EXPERIMENTAL STUDY

Functional characteristics of insulin receptors with a Thr→Ser¹²⁰⁰ mutation overexpressed in Chinese hamster ovary cells

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Abstract

Objective: To investigate the functional properties of insulin receptors with a Thr→Ser¹²⁰⁰-mutation that is associated with severe insulin resistance in humans.

Design and methods: The effect of *in situ* insulin-stimulation on insulin receptor kinase activity was studied in Chinese hamster ovary cells with overexpressed human Ser¹²⁰⁰-mutated, non-mutated, and ATP-binding site-mutated (Lys→Arg¹⁰³⁰) receptors using a microwell-based assay that only detects human (and not hamster) insulin receptors. Moreover, the fraction of anti-phosphotyrosine antibody-binding receptors following *in situ* stimulation was separated, and autophosphorylation and kinase activity resulting from *in situ* and/or *in vitro* activation evaluated in this fraction.

Results: Although insulin-stimulated kinase activity of human-specific anti-insulin receptor antibody-binding receptors in cells with Ser¹²⁰⁰-mutated insulin receptors represented only 3.3% of that reached in cells with non-mutated receptors, a clear insulin-induced increase in kinase activity was observed (3.4-fold; $P < 0.05$). This increase was associated with a $2.3 \pm 0.6\%$ ($P < 0.05$) increase in anti-phosphotyrosine-binding receptors with a kinase activity representing $43 \pm 8\%$ of that found in activated non-mutated receptors. *In vitro* autophosphorylation and kinase activation proceeded much more slowly in Ser¹²⁰⁰-mutated receptors ($t_{1/2}$: 100 min) compared with non-mutated receptors ($t_{1/2}$: 1 min) and were inhibitable by lower alkaline phosphatase concentrations (EC_{50} : 3 U/ml and 70 U/ml respectively). No activation of insulin receptor kinase was observed with Arg¹⁰³⁰-mutated receptors.

Conclusions: Overexpressed Ser¹²⁰⁰-mutated human insulin receptors possess insulin-stimulated kinase activity and can be activated *in situ* and *in vitro*. They are characterized by a markedly slower autophosphorylation reaction, which, in a phosphatase-containing environment, results in a small fraction of phosphorylated and activated receptors.

European Journal of Endocrinology 143 125–131

Introduction

The insulin receptor is a heterotetrameric, transmembrane tyrosine kinase which undergoes ligand-activated autophosphorylation of tyrosine residues on its own β -subunit. This phosphorylation of certain tyrosine residues in the tyrosine kinase domain of the receptor is necessary to allow full activation of the receptor tyrosine kinase activity towards other substrates and to allow normal signaling to downstream effectors (1). Deactivation of the insulin receptor kinase occurs via receptor dephosphorylation by cellular phosphotyrosine-phosphatases (2).

The Trp→Ser¹²⁰⁰ insulin receptor mutation is one of several mutations found in the receptor kinase region that is associated with severe insulin resistance (3–9).

In a previous study, the insulin-stimulated glucose transport and glycogen synthesis were lower in Chinese hamster ovary (CHO) cells with overexpressed Ser¹²⁰⁰-mutated receptors compared with CHO cells with overexpressed non-mutated receptors, but were higher than in CHO cells without insulin receptor overexpression (10). Since this increase by Trp→Ser¹²⁰⁰ insulin-receptor overexpression was observed (although insulin-stimulated receptor kinase activity in partially purified cell extracts was similar to that of extracts from cells without human insulin receptor overexpression (10)), the possibility of receptor kinase-independent insulin signaling was discussed. In the present study, a microwell-based assay specific for human, but not hamster, insulin receptors (11) was used to determine if insulin stimulation activates the mutated receptors in

CHO cells with overexpressed Ser¹²⁰⁰-mutated insulin receptors. We show that the kinase of the Ser¹²⁰⁰-mutated insulin receptors can be insulin-dependently activated *in situ* and *in vitro*. Their autophosphorylation, however, is much slower than that of non-mutated insulin receptors and therefore only a few of the mutated receptors are tyrosine-phosphorylated and activated in the *in situ* environment.

Materials and methods

Materials

Porcine monocomponent insulin was purchased from Novo Biolabs (Bagsvaerd, Denmark), insulin-like growth factor-I (IGF-I) was from Bissendorf (Hannover, Germany), [γ -³²P]ATP (5000 Ci/mmol) was from DuPont-NEN (Dreieich, Germany) and [¹²⁵I-Tyr-A¹⁴]moniodoinsulin and [3-¹²⁵I]iodotyrosyl-IGF-I were from Amersham (Braunschweig, Germany). BSA was from Boehringer Mannheim (Mannheim, Germany) and materials for SDS-PAGE were from Bio-Rad (München, Germany). Goat anti-mouse IgG was from Dianova (Hamburg, Germany), mouse hybridoma cells producing a monoclonal antibody directed against the α -subunit of the human insulin receptor (α IR) were from the American Type Culture Collection (Rockville, MD, USA) and a monoclonal antibody against phosphotyrosine (α PY) was from Upstate Biotechnology (New York, NY, USA). A synthetic polymer with a 4:1 ratio of glutamic acid residues to tyrosine residues (Glu4:Tyr1) and other reagents were from Sigma (München, Germany).

Cell lines

Transfected CHO cells that overexpressed non-mutated human insulin receptors (non-mutated huIRs), human insulin receptors with a Lys→Arg¹⁰³⁰ mutation at the ATP-binding site (Arg¹⁰³⁰-huIRs) or a Thr→Ser¹²⁰⁰ mutation (Ser¹²⁰⁰-huIR) were kindly provided by Moller *et al.* (10). In addition, CHO cells that expressed only the hamster insulin receptors (haIRs) were studied. Cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 800 μ g/ml geneticin at 37 °C under an atmosphere of 95% air and 5% CO₂ in 140-mm dishes.

Incubation and solubilization of cells

Confluent cells were first preincubated with incubation buffer (20 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 130 mmol/l NaCl, 4.8 mmol/l KCl, 1.3 mmol/l KH₂PO₄, 1 g/l D-glucose, 1.3 mmol/l MgSO₄, 1.2 mmol/l CaCl₂, 2% BSA, pH 7.4) for 30 min (37 °C). This buffer was then replaced by fresh incubation buffer, with or without 87 nmol/l

insulin, and the cells incubated for 30 min. The buffer was then quickly removed and the dishes containing the cells were frozen in liquid nitrogen. The thin ice layer that contained the cells was scraped off the dishes at -20 °C and homogenized with a motor-driven homogenizer Potter S (Braun, Melsungen, Germany) in a solution that contained 2% Triton X-100, 5 mmol/l phenylmethylsulfonylfluoride, 800 U/ml aprotinin (trypsin inhibitor), 8 mmol/l EDTA, 30 mmol/l benzamidine, 2.5 μ g/ml pepstatin, 2.5 μ g/ml leupeptin, 160 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 0.2 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid, and 20 mmol/l HEPES, pH 7.4 (final concentrations). Samples were then centrifuged at 10⁵ g and 4 °C to remove insoluble material and stored at -80 °C.

Immobilization of receptors and measurement of receptor kinase and binding activities

This was performed essentially as described previously (11). Briefly, 30 μ l aliquots of solubilized cell samples were pipetted into microwells coated with α IRs. After 16 h at 4 °C, the wells were washed and phosphorylation reactions were initiated in the wells by the addition of a mixture that contained [γ -³²P]ATP (100–200 Ci/mmol) and Glu4:Tyr1 (2 mg/ml). The radioactivity incorporated into Glu4:Tyr1 was then measured (11). Binding of insulin and/or IGF-I to immobilized receptors was measured in the wells, also as described elsewhere (11). Briefly, [¹²⁵I-Tyr-A¹⁴]moniodoinsulin or [3-¹²⁵I]iodotyrosyl-IGF-I (0.2 nmol/l in each case) was added to the wells in the absence or presence of unlabeled insulin or IGF-I. After 16 h at 4 °C, the wells were washed and the amount of bound radioactive ligands determined (11).

Selection of insulin receptors that bound to α PY

This was done essentially as described previously (2). Briefly, cell extracts or receptors that had been partially purified by wheat-germ agglutinin-agarose chromatography were pipetted into α PY-coated microwells. After 16 h at 4 °C, the wells were washed and receptors that had bound to α PY were detached by the addition of 30 μ l buffer containing 100 mmol/l phenylphosphate for 8 h at 4 °C (2). Detached receptors were then transferred to α IR-coated microwells for measurement of kinase activity and/or binding activities.

In vitro autophosphorylation and kinase activity of insulin receptors

To study *in vitro* autophosphorylation and kinase activity of immobilized receptors, wells were incubated with 0.5 mmol/l ³²P labeled or unlabeled ATP, respectively, for 24 h in buffer (0.5% Triton X-100, 100 mmol/l NaCl, 2.5 mmol/l KCl, 1 mmol/l CaCl₂,

20 mmol/l HEPES, 10% glycerol, and 0.5% BSA, pH 7.4). For the detection of insulin receptor autophosphorylation receptors that had bound to α IR-coated microwells were detached by twice adding a solution of 2% SDS for 30 min. Then ³²P incorporation into the insulin receptor β -subunit was measured by autoradiography. Insulin receptor kinase activity was measured as described above.

Results

Insulin activation of insulin receptor kinase

Insulin stimulation of cells with overexpressed non-mutated huIRs resulted in an 8.5-fold increase in kinase activity of the subsequently immuno-immobilized receptors (Fig. 1A). An insulin-stimulated increase in kinase activity was also observed in cells with overexpressed Ser¹²⁰⁰-huIRs (Fig. 1B); this effect, however, was much smaller than in cells with overexpressed non-mutated huIRs (0.01 ± 0.001 compared with 0.3 ± 0.04 fmol P/fmol binding activity/min respectively). To exclude the possibility that this effect on insulin receptor kinase activity could be simply explained by the binding of parental haIRs to α IRs, α IR-coated microwells were also incubated with extracts of insulin-stimulated or non-insulin-stimulated CHO cells without overexpressed huIRs or with Arg¹⁰³⁰-huIRs. In these wells, no insulin-binding activity was detectable (data not shown), indicating that no haIRs bound to the antibody. Moreover, in wells incubated with extracts from Arg¹⁰³⁰-huIR cells, a binding activity was measured which was similar to that found in wells that had been incubated with Ser¹²⁰⁰-huIR cells (data not shown) but, in contrast to the Ser¹²⁰⁰-huIR cells, there was no kinase activation (Fig. 1B). Taken together, these data indicate that the increase in kinase activity observed with the immobilized receptors from Ser¹²⁰⁰-huIR cells

was in fact due to activation of Ser¹²⁰⁰-mutated holoreceptors or possibly hybrids formed with the haIR- (Ser¹²⁰⁰-huIR/haIR) and the haIGF-I receptor (Ser¹²⁰⁰-huIR/haIGF-I).

Number and kinase activity of in situ-activated receptors

To explore whether the small, but significant, kinase activation of receptors from Ser¹²⁰⁰-huIR cells that bound to microwells coated with α IRs was the result of a small number of receptors being activated or a low kinase activity reached by the individual receptors, tyrosine-phosphorylated receptors from insulin-stimulated cells were selected by immobilization to α PY. Only ≈ 2 –3% of the overexpressed insulin receptors from Ser¹²⁰⁰-huIR cells (compared with $\approx 50\%$ of the receptors from non-mutated-huIR cells) bound to α PY (Fig. 2A), but the kinase activity per binding activity of those receptors that bound to α PY represented almost 50% of the kinase activity per receptor of similarly treated non-mutated huIRs (Fig. 2B). This demonstrates that the increase in insulin-stimulated kinase activity that was measured in Ser¹²⁰⁰-huIR cells was derived from a small fraction of tyrosine-phosphorylated receptors with almost half of the insulin-stimulated kinase activity of non-mutated huIRs.

In vitro autophosphorylation and kinase activity of Ser¹²⁰⁰-huIRs

To test whether the human receptors from Ser¹²⁰⁰-huIR cells were able to autophosphorylate, receptors from non-stimulated Ser¹²⁰⁰-huIR cells were immobilized to α IR-coated microwells and exposed to insulin and ATP. This resulted in an autophosphorylation of the receptors (Fig. 3A) and was associated with increased kinase

Figure 1 Insulin-stimulation of insulin-receptor kinase activity in intact CHO cells. Cells that overexpressed non-mutated- (A), Ser¹²⁰⁰- or Arg¹⁰³⁰-huIRs (B) were incubated without (□) or with (■) 87 nmol/l insulin for 30 min at 37 °C and solubilized. Insulin receptors were immobilized to α IR-coated microwells and insulin receptor kinase and binding activities were measured as described in the methods section. Kinase activities were normalized for insulin binding activities in the respective wells and therefore reflect kinase activities per receptor. Microwells coated with α IRs were also incubated with extracts from insulin-stimulated or non-insulin-stimulated CHO cells that only expressed haIRs. In these wells, no insulin-binding activity was detectable (data not shown). Values are means $\pm$ S.E.M.; $n = 6$. * $P < 0.005$ compared with cells without insulin-stimulation (paired t -test).

Figure 2 Number and kinase activities of receptors from insulin-stimulated cells that bound to α PY and subsequently to α IRs. Cells with over-expressed non-mutated-, Ser¹²⁰⁰- or Arg¹⁰³⁰-huIRs were stimulated with 87 nmol/l insulin for 30 min at 37 °C, solubilized and added either directly to α IR-coated wells or previously to wells that were coated with α PY. After immobilization of tyrosine-phosphorylated proteins to the α PY-coated wells, wells were washed and bound proteins were detached with phenylphosphate and then immobilized to α IR-coated wells, as described in the methods section. Subsequently, kinase- and binding activities were measured. (A) Number of insulin receptors that bound to α PY (expressed as percentage of the total amount of insulin receptors that bound to α IRs without prior selection of tyrosine-phosphorylated receptors). (B) Kinase activities per binding activities of insulin receptors that bound to α PY and to α IRs (■) compared with kinase activities per binding activities of receptors that bound directly to α IRs without prior selection on α PY (□). Values are means $\pm$ S.E.M., $n = 6$.

activity towards insulin receptor substrate-1 (IRS-1) (Fig. 3B). Investigations were then directed towards establishing how many of the Ser¹²⁰⁰-huIRs could be activated *in vitro* and what the time course of this activation was. With non-mutated huIRs, a rapid increase in the number of α PY-binding receptors was observed with half-maximal and maximal effects at 1 and 5 min respectively (Fig. 3C). An increase in the number of α PY-binding receptors was also observed in wheat-germ eluates from Ser¹²⁰⁰-huIR cells, and since the α PY-binding receptors were detected after they bound to α IRs they represented receptors with at least one Ser¹²⁰⁰-mutated insulin receptor half. The increase in the number of α PY-binding receptors from Ser¹²⁰⁰-huIR cells was, however, markedly slower than that observed with receptors from non-mutated huIR cells and reached at least 12% of all α IR-binding over-expressed insulin receptors after 48 h (half-maximal effect at $\approx$ 100 min, Fig. 3C).

In vitro autophosphorylation of non-mutated and Ser¹²⁰⁰-huIRs in the presence of alkaline phosphatase

In cells, phosphotyrosine-phosphatases that dephosphorylate phosphorylated insulin receptors are present. It is therefore likely that slow insulin-stimulated receptor autophosphorylation shifts the equilibrium to the dephosphorylated state and thereby leads to the presence of only a few activated receptors. To test this hypothesis in an *in vitro* model, autophosphorylation of non-mutated- and Ser¹²⁰⁰-huIRs was performed in the presence of alkaline phosphatase (Fig. 4). At 20 U/ml alkaline phosphatase, the Ser¹²⁰⁰-huIR reached only $\approx$ 20% of the kinase activity attained in the absence of

alkaline phosphatase, whereas the non-mutated-huIR reached $\approx$ 80%. This indicates that the slow autophosphorylation of the Ser¹²⁰⁰-huIRs severely impairs their activation in a phosphotyrosine-phosphatase-containing environment.

IGF-I/insulin receptor hybrids

It is likely that only one huIR half is sufficient for the binding to α IRs. It was therefore possible that receptors from insulin-incubated Ser¹²⁰⁰-huIR cells that bound to α PY were Ser¹²⁰⁰-huIR/haIGF-I hybrids. In order to differentiate between receptors that contained either two Ser¹²⁰⁰-mutated- or one Ser¹²⁰⁰-mutated huIR and one haIGF-I receptor half, binding of insulin and IGF-I to α IR-coated microwells that had been incubated with the α PY-precipitable fraction of extracts from insulin-stimulated Ser¹²⁰⁰-huIR cells was measured. The data show that those receptors that were phosphorylated in Ser¹²⁰⁰-huIR cells and bound to α IRs had typical binding curves for insulin receptors (Fig. 5). This indicates that the fraction of insulin receptors that were tyrosine-phosphorylated following *in situ* insulin stimulation did not contain a substantial number of Ser¹²⁰⁰-huIR/haIGF-I hybrids.

Discussion

Our data show that insulin stimulation of Ser¹²⁰⁰-huIR cells led to a small, but significant, kinase activation of receptors that bound to α IRs. No increase in insulin-stimulated receptor kinase activity was observed in Arg¹⁰³⁰-huIR cells or in haIR cells. This demonstrates that receptors with the Ser¹²⁰⁰-mutation were insulin-independently activated in the cells. The insulin-stimulated

Figure 3 *In vitro* autophosphorylation and kinase activation of receptors that bound to α IR. Insulin receptors from cells that overexpressed the respective insulin receptors were immobilized to microwells coated with α IRs and subsequently incubated with 0.5 mmol/l ³²P labeled (A) or unlabeled (B) ATP, respectively, for 24 h, as described in the methods section. For the detection of insulin-receptor autophosphorylation (A), ³²P was collected by twice adding a solution of 2% SDS for 30 min. For the measurement of insulin-receptor kinase activity (B), ³²P was collected from the supernatant of *in vitro*-incubated wells after the addition of a mixture that contained [³²P]ATP and recombinant insulin-receptor substrate-1 (IRS-1). Proteins were then separated by SDS-PAGE and ³²P-incorporation into the receptor β -subunit (A, 95 kDa) or IRS-1 (B, 170 kDa) was measured by autoradiography. Counts per minute (c.p.m.), as obtained from Fig. 3A and B respectively were as follows: (1) huIR, 10 c.p.m. at 95 kDa, 17 c.p.m. at 170 kDa; (2) Arg¹⁰³⁰-huIR, 71 c.p.m. at 95 kDa, 32 c.p.m. at 170 kDa; (3) Ser¹²⁰⁰-huIR, 2230 c.p.m. at 95 kDa, 390 c.p.m. at 170 kDa; (4) non-mutated huIR, 15 551 c.p.m. at 95 kDa, 3730 c.p.m. at 170 kDa. To determine the time course of *in vitro* receptor autophosphorylation, solubilized non-mutated- (□), Ser¹²⁰⁰- (●) or Arg¹⁰³⁰-huIRs (◆) were partially purified by wheat-germ agglutinin-agarose chromatography, incubated with 0.5 mmol/l ATP for the indicated times and the reaction stopped by the addition of kinase- and phosphatase-inhibitors (C). The receptors were then either directly immobilized to α IR-coated wells or the α PY-binding fraction was first selected as described in the methods section. The amount of tyrosine-phosphorylated insulin receptors that bound to α PY and α IRs is shown as a percentage of the total amount of receptors that bound directly to α IRs. Values are means of three experiments.

Figure 4 *In vitro* autophosphorylation of Ser¹²⁰⁰- and non-mutated huIRs in the presence of alkaline phosphatase. Receptors from cells that overexpressed non-mutated (□) or Ser¹²⁰⁰-huIRs (●) were immobilized to microwells coated with α IRs. Immuno-immobilized receptors were then incubated for 24 h with 0.5 mmol/l ATP in the presence of the indicated concentrations of alkaline phosphatase. Wells were washed and insulin receptor kinase activity measured as described. Receptor kinase activity is shown in percent receptor kinase activity after *in vitro* incubation of the wells in the absence of alkaline phosphatase. Values are means of two experiments.

increase in kinase activity of the Ser¹²⁰⁰-huIR cells might well explain the increased insulin effect on 2-deoxyglucose uptake and glucose incorporation into glycogen in these cells compared with cells without overexpression of human insulin receptors or expression of the Arg¹⁰³⁰-mutated insulin receptor (10). In fat cells, the half-maximal stimulation of glucose uptake occurred at an insulin concentration at which only 4% of the maximal kinase activity was reached (12), and in cells with overexpressed insulin receptors an even smaller percentage of the maximal insulin-stimulated kinase activity might be sufficient to produce biological effects. Taken together, our data demonstrate that the Ser¹²⁰⁰-huIRs that were overexpressed in CHO cells had insulin-stimulated kinase activity and do not support the hypothesis that the receptor kinase might not be required for the stimulation of biological insulin effects (10).

The slight increase in the overall human insulin receptor kinase activity measured in insulin-stimulated Ser¹²⁰⁰-huIR cells was not the result of a slight increase in the kinase activity of all or most cellular insulin receptors, but rather resulted from the activation of only approximately 2–3% of the cellular receptors that exhibited $\approx$ 50% of the kinase per binding activity of that reached in insulin-stimulated non-mutated huIRs. These results led us to further explore the functional properties of α IR-binding receptors from Ser¹²⁰⁰-huIR cells that were tyrosine-phosphorylated by insulin incubation of the cells. It was possible that these receptors were unable to autophosphorylate and that their kinase activation resulted from intermolecular

Figure 5 Insulin- and IGF-I binding to tyrosine-phosphorylated receptors from Ser¹²⁰⁰-huIR cells. Receptors from insulin-incubated Ser¹²⁰⁰-huIR cells were first immobilized to α PY-coated wells, detached and then transferred to α IR-coated wells as described in the methods section. Subsequently, insulin receptors were incubated for 24 h with 0.2 nmol/l [¹²⁵I]insulin and unlabeled insulin (—), 0.2 nmol/l [¹²⁵I]insulin and unlabeled IGF-I (---) or 0.2 nmol/l [¹²⁵I]IGF-I and unlabeled IGF-I (···). Binding activities were measured as described. Values are means $\pm$ S.E.M., $n = 3$.

phosphorylation by other cellular receptors, as previously described with cells expressing both wild-type and Ile¹¹⁵³-mutated human insulin receptors (13). The Ser¹²⁰⁰-receptors were, however, insulin-dependently phosphorylated *in vitro* following their immuno-immobilization to microwells coated with α IRs and this also resulted in their activation for the phosphorylation of IRS-1. These results demonstrate that these receptors were able to autophosphorylate and indicate that their activation in the cells was at least not dependent on phosphorylation by other receptors.

The rate of *in vitro* autophosphorylation of the Ser¹²⁰⁰-huIR was much slower compared with the non-mutated huIR. After 24 h of *in vitro* incubation with insulin and ATP, $\approx 12\%$ bound to α PY indicating that at least this percentage of the α IR-binding receptors from Ser¹²⁰⁰-huIR cells were able to autophosphorylate. Conversely, only $\approx 2\text{--}3\%$ of the α IR-binding insulin receptors were activated by maximal insulin stimulation of the Ser¹²⁰⁰-huIR cells. This may be explained by the slow autophosphorylation of the mutated receptors in the presence of phosphotyrosine-phosphatases in the cellular environment. Phosphotyrosine-phosphatases strongly counter-balance insulin receptor activation (2) and thus a slow autophosphorylation results in a shift of the equilibrium between receptor autophosphorylation and dephosphorylation. Accordingly, we have demonstrated *in vitro* that in the presence of 20 U/ml alkaline phosphatase Ser¹²⁰⁰-huIR reaches only $\approx 20\%$ of the kinase activity attained in the absence of alkaline phosphatase, whereas non-mutated huIR reaches $\approx 80\%$.

Receptor halves from human insulin receptors that

are expressed in rodent cells can dimerize with endogenous receptor halves (14–16). It is therefore possible that *in situ*-activated receptors that bound to α IRs were not Ser¹²⁰⁰-mutated holoreceptors but represented a subfraction of the α IR-binding receptors, e.g. hybrids that contained one human insulin and one hamster insulin or IGF-I receptor half. Since CHO cells without overexpression of human insulin receptors contained approximately 5000 IGF-I receptors per cell (10), $\approx 10,000$ huIR/IGF-I hybrids per Ser¹²⁰⁰-huIR cell could be expected. Our insulin- and IGF-I-binding data suggest, however, that the activated receptors from Ser¹²⁰⁰-huIR cells that bound to α IRs did not represent hybrids between human insulin receptors and rodent IGF-I receptors. Potentially, no huIR/IGF-I hybrids existed, they existed but were not able to autophosphorylate, or they did not bind to α IRs. Our data indicate that of the activated receptors from Ser¹²⁰⁰-huIR cells that bound to α IRs few (if any) were Ser¹²⁰⁰-huIR/haIGF-I hybrids, and thus the Ser¹²⁰⁰-huIRs that were activated by insulin stimulation of the cells were Ser¹²⁰⁰-mutated holoreceptors or hybrids of human and hamster insulin receptor halves or both. Unfortunately, we were unable to resolve the latter question, since no hamster-specific insulin receptor antibody was available. The CHO cells without insulin receptor overexpression express $\approx 2,000$ endogenous insulin receptors per cell (10). If the same amount of endogenous insulin receptors were expressed in the Ser¹²⁰⁰-huIR cells, up to 4,000 Ser¹²⁰⁰-huIR/haIR hybrids might be present, which would amount to approximately 8% of the overexpressed receptors (10). We found that at least 12% of the overexpressed receptors from Ser¹²⁰⁰-huIR cells were able to autophosphorylate, and therefore, with these estimates, the possibility remains that the receptors from insulin-stimulated Ser¹²⁰⁰-huIR cells that were able to autophosphorylate were Ser¹²⁰⁰-huIR/haIR hybrids. In any case, even if they represented such hybrids, the Ser¹²⁰⁰-huIR/haIR receptors would presumably be functionally similar to the Ser¹²⁰⁰-huIR/non-mutated huIR receptor that probably represents the most abundant insulin receptor structure in the heterozygous patient with this mutation (10).

Most of the naturally occurring point mutations in the regulatory domain of the insulin receptor kinase (1002–1257) were reported to result in a complete loss of the insulin-stimulated receptor activity (3, 7, 17). There are also reports, consistent with our results, that describe mutated receptors that only partially impair the *in vitro* (18) or *in situ* (19) autophosphorylation. The functional characteristics of these mutations, however, have not been evaluated to the same extent as in the present study. In particular, a mutation with a ≈ 100 -fold slower autophosphorylation, but only 50% decreased kinase activity if autophosphorylated, to our knowledge has not been described. It is, however, possible that other insulin receptor mutations with

only partially reduced kinase function have similar functional characteristics.

In summary, our data show that CHO cells with overexpressed Ser¹²⁰⁰-huIRs contain receptors that display insulin-stimulated kinase activity and bind to an antibody that only detects human insulin receptors. This result may explain why overexpression of Ser¹²⁰⁰-huIRs increases the insulin effect of distinct biological activities. Furthermore, we have shown that activated receptors from Ser¹²⁰⁰-huIR cells that bound to α IRs are characterized by a slow, insulin-dependent autophosphorylation and a relatively high kinase activity if activated.

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Received 1 December 1999

Accepted 28 March 2000