Characterization of a Mutant Human Insulin Species*

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We have studied a chemically abnormal insulin species isolated from the pancreas of a patient with diabetes mellitus. The purified pancreatic preparation demonstrated a reduced ability to interact with insulin receptors of IM-9 cultured lymphocytes or isolated rat adipocytes, and its overall binding potency was 46% of normal. The ability of this insulin preparation to stimulate adipocyte glucose transport and oxidation was even further reduced and the overall biologic potency was 12% of normal. Amino acid composition studies have demonstrated a leucine substituted for phenylalanine in position 24 or 25 of the insulin B chain in approximately 60% of the molecules. This finding indicated that the patient's pancreas contained a mixture of normal and abnormal insulin species, such that the normal insulin had normal binding and biologic properties, while the substituted species was highly abnormal. The two insulins were separated based on their differential ability to bind to high affinity insulin receptors. The chromatographic (Sephadex G-50, Bio-Gel P-30) and electrophoretic (polyacrylamide gel) properties of the two separated insulin species and control insulin were identical. Furthermore, the normal insulin species had normal binding and biologic properties. In contrast, the abnormal or mutant insulin (containing the Leu → Phe substitution) was highly abnormal, displaying markedly diminished binding and biologic activity (<10% of normal). To explain the discrepancy between the binding and biologic potency of the insulin mixture, it was necessary to postulate that the mutant insulin could inhibit the biologic effects of normal insulin. This hypothesis was directly tested by mixing the purified separated mutant insulin species with normal insulin. These studies demonstrated that equimolar concentrations (5 × 10⁻¹¹ M) of the mutant insulin inhibited 60 to 70% of the effects of normal insulin to stimulate glucose transport and oxidation by rat adipocytes. This antagonistic effect was not mediated through inhibition of binding or degradation of normal insulin.

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The abbreviation used is: BSA, bovine serum albumin.

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EXPERIMENTAL PROCEDURES

Materials—Porcine monocomponent insulin was generously supplied by Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, Ind.). Na¹⁺ was purchased from New England Nuclear (Boston, Mass.); bovine serum albumin (Fraction V) was from Armour Pharmaceutical Co. (Phoenix, Ariz.); collagenase was from Worthington Biochemical Corp. (Freehold, N. J.); 2-deoxy[¹-¹⁴C]glucose, [¹⁵N]glucose, and L-¹⁴C]glucose were from New England Nuclear.

Induction of Insulin—¹⁵¹I-insulin was prepared at a specific activity of 100 to 150 μCi/μg according to the modification of Freychet et al. (4) of the method of Hunter and Greenwood (5) as described previously (6).

Preparation of Isolated Adipocytes—Animals were stunned by a blow to the head and decapitated, and epididymal fat pads were removed. Isolated fat cells were prepared by shaking at 37°C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (3 mg/ml) and BSA (40 mg/ml) according to the method of Rodbell (7). Cells were filtered through 250-μm nylon mesh, centrifuged at 400 rpm for 4 min, and washed three times in buffer (8). Adipocyte counts were performed according to a modification of Method III of Hirsch and Gallian (9), in which the cells were fixed in 2% osmium tetroxide in 0.05 M collidine buffer (made isotonic with saline) for 24 h at 37°C and then taken up in a known volume of 0.154 M NaCl for counting...
with a Coulter counter model ZB.

**Binding Studies**—Isolated fat cells were suspended in a buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM MgSO₄, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, 5% BSA (10), pH 7.6, and incubated with 115I-insulin and unlabeled insulin in plastic vials in a 24°C shaking water bath as described previously (8, 11, 12). Optimal steady state binding conditions were achieved at 24°C following 90 min of incubation. The incubations were terminated as described by Gorenflof and Glinnem (13) by removing 200 µl aliquots from the cell suspension and rapidly centrifuging the cells in plastic microtubes to which 100 µl of silicone oil had been added. IM-9 cultured lymphocytes were maintained in culture and prepared for binding studies as described by Gavin et al. (10, 14). Studies of insulin binding to these cells were performed according to the method of Gavin et al. (10, 14).

**Glucose Transport Studies**—Transport studies were performed using the same cell centrifugation technique as described for the binding studies; the details of this method have been reported previously (15, 16). Unless otherwise stated, isolated adipocytes were incubated with 2-deoxy-D-[1-14C]glucose (specific activity, 2 mCi/ml) at a concentration of 0.1 mM in Krebs-Ringer bicarbonate, pH 7.4, containing BSA (10 mg/ml) at 24°C. This assay measures the total uptake of the radiolabeled 2-deoxyglucose and is based on the principle that while 2-deoxyglucose is transported and phosphorylated by the same processes as glucose, it cannot be further metabolized (17). The assay was terminated at the end of 3 min by transferring 200 µl aliquots from the assay mixture to plastic microtubes containing 100 µl of silicone oil. The tubes were centrifuged for 30 sec in a Beckman microfuge; the assay is considered terminated when centrifugation begins. In experiments in which the stimulatory effect of insulin on uptake was measured, the cells were preincubated with insulin for 60 min at 24°C. The amount of sugar trapped in the extracellular water space of the cell layers was determined by using 1-14C glucose as described previously (18). Extracellular water space was measured in each experiment, and all data of sugar uptake were corrected for this factor.

**Glucose Oxidation**—The ability of adipocytes to oxidize glucose was determined according to the method of Rodbell (7). Adipocytes were incubated at 37°C with 1-14C glucose in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4% BSA. After 1 h of incubation, the generated 14CO₂ was collected and counted in a liquid scintillation counter.

**Chromatography**—Iodinated insulins and insulin mixtures were chromatographed on a Sephadex G-50 column (50 x 1.2 cm). All columns were then eluted with 1 M acetic acid and standardized with blue dextran and 125I. Recovery was >90% of the total amount of radioactivity applied in all cases. Separated iodinated insulins were also subjected to gel filtration on a column (0.9 x 30 cm) of Bio-Gel P-30 using 3% acetic acid containing 10 µg/ml of bovine serum albumin as the solvent. Electrophoresis of the separated insulins was carried out on polyacrylamide gels at pH 4.5. The sample, containing 10 µg of porcine insulin as carrier, was applied to a 15% acrylamide gel. After electrophoresis, the gels were fractionated into slices and radioactivity or immunoreactivity was determined; pronase Y was used as a tracking dye.

**RESULTS**

The insulin purified from the patient's pancreas yielded single sharp peaks on Sephadex G-50 and on polyacrylamide gel electrophoresis at pH 4.5 and 8.7 (Fig. 1). The purity of this preparation was further established by quantitatively comparing Lowry protein determinations to radioimmunoassay measurements. In all cases, over a range of protein concentrations, these two methods gave identical results (1). Consequently, in all the following studies, the concentration of the purified insulin preparation was determined by radioimmunoassay.

The effectiveness of the pancreatic insulin preparation in inhibiting 2-15P-insulin binding to either rat adipocytes (Fig. 2A) or cultured IM-9 lymphocytes (Fig. 2B) was markedly less than normal at all hormone concentrations, and the overall binding potency was only 46 ± 2% (mean ± S.E., n = 24) of native porcine insulin in both systems. Results with other low affinity insulins, i.e. proinsulin (5% potency) and desoctapeptide insulin (0.4% potency) are shown for comparison. Additionally, three separate human insulin preparations purified from the plasma of three normal individuals yielded results identical with those from porcine insulin (data not shown). Rates of insulin degradation were minimal (<7% of media insulin degraded at the end of the incubation) for both cell systems.

The biologic activity of this insulin preparation was even further reduced. Thus, the ability of this insulin preparation to stimulate either glucose transport (Fig. 3A) or glucose oxidation (Fig. 3B) was markedly impaired. At submaximal concentrations, much more of the patient's insulin was required to achieve a given degree of biologic activity as compared to native insulin. On the other hand, at high concentra-
tions, the patient’s insulin was able to elicit a fully maximal response. The half-maximally effective concentration was 2.5 ng/ml compared to the normal value of 0.3 ng/ml for an overall biologic potency of only 12 ± 1% (n = 29). Again, the low affinity insulins (proinsulin and desoctapeptide insulin) are shown for comparison.

We have demonstrated previously that the insulin purified from the patient’s pancreas contained 0.6 residue of leucine (theoretical 0) and 1.4 residue of phenylalanine (theoretical 2) in the COOH-terminal octapeptide fragment of the insulin B chain (1). The amino acid composition of the desoctapeptide portion was entirely normal. This placed the leucine substitution at either of the normal phenylalanine residues, i.e. B24 or B25. Furthermore, the lack of one-for-one substitution suggested heterogeneity in the insulin species produced by this patient, such that about 40% of the insulin molecules were normal and 60% were mutated, accounting for the reduced biologic properties. In order to explore this finding, it was necessary to devise a method which could separate the two proposed insulin species.

This was accomplished by performing a series of repetitive binding studies using IM-9 cultured lymphocytes. We reasoned that the normal insulin species should bind with normal high affinity to IM-9 lymphocyte receptors, whereas the abnormal mutant insulin species should bind poorly. This strategy is schematized in Fig. 4. The purified pancreatic insulin preparation, containing the theoretical 60:40 mixture of the two species was first iodinated, and the labeled insulin mixture was then exposed to IM-9 lymphocytes. Since the unsubstituted insulin binds normally, while the substituted species does not, the buffer should be relatively depleted of the normal insulin and enriched in the abnormal species. Incubations were performed at 15°C for only 30 min to minimize degradation of the free extracellular insulin. Following the

![Fig. 2. Ability of control porcine insulin (●—●), the purified insulin preparation isolated from the patient’s pancreas (●—●), proinsulin (●—●), and desoctapeptide (DOP) insulin (●—●) to inhibit 125I-porcine insulin binding to IM-9 cultured lymphocytes (A) or isolated rat adipocytes (B).](image)

![Fig. 3. Ability of control porcine insulin (●—●), the purified insulin preparation isolated from the patient’s pancreas (●—●), proinsulin (●—●), and desoctapeptide (DOP) insulin (●—●) to stimulate 2-deoxyglucose uptake (A) or glucose oxidation (B) by isolated rat adipocytes. All data are normalized to a cell concentration of 2 x 10^6 cells/ml.](image)

![Fig. 4. Repetitive receptor binding assay. The iodinated pancreatic insulin preparation containing the mixture of abnormal and normal insulins was incubated with IM-9 lymphocytes (20 x 10^6 cells/ml) at 15°C for 30 min. Following this, the cells were pelleted and the supernatant was exposed to a fresh aliquot of cells (so that the final concentration was again 20 x 10^6 cells/ml) at 15°C for 30 min. This procedure was then repeated once more. The final buffer was then applied to a column (50 x 1.2 cm) of Sephadex G-50. The initial cell pellet was washed twice in ice-cold buffer and the receptor-bound insulin was dissociated at pH 6.0. The insulin which was dissociated from receptors was then submitted to Sephadex G-50 chromatography. This procedure allows the separation of high affinity insulin, which binds to receptors, from low affinity insulin, which does not readily bind to receptors.

The initial incubation, the buffer (containing the free insulin) was transferred to fresh cells and the procedure was repeated twice. The concentration of labeled insulin mixture in the original incubation was 1 ng/ml, and the concentration of IM-9 lymphocytes used (20 x 10^6 cells/ml) will bind 60 to 70% of 125I-porcine insulin at 1 ng/ml. Consequently, it can be calcu-
FIG. 5. Chromatographic and electrophoretic analysis of the separated insulins. Sephadex G-50 gel filtration elution profiles of \textsuperscript{125}I-insulin dissociated from the initial cell pellets (A) and the \textsuperscript{125}I-insulin remaining free in the final buffer (B) from the repetitive binding assay (see the legend to Fig. 4 for details). Columns were calibrated with blue dextran, native insulin, and \textsuperscript{125}I. C, Bio-Gel P-30 chromatography of the insulin peaks (between cross marks in A and B) from the elution profiles of the normal (W) and abnormal (M) insulin species. Arrow, elution point of \textsuperscript{125}I-porcine insulin. D, polyacrylamide gel electrophoresis of the normal (– – –) and abnormal insulin (○ – ○) taken from the insulin peaks between the cross marks from the elution profiles in A and B. Arrow, migration point of \textsuperscript{125}I-porcine insulin. Samples were applied to 15% acrylamide gels and, after electrophoresis, the gels were sliced and radioactivity was determined.

The insulin species prepared in this manner were reconstituted in the appropriate buffer for studies of binding and biologic activity. Fig. 6 illustrates the ability of the two preparations, consisting of the theoretically separated insulin species, to bind to IM-9 lymphocytes. At a total \textsuperscript{125}I-insulin concentration of \(5 \times 10^{-11}\) M, the specific binding ability of the normal insulin species and porcine insulin were comparable. In striking contrast, the mutant insulin displayed only 9 \(\%\) \((n = 14)\) of normal binding potency. For comparison, the binding ability of the unseparated original iodinated mixture is presented and, as indicated earlier, its binding potency was \(46\%\) of normal. Nonspecific binding was minimal (1% of the total radioactivity added) and equal for all preparations. Results identical with those presented in Fig. 6 were obtained using isolated rat adipocytes or isolated circulating human lymphocytes.

It should be noted that, since the abnormal insulin exhibited \(10\%\) of normal binding potency, a small amount should bind to cells during the initial incubation with IM-9 lymphocytes. Thus, the normal insulin which was dissociated from the cells should be contaminated, to a small degree, with the mutant species. The fact that the normal insulin displayed 85 to 95% of normal binding and biologic potency is consistent with this consideration.
might not have been reached under the usual binding incubation conditions, and this could lead to an underestimate of overall binding potency. To evaluate this possibility, the association rates of the labeled mutant and normal species were determined (Fig. 7A) and compared to results with labeled porcine insulin. Although the amount bound was much less with the mutant species, the shapes of the curves were similar, and steady state binding conditions were clearly obtained by 90 min. If the time course of binding was uniquely slow using the iodinated mutant insulin species, then steady state binding might not have been reached under the usual binding incubation conditions, and this could lead to an underestimate of overall binding potency. To evaluate this possibility, the association rates of the labeled mutant and normal species were determined (Fig. 7A) and compared to results with labeled porcine insulin. Although the amount bound was much less with the mutant species, the shapes of the curves were similar, and steady state binding conditions were clearly obtained by 90 min.

Using tracer concentrations ($5 \times 10^{-11}$ M) of iodinated porcine, normal, and mutant insulin, the ability of unlabeled porcine insulin to inhibit binding was comparable (Fig. 7B). The absolute maximal percentages bound were 56, 51, and 4.5 for porcine, normal, and mutant insulin, respectively. Due to insufficient amounts of labeled mutant insulin, we were unable to perform a full competition curve; however, the results indicate that all three labeled insulins (porcine, mutant, and normal) bind to the same population of insulin receptors. In further support of this conclusion, an anti-insulin receptor antibody, which inhibits $^{125}$I-porcine insulin binding by 50%, at a titer of 1:100, had a comparable ability to inhibit binding of the $^{125}$I-mutant or $^{125}$I-porcine insulin.

The biologic activity of these two insulin species was also investigated and further abnormalities were identified. As summarized in Fig. 8, 25 ng/ml of insulin elicits a maximal stimulating effect on rat adipocyte glucose oxidation, and 0.3 ng/ml of insulin is a half-maximally effective concentration. The normal species has a biologic potency comparable to native insulin, whereas the mutant insulin species displays no biologic activity at this concentration. Dose-response studies showed that the normal species was similar to the control over the full concentration range. Identical results were observed when glucose transport was measured (data not shown).

When Figs. 2 and 3 or 6 and 8 are compared, it is obvious that a major discrepancy exists between the biologic activity and receptor-binding potency of this abnormal insulin. Thus, the biologic potency is much less than the binding potency, implying that receptor occupancy is necessary but not sufficient to initiate biologic action. This finding cannot be explained by postulating that the substituted insulin is a partial agonist or even a full antagonist (in the sense that it binds with low affinity to receptors but has no intrinsic biologic potency), since the difference between binding potency (46%) and biologic potency (12%) of the mixture is too great. Therefore, to investigate this discrepancy, the effect of the separated mutant insulin on the biologic action of native insulin was determined. When a submaximal concentration of either native porcine insulin or the patient's normal insulin was mixed with an equimolar amount of the mutant insulin, a marked inhibition of insulin action was observed (Fig. 9). Thus, the ability of a half-maximal concentration (0.3 ng/ml) of native porcine or the patient's normal insulin to stimulate adipocyte glucose oxidation (Fig. 9A) or glucose transport (Fig. 9B) was inhibited by 60 to 70% in the presence of the mutated insulin. This inhibitory property was not observed with a variety of other insulins, such as proinsulin, desoctapeptide insulin, desalanine desasparagine insulin, despentapeptide insulin, catfish insulin, or porcine insulin, carried through the same monocytes (data not shown).

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procedures as outlined in the legend to Fig. 4.

The mutant insulin behaves as an active antagonist of the biologic effects of insulin, and the magnitude of this inhibition over the entire glucose oxidation dose-response curve is demonstrated in Fig. 10. In the presence of a constant amount of mutant insulin (0.3 ng/ml), a marked inhibition of glucose oxidation is seen at submaximal native insulin concentrations, but this effect can be overcome at high levels of native insulin and a fully maximal response can be observed.

The inhibitory effect of the mutant insulin cannot be explained by inhibition of native insulin binding, since the tracer concentrations employed (0.3 ng/ml) were too low to compete with native insulin for receptor binding. This is confirmed by the data in Table I, which demonstrate that the percentage of normal insulin bound to adipocyte insulin receptors is unaffected by the addition of the mutant insulin. Furthermore, the ability of cells to degrade native insulin was not influenced by the addition of the substituted insulin (data not shown).

The unique specificity of the inhibitory effect of the mutant species on the insulin action mechanism is demonstrated in Table II. Spermine is an insulin-like agent which stimulates glucose transport and oxidation via a mechanism independent of the insulin receptor (19). On the other hand, anti-insulin receptor antibodies derived from patients with severe insulin resistance (20, 21) mimic insulin action via the receptor in a manner analogous to the native hormone. As can be seen in

![Fig. 9. Antagonistic effect of the mutant insulin. A, glucose oxidation was measured in the presence of 0.3 ng/ml of native porcine insulin (open bar) and 0.3 ng/ml of native insulin plus 0.3 ng/ml of mutant insulin (hatched bar). Native insulin (0.3 ng/ml) led to an approximately half-maximal stimulation of glucose oxidation. All data represent the mean ± S.E. of 11 separate experiments. B, stimulation of glucose transport by 0.3 ng/ml of native porcine insulin (open bar) and 0.3 ng/ml of native insulin plus 0.3 ng/ml of mutant insulin (hatched bar). All data represent the mean ± S.E. of seven separate experiments.](image)

![Fig. 10. Dose response curve of glucose oxidation by native porcine insulin (●●●) or native porcine insulin (at the indicated concentrations) mixed with a constant amount (0.3 ng/ml) of mutant insulin (---).](image)

Table II, the mutant insulin species inhibits the effects of the antireceptor antiserum on both glucose oxidation (A) and glucose transport (B), but has no effect on the ability of spermine to stimulate these processes. Thus, the mutated insulin is an antagonist which is specific for the mechanism of action of insulin.

**DISCUSSION**

The results of these studies have demonstrated that the insulin purified from the patient's pancreas was functionally abnormal, displaying reduced binding potency and even further reduced biologic activity. Previous results have shown that this insulin contains a leucine substituted for phenylalanine at position 24 or 25 of the insulin B chain in about 60% of the molecules (1). This suggested that the insulin produced in this patient's pancreas contained a mixture of normal, unsubstituted insulin, and an abnormal, substituted species, in an approximate 40:60 ratio. To demonstrate conclusively the presence of two insulin species, a method of physical or chemical separation was necessary. Since the substitution did not result in a molecular weight or charge difference between the two insulins, standard chromatographic or electrophoretic techniques would not be effective. Consequently, we devised a method of separation based on the differential ability of the two insulins to bind to insulin receptors. We reasoned that the normal insulin would bind with normal, high affinity, whereas the mutant insulin would bind with low affinity to insulin receptors of IM-9 cultured lymphocytes. After expo-
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Binding potency, such that if the molecule binds to the receptor, a normal biologic response will ensue; therefore, all insulin-like peptides might be expected to be full agonists and the affinity of the hormone for the receptor should determine its biologic potency. Our findings with the Leu→Phe substituted insulin do not conform to this theory. On the other hand, if this insulin behaved as a partial agonist or was completely devoid of agonist properties, differences in binding and biologic potencies of the purified preparation might be explained. However, careful consideration of all the results indicates that this could not be the case. If one assumes that the original insulin mixture contains 40% normal insulin, then the overall binding potency should be the composite of the normal species and the mutant species and should be no less than 40%. For example, given a mixture of insulins consisting of 40% normal and 60% abnormal insulin (with an intrinsic binding potency of 10%), then the overall binding potency of the mixture should be the sum of these activities, i.e. 40 + 6 or 46%. Similarly, according to standard receptor theory, the biologic potency of the mixture should be the sum of the two activities and should equal the binding potency. Even if the mutant insulin were completely devoid of agonist properties (a full antagonist, in pharmacologic terms), the overall biologic activity of the mixture should not be less than 40%. However, the biologic potency of this preparation was only 12%. This suggested that the abnormal insulin in some way inhibits the biologic activity of the normal insulin, and this hypothesis was confirmed by direct studies of the separated mutant insulin. Thus, mixing studies demonstrated that at equimolar concentrations, the mutant insulin markedly inhibited the ability of normal human or porcine insulin to promote glucose transport and oxidation. Although the mechanisms of this effect are currently unknown, control studies demonstrated that, at these concentrations, the mutant insulin did not alter either the binding or degradation of normal insulin. Therefore, the ability of the mutant insulin to antagonize the biologic activity of normal insulin is not due to inhibition of either the binding of normal insulin to receptors or the subsequent degradation of the hormone. The specificity of this effect for the insulin action mechanism was further demonstrated by finding that the mutant insulin could also inhibit the biologic activity of an anti-insulin receptor antibody, which normally exerts agonistic properties by interacting with the insulin receptor (21), but had no effect on the activity of the insulin mimic (spermagline) which does not work through the insulin receptor (19). It would appear that the antagonistic properties of the mutant insulin species can fully account for the discrepancy between binding and biologic potency of either the insulin mixture or the separated abnormal insulin species. Additionally, this unique inhibitory property also provides a likely explanation for the diabetic syndrome displayed by the patient from whom this insulin was obtained (2, 3). Thus, although the patient’s plasma seemingly contained enough normal insulin to maintain relatively normal glucose homeostasis, the antagonistic properties of the circulating mutant insulin would prevent this, leading to the diabetic state.

Although the mechanism underlying these antagonistic properties has not yet been elucidated, certain inferences seem justified. The concept that all insulin-like peptides are full agonists and that their biologic activity is solely determined by their affinity for the insulin receptor cannot be uniformly applied. Binding to receptors is a necessary, but not sufficient, step in the initiation of biologic activity, and some insulin-like peptides will have a diminished, or absent, ability to activate biologic effector systems following the binding event. Furthermore, as is the case with the currently studied mutant insulin, post-binding inhibitory effects may also be exerted.
Thus, the insulin molecule must contain information in addition to that which is necessary for simple binding. It seems likely that some additional structural components of the insulin molecule, or information imparted by the interaction of the insulin molecule with the insulin receptor, is necessary to allow coupling between the insulin-receptor complex and the biologic effector systems. Furthermore, because of the relative inhibitory effects, regardless of individual receptor sites, then it is possible that the insulin molecule must contain information in addition to that which is necessary for insulin receptors to aggregate or cluster in order to initiate biologic activity. If the mechanism which initiates this receptor-receptor interaction involves coordinated activity of individual receptor sites, then it is possible that the mutant insulin forms an insulin-receptor complex which cannot participate in this receptor-receptor interaction, thus inhibiting the biologic activity of all the insulin-receptor complexes which would normally participate in the interaction with the mutant insulin-receptor complex. This concept is clearly highly speculative, and simply serves as one idea to visualize a model whereby the abnormal insulin could exert inhibitory effects. Regardless of the eventual molecular explanation for this antagonistic effect, any theory for the mechanism of insulin action will have to account for the unusual properties of this mutant insulin species.

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