Rapid Publications

Transcription Factor jun-B Is Target of Autoreactive T-Cells in IDDM

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Target antigens defined by autoantibodies in IDDM include insulin, a putative glycolipid that reacts with islet cell antibodies, and a 64,000-M_r protein recently identified as glutamic acid decarboxylase. In addition, some IDDM sera that contain antibodies to glutamic acid decarboxylase also coprecipitate a 38,000-M_r protein from islets. This study used a high titer anti-38,000-M_r serum to screen bacteriophage λ cDNA expression libraries and identified human islet and placental clones encoding jun-B, the nuclear transcription protein, of predicted 38,000 M_r. Peripheral blood T-cells exhibited significant proliferation in response to a recombinant fragment of jun-B (amino acids 1–180) in 12 of 17 (71%) recent-onset IDDM subjects, 8 of 16 (50%) ICA-positive first-degree relatives of IDDM subjects who were at risk, 3 of 12 (25%) other autoimmune disease subjects, and 0 of 10 healthy control subjects. Proliferation to tetanus toxoid did not differ significantly between the groups. Responses to jun-B were not related to age, sex, or human leukocyte antigen status. Thus, autoreactive T-cells identify a novel antigen, p38 jun-B, in IDDM and appear to indicate subjects at risk for the development of clinical disease. *Diabetes* 42:626–30, 1993

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IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibody; GAD, glutamic acid decarboxylase; HLA, human leukocyte antigen; CMV, cytomegalovirus; JDF U, Juvenile Diabetes Foundation units; IPTG, isopropyl-1-thio-β-D-galactoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione-*S*-transferase; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; SI, stimulation index; CI, confidence interval; IL-6, interleukin-6; HSV1, herpes simplex virus type 1; cpm, counts per minute.

IDDM is the outcome of an autoimmune process that destroys the pancreatic islet β-cells. Evidence includes a mononuclear cell infiltrate of the islets (insulitis) and circulating autoantibodies and T-cells that react to islet antigens (1). Islet antigens that react with autoantibodies (2) include a putative glycolipid (3), which is the target of ICA in frozen sections of human pancreas; insulin (4); and a 64,000-M_r protein GAD (5–7). Recently, T-cells in the peripheral blood of recent-onset IDDM subjects and at-risk ICA-positive first-degree relatives of IDDM subjects have been shown to proliferate to islets (8,9) and specifically to insulin (8,9) and GAD (10,11). In addition, Roep et al. (12,13) generated T-cell lines from peripheral blood of recent-onset IDDM subjects to an uncharacterized 38,000-M_r fraction of insulin secretory granule membranes.

Several other lines of evidence exist for a 38,000-M_r islet antigen. In the first study of the 64,000-M_r antigen (5), some IDDM sera also precipitated a 38,000-M_r protein, which was overlooked. We have found that at least 33% of IDDM sera that immunoprecipitate the 64,000-M_r antigen from [^{35}S]methionine-labeled fetal porcine proislets also precipitate a 38,000-M_r protein (14). In the autoimmune BB rat, a model of IDDM, antibodies to a 38,000-M_r islet protein are reported to precede the onset of diabetes (15). Finally, a mouse monoclonal antibody raised against human CMV by Pak et al. (16) reacted by immunoblotting with a 38,000-M_r human islet-specific protein; Pak et al. (16) referred to unpublished data and claimed that antibodies to a 38,000-M_r islet protein were present in IDDM subjects carrying CMV genome.

By screening human islet and placental cDNA λ-phage expression libraries with an anti–64,000/38,000-M_r IDDM serum, this study identified clones whose sequences were identical with the gene encoding the early response nuclear protein, jun-B (17), of pre-

dicted 38,000 M_r. Because β-cell destruction in IDDM is mediated by T-cells (1), we investigated whether T-cells reactive with recombinant human jun-B are present in the peripheral blood of IDDM subjects and at-risk first-degree relatives.

RESEARCH DESIGN AND METHODS

Sixteen at-risk first-degree relatives of IDDM subjects with ICA ≥20 JDF U (18), 17 with recent-onset IDDM within 6 wk of clinical diagnosis, 8 with Graves' disease, 4 with scleroderma, and 10 healthy, HLA-matched control subjects were studied with their informed consent and approval of the Royal Melbourne Hospital Human Ethics Committee.

Identification and expression of jun-B cDNA clones. Serum from a recent-onset IDDM patient containing high titer antibodies that precipitated 64,000-M_r GAD and a 38,000-M_r protein from islets was used to immunoscreen human islet and placental cDNA expression libraries (islet library a gift from Dr. Alan Permutt, Washington School of Medicine, St. Louis, MO), as described by Young and Davis (19). A total of 27 positive clones identified from the islet library and 18 from the placental library were plaque purified by repeated screening. Phage DNA was purified on CsCl step gradients, and insert sizes were determined by *Eco*RI restriction analysis. Sequencing of the 1.35-kb insert in the strongest immunoreactive clone from the islet library and comparison of its sequence against the Genbank protein data base revealed that the cDNA encoded the COOH-terminal 299 amino acids of the 347 amino acid jun-B. Sequencing of the 1.57-kb insert in the strongest immunoreactive clone from the placental library revealed that it encoded the full-length jun-B protein. Nucleotide sequencing was performed by the dideoxy chain termination method using the M13 vector primers and specific primers from the published DNA sequence of jun-B (17). Full-length jun-B could not be expressed from either pGEX or hexahistidine fusion vectors in *Escherichia coli*. Truncated jun-B DNA encoding amino acids 1–180 was therefore generated by PCR using 5′ and 3′ primers containing *Bam*HI and *Hin*dIII restriction sites, respectively, and cloned into the hexahistidine vector pDS56. After transformation of *E. coli* and inducement with 1 mM IPTG, the his_6-jun-B fusion protein was affinity purified from 6 M guanidine HCl lysates on a nickel tetraacetate column (20). The recombinant fusion protein was further purified by elution from a Bio-Rad (Richmond, CA) 491 preparative electrophoresis column and shown to resolve as a single band by SDS-PAGE (Fig. 1).

As a control recombinant protein, GST from the pGEX vector, was expressed in *E. coli* and affinity purified on glutathione-agarose beads (11). Protein was eluted from the beads with 10 mM reduced glutathione and 50 mM Tris-HCl, pH 8.0, dialyzed against human tonicity PBS, filter sterilized, and stored at −70°C. The eluted protein, apparent 26,000 M_r, was homogeneous by SDS-PAGE.

T-cell proliferation studies. PBMC were prepared from heparinized venous blood by Ficoll-Hypaque density centrifugation, washed twice, and resuspended at

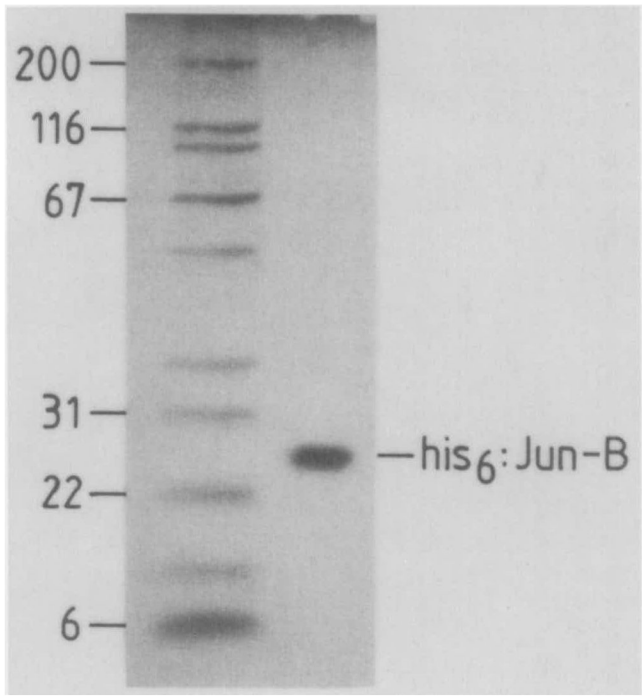

FIG. 1. SDS-PAGE of purified recombinant his^6-jun-B (amino acids 1–80) stained with Coomassie blue.

2×10^6/ml in RPMI-1640 medium with 5% autologous serum and 10^{-5} M mercaptoethanol. Aliquots of 200 μl/well were dispensed into 96-well flat-bottomed trays (Linbro, Flow, McLean, VA). jun-B (final concentrations 10, 1.0, and 0.1 μg/ml) or thiomersal-free tetanus toxoid (CSL, Melbourne, Australia; final concentrations 1.78, 0.18, and 0.018 Lyons flocculating units/ml) were added in 10-ml vol to quadruplicate wells, and the trays were incubated at 37°C in 5% CO_2-humidified air for 7 days. Cells were harvested semiautomatically 7 h after adding 1 μCi/well of [^{3}H]thymidine (Amersham, Sydney, Australia, TRK61) and counted for β-radioactivity. An SI was calculated as the ratio of the median counts per minute for the antigen concentration giving the highest stimulation to the median counts per minute in the absence of antigen. An increased SI was defined as CI >95% (2.1) of the SIs of the healthy control subjects. The SIs of the control subjects were first log transformed because the distribution of SIs appeared to be skewed. Statistical significance between groups was determined by Wilcoxon's rank sum test.

RESULTS

Proliferation of peripheral blood T-cells in the presence of jun-B was greater than basal proliferation or proliferation in the presence of the control recombinant protein GST in most subjects tested (Table 1). Expression of the results as SIs highlighted the differences between each group (Fig. 2). Significant SIs (>2.1) to jun-B were found in 8 of 16 (50%) ICA-positive first-degree relatives, 12 of 17 (71%) recent-onset IDDM subjects, 2 of 8 (25%) Graves' disease subjects, 1 of 4 (25%) scleroderma subjects, and 0 of 10 healthy control subjects. The number of recent-onset IDDM subjects responding to jun-B was

TABLE 1
Clinical characteristics and immunological data of study subjects

	Sex	Age (yr)	HLA-DR; DQ	Peripheral blood T-cell proliferation (cpm)			
				No antigen	jun-B	GST	Tetanus toxoid
ICA-positive first-degree relatives							
1	M	19	—	877	1101	756	30,924
2	F	50	3,4;2,7	6514	5246	10,079	24,101
3	M	16	3,4;2,8 3,—;2,—	403	1578	587	4030
4	M	12	*	8727	21,570	13,513	20,072
5	M	11	3,4;2,8	494	872	416	2074
6	F	44	3,4;2,8	1439	7526	2274	32,653
7	M	14	3,—;2,—	635	2152	1561	—
8	M	15	Z3,4;2,8	2417	9005	4980	17,688
9	F	20	3,4;2,8	42,604	48,818	42,520	68,954
10	F	40	3,12;2,7	6600	6047	6301	7653
11	M	11	—	650	932	785	927
12	M	16	1,3;1,2	1811	10,908	8731	68,731
13	F	35	3,13;1,2	7385	8047	6692	43,155
14	F	35	3,4;2,7	2392	17,279	—	—
15	F	46	3,6;1,2	4467	5446	5151	31,037
16	M	8	1,4;1,8	872	11,352	932	44,923
Recent onset IDDM subjects							
1	F	10	—	2902	4073	2938	9867
2	M	12	—	2314	6534	3489	11,175
3	M	18	3,4;2,8	147	1695	115	1470
4	M	16	3,4;2,8	9555	18,853	12,802	79,281
5	M	20	4,11;7,7	10,130	24,147	5501	59,543
6	M	26	3,4;2,8	4880	12,935	8596	46,031
7	F	20	4,—;7,—	1993	19,161	1813	13,334
8	M	28	4,—;8,—	3878	26,077	4062	58,946
9	F	19	3,—;2,—	3501	7721	4620	60,838
10	F	33	3,4;2,8	4306	37,803	20,991	38,309
11	M	33	3,4;2,8	8990	15,895	14,044	38,998
12	M	16	1,4;1,8	2539	9219	4118	31,340
13	M	40	3,—;2,—	4397	31,397	12,779	16,640
14	M	12	3,4;2,8	7118	17,566	12,790	37,238
15	F	35	3,4;2,9	18,096	28,675	16,356	—
16	F	30	11,13;1,7	1518	10,916	10,305	20,706
17	M	17	—	456	3364	634	11,361
Other autoimmune disease subjects							
Graves' disease							
1	F	30	3,4;2,7	4910	5547	4941	7365
2	F	28	—	2629	10,922	—	53,465
3	F	20	3,—;2,—	650	774	770	872
4	F	52	2,9;1,9	1616	1905	—	2835
5	F	29	—	978	1534	3159	20,949
6	F	47	—	1150	1569	2133	9575
7	F	47	—	1748	8552	1711	59,190
8	F	33	—	6511	6869	—	8059
Scleroderma							
9	M	48	—	1294	4018	—	41,230
10	F	53	1,4;1,8	7125	8228	8476	21,815
11	F	59	3,4;2,7	6339	12,750	—	14,621
12	F	45	4,15;6,7	490	806	692	2887
Healthy control subjects							
1	M	30	3,—;2,—	4992	5836	4542	11,387
2	M	50	1,4;1,8	9854	6958	7262	9778
3	M	34	1,4;1,7	6593	9347	7015	10,226
4	M	36	4,—;8,—	3433	3956	3309	4868
5	F	30	3,4;2,8	4379	4500	5077	14,072
6	M	33	2,4;1,7	7460	16,400	—	43,688
7	F	28	3,4;2,8	24,246	27,006	—	84,285
8	M	39	4,11;7,—	26,080	32,640	—	17,864
9	F	27	1,4;1,8	21,765	33,041	—	49,776
10	F	48	3,—;2,—	1307	1675	—	2389

*One antigen observed at each locus; presumed homozygosity.

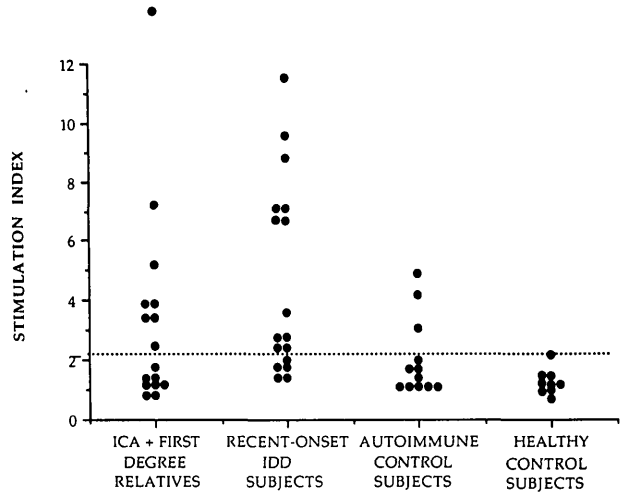

FIG. 2. Proliferation (SI) of peripheral blood T-cells in response to jun-B.

significantly higher than ICA-positive first-degree relatives ($P < 0.05$), subjects with Graves' disease or scleroderma ($P < 0.005$), and healthy control subjects ($P < 0.0005$). The responses to tetanus toxoid did not differ significantly between the groups. No significant differences were observed between responders and nonresponders with respect to age, sex, HLA status, or basal counts per minute.

DISCUSSION

jun-B is a nuclear transcription factor that dimerizes through its leucine zipper with related proteins, e.g., c-fos (21). T-cell responses to jun-B in the majority of subjects with recent-onset IDDM, 50% of the at-risk first-degree relatives of IDDM subjects, and a minority of subjects with other autoimmune disease were specific, in that the groups were not distinguished by different levels of T-cell responses to tetanus toxoid. Furthermore, the apparent lack of association between T-cell responses and specific class II HLA alleles indicates that the differences were not just the result of the presentation of jun-B peptides by IDDM susceptibility alleles (e.g., DR3,4 or DQ2,8) but to an increase in the number or responsiveness or both of T-cells that recognize jun-B peptides. To our knowledge, this is the second example of a nuclear protein autoantigen defined by T-cells in humans, the other being the p70 protein of U1 ribonucleoprotein in mixed connective tissue disease (22).

Two subjects with Graves' disease and one with active scleroderma also had increased responses, as defined. This overlap may not be unexpected as the cutoff for an increased response is arbitrary, and specificity and sensitivity need to be defined from the analysis of much larger disease and control groups. Biologically, it is consistent with the idea that autoreactivity is a continuous variable. Furthermore, a known association, both clinically and serologically, exists between IDDM and other autoimmune diseases, especially Graves' disease. Operationally, our findings indicate that T-cell reactivity to jun-B, or a molecule with crossreactive epitopes, is a risk marker for IDDM. Confirmation requires longitudinal studies of at-risk individuals followed to clinical disease. Not all at-risk ICA-positive first-degree relatives develop diabetes (18). Interestingly, the frequency of T-cell responses to jun-B was higher in recent-onset IDDM subjects than in relatives, suggesting that the relatives with reactivity to jun-B are at higher risk.

We were unsuccessful in expressing the full-length jun-B protein and therefore were unable to investigate its reactivity with a panel of IDDM sera. The truncated recombinant protein (amino acids 1–180) used for the T-cell studies reacted by immunoblotting with ≤10% of IDDM sera (data not shown). Although retaining linear epitopes for autoreactive T-cells, the recombinant proteins may lack dominant antibody epitopes, and autoantibody binding to jun-B may be conformation dependent.

Nuclear proteins are targets of autoantibodies that characterize systemic autoimmune connective tissue diseases such as systemic lupus erythematosus (23) but may also be targeted by autoantibodies in restricted tissue- or organ-specific autoimmune diseases, e.g., the La ribonucleoprotein in Sjögren's syndrome. Although useful as disease markers, the relevance of autoantibodies to these ubiquitous proteins in the pathogenesis of disease remains unclear. Given that jun-B is not specific for β-cells, how can our findings be reconciled with the immunopathology of IDDM and with reports (12–16) of a 38,000-M_r islet antigen in IDDM? jun-B is an early response nuclear protein in many cells (24,25) and, although transiently expressed in response to growth factors, can be expressed in stable form under certain conditions, e.g., in myeloid cells terminally differentiating in response to IL-6 (26) or in sympathetic neurones switched from a noradrenergic to cholinergic phenotype in response to leukemia inhibitory factor (27). By analogy, jun-B might be expressed in stable form in the terminally differentiated β-cell or upregulated by inflammatory cytokines or both including the IL-6 present in the islet lesion (28,29). Specificity of an anti–jun-B T-cell response would require processing and presentation of islet (β-cell)-specific jun-B epitopes or the persistent effect of a β-cell cytotropic virus (30) or both, or chemotoxin (31) to induce aberrant expression of jun-B. Roep et al. (12,13) generated T-cell lines from IDDM subjects to a 38,000-M_r fraction of insulin secretory granules. Although secretory granules would not be expected to contain jun-B, after isolation they appear to be contaminated by a multiplicity of proteins; moreover, the T-cell lines reacted not only with 38,000-M_r granule fraction but also with the pellet fraction that contained endoplasmic reticulum (13). Conceivably, these T-cell lines may have been generated to jun-B, and this possibility should be the subject of future experiments.

Pak et al. (16) described a monoclonal antibody to human CMV that immunoblotted a 38,000-M_r human (but not mouse or rat) islet protein. Molecular mimicry, the sharing of an epitope between a CMV protein and a 38,000-M_r islet protein, could result in anti-CMV immunity being directed against the islet. Data-base analysis (FASTA and T FASTA search programs) reveals that jun-B shares amino acid sequences with human CMV

(AD169 strain) and related herpes viruses. For example, GRAPGGL at positions 21–27 near the NH_2-terminus of jun-B is present in human CMV amino acid 44963–44968, Epstein-Barr virus amino acid 11231–11236, and HSV (replication-associated long unique region) amino acid 32640–32645. The sequence ELERLIV at positions 86–92 of jun-B is present in CMV amino acid 443–449 (glycoprotein-B, neutralization epitope related), and amino acid 1411–1417 (major capsid protein). The sequence REQVAQ at positions 318–323 near the COOH-terminus of jun-B is present in CMV amino acid 50232–50237 and in HSV1-transforming domain shares five of six amino acids in positions 136–141. The first two of these sequences occur in the jun-B fusion protein used here to elicit T-cell reactivity in IDDM subjects. If further studies were to demonstrate that T-cells recognize one or other of these sequences, target cell specificity would require their presentation uniquely on β-cells or antigen-presenting cells in the islet. Although the pathogenic role of T-cell reactivity to jun-B requires further investigation, these findings indicate that jun-B–reactive T-cells are a marker of subjects at risk for clinical disease. These findings raise the possibility that autoimmunity to oncogene products might be more prevalent than realized up to this time.

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