Antibodies to the Extracellular Receptor Domain Restore the Hormone-insensitive Kinase and Conformation of the Mutant Insulin Receptor Valine 382*

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A mutation substituting a valine for phenylalanine at residue 382 in the insulin receptor α-subunit has been found in two sisters with a genetic form of extreme insulin resistance. This receptor mutation impairs the ability of the hormone to activate autophosphorylation of solubilized receptors and phosphorylation of substrates (Accili, D., Mosthaf, L., Ullrich, A., and Taylor, S. I. (1989) J. Biol. Chem. 264, 4303–4310). We have previously demonstrated that in native receptors insulin induces a conformational change in the receptor β-subunit, which is thought to be necessary for receptor activation (Baron, V., Gautier, N., Komoriya, A., Hainaut, P., Scimeca, J. C., Mervic, M., Lavielle, S., Dolais-Kitabgi, J., and Van Obberghen, E. (1980) Biochemistry 29, 4634–4641). Hence, it was thought that a defect in this conformational change might explain the functional defect of the mutant receptor. This appears to be the case, as we demonstrate here that the mutant receptor is locked in its inactive configuration. However, we found two monoclonal antibodies, directed to the extracellular domain, which are capable of restoring the mutant receptor kinase activity. The activation of the mutant receptor was accompanied by restoration of conformational changes in the β-subunit C terminus.

From these data, we draw the following conclusions. (i) A causal link exists between receptor kinase activation and the occurrence of conformational changes. (ii) Ligands other than insulin, such as antibodies, which perturb the extracellular domain, can function as alternative ways to restore the mutant receptor kinase.

Insulin is a key regulator of the metabolism of glucose, lipids, and proteins. The first step of its biological action is binding to a specific cell-surface receptor. This receptor is a

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MATERIALS AND METHODS

Cell Lines—NIH 3T3 fibroblasts transfected with expression plasmids encoding the wild type human insulin receptor or the Val382

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receptor (VaMP) were produced by Accili et al. (11). The wild type and mutant insulin receptors correspond to isoforms lacking the 12 amino acids of the C terminus of the α-subunit (1), but we have used the numbering system including these residues (2). Both cell lines express approximately the same number of cell-surface receptors, i.e., \( 5 \times 10^5 \)/cell.

Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1 mM L-glutamine.

Antibodies to Synthetic Peptides—In this study, we used antipeptide antibodies directed against the following propeptide sequence: (i) 1306–1329, in the β-subunit C terminus; and (ii) 456–465, located in the α-subunit. The antipeptide antibodies were produced as described earlier and were partially purified by chromatography on protein A-Sepharose. The anti-(1306–1329) was previously found to be an excellent choice for the receptor C-terminal, induced by insulin binding or receptor autophosphorylation (14). The sequence 456–465 is located in the domain 450–601, originally described to represent an antigenic region for a series of autoantibodies found in type B insulin-resistant patients (15). The antipeptide does not immunoprecipitate the native insulin receptor but recognizes the denatured receptor in immunoblotting experiments.

Monoclonal Antibodies to the Insulin Receptor—Highly purified insulin receptors were prepared from fresh human placenta by chromatography on insulin bound to Sepharose following a procedure previously described (16). Purified receptors (100 mg/ml) emulsified in Freunds complete adjuvant was used to inject female BALB/c mice. Ten days later, the procedure was repeated using incomplete Freunds’ adjuvant, and two other boosts were performed. The mice splenic lymphocytes were fused to NSI cells according to Milstein (17). Positive cells were expanded, cloned by limited dilution, and subsequently grown in tissue culture and ascites fluid in pristane-primed BALB/c mice. Screening was based on the capacity of antibodies to immunoprecipitate the insulin receptor. Several monoclonal antibodies to the extracellular domain of the insulin receptor were obtained. We used two of them, B6 and αM2, which are IgG1 and IgG2 immunglobulins, respectively. They are specific for the human insulin receptor and do not cross-react with insulin receptors from rat or mouse. These antibodies are able to immunoprecipitate the insulin receptor but do not recognize it in immunoblot experiments. Note that neither B6 nor αM2 recognizes the human IGF-1 receptor.

Receptor Purification—Cells expressing wild type human insulin receptor or VaMP were solubilized in 1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10 µg/ml leupeptin, 1.25 µg/ml bacitracin, 100 µg/ml antipeptide 1306-1329 (50 µg/ml) in buffer

Absence of Insulin-induced Conformational Change in Mutant Insulin Receptor VaMP—First, we searched for a possible link between the defect in the insulin-dependent activation of the receptor tyrosine kinase and structural modifications of the mutant receptor. To do this, we studied the interaction of both receptors with antibodies to the extracellular domain: (i) an antipeptide antibody to receptor sequence 456–465; and (ii) the monoclonal antibodies αM2 and B6.

The effect of these antibodies on insulin binding to wild type and mutant receptors was measured by incubation of antibodies with the receptors before addition of tracer amounts of \( ^{125}\text{I} \)-insulin. In these conditions, an alteration in hormone binding could reflect a modification in receptor affinity for the hormone or in the number of available binding sites. Results obtained with the antipeptide to sequence 456–465 are presented in Fig. 1. This antibody inhibits insulin binding to the mutant receptor, whereas it has no effect on the wild type receptor. Monoclonal antibodies αM2 and B6 also had different effects upon ligand binding to mutant and wild type receptors (Fig. 2). Indeed, αM2 inhibited hormone binding to the wild type receptor more strongly than to the wild type receptor. In contrast, B6 reduced hormone binding to the wild type receptor to a considerable extent but exerted only a slight effect on the mutant receptor. Taken together, these data show that our antibodies distinguish between the two receptors and suggest that the extracellular conformation of the mutant receptor and that of the wild type receptor are different.

We have previously shown that insulin binding induces a
conformational change in the wild type receptor C terminus, and we proposed that this change could be important in the mechanism leading to receptor activation. Then, we hypothesized that the Val^{382} receptor might not undergo such a hormone-induced change, explaining the failure of insulin to stimulate its kinase activity. To test this view, increasing amounts of ^{35}S-receptors were incubated with or without insulin before addition of an antipeptide to the C-terminal domain 1306–1329. This antipeptide is able to detect a conformational change after hormone binding to the wild type receptor. Indeed, insulin binding results in a 50% decrease in immunoprecipitation compared with non-occupied receptors (at saturating receptor concentrations) (19). However, as shown in Fig. 3, immunoprecipitation of the Val^{382} receptor is not significantly affected by insulin binding. Thus, the Val^{382} and the wild type receptors have distinct behaviors with respect to antipeptide recognition. Our data indicate that interaction of the hormone with the mutant receptor does not change the receptor C-terminal configuration in the same way as has been shown for the wild type receptor. We conclude that the Val^{382} receptor is locked in a conformation that cannot be changed by insulin and appears to be different from that of the wild type receptor.

**Monoclonal Antibodies to Receptor Restore the Tyrosine Kinase Activity of the Mutant Insulin Receptor Val^{382}**—The Val^{382} receptor kinase has been shown to be unstimulated by hormone binding to the receptor α-subunit but to be able to undergo activation upon transphosphorylation of its cytoplasmic domain. This indicates that the intrinsic kinase is intact (12). We were therefore interested to assess the effect of our extracellular targeted antibodies on the mutant receptor kinase activity.

In these experiments, antibodies were incubated with Val^{382} receptors and then with or without insulin at saturating concentration. Finally, the substrate poly(Glu-Tyr) and the phosphorylation solution were added. As expected, in the absence of antibody, insulin had little effect on the phosphorylation of poly(Glu-Tyr) (Fig. 4). In the lower panel, results obtained with αM2 in the presence of insulin are shown. The antibody plus the hormone stimulate the Val^{382} receptor about 2-fold (220% of basal at optimal concentration), indicating that combination of the two effectors restores the Val^{382} kinase. Next, we tested the effect of the other monoclonal antibody B6 (upper panel). This antibody also stimulates 2-fold the mutant receptor kinase activity, but, in contrast to αM2, B6 alone appears to be sufficient. The 2-fold stimulation obtained with αM2 plus insulin or with B6 corresponds approximately to the insulin-induced stimulation of the wild type receptor, i.e., 2–3-fold (data not shown).

We then analyzed whether this could be correlated with an increase in receptor autophosphorylation. These experiments were performed as described above, except that no exogenous substrate was added before the phosphorylation mixture. The samples were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography. As seen in Fig. 5, there is no ^{32}P incorporation in the receptor β-subunit in the absence of antibody, whether insulin is present or not. After incubation with αM2 (panel A), only a slight increase in phosphorylation is observed in the absence of insulin, whereas in the presence of the hormone, αM2 enhanced considerably the mutant receptor autophosphorylation. By contrast, B6 alone was able to stimulate the Val^{382} receptor autophosphorylation, and phosphate incorporation was not enhanced by addition of insulin (panel B).

To summarize, we have obtained two monoclonal antibodies capable of restoring the Val^{382} receptor kinase for autophosphorylation and phosphorylation of the synthetic substrate poly(Glu-Tyr). Interestingly, whereas B6 alone is sufficient, αM2 needs insulin to produce the effect. Finally, our results indicated that it is possible to stimulate the mutant receptor by perturbing its extracellular domain.

**Monoclonal Antibodies to Receptor Alter the Conformation of the Mutant Insulin Receptor Val^{382}**—Based on our preceding results, we speculated that the restoration of the kinase activity by monoclonal antibodies could be due to the appearance of C-terminal conformational changes normally induced in the wild type receptor by insulin binding. Therefore, we measured the effect of αM2 and B6 on ^{35}S-receptor recognition by the antipeptide to the C terminus. The antipeptide to sequence 456–465 was used as a control, since it does not stimulate the receptor kinase activity (data not shown). First, we made sure that the three antibodies did not interfere with immunoprecipitation by the antipeptide to the C terminus.

**Fig. 1.** Effect of antipeptide anti-(456–465) on ^{125}I-insulin binding to its receptor. Solubilized wild type (open symbol) or mutant (closed symbol) receptors were incubated for 2 h at 4 °C with increasing concentrations of partially purified antipeptide anti-(456–465). Thereafter, radiolabeled ligand was added at tracer concentration for 2 h at 15 °C. The nonspecific binding was determined in the presence of 0.1 μM unlabeled insulin. ^{125}I-Insulin bound to preimmune Ig-treated receptor was defined as 100%. A representative experiment is shown.

**Fig. 2.** Effect of monoclonal antibodies on ^{125}I-insulin binding to its receptor. Experiments were performed with αM2 and B6 as described in the legend to Fig. 1.
FIG. 3. Anti-(1306–1329)-induced immunoprecipitation of Val382 mutant 35S-receptor occupied or not by insulin. 35S-Receptors were incubated with 0.1 μM insulin (open symbols) or with buffer (closed symbols) for 3 h at 4 °C. Increasing amounts (20–180 fmol/sample) of the 35S-receptors were then added to 50 μg/ml antipeptide to sequence 1306–1329 for 2 h at 4 °C. After precipitation with protein A, the pellets were washed and resuspended in scintillation solution. The data shown correspond to the mean of three independent experiments ± S.E. They are expressed as a percentage of the maximal immunoprecipitation obtained in each experiment.

FIG. 4. Effect of monoclonal antibodies to insulin receptor on the receptor kinase activity. Val382 receptors (20 fmol/sample) were incubated for 2 h at 4 °C either with control Ig or with monoclonal antibodies prior to addition of insulin (0.1 μM) for 1 h at room temperature. Poly(Glu-Tyr) was then added to a final concentration of 0.2 mg/ml, and phosphorylation was performed as indicated under “Materials and Methods.” After 45 min at room temperature, the reaction was stopped, and the samples were analyzed using a filter paper assay. Results are expressed as a percentage of the basal substrate phosphorylation obtained in the absence of effectors. We show the mean of three and two independent experiments (upper and lower panels, respectively) where each point was run in triplicate.

FIG. 5. Effect of monoclonal antibodies to insulin receptor on receptor autophosphorylation. Val382 receptors (40 fmol/sample) were incubated for 2 h at 4 °C with control Ig or with the monoclonal antibodies, αM2 (panel A) or B6 (panel B). After exposure of receptors to insulin (0.1 μM) for 1 h at room temperature, phosphorylation was performed for 15 min. Thereafter, the reaction was stopped with Laemmli sample buffer, and samples were analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiograph of one representative experiment is shown.

The antipeptide to sequence 456–465 does not immunoprecipitate the insulin receptor, and αM2 does not bind to protein A, since it is a murine IgG. However, we had to produce F(ab′)2 fragments of B6, because this antibody is recognized by protein A. Having done this, we confirmed that B6 F(ab′)2 retained the capacity to inhibit ligand binding and to stimulate kinase activity of mutant receptor at the concentration used in the immunoassay (10 μg/ml) (data not shown). The assay was performed as follows. Samples of 35S-receptors at increasing concentrations were incubated with antibodies to the extracellular domain in the condition where they exhibit the optimal stimulation of the kinase (i.e. in the presence of insulin and αM2). The incubation was continued with the antipeptide to the C-terminal receptor sequence 1306–1329, and immunoprecipitation was achieved by addition of protein A. The presence of anti-(456–465) did not interfere with the precipitation of Val382 receptor by anti-(1306–1329), indicating that this extracellular antibody does not provoke C-terminal conformational changes (data not shown). In contrast, αM2 is able to induce an improved recognition of the mutant receptor by the anti-C-terminal antibody (Fig. 6). In the absence of insulin, αM2 has no or little effect on mutant receptor conformation (data not shown). Finally, F(ab′)2 B6 also induces a clear-cut increase in recognition by the anti-C-terminal antipeptide (Fig. 7).

In summary, the monoclonal antibodies αM2 and B6 pro-
Results are expressed as a percentage of the maximal before addition of the scintillation solution for counting. The results of ValsE2 mutant 35S-receptor occupied or not by F(ab')2 6. Results are expressed as a percentage of the maximal immunoprecipitation obtained in the presence of aM2. As a control, the antibodies inhibit insulin binding to a different extent depending on the receptor studied (Figs. 1 and 2).

Further, the mutant receptor appears to be locked in its inactive configuration. This is reflected at the molecular level by the inability of insulin to induce a change in the C terminus of Val382, as illustrated in Fig. 3. The lack of hormone-induced conformational changes probably explains the unresponsiveness of the mutant receptor to insulin and the inability of the hormone to activate the receptor kinase.

As a whole, the data presented in this study are in favor of a transductional defect as we show that the interactions of the mutant and wild type receptors with antipeptide to sequence 1306–1329 or with monoclonal antibodies are distinct. Indeed, the antibodies inhibit insulin binding to a different extent depending on the receptor studied (Figs. 1 and 2). Further, the mutant receptor appears to be locked in its inactive configuration. This is reflected at the molecular level by the inability of insulin to induce a change in the C terminus of Val382, as illustrated in Fig. 3. The lack of hormone-induced conformational changes probably explains the unresponsiveness of the mutant receptor to insulin and the inability of the hormone to activate the receptor kinase.

It has been shown that the receptor domain surrounding the residue glycine 390 is part of the insulin binding site (21). The fact that the Val382 receptor exhibits normal insulin binding suggests that the binding site may be unaltered and that other extracellular domains involved in the signal transduction from the α- to the β-subunit may be affected by the mutation.

Our data emphasize the importance of the molecular processes involving structural changes in signal transmission. To be specific, we find that an alteration in the receptor α-subunit induces inactivation of the receptor, leading at the cellular level to insulin resistance with severe physiological consequences. The correlation that we observed between the capacity of certain antibodies to activate the mutant receptor kinase activity and their ability to induce conformational changes reinforces the notion that this represents a major mechanism for receptor activation.

An intriguing finding of this study concerns the discrepancy between the effect of certain antibodies on the configuration of the Val382 receptor and the effect of insulin on wild type receptor (Figs. 6 and 7). Indeed, the antibodies increase Val382 recognition by the anti-C-terminal antipeptide, whereas for
the wild type receptor, insulin decreases this interaction (19). However, conformational changes in the wild type receptor β-subunit have been found to be insulin- or phosphorylation-induced, in a two-step mechanism (14). The hormone-evoked receptor form is an intermediary one, and the phosphorylation-induced one corresponds to the active receptor. This conformation is accompanied by an increased immunoprecipitation by anti-(1306-1329). Thus, it is possible that our monoclonal antibodies induce a conformational change similar to the change induced by phosphorylation, mimicking the activating process. This could also explain the stimulating properties of these antibodies.

Until now, the only known way of stimulating this mutant receptor was by transphosphorylation of its cytoplasmic domain. Here, we demonstrate that it is possible to achieve activation by interacting at the level of the extracellular domain. The monoclonal antibody B6 stimulates receptor autophosphorylation and kinase activity, without additivity with insulin. In contrast, aM2 needs the hormone to exert a stimulatory effect. Moreover, this is in apparent contradiction with the strong inhibition of this antibody on insulin binding to Val382 receptor. This could be due to our experimental conditions, since hormone binding was measured at tracer ligand concentration, whereas saturating hormone concentration was used in the kinase assay. It seems that aM2 alone is not potent enough to activate, by itself, the mutant receptor.

We conclude from our data that a correlation exists between receptor kinase activation and the occurrence of conformational changes. In such a causal relationship, it would appear that some antibodies to the receptor extracellular domain represent alternative means to activate the mutant Val382 receptor kinase. Cells expressing Val382 receptor do not respond to insulin for a number of biological actions such as thymidine incorporation, c-Jun expression, and receptor autophosphorylation in intact cells (22). Hence, it would be of interest to study whether our antibodies are able to restore such responses.

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REFERENCES