Cloning and Characterization of Islet Cell Antigen-related Protein-tyrosine Phosphatase (PTP), a Novel Receptor-like PTP and Autoantigen in Insulin-dependent Diabetes*

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Cloning of the cDNA encoding a novel human protein-tyrosine phosphatase (PTP) called islet cell antigen-related PTP (IAR) predicts a receptor-like molecule with an extracellular domain of 614 amino acids containing a hydrophobic signal peptide, one potential N-glycosylation site, and an RGDS peptide which is a possible adhesive recognition sequence. The 376-amino acid intracellular region contains a single catalytic domain. Recombinant IAR polypeptide has phosphatase activity. Northern blot analysis shows tissue-specific expression of two IAR transcripts of 5.5 and 3.7 kilobases, which are most abundant in brain and pancreas. The IAR PTP is homologous in its intracellular region to IA-2, a putative PTP that is an insulin-dependent diabetes mellitus (IDDM) autoantigen. IAR is also reactive with IDDM patient sera. IAR and IA-2 may distinguish different populations of IDDM autoantibodies since they identify overlapping but nonidentical sets of IDDM patients. Thus IAR is likely to be an islet cell antigen useful in the preclinical screening of individuals for risk of IDDM.

The co-ordinated actions of protein tyrosine kinases and phosphatases (PTPs) control much of the reversible protein phosphorylation central to eukaryotic cell proliferation and differentiation. As has been found for the protein tyrosine kinases, the ever expanding number and structural diversity of members of the PTP family suggest that these enzymes are critical and specific regulators of cellular processes (1, 2). All PTPs possess one or two conserved catalytic domains. The highly conserved active site within each domain contains an essential cysteine residue that functions as a transient phospho-acceptor during the dephosphorylation reaction (3). This sequence conservation has permitted the PCR-based isolation of many novel PTPases. Nonreceptor PTPs with a single catalytic domain and receptor-like PTPs generally possessing two intracellular catalytic domains have been isolated. The regions flanking the catalytic domain of nonreceptor PTPs often contain structures responsible for subcellular localization and/or enzymatic regulation, for example N-terminal SH2 domains or C-terminal hydrophobic tails (reviewed in Ref. 4). The receptor-like PTPs consist of an intracellular region with the catalytic domains, a transmembrane region, and an extracellular region that often, but not always, contains structural motifs found in cell adhesion such as FN-III and Ig-like repeats. The receptor-like structure of many PTPs suggests that they act as transducers of extracellular signals and that appropriate intracellular signaling pathways are initiated by tyrosine dephosphorylation events.

Several PTPs have been implicated in disease processes. The pathogenic Yersinia bacteria, causing plague or other often fatal gastrointestinal disorders, contain an essential virulence determinant which is a PTP (5). The secreted PTP may act to abrogate the host response to bacterial infection (6). Mice with a defective gene encoding the nonreceptor SH-PTP1 (HCP, PTP-1C) exhibit multiple severe hematopoietic defects (7), which may be due to a lack of negative regulation of cytokine signaling. There is also evidence that some PTPs are potential oncogenes; overexpressed receptor-like PTPα is transforming, and elevated levels of PTPα message are present in 70% of colon carcinomas (8, 9), and one isoform of the PTP cdc25 is transforming while another is overexpressed in 32% of human breast cancers (10).

Insulin-dependent diabetes mellitus (IDDM) is believed to result from the autoimmune-mediated destruction of pancreatic beta cells. Serum antibodies reactive with islet cell components can often be detected months or years before the disease is clinically apparent. These include the unidentified ICA antigen (11), insulin (12), glutamate decarboxylase (13), and 37- and 40-kDa tryptic fragments of islet extracts (14). The use of single antibody markers in preclinical IDDM screening is limited by their comparatively low positive predictive value, but this has been improved by the use of combined markers (15). Identification of novel autoantigens that can be used in combination with existing markers is therefore an important priority. We report here the cloning and characterization of a human receptor-like PTP, termed IAR, which is reactive with IDDM sera. A study with a small group of IDDM patients suggests that IAR reactivity delineates a unique population of autoantibodies.

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† The abbreviations used are: PTP, protein tyrosine phosphatase; FN-III, fibronectin type-III; GAD, glutamic acid decarboxylase; ICA, islet cell antigens; IDDM, insulin-dependent diabetes mellitus; pNPP, para-nitrophenyl phosphate; IAR, islet cell antigen-related PTP; BSA, bovine serum albumin; bp, base pair(s); RACE, rapid amplification of DNA ends; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; kb, kilobase pair(s); Mes, 4-morpholineethanesulfonic acid.
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EXPERIMENTAL PROCEDURES

cDNA Isolation and Sequencing—Primers corresponding to conserved amino acid sequences (DTYNA and VHCSAGV) of the catalytic domain of PTPases were designed (with EcoRI sites added) and used to amplify a human colon carcinoma cell (SW480) cDNA library (Clontech, HL3014b), which contained cDNA from healthy human blood bank donors (41 clones) and PTPases (with added 5′-GGAATTC-CTC-3′). The amplified fragment was cloned into the pGEM-T vector (Promega). The 3′ RACE and 5′ RACE were used to generate the Marathon cDNA Amplification Kit (Clontech), and cDNA was reverse-transcribed from human brain poly(A)+ mRNA (Clontech) using the cDNA synthesis primer provided with the kit (3′ RACE) or random primers or an IAR-specific primer (P5) with the sequence 5′-GGGACATAGGCTGAGGATGCTCTCGGAGAA-3′ (5′ RACE). The PCR amplification was carried out using either a forward or reverse primer corresponding to the ligated adaptor in combination with an IAR-specific forward primer, 5′-CTCTGCTCTGGCGGAGCGAAGA-3′ (5′ RACE) or an IAR-specific reverse primer (P11), 5′-TGCGGAGACCTGCTTGGAGCTCG-3′ (5′ RACE). The amplified fragments were cloned into pGEM-T (Promega) and sequenced along both strands.

Northern Analysis—Human multiple tissue Northern blots (Clontech) were hybridized with labeled IA-2 probe (nucleotides 1665–1906 of IA-2 cDNA according to Ref. 16) and stripped and reprobed with labeled IAR cDNA (nucleotides 1264–1671, Fig. 2).

Analysis of Signal Peptide Function—The cDNA encoding the full-length IAR protein was modified by replacing the 5′-untranslated region with the sequence CCACC and then cloned into pBlueScript SK (+) (Stratagene). In vitro transcription and translation of this construct or the B11 cDNA were carried out according to the manufacturer’s protocol using the TNT Coupled Reticulocyte Lysate Systems (Promega) in the presence of 35S methionine and with or without the addition of canine microsomal membranes (Promega). After translation, the products were treated with or without proteinase K (Boehringer Mannheim) at a final concentration of 0.01 mg/ml on ice for 7.5 min, in the presence or absence of 0.1% Triton X-100. The reaction products were analyzed by 10% SDS-PAGE and autoradiography.

Expression, Purification, and Assay of Recombinant IAR—The primers P1 and P2, 5′-GGGGCTCGAGTGTACAGGCTGAGGAGAGCTCCTC-3′ (with added XhoI and EcoRI sites) and 5′-GGGAATTCCTTCGAGAAGAAGACCGAAGCAG-3′ (with added EcoRI and NcoI sites), were used in a PCR with the 5′-3′ clone as template to amplify the intracellular region of IAR encoding amino acids 646–1015. The amplified fragment was cloned into the XhoI and EcoRI sites of pGEX-3C (17). Sequencing of the insert confirmed that no mutations had resulted from the PCR. Mutant IAR (C945S) was generated by PCR using overlapping forward and reverse primers corresponding to the desired mutation and surrounding sequence encoding HVIHSSDG; 5′-ATAATGTGTCCATCTGAGGGGACAGGAAT-3′ and 5′-TGCAACCTGATCCTTGGAAITGCAAATTAT-3′. Each primer was used in combination with one of the overlapping primers (used to amplify the intracellular region of IAR) to generate two overlapping DNA fragments corresponding to nucleotides 1993–2904 and 2878–3197 of IAR. These fragments were mixed and used as template for a PCR reaction with primers P1 and P2. The amplified fragment was cloned into pGEX-3C and verified by sequencing. Soluble GST-IAR fusion proteins were produced after isopropyl-1-thio-β-D-galactopyranoside induction (0.15 mm) of Escherichia coli (DH5αF) transformed with the above plasmids. The GST-IAR was purified from bacterial lysates (17), and the IAR cleaved from GST (with added 5′-GGGAATTC-CTC-3′) was used to generate a human pancreas cDNA library. Several positive clones were isolated and identified as IA-2 cDNA by sequencing. Other clones encoded a distinct cDNA that contained r75 sequence. The largest of these, C3, was sequenced on both strands and found to contain an open reading frame of about 2.4 kb (Fig. 1). The C3 clone contained a coding sequence missing initiation and termination signals and was significantly smaller than the transcripts detected by Northern blotting with r75, suggesting that it was incomplete at both the 5′ and 3′ ends. To obtain the 3′ end of the clone, 3′ RACE was used on cDNA reverse-transcribed from human brain mRNA. Two different clones were obtained (Fig. 1). Clone 3–7 had a 5′ region of identical overlap with C3 and additional sequence containing a stop codon and polyadenylation signal. Clone 3–10 was identical to 3–7 from the 5′ end up to the 3′ polyadenylation site, but was followed by approximately 2 kb of additional untranslated sequence. To obtain the 5′ end of the cDNA, the pancreas library was rescreened with a 300-bp probe close to the 5′ end of C3. No IA-2 clones were found. Of the positive clones, clone B11 appeared to have the longest 5′ extension to C3 and was selected for further sequencing along both strands. The 5′ end of B11 was identical to C3 sequence over a length of about 2 kb, but the 5′ ends of B11 and C3 were different (Fig. 1). B11 had a unique 5′ sequence of 412 bp, followed by a 54-bp sequence that is found in the opposite
orienation in C3. The sequence of C3 found 5’ to this inverted region was shorter and different from that of B11. RACE was used to determine which clone contained the correct 5’ sequence and to try to obtain additional 5’ sequence to the open reading frames of B11 and C3. Template cDNA was prepared from human brain poly(A)+ mRNA using random primers or a primer (P5) corresponding to shared B11/C3 sequence located about 170 bp 3’ to the invert. The 5’ RACE used a primer to the ligated adaptor sequence at the ends of the cDNA and a primer (P11) to a region of shared B11/C3 sequence located about 90 bp 3’ to the invert. Cloning and sequencing of the 5’ RACE PCR products from both types of template cDNA showed that all had B11 sequence. Similarly, PCR amplification of the pancreas cDNA library using a primer to gt11 and either P5 or P11 primers gave DNA fragments that all corresponded to the unique B11 sequence. Thus, the B11 sequence is correct, and the 5’ end of the C3 clone is likely a library artifact. No PCR products were found with a 5’ extension of the B11 sequence, and no 5’-extended clones were identified by further screening of human brain and pancreas cDNA libraries.

The complete cDNA is predicted to encode a receptor-like PTP of 1015 amino acids. This includes a 614-amino acid extracellular region, a 25-residue hydrophobic transmembrane segment, and a 376-amino acid intracellular region containing a single PTP catalytic domain (Fig. 2). This protein has about 43% overall sequence identity to the putative PTP IA-2 (16, 22), a protein identified as an islet cell autoantigen in IDDM, and another PTP-like molecule called PTPLP (24). We have thus named the novel PTP as islet cell antigen-related PTP or IAR PTP. An alignment of IAR and IA-2 amino acid sequences is shown in Fig. 3. The intracellular domain of IAR is 73% identical to that of IA-2; however, the identity falls to 24% in the extracellular regions of these proteins. The IAR extracellular region does not contain FN-III or Ig-like repeats but has the adhesion recognition peptide sequence RDGS (amino acids 372–375). There is one potential site for N-linked glycosylation. The catalytic domain of IAR has conserved sequences typical of other PTPases, although the active site is unusual in having an aspartate residue at position 947 (IVHCSDGAGRTG) in place of a conserved alanine residue (Fig. 2).

Although no in-frame stop codons were found 5’ to the first ATG codon in the B11 sequence, we propose that this is the initiation codon for the following reasons. 1) A purine (G) in the 2’ position conforms with the Kozak rules of initiation (25). 2) This methionine is followed by a 20-residue hydrophobic sequence that has the features of a signal sequence (26). To test for signal sequence function, a cDNA encoding the predicted full-length protein was transcribed and translated in vitro in the presence of [35S]methionine and in the presence or absence of microsomal membranes. The protein synthesized in the absence of microsomal membranes (Fig. 4, lane 1) was completely digested upon subsequent treatment with proteinase K (Fig. 4, lane 2).

**Fig. 2.** Nucleotide and predicted amino acid sequence of IAR. The signal peptide is underlined with a bold line, and the transmembrane region is boxed. An extracellular RGDS sequence and a potential site of N-linked glycosylation are underlined. The active site of the phosphatase is found at amino acids 942–954.
Expression of IAR—In view of the homology of IAR and IA-2, nonhomologous fragments of these cDNAs were selected to use as specific probes on Northern blots (Fig. 5). The two probes recognize distinct transcripts in terms of size and tissue specificity. The IA-2 probe detects a single transcript of about 3.9 kb, in accord with the results of Lan et al. (16). The highest expression of IA-2 is detected in brain, followed by spinal cord and pancreas, with low levels expressed in small intestine and adrenal gland. The IAR probe detects two transcripts of about 3.7 and 5.5 kb, with the highest expression in pancreas and brain, followed by trachea, prostate, stomach, and spinal cord with low levels detectable in small intestine and adrenal gland.

It is possible that the 3.7-kb transcript has a 3′-untranslated sequence corresponding to that of the shorter 3–7 clone, whereas the 5.5-kb transcript represents an alternative 3′-untranslated sequence corresponding to that of the longer 3–10 clone.

Catalytic Activity of IAR—The intracellular region of IAR (amino acids 646-1015) was expressed as a GST-fusion protein and purified following 3C protease cleavage from GST. The IAR migrated on SDS-PAGE as a major protein band of about 41 kDa (Fig. 6a, lane 2), in accord with the predicted size of 41.7 kDa. The purified IAR possessed low but detectable phosphatase activity toward p-NPP. The IAR is active over a narrow pH range, with optimal activity at pH 4.5, and is essentially inactive at pH 5.5 (Fig. 6b). IAR catalyzes the time-dependent dephosphorylation of p-NPP (Fig. 6c) with a specific activity of 21 nmol/min/mg. Like many PTPs, IAR activity is sensitive to inhibition by vanadate, and 1 mM sodium orthovanadate completely abolished activity (Fig. 6c). Site-directed mutagenesis of the essential cysteine residue (to a serine) in the active site resulted in the expression of an IAR intracellular region polypeptide which did not catalyze p-NPP hydrolysis (Fig. 6c), indicating that the phosphatase activity measured with the wild type polypeptide was unlikely due to contaminants in the protein preparation. IAR dephosphorylated phosphotyrosyl casein in a time-dependent manner, although with extremely low specific activity (2–3 pmol/min/mg), and about equivalent optimal activities were observed at pH 5.5 in sodium acetate buffer or at pH 6.5 in Mes buffer (data not shown). Phosphotyrosyl casein was not detectably dephosphorylated by IAR.

Reactivity of IAR with IDDM Patient Sera—Sera from 10 healthy blood bank control and 20 recent-onset IDDM subjects (ICA, GAD antibodies, age, and sex of subjects are shown in Table 1) were incubated with 

\[ ^{35} \text{S} \text{methionine-labeled IAR (amino acids 646-1015)} \]

Immunoprecipitates were analyzed by SDS-PAGE, where the translated IAR protein appeared as a band of 41 kDa with an associated band of 32 kDa. The latter is likely a proteolytic breakdown product of the 41-kDa band.

Representative immunoprecipitations of
The amino acid sequence of IAR is most distinct from that of IA-2 and PTPLP in its extracellular region, with 24.4 and 22.9% identity, respectively. However, all three PTPs are structurally alike in that they lack the FN-III and Ig-like repeats characteristic of many other PTPs. The low specific activity of IAR toward this sequence can mediate cell attachment of IAR is more closely related to those of IA-2 (73.4% identity) and PTPLP (74.4% identity). All have a single catalytic domain, within which the active site has an aspartate residue substituted for a conserved alanine (VHCS to VHCS). In the second (membrane distal) catalytic domain of CD45 (30). No phosphatase activity of IA-2 or PTPLP has been reported, and this has been suggested to be due to this alteration (22, 24, 31). However, IAR does have phosphatase activity, albeit low relative to that of many other PTPs. The low specific activity of IAR toward p-NPP may reflect a narrow substrate specificity of this phosphatase. Several nonreceptor PTPs have substitutions at the same position in the active site, for example BVH and cdc25 have a histidine residue in this position, whereas human VHR has a glutamic acid residue (32–34). These substitutions do not preclude catalysis, since all are active PTPs.

IAR is reactive with IDDM patient sera, identifying an overlapping set of subjects as does IA-2. Antibodies (termed ICA or islet cell antibodies) in the sera of IDDM patients react with islet cells in frozen sections of pancreas (11), and the nature of
the reactive antigen(s) has long been sought. Autoantibodies recognize a 64-kDa polypeptide in detergent extracts of islet cells that was identified as glutamic acid decarboxylase (GAD) (13). Tryptic digestion of islet cell lysates reveals 50-, 37-, and 40-kDa IDDM-reactive antigens, of which only the 50-kDa form is reactive with anti-GAD antibody (14, 35). Anti-GAD antibodies do not strictly correlate with IDDM, being found in patients with other autoimmune diseases (36). Serum antibodies to the 37/40-kDa antigens are strongly correlated with the development of IDDM in ICA-positive subjects, whether these be first degree relatives (15) or non-diabetic twins of IDDM patients (37), patients with polyendocrine autoimmunity (36), or selected by screening from the general population of schoolchildren (38). Recent efforts to identify the 37/40-kDa antigens have found that IA-2 polypeptide can block precipitation of the 40-kDa fragment by antibodies from non-diabetic relatives of IDDM patients, and partially block precipitation of the 37-kDa fragment, indicating that the antibodies can distinguish between what appear to be two related fragments (39, 40). Furthermore, trypsin treatment of IA-2 generates a 40-kDa, but not a 37-kDa, fragment (40). This suggests that the 40-kDa fragment is IA-2, and the 37-kDa fragment is derived from a distinct but related protein, likely also to be a PTP. The recognition of IAR by IDDM antibodies and the identification of an overlapping yet distinct set of patients from IA-2, the sequence homology between IA-2 and IAR, particularly in the intracellular region that appears to be the antigenic region, and the expression of IAR in pancreas, all support the possibility that IAR is the precursor of the islet 37-kDa antigenic fragment.

The availability of recombinant antigenic polypeptides will greatly enhance the ease of screening general or IDDM-risk populations, and the sensitivity of such screens will be improved by employing more than one antigen. The IA-2 and IAR PTPs may serve such a purpose. Also of interest is the investigation of the cellular role of these PTPs in the pancreas, and, if any, in IDDM development.

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| Patient | Age (y) | Sex | ICA | GAD | IAR | IA-2 |
|---------|---------|-----|-----|-----|-----|------|
| Control |         |     |     |     |     |      |
| 1       | 12.1    | F   | 0   | 209 | 1092| 508  |
| 2       | 5.5     | M   | 160 | 522 | 1084| 1326 |
| 3       | 6.0     | M   | 140 | 180 | 312286 | 13491 |
| 4       | 13.1    | F   | 42  | 272 | 7963 | 318  |
| 5       | NA      | NA  | NA  | NA  | 2148 | 12990 |
| 6       | 4.0     | F   | 270 | 3291| 365 | 170  |
| 7       | 12.9    | F   | 0   | 238 | 428 | 691  |
| 8       | 13.4    | M   | 84  | 184 | 376 | 470  |
| 9       | 12.8    | F   | 270 | 3335| 21802 | 18732 |
| 10      | 10.7    | M   | 63  | 283 | 5392 | 6526 |
| 11      | 12.5    | F   | 32  | 1713| 631 | 567  |
| 12      | NA      | NA  | NA  | NA  | 150105 | 16493 |
| 13      | 9.8     | F   | 120 | 839 | 4344 | 140  |
| 14      | 10.5    | M   | 270 | 167 | 17107 | 37610 |
| 15      | 3.8     | F   | 270 | 485 | 5564 | 10156 |
| 16      | 7.0     | M   | 270 | 199 | 160 | 1365 |
| 17      | NA      | NA  | NA  | NA  | 19517 | 11265 |
| 18      | 4.6     | M   | 270 | 126 | 21006 | 12713 |
| 19      | 9.6     | M   | 26  | 235 | 1232 | 1726 |
| 20      | 15.7    | F   | 160 | 1959| −378 | 4717 |

Fig. 7. Reactivity of IAR with IDDM sera. A, immunoprecipitation of IAR with sera from control and recent-onset IDDM subjects. In vitro translated [35S]methionine-labeled IAR was incubated with sera (prebound to protein A-Sepharose) from control (subjects 5 and 6 in Table I) (lanes 1 and 2) and IDDM subjects (subjects 12, 3, and 18 in Table I) (lanes 3–5). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The 41- and 32-kDa bands (arrowed) are IAR and a likely IAR proteolytic product, respectively. B, graphical depiction of control and IDDM subject sera reactivity with IAR. The exact values of the data presented here and further subject information are given in Table I. Experiments were carried out as in A, and the immunoprecipitated IAR was resolved by SDS-PAGE. The signal from the 41-kDa IAR band was quantitated (in arbitrary density units) using a phosphorimager.
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