Deletion of Exon 21 of the Insulin Receptor Eliminates Tyrosine Kinase Activity but Preserves Mitogenic Signaling†

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ABSTRACT: To study the function of exon 21 of the insulin receptor, a mutant human insulin receptor lacking this domain was constructed. The mutant HIRΔE21 cDNA was transfected into Rat-1 fibroblasts and stable cell lines were selected. The HIRΔE21 receptors were expressed on the cell surface, and they bound insulin with the same affinity as did the wild-type-expressing cell line, hIRcB. The HIRΔE21 receptors did not display detectable autophosphorylation or kinase activity, and as expected, internalization was impaired and metabolic signaling properties were absent. Unexpectedly, insulin's ability to stimulate DNA synthesis in cells expressing HIRΔE21 receptors was far greater than that in the parental Rat-1 cells and equal to that measured in the hIRcB cell line. The enhanced mitogenic signaling properties of the HIRΔE21 receptors was confirmed by showing that treatment of HIRΔE21 cells with a human-specific insulin–mimetic anti-insulin receptor antibody also led to enhanced DNA synthesis. Thus, although no insulin receptor autophosphorylation or kinase activity was detectable in HIRΔE21 cells, these cells displayed enhanced insulin-induced mitogenic signaling. These results suggest that an alternative non-kinase-dependent stimulus-response pathway exists for the long-term biological effects of insulin.

The insulin receptor (IR) is a heterotetrameric transmembrane glycoprotein consisting of two α and two β subunits. The extracellular domain contains the insulin binding site(s) and the cytoplasmic region of the receptor contains the tyrosine kinase domain. After insulin binds to the IR a conformational change occurs, leading to autophosphorylation of tyrosine residues (Kasuga et al., 1982; Rosen et al., 1983; Yu & Czech, 1984) and activation of tyrosine kinase activity. The activated receptor can then associate with and phosphorylate endogenous substrates, such as pp185/IRS-I (Maegawa et al., 1998a; Olefsky, 1990; Rosen, 1987; White et al., 1985). This tyrosine kinase activity is one of the earliest steps in the insulin action cascade, and much evidence exists indicating that it is essential for most, if not all, of insulin's biological effects (Cadena & Gill, 1992; Maegawa et al., 1998a; Olefsky, 1990; Rosen, 1987; White et al., 1985).

Within the insulin receptor cytoplasmic domain, various regions can modify discrete aspects of receptor function. For example, 43 C-terminal amino acids of the insulin receptor are poorly homologous to the same region of the insulin-like growth factor 1 receptor (IGF-IR), and it has been reported that a truncated IR missing the C-terminus displays decreased metabolic and increased mitogenic signaling (Thies et al., 1989). This C-terminal region contains two tyrosine autophosphorylation sites (residues 1316 and 1322), and mutation of these tyrosines to phenylalanine produces an insulin receptor which displays enhanced insulin stimulation of S6 kinase, MAP kinase, and DNA synthesis (Ando et al., 1992; Takata et al., 1991, 1992). Immediately upstream of this region the IR contains an acidic domain (residues 1262–1291) that is highly homologous to the IGF-IR. As we demonstrated recently, deletion of this region results in an insulin receptor with increased metabolic signaling properties (Rolband et al., 1993). Finally, various deletions within the juxtamembrane region of the insulin receptor can result in compromised ligand-mediated endocytosis (Thies et al., 1990) and/or impaired phosphorylation of pp185/IRS-I (Backer et al., 1992).

The amino acid residues encoded by exon 21 of the insulin receptor (residues 1186–1241) lie within the putative kinase domain, although downstream from the crucial tyrosine autophosphorylation sites, 1146, 1150, and 1151 (Hanks et al., 1988). This region is highly homologous to the same domain within the IGF-I receptor, and is immediately N-terminal to the previously mentioned acidic domain of the IR. On the basis of the crystal structure of the cAMP-dependent protein kinase catalytic subunit, this region is predicted to contain the three α-helices that form the scaffold of the substrate binding site. Deletion of this region would thus impair substrate recognition, but should leave the ATP binding site and catalytic loop untouched. To explore the potential properties of the exon 21 domain in modulating receptor function, we constructed a mutant cDNA lacking this exon, expressed it stably in Rat-1 fibroblasts (HIRΔE21 cells), and studied its biochemical and biological characteristics.

MATERIALS AND METHODS

Materials. Porcine insulin and ^{125}I-A^{14}-monoiodinated insulin were kindly provided by Eli Lilly Inc. Cell culture reagents were from Gibco (Grand Island, NY), and fetal calf serum was from Gemini Bioproducts Inc. (Calabasas, CA). [^{14}C]-D-Glucose (320 mCi/mM), [^{32}P]ATP (6000 Ci/mmol), labeled in the γ position, and [^{3}H]thymidine (83 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA); PY20, a monoclonal anti-phosphotyrosine antibody,

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was from ICN (Costa Mesa, CA). Other routinely used reagents were purchased from Sigma (St. Louis, MO).

Plasmid Construction. Deletion of exon 21 (amino acid residues 1262–1291) was accomplished by site-directed mutagenesis as previously described (Williams et al., 1990). A synthetic oligonucleotide DNA primer (5′-ACTTCTTCT-GACATGTGGACTGACCTCATGCGCATG-3′) was prepared that spanned the exon 20–22 junction and deleted exon 21 of the hIR cDNA while maintaining the correct reading frame. The mutation was confirmed by dideoxy sequencing. Then a 2.5-kb *Bst*EII–*Bst*EII fragment (nucleotides (nt) 1836–4325) was subcloned in place of the wild-type *Bst*EII–*Bst*EII fragment in the hIR/RLDN expression vector (Rolband et al., 1993).

Cell Transfection. Stable cell lines were prepared by transfection of Rat-1 fibroblasts with the hIRΔE21/RLDN cDNA by the calcium phosphate coprecipitation technique (Williams et al., 1990). Cells were selected in 400 μg/mL G-418, cloned by limiting dilution, and screened for expression of HIRΔE21 by measurement of insulin binding. Three HIRΔE21 cell lines, expressing 5.5×10^4, 2×10^5, or 4.5×10^4 receptors/cell were isolated for further studies. Rat fibroblasts expressing 1.2×10^6 (hIRcB) or 2.7×10^5 (HIR270) receptors per cell were studied for comparative purposes.

Insulin Binding. Insulin binding was measured as previously described (Williams & Olefsky, 1990) by incubating cells with 35 pM [^{125}I]insulin for 3 h at 12 °C, followed by five rinses with ice-cold PBS. Cells were then solubilized and the radioactivity was measured. Binding affinity and receptor number were estimated by Scatchard analysis of the equilibrium binding data.

Insulin Receptor Autophosphorylation. To measure *in vitro* autophosphorylation, receptors were partially purified by wheat germ agglutinin affinity chromatography. Twenty femtomoles purified receptor was incubated with or without insulin for 16 h at 4 °C, followed by the addition of 20 μCi of [^{32}P]ATP in reaction buffer (25 mM HEPES (pH 7.4), 50 μM ATP, 5 mM $MgCl_2$, and 10 mM $MnCl_2$) for 45 min. The reaction was terminated by the addition of 50 mM ATP and 2 mM sodium orthovanadate in 25 mM HEPES (pH 7.4). The receptors were immunoprecipitated with the anti-IR antibody 83–14 (kindly provided by Dr. Ken Siddle, Cambridge University) for 4 h followed by precipitation with protein A. Phosphorylated β subunits were visualized by SDS–PAGE and autoradiography as previously described (Freidenberg et al., 1987).

Autophosphorylation in intact cells was assessed by photoaffinity-labeling cells with 3.6 nM [^{125}I]NAPA–insulin, as described previously (Williams & Olefsky, 1990), followed by incubation at 37 °C for 10 min to allow autophosphorylation. Cells were then solubilized in the presence of phosphatase inhibitors, and the receptors were immunoprecipitated and analyzed by SDS–PAGE. Immunoprecipitation was performed either with the specific anti-IR antibody 83-14 to measure total insulin receptors or with PY20, a monoclonal anti-phosphotyrosine antibody, to measure phosphorylated insulin receptors.

In Vitro Kinase Activity. The ability of insulin receptors to phosphorylate an exogenous substrate, histone 2B, was assessed as previously described (Freidenberg et al., 1987). Briefly, cells were incubated with 83 nM insulin for 1 min and then solubilized and subjected to wheat germ agarose affinity chromatography in the presence of phosphatase inhibitors (2 mM sodium orthovanadate, 1 M sodium fluoride, and 10 mM sodium tetrapyrophosphate). Forty femtomoles of purified receptors was incubated with insulin–agarose beads for 16 h at 4 °C. The beads were pelleted, washed three times and then incubated in a reaction mixture containing 1 mg/ml histone 2B, 10 μCi of [^{32}P]ATP, 0.5 μM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$, and 25 mM HEPES, (pH 7.4) for 20 min on ice. The reaction was terminated by the addition of 2× SDS–sample buffer and boiled for 3 min prior to separation by 13% SDS–PAGE.

Immunoblotting with Anti-Phosphotyrosine Antibodies. Tyrosine phosphorylation of the insulin receptor and insulin-stimulated tyrosine phosphorylation of other proteins were detected by anti-phosphotyrosine immunoblotting using PY20, as previously described (Thies et al., 1989).

Receptor Internalization. Receptor internalization was measured as previously described (Williams & Olefsky, 1990) using [^{125}I]NAPA–insulin. Washed cells were incubated with 40 ng/mL [^{125}I]NAPA–insulin for 1 h at 4 °C and cross-linked by exposure to 336-nm UV light for 3 min on ice. Noncovalently bound material was removed by washing with DMEM and 0.1% BSA (pH 4.0) for 3 min followed by three ice-cold PBS rinses. The labeled cells were then incubated at 37 °C for 5–60 min to allow internalization to occur and then returned to 0 °C. Cell surface [^{125}I]NAPA–insulin receptor complexes were digested with trypsin. Trypsin-resistant (intracellular) complexes were assayed by SDS–PAGE and autoradiography.

[^{14}C]Glucose Incorporation into Glycogen. Insulin's metabolic effects were determined by measuring glucose incorporation into glycogen (Anderson & Olefsky, 1991). Confluent cells in 35-mm dishes were incubated for 2 h at 37 °C in serum-free media containing 5 mM glucose. Insulin and D-[U-^{14}C]glucose (0.7 μCi/well) were then added. The assay was terminated by washing four times with ice-cold PBS. The cells were dissolved in 30% KOH, transferred to glass tubes containing 4 mg of glycogen, and then boiled for 30 min. After boiling, glycogen was precipitated by the addition of ice-cold ethanol. After incubation for 16 h at 4 °C, the samples were centrifuged and the precipitated glycogen was dissolved in water. The sample was added to 10 mL of aqueous scintillation fluid and counted (Anderson & Olefsky, 1991).

DNA Synthesis. Cells were grown for 3 days in 35-mm dishes (~60% confluent) and then serum-starved in medium containing 0.05% fetal calf serum for 24 h. At this point insulin was added, and 20 h later [^{3}H]thymidine (0.5 μCi/dish) was added for 3 h. The reaction was terminated by sequential washing with ice-cold PBS, 10% TCA, and once with ethanol (Williams et al., 1990). Cells were solubilized in 1 N NaOH, neutralized with 1 N HCl, transferred to scintillation vials, and counted.

RESULTS

Insulin Binding. The insulin binding affinity of HIRΔE21 cells (0.9 nM) was comparable to that of hIRcB cells (data not shown), and Scatchard analysis showed that the three hIRΔE21 clones studied expressed 4.4×10^4, 5.5×10^4, and 2.7×10^5 receptors/cell.

Insulin Receptor Autophosphorylation. To begin to characterize the ΔE21 receptor, cells were incubated with photoreactive [^{125}I]NAPA–insulin, cross-linked, and warmed to 37 °C. The cells were lysed in the presence of phosphatase inhibitors and immunoprecipitated either with a human-specific monoclonal anti-IR antibody (Figure 1, lanes 1, 4, and 7) or with a monoclonal anti-phosphotyrosine antibody (Figure 1, lanes 2, 5, and 8). Lanes 1 and 4 demonstrate that

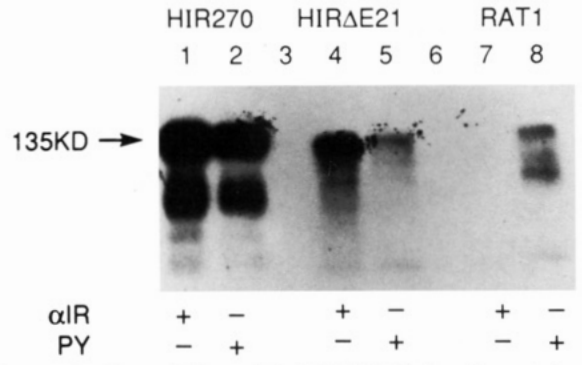

FIGURE 1: Cross-linking with [^{125}I]NAPA–insulin and *in vivo* autophosphorylation. Cells were incubated with 20 ng/mL [^{125}I]-NAPA–insulin followed by incubation at 37 °C, as described in Materials and Methods. Receptors were then solubilized in the presence of phosphatase inhibitors and immunoprecipitated with a human-specific anti-insulin receptor antibody (lanes 1, 4, and 7) or an anti-phosphotyrosine Ab (lanes 2, 5, and 8). Shown here are the results for HIR270 cells (lanes 1 and 2), HIRΔE21 cells (lanes 4 and 5), and parental Rat-1 fibroblasts (lanes 7 and 8). Precipitates from one 35-mm well were used for the HIR270 cells and from two 35-mm wells for the HIRΔE21 and Rat-1 cells. The labeled IR α subunits were cut out of the gel and counted (HIR270: αIR, 930 cpm; PY, 490 cpm. HIRΔE21: αIR, 97 cpm; PY, 30 cpm. Rat-1: αIR, 4 cpm; PY, 53 cpm). This experiment was repeated four times with comparable results.

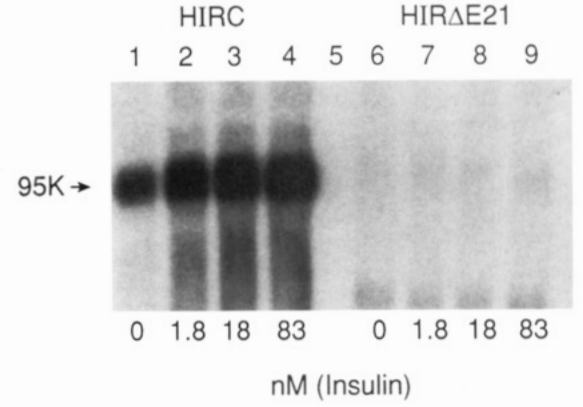

FIGURE 2: *In vitro* autophosphorylation. Lectin affinity purified insulin receptors were incubated with or without insulin for 16 h at 4 °C and then labeled by the addition of 20 of μCi of [^{32}P]ATP in reaction buffer. The receptors were immunoprecipitated with a human-specific insulin receptor monoclonal antibody. Phosphorylated β subunits are visualized by SDS–PAGE and autoradiography.

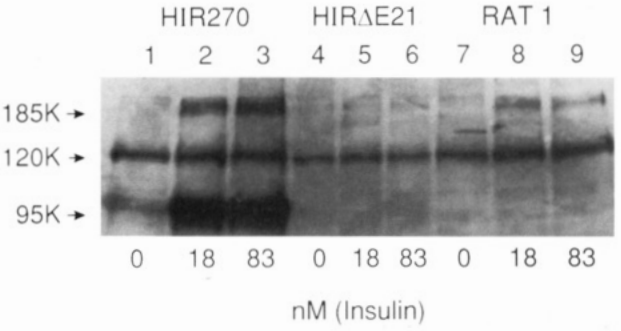

FIGURE 3: *Invivo* autophosphorylation and substrate phosphorylation. Confluent monolayers of Rat-1, HIRΔE21, and HIR270 cells were serum-starved for 16 h and then stimulated with the indicated concentration of insulin for 1 min at 37 °C. The samples were separated by SDS–PAGE and immunoblotted with an anti-phosphotyrosine antibody. In HIR270 cells (lanes 1–3), insulin stimulated the phosphorylation of pp185/IRS-1 (185 kDa) and the insulin receptor β subunit (95 kDa) in a dose-responsive manner. Much less insulin-stimulated phosphorylation of pp185/IRS-1 is seen in HIRΔE21 cells (lanes 4–6) and Rat-1 cells (lanes 7–9), and no insulin receptor β-subunit phosphorylation was detectable. The results are representative of three separate experiments with comparable results.

receptors from the HIR270 and HIRΔE21 cells were recognized by the human-specific antibody, while endogenous receptors from Rat-1 fibroblasts were not (lane 7). For HIR270, the phosphorylated receptors (lane 2) constituted ~50% of the total receptor pool (lane 1). In contrast, only ~ 30% of the insulin receptors from HIRΔE21 cells were autophosphorylated (lane 5). However, since the degree of autophosphorylation in ΔE21 cells was comparable to that seen in Rat-1 cells (compare lanes 5 and 8), a sizeable proportion of the receptors in lane 5 represents endogenous rat receptors rather than ΔE21 receptors. If one subtracts the contribution of the rat receptors from the total in lane 5, then only a small fraction of ΔE21 receptors was autophosphorylated.

To confirm the apparent lack of autophosphorylation of HIRΔE21 receptors, *invitro* autophosphorylation studies were performed on receptors partially purified using wheat germ agarose. Receptors were incubated with [^{32}P]ATP after a 16-h treatment with insulin and then immunoprecipitated with the human-specific antibody prior to analysis by SDS–PAGE. As demonstrated in Figure 2, no phosphorylation of the HIRΔE21 receptor was apparent.

A third method was utilized to confirm the absence of ΔE21 receptor autophosphorylation. Cells were grown to confluence in 35-mm dishes and stimulated with insulin at 37 °C for 1 min. The cells were lysed in the presence of phosphatase inhibitors, immunoprecipitated with the anti-phosphotyrosine antibody, followed by SDS–PAGE separation, and immunoblotting with an anti-insulin receptor antibody (C-terminal specific). The wild-type receptor was readily visualized, whereas HIRΔE21 receptors were not detected (data not shown).

Anti-Phosphotyrosine Immunoblotting. ΔE21 cells (2.0×10^5 receptors/cell) were treated with insulin for 1 min prior to lysis and anti-phosphotyrosine immunoblotting. As seen in Figure 3, two major insulin-stimulated phosphoproteins are visualized in HIR270 cells (lanes 1–3) migrating at 95 and 185 kDa. The 95-kDa band represents autophosphorylation of the receptor β subunit, and the 185-kDa band shows phosphorylation of the endogenous substrate IRS-I. Phosphorylation of these two bands is negligible in ΔE21 cells (lanes 4–6) and is similar to the results obtained with the untransfected parental Rat-1 cells (lanes 7–9).

In Vitro Receptor Kinase Activity. To confirm the absence of kinase activity for the ΔE21 receptor, phosphorylation of the exogenous substrate histone 2B was assessed. HIRcB, ΔE21 cells, and Rat-1 fibroblasts were stimulated with insulin, followed by solubilization. Receptors were purified by lectin affinity chromatography in the presence of phosphatase inhibitors. The purified receptors were then immobilized on insulin affinity beads and used to measure *in vitro* histone phosphorylation. On a per receptor basis, histone kinase activity was only 24% as great for ΔE21 receptor preparations compared to HIRc. When compared to Rat-1 cells on a per protein basis (to equalize for cell number), histone kinase activity was comparable between ΔE21 and Rat-1 fibroblasts, despite the fact that ΔE21 cells express many more insulin receptors. Thus, the kinase activity of E21 receptors is even more reduced than the measured 24% of control, since a large component of this residual activity represents histone kinase activity endogenous to the Rat-1 fibroblasts, rather than to the ΔE21 receptors.

Insulin Receptor Internalization. Insulin receptor internalization was measured by cross-linking [^{125}I]NAPA–insulin

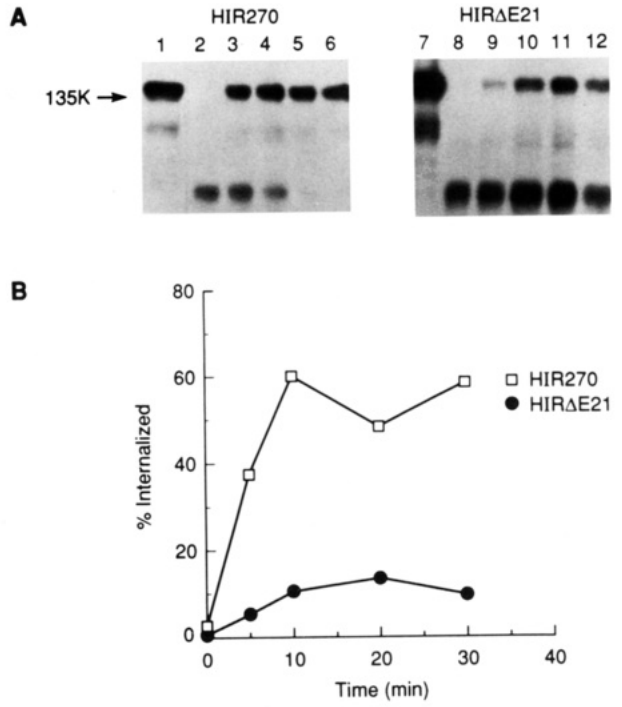

FIGURE 4: Internalization of insulin receptors. (A) Receptor internalization was measured using [^{125}I]NAPA–insulin. Cells were incubated with 6.6 nM [^{125}I]NAPA–insulin for 1 h at 4 °C. After cross-linking, the labeled cells were incubated at 37 °C for 0–30 min to allow internalization to occur and then placed on ice. Cell surface [^{125}I]NAPA–insulin receptor complexes were destroyed by treatment with trypsin. Total and trypsin-resistant (intracellular) complexes were assayed by SDS–PAGE and autoradiography. (B) The bands from A were cut and counted in a gamma counter to quantitate the percentage of receptors internalized at each time point.

to the cell surface receptors and then warming the cells to 37 °C for various times followed by trypsinization to distinguish surface from intracellular receptors (Thies et al., 1990). The autoradiographs and the plotted data in Figure 4 demonstrate rapid internalization of the wild-type receptor (HIR270 cells) with diminished internalization of the ΔE21 receptor. These findings were confirmed by measurements of [^{125}I]insulin internalization, which showed markedly reduced [^{125}I]insulin uptake in ΔE21 cells compared to HIRcB cells (data not shown).

Glucose Incorporation into Glycogen. The metabolic signaling of the HIRΔE21 receptor was studied by measuring the ability of insulin to stimulate glucose incorporation into glycogen in HIRcB cells versus HIRΔE21 cells. As previously reported, the wild-type HIRcB cells are more responsive to insulin then are the parental Rat-1 cells. The response of HIRΔE21 cells to insulin stimulation was identical to that of the parental Rat-1 cells, indicating an absence of signaling from the mutant human receptor (Figure 5). Interestingly, the dose-response curve for the HIRΔE21 cells was not right-shifted compared to that of the Rat-1 cells, indicating that the HIRΔE21 receptor does not exert a "dominant negative" effect on endogenous receptor function (Maegawa et al., 1988b). Comparable data were obtained using two independently isolated clonal cell lines (data not shown).

DNA Synthesis. [^{3}H]Thymidine incorporation into DNA was measured as an index of insulin-stimulated mitogenesis. Seventy-two hours after cells were plated onto 12-well dishes, the cells were serum-starved for 24 h, stimulated by insulin for 20 h, and then incubated for an additional 3 h with [^{3}H]-thymidine. HIRcB cells were markedly more responsive to insulin than Rat-1 cells. Surprisingly, the effect of insulin to

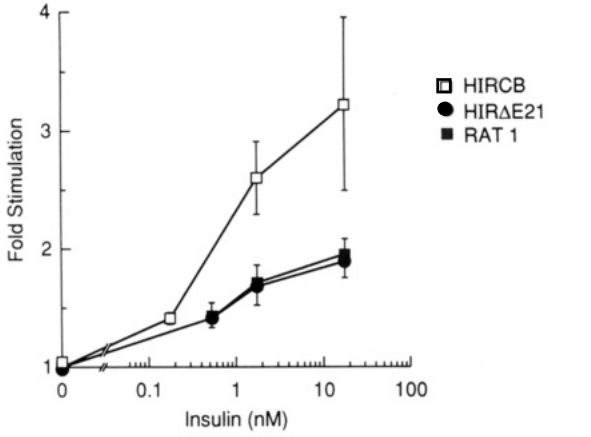

FIGURE 5: [^{14}C]Glucose incorporation into glycogen. Cells were incubated with the indicated insulin concentrations, and the incorporation of labeled glucose into glycogen was measured. The data are presented as the fold stimulation over basal against insulin concentration. Results are the mean ± SEM of five experiments for each cell line performed in duplicate.

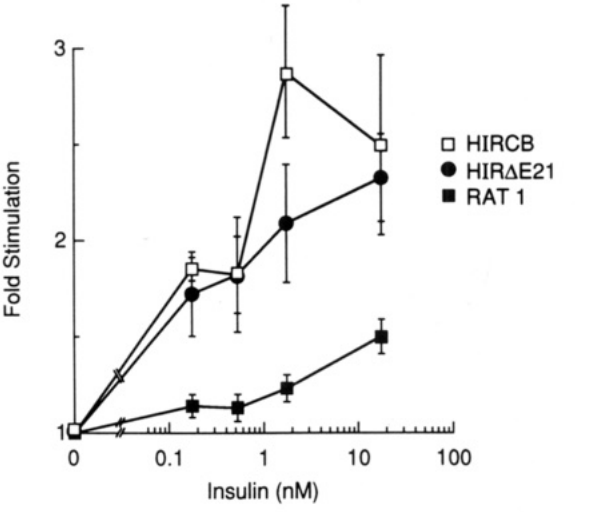

FIGURE 6: Insulin-stimulated [^{3}H]thymidine uptake. Cells were incubated with the indicated insulin concentrations, and the incorporation of labeled thymidine was measured. Data are expressed as the fold stimulation over basal [^{3}H]thymidine uptake, and comparable data were obtained in two clonal lines. The results are the mean ± SEM of five experiments for each cell line. All cell lines gave a similar response to serum (fold induction ± SEM: Rat-1, 4.7 ± 0.8; hIRcB, 4.8 ± 0.7; HIRΔE21, 4.0 ± 0.7).

stimulate DNA synthesis was greatly enhanced in the ΔE21 cells, and the dose-response curve was comparable between HIRcB and ΔE21 cells (Figure 6).

To verify that the increased insulin-stimulated DNA synthesis in ΔE21 cells represented insulin signaling through the mutant human insulin receptor, a monoclonal human-specific, insulinomimetic anti-receptor antibody was used. This antibody does not cross-react with the rat insulin or IGF-1 receptor (Figure 1; Brindle et al., 1990), and it does not stimulate biological activity in Rat-1 fibroblasts (Grako et al., 1992; Sasaoka et al., 1993). This antibody, which can only stimulate mitogenesis through the human insulin receptor, increased [^{3}H]thymidine uptake equally in HIRcB cells and HIRΔE21 cells (Table I).

DISCUSSION

In the present report, we have studied a mutant insulin receptor in which the amino acids corresponding to exon 21 have been deleted by site-directed mutagenesis. This receptor

Table I: Antibody-Stimulated DNA Synthesis[a]

monoclonal antibody (dilution)	[3H]thymidine uptake (% of insulin effect)	
	HIRcB	HIRΔE21
1:25000	42 ± 21	68 ± 13
1:5000	71 ± 6	51 ± 10
1:1000	101 ± 21	115 ± 31

[a] [3H]Thymidine incorporation was stimulated by the human-specific agonistic insulin receptor monoclonal antibody. Thymidine uptake was compared to the response produced by a maximally effective insulin level (18 nM), and the data are reported as a percent of the maximal insulin effect. Results (mean ± SEM) represent the mean of three separate experiments on each of two independent HIRΔE21 clonal lines.

was overexpressed in the Rat-1 fibroblast background, and its biological properties were characterized. The exon 21 domain does not contain the ATP-binding site nor the catalytic loop nor any tyrosine autophosphorylation sites, but it does comprise the C-terminal portion of the kinase domain including three predicted α-helices that form the scaffold of the peptide binding site. Deletion of exon 21 resulted in a receptor with undetectable autophosphorylation/kinase activity and a severely compromised ability to undergo insulin-stimulated internalization. Consistent with the above data, the HIRΔE21 receptors did not appear to transduce insulin's metabolic signals, since dose-response curves for insulin stimulation of glucose incorporation into glycogen were the same in HIRΔE21 cells and parental Rat-1 fibroblasts. Surprisingly, however, despite the absence of detectable autophosphorylation/kinase activity, insulin-stimulated DNA synthesis was greatly enhanced in HIRΔE21 cells to a degree comparable to cells overexpressing wild-type insulin receptors.

It is thought that tyrosine kinase activity is key to many, if not all, of insulin's actions (Olefsky, 1990). A number of mutations in either the ATP binding site or the tyrosine kinase domain have been described in insulin receptors derived from patients with extreme insulin resistance (Cama et al., 1992; Moller et al., 1990a; Taira et al., 1989; Yamamoto-Honda et al., 1990). Generally, these mutant receptors lack tyrosine kinase activity and are unable to transduce an insulin signal when overexpressed in transfected cells. However, as recently reviewed by Sung (1992), evidence exists to suggest that the receptor tyrosine kinase activity may not be essential in all cases for insulin receptor signaling.

Consistent with this idea are recent data from Moller et al. describing a naturally occurring point mutation, Trp^{1200} to Ser^{1200}, in exon 21 from a patient with severe insulin resistance (Moller et al., 1990a, 1991). When this mutant receptor was expressed in CHO cells, it did not display kinase activity and did not mediate mitogenic signaling, but did exhibit normal metabolic responses to insulin. Trp^{1200} is highly conserved in receptor tyrosine kinases and lies in one of the three predicted α-helices. Substitution of a hydrophilic serine residue for a hydrophobic tryptophan would be expected to disrupt the local secondary structure. Other examples of insulin receptors lacking measurable tyrosine kinase activity, but still transmitting certain biological signals of insulin, have been reported (Desbois et al., 1992; Wilden et al., 1990).

Given that the HIRΔE21 receptor did not exhibit detectable autophosphorylation or kinase activity and did not mediate metabolic signaling, it was surprising that its mitogenic signaling properties were preserved. Several possible explanations can be proposed to reconcile these findings. The insulin-induced mitogenic signaling in the HIRΔE21 cells could be mediated by (1) IGF-I receptors, (2) hybrid IGF-I:HIRΔE21 receptors, (3) endogenous rodent insulin receptors, or (4) the transfected HIRΔE21 receptors.

It is unlikely that IGF-I receptors mediate insulin-stimulated mitogenesis in HIRΔE21 cells, since stimulation was observable at low insulin concentrations which would cause negligible cross-reactivity with IGF-I receptors. Similar reasoning argues against a role for hybrid insulin–IGF-I receptors in insulin-induced mitogenic stimulation, since hybrid receptors bind insulin with low affinity and IGF-I with high affinity (Moxham & Jacobs, 1992). Finally, a monoclonal human-specific agonistic antibody, which does not recognize IGF-I receptors, was able to stimulate mitogenesis in HIRΔE21 cells. Since this antibody stimulated mitogenesis in HIRΔE21 and HIRcB cells in a comparable manner, whereas it has no effect in parental Rat-1 cells, it is also very unlikely that the endogenous rodent insulin receptors mediated the enhanced mitogenic signal.

On the basis of the above observations, we conclude that the augmented insulin-stimulated mitogenic signaling in HIRΔE21 cells was most likely mediated by the mutant HIRΔE21 receptor itself. This implies either that autophosphorylation/kinase activity is not essential for mitogenic signaling in the context of the HIRΔE21 cell line or that these cells display a level of kinase activity that is below the limit of detection of our assays, yet sufficient for mitogenesis. We think the latter possibility is unlikely since, at insulin concentrations which cause submaximal stimulation of thymidine uptake, receptor autophosphorylation and kinase activity can be easily measured in our hands (Freidenberg et al., 1987).

This raises the possibility that a secondary, or bypass, pathway has been activated in the HIRΔE21 cells for which receptor tyrosine phosphorylation is not essential. One potential pathway could be via G-proteins. The IR has two potential G-protein binding sites (amino acids 1135–1156 and 1319–1333), which coincide with the autophosphorylation sites. Perhaps deletion of exon 21 has exposed one of these sites, allowing the IR to couple to a G-protein-mediated pathway. If such a bypass, or redundant, pathway exists, it will be important to determine whether it is normally present in all insulin-sensitive cells or whether it is an adaptive insulin-response pathway present in these cells expressing kinase-incompetent receptors.

Another theoretical possibility is that HIRΔE21 receptors can activate the traditional kinase pathway without inducing tyrosine phosphorylation. For example, Rothenberg and coworkers have recently shown that certain cellular proteins physically associate with IRS-I in its non-tyrosine phosphorylated form (Rothenberg et al., 1993). Also along these lines, it has been shown that insulin binding and autophosphorylation lead to a conformational change in the β subunit C-terminus. Mutant receptors lacking the two C-terminal tyrosine residues (positions 1316 and 1322) display increased mitogenic signaling and in the unstimulated state are already in the active conformation associated with ligand occupancy and autophosphorylation (Kaliman et al., 1993). Conceivably, the HIRΔE21 receptors, which do not autophosphorylate, are already in an active conformation that allows for productive interactions with downstream effectors.

In summary, we have studied an insulin receptor mutant in which the domain encoded by exon 21 has been deleted. This receptor is processed and inserted into the plasma membrane where it binds insulin in an apparently normal fashion. This receptor did not display detectable autophosphorylation and kinase activity, and its ability to undergo ligand-mediated endocytosis was severely compromised. Interestingly, while insulin's ability to signal metabolic events

(glucose incorporation into glycogen) was impaired, insulin-induced mitogenesis through the HIRΔE21 receptor was apparently normal. These results demonstrate a divergence of these two insulin signaling pathways and suggest the presence of an alternative, bypass pathway in these cells, whereby insulin can stimulate mitogenesis independent of receptor or IRS-I phosphorylation.

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