from both panels. Sera from 25 non-diabetic subjects (18 males, 7 females: average age, 12) that were negative for autoantibodies to IA-2 and GAD65 served as controls. For the confirmation study, 200 sera from patients with type 1 diabetes that had been screened for autoantibodies to IA-2, GAD65 and insulin were used. Sera from 200 age-matched non-diabetes subjects served as controls.

Liquid phase radioimmunoassays

In vitro translated radiolabeled proteins (approximately 20,000 cpm of trichloroacetic acid precipitable protein) were incubated with 5 μl of serum overnight at 4 °C on a rotating platform in 100 μl of Tris-buffered saline/Tween 20 (TBST; 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% BSA, 0.15% Tween 20). The reaction mixtures then were transferred to a MultiScreen-DV 96-well filtration plate (Millipore, Burlington, MA). Fifty μl of 35% protein A Sepharose (Amersham Bioscience, Uppsla, Sweden) in TBST then was added to each well, incubated for 45 min at 4 °C and washed eight times with cold TBST using a Millipore vacuum-operated 96-well plate washer (Millipore). After washing, 50 μl scintillation liquid (Research Product International, Mount prospect, IL) was added to each well and precipitated counts were determined directly with a 96-well plate MicroBeta counter (PerkinElmer Life and Analytical Sciences, Boston, MA). For the confirmation studies, positive and negative control sera were included on each plate, and the antibody levels were expressed in arbitrary units (AU) defined as: [(cpm in the unknown sample − negative control) / (positive control − negative control)] × 100.

Statistical analysis

Mean ± SD of precipitated counts from duplicate wells was determined and the 3SD and 5 SD cutoff points calculated. For most subjects, the coefficient of variation (CV) from duplicate samples was within 20%. When CV was 50% or greater in the screening study or 25% or greater in the validation study, the data were either discarded or the assay repeated. In the screening study to exclude chance positivity, a serum was considered antibody positive for a particular protein only when the precipitated counts were 5 SD or greater above the mean of the controls. In the confirmation study a serum was considered autoantibody positive when the AU was 2SD or greater above the mean of the nearly 200 control sera. Frequency of antibody positivity in diabetes and control groups was compared using Fisher's exact test.

RESULTS

Fifty-six different recombinant proteins (Table 1) were screened for reactivity with type 1 diabetes sera. Thirty-seven of these proteins are associated with secretory vesicles or their pathways, two are major autoantigens in type 1 diabetes, ten are putative, but not validated, type 1 diabetes autoantigens, six are members of the PTP family and five have other functions. After PCR amplification, the length of each PCR product was determined on agarose gel and its sequence compared to that in the Genbank (Table 1). The PCR products then were used to make proteins in an in vitro transcription/translation system and the protein size was determined and compared to the expected size (Table 1). Fifty-six of 74 initially prepared PCR products gave proteins of the expected size and were used to screen for autoantibodies with panels of approximately 24 diabetes and 23 control sera. The data in Figure 1 show, as proof of principle, that if IA-2 and GAD65 had not previously been identified as autoantigens they would have been readily identified as autoantigens by the screening procedure used here. All of the diabetes sera reacted with IA-2 and 75% with GAD65 at the 5 SD cutoff.

Ten putative minor autoantigens also were screened. Three of these 10 proteins (CPE, Sox13 and TOP2) were reported to react with autoantibodies [12–16], three (Imogen38, IGRP and S100β) with T cells [17–19] and four (GFAP, HSP70, ICA69 and JunB) with both autoantibodies and T cells [18,20–23]. In our hands (Fig. 1), at 5 SD cutoff, approximately 17% of the diabetes sera reacted with Sox13, similar to the findings from other laboratories [13–15], and 17% reacted with JunB, but none of the diabetes sera reacted with CPE, GFAP, HSP70, ICA69 or TOP2 (5′). Of the three T cell-reactive autoantigens, 4% or less of the diabetes sera reacted with Imogen38, IGRP and S100β. Thus, of the 10 putative, but not previously validated, minor autoantigens, eight did not react with type 1 diabetes sera in this screening study.

Since type 1 diabetes sera react with two members of the PTP family (i.e., IA-2 and the closely related protein IA-2β) [9], six other PTPs were screened. As seen in Figure 2, none of the diabetes sera reacted with PTP-α, PTP-δ or PTP-γ, 4% reacted with LAR and PTP-ζ and 8% reacted with PTP-ρ. Of the five miscellaneous proteins, none of the diabetessera reacted with GCK, GAPDH, Hoxb13 or P-Selectin and only one serum (4%) reacted with GLUT4.

To test the hypothesis that the autoimmune response in type 1 diabetes might be directed not only to IA-2, IA-2β, GAD65 and insulin, but also to other secretory vesicle-associated proteins, we screened 33 proteins that were directly or indirectly associated with secretory vesicles (Fig. 3). Of these 33 proteins, at 5 SD cutoff, 27 showed no reactivity with type 1 diabetes sera, four reacted only with 4% of the diabetes sera and two, NPY and VAMP2, reacted with 25% and 23%, respectively, of the diabetes sera. Thus, based upon the screening protocol, VAMP2 and NPY would fit into the category of potential new candidate autoantigens associated with secretory vesicles.

To confirm the observation that VAMP2 and NPY are new autoantigens in type 1 diabetes, we cloned their cDNA into plasmids and tested nearly 200 sera from patients with type 1 diabetes who previously had been screened for autoantibodies to three of the major diabetes autoantigens: IA-2, GAD65 and insulin. Autoantibodies to VAMP2 were detected in 41 of 194 diabetes patients (21.1%) as compared to 8 of 192 control subjects (4.2%), whereas autoantibodies to NPY were detected in 16 of 181 diabetes patients (8.8%) as compared to 4 of 188 control subjects (2.1%) (Fig. 4). Thus, the frequency of autoantibodies to VAMP2 and NPY were significantly higher in sera from patients with type 1 diabetes than controls ($p<0.0001$, $p=0.017$, respectively), although the dynamic range of the autoantibody response was not as great as with IA-2 or GAD65.

Further analysis of the data revealed a positive correlation between the prevalence of autoantibodies to VAMP2 and autoantibodies to the three major diabetes autoantigens. Approximately 45% of the subjects with autoantibodies to the three major diabetes autoantigens also had autoantibodies to VAMP2 (Table 2), whereas, only 13% and 20%, respectively, of the subjects who were single or double autoantibody-positive also had autoantibodies to VAMP2. Subjects with autoantibodies to the three major diabetes autoantigens also showed a higher prevalence of autoantibodies to NPY (18%), but the trend was not statistically significant.

DISCUSSION

In the current study we evaluated the reactivity of 56 different proteins with diabetes sera. We found that a number of diabetes sera reacted with one or more of the proteins at a level just barely 3 SD above the mean of the controls. Therefore, 5 SD rather than 3 SD seemed to be a more reliable cutoff point for identifying candidate autoantigens in our screening assay. In the literature, well over a dozen different proteins have been reported to be autoantigens in type 1 diabetes [12–32]. Most of these have been described as minor autoantigens, but there has never been a formal definition of what is meant by a minor or major autoantigen. Only for the purpose of categorizing our screening results, we viewed any protein that reacted with 35% or more of the diabetes sera as a potential major autoantigen and any protein that reacted with more than 10%, but less than 35%, of the diabetes sera as a potential minor autoantigen. Proteins that reacted with less than 10% of the diabetes sera on the initial screening were not studied further. Accordingly, VAMP2 [33,34] and NPY [35,36] were viewed as potential autoantigen candidates.

Proof that the screening strategy used here truly can identify new autoantigens was obtained from further studies on approximately 200 diabetes and 200 control sera which showed that the prevalence of autoantibodies to VAMP2 and NPY in sera from patients with type 1 diabetes was 21% and 9%, respectively. Analysis of the data also revealed that the prevalence of autoantibodies to VAMP2 was highest in sera that had autoantibodies to three of the major diabetes autoantigens. This finding provides support for the idea that individuals with multiple autoantibodies are more likely to possess autoantibodies to still undiscovered autoantigens because of a more severe or advanced form of their autoimmune disease. Of particular interest is the fact that VAMP2 is a secretory vesicle membrane protein and NPY a hormone secreted by secretory vesicles. Thus, these two new, but minor, autoantigens and the four known major diabetes autoantigens (i.e., IA-2, I-2β, GAD65 and insulin) together with the recently reported ZnT8 autoantigen [37] are all associated with secretory vesicles or their pathways arguing that secretory vesicle-associated proteins are particularly important, although not the exclusive, targets of the autoimmune response in type 1 diabetes. Why secretory vesicle proteins should be important targets of the immune response in this disease is not known.

Taken together with earlier reports [38–40], our studies suggest that a number of minor autoantigens are associated with type 1 diabetes. These autoantigens might provide additional diagnostic and predictive markers and also might be an explanation for the reported residual beta cell-staining capacity of some diabetes sera after adsorption with IA-2 and GAD65 [3,41]. In addition, minor autoantigens might be an explanation for the occasional ICA positive, but GAD65 and IA-2 autoantibody negative, sera observed in a number of studies [42,43].

Since the sequence of the genes encoding most of the human proteins is now known, thousands of proteins can be readily prepared in recombinant form and screened for autoantigens by incubation with sera from patients with type 1 diabetes. Although proteins placed on solid phase microchips may lend themselves more readily to high through-put screening [2] than proteins in liquid phase assays, the latter is generally more sensitive and less likely to give false positive or negative results. Using this approach, it now should be possible to screen many of the thousands of proteins in the human proteome for autoantigens with sera from each of the 40 or more different autoimmune diseases. Although this proteomic screening approach will not detect autoantigens related to lipids or nucleic acid and may miss proteins resulting from post-translational modifications, it will almost certainly lead to the discovery of new autoantigens and help in characterizing the “autoantigenome” of human autoimmune diseases.

Acknowledgments

We thank Dr. Yasuko Uchigata for providing sera used in the initial screening phase of our study. This research was supported by the Intramural Research Program of the National Institute of Dental and Craniofacial Research, the National Institutes of Health.

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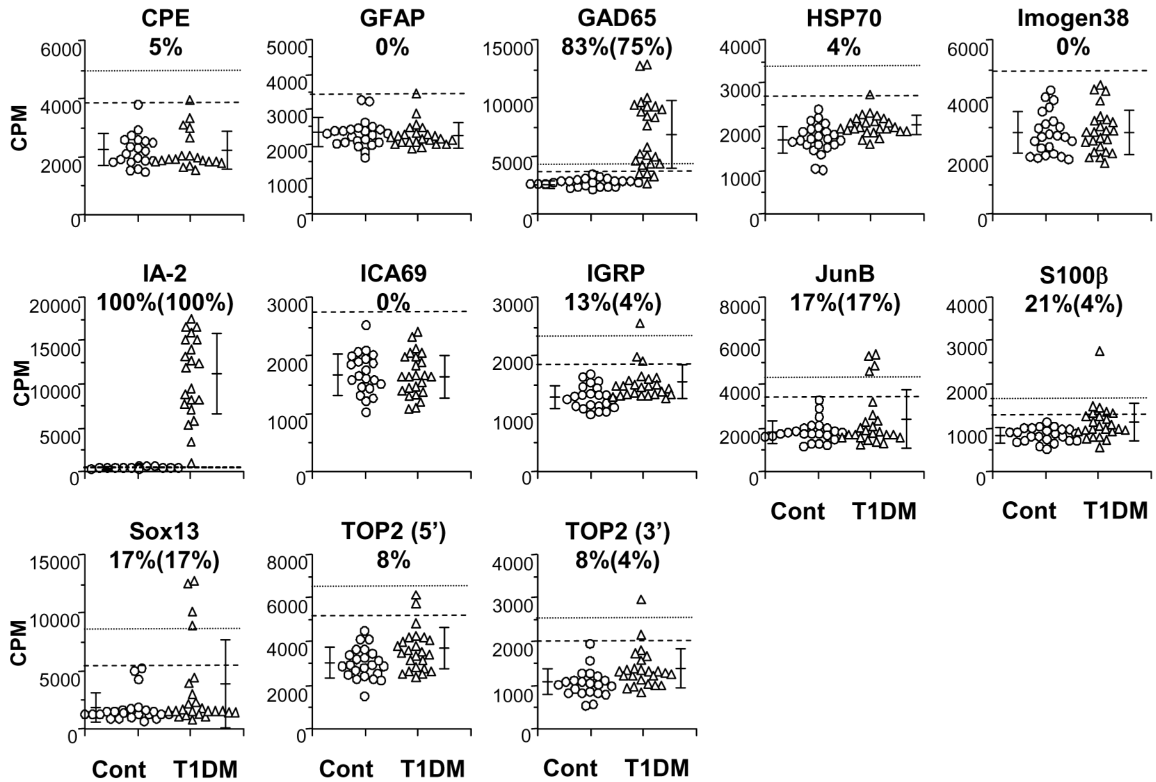

Figure 1.

Percentage of sera reacting with radiolabeled recombinant putative autoantigens. Results are expressed as cpm precipitated. Lower dashed lines and upper dotted lines represent, respectively, 3SD and 5SD above the mean of control sera. Numbers at top of each panel indicate percent of diabetes sera positive at 3 SD (no parenthesis) and at 5 SD (parenthesis). Information about each protein is given alphabetically in Table 1.

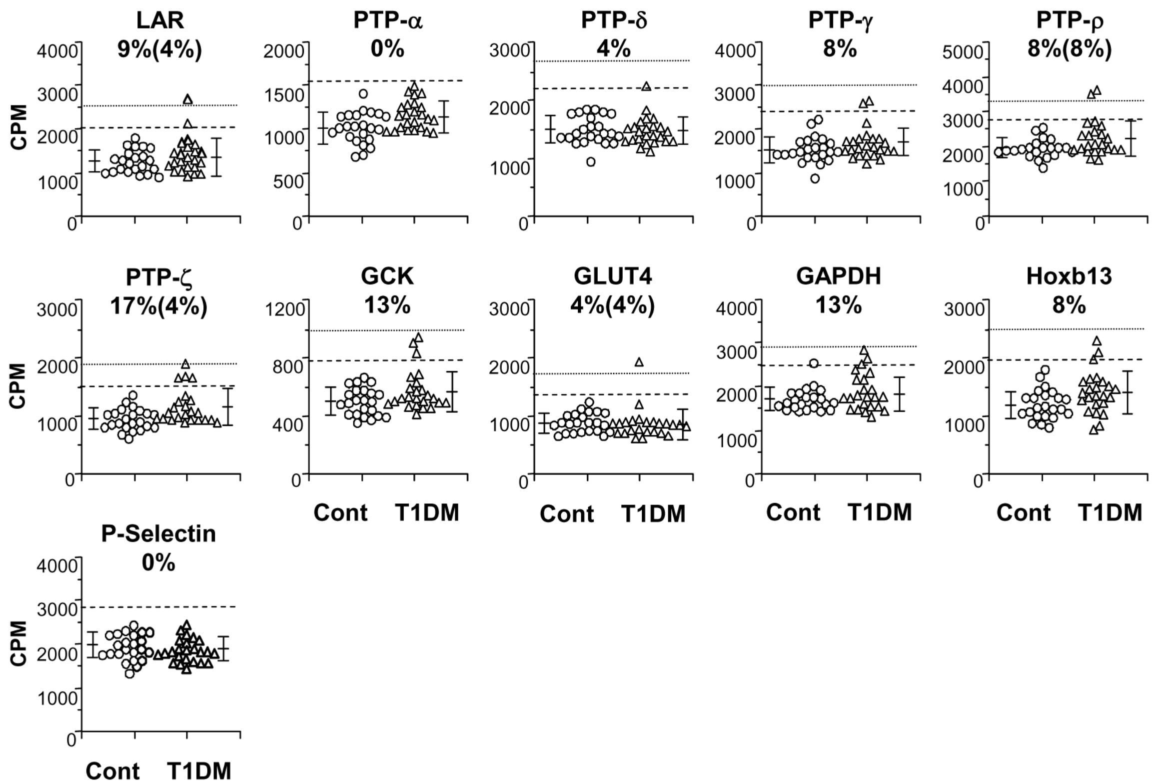

Figure 2.
Percentage of sera reacting with radiolabeled recombinant PTPs and miscellaneous proteins. See legend to Figure 1.

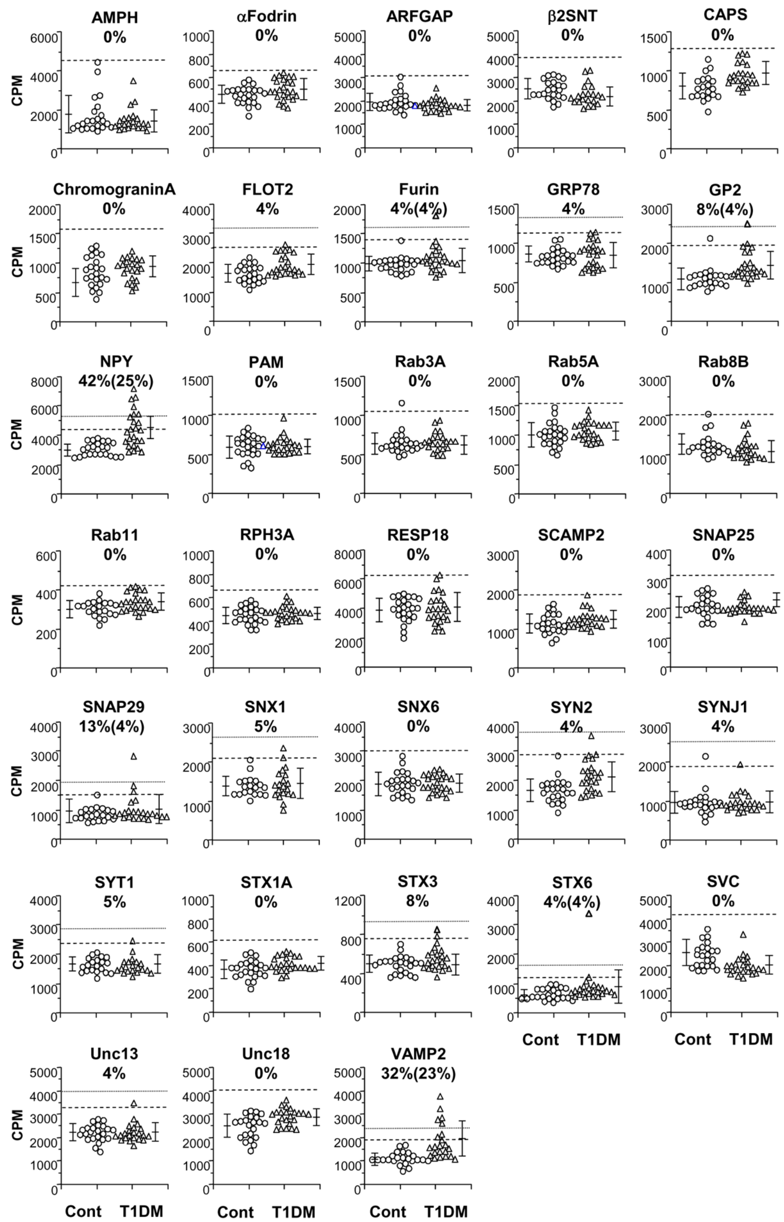

Figure 3.

Percentage of sera reacting with radiolabeled recombinant secretory vesicle-associated proteins. See legend to Figure 1.

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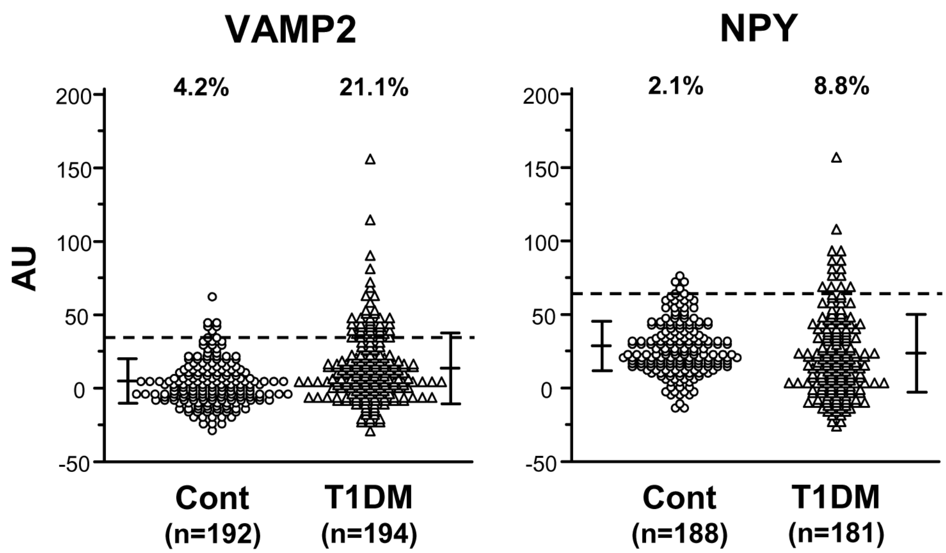

Figure 4.

Percentage of patients with type 1 diabetes who have autoantibodies to VAMP2 and NPY. Results are expressed in arbitrary units (AU). Dashed lines represent 2SD above the mean of control sera. Numbers at top of each panel indicate percentage of autoantibody-positive sera.

Table 1

Candidate Autoantigens in Type 1 Diabetes Mellitus

Molecule	Amplified Region (bp)	Predicted Protein Size (kD)	Group[a]	GenBank Ref.
Amphiphysin (AMPH)	115–2195	76.3	S	U07616
α-Fodrin	107–1887	69.6	S	NM_003127
ADP ribosylation factor GTPase activating protein (ARFGAP)	1064–2379	45.0	S	BC062366
β2-Syntrophin (β2-SNT)	585–1571	36.5	S	BC048215
Calcium-activated protein for secretion (CAPS)	107–771	25.8	S	U36448
Carboxypeptidase E (CPE)	559–1718	43.4	S, A	NM_001873
Chromogranin A	199–1574	50.7	S	BC006459
Flotillin (FLOT)	91–1232	41.7	S	BC017292
Furin	78–2387	83.7	S	BC012181
Glial fibrillary acidic protein (GFAP)	15–1311	49.9	M, A	NM_002055
Glucokinase (GCK)	335–1727	52.2	M	M88011
Glucose-regulated protein 78 (GRP78)	260–2151	69.9	S	NM_005347
Glucose transporter type 4 (GLUT4)	159–1672	54.4	M	M20747
Glutamic acid decarboxylase 65 (GAD65)	75–1810	64.8	S, A*	M81882
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	57–1060	36.1	M	BC083511
Glycoprotein 2 (GP2)	59–1201	42.1	S	BC032693
Heat shock protein 70 (HSP70)	182–2100	70.1	M, A	BC002453
Hoxb13	59–906	30.6	M	U57052
Imogen 38	10–1190	45.3	M, A	Z68747
Insulinoma-associated protein 2 ic (IA-2)	1891–3026	42.8	S, A*	NM_002846
Islet cell autoantigen 69 (ICA69)	179–1630	54.7	S, A	L01100
Islet-specific glucose-6-phosphatase subunit-related protein (IGRP)	1–1068	40.6	M, A	NM_021176
JunB	281–1320	35.9	M, A	NM_002229
Leukocyte antigen related protein (LAR)	4077–6064	79.2	P	Y00815
Neuropeptide Y (NPY)	82–377	10.9	S	NM_000905
Peptidylglycine alpha-amidating monooxygenase (PAM)	374–2974	96.3	S	BC018127
Protein Tyrosine Phosphatase α (PTPα)	38–2409	90.6	P	X54130
Protein Tyrosine Phosphatase δ (PTPδ)	3971–5889	73.6	P	L38929
Protein Tyrosine Phosphatase γ (PTPγ)	2328–4414	79.4	P	L09247
Protein Tyrosine Phosphatase ρ (PTPρ)	2499–4561	78.7	P	AF043644
Protein Tyrosine Phosphatase zeta; (PTPzeta;)	5042–7080	77.0	P	M93426
P-selectin	100–2546	89.5	M	NM_003005
Rab3A	145–770	24.7	S	NM_002866
Rab5A	381–1021	23.7	S	NM_004162
Rab8B	96–712	23.6	S	NM_016530
Rab11	54–704	24.4	S	X56740
Rabphilin 3A (RPH3A)	226–2282	76.2	S	NM_014954
Regulated endocrine specific protein 18 (RESP18)	5–558	19.0	S	NM_001007089

Molecule	Amplified Region (bp)	Predicted Protein Size (kD)	Group[a]	GenBank Ref.
S100β	73–351	10.7	M, A	NM_006272
Secretory carrier membrane protein 2 (SCAMP2)	75–1064	36.6	S	NM_005697
Synaptosomal-associated protein 25 (SNAP25)	217–830	23.3	S	NM_003081
Synaptosomal-associated protein 29 (SNAP29)	112–881	29.0	S	NM_004782
Sox13 (SRY-related HMG box) / ICA12	194–1869	62.6	M, A	AF098915
Sorting nextin 1 (SNX1)	198–1576	53.0	S	BC000357
Sorting nextin 6 (SNX6)	34–1256	46.6	S	AF121856
Synapsin II (SYN2)	82–1394	48.7	S	BC051307
Synaptojanin 1 (SYNJ1)	2403–4762	84.7	S	NM_003895
Synaptotagmin I (SYT1)	32–1293	46.3	S	NM_005639
Syntaxin 1A (STX1A)	27–763	28.2	S	BC003011
Syntaxin 3A (STX3A)	195–1057	33.2	S	NM_004177
Syntaxin 6 (STX6)	188–936	28.5	S	NM_005819
Synaptic vesicle glycoprotein 2C (SV2C)	205–2381	82.3	S	XM_043493
Topoisomerase II (TOP2) (5′)	41–2454	92.1	M, A	J04088
Topoisomerase II (TOP2) (3′)	2242–4629	82.4	M, A	J04088
Unc13	2521–4985	93.7	S	NM_006377
Unc18	74–1851	67.6	S	D63851
Vesicle-associated membrane protein 2 (VAMP2)	92–445	12.6	S	NM_014232

[a](A), putative minor autoantigen; (A*), major autoantigen; (S), protein associated with secretory vesicles; (P), PTP family members; (M), miscellaneous

Table 2

Autoantibodies to VAMP2 and NPY: Relationship to the three major diabetes autoantigens

	Autoantibodies				Anti-VAMP2		Anti-NPY	
Number of Autoantibodies	**IA-2**	**GAD65**	**Insulin**	**Number**	**Number**	**Percent**	**Number**	**Percent**
Three	+	+	+	33	15	45	6	18
Two	+	+	−	30	8	27	2	7
	+	−	+	26	5	19	3	12
	−	+	+	18	2	11	1	6
	Total			74	15	20	6	8
One	+	−	−	24	4	17	2	8
	−	+	−	26	4	15	1	4
	−	−	+	17	1	6	1	6
	Total			67	9	13	4	6
None	−	−	−	24	2	8	0	0

Autoantiboies to VAMP2 were significantly associated with autoantibodies to IA-2 (p=0.004) and GAD65 (p=0.022).