We have examined the role of autophosphorylation in insulin signal transmission by oligonucleotide-directed mutagenesis of seven potential tyrosine autophosphorylation sites in the human insulin receptor. Chinese hamster ovary cells transfected with these receptors were analyzed for insulin-stimulated 2-deoxyglucose uptake, thymidine incorporation, endogenous substrate phosphorylation, and in vitro kinase activity. We found that phosphorylation on tyrosine residues 953, 1316, and 1322 were not necessary for receptor-mediated signal transduction. Mutation of tyrosine 960 reduced but did not abolish the signaling capabilities of the receptor. Finally, the simultaneous mutation of tyrosine residues 1146, 1150, and 1151 resulted in a receptor that was unable to mediate any of the insulin stimulated responses examined. In contrast to a previous study, we found that the mutation of tyrosine residue 960 in the juxtamembrane domain of the hIR was reported to eliminate both metabolic and mitogenic responses (20). Although some studies did not detect autophosphorylation sites in the juxtamembrane domain of insulin receptors, others have evidence that this region is involved in kinase activation (22) and that tyrosine residues in this region may be autophosphorylated (14, 29).

In order to analyze the role of autophosphorylation in the transduction of insulin mediated responses, seven putative autophosphorylation sites of the hIR were mutated to phenylalanine residues (16, 17). The mutation of tyrosine residues 953, 1316, and 1322 had no discernable effect on signaling by the receptor. Although some studies did not detect autophosphorylation sites in the juxtamembrane domain of insulin receptors, others have evidence that this region is involved in kinase activation (22). The simultaneous mutation of the three tyrosine residues at positions 953, 1316, and 1322 resulted in a receptor that was able to mediate any of the insulin stimulated responses examined. In contrast to a previous study, we found that the mutation of tyrosine residue 960 in the juxtamembrane domain of the hIR was reported to eliminate both metabolic and mitogenic responses (20).

The Role of Insulin Receptor Autophosphorylation in Signal Transduction*

(Received for publication, March 11, 1991)

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The effects of insulin are mediated through a transmembrane receptor, a glycosylated tetramer composed of two extracellular α subunits linked by disulfide bonds to two transmembrane β subunits. After insulin binds to the α subunits, the cytoplasmic protein tyrosine kinase encoded by the β subunit is activated (1). The tyrosine kinase activity was abolished by the mutation of the lysine (at position 1018) in the ATP-binding domain. This mutation rendered the receptor biologically inactive; the receptor no longer mediated insulin-stimulated glucose uptake, thymidine incorporation, S6 kinase activation, receptor down-regulation, or any insulin-mediated response examined (2–6). These studies demonstrated that the protein tyrosine kinase activity was essential for mediating the effects of insulin. The activated kinase phosphorylates itself exclusively on tyrosine residues (7–9). This autophosphorylation enhances protein tyrosine kinase activity toward exogenous substrates and renders the kinase active in the absence of insulin (10). The major sites of autophosphorylation, tyrosine residues 1146, 1150, 1151, 1316, and 1322, were identified by protein microsequencing of phosphorylated tryptic peptides (12, 13). Phosphorylation of tyrosine residues 1146, 1150, and 1151 was correlated with the activation of the human insulin receptor (hIR) protein tyrosine kinase (14, 15). Tyrosine residues 1146 and 1151 have been mutated to phenylalanine residues individually (16, 17), and tyrosine residue 1150 has been mutated in combination with tyrosine residue 1151 (17). Mutations in this region cause aberrant kinase activity and result in partially defective receptors. The mutation Y1146F impairs the mitogenic, but not metabolic responses, whereas the mutation Y1150/1151F impairs the metabolic, but not the mitogenic responses (16–18). Removal of 43 amino acids at the carboxyl terminus (including tyrosine residues 1316 and 1322) resulted in a receptor that was defective for metabolic responses. Interestingly, this receptor appeared to augment mitogenic responses (19). In contrast to these partially defective receptors, mutation of the ATP binding domain (Lys-1018 to Ala) abolished kinase activity and created a biologically inactive receptor (2, 3).

Mutation of tyrosine residue 960 in the juxtamembrane domain of the hIR was reported to eliminate both metabolic and mitogenic responses (20). Although some studies did not detect autophosphorylation sites in the juxtamembrane domain (12, 13, 20, 21), others have evidence that this region is involved in kinase activation (22) and that tyrosine residues in this region may be autophosphorylated (14, 29).

Experimental Procedures

Construction of Mutants—A 1592-bp PstI to SpeI (bp 2742 to bp 4334 in the hIR cDNA) fragment was subcloned into M13 mp19.

This work was supported by the National Institutes of Health Grant DK35158 (to O. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ This paper is dedicated to the memory of Ora M. Rosen (1935–1980).
Insulin Receptor Autophosphorylation

Oligonucleotide-directed mutagenesis was carried out as described by Kunkel et al. (24). Chemically synthesized oligonucleotides (MSKCC core facility) were used for the mutagenesis as are follows, mismatches are underlined: Y853F, 5 ' GGC ACT GAG ATA CTC AGG GTT TGA AGA GAA AAG CCC CCC CCC GCC CCC CCC TTC CGT CCT TTT (resulting in a 28 nucleotide site at bp 853); CY-1, 5 ' GTC CTC GCC CTC GTT CAT GCT TGG CAT AGG CAT GTC TCC GCT GAA GGA CCA CGC CCT TTT CCG GAA GAT CCT AAA AAT GTA GTC CTC TAT ACC CTT GCC (BamHI site at bp 3581); CY-2, 5 ' GTC CTC GCC CTC GTT CAT GCT TGG CAT AGG CAT GTC TCC GCT GAA GGA CCA CGC CCT TTT CCG GAA GAT CCT AAA AAT GTA GTC CTC TAT ACC CTT GCC (BamHI site at bp 3581). Deoxyuridine-containing single-stranded DNA templates were grown in Escherichia coli strain C3316 (dat ' -mutant), and mutagenesis was accomplished in vitro as described in Ref. 24. Mutated templates were selected in E. coli strain DH5α. Phage were screened for the presence of mutations by restriction digestion of the plasmid replicative form I DNA and then verified by single-stranded DNA sequencing (Sequenase, U. S. Biochemical Corp.). A 1373-bp BglII-Spe I fragment was isolated from the mutated M13 replicative form I DNA and subcloned into pCvSVHRIICc (2). The presence of the mutation was verified by restriction digestion of the expression vector (each mutation introduced into the same site). All cDNAs were inserted into the wild type hIR (2), were cotransfected with pSV3-neo (2, 10, and 0.5 μg/μl, respectively) into CHO cells obtained from ATCC. Neomycin-resistant cells were selected in DME supplemented with methionine (10% fetal calf serum) and glutamine. Cells were maintained in DME containing methionine and 10% fetal calf serum. Preparation and manipulation of DNA was done as described in Maniatis et al. (25).

**Metabolic Labeling**—Confluent monolayers (approximately 1×10⁵ cells) were incubated in 3 ml of cysteine and methionine-free DME, 0.5% dialyzed BSA for 1 h. Cells were labeled with [35S]cysteine and [35S]methionine (200 μCi/ml, Trans-S-Label™), ICN, 1026 Ci/mmol methionine) for 16 h in 3 ml of DME (Cys/Met-free) with 10% fetal calf serum and 50 μg/ml leupeptin (Sigma). The cultures were harvested with sheep anti-mouse antibody and mouse monoclonal antibody 5D9 (a gift from Dr. R. Roth, Stanford University) which recognized the extracellular domain of the hIR. Cell monolayers were isolated with cloning rings. Cells were maintained in DME containing glutamine and 10% fetal calf serum. Preparation and manipulation of DNA was done as described in Maniatis et al. (25).

**Assays of Receptor Protein Tyrosine Kinase Activity**—Approximately 5×10⁵ cells were solubilized in 5 ml of S buffer and prepared as described above. The high speed (100,000 × g) supernatants were applied to wheat germ agglutinin (WGA)-agarose (Vector Laboratory). High speed supernatants were applied to the WGA-agarose, and the column was washed with 12 ml of WGA wash buffer. Proteins were eluted from the column in 0.5 ml of WGA wash buffer containing 0.5 M N-acetylglycine (Sigma). Determination of insulin binding activity in the WGA eluates is described below. Protein tyrosine kinase assays were performed as described in Ref. 9. Each 60 μl reaction contained 20 ml WGA HEPES, pH 7.4, 150 mM NaCl, 50 μM MgCl₂, 5 μM Mg⁰²⁺ orthovanadate, 20 μM [γ³²P]ATP at 8 C/ml (Du Pont-New England Nuclear, 3000 Ci/mmol), 1 mM dithiothreitol, 100 μM/ml leupeptin, 10 μg/ml aprotinin, and 2 μg/ml pepstatin). After incubation for 4 h at 2 °C, the reaction was clarified by centrifugation at 4 °C for 20 min at 100,000 × g. Inusin receptors were immunoprecipitated from the supernatants with 2 μg of monoclonal antibody CII 25.3 (which recognizes the extracellular α subunit of the hIR, Ref. 26) covalently cross-linked to protein A-Sepharose 6B (Pharmacia LKB Biotechnology Inc.). The Dynabeads were washed with 1 ml of binding solution (DME, 0.5% dialyzed BSA, 50 mM HEPES, pH 7.45, and 125I-insulin (150,000 cpm/ml, 1450 cpm/nmol, iodinated by chloramine T, see Ref. 2) for 4 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled insulin (<10% of specific binding). Insulin binding activity was quantitated by immunoprecipitation (12 h, 4 °C) with monoclonal antibody CII 25.3 (this antibody does not interfere with insulin binding, Ref. 2). Immune complexes were washed three times with 1 ml of ice-cold PBS, and the associated radioactivity was quantitated in a Beckman γ counter.

Cell surface insulin binding assays were performed in triplicate on subconfluent monolayers of cells (approximately 5×10⁵ cells/well, six-well plates). Cells were incubated in 1 ml of binding solution (described above) in the presence of 0.1 nM unlabeled insulin for 4 h at 4 °C. Cells were washed three times with 5 ml of ice-cold PBS, and the radioactivity was determined by incubation with [35S]sulfuric acid (approximately 30 pg of protein as determined by the method described in Ref. 27). The partially purified insulin receptors were incubated in a reaction mixture with or without insulin for 20 min on ice after which autophosphorylation was assayed for 5 min at 30 °C. The reactions were terminated by diluting the reaction mixture to 500 μl with EDTA and sodium orthovanadate (final concentrations of 1 mM and 10 μM, respectively). Immunoprecipitations were carried out from this diluted reaction with the monoclonal antibody CII 25.3 protein-A-Sepharose complex, and immune complexes were washed and prepared for SDS-PAGE as described above. Histone kinase activity was determined by incubating approximately 20 fmol of insulin binding activity (WGA eluate) in a reaction mixture with or without insulin (30 nM) for 30 min on ice; receptors were autophosphorylated by incubation at 30 °C for 5 min, then returned to ice, and washed 3 times with 500 μl of ice-cold PBS. The radioactivity associated with the histone H2B (5 μg in 5 μl) was added, and assays were completed at 30 °C for 5 min. Reactions were terminated by the addition of Laemmli sample buffer containing SDS, dithiothreitol, and EDTA (2%, 100 mM, and 50 mM, respectively) and then subjected to 20% SDS-PAGE (28). Polyacrylamide gels were subjected to alkali treatment (1 N NaOH, 55 °C for 1 h). ³²P incorporation into the β subunit of the hIR and histone was resistant to this treatment, indicating phosphorylation on tyrosine residues (29). Quantitation of ³²P incorporation was accomplished with a Molecular Dynamics Phosphor Imager (laboratory equipment). The autoradiographic films containing the precipitated immune complexes were developed and analyzed as described above.

**Insulin Binding and Internalization**—Insulin binding activity in the WGA eluate was determined by incubating approximately 10 μl of WGA eluate with 1 ml of binding solution (DME, 0.5% dialyzed BSA, 50 mM HEPES, pH 7.45, and 125I-insulin (150,000 cpm/ml, 1450 cpm/nmol, iodinated by chloramine T, see Ref. 2) for 4 h at 4 °C. The radioactivity (resistant to 0.1% SDS, and the radioactivity present in the lysates was quantitated in a Beckman γ counter. Scatchard analysis of binding data was carried out as described in Ref. 6.

Internalization experiments were performed essentially as given in Ref. 6. Approximately 1×10⁵ cells/well (six-well plate) were incubated in 1 ml of binding solution (150,000 cpm/ml, 125I-insulin) for 4 h at 4 °C. Cells were warmed to 37 °C for 30 min to permit receptor-mediated endocytosis of the radioiodinated ligand. The cells were washed twice either with ice-cold PBS or with ice-cold 0.3 M sodium acetate, pH 4.5, containing 0.15 M NaCl. The cells were washed twice more with ice-cold PBS and then solubilized in 1 ml of 0.1% SDS, and the radioactivity was determined in a Beckman γ counter. Scatchard analysis of binding data was carried out as described in Ref. 6.

**2-Deoxyglucose Uptake**—Insulin stimulation of 2-deoxyglucose uptake was assayed (3) by incubating subconfluent cells (1×10⁵ cells/well, six-well plate) in DME with 0.5% dialyzed BSA and 10 mM 2-deoxyglucose for 5 h (17). Cells were washed with PBS and incubated
The nomenclature and positions of the tyrosine to phenylalanine mutations in the β subunit of the hIR are shown schematically in Fig. 1. The 2 juxtamembrane tyrosine residues, 953 and 960, were mutated independently (Y953F, Y960F) or together (JMY-2). Tyrosine residues 1146, 1150, and 1151, in the regulatory domain, were simultaneously mutated to phenylalanine residues; this mutation is referred to as RY-3. Finally, the C Y-2 receptor has tyrosine residues 1316 and 1322 in the carboxyl terminus mutated to phenylalanine residues. K1018A is a lysine to alanine mutation at residue 1018 in the ATP binding domain (2). The cell line referred to as Y960F (W) or Y960F (MW) was a generous gift from Dr. M. F. White, Harvard University. Y960F cDNA (obtained from Dr. White) was subsequently transfected into CHO cells in our laboratory; this Y960F cell line is referred to as Y960F (R). All assays are done with the Y960F (R) cell line; exceptions are indicated.

Expression of Mutant Receptors—CHO cells were stably transfected with wild type and mutated hIR cDNAs. Clonal cell lines were examined for expression of the mutated receptors. Extracts were prepared from cells metabolically labeled with Translabel. The radiolabeled insulin receptors were immunoprecipitated with one of four antibodies (Fig. 2). Antibodies P2, P4, and P5 (indicated 2, 4, and 5 above the lanes) are antipeptide antibodies directed against amino acids 1143-1162, 952-967, and 1328-1343, respectively (22, 31). Monoclonal antibody CII 25.3 (indicated M above the lane) is directed against the extracellular domain of the hIR (26). In cell lines transfected with wild type or mutated cDNAs, all four antibodies specifically immunoprecipitated two proteins with Mr values of 135,000 and 95,000, corresponding to the α and β subunits of the hIR, respectively. Monoclonal antibody CII 25.3, which recognizes only the human and not the hamster receptor (26), efficiently precipitated receptors from all cell lines except the untransfected CHO cell line (Fig. 2, lane 1). Antibody P2 precipitated receptors least efficiently, this may be explained by the preference of antibody P2 for the autophosphorylated form of the receptor (14). The mutations did not alter the affinity of the antibodies for the hIR variants, although some of the antibodies were directed against regions that were altered by the mutations (e.g. Ab P4 is directed against amino acids 952-967, and JM Y-2 is mutated at residues 953 and 960). The rate of insulin receptor biosynthesis was examined by labeling the cells metabolically with [35S]methionine and [35S]cysteine for 15 min, followed by incubation with unlabeled amino acids for varying lengths of time (pulse-chase analysis). All mutated receptors were synthesized at approximately the same rate. Each receptor was initially made as a 200-kDa precursor that was subsequently processed into a mature receptor comprised of a 135-kDa α subunit and a 95-kDa β subunit (data not shown).

Insulin Binding and Receptor Number—The affinity of the receptors for insulin was assessed by Scatchard analysis of equilibrium 125I-insulin binding data (Table I). All receptors displayed dissociation constants (Kd) for insulin between 0.72 and 0.36 nM (Table I, column 2). These values agree with previously reported values between 0.25 and 0.6 nM (17). Scatchard analysis also indicated the approximate number of cell surface receptors. The parental CHO cells expressed 

![Fig. 2. Immunoprecipitation of [35S]methionine- and [35S]cysteine-labeled insulin receptors](image-url)
**Table I**

Scatchard analysis of insulin binding data

| Cell line   | K<sub>d</sub> (nM) | Receptors/cell |
|-------------|--------------------|----------------|
| Wild type   | 0.62               | 160,000        |
| K1018A      | 0.72               | 120,000        |
| Y953F       | 0.62               | 120,000        |
| JM Y-2      | 0.49               | 180,000        |
| Y960F       | 0.52               | 160,000        |
| R Y-3       | 0.36               | 300,000        |
| C Y-2       | 0.67               | 160,000        |

**Table II**

Insulin-stimulated histone kinase activity of mutated insulin receptors

| Cell line | No insulin | 30 nM insulin | -Fold activation |
|-----------|------------|----------------|-----------------|
| Wild type | 11.1       | 81.4           | 7.3             |
| K1018A    | 5.1        | 6.8            | 1.3             |
| Y953F     | 11.2       | 51.2           | 4.6             |
| Y960F     | 9.1        | 37.8           | 4.2             |
| JM Y-2    | 6.3        | 31.3           | 4.9             |
| R Y-3     | 9.7        | 17.1           | 1.7             |
| C Y-2     | 5.1        | 15.4           | 3.0             |

Fig. 3. Insulin-stimulated autophosphorylation of partially purified insulin receptors. Insulin receptors partially purified by wheat germ agglutinin-agarose chromatography (15–20 fmol of insulin binding activity) were assayed for kinase activity with [γ-<sup>32</sup>P]ATP in the presence or absence of 30 nM insulin (see “Experimental Procedures”). Autophosphorylation assays were done for 5 min at 30 °C. Autophosphorylated receptors were immunoprecipitated in the presence of sodium orthovanadate and EDTA with 2 μg of monoclonal antibody CI1 25.3. Immuno precipitates were subjected to 7.5% SDS-PAGE. The autoradiogram was exposed 12 h at -70 °C.

- Approximately 2000 endogenous hamster receptors/cell (6).
- Cells transfected with wild type and mutated insulin receptor cDNAs expressed 120,000–300,000 receptors/cell (Table I, column 3). Thus, all cell lines transfected with hIR cDNAs expressed high levels of receptors with normal insulin binding affinities.

**Insulin-dependent Protein Tyrosine Kinase Activity**—The protein tyrosine kinase activity of the mutated receptors was measured *in vitro* as insulin-dependent <sup>32</sup>P incorporation into the β subunit of the hIR or <sup>32</sup>P incorporation into the exogenous substrate histone H2B. Quantitation of the <sup>32</sup>P incorporation was accomplished using a Molecular Dynamics Phosphor Imager and was normalized to cell-associated histone H2B. The autoradiogram was exposed 12 h at -70 °C.

- Antibody P4, which is directed against amino acids 952–967, was shown to inhibit insulin stimulated autophosphorylation of wild type insulin receptors (22). To further characterize the mutated receptors, the partially purified receptors were preincubated with either antibody P4 or antibody P5 (antibody P5 does not affect kinase activity). Antibody P4 dramatically inhibited the insulin stimulated autophosphorylation of the wild type, Y953F, Y960F, JM Y-2, and CY-2 receptors (10–20-fold, insulin and Ab 5 versus insulin and Ab 4, Fig. 4) but weakly inhibited the activity of the R Y-3 receptor (2.7-fold, Fig. 4). The inhibitory effect of antibody P4 was specifically relieved by preincubation with an excess of the synthetic peptide (peptide 4) used to raise antibody P4 (Fig. 4). Thus, we conclude that all of the mutated receptors, except R Y-3, had essentially normal *in vitro* protein tyrosine kinase activity as judged by their autophosphorylation activity, histone kinase activity, and inhibition of autophosphorylation by antibody P4. In contrast, the R Y-3 receptor was observed to phosphorylate itself but not histone in the absence of insulin; the kinase activity of this receptor was minimally stimulated by insulin, and antibody P4 did not inhibit autophosphorylation of this receptor.

**<sup>125</sup>I-Insulin Internalization**—The ability of the mutated receptors to mediate endocytosis of radiolabeled insulin was measured by loading cell surface receptors with <sup>125</sup>I-insulin for 4 h at 4 °C. The cells were warmed to 37 °C for 30 min, allowing receptor mediated endocytosis to occur. Cells were washed with PBS (total counts/min) or with 0.3 M sodium acetate, pH 4.5, and 150 mM NaCl (acid-resistant counts/min) which released the radiolabeled insulin from cell surface receptors (6). The acid-resistant radioactivity is taken to represent the number of receptors internalized during the 30-min incubation at 37 °C and was normalized to total cell-associated radioactivity. 40–50% of the total cell-associated radioactivity was resistant to the acid treatment in the wild type, Y953F, Y960F, and CY-2 cell lines (Table III). 35% of the total cell-associated radioactivity was resistant to the acid treatment in cells expressing the JM Y-2 receptor. In cells...
essentially normal levels of insulin internalization except the K1018A and the R Y-3 receptors, which permit minimal levels of insulin internalization.

**Biological Activity**—The stimulation of 2-deoxyglucose uptake by insulin was examined by treating the cell lines with various concentrations of insulin for 30 min. The amount of [3H]2-deoxyglucose incorporated into the cells during a 10-min period was expressed as percent of maximal incorporation. Previous studies (2–6) demonstrated that high levels of wild type receptor expression increased the sensitivity of CHO cells to insulin, whereas high levels of K1018A receptor expression did not. Untransfected parental CHO cells and cells expressing the K1018A mutant (data not shown) were half-maximally stimulated at 40 nM insulin (Fig. 5). Cells expressing wild type, Y953F, and C Y-2 receptors were half-maximally stimulated by approximately 1 pM of insulin (Fig. 5, panel A). Cells expressing the Y960F (R) and JM Y-2 receptors were half-maximally stimulated by approximately 0.5 nM insulin (Fig. 5, panel B). Cells expressing the R Y-3 receptor were half-maximally stimulated at 14 nM, approximately the same concentration at which half-maximal stimulation occurred for untransfected parental CHO cells (Fig. 5, panel C) and cells expressing K1018A receptors (data not shown).

The stimulation of [3H]thymidine incorporation by insulin was measured after exposing the cells to insulin for 16 h. Cells expressing wild type, Y953F, and CY-2 receptors were half-maximally stimulated by approximately 1 pM insulin (Fig. 6, panel A). Expression of similar numbers of Y960F and JM Y-2 receptors resulted in half-maximal stimulation by 0.15 and 0.01 nM insulin (Fig. 6, panel B). Parental CHO cells, K1018A, and R Y-3-expressing cells were stimulated half-maximally by approximately 1 nM insulin (Fig. 6, panel C). Insulin stimulated tyrosine phosphorylation of the hIR β subunit and pp185, a well characterized endogenous substrate (20), was analyzed in vivo. These results paralleled the results for insulin stimulation of thymidine incorporation and 2-deoxyglucose uptake (Fig. 7). Cells were treated with varying concentrations of insulin. The extracts were immunoblotted and reacted with a monoclonal anti-phosphotyrosine antibody pY20. Fig. 7 shows the tyrosine phosphorylation of the hIR β subunit and pp185 at 1 nM insulin in cells which expressed wild type, Y953F, Y960F(MW), and CY-2 receptors. Cells expressing the JM Y-2 receptor show tyrosine phosphorylation of the hIR β subunit and pp185 at 10 nM insulin. The cells expressing the K1018A and CY-3 receptor show tyrosine phosphorylation of pp185 at 1 μM insulin; however, these cells do not show tyrosine phosphorylation of the hIR β subunit at that insulin concentration.

In summary, for all in vivo responses examined, cell lines which expressed the Y953F and the CY-2 receptors responded to insulin with the same sensitivity as cells which expressed the wild type receptors. Cell lines which expressed the JM Y-2 and Y960F receptors were 10-fold less sensitive than cells lines which expressed the wild type receptor; however, they were 10-fold more sensitive to insulin than untransfected CHO cells or cells expressing the K1018A receptors. Expression of high levels of the R Y-3 receptors did not enhance the sensitivity of transfected cells to insulin.

**DISCUSSION**

Autophosphorylation of the insulin receptor activates the intrinsic protein tyrosine kinase and renders the kinase independent of insulin (1). In order to determine the role of autophosphorylation in insulin action, we mutated seven putative sites of tyrosine autophosphorylation to phenylalanine

| Cell line | Acid-resistant [125I]-insulin (cpm) | Total [125I]-insulin (cpm) | Percent internalized |
|-----------|----------------------------------|--------------------------|---------------------|
| Wild type | 4,674 8,949 52                   |                          |                     |
| K1018A    | 2,772 15,072 18                  |                          |                     |
| Y953F     | 4,715 12,612 37                  |                          |                     |
| Y960F(W)  | 6,559 13,158 48                  |                          |                     |
| Y960F(R)  | 3,708 8,244 45                   |                          |                     |
| JM Y-2    | 3,125 8,388 35                   |                          |                     |
| R Y-3     | 2,964 16,602 18                  |                          |                     |
| CY-2      | 4,638 10,375 44                  |                          |                     |

expressing the K1018A and the R Y-3 receptors only 18% of the total cell-associated radioactivity was resistant to the acid-treatment. The percent internalized values for the wild type and the K1018A cell lines agree with previous reports (30). Kinetic analysis showed no difference in the rate of internalization of the Y953F, Y960F (W), Y960F (R), and CY-2 receptors (data not shown). The R Y-3 receptor was not internalized at any (15, 30, 45, or 60 min) time point (data not shown). In summary, all mutated receptors mediated
FIG. 5. Insulin-stimulated 2-deoxyglucose uptake. Cells were incubated with transport buffer containing the indicated concentrations of insulin for 30 min as described under "Experimental Procedures." [3H]2-Deoxyglucose was added to 1 μCi/ml, and incorporation was measured for 10 min at 37°C. All assays were carried out in triplicate. Nonspecific uptake was measured in the presence of 10 μM unlabeled 2-deoxyglucose. For each cell line, basal incorporation (incorporation in the absence of insulin) was subtracted from each data point. Data are presented as percent of maximal incorporation. Values for minimal (0%) and maximal incorporation (100%) are given below.

Panel A, untransfected CHO cells (4269-7940 cpm), Y953F (5460-14,361 cpm), CY-2 (9037-17,348 cpm), and wild type (3352-8537 cpm).
Panel B, Y960F (4231-10,901 cpm) and JM Y-2 (5093-15,537 cpm).
Panel C, R Y-3 (6037-13,983 cpm).

residues. Cell lines transfected with the mutated cDNAs were isolated and shown to express similar numbers of insulin receptors. The mutations did not affect the affinity of the receptor for insulin or the rate of receptor synthesis. The mutated receptors fell into three groups: those that showed wild type signaling (Y953F and CY-2), those that showed reduced signaling (Y960F and JM Y-2), and those that did not signal (R Y-3).

The mutation of tyrosine residues 1316 and 1322 at the carboxyl terminus (C Y-2) did not alter any of the insulin-stimulated responses examined. Insulin stimulation of 2-deoxyglucose uptake, DNA synthesis, and kinase activity in vitro and in vivo were similar to that observed for cells expressing the wild type receptor. These results suggest that tyrosine residues 1316 and 1322 are not involved in mitogenic or metabolic signaling in CHO cells. This concurs with the findings of Myers et al. (32) who have shown that the removal of 43 amino acids at the carboxyl terminus (including tyrosine residues 1316 and 1322) resulted in a receptor that functioned normally in CHO cells. However, Maegawa et al. (19) have studied the same carboxyl-terminal truncation in Rat 1 fibro-
blasts. They observe these receptors to have increased sensitivity to insulin for mitogenic signaling, yet they are defective for metabolic signaling (19). Additionally, Takeya et al. (33) have shown that mutation of tyrosine residues 1316 and 1322 (identical to our CV-2 mutation) had no effect on metabolic signaling, yet augmented mitogenic signaling in Rat 1 fibroblasts. It seems that the carboxyl terminus of the insulin receptor plays a role in signal transduction in Rat 1 fibroblasts but not CHO cells. It would be interesting to determine the function of the carboxyl-terminal region in a human cell line.

Autophosphorylation of residues 1316 and 1322 may be important for aspects of insulin action not examined in our study, since these tyrosine residues are phosphorylated in vivo (21, 34). Of note, tyrosine residue 1322 of the hIR is located in a putative consensus sequence involved in binding phosphatidylinositol 3-kinase (PtdIns 3-kinase) (35). The association of tyrosine kinases with PtdIns 3-kinase was correlated with the ability to mediate transformation (35). PtdIns 3-kinase was shown to associate with the autophosphorylated form of several tyrosine kinases, and autophosphorylation was essential for this association (35). The C Y-2 receptor, however, is able to activate and associate with PtdIns 3-kinase as well as the wild type receptor. It seems that these tyrosine residues are not necessary for the association or activation of PtdIns 3-kinase. This agrees with functional studies presented here.

Antipeptide antibody P4, directed against the juxtamembrane region, inhibits insulin-stimulated autophosphorylation. This suggests that this region plays a role in kinase activation (22). It was postulated that antibody P4 mediated inhibition by blocking autophosphorylation of tyrosine residue 953 or 960. However, we find that receptors mutated at those residues (Y953F, Y960F, and JM Y-2) are still inhibited by antibody P4. This suggests that the inhibitory effects of antibody P4 are not mediated by blocking phosphorylation of those residues. In addition, the fact that the Y960F and the JM Y-2 mutations did not affect the ability of the receptor to be inhibited by the antibody emphasizes their similarity to wild type receptors with regard to kinase activity.

Mutation of tyrosine residue 953 caused a slight decrease in insulin internalization; however, this did not affect the ability of the Y953F receptor to transduce insulin mediated responses. In contrast to the mutation of tyrosine residue 953, which had no demonstrable effect on the receptor, mutation of tyrosine residue 960 resulted in a receptor with reduced signaling capabilities (Ref. 20 and this study). Cell lines expressing the Y960F receptors were previously reported to be biologically inactive (20). Our evaluation of the same Y960F cell line (obtained from Dr. White) suggested that the mutation of tyrosine residue 960 reduced, but did not abolish, the signaling capabilities of the insulin receptor. This concurs with our results on the JM Y-2 receptor, which also showed reduced signaling capabilities. It should be noted, however, that the Y960F receptor cDNA obtained from Dr. White was shown to contain a second mutation, serine residue 962 to threonine. The behavior of the Y960F receptor, however, is identical to our JM Y-2 receptor (Y953/960F), and mutation of tyrosine 953 alone does not alter signaling. Taken together, these results suggest that the signaling defects of the JM Y-2 receptor (Y953/960F) and the Y960F (Y960F/S962T) receptor are due to the mutation at tyrosine residue 960. Since mutation of tyrosine residue 960 reduced the signaling capabilities of the receptor without altering kinase activity, this region must have a role in signal transmission that is independent of its role in kinase activation. Furthermore, this demonstrates that normal signaling by the insulin receptor involves processes other than kinase activity. However, it is important to emphasize that signaling by the Y960F and JM Y-2 receptors was reduced, not abolished. We observe signaling to be abolished only by mutations that interfere with kinase activity (K1018A and R Y-3).

The third group contains the R Y-3 mutation which rendered the receptor biologically inactive. We suggest that the R Y-3 mutant is defective because it is unable to phosphorylate the critical tyrosine residues needed to activate the kinase toward endogenous substrates and that this activation is necessary for signaling to occur. In support of this hypothesis, we observe diminished histone kinase activity in the R Y-3 receptor. Autophosphorylation of the receptor has been demonstrated to increase the kinase activity toward exogenous substrates (10), and phosphorylation of tyrosine residues 1146, 1150, and 1151 was shown to correlate with this activation (14, 15). We believe it is unlikely that the R Y-3 receptor is defective because it fails to internalize. Data from other laboratories (36) suggest that internalization-deficient receptors still mediate insulin stimulated glucose uptake and thymidine incorporation. It is interesting that two signals appear to be involved in insulin receptor internalization; one signal involves the juxtamembrane region, since deletions in this region (30, 37) result in receptors unable to internalize. The second signal requires either kinase activity (6) or autophosphorylation on tyrosine residues 1146, 1150, and 1151 or both (Ref. 38 and data presented here).

Both the R Y-3 receptor and the K1018A receptor (mutated in the ATP binding domain) are biologically inactive; however, only the R Y-3 receptors have elevated basal in vitro autophosphorylation activity (2, 3). Curiously, the R Y-3

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3 R. Kapeller and L. Cantley, personal communication.
receptor has no concomitant increase in basal autophosphorylation in vivo. Similar findings were reported by Ellis et al. (17) and Zhang et al. (39) using receptors mutated at two or three of these tyrosine residues (1146, 1150, and 1151). High levels of basal in vitro autophosphorylation suggest that the kinase has become insulin-independent. This may be caused by a conformational change generated by the mutation of tyrosine residues 1146, 1150, and 1151. In support of this hypothesis, the basal in vitro autophosphorylation activity of the mutated (RY-3) receptor was not efficiently inhibited by antibody P4, suggesting an altered receptor structure. Antibody P4 prevents the insulin-stimulated activation of unphosphorylated receptors, but it does not inhibit kinase activity if receptors are autophosphorylated prior to incubation with antibody (22). We cannot rule out the possibility that the mutations in RY-3, which cause increased basal autophosphorylation, produce a constitutively active receptor. This constitutive activity may lead to a down-regulation of subsequent signaling events, thereby creating an insulin insensitive cell. If down-regulation involved the activation of a tyrosine phosphorylated receptor, but it does not inhibit kinase activity if receptors are autophosphorylated prior to incubation with antibody (22). We cannot rule out the possibility that the mutations in RY-3, which cause increased basal autophosphorylation, produce a constitutively active receptor. This constitutive activity may lead to a down-regulation of subsequent signaling events, thereby creating an insulin insensitive cell. If down-regulation involved the activation of a tyrosine phosphorylated receptor, but it does not inhibit kinase activity if receptors are autophosphorylated prior to incubation with antibody (22).