The Insulin Receptor Functions Normally in Chinese Hamster Ovary Cells After Truncation of the C Terminus*

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Martin G. Myers, Jr.*, Jonathan M. Backer§, Kenneth Siddle, and Morris F. White

From the Research Division, Joslin Diabetes Center, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

We studied the structure and function of the human insulin receptor (IR) and a mutant which lacked the last 43 amino acids of the β-subunit (IRact). This deletion removed tyrosine (Tyr114), threonine (Thr139) and serine (Ser135) autophosphorylation sites. In Chinese hamster ovary (CHO) cells, insulin binding to the mutant receptor was normal, and [3H]methionine labeling indicated that both the IR and IRact were processed normally; however, the β-subunit of IRact was 5 kDa smaller than that of the IR. The time course of insulin-stimulated autophosphorylation of the partially purified IRact was normal, but the maximum autophosphorylation was reduced 20-30%. Tryptic phosphopeptide mapping confirmed the absence of the C-terminal phosphorylation sites and indicated that phosphorylation of portions of the juxtamembrane region which is not found in the insulin receptor. Another possible regulatory mechanism involving the C terminus of the IR appears to be essential for interactions between the kinase-active insulin receptor and certain signal-transducing molecules.

Insulin alters cellular growth and metabolism by interacting with specific receptors present on the surface of most cells. Insulin binding to the α-subunit of the insulin receptor (1)

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† Recipient of a Medical Scientist Training Program Award.

‡ Recipient of National Research Service Award DK08126.

§ Scholar of the Pew Foundation, Philadelphia. To whom correspondence should be addressed: Research Div., Joslin Diabetes Center, 1 Joslin Pl., Boston, MA 02215.

The abbreviations used are: IR, insulin receptor; IRact, truncated IR lacking the last 43 amino acids at the C terminus of the β-subunit; IRαβ116, insulin receptor with phenylalanine substitution at position 116; α-FY, anti-phosphotyrosine antibody; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; 83-14, a monoclonal anti-insulin receptor antibody that mimics insulin action; PtdIns, phosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.

Immediately stimulates tyrosyl autophosphorylation of the β-subunit (1), which activates the tyrosine-specific phosphotransferase (2-4). The kinase activity is essential for insulin action, as kinase-deficient receptor molecules are biologically inactive (5, 6). Mutation of the tyrosine autophosphorylation sites in the regulatory region of the IR (Tyr113, Tyr115, and Tyr135) variably alters kinase activity and biological activity, suggesting that insulin responses are mediated by multiple transduction pathways (7-9). Point mutations or deletions of portions of the juxtamembrane region block insulin-stimulated phosphorylation of pp185 and the activation of the PtdIns 3-kinase, two proteins thought to be involved in signal transmission (10-12). Since autophosphorylation and in vitro kinase activity are apparently normal, the juxtamembrane region of the IR appears to be essential for interactions between the kinase-active insulin receptor and certain signal-transducing molecules.

Several studies suggest various roles for the C-terminal domain of the IR. In Rat-1 fibroblasts, the IRact does not fully stimulate glycogen synthesis, whereas it stimulates DNA synthesis more strongly than the wild-type IR (13-15). Moreover, it is thought that the C terminus exerts an inhibitory effect on the mitogenic activity of the IR, such that its removal releases the IR from regulatory constraints (15). A similar mechanism has been proposed for several src-like tyrosine kinases, but it appears to require an interaction between C-terminal phosphotyrosine residues and the src homology-2 domain which is not found in the insulin receptor. Another possible regulatory mechanism involving the C terminus of the insulin receptor is the phosphorylation of Thr139 by the protein kinase C (16).

In order to further define the function of the C-terminal domain of the insulin receptor in the context of the other mutant receptors, we have studied the enzymatic and biological effect of removing 43 amino acids including two tyrosine phosphorylation sites from the C terminus of insulin receptor β-subunit. In contrast to previous reports, function of the truncated receptor (IRact) is identical to the intact receptor by several criteria, and in CHO cells the C-terminal phosphorylation sites are not required for several signaling pathways.

MATERIALS AND METHODS

Transfection of CHO Cells and Insulin Binding—Subconfluent CHO cells (106) grown in F-12 medium containing 10% fetal bovine serum (GIBCO) were transfected by calcium phosphate precipitation with 1 μg of pSVEno alone or together with 10 μg of pCVSVHRC or pCVSVHRC/Δact as previously described (13, 17). After 72 h, geneticin (GIBCO) was added to the medium (800 μg/ml) to select the neomycin-resistant cells. Surviving CHO cells were selected for high levels of surface IR expression by fluorescence-activated cell sorting, and clonal cell lines were obtained by plating at limiting dilution (10).

10616
Insulin binding to the transfected CHO cells was measured on confluent monolayers in 24-well plates (Costar) as previously described (10). CHO.neo cells express approximately 30,000 hamster insulin receptors/cell. Analysis of equilibrated cell sorting, cloned lines of CHO/IR cells used in this study with the LIGAND system using a two-site binding model, the high affinity identical to that of the normal IR. When analyzed together chard binding analysis also revealed that IR, bound insulin with an affinity identical to that of the normal IR. When analyzed together chard binding analysis also revealed that IR, bound insulin with an affinity identical to that of the normal IR. 

Glucose Incorporation into Glycogen and Thymidine Incorporation into DNA—Confluent CHO cells were washed in PBS and incubated for 3 h at 37°C in F-12 medium containing 2.5 mM glucose, 0.1% BSA, and 25 mM HEPES (pH 7.4); the medium was then aspirated and replaced with similar medium containing various concentrations of insulin or anti-IR 83-14 antibody, and incubation was continued for 30 min at 37°C. Glucose (Sigma) (1 µCi/ml final concentration) was added, and the cells were incubated for 90 min at 37°C. Lysates were prepared as described (1) and washed in an equal volume of 70% ethanol. Radioactivity was quantified by scintillation counting.

DNA synthesis was measured in subconfluent CHO cells. The cells were washed in PBS and incubated without serum for 24 h at 37°C in F-12 medium. The cells were washed in PBS and incubated at 37°C for exactly 15 h in F-12 medium containing 0.1% BSA and various concentrations of insulin, monoclonal anti-IR antibody 83-14, or 19% fetal bovine serum. The cells were then incubated 1.5 h in F-12 medium containing 0.1% BSA, 25 mM HEPES (pH 7.4) and 0.5 µCi/ml [3H]thymidine (Du Pont-New England Nuclear). The cells were washed three times in ice-cold PBS, and solubilized in 0.1% SDS at 37°C for 1 h. DNA was precipitated with ice-cold trichloroacetic acid (>2.5% final concentration), collected on glass filters (Whatman), washed three times with ice-cold 10% trichloroacetic acid, and washed once with ice-cold ethanol. Radioactivity was quantified by scintillation counting.

PtdIns 3-Kinase Activity—PtdIns 3-kinase activity in α-PY immunoprecipitates was determined as previously described (1). Subconfluent CHO cells were incubated overnight in F-12 medium containing 0.5% BSA. The cells were then incubated with 10 min and washed once in ice-cold PBS and twice in 10 mM Tris (pH 7.5), containing 137 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, and 100 µM Na3VO4 (Buffer A). The cells were solubilized in Buffer A containing 1% Nonidet P-40 (Sigma), and 10% glycerol and insoluble material was removed by centrifugation at 13,000 × g for 10 min. Tyrosyl phosphorylated proteins were immunoprecipitated from the supernatant with α-PY and protein A-Sepharose (Pharmacia LKB Biotechnology, Inc.). The immunoprecipitates were washed successively three times in PBS containing 1% Nonidet P-40 and 100 µM Na3VO4, three times in 100 mM Tris (pH 7.5) containing 500 mM LiCl and 100 µM Na3VO4, and twice in 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 µM Na3VO4. Finally, the pellets were resuspended in 10 mM Tris (pH 7.5) containing 106 µCi/ml [3H]thymidine (Du Pont-New England Nuclear) (75 µCi/mM) was added to a final concentration of 40 µM, and the reaction was allowed to proceed for 10 min at 22°C. Phosphorylation was stopped by the addition of 0.2 volume of 8 N HCl and 1.5 volumes of CHCl3:MeOH (1:1). After centrifugation the lower phase was removed and applied to a TLC plate (Merck) coated with phosphatidylinositol previously sonicated in 10 mM Tris (pH 7.5) containing 137 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, and 100 µM Na3VO4. The plate was developed in CHCl3:CH2OH:H2O:NH4OH (60:47:11:3.2), dried, and visualized by autoradiography. The radioactivity in spots co-migrating with PtdIns 4-monophosphate standard (Sigma) was measured by Cerenkov counting.
Expression of Normal IR and IRAct in CHO Cells—The structure of the wild-type and mutant insulin receptors was analyzed in CHO/IR and CHO/IRact, cells, respectively, labeled for 18 h with \(^{35}\)Smethionine. The receptors were immunoprecipitated with anti-insulin receptor antibodies and analyzed by SDS-PAGE (Fig. 1). The a-subunit from both cell lines migrated at 135 kDa, and the b-subunit of the wild-type IR migrated at 95 kDa. In contrast, the b-subunit of IRact migrated at about 90 kDa owing to the deletion of 43 C-terminal amino acids. The wild-type and truncated b-subunits contained approximately twice as much incorporated \(^{35}\)S methionine as the a-subunit, consistent with a 2:1 ratio of methionine residues for the b-subunits to a-subunits (20). These data suggest that the IR and IRact are translated, processed, and directed to the cell membrane normally in CHO cells.

Autophosphorylation of the Partially Purified IR and IRAct—To assess autophosphorylation of IR and IRact purified on immobilized wheat germ agglutinin, equal concentrations of receptor determined from Scatchard analysis were autophosphorylated with \(^{32}\)PATP in the presence or absence of 100 nM insulin for various time intervals and resolved by SDS-PAGE. Insulin stimulated the rate of autophosphorylation on both receptors about 20-fold (Fig. 2). The wild-type and mutant receptors showed similar kinetics, and reached maximum \(^{32}\)P phosphate incorporation in 10–20 min. However, the level of autophosphorylation of IRact during insulin stimulation was 25% lower than wild-type IR at all time points.

Separation of tryptic phosphopeptides from the b-subunit of the IR phosphorylated in vitro for 5 min revealed an elution profile as previously described (Fig. 3A) (4, 21). This included a doublet (pY1 and pY1a) attributed to the tris-phosphorylated regulatory region, a single peptide (pY4) attributed to the bis-phosphorylated regulatory region, and two peaks (pY2 and pY3) corresponding to phosphorylation of the C-terminal domain. Only the pY2 and pY3 were absent form the peptide maps of the IRact, which is consistent with removal of the C-terminal domain (Fig. 3C). However, truncation does not alter the pattern of phosphorylation in the regulatory region as indicated by the normal appearance of pY1, pY1a, and pY4 in the IRact.

Tris-phosphorylation of the regulatory region and phosphorylation of the C-terminal sites is inhibited by the inclusion of a-PY during the in vitro autophosphorylation reaction (4, 21). In the presence of \(\alpha\)-PY, phosphopeptides pY1, pY1a (regulatory region), and pY2 and pY3 (C terminus) are significantly inhibited during insulin-stimulated autophosphorylation (Fig. 3B). Moreover, as previously shown, the presence of \(\alpha\)-PY caused the accumulation of the bis-phosphorylated regulatory region in the IR as shown by increased pY4 and the appearance of pY5 (Fig. 3B) (4). Similarly, the \(\alpha\)-PY inhibited tris-phosphorylation of the regulatory region of the IRact and caused the accumulation of the bis-phosphorylated region (Fig. 3D) In the absence of the C-terminal peptides an additional phosphopeptide labeled pY4a was also detected during incubation with the \(\alpha\)-PY, but its identity is unknown; it appears to be obscured by pY2 in the elution profile of the wild-type receptor. Thus, truncation of the IR does not alter the cascade of autophosphorylation in the regulatory region of the IR.

Kinase Activity of the Purified IR and IRact—Insulin stimulates the phosphotransferase activity of the IR during in vitro incubation with synthetic substrates. Before insulin stimulation, both the IR and IRact poorly phosphorylated the substrate Thr-12-Lys, even when the receptor was allowed to undergo basal autophosphorylation for 30 min before adding the substrate (Fig. 4). In contrast, insulin-stimulated kinase activity was strongly dependent on prior autophosphorylation. After insulin stimulation but before autophosphorylation, the activity of the IR and IRact was barely increased. However, 5 min of insulin-stimulated autophosphorylation completely activated the kinase of the IR and IRact. The time courses for kinase activation and receptor autophosphorylation were similar (compare Figs. 2 and 4). Thus, kinase activity is regulated by insulin binding and phosphorylation of the regulatory region (4), and is entirely independent of autophosphorylation in the C terminus.

Phosphorylation of the IR and IRact in CHO Cells—In the absence of insulin, tyrosyl autophosphorylation of IR in \(^{32}\)P phosphate-labeled CHO/neo, CHO/IR, and CHO/IRact cells was undetectable. However, after a 1-min insulin stimulation, autophosphorylation of the b-subunit increased in CHO/IR and CHO/IRact, but little stimulation was observed in CHO/neo cells (Fig. 5A). After normalizing for receptor levels by \(^{35}\)S methionine labeling and confirming this by Scatchard binding analysis, the amount of \(^{32}\)P phosphate incorporated into IRact was 70–80% of wild-type IR (Fig. 5B). The reduced autophosphorylation of the IRact is consistent with the deletion of the two C-terminal autophosphorylation sites; however, peptide mapping has not been done to confirm this conclusion.

In addition to tyrosine phosphorylation, insulin stimulates serine and threonine phosphorylation of the insulin receptor (6, 22). IR molecules containing mainly Ser(P) and Thr(P) are not immunoprecipitated from \(^{32}\)P phosphate-labeled cell extracts with \(\alpha\)-PY, and the supernatant is enriched in Ser(P)/Thr(P)-containing receptors. To study insulin-stimulated Ser/Thr phosphorylation directly by SDS-PAGE, phosphotyrosine-containing receptors were immunodepleted with \(\alpha\)-PY from extracts of \(^{32}\)P phosphate-labeled CHO/neo, CHO/IR, and CHO/IRact cells, and the remaining IR and IRact molecules were immunoprecipitated with anti-insulin receptor antibody. The Tyr(P)-free b-subunit of the endogenous hamster receptors in CHO/neo cells was undetectable. In both CHO/IR and CHO/IRact cells, Tyr(P)-free b-subunit was detected before insulin stimulation, and the level of phosphorylation was strongly stimulated by insulin (Fig. 6A); phosphorylation of the b-subunit of IRact was 75–85% of that in IR, at equal receptor numbers. Qualitative phosphoamino acid analysis of the IR and IRact revealed that under these conditions...
Truncated Insulin Receptor Functions Normally

FIG. 2. Insulin-stimulated autophosphorylation of WGA-purified receptors. Equal amounts of wild-type and mutant insulin receptors partially purified on WGA were incubated at 25 °C in the absence or presence of 100 nM insulin for 30 min; [γ-32P]ATP was added to a final concentration of 50 μM, and receptor was allowed to autophosphorylate for various times. Reactions were stopped by boiling for 3 min in Laemmli sample buffer. Samples were resolved by SDS-PAGE under reducing conditions and visualized by autoradiography. A, autoradiogram of wild-type IR time course, B, autoradiogram of IR_{Δct} time course. C, incorporated [32P]phosphate in β-subunit bands from gels in A and B were quantitated by Cerenkov counting and plotted relative to the maximum value for wild-type IR.

FIG. 3. Reverse-phase HPLC analysis of tryptic peptides derived from autophosphorylated WGA-purified IR and IR_{Δct}. The IR (A and B) and IR_{Δct} (C and D) partially purified on WGA were stimulated with 100 nM insulin in the absence (A and C) or presence (B and D) of α-PY and allowed to autophosphorylate in the presence of [γ-32P]ATP. Gel fragments containing autophosphorylated receptor were excised, digested with 0.1 mg/ml trypsin for 12 h, and resolved by reverse-phase HPLC.

conditions, the insulin-stimulated β-subunit contained mainly Ser(P) and Thr(P), with only trace amounts of Tyr(P) (Fig. 6B). Therefore, insulin stimulated Ser/Thr phosphorylation of the IR_{Δct}, suggesting that this mutant receptor activates the appropriate Ser/Thr kinases normally, and retains most sites of Ser/Thr phosphorylation. The apparent reduction in β-subunit phosphorylation may be attributed to the removal of Thr^{116}(16).

Stimulation of Phosphatidylinositol 3-Kinase by Insulin in CHO/IR and CHO/IR_{Δct} Cells—Insulin stimulates phosphatidylinositol 3-kinase (PtdIns 3-kinase) in cells expressing insulin receptors (11, 12). The PtdIns 3-kinase is found in α-PY immunoprecipitates from insulin-stimulated cells, suggesting that it may be activated by tyrosyl phosphorylation. PtdIns 3-kinase activity in α-PY immunoprecipitates from CHO/neo cells was poorly stimulated by insulin (Fig. 7). In both CHO/IR and CHO/IR_{Δct} cells, insulin stimulated PtdIns 3-kinase was 8–10-fold above the basal level with similar dose responses. These data indicate that the C-terminal region of the IR is not necessary for stimulation of the PtdIns 3-kinase. Whether the PtdIns 3-kinase binds to the regulatory region of the receptor, to some other phosphotyrosine-containing
Truncated Insulin Receptor Functions Normally

**Fig. 5.** Tyrosine phosphorylation of wild-type and mutant IR in CHO cells. Confluent monolayers of control CHO cells (neo) or CHO cells expressing wild-type (IR) or mutant (IR₅₆) insulin receptors were incubated at 37 °C in medium containing ³²P, for 2 h, incubated in the presence or absence of insulin (1 μM) for 1 min at 37 °C, frozen in liquid nitrogen, and solubilized. A, tyrosyl phosphoproteins were immunoprecipitated twice with anti-phosphotyrosine antibodies, resolved by reducing SDS-PAGE, and visualized by autoradiography. B, relative receptor numbers per cell were determined by Scatchard binding analysis (a) and by quantitating [³⁵S]methionine-labeled IR and IRA₅₆ in bands excised from gels such as in Fig. 1. In multiple experiments similar to that described in A, bands containing ³²P-labeled IR and IRA₅₆ were excised and incorporated counts quantitated by Cerenkov counting. Relative incorporation was calculated per receptor.

**Fig. 6.** Visualization and analysis of wild-type and mutant insulin receptors from CHO cells remaining after depletion with anti-phosphotyrosine antibody. CHO cells expressing IR, IRA₅₆, or control (neo) were labeled, stimulated, and immunoprecipitated with anti-phosphotyrosine antibodies as described in Fig. 5. Supernatants were then precipitated with anti-insulin receptor antibodies, and immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography (A). Bands corresponding to insulin-stimulated IR and IRA₅₆ were then excised, digested twice for 12 h at 37 °C in 0.1 mg/ml trypsin, hydrolyzed in 6 N constant boiling HCl at 110 °C for 2 h, resolved by high voltage thin-layer electrophoresis, and visualized by autoradiography (B). Positions of phosphoserine, phosphothreonine, and phosphotyrosine standards are indicated.

protein, or directly to the a-PY is currently unknown.

**Biological Activity of the IR₅₆**—The biological activity of the wild-type and mutant insulin receptor in CHO/IR and CHO/IR₅₆ cells was studied during stimulation with insulin or a biologically active human-specific anti-insulin receptor antibody 83-14. The use of anti-insulin receptor antibody is valuable because the CHO.neo cells that contain rodent insulin receptors are unstimulated by this agonist, eliminating the problem of background biological activity. Insulin stimu-
Truncated Insulin Receptor Functions Normally

A) B)

Fig. 8. Stimulation of glycogen synthesis in CHO cells expressing IR and IR_{ct}. Confluent monolayers of control CHO cells (neo) or CHO cells expressing IR or IR_{ct} were preincubated at 37 °C in medium containing 2.5 mM glucose and 0.1% BSA. The medium was replaced after 3 h with fresh medium containing ligand (A, insulin; B, anti-IR antibodies) of various concentrations; cells were incubated at 37 °C for 30 min. [\textsuperscript{14}C]Glucose was then added, and incubation was continued for another 90 min at 37 °C. Cells were then washed, lysed in 20% KOH, and glycogen was precipitated in 70% ethanol for 1 h at 4 °C and collected by filtration; precipitated glycogen was counted in a liquid scintillation counter. Data represent averages of triplicate points (± S.E.). Experiments were performed at least three times.

Fig. 9. Stimulation of thymidine incorporation in CHO cells expressing IR and IR_{ct}. Subconfluent monolayers of control CHO cells (neo) or CHO cells expressing IR or IR_{ct} were serum starved at 37 °C in medium containing 0.5% FBS for 24 h. Cells were then grown at 37 °C in media containing either 10% FBS or 0.1% BSA and various concentrations of ligand (A, insulin; B, anti-IR antibodies). The medium was removed after 15 h and replaced with medium containing 0.1% BSA and [\textsuperscript{3}H]thymidine for 90 min at 37 °C. Cells were then chilled at 4 °C, washed, and lysed in 0.1% SDS. DNA was precipitated with TCA (10% final concentration), collected by filtration, and counted in a liquid scintillation counter. Data represent averages (± S.E.) of triplicate data. Experiments were performed three times with identical results.

labeled glycogen synthesis with an ED_{50} of 20 nM in CHO/neo cells (Fig. 8A). CHO/IR and CHO/IR_{ct} were 100-fold more sensitive to insulin. Consistent with this result, glycogen synthesis was not significantly stimulated by anti-insulin receptor antibodies in CHO/neo cells, whereas it was stimulated 2-fold in both CHO/IR and CHO/IR_{ct}, with an ED_{50} of 1.0 nM IgG (Fig. 8B). Thus, the ability of IR_{ct} to stimulate glycogen synthesis in response to insulin or stimulatory antibody 83-14 does not differ from that of the wild-type IR.

The effect of insulin and anti-receptor 83-14 antibodies on the incorporation of [\textsuperscript{3}H]thymidine into DNA in CHO/neo, CHO/IR and CHO/IR_{ct} cells was assessed as a measure of growth response. Insulin stimulated thymidine incorporation in CHO/neo cells with an ED_{50} of 0.5 nM, whereas the ED_{50}
**DISCUSSION**

We have examined the function of an insulin receptor molecule which is truncated at the C terminus of the β-subunit by the removal of 43 amino acids. This truncation removes two sites of insulin-stimulated tyrosyl phosphorylation (Tyr<sup>1316</sup>, Tyr<sup>1322</sup>) and a site of threonine phosphorylation (Thr<sup>1336</sup>) (4, 16, 23). Previous studies with this mutant receptor carried out in transfected Rat-1 fibroblasts suggest that the C terminus is required for signaling metabolic actions of insulin and functions as an inhibitory regulator of insulin-stimulated mitogenesis (14, 15). In contrast, behavior of the IR<sub>ΔCT</sub> in intact cells and with WGA-purified receptors, the maximum autophosphorylation of IR<sub>ΔCT</sub> was 20–30% below that of the wild-type IR, although the time course of IR<sub>ΔCT</sub> autophosphorylation in vitro was similar to that of the wild-type. This reduced level of autophosphorylation is consistent with the removal of the C-terminal autophosphorylation sites. Relative amounts of [<sup>32</sup>P]phosphate in tryptic phosphopeptides pY1, pY1α (tris-phosphorylated regulatory region), and pY4 (bis-phosphorylated regulatory region) were similar in the IR<sub>ΔCT</sub> and the wild-type IR, whereas the peaks corresponding to C-terminal site (pY2 and pY3) were absent in IR<sub>ΔCT</sub>. The peptides derived from the regulatory region were unaltered in the IR<sub>ΔCT</sub>, suggesting that the autophosphorylation cascade in the regulatory region is independent of the C-terminal sequences.

The insulin receptor is strongly activated by insulin-stimulated tyrosine autophosphorylation (2, 3). Although mutations in the juxtamembrane and regulatory regions of the β-subunit greatly reduce the ability of the IR to phosphorylate endogenous substrates on tyrosine in response to insulin stimulation (Fig. 10), we saw no reduction of the ability of IR<sub>ΔCT</sub> to stimulate DNA synthesis in CHO cells. However, before insulin stimulation, the C terminus does not serve to interfere with the enzymatic activity of the insulin receptor, since C-terminal phosphorylation always follows phosphorylation of the activation of the PtdIns 3-kinase, are stimulated normally in the IR<sub>ΔCT</sub> but are blocked in the IR<sub>ΔβH60</sub> and the IR<sub>ΔβH10</sub>. Moreover, based on the results in Fig. 10, the phosphorylation of pp185 is closely associated with the effect of insulin on DNA synthesis, but not on glycogen synthesis. Thus by several criteria the C terminal 43 amino acids of the β-subunit show no significant role in biological signaling. Of course it is possible that unidentified signaling pathways or other biological responses are altered in the IR<sub>ΔCT</sub> and their identification will be made in the future.

In the intact cells and with WGA-purified receptors, the maximum autophosphorylation of IR<sub>ΔCT</sub> was 20–30% below that of the wild-type IR, although the time course of IR<sub>ΔCT</sub> autophosphorylation in vitro was similar to that of the wild-type. This reduced level of autophosphorylation is consistent with the removal of the C-terminal autophosphorylation sites. Relative amounts of [<sup>32</sup>P]phosphate in tryptic phosphopeptides pY1, pY1α (tris-phosphorylated regulatory region), and pY4 (bis-phosphorylated regulatory region) were similar in the IR<sub>ΔCT</sub> and the wild-type IR, whereas the peaks corresponding to C-terminal sites (pY2 and pY3) were absent in IR<sub>ΔCT</sub>. The peptides derived from the regulatory region were unaltered in the IR<sub>ΔCT</sub>, suggesting that the autophosphorylation cascade in the regulatory region is independent of the C-terminal sequences.

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We originally suggested that the activation of the IR kinase occurs upon triphosphorylation of the regulatory region (4, 24). Consistent with this hypothesis, point mutations at these tyrosine residues alter receptor activation (7, 9). Moreover, removal of the C-terminal region of the IR by mild trypsin digestion has no effect on the activation of the IR kinase (23), and autophosphorylation occurs normally in the regulatory region of the IR truncated by trypsinization (4). By tryptic peptide mapping, insulin-stimulated triphosphorylation of the regulatory region occurs normally in the IR<sub>ΔCT</sub>. Moreover, the anti-phosphotyrosine antibody inhibits triphosphorylation and causes the accumulation of the bis-phosphorylated region. This is consistent with our previous results using the trypsin-truncated IR (4). Thus, the pattern of autophosphorylation observed in the IR<sub>ΔCT</sub> is consistent with normal activation of the tyrosine kinase.

Our experiments with the IR<sub>ΔCT</sub> suggest that signal transmission in the intact CHO cells is normal. During insulin binding to the IR<sub>ΔCT</sub>, glycogen and DNA synthesis are stimulated normally. Expression of the IR and IR<sub>ΔCT</sub> reduced the ED<sub>50</sub> for insulin-stimulated glycogen synthesis from 20 nM in CHO/neo cells to 0.2 nM in the transfected cells. Similarly, the ED<sub>50</sub> for thymidine incorporation was reduced from 0.5 nM in the CHO/neo cells to 0.025 nM in the CHO/IR and

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**Fig. 10. Functional and biological characteristics of mutant insulin receptors. Upper part, a linear model of the intracellular domain of the β-subunit indicates the relative position of the transmembrane region (TM) and the juxtamembrane, regulatory, and C-terminal regions. Lower part, the activities listed are qualitatively interpreted against the wild-type insulin receptor. A + sign indicates that the wild-type and the corresponding mutant gave the same results, whereas a +/− and a − sign indicate that the particular activity of the mutant was partially or fully reduced, respectively. This summary was prepared from published data for the IR<sub>ΔβH60</sub> (10), IR<sub>ΔβH10</sub> (6), and IR<sub>ΔCT</sub> (9); the IR<sub>ΔCT</sub> taken from this paper, and the IR<sub>ΔβH60</sub> is from our unpublished results.**

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<sup>2</sup>J. M. Backer and M. F. White, unpublished results.
CHO/IRacet cells. Moreover, human monoclonal anti-insulin receptor 83-14 antibodies, which mimic insulin action, stimulate CHO and CHO/IRacet cells; the antibodies have no effect in CHO/neo cells, because they do not react with the hamster IR or the endogenous IGF-1 receptor. The 83-14 antibody provides a very sensitive assay for biological activity of the IRacet in CHO cells, as the endogenous background due to the hamster IR is eliminated. The mechanism by which the 83-14 antibody activates the insulin receptor is controversial, and several studies suggest that it is independent of phosphorylation (25–27). However, recent results show that 83-14 stimulates β-subunit tyrosine autophosphorylation and the phosphorylation of pp185, suggesting that it may use the same mechanism as insulin (18, 29).

Maegawa et al. (14) and Thies et al. (15) measured several biological effects of the IRacet expressed in Rat-1 fibroblasts. The mutant receptor showed a low activity for stimulation of 2-deoxyglucose uptake or glycogen synthesis. The ED50 for insulin-stimulated 2-deoxyglucose uptake was 0.065 nM in Rat-1/IR cells, whereas the ED50 was 0.375 nM in the Rat-1 and Rat-1/IRacet cells. A similar result occurred for insulin stimulation of glycogen synthase. In contrast, the ability of the IRacet to stimulate DNA synthesis was more sensitive than the wild-type IR, as the ED50 values for insulin stimulation of thymidine incorporation were 3.9 nM for Rat-1/IR cells and 1.3 nM for the Rat-1/IRacet. Rat-1 cells were slightly less sensitive with an ED50 of 9.8 nM (15). These cell lines were also tested with the IRC-terminal region than was obtained with the IRacet, including removal of serine phosphorylation sites 1293 and 1294, may not be required for tyrosine kinase activity or biological signaling. Thus, sequences in the C terminus may play an important regulatory role, but they are not required for tyrosine kinase activity or biological signaling.

Furthermore, we have not directly addressed in this study the possibility that the C terminus functions as a negative regulator of insulin receptor action after phosphorylation by another kinase system. Karasik et al. (31) recently demonstrated that insulin-stimulated autophosphorylation of the WGA-purified hepatic insulin receptor was decreased by 45% in starved rats as compared with fed controls. This negative regulation was entirely reversed by dephosphorylation of serine and threonine residues with alkaline phosphatase. Moreover, removal of about 10 kDa of the C-terminal region of the β-subunit by mild trypsin digestion also reversed the inhibition. Therefore, removal of a larger portion of the C-terminal region than was obtained with the IRacet, including removal of serine phosphorylation sites 1293 and 1294, may show important regulatory effects. Thus, sequences in the C terminus may play an important regulatory role, but they are not required for tyrosine kinase activity or biological signaling.

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