Expression, characterization, processing and immunogenicity of an insulin-dependent diabetes mellitus autoantigen, IA-2, in Sf-9 cells

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SUMMARY

Autoantibodies to a 64-kD protein and a 40-kD tryptic fragment from pancreatic islets have been detected at high frequency in the sera of patients with insulin-dependent diabetes mellitus (IDDM). IA-2, a newly isolated transmembrane protein tyrosine phosphatase, is a major islet cell autoantigen in IDDM and the precursor of a 40-kD tryptic fragment. To express large quantities of recombinant IA-2 protein and analyse post-translational modifications we expressed full-length human IA-2 in baculovirus-infected Sf-9 cells. IA-2 expression was analysed by Western blot and by immunoprecipitation of ^{35}S-methionine-radiolabelled proteins with rabbit antisera or IDDM sera. A 120-kD IA-2 protein was detected during the early, but not the late, phase of the infection. Pulse-chase experiments showed that the 120-kD protein was processed into fragments of 64 kD and smaller fragments of ≈ 50 kD, 38 kD and 32 kD. The 64-kD fragment appeared as a doublet. Tunicamycin and PNGase F treatment down-shifted the 120-kD protein and the 64-kD doublet into lower molecular weight bands, suggesting that both were glycosylated. Trypsin treatment converted the 120-kD protein and the 64-kD doublet into a 40-kD fragment. Baculovirus-expressed IA-2 was as sensitive or slightly more sensitive than *in vitro* translated IA-2 in detecting autoantibodies to IA-2: 66% of sera from newly diagnosed IDDM patients reacted with baculovirus-expressed IA-2 compared with 59% of the same sera which reacted with *in vitro* translated IA-2. It is concluded that baculovirus-expressed IA-2 is a good source of autoantigen and that a number of lower molecular weight fragments with which IDDM autoantibodies react are derived from the 120-kD full-length IA-2 molecule.

Keywords IA-2   protein tyrosine phosphatase   post-translational modification   baculovirus expression   autoantibodies

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is characterized by gradual destruction of pancreatic islets [1]. Autoantibodies to islet cells (ICA) are found many years before the onset of clinical diabetes and serve as predictive markers [1–5]. One of the autoantigens to which ICA react is IA-2, a transmembrane protein tyrosine phosphatase (PTP) [6–9]. A high percentage of prediabetics and diabetics have autoantibodies to IA-2 and these autoantibodies are useful markers for predicting and monitoring IDDM [7–14]. Full-length IA-2 cDNA encodes a protein of 979 amino acids with a molecular mass of 105 847 [6]. The protein has an intracellular, transmembrane and extracellular domain with a signal peptide. IA-2 is expressed primarily in neuroendocrine

cells including the pancreatic islets [6,15], and appears to be an intrinsic membrane protein of neuroendocrine secretory granules [16]. Trypsin treatment and blocking experiments revealed that IA-2 is the precursor of a 40-kD fragment which is immunoreactive with IDDM sera [10,12,13]. Studies with deletion mutants showed that the autoantibodies in IDDM sera are directed to the intracellular and not extracellular domain of IA-2 and primarily to the COOH-terminus of the intracellular domain [17,18]. Immunoprecipitation with IDDM sera of cell lysates expressing IA-2, however, failed to detect autoantigens in the expected molecular weight range of the full-length IA-2 molecule (106 kD) [12,19,20]. Instead, autoantigens in the 64-kD and lower ranges were found.

The present experiments, in the baculovirus system, were initiated to obtain large amounts of full-length IA-2 protein, examine the possibility that the full-length molecule was processed into smaller fragments within the cell, determine if these fragments could be detected by IDDM autoantibodies and compare the

immunoreactivity of baculovirus-expressed IA-2 with *in vitro* translated IA-2.

MATERIALS AND METHODS

Expression of human full-length IA-2 autoantigen in baculovirus-infected Sf-9 insect cells

An IA-2 recombinant transfer vector, designated pAc-hIA-2, was constructed by inserting a 3·2-kb SacI-KpnI fragment of full-length human IA-2 cDNA into the pAcSG-His NTA transfer vector (PharMingen, San Diego, CA). The reading frame of cloning sites was verified by sequence analysis. IA-2 recombinant transfer vector, pAc-hIA-2, was co-transfected into Sf-9 insect cells with BaculoGold baculovirus DNA using the calcium phosphate transfection method. After 6 days of transfection, the supernatant from the transfection plate was collected for plaque assay. In total, 15 plaques were selected for human IA-2 expression by Western blot analysis. The recombinant baculovirus, named Bac-hIA-2, was prepared through three cycles of infection of Sf-9 cells. Sf-9 cells were cultured as a monolayer in TN-MFH medium supplemented with 10% fetal bovine serum (FBS) at 27°C.

Antiserum and immunostaining

The intracellular domain of IA-2 protein was expressed in bacteria with glutathione S-transferase in pGEX vector (Pharmacia, Arlington Heights, IL). The GST-IA-2 fusion protein was induced with 0·1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and purified through a glutathione-agarose affinity column [21]. Recombinant IA-2 protein was used to immunize rabbits to generate rabbit polyclonal antiserum [10]. Preimmune rabbit serum served as the negative control. IDDM sera and age-matched normal control sera were kindly provided by Dr N. K. Maclaren (University of Florida, Gainesville, FL). For immunostaining, Bac-hIA-2 transfected Sf-9 insect cells were fixed at 4°C in acetone for 15 min, dried, and rehydrated in PBS. Cells were blocked with 10% normal goat serum for 30 min. Hyperimmune rabbit serum (1:400) or preimmune serum was then added to the cells at room temperature for 1 h. Fluorescence-conjugated goat anti-rabbit secondary antibody (1:500 dilution) was used for immunostaining.

Western blot analysis

Sf-9 cells were infected with Bac-hIA-2 recombinant virus (5–10 plaque-forming units (PFU)/cell) or wild-type virus at 27°C for 24–96 h. Whole cell lysate was prepared by extraction of Sf-9 cells with lysis buffer (20 mM Tris pH 8·0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 16 μg/ml benzamidine, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). Lysates were centrifuged for 30 min at 4°C and supernatants collected for analysis. The protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). Lysates (20 μg) were subjected to SDS–PAGE and transferred to a nitrocellulose membrane. Western blot analysis was carried out by blocking the membrane with 5% non-fat dry milk in PBS for 1 h, incubation with primary antibody for 1 h (rabbit antiserum 1:500 dilution), and then horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2000 dilution) for an additional hour. The membrane was developed in the ECL system (Amersham).

Metabolic labelling and immunoprecipitation

To prepare ^{35}S-methionine-labelled IA-2 recombinant protein, 2-day infected Sf-9 cells were starved with methionine-free Grace

medium for 1 h followed by incubation with ^{35}S-methionine at a concentration of 200 μCi/ml for 5 h. The cells were then washed with cold PBS and solubilized in 1% Triton X-100 lysis buffer. For the pulse-chase experiment, cells were pulsed with ^{35}S-methionine for 30 min, washed three times with cold medium, and then chased for various time intervals. Prior to immunoprecipitation, 10×10^6 ct/min extracts were pre-cleared with 10 μl of protein A-agarose for 1 h, followed by the addition of 5 μl of either anti-human IA-2 rabbit antiserum or IDDM sera at 4°C overnight. Immunocomplexes were precipitated by adding 50 μl of protein A-agarose for 1 h. The beads were washed twice with immunoprecipitation buffer (10 mM Tris pH 8·0, 150 mM NaCl, 1% Triton X-100) and twice with PBS. Immunoprecipitates were separated on 10% SDS–PAGE, fixed, dried, and exposed to x-ray film.

Tunicamycin and peptide: N-glycosidase treatment

Tunicamycin was dissolved in DMSO and added to Sf-9 cells as a final concentration of 10 μg/ml. During tunicamycin treatment, cells were labelled with ^{35}S-methionine for 5 h. Peptide:N-glycosidase F (PNGase F; Boehringer Mannheim, Indianapolis, IN) treatment was performed by washing immunoprecipitation complexes twice with PNGase F buffer containing 20 mM sodium phosphate buffer pH 7, 10 mM EDTA, 1% Triton X-100, 0·2% SDS and 1% 2-mercaptoethanol, followed by treatment with 10 μl of PNGase F (20 U/100 μl) for 20 h at 37°C.

Detection of IA-2 autoantibodies by radioimmunoprecipitation

Immunoprecipitation was performed as described [7]. Either ^{35}S-labelled baculovirus-expressed IA-2 or *in vitro* translated human IA-2 was incubated at 4°C overnight with 5 μl of sera in 100 μl of precipitation buffer (20 mM Tris–HCl, 1% Triton X-100, 0·15 M NaCl). Fifty microlitres of 50% (v/v) proteinA-agarose (Life Technologies, Rockville, MD) were then added to the reaction mixture for an additional hour. The immunoprecipitation mixture was washed four times with immunoprecipitation buffer and separated on 10% SDS–PAGE gel. The intensity of the IA-2 bands was scored independently by two investigators [7,8].

RESULTS

Expression of Bac-hIA-2 in Sf-9 insect cells

To study the expression and processing of IA-2 in eukaryotic cells, Sf-9 insect cells were infected with the recombinant baculovirus, designated Bac-hIA-2, which contained the IA-2 insert. Cells were then stained with a rabbit polyclonal antiserum raised against bacteria-expressed IA-2 intracellular domain. Figure 1b shows that Bac-hIA-2-infected cells stained brightly with antibody to IA-2 compared with the uninfected Sf-9 cells (Fig. 1a). Preimmune sera failed to stain infected or uninfected cells.

To study in greater depth the time course of IA-2 protein expression, lysates from Bac-hIA-2-infected, wild-type-infected and uninfected cells were examined by SDS–PAGE and Western blot at 24, 48, 72 and 96 h after infection. Figure 2 shows a strong 120-kD band at 48 h which gradually decreased in intensity. Beginning at 48 h, bands representing degradation and/or cleavage products of lower molecular weight (e.g. 90, 80 and 64 kD) increased in intensity, peaking at 96 h. At 6 days (data not shown) all of the bands except for the 64-kD band had disappeared.

Pulse-chase analysis of Bac-hIA-2

Sf-9 cells, infected with Bac-hIA-2, were pulsed for 30 min with
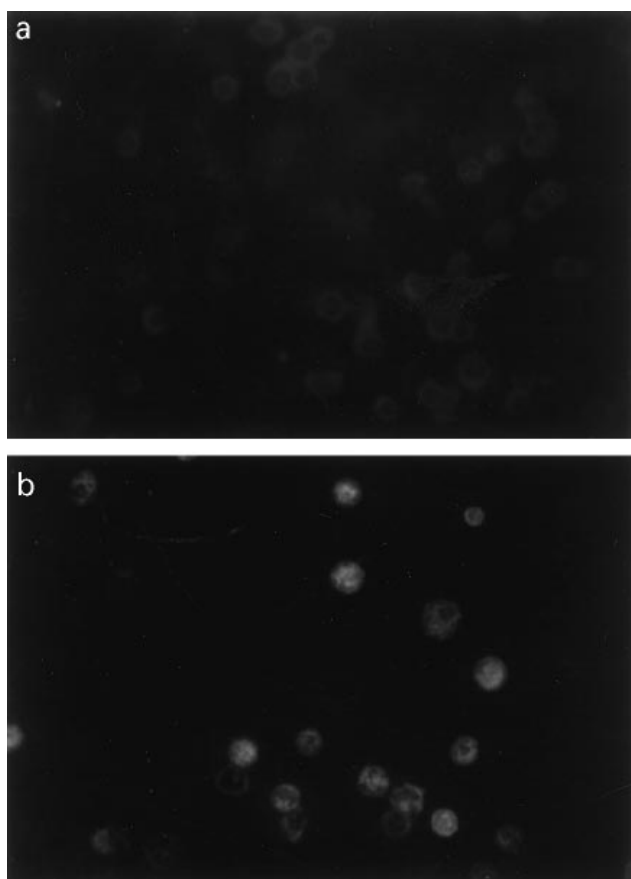
Fig. 1. Expression of human IA-2 protein in Bac-hIA-2-infected Sf-9 cells. Uninfected (a) and 2 day-infected (b) Sf-9 cells were stained with rabbit antisera to IA-2 intracellular domain.

[35]S-methionine and chased for 24 h. As seen in Fig. 3, at the end of the 30-min pulse (zero time), a broad 120-kD band was present, probably due to the multiple initiation sites at the $5'$ end of the IA-2 cDNA [6]. The intensity of the band increased over the next 4 h. By 9 h the band had markedly decreased in intensity and was absent at 24 h. In contrast, the 64-kD IA-2 band, which appeared as a doublet, was still prominent at 9 and 24 h. Bands representing smaller fragments of ≈ 50, 38 and 32 kD were most pronounced at 2 and 4 h, decreased in intensity by 9 h and were absent by 24 h.

Bac-hIA-2 is a glycoprotein

Based on the predicted protein sequence, IA-2 contains two potential N-linked glycosylation sites. IA-2 protein isolated from a rat insulinoma cell line bound wheat germ agglutinin lectin, suggesting that IA-2 is a glycoprotein [12]. To substantiate this possibility, we treated infected Sf-9 cells with tunicamycin which inhibits N-linked glycosylation. As seen in Fig. 4, both the 120-kD and the 64-kD proteins showed a small but reproducible down-shift in their molecular mass, whereas the 50-kD and 38-kD proteins retained the same molecular mass. Similarly, a small down-shift in molecular mass was noted when we treated infected Sf-9 cell lysates with PNGase F, which cleaves N-glycan sugar chains on glycopeptides and glycoproteins [22,23].
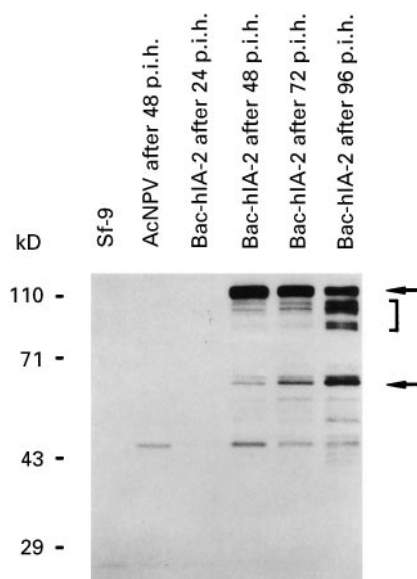
Fig. 2. Time course of Bac-hIA-2 expression in Sf-9 cells. At 24, 48, 72 and 96 h post-infection cellular lysates were subjected to 10% SDS–PAGE and immunoblotted with rabbit antiserum to the intracellular domain of human IA-2. The arrows point to the 120-kD full-length human IA-2 protein and the 64-kD IA-2 doublet. The bracket shows degradation products of IA-2. Wild-type baculovirus (AcNPV).

Conversion of 120-kD and 64-kD IA-2 proteins into a 40-kD tryptic fragment

As shown in Figs 3 and 4, IA-2 expressed in Bac-hIA-2-infected Sf-9 cells undergoes autoprocessing into multiple fragments (e.g. 64 and 50 kD). To determine whether these fragments are the precursors of the 40-kD tryptic fragment, we treated the infected cell lysates with trypsin. As seen in Fig. 5, trypsin converted the 120-, 64- and 50-kD fragments into a distinct 40-kD tryptic fragment.
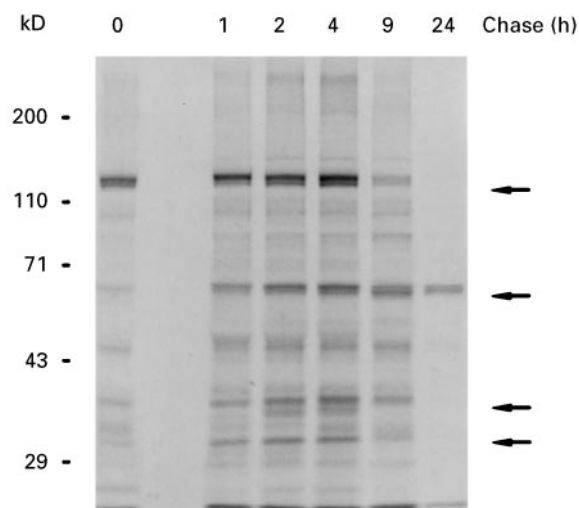
Fig. 3. Molecular processing of Bac-hIA-2 protein in Sf-9 cells. Sf-9 cells were pulsed for 30 min with ^{35}S-methionine (zero time) and chased for 1, 2, 4, 9 and 24 h. Cellular lysates were then immunoprecipitated with anti-IA-2^{+} insulin-dependent diabetes mellitus (IDDM) sera and subjected to 10% SDS–PAGE. Arrows point to the 120-kD and 64-kD IA-2 proteins and the various fragments of IA-2 being processed during the chase.
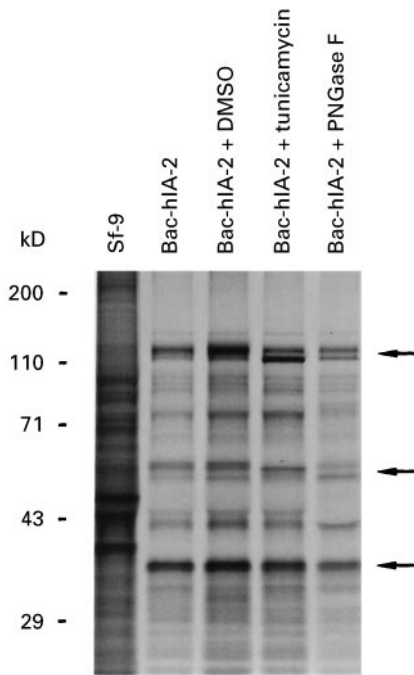
Fig. 4. Effect of tunicamycin and peptide:N-glycosidase F (PNGase F) on Bac-hIA-2 protein. Sf-9 cells were treated with 0·1% DMSO or tunicamycin (10 μg/ml) before being infected with Bac-hIA-2 recombinant virus and labelled with ^{35}S-methionine. The cell lysates then were immunoprecipitated with insulin-dependent diabetes mellitus (IDDM) sera and subjected to 10% SDS–PAGE. Immunoprecipitated proteins were treated with PNGase F (lane 5) before being subjected to gel separation. The arrows show the molecular mass and down-shift of the 120- and 64-kD proteins.

Radioimmunoprecipitation of baculovirus-expressed human IA-2

To determine whether baculovirus-expressed IA-2 protein reacts with IDDM autoantibodies, radiolabelled cell lysates were precipitated with either IDDM or control sera. Figure 6 shows that a prominent band of 120 kD was precipitated by IDDM sera but not by control sera. A large number of IDDM and control sera were then tested for IA-2 autoantibodies using radiolabelled *in vitro* translated IA-2 [7] or radiolabelled baculovirus-expressed IA-2. As seen in Fig. 7, 66% of IDDM sera reacted with baculovirus-expressed IA-2, compared with 59% with *in vitro* translated IA-2. None of the 46 control sera reacted with recombinant IA-2. The correlation coefficient between the assays using baculovirus-expressed IA-2 and *in vitro* translated IA-2 (Fig. 8) was $r = 0·89$ ($P < 0·0001$). Four samples which were negative in the *in vitro* translated IA-2 assay were positive in the baculovirus-expressed IA-2 assay.

DISCUSSION

In an earlier study we expressed IA-2 in bacteria [10,24]. Baculovirus-infected insect cells, however, have a number of advantages over bacteria. First, they are eukaryotic cells and phosphorylate and glycosylate proteins [25–29]. Second, they correctly process proteins into bioactive peptides and target them to the nucleus or plasma membrane or allow them to be secreted into the media [30–32]. For example, the neuroendocrine protein, synaptophysin, is expressed as an N-glycosylated integral membrane protein in small synaptophysin-rich vesicles in Sf-9 insect
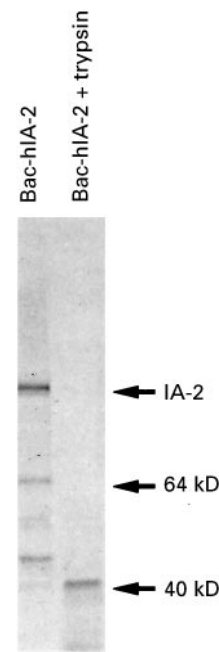

Fig. 5. Trypsin treatment of Bac-hIA-2 protein. Infected Sf-9 cell lysates were radiolabelled with ^{35}S-methionine, immunoprecipitated with insulin-dependent diabetes mellitus (IDDM) sera, treated with trypsin (50 μg/ml) for 30 min, and then subjected to 10% SDS–PAGE. The 120-, 64- and 50-kD IA-2 proteins were converted into a 40-kD tryptic fragment.

cells [30]. In the study reported here, we showed that large amounts of IA-2 were expressed in baculovirus-infected cells, and that the expressed protein retained its ability to bind autoantibodies. Moreover, radioimmunoprecipitation of baculovirus-expressed IA-2 protein with IDDM sera showed that it was as sensitive if not more sensitive than *in vitro* translated IA-2 protein. Baculovirus-expressed IA-2 should be a good source of antigen for cell-mediated autoimmune studies in patients with IDDM.
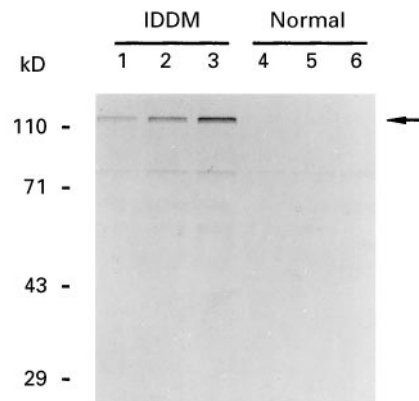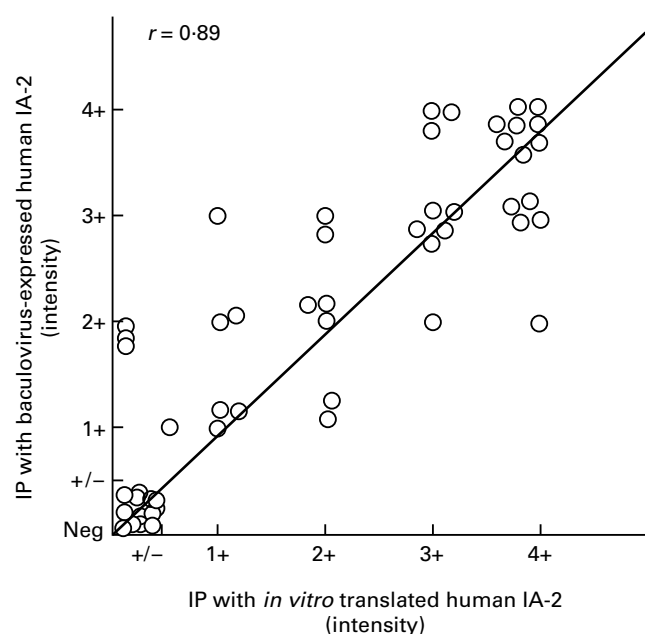

Fig. 6. Immunoprecipitation of baculovirus-expressed human IA-2 with insulin-dependent diabetes mellitus (IDDM) serum. Sf-9 cells were labelled with ^{35}S-methionine 48 h after infection with Bac-hIA-2 recombinant virus. Various amounts of labelled cell lysates (lanes 1 and 4, 40 000 ct/min; lanes 2 and 5, 80 000 ct/min; and lanes 3 and 6, 60 000 ct/min) were immunoprecipitated either with IDDM sera (lanes 1–3) or control sera (lanes 4–6) and then subjected to 10% SDS–PAGE.
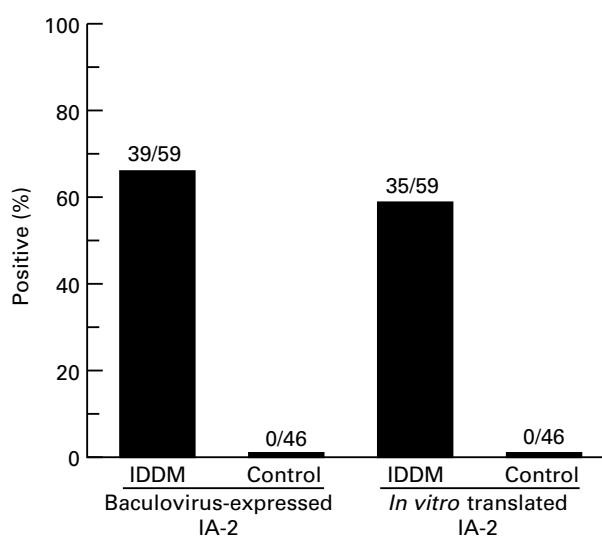
Fig. 7. Comparison of the reactivity of baculovirus-expressed IA-2 and *in vitro* translated IA-2 with insulin-dependent diabetes mellitus (IDDM) sera. Sera from 59 new-onset IDDM patients and 46 healthy controls were tested by radioimmunoprecipitation with either baculovirus-expressed IA-2 (80 000 ct/min) or *in vitro* translated IA-2 (100 000 ct/min).

The full-length IA-2 molecule was detected early in the course of the infection, but gradually disappeared, being replaced by a 64-kD doublet and a number of fragments of lesser molecular mass. The full-length molecule (approx. 120 kD) was, in fact, larger than that predicted on the basis of its amino acid composition (106 kD). In this context, analysis of the amino acid sequence revealed two potential N-linked glycosylation sites in the extracellular domain of IA-2 [6]. Treatment of the baculovirus-infected cells with tunicamycin or the 120-kD IA-2 molecule with PNGase F down-
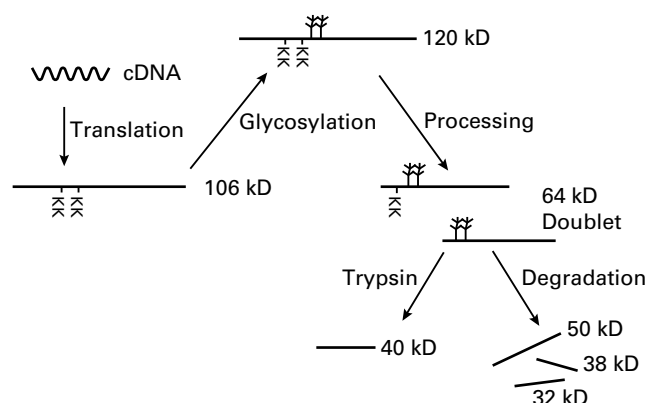
Fig. 9. Post-translational modification of IA-2. Full-length IA-2 is processed by glycosylation and protease cleavage. The dibasic amino acids (KK) represent the potential cleavage sites from which the 64-kD doublet is generated. Trypsin treatment converts the 120-kD and 64-kD proteins into a 40-kD fragment.

shifted the molecular mass of IA-2, suggesting that the larger than expected molecular mass was due, at least in part, to glycosylation.

Pulse-chase experiments support the argument that the full-length 120-kD molecule is the precursor of the 64-kD doublet. Dibasic amino acids are a common site for protease cleavage [33] and consecutive lysines are found in IA-2 at amino acid positions 386–387 and 448–449. Cleavage at these sites would result in a doublet in which the two bands would differ from each other by 62 amino acids. Solimena *et al.* [16] recently isolated a 70-kD fragment from bovine pituitary and showed that the fragment began at position 449 immediately after the dibasic lysines. They did not, however, observe a doublet. The difference in the molecular mass of the IA-2 fragment from baculovirus-infected cells (64 kD) compared with bovine pituitary (70 kD) probably represents a difference in the degree of glycosylation of IA-2 in the two cell types. Similarly, the demonstration of a doublet in baculovirus-infected cells, but not in bovine pituitary, could be due to differences in the expression and abundance of proteases in the different cell types.

Earlier studies, using recombinant IA-2, showed that trypsin treatment of the full-length 106-kD molecule resulted in a 40-kD fragment which retained its immunoreactivity with sera from IDDM patients [8,10,12–14]. The current experiments show that the 120-kD, 64-kD and smaller 50-kD molecules also can be converted, upon treatment with trypsin, into immunoreactive fragments of ≈ 40 kD. From the studies described here (Fig. 9), we conclude that post-translational modification and processing of IA-2 is responsible for the failure to detect the 106-kD or 120-kD autoantigens in the cell types examined thus far, and that the 64-kD fragment is probably the major naturally occurring cleavage product of IA-2 to which autoantibodies in IDDM sera react.

Fig. 8. Correlation between the intensity of immunoprecipitated IA-2 bands with baculovirus-expressed IA-2 and *in vitro* translated IA-2 ($r = 0.89$, $P < 0.0001$).

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