

Insulin as a Surface Marker on Isolated Cells from Rat Pancreatic Islets

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ABSTRACT Immunoreactive insulin was shown to exist as a surface molecule in the plasma membrane of dispersed rat pancreatic islet cells. The intact cells were stained by immunofluorescence with a guinea pig antisera specific for insulin. The hormone on the cell surface could not be accounted for by insulin bound to specific receptors or nonspecifically adsorbed to cells. Thus, surface insulin was demonstrated to be a specific membrane antigen for islet cells. Furthermore, the proportion of islet cells with insulin on the cell surface was directly correlated with insulin secretion in several different settings. This correspondence was demonstrated by varying the glucose concentration in the medium, by withholding Ca^{2+} , which inhibits secretion, and by adding theophylline, which potentiates secretion. Consequently, these results suggested that insulin as a membrane protein was a marker for cells that actively secreted the hormone and may have been derived in the fusion process of secretory granules with the plasma membrane.

Plasma membranes form the boundary between the internal biochemical machinery of a cell and its immediate milieu. In this pivot of importance the lipid bilayer with its constituent polypeptide and polysaccharide components mediates essential interactions that determine the functioning of the cell and thereby which ultimately affect the physiology of the organism. Thus, the plasma membrane plays a vital role in immune regulation, cellular differentiation, ligand internalization, regulation of cellular metabolism, maintenance of electrochemical differences, and mitotic activity. Although cell surface markers of plasma membranes from freely circulating blood cells have been extensively investigated, markers on cells from solid organs including the mammalian endocrine system have not.

β cells from the pancreatic islet produce and secrete insulin. The secreted hormone consists of two polypeptides, the A- and B-chains, joined by two disulfide bonds; however, like other peptide hormones, it is initially synthesized in a larger precursor form (1). Preproinsulin has 25 additional amino acids on the N-terminus and a polypeptide chain, C peptide, which connects the A-chain and the B-chain. Because of its hydrophobicity, the additional N-terminal amino acids are considered to act as a leader sequence in the vectorial translocation during synthesis. Preproinsulin has been synthesized in cell-free translation of mRNA that was extracted from islet cells and enriched by affinity of the polyadenosine 3' tail to deoxythymidine immobilized on a cellulose column (2–4).

Consequently, cDNA to rat proinsulin mRNA was used to isolate rat insulin genes, and two nonallelic genes were found and sequenced (5). Furthermore, studies of β cells have characterized the physiologic factors and pharmacologic agents that influence insulin synthesis and secretion (6). Histologic techniques have revealed that secretory granules contain insulin, and it is thought that release of the hormone occurs via fusion of this organelle with the plasmalemma (7). Thus, the biosynthesis and cellular biology of the secreted insulin hormone have been intensively investigated.

In this paper the possibility that insulin exists as a surface marker on pancreatic islet cells was tested. It was determined that insulin was expressed in the plasma membrane as a consequence of the secretory process, and the biology of this expression was investigated.

MATERIALS AND METHODS

Materials: Male Sprague-Dawley rats (BW 180–200 g) were purchased from Sasco (O'Fallon, MO), collagenase (Type IV) was purchased from Worthington Biochemical Corp. (Bedford, MA), and culture medium (1066) was purchased from Gibco Laboratories (Grand Island, NY). Lyophilized guinea pig antiporcine insulin antiserum (AIS¹, Lot 523) was a gift of Peter Wright (Indiana University); also, guinea pig antiporcine insulin antiserum was purchased from Linco (Eureka, MO). Guinea pig antibovine insulin antiserum was purchased from Miles Laboratories, Inc. (Naperville, IL). Porcine insulin

¹Abbreviations used in this paper: AIS, anti-insulin antiserum; KRB, Krebs's Ringer Bicarbonate buffer; NGPS, normal guinea pig sera.

was a gift of Lilly, Eli, & Co. (Indianapolis, IN). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation: Islets from 5–6 rats were isolated by collagenase digestion (8) and purified on Ficoll gradients. The freed islets were then washed in Hanks' solution and individually selected under a stereo microscope. The islets were then suspended in Ca^{2+} - and Mg^{2+} -free Hanks' solution and dispersed into isolated cells with dispase (9). The dispersed cells were resuspended in CMRL 1066 containing 150 mg% glucose, 1% glutamine, 10% inactivated fetal calf serum, and 1% penicillin and streptomycin, and cultured for various times prior to staining for surface insulin. Rat hepatocytes were isolated by the collagenase perfusion technique (10).

Cell Surface Immunofluorescence: After incubation in culture medium, $1\text{--}2 \times 10^5$ islet cells were washed thoroughly in phosphate-buffered saline, pH 7.2, 5 g% BSA, 0.1% sodium azide. Incubation with AIS was performed with varying amounts of guinea pig anti-insulin antisera or normal guinea pig serum in phosphate buffered saline, pH 7.2, 0.5 g% BSA, and 0.15% sodium azide. After 30 min at 4°C the cells were washed three times and incubated for another 30 min at 4°C in 0.1 ml with 10% fluoresceinated rabbit anti-guinea pig IgG (Miles Laboratories, Inc.) in diluent. Cells were washed and then scored for surface fluorescence with a Leitz Orthoplan microscope. Dead cells demonstrated homogeneous uptake of the stain and were not counted. Surface fluorescence was defined as staining coincident with the plasma membrane. At least 100 cells were counted for each determination. Maximal surface staining reached a plateau at an antisera dilution of 1:20, and this level remained constant up to a dilution of 1:2. In these experiments, either a 1:10 or a 1:2 dilution of guinea pig anti-insulin antisera was used. Normal guinea pig serum controls were always incubated with a 1:2 dilution. For the experiments demonstrating cap formation, the cells were incubated with AIS at 37°C in culture medium without sodium azide, and they were stained with the fluoresceinated antibodies as described above.

Measurement of Insulin Secretion: Supernatants from cell incubation were frozen at -60°C prior to assay for insulin. Immunoreactive insulin was determined by alcohol precipitation using porcine insulin as the standard (11).

RESULTS

Demonstration of Cell Surface Insulin

Rat pancreatic islets were isolated and then dispersed into a suspension of individual cells. After these cells were incubated in CMRL 1066 culture medium for several hours, they were stained in the presence of azide and at 4°C with antisera specific for insulin (AIS) and then with a fluoresceinated second antibody. Fig. 1*a* shows an example of a cell exhibiting surface fluorescence. Grains of fluorescent stain outlined the plasma membrane of the islet cell. There was no internal stain; shadows of fluorescence seen within the boundary of the cell were actually out-of-focus grains present on the surface in another plane. Besides the morphological appearance of the staining, evidence that the fluorescence was a cell surface phenomenon was obtained by staining the islet cells with AIS at 37°C in the absence of azide (Fig. 1*b*). This protocol allows for patching and capping of cell surface molecules, a process that requires connections to the microfilaments of the cytoskeleton (12–13). In Fig. 1*b*, two contiguous cells can be seen. One cell had developed a cap and the other several patches. These cells were representative of the positive cells stained with AIS at 37°C; the percent stained was the same with either protocol. Thus, the fluorescent staining was characteristic of a cell surface event.

The specificity of the AIS used in these experiments had been previously determined in a radioimmunoassay (11); the specificity of the islet cell staining with AIS was also ascertained. The data shown in Table I demonstrate that normal guinea pig sera (NGPS) did not contain any immunoglobulin that could bind to islet cells. Moreover, the antibody in AIS that stained the cells could be absorbed by insulin conjugated to a gel but not by the gel left unconjugated. Furthermore, insulin added to the islet cells during incubation with AIS

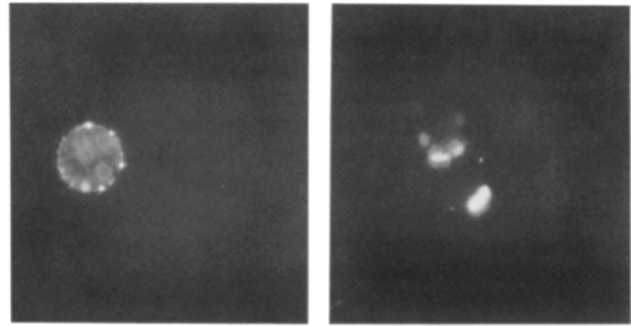


FIGURE 1 Surface insulin on islet cells. As described in Materials and Methods, pancreatic islets were isolated and stained sequentially with AIS and fluoresceinated rabbit anti-guinea pig IgG (left) at 4°C in the presence of sodium azide and (right) at 37°C in the absence of sodium azide. Cells were then washed and scored for surface fluorescence with a Leitz Orthoplan microscope.

TABLE I
Specificity of Antisera

Antisera	Immunoabsorbent*	% Cells with surface fluorescence†
AIS	—	73
NGPS	—	5
AIS	Unsubstituted	61
AIS	Insulin	11

* Antisera was incubated overnight at 4°C with the immunoabsorbent affigel (Bio-Rad Laboratories, Richmond, CA) conjugated with porcine insulin according to manufacturers specifications.

† Cells were incubated in tissue culture medium for 20 h at 37°C. Cell surface insulin was assayed as in Fig. 1*a*.

competitively inhibited the binding of AIS to the islet cell surface (Fig. 2). The pertinent specificity in this reaction was insulin. Consequently, insulin existed on the cell surface of islet cells.

Immediately after dispersion only a few of the islet cells exhibited surface fluorescence; however, this parameter markedly increased with incubation at 37°C in complete medium (Fig. 3). In the experiment presented, the percent cells with surface fluorescence gradually increased for several hours and then reached a plateau of 76% at 6 h. The staining intensity varied considerably among the positive cells and in time it tended to increase. The percent cells staining at the plateau was stable; it was maintained for up to 48 h.

Since the percent islet cells with surface insulin increased with incubation at 37°C, the temperature dependence of this effect was studied by incubating the cells at 4°C (Fig. 4). At this lower temperature there was only a marginal increase in the cells with surface fluorescence, and the addition of exogenous insulin to account for the lack of secretion at 4°C did not change that result. Thus, the increase of surface insulin with time was temperature-dependent, and this requirement was not fulfilled simply by the presence of insulin in the medium. The addition of exogenous insulin to the incubation medium at either 4 or at 37°C did not affect the surface fluorescence indicating that insulin per se did not bind to the cell surface.

Further indication that the surface staining did not represent insulin adsorbed to the surface was obtained by using two media that abrogate effects of nonspecific binding; a hypertonic saline solution and Ca^{2+} -free medium were used. Cells that were incubated for 1 h in CMRL 1066 complete

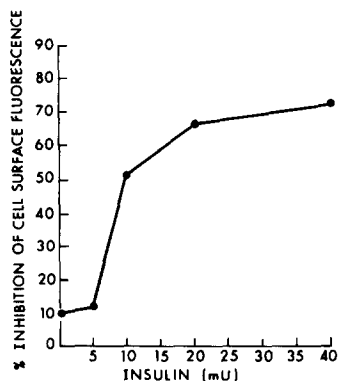


FIGURE 2. Insulin inhibition of cell surface fluorescence. Cells were obtained as in Fig. 1 and incubated for either 4 or 20 h. Staining with AIS was accomplished as in Fig. 1a; varying amounts of porcine insulin were added to the cells together with the AIS. The results were similar regardless of incubation time, and they are combined in this figure.

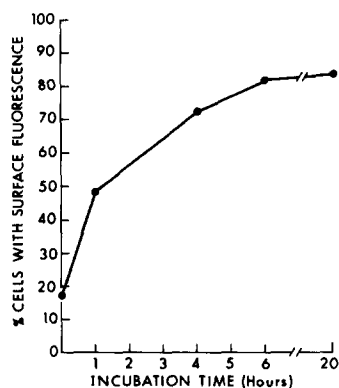


FIGURE 3. Time dependence of cell surface fluorescence. Cells were obtained as in Fig. 1 and incubated in tissue culture medium for varying times. Staining with AIS was accomplished as in Fig. 1a.

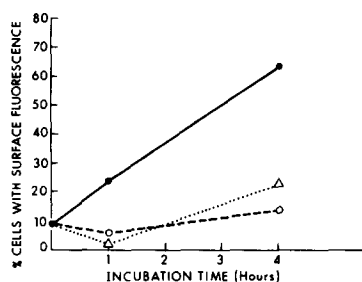


FIGURE 4. Temperature dependence of cell surface fluorescence. Cells were obtained as in Fig. 1 and incubated for varying times at either 4 (Δ) or 37°C (\bullet); 20 mU of porcine insulin was added to one group incubated at 4°C (\circ); the reaction volume was 0.1 ml. Staining for surface insulin was accomplished as in Fig. 1a.

medium with 5 mM theophylline (see below for the effect of theophylline) showed 49% surface staining, and an aliquot of these cells that was washed in 1 M saline immediately before the assay stained at a 44% level. Likewise, cells incubated for 3 h in the same medium demonstrated 54% surface staining, and this proportion of cells with surface insulin was decreased marginally to 50% by prior washing in 1 M saline. Moreover, the absence of calcium in the medium did not decrease the staining of positive cells. Rat pancreatic islet cells were dispersed into a single cell suspension, and then incubated overnight in CMRL 1066 complete medium. An aliquot of the cells was removed and 3 mM EGTA, which is sufficient to chelate all of the Ca^{2+} in the medium, was added. After an additional hour of incubation at 37°C, the cells with and without EGTA were tested for surface insulin. The proportion of cells staining in the group without EGTA was 41% and in the group with EGTA 44%. Similarly, after 2 h of incubation there was no decrease in the proportion of cells staining or the intensity of staining in the group incubated in 3 mM EGTA. Thus, these results suggested that surface insulin was not adsorbed to the cell surface but that it was incorporated in the plasmalemma as an integral protein.

Distinction between Surface Insulin on Islet Cells and Insulin Bound to Receptors

Because it has been shown that there are cells within isolated pancreatic islets that possess insulin receptors and that dispersed cells of an insulinoma also have insulin receptors (14–16), a series of experiments were designed to see if the fluorescence on the surface of islet cells could represent insulin bound to an insulin receptor. Two schemes were pursued. First, other cells that possess insulin receptors were investigated with the assay to detect surface insulin. Human transformed B lymphocyte lines and the human line IM-9, which are known to possess a large number of insulin receptors (17), were incubated with insulin. After incubation with insulin these cells did not bind the specific antisera more than the nonimmune sera (data not shown). Moreover, isolated rat hepatocytes were incubated for 0.5–2 h in medium without insulin, then different amounts of insulin were added either at 37 or at 4°C, either with or without azide; in no case were hepatocytes stained with AIS (Table II). Thus, the antisera used in these experiments did not recognize insulin bound to insulin receptor. The second protocol relied on the finding by several investigators using different systems that tunicamycin, a glycosylation inhibitor, totally inhibits the formation of functional insulin receptors (18–20). It is shown in Table II that the addition of tunicamycin to the incubation medium of the dispersed islet cells resulted in an increase in the percent cells with surface insulin. Besides the one-third increase in the number of cells stained with AIS, the brightness of fluorescence per cell was noticeably greater in the cells incubated with tunicamycin than those incubated in the control medium. This increased fluorescence in the tunicamycin-treated islet cells was observed in three consecutive experiments. Insulin bound to insulin receptor on the surface of islet cells did not account for the surface insulin detected with the fluorescence assay.

Correlation of Surface Insulin and Insulin Secretion

Nutrients in the islet cell milieu stimulate secretion of insulin. The relationship between surface insulin and insulin

TABLE II
Antisera Does Not Bind to Insulin Bound to Insulin Receptor

Antisera	Tissue origin of dispersed cells	Insulin incubation*	Glycosylation inhibitor	% Cells with surface fluorescence
AIS	Pancreatic islet	—	—	51
NGPS	Pancreatic islet	—	—	3
AIS	Pancreatic islet	—	Tunicamycin [†]	68
NGPS	Pancreatic islet	—	Tunicamycin [†]	1
AIS	Liver	+	—	0

* Rat liver cells were isolated (10) and incubated in medium with or without serum for 1–2 h. Then, the cells were incubated in the presence of insulin. This incubation has been attempted with eight different protocols that varied the amount of insulin from 1 mU/ml to 1 U/ml, the time of incubation from 0.5–2 h, the presence of azide, and the temperature from 4 to 37°C. Each experiment produced identical results.

[†] 1 $\mu\text{g/ml}$ tunicamycin was added to the culture at the beginning of the 48-h. incubation. Equivalent amounts of alcohol vehicle were added to the control cultures.

secretion was investigated by incubating the islet cells for 3 h in Krebs Ringer Bicarbonate buffer (KRB) with variable concentrations of glucose, a potent secretagogue. As shown in Fig. 5, the proportion of islet cells expressing surface insulin increased with increasing glucose concentrations. Without glucose in the medium cell surface insulin was not seen; a maximal proportion of cells was stained at 11 mM glucose and there was no further increase at 28 mM. The supernatants of these cultures were assayed for insulin, and the levels of secretion for the different glucose concentrations correlated to the proportion of cells with surface insulin (Fig. 5). Islet cells cultured in CMRL medium, which contains both glucose and amino acids, had more cells staining with anti-insulin antibodies than cells cultured in KRB medium at any glucose concentration, and this larger proportion of cells with surface insulin was also reflected in an increased amount of insulin secreted into the medium. Moreover, cells incubated at a high glucose concentration but without Ca^{2+} showed depressed insulin secretion, and the proportion of cells with surface insulin was also depressed. Thus, there was a positive correlation between the level of insulin secretion by islet cells and the expression of insulin on the cell surface.

Further characterization of the correspondence between insulin secretion and surface insulin was achieved by exposing the islet cells to an active pharmacologic agent during the 3 h-incubation after dispersion. Theophylline, a drug that increases intracellular cAMP levels, was used because it is known to potentiate insulin release by islet cells (21). Fig. 6 demonstrates the effects of the drug on both secretion and surface fluorescence. With 5 mM theophylline in the medium there was an augmentation of insulin secretion and the proportion of cells stained with AIS at every glucose concentration tested.

The longevity of insulin on the surface of islet cells was estimated by utilizing the different stimulatory capacities of the various media. Islet cells were cultured overnight in complete CMRL 1066 medium and then half of the cells was transferred to KRB without glucose. Aliquots of the cells were assayed for staining with AIS at various times afterwards. Surface fluorescence of cells in the medium without glucose decreased to half the value of the plateau level maintained by the cells in complete medium in ~ 2 h (data not shown).

DISCUSSION

Insulin has been shown to be a cell surface marker on dispersed cells from rat pancreatic islets. Because capping has been demonstrated, it seems that surface insulin was a membrane protein connected to the internal cytoskeleton. Its presence was correlated to insulin secretion and could not be accounted for by binding to receptors or by nonspecifically associating with membrane components. The failure to detect insulin bound to receptor indicated that bound insulin no longer displayed the antigenic determinants that AIS bound, probably a consequence of a steric change in receptor-bound insulin or a masking of the relevant portions of the molecule.

This demonstration that insulin was a surface molecule for islet cells is the first example of dual localization of a hormone in secretory granules and in plasma membrane. One possible explanation for surface insulin is an alteration of the posttranslational cleavage of preproinsulin into proinsulin. The *N*-terminal portion of preproinsulin is hydrophobic. If it was not cleaved, the molecule would be expected to remain

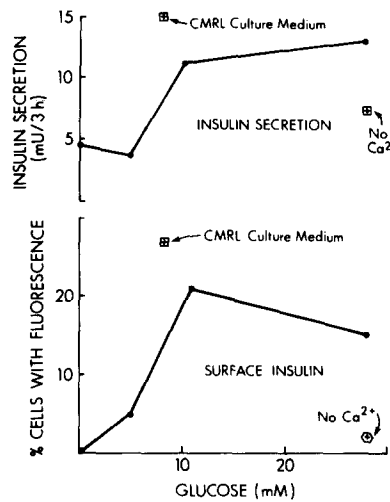


FIGURE 5. Correlation of surface insulin with insulin secretion. Islet cells were obtained as in Fig. 1 and incubated for 3 h in KRB medium with various concentrations of glucose. Cultures were also established in CMRL medium and in KRB medium with glucose but without Ca^{2+} . The upper panel represents a radioimmunoassay for insulin in the culture supernatants (11). The lower panel shows cell surface staining with AIS performed as in Fig. 1a.

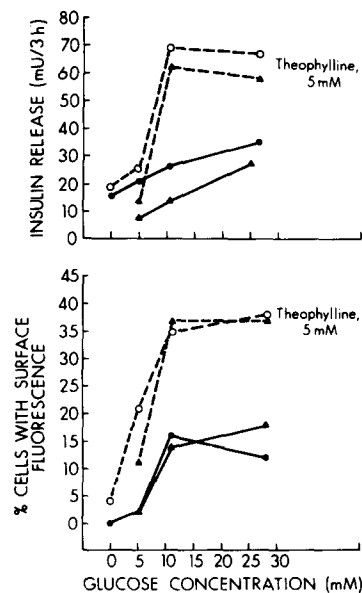


FIGURE 6. Theophylline potentiation of surface insulin and insulin secretion. Cells were obtained and treated as in Fig. 5 with or without the addition of 5 mM theophylline. Assays were performed as in Fig. 5. Results of two experiments are shown (circles and triangles). Points connected with a dashed line indicate the addition of theophylline.

anchored in the rough endoplasmic reticulum, to be included in the membrane that constituted the secretory granules, and to eventually reach the plasma membrane as a consequence of emiocytosis of this organelle. Failure to cleave preproinsulin could result from sequestration of the necessary enzymes or simply the level of efficiency of the reaction. Immunofluorescence is capable of detecting as few as 10^5 molecules on the cell surface. If this explanation is correct, then the presence of membrane-bound insulin might be used as a marker to study the kinetics and dynamics of intracellular membranes and organelles (22). An alternative explanation is either a covalent or noncovalent association of cellularly derived insulin with another polypeptide. The prototype of a covalent association is the B lymphocyte that produced immunoglobulin for secretion as well as for the cell surface. In the case of IgM, two species of mRNA exist: they are identical except for an extra piece that codes for the C-terminal membrane portion of the molecule (23). Surface insulin might also have resulted from two species of mRNA; however, an extra membrane piece has not been described for insulin. Finally, because surface insulin was correlated with secretion, it is conceivable that insulin was derived from a portion of the secre-

tory granular core. This possibility cannot be excluded; however, thorough washing of the cells with 5 g% BSA or 1 M saline before staining, an approximate membrane insulin half-life of 2 h, and failure to detect fixed portions of the granular core on the cell surface in electron micrographs (data not shown) made this possibility less likely. Preliminary data indicate that parathormone is also a cell surface molecule among cells of the parathyroid (unpublished data), and recently, other experiments have suggested that luteinizing hormone on the cell surface identifies a subpopulation of anterior pituitary cells (24). Thus, this phenomenon may be a general occurrence in secretory cells.

The correspondence between surface insulin and insulin secretion was demonstrated in several different settings. It is clear that the functional event of hormone release correlated with this structural property that could be observed at the cellular level. Consequently, it seems that surface insulin was a marker of cells that actively secreted the hormone. Although the basis of this structure-function relationship has not been determined, testing for membrane-bound insulin in the internal membrane of secretory granules could help to explain the correlation.

Islet cells stained by AIS have not formally been demonstrated to be β cells. Dispersed cells from pancreatic islets are 80–90% β cells, and this value corresponded to the percent cells with surface insulin. Furthermore, the proportion of islet cells with surface insulin is closely correlated to the level of insulin secretion. Since insulin was secreted by β cells, it seems likely that the majority of the stained cells were also β cells. Testing of this hypothesis could be achieved by combining the technologies of fluorescence-activated cell sorting and histology. Such technology may also be utilized to yield a purified population of β cells.

Theophylline potentiated insulin secretion and augmented the proportion of cells that stained with AIS. Because insulin secretion and the percent cells demonstrating surface insulin were positively correlated, it seems likely that theophylline acted by increasing the proportion of cells secreting the hormone. However, the possibility that theophylline also increased the secretion of insulin per cell has not been excluded.

Heterogeneity of β cells has been proposed as a possible explanation for the multiphasic nature of glucose-induced insulin secretion that occurs in perfused pancreas and perfused islets (25). Since a definitive approach to heterogeneity has not yet been developed, kinetic analysis of surface insulin might be appropriately used to dissect the various components in the insulin response.

Islet cells constitute an important organ for the physiologic response to nutrition. Their importance is underscored by the widespread pathology that results from their destruction. Because of the possibility of an autoimmune etiology in diabetes mellitus, studies of the islet cell surface have been undertaken. Indeed, investigators have demonstrated antibodies specific for the islet cell surface in approximately a third of patients with insulin-dependent diabetes mellitus; however, the target molecules for the antibodies in these studies, as well as for antibodies raised xenogeneically against islet cell plasma membranes, were not determined (26, 27). In a more defined study, Class I but not Class II antigens of the major histocompatibility complex were demonstrated on the surface of murine pancreatic β -cells (28). These antigens have been biochemically characterized; nevertheless, they are not specific for islet cells. In the present study a cell surface molecule

specific for β cells of the pancreatic islet has been described. Thus, it seems that surface insulin is antigenically intact and available only on β cells of the pancreatic islet. Since autoimmune destruction of islets may cause diabetes mellitus, it is possible that this process could occur via surface insulin.

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