The Gly40Ser Mutation in the Human Glucagon Receptor Gene Associated With NIDDM Results in a Receptor With Reduced Sensitivity to Glucagon

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The pancreatic islet hormone, glucagon, stimulates hepatic glucose production and has also been shown to potentiate glucose-induced insulin secretion. Because glucagon is a key regulator of glucose homeostasis, its receptor, which mediates the actions of glucagon, was considered a candidate gene involved in the pathogenesis of NIDDM. We have previously reported that a single heterozygous missense mutation in exon 2 of the glucagon receptor gene, which changes a glycine to a serine (Gly40Ser), is associated with NIDDM in a French population. In the present study, the signaling properties of this mutant receptor were examined in baby hamster kidney cells and rat insulinoma cells (RIN-5AH) stably transfected with either the wild type or Gly40Ser mutant human glucagon receptor cDNAs. Competition assays using ^{125}I-labeled glucagon were performed, and in both cell types, the Gly40Ser mutant receptor was found to bind glucagon with an approximately threefold lower affinity compared with the wild type receptor. In both cell types, the production of cAMP in response to glucagon was decreased in cells expressing the mutant receptor compared with those expressing the wild type. Finally, glucagon-stimulated insulin secretion by RIN cells expressing the mutant receptor was decreased such that the dose-response curve was shifted to the right in comparison to that obtained with cells expressing the wild type receptor. These results indicate that this single-point mutation located in the extracellular region of the glucagon receptor decreases the sensitivity of target tissues to glucagon. *Diabetes* **45:725–730, 1996**

Glucagon, secreted from the pancreatic islet α-cells in response to low blood glucose levels, is a key hormone involved in maintaining euglycemia primarily by stimulating hepatic glucose production (1,2) and also by potentiating glucose-induced insulin secretion (3–5). The biological effects of glucagon are mediated by its binding to specific GTP-binding protein (G-protein)–coupled receptors expressed on the plasma membrane of target tissues. Activation of these receptors by glucagon results in the production of cAMP as well as a rise in cytosolic free Ca^{2+}, which then mediate the physiological response to the hormone. The expression cloning of the rat hepatic glucagon receptor revealed that it indeed is a member of the superfamily of G-protein–coupled receptors having the characteristic seven transmembrane domains (6). In addition, it was shown that the cloned receptor could activate both cAMP-dependent and calcium-dependent intracellular signaling pathways. The human glucagon receptor gene, which has subsequently been cloned, contains multiple introns and is located on chromosome 17q25 (7,8).

In view of glucagon's central role in maintaining glucose homeostasis as well as our recent studies demonstrating the upregulation of glucagon receptor expression in both hepatocytes (9) and pancreatic islets (10) by high glucose concentrations, the glucagon receptor has been considered a likely candidate gene in the development of NIDDM. Because it is a heterogeneous multifactorial disease arising from genetic as well as environmental factors, the molecular mechanisms involved in the development of hyperglycemia in NIDDM have been difficult to elucidate. The candidate gene approach has thus proven useful in the detection of mutations in genes involved in regulating blood glucose in subsets of diabetic subjects. In a previously reported study, we scanned the glucagon receptor gene for mutations by single-stranded conformational polymorphism analysis in a cohort of French and Sardinian NIDDM patients from multiple case families and identified a single heterozygous missense mutation that changed GGT40 (Gly) to AGT40 (Ser) (Gly40Ser) (11). Furthermore, an association between this mutation and diabetes was also observed in the French population and confirmed among German diabetic subjects (W. Poller, C. C. Bollen, F. Merklein, P. Froguel, and J. Hager, unpublished observations).

Since this Gly40Ser mutation in exon 2 of the glucagon receptor gene is located in what corresponds to the extracellular domain of the receptor (Fig. 1), the question was raised as to whether such a point mutation would have any effect on the signaling capacity of the receptor and ultimately the biological response to glucagon. Therefore, in this study, we have determined the functional consequences of this mutation by introducing the Gly40Ser mutation into the human glucagon receptor cDNA and by establishing baby hamster kidney (BHK) fibroblast and rat insulinoma (RIN)-derived 5AH cell lines stably expressing the wild type or

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BHK, baby hamster kidney; EC_{50}, effective concentration giving 50% activity; FCS, fetal calf serum; GIP, gastric inhibitory polypeptide; GLP-I, glucagon-like peptide I; PCR, polymerase chain reaction; RIN, rat insulinoma; WT, wild type.

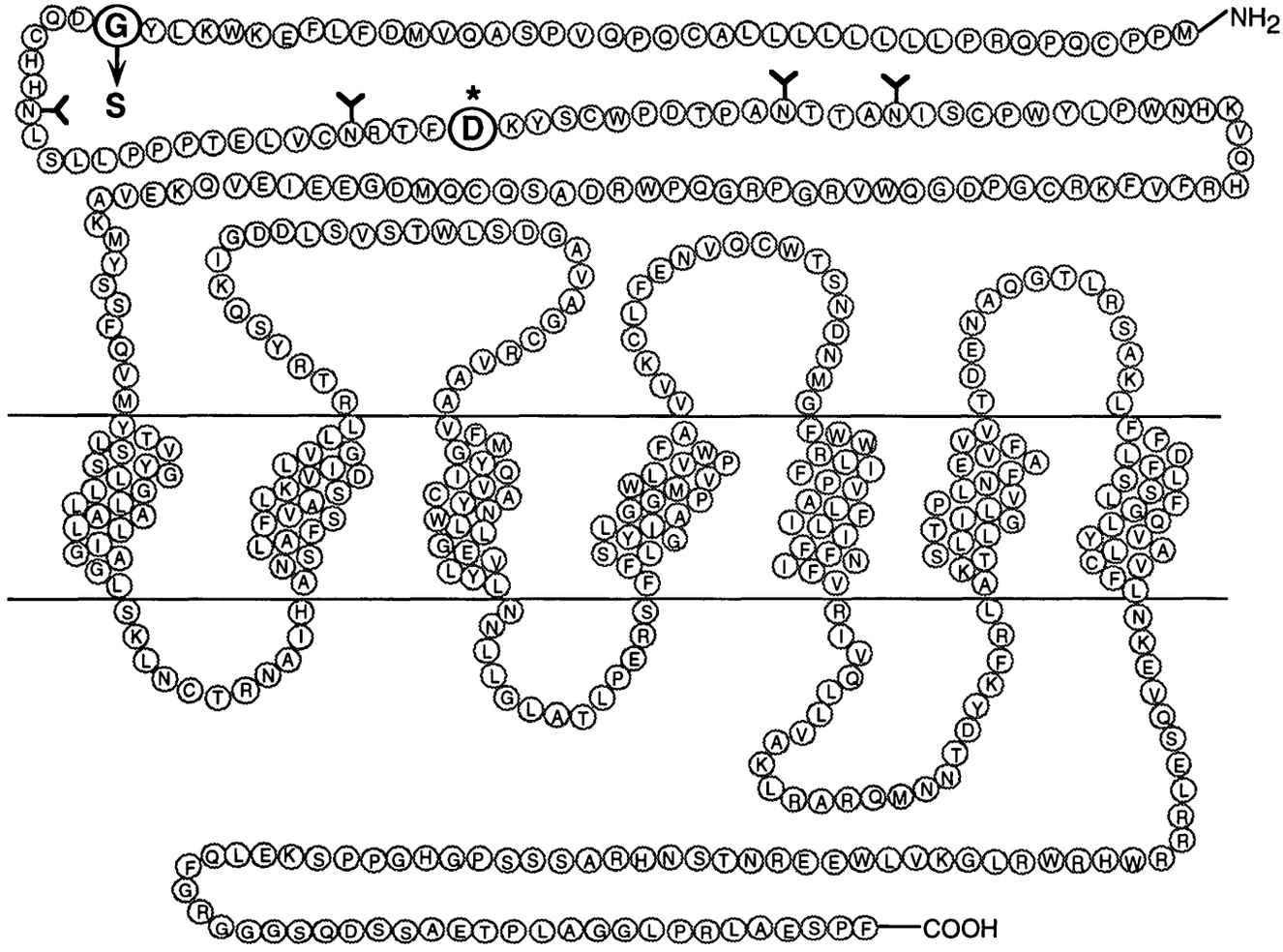

FIG. 1. Schematic representation of the predicted topological structure of the human glucagon receptor. The amino terminal is extracellular while the COOH-terminal is intracellular. The Gly40Ser mutation is denoted by the arrow, and the residue corresponding to the highly conserved aspartic acid at position 64 in the rat glucagon receptor (63 in the human) is marked by an asterisk. Four putative N-linked glycosylation sites are indicated by Y.

mutated receptor. As a means of determining receptor function, we have compared the glucagon binding and glucagon-induced cAMP production mediated by the wild type or mutant receptors expressed in these cell lines. Finally, as a measure of a biological endpoint, glucagon-stimulated insulin secretion from RIN cells expressing either the wild type or mutant receptor was compared.

RESEARCH DESIGN AND METHODS

Site-directed mutagenesis. The Gly40Ser mutation was introduced into the human glucagon receptor cDNA pLJ6 (7), a plasmid suitable for expression studies, according to the previously described polymerase chain reaction (PCR) procedure for site-directed mutagenesis by overlap extensions (12). Using pL J6 cDNA as the template for the initial reaction, the PCR-generated fragment containing the mutant sequence was then subcloned into pBhGR, which is the *Eco*RI/*Xba* I fragment representing the full-length human glucagon receptor cDNA in pBluescript SK$^+$ (Stratagene). The *Eco*RI/*Xba* I fragment containing the Gly40Ser mutation was then ligated into the corresponding region of pL J6 and referred to as pL J6G40S. Finally, the entire region of pL J6G40S, which was generated by PCR, was sequenced, confirming the presence of the G-to-A substitution and assuring that no other mutations were created during any of the PCR reactions.

Cell culture and cell transfections. BHK cells were cultured in Dulbecco's modified Eagle's medium DMEM (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine, while RIN-5AH cells were cultured in RPMI 1640 media (Gibco BRL) with the same above mentioned additions. BHK cells were transfected with the wild type (pL J6) and mutant (pL J6G40S) glucagon receptor cDNAs using the Lipofectamine Reagent (Gibco BRL) essentially as described by the manufacturers. Cotransfection with the pRSVneo plasmid, which carries the dominant selection marker for neomycin resistance, was performed to select for transfected cells by adding 0.6 mg/ml geneticin G418 2 days after the transfection. At this time, the cells were split 1:1,000 or 1:2,000 and after 7 days in culture, individual colonies were selected and independent lines were established and checked for glucagon receptor expression by ^{125}I-labeled glucagon binding. Stable transfections of RIN cells with pL J6 and pL JG40S were performed by electroporation as previously described (13). Again, pRSVneo was cotransfected for selection purposes, although in this case, the RIN cells were cultured for 6 days after transfection before 0.25 mg/ml G418 was added. After a further 6 days, the G418 concentration was increased to 0.6 mg/ml. The surviving colonies were picked, and independent lines were established and screened for ^{125}I-glucagon binding.

Ribonuclease protection assay. The procedure described previously for glucagon and glucagon-like peptide I (GLP-I) receptor ribonuclease protection assay was followed (10). For each assay, 10 μg of total RNA, isolated from 10^8 nontransfected RIN cells or those transfected with the rat glucagon receptor cDNA (pL J4) (6), was used.

Binding assay. Competition binding analyses were performed in six-well plates. Nontransfected BHK and RIN cells or those transfected with the wild type or mutant glucagon receptors were seeded out (200,000 cells/well) and cultured overnight (BHK cells) or for 2 days (RIN cells). Before binding, the culture medium was removed and the cells were washed with 3 ml of binding buffer (25 mmol/l HEPES, 124 mmol/l NaCl, 4 mmol/l KCl, 2 mmol/l K_2HPO_4, 1.5 mmol/l $MgCl_2$, and 1 mmol/l $CaCl_2$). The cells were incubated at room temperature in 1 ml of binding buffer

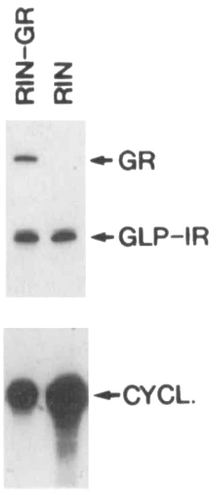

FIG. 2. Autoradiogram from a ribonuclease protection assay for glucagon and GLP-I receptor mRNA detection in RIN cells. Ten micrograms of total RNA from RIN cells transfected with the rat glucagon receptor cDNA (RIN-GR) or from nontransfected RIN cells were examined. The arrows labeled GR, GLP-IR, and CYCL indicate glucagon receptor, GLP-I receptor, and cyclophilin-specific protected bands, respectively.

containing 2.5% bovine serum albumin (Sigma), ~50,000 cpm ^{125}I-glucagon (specific activity ~400 μCi/μg) (Novo Nordisk), and varying concentrations of unlabeled glucagon. After 120 min, the cells were washed three times in ice-cold binding buffer, then removed from the wells with 1 ml of 1 mol/l NaOH and finally counted in a γ-counter. Competition data were fitted to a one-site binding model using a Marquardt nonlinear fitting algorithm (14), and the resulting fitted curves were displayed in Scatchard coordinates.

cAMP measurements. Cells were seeded out in six-well plates and grown as described in the previous section for the binding assays. Before stimulation, the cells were washed once in Hanks' balanced salt solution (Sigma) and once in RPMI containing 0.5% FCS. Subsequently, 1 ml of RPMI with 0.5% FCS and 0.45 mmol/l isobutyl-1-methyl-xanthine (IBMX) was added to each well along with varying concentrations of glucagon (in duplicate). After a 20-min incubation at 37°C, 750 μl of ice-cold 65% ethanol was added to each well, and the cells were scraped off and the suspension transferred to eppendorf tubes. An additional 500 μl ethanol was used to wash the well and the 2 vol were combined and centrifuged at 300*g* for 15 min. The supernatants were dried down overnight in a speed-vac, and the resulting pellets were stored at −20°C until assayed. Intracellular cAMP concentrations were measured using the cAMP ^{125}I scintillation proximity assay system (Amersham). The acetylation protocol described by the manufacturer was used. The dried cell extracts were resuspended in 1 ml assay buffer and further diluted 1:200 for BHK cells and 1:100 for RIN cells before assay. The cAMP determinations were normalized to cell number.

Insulin measurements. RIN cells were seeded out, grown, and washed as described in the previous section for the cAMP measurements. The cells were incubated for 30 min at 37°C in 1 ml of RPMI medium containing 0.1% bovine serum albumin and varying concentrations of glucagon (in duplicate). The medium was then removed and stored at −20°C until assayed. Cells in two wells were counted for an estimate of cell number. For the radioimmunoassay, rat insulin was used as the standard (Novo Nordisk), and the ethanol precipitation method described previously (15) was followed. The samples were diluted 1:10 in assay buffer and assayed in duplicate.

RESULTS

Receptor mRNA expression in RIN-5AH cells. To check for the expression of endogenous glucagon receptors in RIN-5AH cells, we used the highly sensitive ribonuclease protection assay to detect the presence of glucagon receptor mRNA and also the related GLP-I receptor mRNA in nontransfected RIN cells and those transfected with the rat glucagon receptor cDNA. As shown in Fig. 2, no glucagon receptor message could be observed in the nontransfected RIN cells, whereas GLP-I receptor mRNA was abundant. The expression of glucagon receptor mRNA in the RIN cells transfected with the glucagon receptor cDNA was also confirmed.

Effect of the Gly40Ser mutation on glucagon binding. The ^{125}I-glucagon binding displacement curves presented in Fig. 3 demonstrate that the affinity of the Gly40Ser mutant receptor is lowered compared with that of the wild type (WT) receptor when expressed in either BHK or RIN cells. The mean ± SD of six individual binding experiments are plotted. Two clonal lines expressing the Gly40Ser mutation are presented for both the BHK and RIN cells. All clones selected express a similar number of receptors per cell. For the clones shown in Fig. 3, the receptor numbers per cell were as follows: BHK/WT = 3×10^6, BHK/Gly40Ser = 4.8 and 4×10^6, RIN/WT = 2×10^5, and RIN/Gly40Ser = 2 and 3×10^5. In addition, we have tested four other mutant and two WT clones, all demonstrating this approximately three- to fivefold difference in binding affinity. For the particular clones shown in Fig. 3 from Scatchard analysis, the K_d values were calculated as 39.0 and 32.6 nmol/l for the two BHK clones expressing the mutant receptor, while the K_d value for the WT receptors was 10.3 nmol/l. In RIN cells, the K_d was 12.9 nmol/l for the WT receptors, whereas for the mutant receptors, it was increased to 69.5 and 88.7 nmol/l. Although there appears to be a slightly greater difference in K_d of the mutant receptors when expressed in RIN compared with BHK cells, this is most likely due to differences in cell types. To examine the specificity of binding, GLP-I and gastric inhibitory polypeptide (GIP), hormones structurally related to glucagon, were used to displace ^{125}I-glucagon binding to BHK cells transfected with either the WT or mutant glucagon receptor cDNAs. At concentrations of 300 or 1,000 nmol/l, there was no significant displacement by GLP-I or GIP in either the WT or mutant expressing BHK cells (Fig. 3*A*).

Effect of the Gly40Ser mutation on glucagon-stimulated cAMP production. As shown in Fig. 4, the cAMP

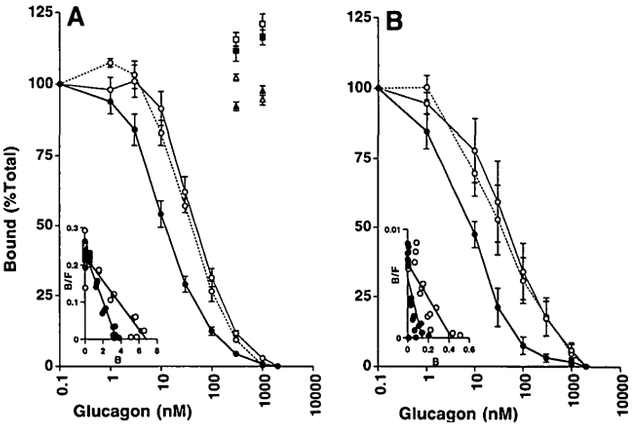

FIG. 3. Competition binding of ^{125}I-glucagon to cells transfected with the wild type or Gly40Ser mutant glucagon receptor cDNAs. *A*: one clone of BHK cells expressing the wild type glucagon receptors (●) and two independent clones expressing mutant receptors (○) are presented. Competition of ^{125}I-glucagon binding by GLP-I (■, □) and GIP (▲, △) are shown. The filled symbols are for BHK cells expressing the wild type and the open symbols for those expressing the mutant receptor. *B*: one clone of RIN cells expressing the wild type receptor (●) and two independent clones expressing the mutant receptor (○) are shown. Each point represents the mean ± SD of six independent experiments, each performed in triplicate. The insets show Scatchard analyses of wild type (●) and Gly40Ser mutant (○) glucagon receptor expressing BHK (*A*) and RIN (*B*) cells.

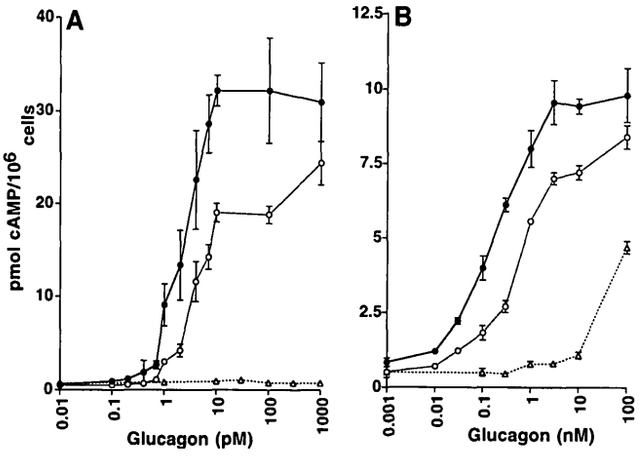

FIG. 4. Glucagon-stimulated cAMP production in BHK (*A*) and RIN (*B*) cells expressing the wild type or Gly40Ser mutant receptor. *A*: nontransfected BHK cells (△) or those transfected with the wild type (●) or mutant (○) glucagon receptor cDNAs. *B*: nontransfected RIN cells (△) or those transfected with the wild type (●) or mutant (○) glucagon receptor cDNAs. Each graph is a representative of at least three experiments, and each point is the mean of 4 determinations ± SD.

production in response to glucagon is reduced in both BHK and RIN cells expressing the mutant glucagon receptor. Here we present a representative experiment comparing a WT and mutant clone. At least two other clones for each have been tested with similar results in that there is a shift in the dose response curve such that the effective concentration of glucagon required to give 50% stimulation of adenylate cyclase (EC_{50}) is increased in cells (both BHK and RIN) expressing the Gly40Ser mutant glucagon receptor. For the particular clones shown in Fig. 4, EC_{50} values were calculated as being 2 (WT) and 5 (Gly40Ser) pmol/l in the BHK cells and 0.2 (WT) and 0.8 (Gly40Ser) nmol/l in RIN cells. It is interesting to note that we consistently observed a reduction in the maximum cAMP production in cells expressing the mutant receptor when compared with the WT receptor. A difference in the level of receptor expression cannot account for the decreased cAMP production in cells expressing the mutant receptors, since the number of WT and mutant receptors is comparable as described above. No glucagon-stimulated cAMP could be detected in the nontransfected BHK control cells (Fig. 4*A*); however, we did observe a significant increase in intracellular cAMP levels in nontransfected RIN cells with high concentrations of glucagon (Fig. 4*B*).

Effect of the Gly40Ser mutation on glucagon-induced insulin secretion. In Fig. 5, the glucagon-stimulated insulin secretion by RIN cells expressing either the WT or mutant glucagon receptor is shown and demonstrates that RIN cells expressing the mutant receptor are less sensitive to glucagon since the dose-response curve is shifted to the right. However, since these RIN cells express both the endogenous GLP-I receptor and the transfected glucagon receptor, it was not possible to distinguish the effects contributed by these receptors; therefore, EC_{50} values could not be calculated. Furthermore, it appears that glucagon concentrations higher than those required for maximal cAMP production (10 nmol/l, Fig. 4) are necessary for stimulating insulin secretion. This may be attributed to the fact that second messengers other than cAMP (for example Ca^{2+}) are also involved in the stimulation of insulin secretion from islet β-cells. No consistent glucagon-stimulated insulin secretion could be measured from the nontransfected RIN cells. In addition to the clones shown in Fig. 4, we have examined two other independent clonal lines expressing either the WT or Gly40Ser mutant receptors and found a similar difference—a right-shift of the dose-response curve with the mutant receptor.

DISCUSSION

It is apparent from these studies that this single Gly40Ser mutation in the glucagon receptor impairs glucagon-mediated signaling and consequently reduces the sensitivity of target tissues to glucagon. Not only is the binding affinity of the mutant receptor decreased when expressed in BHK cells as we have previously reported (11), but we have now shown that this is also the case when the Gly40Ser mutant receptor is expressed in RIN cells. Because the extracellular NH_2-terminal domain of the glucagon receptor has been found to be required for ligand binding (16), it is possible that a change in a single amino acid may result in the perturbation of the tertiary structure, thus causing a lowered binding affinity. Although it can be speculated that this glycine residue at position 40 may be directly involved in ligand binding, it is most likely that the overall structure of the extracellular domain of the glucagon receptor is important for high affinity binding. As can be seen in Fig. 1, there are potential N-linked glycosylation sites located in the area of the Gly40Ser mutation that are important for structural determination. Furthermore, in a study by Carruthers et al. (17), substitutions of Asp^{64} in the rat glucagon receptor (Fig. 1) with Glu, Asn, Lys, or Gly all resulted in receptors that were completely unable to bind ^{125}I-glucagon, suggesting that the structural integrity of this region in the extracellular domain of the glucagon receptor is indeed essential for glucagon binding.

As expected from the reduced binding affinity in cells expressing the Gly40Ser mutant receptor, glucagon-stimu-

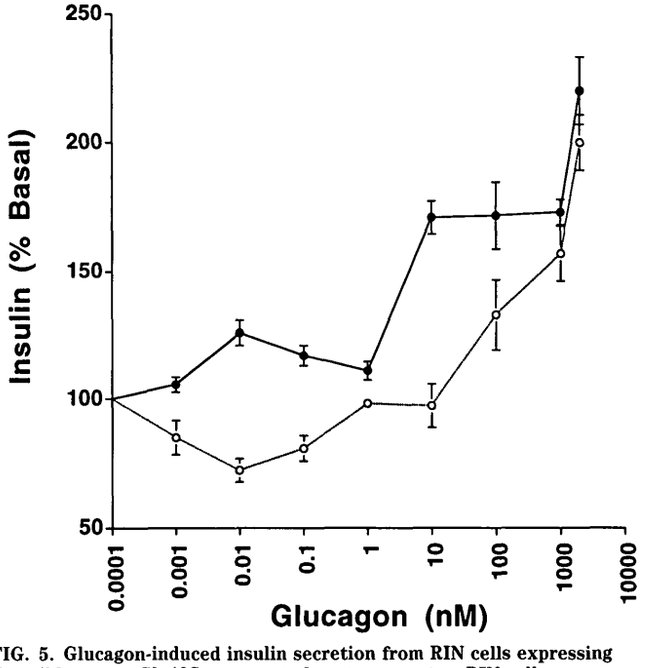

FIG. 5. Glucagon-induced insulin secretion from RIN cells expressing the wild type or Gly40Ser mutant glucagon receptor. RIN cells transfected with the wild type receptor cDNA (●) and RIN cells transfected with the mutant glucagon receptor cDNA (○) are represented. This is representative of three experiments, and each point is a mean of 4 determinations ± SD.

lated cAMP production mediated by the mutant receptor was found to be likewise decreased in both the BHK and RIN cells. The increase in cAMP production observed in the nontransfected RIN cells when stimulated with high glucagon concentrations is most likely due to the activation of endogenous GLP-I receptors, which are abundant in these cells as can be seen by the high level of GLP-I receptor mRNA expression in RIN cells (Fig. 2). We have also demonstrated that the RIN-5AH cells do not express endogenous glucagon receptor mRNA (Fig. 2), which makes the interpretation of transfection studies with the glucagon receptor cDNAs uncomplicated in these cells. Therefore, to measure a biological response to glucagon, we examined the insulin secretion in RIN cells transfected with the wild type or mutant glucagon receptors, since it has been shown that glucagon can stimulate insulin secretion by activating specific glucagon receptors on β-cells (5,18). The reduction in cAMP production in response to glucagon in cells expressing the mutant receptors was reflected by a lowered sensitivity to glucagon in RIN cells expressing the Gly40Ser receptors in terms of insulin secretion. The increase in insulin secretion observed when a high (2 μmol/l) concentration of glucagon was used is again assumed to be a result of the activation of endogenous GLP-I receptors expressed on the RIN cells.

Recently, Gough et al. (19) found not only a similar association between this Gly40Ser mutation and NIDDM, but also an increased frequency in probands of IDDM families, which strengthens the suggestion that this mutation may in some manner predispose carriers to diabetes. However, other recent studies demonstrating that this Gly40Ser mutation in the glucagon receptor is absent in Japanese (20) and Finnish (21) populations of NIDDM patients exemplify the genetic heterogeneity of diabetes and also suggest that the contribution of this mutation to the development of diabetes may be limited. Furthermore, because the Gly40Ser mutation has only been observed so far as a heterozygous mutation, it is uncertain as to what the functional consequences would be in the situation where there are 50% wild type and mutant receptors expressed on the same cell. Thus, whether this Gly40Ser mutation in the glucagon receptor contributes to the pathogenesis of NIDDM, and if so, understanding the molecular basis, is certainly of primary concern. Our present study describing a clear functional effect of the Gly40Ser mutation on glucagon receptor–mediated signal transduction suggests that this mutation may in some manner contribute to the development of hyperglycemia that is characteristic of NIDDM, but we can only speculate as to the mechanisms involved.

One possibility that we had considered was that the mutation may increase the binding affinity of glucagon receptors for structurally related hormones such as GLP-I and GIP, thus leading to inappropriate activation of the glucagon receptor. However, we did not observe any affect of the Gly40Ser mutation on the ability of either GLP-I or GIP to displace ^{125}I-glucagon binding, which argues against this possibility. Taking into consideration that specific glucagon receptors are present in pancreatic islets (10) and β-cells (5,18) and that glucagon's primary target organ is the liver, it can be postulated that a mutant glucagon receptor could lead to β-cell and/or hepatic defects, which may contribute to the development of NIDDM. In view of glucagon's described insulinotropic effects (3,5) and our findings of reduced glucagon-mediated insulin secretion from RIN cells expressing the mutant glucagon receptor, this Gly40Ser mutation, which causes a lowered sensitivity to glucagon, may be considered to be involved in the development of islet β-cell dysfunction, leading to an insufficient insulin response that occurs in some NIDDM patients. The role this mutation may play in terms of causing hepatic dysfunction, however, is not readily apparent because a decrease in glucagon receptor sensitivity in the liver would be expected to result in a reduction in glucose production, a situation that is contrary to what is observed in many NIDDM patients. We can thus offer one possibility based on our previous studies showing that glucagon is an important factor in downregulating its own receptor expression in cultured rat hepatocytes (9). It is conceivable that the Gly40Ser mutation decreases the sensitivity of the glucagon receptor to glucagon such that the receptor cannot be sufficiently downregulated, thereby leading to inappropriate hepatic glucose production.

Although several speculations can be made as to if and how a mutation that causes a reduction in glucagon receptor function can contribute to the pathogenesis of NIDDM, it is clear that further studies are necessary to directly address this issue. Thus, thorough examination of the clinical characteristics of subjects carrying this Gly40Ser mutation in the glucagon receptor gene would certainly provide useful information.

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