Autoantibodies to IA-2 and IA-2β in Insulin-Dependent Diabetes Mellitus Recognize Conformational Epitopes

Location of the 37- and 40-kDa Fragments Determined

Hong Xie, Baowei Zhang, Yasunobu Matsumoto, Qing Li, Abner L. Notkins, and Michael S. Lan[1]

IA-2 and IA-2β are major autoantigens in insulin-dependent diabetes mellitus (IDDM) and the precursors, respectively, of a 40- and 37-kDa tryptic fragment that reacts with IDDM sera. In the present study, by amino acid sequencing of recombinant IA-2 and IA-2β, we determined the tryptic cleavage sites involved in the generation of these fragments. Both cleavage sites are immediately after an arginine residue at position 653 for IA-2 and position 679 for IA-2β. The resulting tryptic fragments are 326 and 307 amino acids in length and retain their ability to react with IDDM sera. In contrast to IA-2 and IA-2β, other members of the protein tyrosine phosphatase (PTP) family (i.e., RPTPκ, RPTPμ, NU-3, SHP, and 3CH134) are completely susceptible to digestion by trypsin. Sequence analysis revealed five conserved cysteine residues in IA-2 and IA-2β that are not present in other PTPs. Reduction and alkylation of IA-2 and IA-2β recombinant proteins resulted in loss of both resistance to digestion by trypsin and reactivity with autoantibodies in IDDM sera. It is concluded that disulfide bond formation plays a critical role in the maintenance of antigenic structure and that the autoantibodies to IA-2/IA-2β in IDDM sera recognize conformational epitopes. ***The Journal of Immunology,*** **1997, 159: 3662–3667.**

IA-2 and IA-2β are members of the protein tyrosine phosphatase (PTP)[2] family and major autoantigens in insulin-dependent diabetes mellitus (IDDM) (1–7). Approximately 70% of patients with newly diagnosed IDDM have autoantibodies to IA-2. In otherwise normal individuals, autoantibodies to IA-2 in combination with autoantibodies to glutamic acid decarboxylase (GAD_{65}) and/or insulin are highly predictive of the subsequent development of clinical diabetes (8–13). IA-2 and IA-2β are expressed and processed as transmembrane proteins of secretory granules and are found in cells of the neuroendocrine system, including the β cells of the pancreas (1, 2, 5, 14, 15). Both molecules have extracellular, transmembrane, and intracellular domains. The autoantibodies in IDDM sera appear to react exclusively with the intracellular, and not the extracellular, domain of these molecules (16–19). Within the intracellular domain, the major antigenic determinants reside at the COOH-terminus. Some IDDM sera also react with the juxtamembrane region of the intracellular domain of IA-2, but show little if any reactivity with the juxtamembrane region of IA-2β (17–19).

In the early 1990s, Christie et al. found that trypsin treatment of radiolabeled β cells resulted in the generation of two fragments, one 37 kDa and the other 40 kDa, which could be immunoprecipitated by sera from patients with IDDM (20–22). The identity of these fragments, however, remained obscure. With the cloning, sequencing, and availability of recombinant IA-2 and IA-2β, it soon became apparent that IA-2 was the precursor of the 40-kDa fragment, and IA-2β was the precursor of the 37-kDa fragment (2, 16, 23). The precise location of the 40-kDa fragment within the IA-2 molecule and the 37-kDa fragment within the IA-2β molecule, however, was not known.

In the present experiments, by amino acid sequencing, we determined the trypsin cleavage site and thereby the location of the 40-kDa and 37-kDa fragments within IA-2 and IA-2β, respectively. In addition, we showed that the major antigenic determinants of IA-2 and IA-2β are conformational and highly dependent on disulfide bond formation.

Materials and Methods

Bacterial expression of IA-2 and IA-2β fusion proteins

The intracellular domain of IA-2 (amino acid 604–979) and IA-2β (amino acid 582–986) were subcloned into a pGEX bacterial expression vector (Pharmacia, Piscataway, NJ). Glutathione S-transferase (GST) fusion proteins were produced and purified on a glutathione-agarose affinity column (24).

Microsequencing of 37- and 40-kDa tryptic fragments

Five micrograms of purified recombinant GST-IA-2 and GST-IA-2β fusion proteins were treated with trypsin (Washington Biomedical Corp., Freehold, NJ) at a final concentration of 50 μg/ml for 30 min on ice. The reactions were stopped by adding equal amounts of trypsin inhibitor mixture including lima and soybean trypsin inhibitors (Washington Biomedical Corp.). Both untreated and treated IA-2 and IA-2β were separated on a 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Novex, San Diego, CA). The membrane was stained with amido black for 20 min at room temperature. The tryptic fragments on the PVDF membrane were cut out and washed four times with 1× PBS. Membrane-bound proteins were subjected to amino acid microsequencing by Bio-synthesis Inc. (Lewisville, TX).

Oral Infection and Immunity Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892

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[1] Address correspondence and reprint requests to Dr. Michael S. Lan, Oral Infection and Immunity Branch, NIDR/NIH, Building 30, Room 124, 30 Convent Dr. MSC 4322, Bethesda, MD 20892-4322.

[2] Abbreviations used in this paper: PTP, protein tyrosine phosphatase; IDDM, insulin-dependent diabetes mellitus; GST, glutathione S-transferase; ID, intracellular domain; PVDF, polyvinylidene difluoride.

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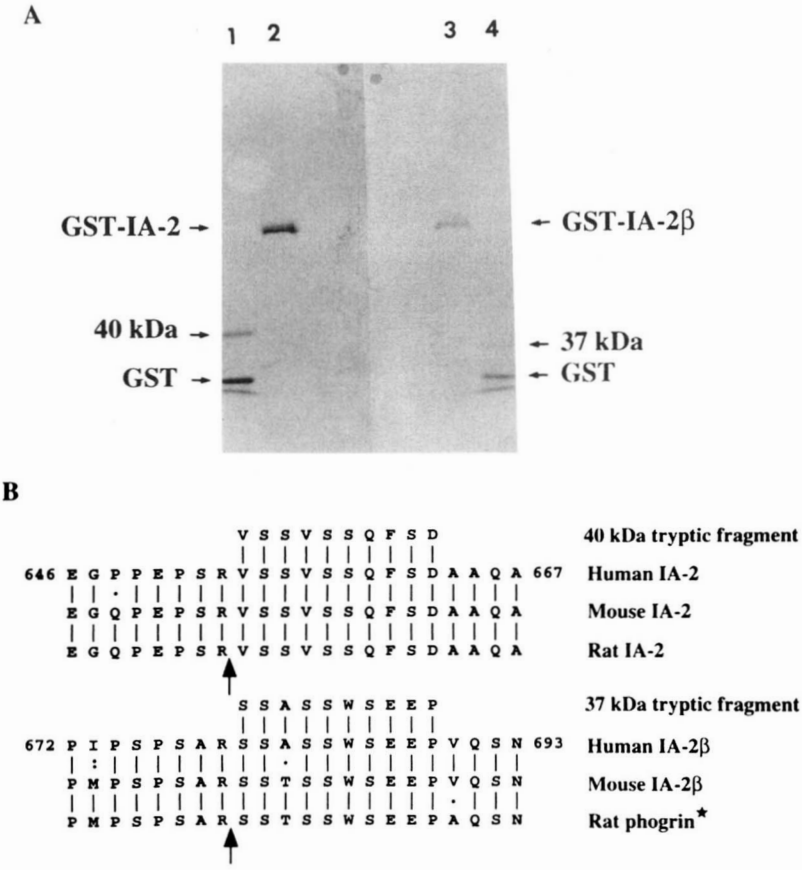

FIGURE 1. Microsequencing of 37- and 40-kDa tryptic fragments. *A*, Gel electrophoretic separation of recombinant IA-2 (*lanes 2* and *1*) and IA-2β (*lanes 3* and *4*) before and after trypsin digestion. Arrows indicate the proteins that were visualized after amido black staining on a PVDF membrane. *B*, Protein sequence analysis of 37- and 40-kDa fragments. Amino acid sequences obtained from microsequencing were aligned with IA-2 and IA-2β from human, mouse, and rat. *, Rat homologue of human IA-2β.

Construction of different domains of IA-2/IA-2β and other members of the PTP family

PCR was used to amplify various domains of IA-2 and IA-2β as described previously (17). Briefly, PCR reaction was performed by using 10 ng of IA-2 or IA-2β plasmid DNA as a template. Reaction conditions were as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles. Forward and reverse primers are listed below. The lowercase letters represent nucleotides that were added either as a Kozak consensus sequence (25) or stop codon. The PCR products were subcloned into pCRII vector (Invitrogen Corp., San Diego, CA), the cloning site of which is flanked by T7 and SP6 promoters. The amplified products were verified by sequence analysis.

IA-2 primers

Forward. 5′-gccgccaccatgGCGCGGCAGCAAGACAAG-3′
5′-gccgccaccatggTTATTGAGCATGACCCTCG-3′
Reverse. 5′-GCTGCCCGCCAAGGGGCCCCA-3′

IA-2β primers

Forward. 5′-gccgccaccatgGACTCCACCAAGTTCATCG-3′
5′-gccgccaccATGGTGTGGGAGAGC-3′
5′-gccgccacCATGGATCACGACCCGAG-3′
5′-gccgccaccATGGACATCTCCACCG-3′
Reverse. 5′-TGAGGCTGCCCGCTCACT-3′

The PTP domains of five PTPs, including RPTPμ (26), RPTPκ (27), NU-3 (28), SHP (29), and 3CH134 (30) were generated from mouse tissues by the RT-PCR method. RT-PCR amplification was performed using the GeneAmp XL PCR kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. The PCR products were subcloned into pCR2.1 or pBluescript SK$^+$ vector (Stratagene, La Jolla, CA). The forward and reverse primers are indicated below.

RPTPκ: 5′-ggccgccaccatgggaTTGCCCAATGATCCACTTGTGCCG-3′
5′-ggggaattcctcacatCAGGCAGGCTATGCTGCAGTCTTC-3′
RPTPμ: 5′-ggccgccaccatgggaAGCACATCGGTGCCGAATTCCTAT-3′
5′-ggggaattcctcacatTAGGAGTGCGATGCTGCAGTCCTC-3′
NU-3: 5′-ggccgccaccatgggaAAACCTGACAGCAAACGCAAGGAC-3′
5′-ggggaattcctcacatAGGCAGGCTGGCGGTGATGAAGCG-3′
SHP: 5′-ggccgccaccatgggaGAGTCGGAGGACACAGCCAAGGCT-3′
5′-ggggaattcctcacatCTTCCTCTTGAGAGAACCTTTGTT-3′
3CH134: 5′-ggccgccaccatgggaATGGTGATGGAGGTGGGCATCCTG-3′
5′-ggggaattcctcacatGCAGCTTGGAGAGGTGGTGATGGG-3′

TNT-coupled in vitro translation and trypsin treatment

Various domains of different members of the PTP family, including IA-2 and IA-2β, were in vitro translated in a TNT-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]methionine (Amersham, Arlington Heights, IL) at 30°C for 2 h. Trypsin digestion was performed on ice for 30 min at a final concentration of 50 μg/ml. The reaction mixture was subjected to SDS-PAGE separation.

Reduction and alkylation of IA-2/IA-2β

Radiolabeled in vitro-translated IA-2/IA-2β lysates were incubated with 50 mM DTT in 10 mM phosphate buffer (pH 7.4) at room temperature for 2 h and then passed through a Sephadex G-50 spin column to remove excess reducing reagent. An alkylating reagent, *N*-ethylmaleimide, was added to the reduced lysates to a final concentration of 2 mM at room temperature for 2 h. The reduced and alkylated lysates were purified on a Sephadex G-50 spin column. The quantities of -SH groups blocked by *N*-ethylmaleimide was followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacted and disappeared (31).

IDDM sera and immunoprecipitation

Seven IDDM sera that previously tested strongly positive for IA-2 and IA-2β autoantibodies were used in this study (kindly provided by Dr. Maclaren, University of Florida, Gainesville, FL). Both native and modified IA-2 and IA-2β lysates were immunoprecipitated with IDDM sera as described (3).

Results and Discussion

Microsequencing of 40- and 37-kDa tryptic fragments

To determine the site at which trypsin cleaves IA-2 and IA-2β, the intracellular domain of these molecules was expressed in a pGEX

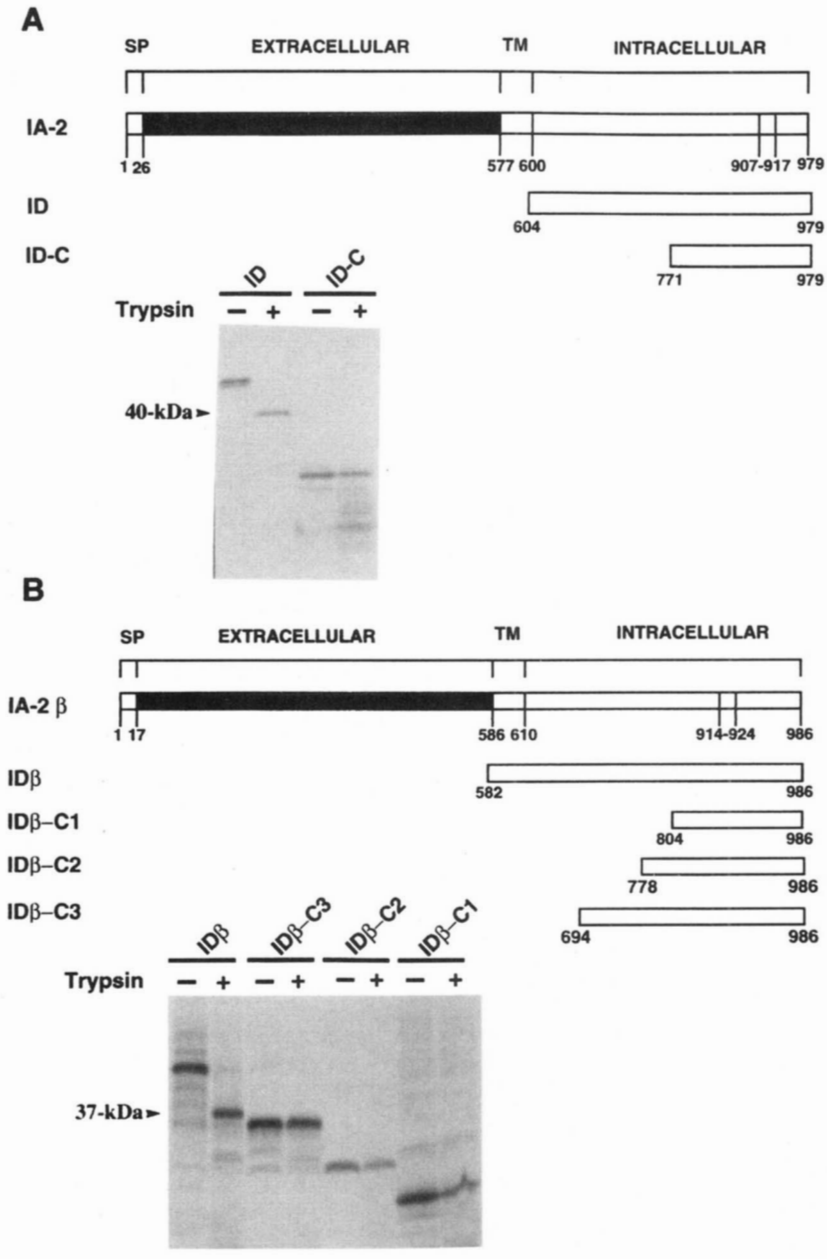

FIGURE 2. Trypsin treatment of the intracellular domains of IA-2 and IA-2β. IA-2 (*A*) and IA-2β (*B*) fragments were in vitro translated in the presence of [^{35}S]methionine, subjected to trypsin digestion, and separated by SDS-PAGE.

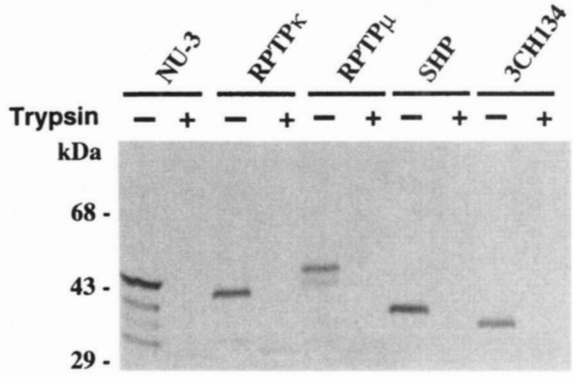

FIGURE 3. Trypsin treatment of the PTP domains of five other members of PTP family. In vitro-translated PTP domains were treated with trypsin and separated on a 10% SDS-PAGE.

vector as a GST-fusion protein and purified to homogeneity by a glutathione-agarose affinity column (24). The recombinant proteins (IA-2 and IA-2β) were treated with trypsin and separated by SDS-PAGE. As seen in Figure 1*A*, mild trypsin treatment converted GST-IA-2 into 40- and 26-kDa fragments and GST-IA-2β into 37-kDa and 26-kDa fragments. Western blot analysis using anti-GST Ab revealed that the 26-kDa fragments were derived from GST (not shown). The PVDF membrane-bound 40- and 37-kDa fragments were then excised and subjected to protein analysis, which yielded a 10-amino acid sequence from each fragment (Fig. 1*B*). The 10 amino acids from IA-2 and IA-2β aligned perfectly with the amino acids at positions 654–663 of IA-2 and 680–689 of IA-2β, immediately after arginine residues at positions 653 and 679, respectively. The 40-kDa fragment of IA-2 is 19 amino acids longer than the 37-kDa fragment of IA-2β.

Comparison of IA-2/IA-2β with other members of the PTP family upon trypsin treatment

To study the trypsin sensitivity of different regions of the intracellular domains of IA-2 and IA-2β, radiolabeled recombinant molecules prepared in the reticulocyte transcription translation system were subjected to trypsin treatment. As seen in Figure 2*A*, the intracellular domain (ID) is sensitive to trypsin yielding a 40-kDa

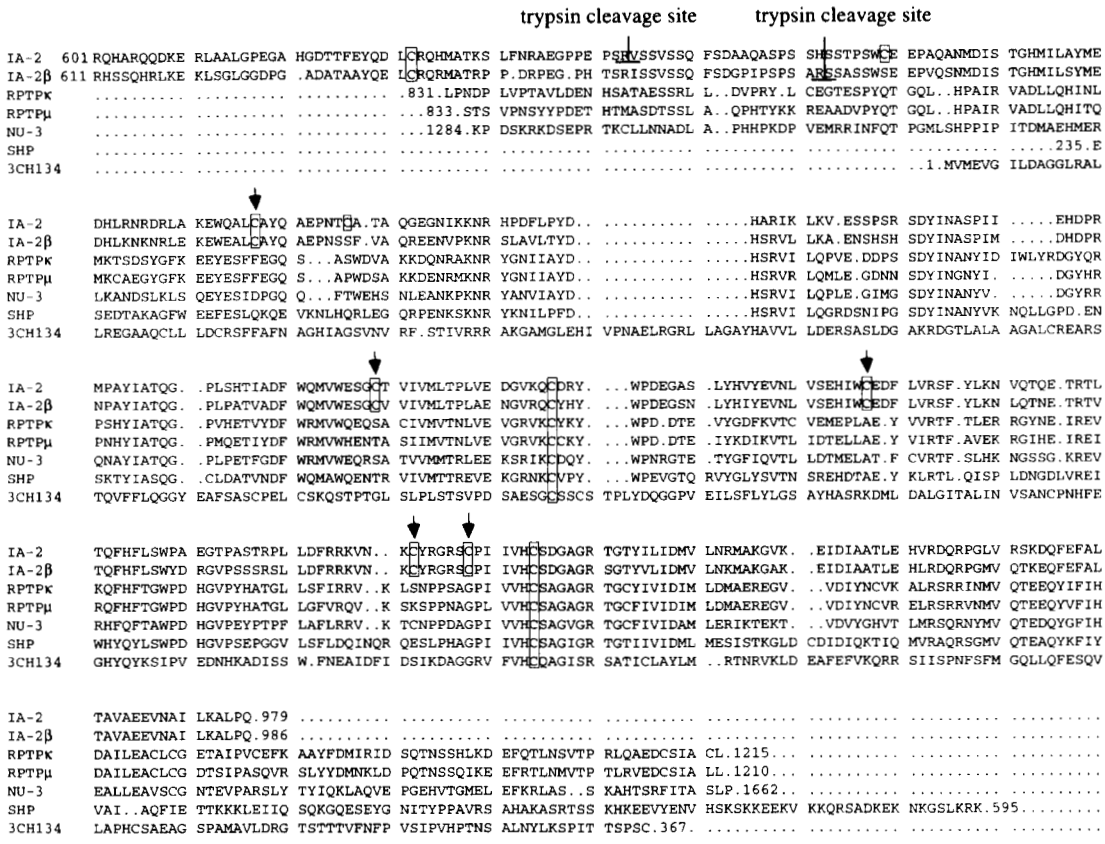

FIGURE 4. Alignment of IA-2/IA-2β with five other members of the PTP family. The PTP domain of five different members of the PTP family were piled up with IA-2 and IA-2β intracellular domain. Cysteine residues in IA-2 and IA-2β are boxed. Conserved cysteine residues only in 37- and 40-kDa fragments are indicated by arrows.

fragment, whereas the COOH-terminus (ID-C) is resistant to the action of trypsin. Similarly, as seen in Figure 2*B*, the intracellular domain of IA-2β (IDβ) is sensitive to trypsin yielding a 37-kDa fragment, whereas the smaller COOH-terminus fragments (IDβ-C1, IDβ-C2, and IDβ-C3) are resistant to the action of trypsin. These findings strongly suggest that the intracellular domains of IA-2 and IA-2β fold into unique configurations that make them resistant to the action of trypsin.

For comparison, five other members of the PTP family were subjected to trypsin digestion. Figure 3 shows that all these PTPs (i.e., NU-3, RPTPκ, RPTPμ, SHP, and 3CH134) were digested to completion by trypsin under the same conditions that produced the 40- and 37-kDa fragments of IA-2 and IA-2β, respectively. It should be noted that although IA-2 and IA-2β are members of the PTP family, they differ considerably from other known PTPs, showing only 35 to 45% sequence similarity (1, 19).

Disulfide bonds play an important role in resistance to trypsin and autoantibody binding

Alignment of the intracellular domains of IA-2 and IA-2β with other members of the PTP family revealed five conserved cysteine residues in IA-2 and IA-2β, not found in the other trypsin-sensitive PTPs (Fig. 4). This raised the possibility that the conserved cysteine residues might form disulfide bonds and fold IA-2 and IA-2β in such a way as to make them resistant to the action of trypsin. To test this hypothesis, we reduced the disulfide bonds of IA-2 and IA-2β with DTT and alkylated the free -SH groups with *N*-ethylmaleimide (32). As seen in Figure 5, the reduced and alkylated IA-2 and IA-2β molecules became susceptible to digestion by trypsin. Moreover, IDDM sera showed no reactivity with reduced and alkylated IA-2β and only weak reactivity (except for sera no. 6) with reduced and alkylated IA-2 (Table I). The weak reactivity of IDDM sera with reduced and alkylated IA-2 may be due to the presence of some nonconformationally-dependent epitopes, perhaps in the juxtamembrane region of IA-2, since IDDM sera react with both the COOH-terminus and juxtamembrane regions of IA-2, but only with the COOH-terminus of IA-2β (19). It is concluded that disulfide bond formation plays an important role in maintaining the conformation required for autoantibody binding and trypsin resistance.

Implications

Recently we and others showed that IDDM autoantibodies react exclusively with the intracellular domain of IA-2 and IA-2β and particularly with the COOH-terminus (16–19). The present study shows that the 40- and 37-kDa tryptic fragments, to which autoantibodies also react, encompass most of the intracellular domain. The 40-kDa fragment lacks the first 54 amino acids of the intracellular domain of IA-2 and begins at amino acid 654, whereas the 37-kDa fragment lacks the first 70 amino acids of the intracellular domain of IA-2β and begins at amino acid 680. Since the majority of IDDM sera react with the COOH-terminus of IA-2 and IA-2β, Ab binding assays using either the recombinant intracellular domain (i.e., IA-2, 601–979; IA-2β, 611–986) or the 37-kDa/40-kDa tryptic fragments (i.e., 40 kDa, 654–979; 37 kDa, 680–986) derived from radiolabeled islet cell lysates would yield very similar results (8, 33).

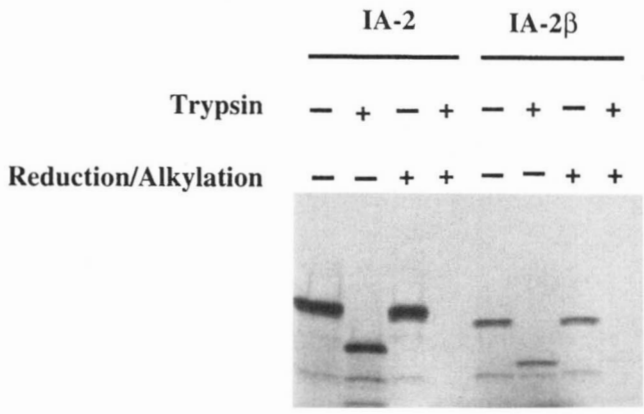

FIGURE 5. Trypsin digestion of reduced and alkylated in vitro-translated IA-2 and IA-2β separated by 10% SDS-PAGE.

Table I. *Radioimmunoprecipitation of IA-2 and IA-2β with IDDM sera*

	IA-2		IA-2β	
IDDM Sera	Native	Red/Alk[a]	Native	Red/Alk
1	++++[b]	++	+++	NR[c]
2	++++	+	++++	NR
3	++++	+	++++	NR
4	++++	+	++++	NR
5	++++	+	++++	NR
6	++++	+++	++++	NR
7	++++	+	+++	NR

[a] Red/Alk = reduction and alkylation.
[b] The intensity of precipitated bands by autoantibodies (3).
[c] NR = no reactivity.

Post-translational modifications, such as glycosylation and protein folding, are known to influence the recognition of autoantigens by autoantibodies. For example, the glycosylated, but not the nonglycosylated, form of the thyrotropin receptor is recognized by autoantibodies (34, 35). In contrast, our studies show that the nonglycosylated form of IA-2/IA-2β from bacteria or from the in vitro transcription/translation system is readily recognized by autoantibodies in IDDM sera (23). Of more importance for the recognition of IA-2/IA-2β is protein folding. The identity of five cysteines in the intracellular domain of IA-2 and IA-2β and the demonstration that the reduced and alkylated molecules do not react or react only minimally with IDDM sera argue that disulfide bond formation is important in maintaining the major antigenic configuration for autoantibody binding. This might explain why liquid phase assays, which tend to preserve antigenic conformation, are more sensitive for detecting autoantibodies to IA-2 and IA-2β than assays such as enzyme-linked immunoassay and Western blots, which are more likely to recognize linear over conformation epitopes. Our findings also suggest that it is highly likely that T cells, which react with linear peptides, will recognize very different antigenic determinants on IA-2 and IA-2β than autoantibodies, which, as we showed here, react with conformational determinants.

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