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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 ($IR_{\Delta ct}$). This deletion removed tyrosine (Tyr^{1322}, Tyr^{1316}) and threonine (Thr^{1336}) phosphorylation sites. In Chinese hamster ovary (CHO) cells, insulin binding to the mutant receptor was normal, and [^{35}S]methionine labeling indicated that both the IR and $IR_{\Delta ct}$ were processed normally; however, the β-subunit of $IR_{\Delta ct}$ was 5 kDa smaller than that of the IR. The time course of insulin-stimulated autophosphorylation of the partially purified $IR_{\Delta ct}$ 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 the regulatory region (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}) occurred normally; kinase activity of the IR and $IR_{\Delta ct}$ was activated normally by insulin-stimulated autophosphorylation. In the intact CHO cells, insulin-stimulated serine and threonine phosphorylation of the $IR_{\Delta ct}$ was reduced 20%, suggesting that most Ser/Thr phosphorylation sites are located outside of the C terminus. During insulin stimulation, the wild-type and mutant insulin receptor activated the phosphatidylinositol 3-kinase. Moreover, insulin itself or human-specific anti-insulin receptor antibodies stimulated glycogen and DNA synthesis equally in both CHO/IR and CHO/$IR_{\Delta ct}$ cells. These data suggest that the C terminus plays a minimal role in IR function and signal transmission in CHO cells.

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 (IR)[1] 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 (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) 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 $IR_{\Delta ct}$ 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 Thr^{1336} 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 ($IR_{\Delta ct}$) 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 (10^6) grown in F-12 medium containing 10% fetal bovine serum (GIBCO) were transfected by calcium phosphate precipitation with 1 μg of pSVEneo alone or together with 10 μg of pCVSVHIRc or pCVSVHIRc/Δct 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).

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§ 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.

[1] The abbreviations used are: IR, insulin receptor; $IR_{\Delta ct}$, truncated IR lacking the last 43 amino acids at the C terminus of the β-subunit; IR_{F1146}, insulin receptor with phenylalanine substitution at position 1146; α-PY, 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.

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. After transfection and selection by fluorescence-activated cell sorting, cloned lines of CHO/IR cells used in this study express about 10^6 human insulin receptors/cell. Analysis of equilibrium insulin binding using the LIGAND system (18) revealed that the selected clones of CHO/$IR_{\Delta ct}$ cells expressed 1–3 times more receptors than CHO/IR. When essential for the interpretation of the results, the number of receptors was quantified by [^{35}S]methionine labeling and Scatchard analysis at the time of the experiment. Scatchard binding analysis also revealed that $IR_{\Delta ct}$ bound insulin with an affinity identical to that of the normal IR. When analyzed together with the LIGAND system using a two-site binding model, the high affinity dissociation constant ± S.E. for wild-type and mutant IR was 2.9 ± 0.3 nM, and the low affinity dissociation constant was 130 ± 40 nM.

Metabolic Labeling of CHO/neo, CHO/IR, and CHO/$IR_{\Delta ct}$ Cells with [^{35}S]Methionine or [^{32}P]Phosphate—Confluent monolayers of transfected CHO cells were labeled for 18 h at 37 °C in methionine-free RPMI 1640 medium (GIBCO) containing 0.2 mCi/ml [^{35}S]methionine (Amersham Corp.). For [^{32}P]phosphate labeling, the cells were incubated for 2 h with 0.2 mCi/ml [^{32}P]phosphate (Du Pont-New England Nuclear) at 37 °C (1). The labeled cells were either stimulated with insulin (100 nM) for 1 min or not, and then rapidly frozen with liquid nitrogen and thawed into 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, 2 mM Na_3VO_4, 4.2 mg/ml NaF, 1.5 mg/ml EDTA, 0.1 mg/ml aprotinin, and 0.34 mg/ml phenylmethanesulfonyl fluoride. Insoluble material was removed by ultracentrifugation, and the supernatant was twice immunoprecipitated with anti-phosphotyrosine (α-PY) or anti-insulin receptor antibody. Immunoprecipitated proteins were reduced with 100 mM dithiothreitol, separated by SDS-PAGE (7.5% resolving gel), and visualized by autoradiography, and radiation in the β-subunit of the IR was quantitated by Cerenkov counting for [^{33}P]P_i or scintillation counting for [^{35}S]methionine.

In order to determine stimulation of serine and threonine phosphorylation by insulin, the non-tyrosine-phosphorylated IR and $IR_{\Delta ct}$ remaining in solution after immunoprecipitation with the α-PY were immunoprecipitated with anti-insulin receptor antibody. These immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography, and the phosphoamino acid composition of the β-subunit was determined as previously described (19).

Insulin Stimulation of Autophosphorylation and Kinase Activity of the WGA-purified IR and $IR_{\Delta ct}$ in Vitro—Wild-type IR and mutant $IR_{\Delta ct}$ receptors from transfected CHO cells were partially purified on WGA-agarose (Vector) as previously described (4). Equivalent amounts of WGA-purified IR and $IR_{\Delta ct}$ were incubated for 30 min at 25 °C in 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100 and 5 mM $MnCl_2$ in the presence or absence of 100 nM insulin. [γ-^{32}P] ATP (Du Pont-New England Nuclear) (0.8–1.0 μCi/mM) and 50 μM ATP (Sigma) were added, and receptors were allowed to autophosphorylate for various time intervals. Reactions were stopped by boiling for 3 min in Laemmli sample buffer. Samples were loaded directly onto SDS-PAGE (6% resolving gels) and subsequently visualized by autoradiography. Radioactivity in the β-subunit was determined by Cerenkov counting.

Tyrosine kinase activity of the insulin receptor was measured during a 3-min incubation with 1 mM Thr-12-Lys, a dodecapeptide which contains the three phosphorylation sites between amino acid residues 1143–1152 of the human insulin receptor as described by Ullrich *et al.* (20). Equivalent amounts of WGA-purified IR or $IR_{\Delta ct}$ in 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100 and 5 mM $MnCl_2$ were incubated in the absence or presence of 100 nM insulin for 30 min at 25 °C. Autophosphorylation was initiated by adding 50 μM ATP (containing 1.0 μCi/mmol [γ-^{32}P]ATP) for 5, 10, 20, or 30 min followed immediately with Thr-12-Lys for 3 min. Peptide phosphorylation was stopped by the addition of 30% trichloroacetic acid and 10% BSA, incubation at 4 °C, and removal of precipitated protein by centrifugation. The supernatants containing peptide substrate were spotted on phosphocellulose papers (Whatman), washed repeatedly in 75 mM phosphoric acid, and counted (Cerenkov) (9).

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis—WGA-purified IR and $IR_{\Delta ct}$ were stimulated with insulin and allowed to autophosphorylate for 5 min in the presence of 100 nM insulin, 50 mM γ-[^{32}P]ATP, and in the absence or presence of 60 μg/ml antiphosphotyrosine antibody. Tryptic phosphopeptides were obtained from the β-subunit of the IR and the $IR_{\Delta ct}$ as previously described (4). The phosphopeptides were separated with a Waters HPLC system equipped with an RP-318 (Bio-Rad) reverse-phase column. Phosphopeptides applied to the column were eluted at a flow rate of 1 ml/min with a mobile phase consisting of water and 0.05% trifluoroacetic acid and an increasing concentration of acetonitrile. Fractions were collected at 30-s intervals, and the amount of radioactivity in each was measured by Cerenkov counting.

The phosphoamino acid composition of the β-subunit was determined as previously described (19). Tryptic peptides were dried, dissolved in 6 N constant boiling HCl (Pierce Chemical Co.) and hydrolyzed for 2 h at 110 °C. Phosphoamino acid standards were added, and the amino acid mixture was spotted onto cellulose thin-layer plates (Analtech), and separated for 1 h at 1000 V in CH_3COOH:H_2O:pyridine (89:10:1). Phosphoamino acid standards were visualized by treatment with ninhydrin, and IR-derived phosphoamino acids were visualized by autoradiography.

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. [^{14}C]Glucose (Du Pont-New England Nuclear) (1 μCi/ml final concentration) was added, and the cells were incubated for 90 min at 37 °C. The cells were washed three times in PBS and lysed by incubation in 20% KOH at 37 °C for 1 h. Lysates were transferred to glass tubes, cold carrier glycogen (1 mg/ml final concentration) was added, and the tubes were boiled for 30 min. Glycogen was precipitated from the extract during a 1-h incubation with 70% ethanol at 4 °C, collected on glass filters (Whatman), and washed with ice-cold 70% EtOH. 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 10% 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 [^{3}H]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 (>12.5% 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 quantitated by scintillation counting in ACS mixture (Amersham).

PtdIns 3-Kinase Activity—PtdIns 3-kinase activity in α-PY immunoprecipitates was determined as previously described (11). Subconfluent CHO cells were incubated overnight in F-12 medium containing 0.5% BSA. The cells were then incubated with insulin for 10 min and washed once in ice-cold PBS and twice in 20 mM Tris (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM Na_3VO_4 (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 phosphoproteins 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 Na_3VO_4, three times in 100 mM Tris (pH 7.5) containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4, and twice in 10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4. Finally, the pellets were resuspended in 10 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EDTA. To each pellet was added 10 mM $MnCl_2$ and 0.3 μg/μl phosphatidylinositol previously sonicated in 10 mM Tris (pH 7.5) containing 1 mM EGTA. [γ-^{32}P]ATP (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 $CHCl_3$:MeOH (1:1). After centrifugation the lower organic phase was removed and applied to a TLC plate (Merck) coated with 1% potassium oxalate. The plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (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.

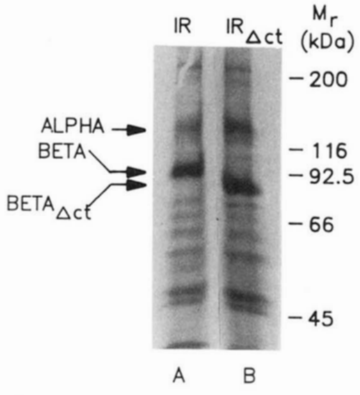


FIG. 1. **[^{35}S]Methionine-labeled insulin receptors from CHO cells expressing wild-type and mutant insulin receptors.** Confluent monolayers of CHO/IR and CHO/IR$_{\Delta ct}$ were incubated overnight at 37 °C in medium containing [^{35}S]methionine. Cells were washed, solubilized at 4 °C, and immunoprecipitated with anti-insulin receptor antibodies. Precipitated materials were resolved by reducing SDS-PAGE and detected by autoradiography.

RESULTS

Expression of Normal IR and IR$_{\Delta ct}$ in CHO Cells—The structure of the wild-type and mutant insulin receptors was analyzed in CHO/IR and CHO/IR$_{\Delta ct}$ cells, respectively, labeled for 18 h with [^{35}S]methionine. The receptors were immunoprecipitated with anti-insulin receptor antibodies and analyzed by SDS-PAGE (Fig. 1). The α-subunit from both cell lines migrated at 135 kDa, and the β-subunit of the wild-type IR migrated at 95 kDa. In contrast, the β-subunit of IR$_{\Delta ct}$ migrated at about 90 kDa owing to the deletion of 43 C-terminal amino acids. The wild-type and truncated β-subunits contained approximately twice as much incorporated [^{35}S] methionine as the α-subunit, consistent with a 2:1 ratio of methionine residues for the β-subunits to α-subunits (20). These data suggest that the IR and IR$_{\Delta ct}$ are translated, processed, and directed to the cell membrane normally in CHO cells.

Autophosphorylation of the Partially Purified IR and IR$_{\Delta ct}$—To assess autophosphorylation of IR and IR$_{\Delta ct}$ purified on immobilized wheat germ agglutinin, equal concentrations of receptor determined from Scatchard analysis were autophosphorylated with [^{32}P]ATP 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 IR$_{\Delta ct}$ during insulin stimulation was 25% lower than wild-type IR at all time points.

Separation of tryptic phosphopeptides from the β-subunit of the IR phosphorylated *in vitro* for 5 min revealed an elution profile as previously described (Fig. 3*A*) (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 IR$_{\Delta ct}$, which is consistent with removal of the C-terminal domain (Fig. 3*C*). 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 IR$_{\Delta ct}$.

Tris-phosphorylation of the regulatory region and phosphorylation of the C-terminal sites is inhibited by the inclusion of α-PY during the *in vitro* autophosphorylation reaction (4, 21). In the presence of the α-PY, phosphopeptides pY1, pY1a (regulatory region), and pY2 and pY3 (C terminus) are significantly inhibited during insulin-stimulated autophosphorylation (Fig. 3*B*). Moreover, as previously shown, the presence of α-PY caused the accumulation of the bis-phosphorylated regulatory region in the IR as shown by increased pY4 and the appearance of pY5 (Fig. 3*B*) (4). Similarly, the α-PY inhibited tris-phosphorylation of the regulatory region of the IR$_{\Delta ct}$ and caused the accumulation of the bis-phosphorylated region (Fig. 3*D*) In the absence of the C-terminal peptides an additional phosphopeptide labeled pY4a was also detected during incubation with the α-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 IR$_{\Delta ct}$—Insulin stimulates the phosphotransferase activity of the IR during *in vitro* incubation with synthetic substrates. Before insulin stimulation, both the IR and IR$_{\Delta ct}$ 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 IR$_{\Delta ct}$ was barely increased. However, 5 min of insulin-stimulated autophosphorylation completely activated the kinase of the IR and IR$_{\Delta ct}$. 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 IR$_{\Delta ct}$ in CHO Cells—In the absence of insulin, tyrosyl autophosphorylation of IR in [^{32}P] phosphate-labeled CHO/neo, CHO/IR, and CHO/IR$_{\Delta ct}$ cells was undetectable. However, after a 1-min insulin stimulation, autophosphorylation of the β-subunit increased in CHO/IR and CHO/IR$_{\Delta ct}$, but little stimulation was observed in CHO/neo cells (Fig. 5*A*). 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 IR$_{\Delta ct}$ was 70–80% of wild-type IR (Fig. 5*B*). The reduced autophosphorylation of the IR$_{\Delta ct}$ 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 α-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 α-PY from extracts of [^{32}P]phosphate-labeled CHO/neo, CHO/IR, and CHO/IR$_{\Delta ct}$ cells, and the remaining IR and IR$_{\Delta ct}$ molecules were immunoprecipitated with anti-insulin receptor antibody. The Tyr(P)-free β-subunit of the endogenous hamster receptors in CHO/neo cells was undetectable. In both CHO/IR and CHO/IR$_{\Delta ct}$ cells, Tyr(P)-free β-subunit was detected before insulin stimulation, and the level of phosphorylation was strongly stimulated by insulin (Fig. 6*A*); phosphorylation of the β-subunit of IR$_{\Delta ct}$ was 75–85% of that in IR, at equal receptor numbers. Qualitative phosphoamino acid analysis of the IR and IR$_{\Delta ct}$ revealed that under these

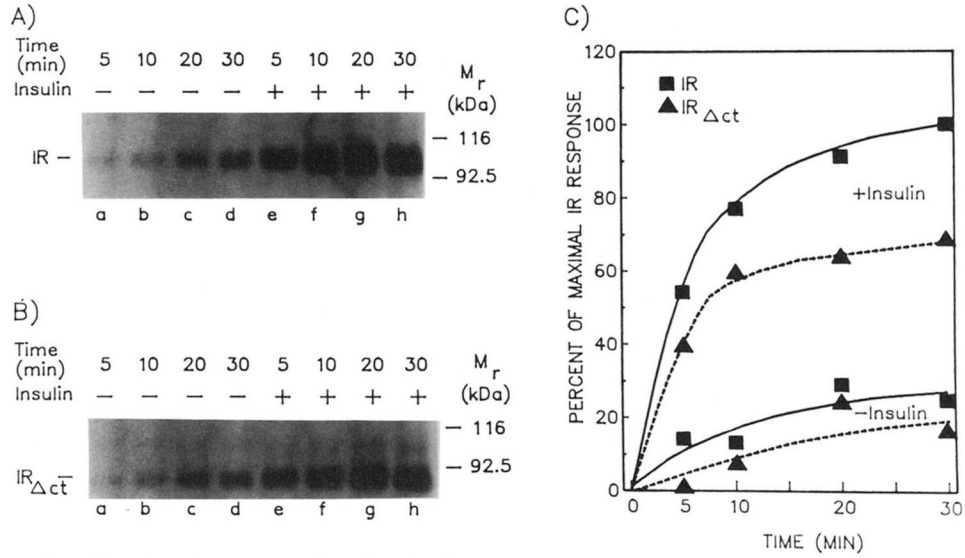


FIG. 2. **Insulin-stimulated autophosphorylation of WGA-purified receptors.** Equal amounts of wild-type and mutant insulin receptors partially purified on WGA were incubated at 25 °C in the absence or presence of 100 nM insulin for 30 min; [γ-^{32}P]ATP was added to a final concentration of 50 μM, and 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 $IR_{\Delta ct}$ time course. *C*, incorporated [^{32}P]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.

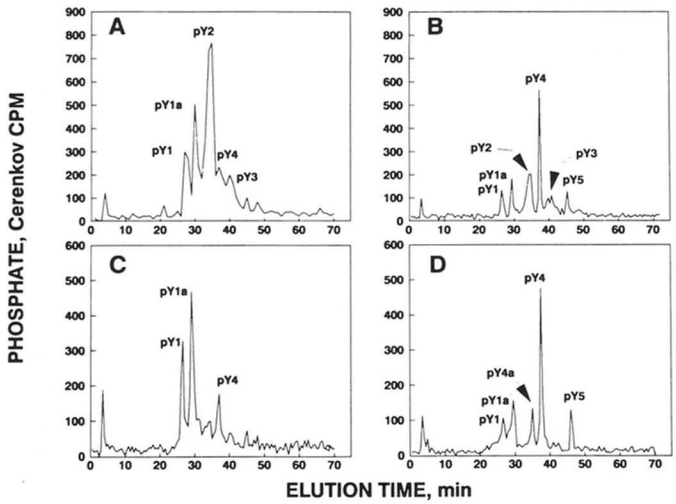


FIG. 3. **Reverse-phase HPLC analysis of tryptic peptides derived from autophosphorylated WGA-purified IR and $IR_{\Delta ct}$.** The IR (*A* and *B*) and $IR_{\Delta ct}$ (*C* and *D*) partially purified on WGA were stimulated with 100 nM insulin in the absence (*A* and *C*) or presence (*B* and *D*)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

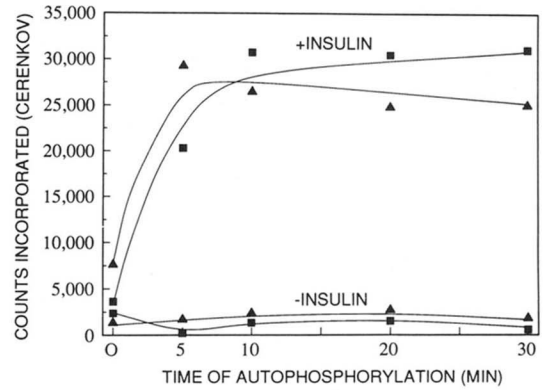


FIG. 4. **Activity of WGA-purified IR and $IR_{\Delta ct}$ toward synthetic substrate Thr-12-Lys.** Equivalent amounts of WGA-purified wild-type (■) and mutant (▲) insulin receptors were incubated without or with 100 nM insulin for 30 min. Before adding 1 mM Thr-12-Lys, the receptor was incubated without or with 50 μM [γ-^{32}P] ATP for 5, 10, 20 and 30 min. Phosphorylation of Thr-12-Lys was measured for 3 min, and the reactions were stopped by the addition of 30% trichloroacetic acid and 10% BSA. Tubes were incubated at 4 °C for 15 min and spun, and supernatants were applied to phosphocellulose papers, which were washed several times in 75 mM phosphoric acid and counted (Cerenkov). Each point is the average of a duplicate determination which represents the results of several similar experiments.

conditions, the insulin-stimulated β-subunit contained mainly Ser(P) and Thr(P), with only trace amounts of Tyr(P) (Fig. 6*B*). Therefore, insulin stimulated Ser/Thr phosphorylation of the $IR_{\Delta ct}$, 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[1336](16.)

Stimulation of Phosphatidylinositol 3-Kinase by Insulin in CHO/IR and CHO/$IR_{\Delta ct}$ 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/$IR_{\Delta ct}$ 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

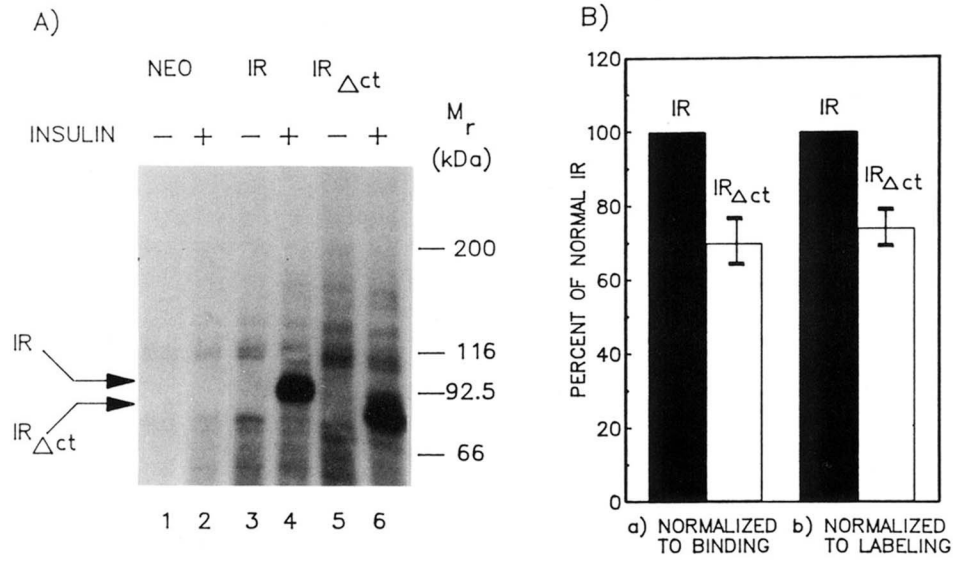


FIG. 5. **Tyrosine phosphorylation of wild-type and mutant IR in CHO cells.** Confluent monolayers of control CHO cells (*neo*) or CHO cells expressing wild-type (*IR*) or mutant ($IR_{\Delta ct}$) insulin receptors were incubated at 37 °C in medium containing $^{32}P_i$ for 2 h, incubated in the presence 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 [^{35}S]methionine-labeled IR (*b*) in bands excised from gels such as in Fig. 1. In multiple experiments similar to that described in *A*, bands containing $^{32}P_i$-labeled IR and $IR_{\Delta ct}$ were excised and incorporated counts quantitated by Cerenkov counting. Relative incorporation was calculated per receptor.

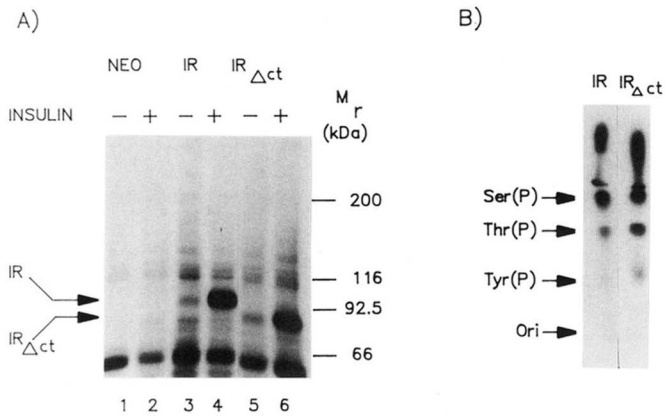


FIG. 6. **Visualization and analysis of wild-type and mutant insulin receptors from CHO cells remaining after depletion with anti-phosphotyrosine antibody.** CHO cells expressing IR, $IR_{\Delta ct}$, or control (*neo*) were labeled, stimulated, and immunoprecipitated with anti-phosphotyrosine antibodies 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 $IR_{\Delta ct}$ 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.

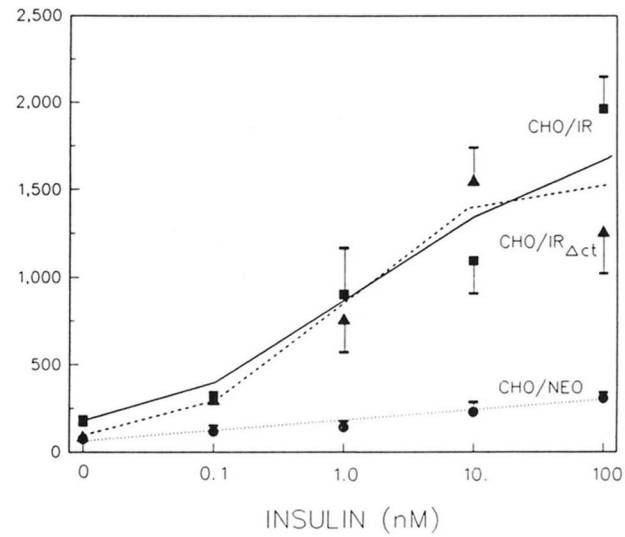


FIG. 7. **Stimulation of phosphatidylinositol 3-kinase activity in CHO cells expressing IR and $IR_{\Delta ct}$.** Subconfluent monolayers of control CHO cells (*neo*) or CHO cells expressing IR or $IR_{\Delta ct}$ were serum-starved at 37 °C overnight in medium containing 0.5% BSA. The cells were then incubated for 10 min with various concentrations of insulin, washed, and solubilized in buffer containing 1% Nonidet P-40. Proteins containing phosphotyrosine were immunoprecipitated with anti-phosphotyrosine antibody. Immunoprecipitated material was washed extensively, added to buffer containing phosphatidylinositol, Mg^{2+}, and [γ^{32}P]-ATP, and incubated for 10 min at 22 °C. The reaction was stopped by the addition of 8 N HCl and lipids extracted in $CHCl_3$:CH_3OH (1:1). Samples were centrifuged and the lower organic phase spotted to TLC plates and resolved. The plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2) and visualized by autoradiography. Spots which co-migrated with phosphatidylinositol 4-monophosphate were scraped and counted (Cerenkov). Trends for the dose response were drawn by hand.

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

Biological Activity of the $IR_{\Delta ct}$—The biological activity of the wild-type and mutant insulin receptor in CHO/IR and CHO/$IR_{\Delta ct}$ 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-

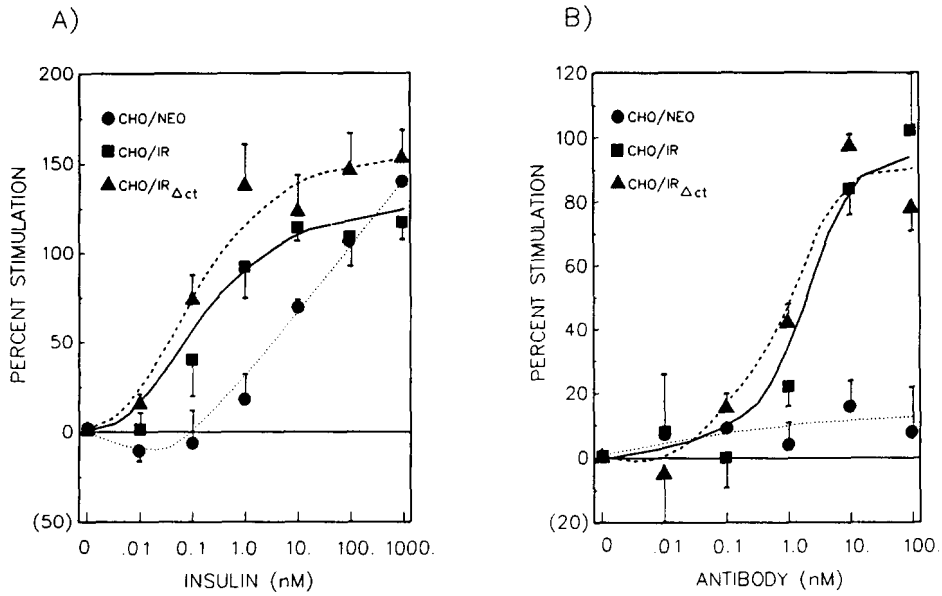


FIG. 8. **Stimulation of glycogen synthesis in CHO cells expressing IR and $IR_{\Delta ct}$.** Confluent monolayers of control CHO cells (*neo*) or CHO cells expressing IR or $IR_{\Delta ct}$ were preincubated at 37 °C in medium containing 2.5 mM glucose and 0.1% BSA. The medium was replaced after 3 h with fresh medium containing ligand (*A*, insulin; *B*, anti-IR antibodies) of various concentrations; cells were incubated at 37 °C for 30 min. [^{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.

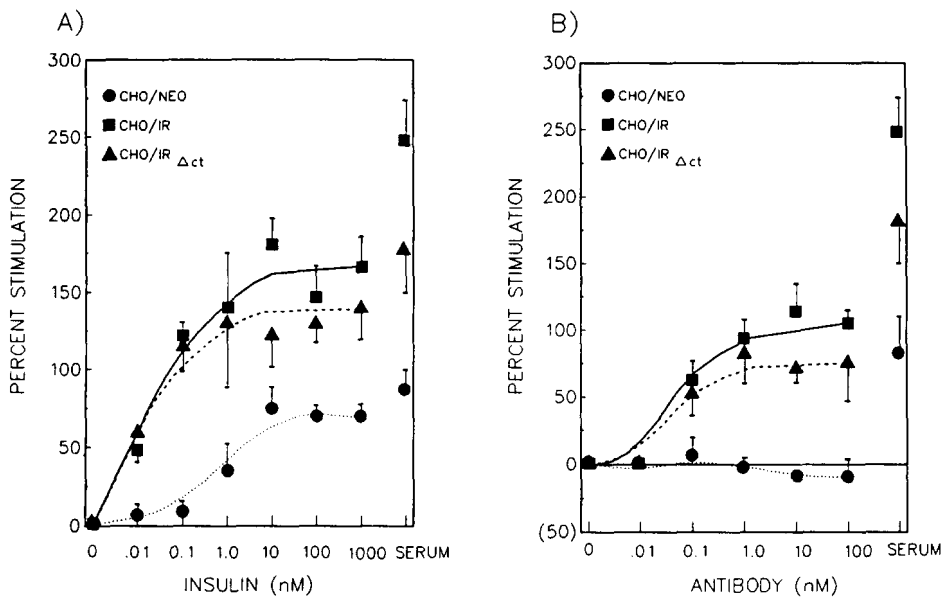
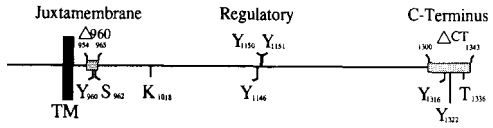


FIG. 9. **Stimulation of thymidine incorporation in CHO cells expressing IR and $IR_{\Delta ct}$.** Subconfluent monolayers of control CHO cells (*neo*) or CHO cells expressing IR or $IR_{\Delta ct}$ serum starved at 37 °C in medium containing 0.5% FBS for 24 h. Cells were then grown at 37 °C in media 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 [^{3}H]thymidine for 90 min at 37 °C. Cells were then chilled to 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.

lated glycogen synthesis with an ED_{50} of 20 nM in CHO/neo cells (Fig. 8*A*). CHO/IR and CHO/$IR_{\Delta ct}$ 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/$IR_{\Delta ct}$ with an ED_{50} of 1.0 nM IgG (Fig. 8*B*). Thus, the ability of $IR_{\Delta ct}$ 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 [^{3}H]thymidine into DNA in CHO/neo, CHO/IR and CHO/$IR_{\Delta ct}$ 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}



	IR_{F960}	$IR_{\Delta960}$	IR_{A1018}	IR_{F1146}	$IR_{\Delta CT}$
Insulin Binding	+	+	+	+	+
Autophosphorylation	+	+	-	+/-	+
Kinase Activation by Insulin	+	+	-	+	+
Kinase Activation by Autophosphorylation	+	+	-	-	+
pp185 Phos	-	-	-	-	+
PtdIns 3-kinase	+/-	-	-	+/-	+
Glycogen synthase	-	-	-	+	+
Thymidine Incorporation	-	-	-	-	+

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_{F960} (10), IR_{1018} (6), and IR_{F1146} (9); the $IR_{\Delta ct}$ taken from this paper, and the $IR_{\Delta 960}$ is from our unpublished results.[2]

for both CHO/IR and CHO/$IR_{\Delta ct}$ cells was 20-fold lower (Fig. 9*A*). Anti-insulin receptor antibodies failed to stimulate thymidine incorporation in CHO/neo cells, whereas anti-insulin receptor antibody stimulated DNA synthesis approximately 2-fold in CHO/IR and CHO/$IR_{\Delta ct}$ cells with an ED_{50} of 0.1 nM (Fig. 9*B*). Thus, the ability of the mutant $IR_{\Delta ct}$ to stimulate DNA synthesis is identical to that of the wild-type IR.

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^{1316}, Tyr^{1322}) and a site of threonine phosphorylation (Thr^{1336}) (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 $IR_{\Delta ct}$ in our transfected CHO cells is indistinguishable from the wild-type IR under several experimental conditions. Therefore we suggest that the C terminus plays a minimal role in IR signal transduction in CHO cells. The reason for a difference between our results and the previous studies is unknown, as we used the same cDNA construct at a similar expression level; however, it may be due to the use of different cell backgrounds or subtle differences in the biological assays.

Recently, we have examined several mutant insulin receptor molecules in CHO cells in which changes were made in the juxtamembrane region and the regulatory region (9, 10).[2] Some of the characteristics of these cell lines are compared with those reported here for the $IR_{\Delta ct}$ (Fig. 10). Whereas point mutations or deletions of a portion of the juxtamembrane region of the β-subunit have no effect on autophosphorylation, they block the stimulation of glycogen synthase and thymidine incorporation. This result is in striking contrast to the $IR_{\Delta ct}$. Consistent with this finding, potential signal transduction pathways, such as tyrosine phosphorylation of pp185 and the activation of the PtdIns 3-kinase, are stimulated normally in the $IR_{\Delta ct}$ but are blocked in the IR_{F960} and the $IR_{\Delta 960}$. 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 $IR_{\Delta ct}$, and their identification will be made in the future.

In the intact cells and with WGA-purified receptors, the maximum autophosphorylation of $IR_{\Delta ct}$ was 20–30% below that of the wild-type IR, although the time course of $IR_{\Delta ct}$ 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 [^{32}P]phosphate in tryptic phosphopeptides pY1, pY1a (tris-phosphorylated regulatory region), and pY4 (bis-phosphorylated regulatory region) were similar in the $IR_{\Delta ct}$ and the wild-type IR, whereas the peaks corresponding to C-terminal sites (pY2 and pY3) were absent in $IR_{\Delta ct}$. The peptides derived from the regulatory region were unaltered in the $IR_{\Delta ct}$, 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 $IR_{\Delta ct}$ in intact cells to phosphorylate pp185 (data not shown and Ref. 15). Furthermore, the WGA-purified IR and $IR_{\Delta ct}$ were activated identically by insulin-stimulated autophosphorylation. These data imply that the autophosphorylation of tyrosines 1316 and 1322 does not act to decrease the enzymatic activity of the receptor, nor is the C terminus important for receptor autophosphorylation. Moreover, 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 tyrosine residues in the regulatory region.

We originally suggested that activation of the IR kinase occurs upon tris-phosphorylation 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 tris-phosphorylation of the regulatory region occurs normally in the $IR_{\Delta ct}$. Moreover, the anti-phosphotyrosine antibody inhibits tris-phosphorylation 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 $IR_{\Delta ct}$ is consistent with normal activation of the tyrosine kinase.

Our experiments with the $IR_{\Delta ct}$ suggest that signal transmission in the intact CHO cells is normal. During insulin binding to the $IR_{\Delta ct}$, glycogen and DNA synthesis are stimulated normally. Expression of the IR and $IR_{\Delta ct}$ reduced the ED_{50} for insulin-stimulated glycogen synthesis from 20 nM in CHO/neo cells to 0.2 nM in the transfected cells. Similarly, the ED_{50} for thymidine incorporation was reduced from 0.5 nM in the CHO/neo cells to 0.025 nM in the CHO/IR and

[2] J. M. Backer and M. F. White, unpublished results.

CHO/$IR_{\Delta ct}$ cells. Moreover, human monoclonal anti-insulin receptor 83-14 antibodies, which mimic insulin action, stimulate these responses equally in the CHO/IR and CHO/$IR_{\Delta ct}$ cells; the antibodies have no effect in CHO/neo cells, because they do not react with the hamster IR nor the endogenous IGF-I receptor. The 83-14 antibody provides a very sensitive assay for biological activity of the $IR_{\Delta ct}$ 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 $IR_{\Delta ct}$ expressed in Rat-1 fibroblasts. The mutant receptor showed a low activity for stimulation of 2-deoxyglucose uptake or glycogen synthesis. The ED_{50} for insulin-stimulated 2-deoxyglucose uptake was 0.065 nM in Rat-1/IR cells, whereas the ED_{50} was 0.375 nM in the Rat-1 and Rat-1/$IR_{\Delta ct}$ cells. A similar result occurred for insulin stimulation of glycogen synthase. In contrast, the ability of the $IR_{\Delta ct}$ to stimulate DNA synthesis was more sensitive than the wild-type IR, as the ED_{50} values for insulin stimulation of thymidine incorporation were 3.9 nM for Rat-1/IR cells and 1.3 nM for the Rat-1/$IR_{\Delta ct}$; Rat-1 cells were slightly less sensitive with an ED_{50} of 9.8 nM (15). These cell lines were also tested with the 83-14 anti-insulin receptor antibody. The 83-14 stimulated 2-deoxyglucose uptake in both Rat-1/IR and Rat-1/$IR_{\Delta ct}$ cells, but it had no effect on Rat-1 cells. However, the stimulation of Rat-1/$IR_{\Delta ct}$ cells was about 50% of that in the Rat-1/IR cells, indicating that this metabolic response is partially lost. Thus, it is possible that the coupling mechanisms between the IR and cellular enzymes are slightly different between the Rat-1 and CHO cells such that the C terminus is essential for stimulation of metabolic effects in Rat-1 cells.

Thr^{1336} in the human insulin receptor was recently identified by Lewis *et al.* (16, 30) as a major site of phosphorylation by the protein kinase C and by the insulin receptor-stimulated kinase. Although this residue is absent from the $IR_{\Delta ct}$, our results indicate that insulin stimulates threonine phosphorylation of the $IR_{\Delta ct}$ in [^{32}P]phosphate-labeled CHO cells. Presumably, an additional threonine residue outside of the C terminus serves as a phosphorylation site; perhaps Thr^{1336} is not the major phosphorylation site on the insulin receptor in CHO cells. Alternatively, a new site of threonine phosphorylation may only be accessible to kinases after C-terminal truncation. Complete tryptic peptide mapping will be necessary to clarify this point. Insulin also stimulates serine phosphorylation of the IR and the $IR_{\Delta ct}$ in [^{32}P]phosphate-labeled CHO cells. In this case, the major sites of serine phosphorylation proposed by Lewis *et al.* (Ser^{1293} or Ser^{1294}) are retained in the $IR_{\Delta ct}$. The finding that insulin stimulates serine phosphorylation of the truncated receptor suggests that the C-terminal 43-amino acid tail does not play an essential role in this process by either affecting receptor signaling to the serine kinase or affecting recognition of the phosphorylation site.

We are still unable to establish a definitive role for the two major autophosphorylation sites in the C-terminal region. 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 $IR_{\Delta ct}$, 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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