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Biochemistry

Direct demonstration that receptor crosslinking or aggregation is important in insulin action

(insulin receptor/receptor antibody/competitive antagonist/insulin antibody/membranes)

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ABSTRACT **Exposure of adipocytes to antibodies to the insulin receptor results in a blockade of ^{125}I-labeled insulin binding, stimulation of glucose oxidation, and many more insulin-like effects. Allowing for differences in purity, antireceptor antibody is equipotent with insulin on a molar basis. Both the bivalent F(ab')$_2$ and monovalent Fab' fragments of the antireceptor antibody are fully active in inhibiting ^{125}I-labeled insulin binding. Bivalent F(ab')$_2$ also retains its insulin-like effects. In contrast, the monovalent Fab' loses almost all ability to stimulate glucose oxidation and acts as a competitive antagonist of insulin-stimulated glucose oxidation. Addition of anti-F(ab')$_2$ antisera, which crosslink the Fab'-receptor complexes, results in a restoration of the insulin-like activity of the antibody. Similarly, when cells are exposed to submaximal doses of insulin, addition of anti-insulin antibodies at low concentration enhances the biological activity of insulin. These data suggest that receptor occupancy by ligand is not sufficient for signal generation and that the insulin-like effects of antireceptor antibody (and perhaps insulin itself) require receptor aggregation or clustering. This aggregation, however, appears to be independent of microfilaments or microtubules because the insulin-like effects of antireceptor antibody, and in fact, of insulin itself, are unaffected by agents that are known to disrupt these structures.**

The first step in insulin action is binding to a receptor site on the plasma membrane of the cell (1). Exactly how this interaction of the hormone with its receptor is transformed into a transmembrane message, however, remains unknown. Most attention has focused on the possibility that the interaction of insulin with its receptor activates some membrane-associated enzyme or transport system, which in turn generates a second intracellular messenger of hormone action, perhaps analogous to cyclic AMP (2). Recently, several investigators have presented data that insulin or one of its degradation fragments may actually enter the cell (3–5), and these workers have postulated that this entry may be important for some of insulin's biological effects.

The discovery of autoantibodies to the insulin receptor in some patients with insulin-resistant diabetes has made available a new tool for the study of insulin action (6, 7). We have shown that these antibodies will bind to the insulin receptor (8), block insulin binding (9, 10), and initiate many of insulin's biological effects (10, 11). In the present study we have prepared monovalent fragments of these antireceptor antibodies and compared their effects to those of the bivalent antibody. Like the bivalent antibody, monovalent antireceptor antibodies compete for insulin binding to the receptor. The monovalent antibodies, however, are unable to initiate a biological response, and behave as a competitive antagonist of insulin action at the receptor level. The insulin-like activity of the monovalent antireceptor antibody can be restored by addition of a second antibody to crosslink the Fab'-receptor complexes. In addition, the activity of insulin itself is enhanced by crosslinking with anti-insulin antibody. These data provide a direct demonstration of a competitive antagonist of insulin action at the receptor level and suggest that receptor crosslinking or aggregation is important for insulin action.

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MATERIALS AND METHODS

Materials. Porcine insulin (lot 7GUHSL) was purchased from Elanco Company, bovine serum albumin (Fraction V, Lot N53309) from Armour and Company, and crude collagenase (CLS45K137) and pepsin (2682 U/mg; lot PM35B735) from Worthington Biochemical Corporation. Cytochalasins B and D and colchicine were purchased from Aldrich Chemical Company, vincristine and vinblastine from Eli Lilly Company, and dinonylphthalate from Eastman Chemical Company. ^{125}I-labeled insulin (^{125}I-insulin) was prepared by a modification of the chloramine-T method (12) at specific activities of 100–200 μCi/μg.

The IgG fraction of serum from the patient with the highest concentration of antireceptor antibody activity (B-2) was prepared from the ammonium sulfate precipitate by ion exchange chromatography of DEAE-cellulose (7, 8). Bivalent F(ab')$_2$ fragment was prepared from the IgG by pepsin digestion (13) and purified by gel filtration on Sephadex G-200. To prepare monovalent Fab' fragments (14), the F(ab')$_2$ was concentrated and adjusted to pH 8.6 with 0.2 M Tris·HCl. This solution was then incubated at room temperature for 60 min with 0.01 M dithiothreitol. Iodoacetamide was added to give a final concentration of 0.022 M, and the sample was incubated for 15 min at room temperature. The sample was then dialyzed at 4°C overnight against 0.01 M potassium phosphate, pH 8.0.

Guinea pig anti-insulin serum was purchased from Peter Wright and used without further purification. Goat antibodies to human F(ab')$_2$, a generous gift of Warren Strober, were partially purified by ammonium sulfate precipitation of the antiserum prior to use.

Binding Studies and Glucose Oxidation Bioassay. Isolated adipocytes were prepared from epididymal fat pads of 100–160 gm Sprague–Dawley rats as described by Rodbell (15). Unless otherwise noted, all studies of ^{125}I-insulin binding were performed in the Krebs–Ringer buffer with albumin, pH 7.4 at 37°C as described (10, 15). Glucose oxidation was studied by measuring the conversion of [*U*-^{14}C]glucose to $^{14}CO_2$ (16) with an incubation period of 30–60 min as indicated. All glucose oxidation assays were performed in triplicate.

RESULTS

Fig. 1 shows the effects of the purified IgG fraction and bivalent $F(ab')_2$ and monovalent Fab' fragments of antireceptor antibodies on both the insulin binding and glucose oxidation by isolated adipocytes. All three preparations were able to inhibit ^{125}I-insulin binding and were approximately equipotent in this effect. Significant inhibition of binding was observed with concentrations as low as 1 μg/ml. As we have previously reported (10), both the purified IgG and the $F(ab')_2$ fragment also produced insulin-like bioeffects in these cells. In both cases significant stimulation of glucose oxidation was observed at 0.3 μg/ml. Based on our earlier studies (8) which showed that approximately 1% of the total IgG are antireceptor antibodies, the antireceptor antibody is approximately equipotent with insulin on a molar basis in both binding inhibition and bioactivity.

By contrast, the monovalent Fab' has little insulin-like effect on the adipocyte. Thus at 5 μg/ml, a concentration at which bivalent $F(ab')_2$ produces full stimulation of glucose oxidation, the monovalent Fab' produces only about 5% stimulation. At higher concentrations of Fab', some stimulation of glucose oxidation does occur. This is probably due to a small contamination of the preparation with $F(ab')_2$ fragments that have not been successfully reduced. In three different preparations of monovalent Fab' fragments, the insulin-like bioactivity varied from 1% to <0.01% of that of the bivalent antibody.

The finding that the monovalent Fab' fragments block insulin binding although possessing little bioactivity suggested that the monovalent Fab' may be able to serve as a competitive antagonist of insulin at the receptor level, and this was confirmed by studying the effect of the Fab' fragment on insulin-stimulated glucose oxidation. When cells pretreated with Fab' at 10 μg/ml were subjected to further stimulation by insulin, the dose-response of glucose oxidation was clearly shifted to the right (Fig. 2). The dose of insulin producing half-maximal stimulation of the antibody-treated cells was 1.4 ng/ml, as compared with about 0.4 ng/ml for the control cells. The 3-fold shift in insulin sensitivity correlates well with the fact that pretreatment of cells with this concentration of Fab' produced about a 60–70% reduction in insulin binding (17).

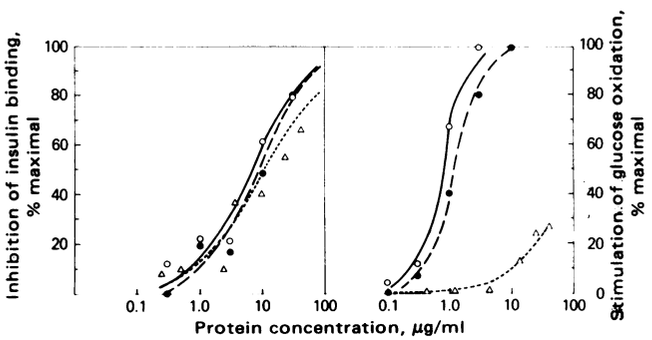

FIG. 1. Comparison of the effects of anti-insulin receptor antibody and its fragments on insulin binding and glucose oxidation by isolated adipocytes. Isolated rat adipocytes were incubated with buffer or the indicated concentrations of the IgG (●), $F(ab')_2$ (○), or Fab' (△) prepared from serum B-2 for insulin binding or glucose oxidation. The inhibitory effect of each fraction on binding and the stimulatory effect on glucose oxidation were calculated as a percentage of the maximal effect produced by insulin in each system. In the glucose oxidation experiments, the maximal effect of insulin occurred at a concentration of 2–3 ng/ml (0.3–0.5 nM) and varied between a 5- and 10-fold stimulation over the basal level. In the binding experiments, 10 μM insulin inhibited ^{125}I-insulin binding by about 90%. The data shown were obtained with antibody fractions of one sequential purification and are representative of four experiments done with three different preparations of each of the immunoglobulin fractions.

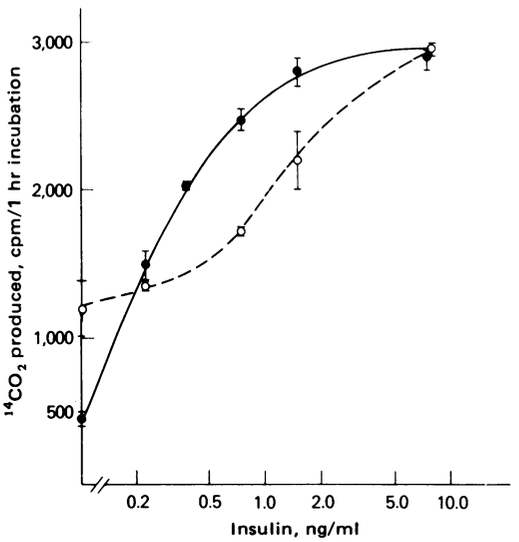

FIG. 2. Effect of monovalent antireceptor antibody on glucose oxidation by isolated adipocytes. Isolated adipocytes were incubated with buffer (●) or Fab' fragments (○) (30 μg/ml) of antireceptor antibody and the indicated concentrations of insulin. Glucose oxidation was measured. This concentration of Fab' produced a 60–70% reduction in specific insulin binding to adipocytes (see Fig. 1).

The loss of bioactivity in the monovalent Fab' fragment could be the result of the change in valency of the ligand or could be the result of chemical modifications that occur with reduction and alkylation. To explore this possibility, reconstitution of the valency was attempted by exposing the cells to the monovalent antibody and then crosslinking these by addition of second antibody. Addition of either anti-$F(ab')_2$ serum (Fig. 3) or anti-human IgG (data not shown) produced a dose-dependent

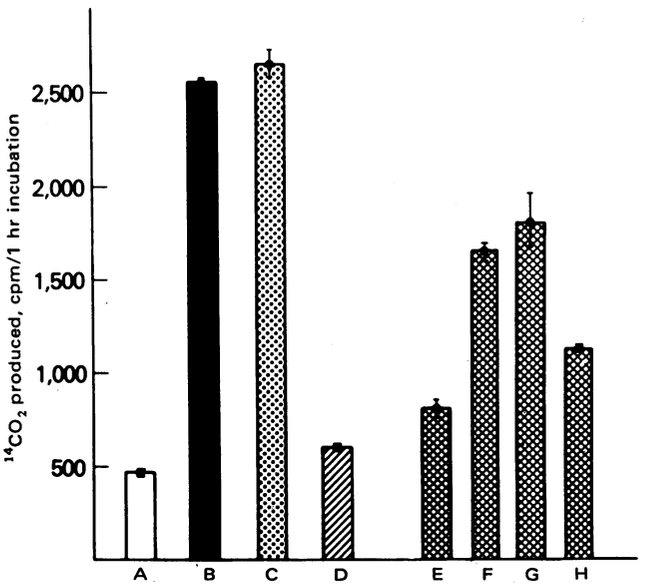

FIG. 3. Effect of crosslinking on the bioactivity of monovalent antireceptor antibodies. Isolated adipocytes were exposed to buffer, insulin (1.5 ng/ml), $F(ab')_2$ (5 μg/ml), or Fab' (5 μg/ml) for 30 min at 22°C. Anti-Fab_2 serum was then added at the indicated dilutions to some of the flasks containing buffer or the Fab' fragments. Glucose oxidation was then measured for 1 hr. Anti-$F(ab')_2$ serum alone had no effect on glucose oxidation at any of the concentrations used. Bars: A, basal; B, insulin at 1.5 ng/ml; C, $F(ab')_2$ at 5 μg/ml; D, Fab' at 5 μg/ml; E, F, G, and H, Fab' plus anti-$F(ab')_2$ at 1:1000, 1:200, 1:50, and 1:20, respectively.

restoration of the bioactivity of the monovalent antibody fragment. At a dilution of 1:50 of the second antibody, the effect of the monovalent Fab' reached a maximum, which was about 60% of the effect that was observed with the $F(ab')_2$. At none of the concentrations tested did anti-$F(ab')_2$ serum alone produce any insulin-like effects.

In an attempt to see if this effect of ligand crosslinking was a general factor in insulin action, experiments were performed in which the effect of anti-insulin antibodies in insulin action was studied. In these experiments, cells were exposed first to a concentration of insulin that produces a submaximal stimulation of glucose oxidation (0.2–0.3 ng/ml) and then anti-insulin antibodies were added at high dilution in hopes of crosslinking some of the insulin bound to the receptors on the cell. Consistent with the effects of anti-$F(ab')_2$ serum on Fab', low concentrations of anti-insulin serum enhanced the activity of these submaximal concentrations of insulin by about 30% (Fig. 4). Although the effect is small, it was highly reproducible and clearly significant ($p < 0.05$). The loss of the potentiating effect that occurs as the antibody concentration is increased could be simply due to the fact that the antibodies bind free insulin in solution and thus decrease its concentration or due to the fact that the antibody is more likely to act as a monovalent reagent when excess amounts are present. Taken together, these data suggest that receptor crosslinking or aggregation is important in insulin action.

A possible role of microtubules and microfilaments in this aggregation might be suggested by effects on insulin binding of the various agents known to alter these structures (18). However, pretreatment of cells with colchicine, vincristine, and vinblastine, agents which alter microtubular function (19), at concentrations of 10 μM had no effect on basal, insulin-stimulated, or antibody-stimulated glucose oxidation (Table 1). As previously described, cytochalasin B, an antimicrofilament agent (20), at 1 μg/ml lowers basal glucose oxidation (transport)

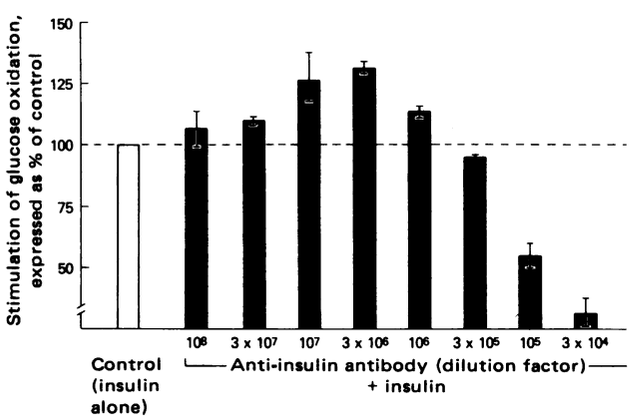

FIG. 4. Effect of anti-insulin antibodies on insulin-stimulated glucose oxidation. Isolated adipocytes were incubated with 0.2–0.3 ng of insulin/ml for 30 min at 22°C. Anti-insulin serum was then added at the indicated dilutions and glucose oxidation was measured for the next 20–60 min. Insulin alone at this concentration produced about a doubling of basal glucose oxidation. The data are expressed as a percent of the control (insulin alone) glucose oxidation and were calculated as:

$$\left(\frac{\text{experimental} - \text{basal}}{\text{control} - \text{basal}}\right) \times 100.$$

The data are presented as the mean ± SEM for three experiments, each done in triplicate. The increase in glucose oxidation observed with anti-insulin antibody at a dilution of $1:3 \times 10^6$ is significant at the $p < 0.05$ level. Anti-insulin antibody alone produced no systematic change in glucose oxidation (data not shown).

Table 1. Effect of antimicrotubular and antimicrofilament agents on stimulation of glucose oxidation by insulin and anti-insulin antibody

Addition	$^{14}CO_2$ produced, cpm/hr of incubation: Basal	Insulin (1.0 ng/ml)	IgG B-2 (5 μg/ml)
None	101 ± 10	745 ± 47	984 ± 17
Colchicine, 10 μM	97 ± 14	639 ± 41	860 ± 47
Vinblastine, 10 μM	98 ± 7	636 ± 63	868 ± 3
Vincristine, 10 μM	98 ± 7	670 ± 38	923 ± 66
None	302 ± 28	1732 ± 196	3019 ± 51
Cytochalasin B			
1 μg/ml	24 ± 5	143 ± 20	167 ± 17
10 μg/ml	6 ± 4	14 ± 5	13 ± 2
Cytochalasin D			
1 μg/ml	256 ± 16	1445 ± 73	2995 ± 23
10 μg/ml	228 ± 52	1377 ± 179	2806 ± 169

by over 90%. Despite this, stimulation by both insulin and IgG can still be observed, and approximately the same ratio of stimulated/basal occurs. Cytochalasin D, which also disrupts microfilaments, has little direct effect on glucose transport (21) and produced minimal inhibition of basal, insulin-stimulated or antibody-stimulated glucose oxidation.

DISCUSSION

Insulin action at the cellular level can be considered to reside at four distinct biochemical levels: (*i*) the binding of the hormone to its membrane receptor; (*ii*) transformation of this hormone-receptor interaction into some form of transmembrane signal; (*iii*) generation of an intracellular message (or messenger), and; (*iv*) subsequent chemical modification of various enzymes and transport systems all of which result in the final biological effects of insulin on carbohydrate, lipid, and protein metabolism. Although much effort has been devoted to exploring the mechanism of insulin action, most of our knowledge is limited to some understanding of the first and last steps in this process. Candidates for the role of intracellular messenger have included calcium (2), the cyclic nucleotides (2), inhibitors of protein kinase (22), and recently, several workers have suggested that insulin or one of its degradation fragments may enter the cell and act directly as a second messenger (23, 24).

Almost no clues exist as to the nature of the transmembrane signal itself. A possible role for movement of receptors in the plane of the membrane has been suggested by the finding that insulin receptors appear to be clustered (25, 26), and a temperature-dependent delay in the onset of insulin action has been noted (27). Using fluorescent derivatives of insulin, Schlessinger *et al.* (28) have shown that insulin on fibroblasts can move laterally with a diffusion coefficient ($3–5 \times 10^{-10}$ cm^2/sec) similar to that for other mobile membrane proteins.

In 1975 we discovered that sera of some patients with insulin-resistant diabetes contain autoantibodies to the insulin receptor (6, 7, 29) and this has provided new probes for the study of insulin action. These antibodies, which were initially found by their ability to inhibit insulin binding to its receptor (6), also produce insulin-like biological effects when exposed to tissues *in vitro* (10, 11). By using both adipocytes and isolated soleus muscle, we and others have shown that these antibodies stim-

ulate glucose transport, glucose incorporation into glycogen and lipid, and glucose metabolism to CO_2. In addition, antireceptor antibodies stimulate amino acid transport (30) and mimic the antilipolytic effect of insulin (30, 31). In collaboration with J. Lawrence and J. Larner, we have found that these antibodies will also inhibit phosphorylase activity and activate glycogen synthase both in the presence and in the absence of glucose, two changes in activity of cytoplasmic enzymes characteristic of insulin action (unpublished data).

Although many other factors, such as lectins (32) and polyamines (33), have been shown to mimic some of insulin's actions, only the antireceptor antibody appears equipotent with insulin, and in contrast to many of these other agents, its bioeffects seem to be due to direct and specific interaction with the insulin receptor. By using ^{125}I-labeled antireceptor antibody, we have shown that these antibodies bind to cells in direct proportion to the concentration of insulin receptors and that the labeled antibody binding can be inhibited by insulin and insulin analogues in proportion to their affinity for the receptor (8). In addition, the antireceptor antibodies will specificially immunoprecipitate solubilized insulin receptors*.

The facts that some of the compounds with insulin-like activity, such as lectins, are multivalent and that the antireceptor antibodies are bivalent suggested a possible role for receptor aggregation or crosslinking in insulin action. To test this, monovalent Fab' fragments of antireceptor IgG were prepared. Although these retained full ability to inhibit insulin binding, the monovalent antibody fragment lost almost all bioactivity. This change in bioactivity appears to be due to the change in valence rather than to the reduction and alkylation, because activity can be restored by crosslinking the monovalent Fab' with a second antibody (Fig. 5*A*). In addition, Fab fragments produced by papain digestion without alkylation also lose bioactivity while retaining their ability to inhibit binding (F. A. Karlsson, K. L. Baird, and C. R. Kahn, unpublished observation).

Although insulin in solution at concentrations at which it induces most of its biological responses is monomeric and presumably "monovalent," these observations, together with the previous findings that insulin receptors are mobile (28) or clustered (25, 26), suggested a possible role for receptor aggregation or crosslinking in the action of insulin itself. To explore this possibility, we exposed cells to a submaximal dose of insulin and then to low concentrations of anti-insulin antibody (about 0.5–1.0 mol of antibody per mol of insulin). Under these circumstances anti-insulin antibodies actually enhanced insulin's effect, consistent with an effect of the insulin antibody to crosslink the insulin-receptor complexes (Fig. 5*B*).

Since all insulin analogues are agonists, presumably insulin is able to either initiate the biological response in the absence of receptor aggregation or induce receptor aggregation independent of external crosslinking (Fig. 5*B*) (34). Interestingly, when insulin binds to the cell membrane, its local concentration may approach 10^{-6} M (J. Schlessinger, personal communication), a concentration at which insulin in solution dimerizes or aggregates (35). Schlessinger *et al.* (36) have also shown that insulin on fibroblasts can form microscopically visible patches. If this aggregation is important for activity, however, it must differ from aggregation in solution, because there are several insulins which do not dimerize but are biologically active (35, 37). Perhaps hagfish insulin, which has a lower bioactivity than affinity for the insulin receptor (38), is unable to fully induce receptor aggregation.

* Harrison, L. C., Flier, J. S., Kahn, C. R. & Roth, J. (1978) *Abstracts of the 60th Annual Meeting of the Endocrine Society*, June 14–16, p. 331.

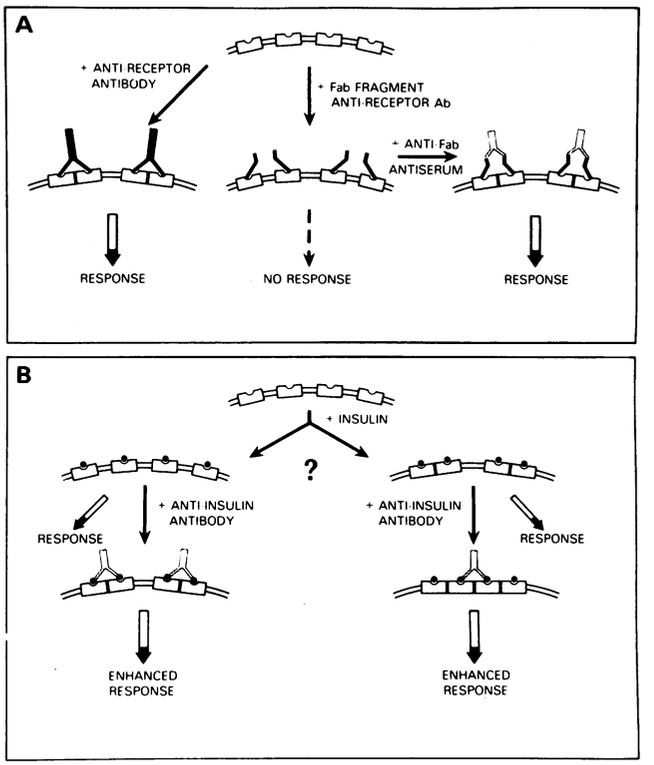

FIG. 5. Diagrammatic representation of crosslinking of receptor by the various ligands. (*A*) Represents bivalent antireceptor antibodies or the combination of monovalent antireceptor antibodies and a second antibody. (*B*) Represents crosslinking of insulin upon addition of anti-insulin antibodies.

Proper orientation and spacing between the insulin monomers may also be important for the observed effect. Covalent insulin dimers linked by a short bridge between the A-1 phenylalanine and the B-29 lysine have an activity which is approximately an average of the activities of the two chemically modified monomers (39). Covalent dimers linked between B-1 and B-29, on the other hand, have the full biological activity of both insulins (D. Brandenberg, personal communication). Increasing chain length and flexibility could possibly lead to analogues that could bind with an affinity even higher than that of native insulin and could have increased activity.

The enhancement of insulin activity by anti-insulin antibodies may be important in one or more clinical situations. Several patients have been reported with a syndrome characterized by hypoglycemia and spontaneous development of insulin autoantibodies (40). It is interesting to speculate that perhaps these patients have developed insulin antibodies which bind to insulin in such a way that the potentiating effect predominates over the blocking activity. Such antibody modulation of insulin activity may also play a role in the "brittle" diabetic where the antibodies might enhance or inhibit insulin's action depending on the type of antibodies present and the ratio of antibody to insulin.

The data of this study also provide several important insights into the mechanism of insulin action. The first is that many, and perhaps all, of insulin's actions can be initiated by the interaction of ligands other than insulin with the insulin receptor. This suggests that the receptor, when properly triggered, contains all the biochemical attributes necessary to initiate hormone action. This argues against the theories that insulin degradation and internalization of insulin (or one of its degradation fragments) are important for all of insulin's actions. It is possible of

course, that the latter are required for some of the long-term growth stimulating effects of insulin. On the other hand, it is not clear if the latter effects are mediated via the insulin receptor or one of the receptors for the insulin-like growth factors.

The second major point is that "occupancy" of the receptor is not sufficient for signal generation. Thus, monovalent Fab' fragments of antireceptor antibody can "occupy" the insulin receptor, at least measured by their ability to block insulin binding and insulin action, without generating much insulin-like effect. A similar situation exists for the IgE receptor of the basophil in which crosslinking of receptors can be accomplished by IgE plus a second antibody, antibodies to the IgE receptor, or chemically crosslinked IgE dimers (41, 42). Recently, Drachman *et al.* (43), have shown that bivalency is also required for the accelerated receptor degradation produced by antibodies to the acetylcholine receptor from patients with myasthenia gravis. Whether a hormone such as insulin is able to induce receptor aggregation without external crosslinking or exerts its signal without receptor aggregation is unclear (34); however, some enhancement of the biological activity of insulin can be obtained by crosslinking insulin-receptor complexes under some circumstances. If aggregation does occur, neither microfilaments nor microtubules appear to be important. Whether some other membrane-associated proteins are required, however, is uncertain.

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