Deletion of 343 Amino Acids from the Carboxyl Terminus of the β-Subunit of the Insulin Receptor Inhibits Insulin Signaling

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ABSTRACT: Naturally occurring mutations in the insulin receptor gene that impair the receptor tyrosine kinase activity cause insulin resistance *in vivo* in a dominant fashion. Previously, two unrelated families have been described that express an insulin receptor with a truncation due to a premature chain termination at codon 1000 (Δ1000), thereby deleting 343 amino acids from the carboxyl terminus of the β-subunit. While clinical findings suggest that the truncated receptor does not mediate insulin action *in vivo*, a recent study suggested that a similarly truncated receptor enhanced insulin sensitivity in transfected cells by augmenting the signaling by endogenous receptors [Sasaoka, T., Takata, Y., Kusari, J., Anderson, C. M., Langlois, W. J., & Olefsky, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 4379–4383]. To investigate these paradoxical data, we studied the structure and function of Δ1000 truncated insulin receptors when expressed in NIH-3T3 cells. We found that, despite the deletion of most of the tyrosine kinase domain and all of the C-terminal domain of the β-subunit of the insulin receptor, the Δ1000 mutant receptors were processed normally and were transported to the plasma membrane where they bind insulin with high affinity. Following ligand addition, the truncated receptors are degraded with a normal half-life. However, they fail to undergo insulin-stimulated internalization, do not regulate the phosphorylation of insulin receptor substrate 1, and are unable to mediate an insulin-stimulated increase in DNA synthesis and c-*jun* and c-*fos* expression. These results demonstrate that the Δ1000 truncated receptors, expressed in our *in vitro* model system, faithfully mirror the *in vivo* findings that this mutation causes insulin resistance.

The insulin receptor is a heterotetrameric membrane protein that belongs to the family of ligand-activated protein tyrosine kinases (Ullrich et al., 1985; Ebina et al., 1985; Hanks et al., 1988). Insulin binding to the α-subunit of its receptor stimulates autophosphorylation of multiple tyrosine residues (Kasuga et al., 1982a,b; Tornqvist et al., 1987, 1988; Tornqvist & Avruch, 1988) and activates the tyrosine kinase activity of the receptor β-subunit (Herrera & Rosen, 1986; Rosen, 1987). Following these events a variety of cellular substrates are phosphorylated, including insulin receptor substrate 1 (IRS-1),[1] and numerous biologic effects are initiated (Chou et al., 1987; Ellis et al., 1986; Sun et al., 1991; Backer et al., 1993). At present, the structural features of the insulin receptor molecule that mediate its interactions with cellular substrates, such as Shc and IRS-1, remain poorly defined.

Several types of mutations in the insulin receptor gene have been identified in patients with inherited syndromes of extreme insulin resistance (Taylor et al., 1992). Some of these severely insulin-resistant patients are compound heterozygotes, possessing two different mutant alleles of the insulin receptor gene (Kadowaki et al., 1988, 1990a; Barbetti et al., 1992; Kusari et al., 1991). Others are simple heterozygotes, possessing one normal allele and one mutant allele. Many of these simple heterozygotes possess one insulin receptor allele that is defective in its tyrosine kinase domain. Futhermore, patients who are heterozygous for a kinase defective receptor appear to be more severely insulin-resistant than patients who are heterozygous for a null allele (Moller & Flier, 1988; Odawara et al., 1989; Cama et al., 1991, 1993). At present, the mechanism underlying this dominant negative effect is unclear. It has been suggested that mutant receptors may form hybrids with wild-type receptors leading to inactive oligomers (Frattali, et al., 1992; Chin et al., 1991). It is also possible that mutant receptors compete with wild-type receptors for a limited number of cellular substrate molecules involved in the insulin signal transduction cascade (Maegawa et al., 1988; Levy-Toledano et al., 1994).

Previously, two unrelated insulin-resistant patients have been identified that possess a missense mutation in one of their insulin receptor alleles and the same nonsense mutation at codon 1000 in their second allele (Moncada et al., 1986; Kadowaki et al., 1990a; Kusari et al., 1991). The truncated receptors encoded by the allele containing the opal stop codon at position 1000 lack the consensus sequence for ATP binding as well as most of the tyrosine kinase domain. From studies carried out with lymphocytes and fibroblasts from these patients, it was unclear whether the truncated receptors were normally synthesized, processed, and transported to the cell surface. It was also unknown whether the truncated molecules had normal half-lives or if deletion of the C-terminal and tyrosine kinase domains resulted in altered receptor turnover. In addition, it was unknown whether, following insulin binding, the mutant receptors were phosphorylated by receptors synthesized from the other allele of the insulin receptor gene, thus allowing them to interact with cellular substrates involved in mediating insulin's actions. To address these points, we have characterized the Δ1000 truncated receptor by transfection of the mutant cDNA into NIH-3T3 cells.

We report here structural and functional studies of recombinant human insulin receptors truncated at codon 1000, resulting in deletion of 343 carboxyl-terminal amino acids.

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[1] Abbreviations: IRS-1, insulin receptor substrate 1; Shc, SH_2-containing sequence; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ECL, enhanced chemiluminescence.

Despite this severe truncation, the Δ1000 mutant receptors are processed normally and are transported to the plasma membrane where they bind insulin with normal affinity. Following addition of ligand, the truncated receptors are degraded with a normal half-life. However, these truncated receptors fail to undergo insulin-stimulated internalization, do not mediate phosphorylation of IRS-1, and are unable to mediate an insulin-stimulated increase in DNA synthesis.

EXPERIMENTAL PROCEDURES

Construction of a Human Insulin Receptor cDNA Truncated at Codon 1000. A fragment containing nucleotides 2805–3009 of the human insulin receptor cDNA was amplified by PCR using a full-length insulin receptor cDNA as template and the following oligonucleotide primers: 5′-GTATTTATCTATTCCTGAGA (nucleotides 2805–2824 of the sense strand of exon 15) and 5′-GGATCACTAGTGGGCCCAGCTCTAGAAGGAGGT (*Spe*I and *Apa*I restriction sites followed by nucleotides 2989–3009 of the antisense strand of the insulin receptor that contains a substitution of **A** for **C** at nucleotide 2999). The amplified PCR product was digested with *Bgl*I and *Spe*I to yield a 166-base-pair fragment. The *Bgl*I/*Spe*I fragment was then substituted for the *Bgl*I/*Spe*I segment of wild-type insulin receptor cDNA. Introduction of the point mutation abolishes as *Xho*I site in the wild-type sequence and generates a termination codon at amino acid 1000 (Ebina et al., 1985; Ullrich et al., 1985). The construction was verified by restriction digestion with *Xho*I and by determination of the nucleotide sequence.

Expression of Receptors by Transfection of cDNAs into NIH-3T3 Cells. The truncated insulin receptor cDNA was ligated into a bovine papilloma virus-based expression vector (pBPV, Pharmacia, Piscataway, NJ) in which insulin receptor cDNA expression is driven by the murine metallothionein promoter (Pavlakis & Hamer, 1983; Kadowaki et al., 1988).

NIH-3T3 cells (~2 × 10^6 cells) were transfected as described by the manufacturer using 50 μL of Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) and a mixture of 1 μg of pRSV-Neo, a plasmid encoding neomycin resistance, plus 20 μg of pBPV containing the mutant receptor. After selection for resistance to the antibiotic G418 (0.6 mg/mL), stable transfectants were isolated and cloned. Construction of a pBPV-based expression plasmid containing the intact human insulin receptor cDNA and transfection of NIH-3T3 cells has been described previously (Kadowaki et al., 1988).

^{125}I-Insulin Binding. Transfected cells were grown to confluence in 24-well plates and [^{125}I]insulin binding was carried out as described previously (Kadowaki et al., 1990b; Cama et al., 1992).

Endogenous Substrate Phosphorylation. Transfected cells were grown to confluence in 10-cm dishes. Following 12–18 h of serum starvation, cells were incubated in the absence or presence of insulin (0–10^{-6} M) for 1 min at 37 °C. The incubation medium was then removed and the cell monolayers were rapidly frozen with liquid nitrogen. The cells were solubilized on ice with 400 μL of Laemmli buffer or 1 mL of solubilization buffer (NaCl, 150 mM; octyl β-glucoside, 20 mM; Triton X-100, 0.5% (v/v); sodium phosphate, 0.25 M; pH 7.4) containing protease and phosphatase inhibitors (antipain, 1.7 mM; leupeptin, 2 mM; phenylmethanesulfonyl fluoride, 1 mM; sodium pyrophosphate, 10 mM; sodium fluoride, 100 mM; sodium orthovanadate, 2 mM). Proteins containing phosphotyrosine were then detected by immunoblotting or by immunoprecipitation followed by immunoblotting as described below.

Immunoblotting. Proteins were solubilized by boiling for 3 min in Laemmli sample buffer in the presence of 3 mg/mL dithiothreitol, separated on SDS–7.5% polyacrylamide gels and transferred to nitrocellulose as described previously (Renfrew & Hubbard, 1991). Thereafter, the nitrocellulose membranes were incubated for 2 h at room temperature in PBS containing 0.1% (v/v) Tween-20 (Bio-Rad, Hercules, CA) and 10% (w/v) nonfat dry milk. The membranes were then incubated overnight at 4 °C in PBS containing 0.1% (v/v) Tween-20 and one of the following antibodies: (1) a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) at a 1/4000 dilution, (2) a rabbit polyclonal antibody directed against a peptide corresponding to amino acids 657–670 of the α-subunit of the human insulin receptor (Rosenzweig et al., 1990) (Upstate Biotechnology Inc., Lake Placid, NY) at a 1/500 dilution, (3) a rabbit polyclonal antibody directed against a peptide corresponding to amino acids 1142–1157 of the β-subunit of the human insulin receptor at a 1/2000 dilution. The next day, membranes were washed three times for 10 min at room temperature with PBS containing 0.1% (v/v) Tween-20 and then incubated for 30 min with either horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (Amersham, Arlington Heights, IL) at a 1/5000 dilution. After the membranes were washed 3 times for 10 min with PBS containing 0.3% (v/v) Tween-20 and then with PBS containing 0.1% (v/v) Tween-20, ECL detection was performed according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Immunoprecipitation of Insulin Receptors. Cell monolayers were extracted for 30 min at 4 °C in solubilization buffer and centrifuged for 30 min at 12000*g*. The supernatant was then precleared for 1–2 h at 4 °C by incubating with 70 μL of protein A–Sepharose CL-4B beads (Life Technologies, Inc., Grand Island, NY). The beads were sedimented and the supernatants were subsequently incubated with 4 μL of anti-insulin receptor antibody (B7/B10 mixture; Levy-Toledano et al., 1993) for 15–18 h. The beads were then washed, and the immunoprecipitates released and prepared for SDS–PAGE as described (Bartles et al., 1987).

Biotinylation of Cell Surface Insulin Receptors. Transfected cells were grown to confluence in 10-cm dishes and cell surface proteins were biotinylated with a solution containing 0.5 mg/mL long-chain *N*-hydroxysuccinimide (NHS-LC-biotin; Pierce, Rockford, IL) in DPBS as described previously (Levy-Toledano et al., 1993). After biotinylation, the monolayers were solubilized in 1 mL of solubilization buffer containing glycine and insulin receptors were immunoprecipitated as described above. Following transfer to nitrocellulose, streptavidin blotting was performed (Levy-Toledano et al., 1993).

Degradation of Insulin Receptors. Transfected cells were grown to confluence in 6-cm dishes and labeled overnight in DMEM containing 0.4 mCi/mL Tran^{35}S-Label (1060 Ci/mmol; ICI Biomedicals, Irvine, CA). Thereafter, the cells were washed once with PBS and incubated in medium containing 0.6 mM methionine, 0.9 mM cysteine, and 10^{-5} M insulin for 0–24 h. The cells were then washed once with ice-cold PBS, frozen in liquid nitrogen, and then solubilized and immunoprecipitated as described above with polyclonal anti-insulin receptor antibody B7/B10. Following SDS gel electrophoresis, the gels were fixed and the radioactive signal was enhanced by treatment with sodium salicylate (Chamberlin, 1979).

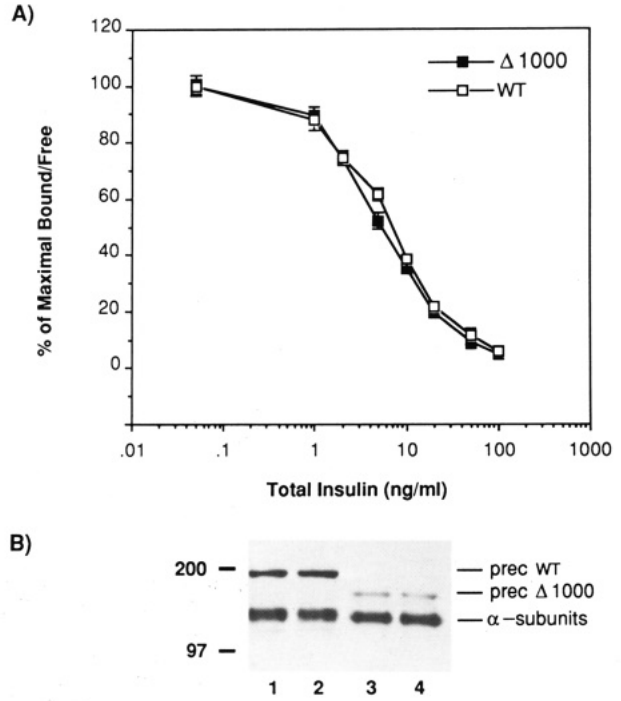

FIGURE 1: Insulin binding to NIH-3T3 cells expressing wild-type (WT) or truncated (Δ1000) insulin receptors. Panel A: [^{125}I]Insulin binding to NIH-3T3 cells transfected with wild-type (WT, open squares) or truncated (Δ1000, closed squares) insulin receptors was carried out as described in Experimental Procedures. The results of the binding studies are expressed as the percentage of the maximal ratio of bound/free insulin plotted as a function of the total insulin concentration. Each point represents the mean ±SEM of four replicate determinations in each of two independent experiments. Panel B: Immunoblotting with an antibody directed against the α-subunit of the insulin receptor was performed on cell extracts from parallel cultures of wild-type cells (WT, lanes 1 and 2) and cells expressing mutant insulin receptors (Δ1000, lanes 3 and 4). Apparent molecular masses (kilodaltons) are shown.

Measurement of Insulin Internalization and Degradation. Transfected cells were grown to confluence in 6-well plates, washed twice with ice-cold PBS, and incubated overnight at 4 °C in 1 mL of internalization buffer [RPMI 1640 medium; HEPES, 25 mM; BSA, 0.1% (w/v)] containing 0.1 ng/mL [^{125}I]insulin (20 000–50 000 dpm/mL). Thereafter, the cells were washed to remove unbound insulin and incubated at 37 °C in prewarmed incubation buffer for 2–60 min. At the designated times, the amounts of insulin bound at the cell surface, dissociated, internalized and degraded were determined as described previously (Kadowaki et al., 1990b; Cama et al., 1992).

[^{3}H]Thymidine Incorporation. Measurements of thymidine incorporation were performed as described previously (Quon et al., 1992).

Induction of c-jun and c-fos Expression by Insulin. Measurements of c-*jun* and c-*fos* expression were performed as described previously (Quon et al., 1992; Kato et al., 1994).

RESULTS

Insulin Binding to Transfected NIH-3T3 Cells. NIH-3T3 cells were stably transfected with cDNA encoding either wild-type (WT) or mutant insulin receptors (Δ1000). The mutant insulin receptors were truncated at amino acid 1000, thereby deleting 343 amino acids from the carboxyl terminus of the β-subunit. This large deletion removes most of the tyrosine kinase domain and all of the C-terminal domain of the β-subunit.

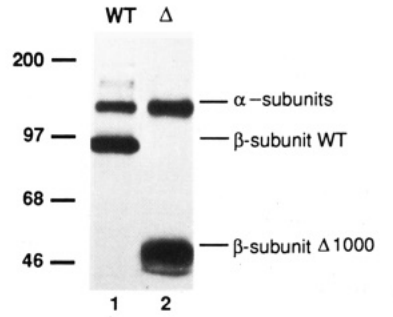

FIGURE 2: Detection of biotinylated cell surface insulin receptors by streptavidin blotting. Cell surface proteins of confluent monolayers of NIH-3T3 cells expressing either WT (lane 1) or Δ1000 mutant (lane 2) insulin receptors were biotinylated as described in Experimental Procedures. Cell surface receptors were immunoprecipitated with a mixture of antibodies (B7 and B10) against the α-subunit. The immunoprecipitates were analyzed by SDS–PAGE (7.5%) under reducing conditions and the proteins were transferred to nitrocellulose. The resulting blot was then probed with horseradish peroxidase-labeled streptavidin. Apparent molecular masses (kilodaltons) are shown.

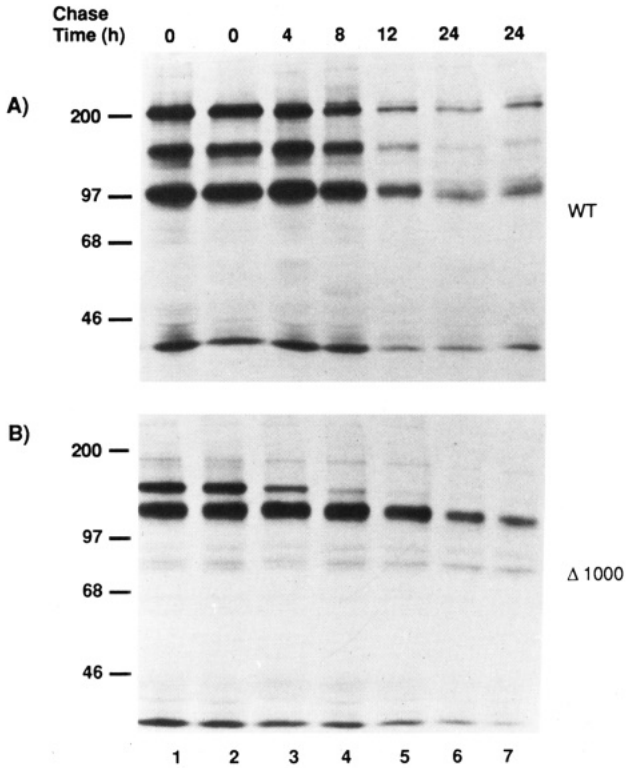

FIGURE 3: Insulin-stimulated degradation of wild-type (WT) and truncated (Δ1000) insulin receptors. Confluent monolayers of NIH-3T3 cells transfected with wild-type (panel A) or truncated insulin receptors (panel B) were incubated for 14 h in DMEM containing [^{35}S]methionine and [^{35}S]cysteine. Thereafter, the cells were transferred to medium containing 10^{-5} M insulin and unlabeled methionine and cysteine for 0–24 h. The cells were then solubilized and the resulting detergent extracts were immunoprecipitated with a mixture of anti-insulin receptor antibodies (B7 and B10). Finally, the immunoprecipitates were analyzed by SDS–PAGE, followed by fluorography. The X-ray films were exposed for 1 day.

When cells expressing WT and truncated receptors were incubated with tracer concentrations of [^{125}I]insulin (0.05 ng/mL), bound/free ratios of 0.88 ± 0.04 for Δ1000 mutant cells and 0.89 ± 0.04 for WT cells were seen. Likewise, binding studies carried out using increasing concentrations of insulin (0.05–100 ng/mL) showed that the two cell lines expressed similar numbers of insulin receptors with similar affinities for insulin (Figure 1A). Immunoblotting studies, with an antibody directed against the receptor α-subunit, confirmed the finding

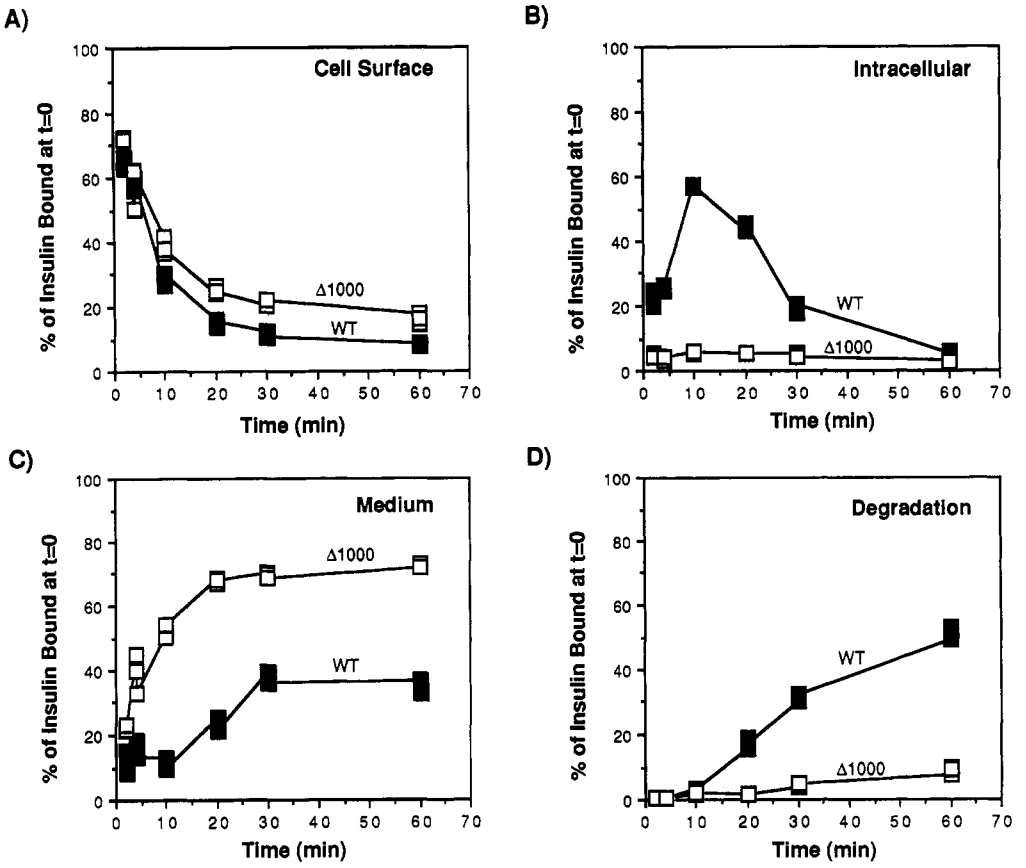

FIGURE 4: Insulin internalization by NIH-3T3 cells expressing wild-type and or truncated insulin receptors. [^{125}I]Insulin was allowed to bind overnight at 4 °C to NIH-3T3 cells expressing either wild-type (WT, solid squares) or truncated (Δ1000, open squares) insulin receptors. Following removal of unbound [^{125}I]insulin, fresh medium prewarmed to 37 °C was added and the fate of the prebound [^{125}I]insulin was determined. Panel A: The [^{125}I]insulin bound to the cell surface was removed by acid-washing the cells. Acid-dissociable radioactivity is plotted as a function of time. Panel B: Internalized [^{125}I]insulin was estimated by measuring cell-associated radioactivity that was not released by the acid wash. Panel C: Intact [^{125}I]insulin that dissociated from the cells was estimated by measuring the trichloroacetic acid-precipitable radioactivity in the medium as a function of time. Panel D: Degraded [^{125}I]insulin released from the cells was estimated by measuring the trichloroacetic acid-soluble radioactivity generated. The data shown are triplicate determinations from a representative experiment.

that the two transfected cell lines express similar numbers of receptor molecules (Figure 1B).

Biotinylation of Cell Surface Insulin Receptors. To determine if the Δ1000 mutant insulin receptors were processed correctly and then transported to the plasma membrane, proteins on the surface of NIH-3T3 cells transfected with WT and truncated insulin receptors cells were labeled by NHS-LC-biotin. Insulin receptors were then immunoprecipitated with a receptor α-subunit antibody and detected by streptavidin blotting (Figure 2). A band of about 135 kDa corresponding to the α-subunit was detected in both cell lines. For cell lines expressing the WT receptor, a band of about 95 kDa corresponding to the β-subunit was seen (Figure 2, lane 1). As expected, a band of about 45–50 kDa corresponding to the truncated β-subunit was seen for the mutant cell line (Figure 2, lane 2). Bands corresponding to the proreceptors were not detected in either cell line, suggesting that unprocessed receptors did not reach the cell surface.

Insulin Receptor Biosynthesis and Degradation. To evaluate the effects of the deletion of the tyrosine kinase domain upon the biosynthesis and insulin-stimulated degradation of the insulin receptor, pulse-chase experiments were performed (Figure 3). Transfected cells expressing either WT (Figure 3A) or truncated receptors (Figure 3B) were labeled overnight to near steady-state with [^{35}S]methionine and [^{35}S]cysteine, followed by incubation with 10^{-5} M insulin and excess unlabeled methionine and cysteine during the chase period (0–24 h). At the beginning of the chase period, the major labeled bands in cells expressing the WT receptor correspond to the 190-kDa proreceptor and the mature α- and β-subunits (Figure 3A, lanes 1 and 2). As expected, the α-subunit found in the mutant cell line has mobility similar to that of the α-subunit found in WT cells. In contrast, the mutant proreceptor is approximately 40–45 kDa smaller than the WT proreceptor, whereas the mutant β-subunit is undetectable. This is presumably due to the small number of cysteine and methionine residues in the β-subunit of the truncated molecule (Figure 3B, lanes 1 and 2). At later time points in the chase, both proreceptors are processed into mature subunits and the mature subunits are degraded (panels A and B, lanes 3–7). In two of three experiments, the proreceptor in the mutant cell line appeared to be more rapidly processed than the WT proreceptor and the mutant α-subunit appeared to have a slightly increased half-life (WT, 10–14 h; Δ1000, 12–16 h). However, no major differences were observed in the insulin-stimulated turnover of the two receptor molecules.

Receptor-Mediated Endocytosis. Previous studies with site-directed mutants of the insulin receptor have suggested that receptor tyrosine kinase activity is required for ligand-stimulated internalization of receptors (Backer et al., 1991; Cama et al., 1992; Chou et al., 1987; Carpentier et al., 1992, 1993; Levy-Toledano, 1993). We therefore examined whether the truncated insulin receptor internalized [^{125}I]insulin. Stably transfected cells were incubated at 4 °C with [^{125}I]insulin. Unbound insulin was then washed away and the cells were warmed to 37 °C for 2–60 min. [^{125}I]Insulin disappeared

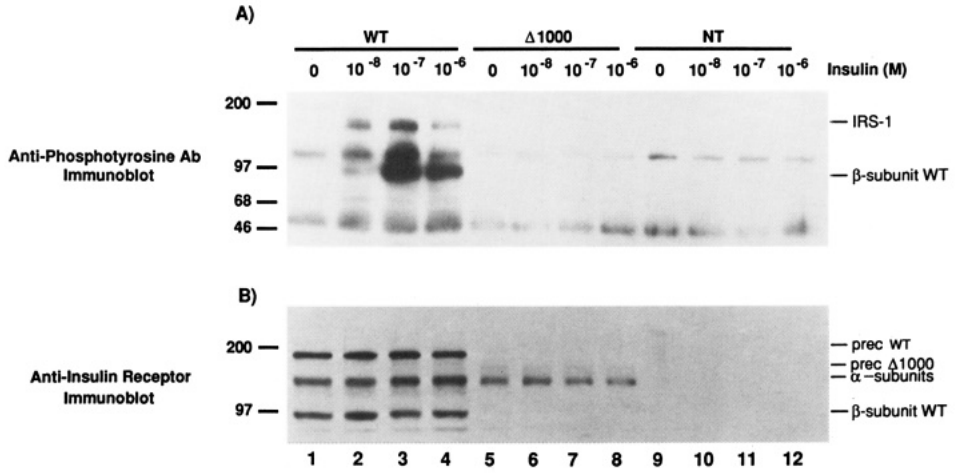

FIGURE 5: Insulin-stimulated autophosphorylation of insulin receptors and phosphorylation of endogenous substrates in intact cells. Untransfected NIH-3T3 cells (NT) and NIH-3T3 cells expressing either wild-type (WT) or truncated (Δ1000) insulin receptors were incubated in the absence or presence of insulin (0–10^{-6} M) for 1 min at 37 °C, and the monolayers were frozen in liquid nitrogen and then lysed in Laemmli buffer. The extracts were analyzed by SDS–polyacrylamide (7.5%) gel electrophoresis in the presence of dithiothreitol, followed by electroblotting onto a nitrocellulose membrane. The blots were then probed with either an anti-phosphotyrosine antibody (panel A) or a mixture of anti-insulin receptor antibodies against the α- and β-subunits (panel B). Autoradiograms representative of three experiments are shown.

from the cell surface at approximately the same rate in cells expressing WT (closed squares) and Δ1000 mutant (open squares) receptors (Figure 4, upper left panel). After 10 min of incubation at 37 °C, cell surface radioactivity had declined to approximately 35% of its initial value. However, the fate of the insulin initially bound at the cell surface was quite different in the two cell lines. After 10 min, approximately 60% of the [^{125}I]insulin was internalized into cells expressing the WT insulin receptors, while only approximately 5% of the initially bound [^{125}I]insulin was internalized by cells expressing the truncated receptor (Figure 4, upper right panel). Furthermore, by 60 min approximately 50% of the initially bound [^{125}I]insulin was released into the medium of cells expressing the WT receptor in a degraded form (Figure 4, lower right panel). In contrast, in cells expressing the Δ1000 mutant receptor, large amounts of insulin were released into the medium in a form precipitable with trichloroacetic acid, suggesting that it was intact. The small amounts of trichloroacetic acid-soluble material present in the medium of cells expressing the truncated receptor suggest that most of the initially bound [^{125}I]insulin dissociated from its cell surface receptors intact rather than being internalized and subsequently degraded (Figure 4, lower panels).

Phosphorylation of Insulin Receptors and Endogenous Substrates in Intact Cells. The Δ1000 mutation deletes the entire C-terminus, most of the tyrosine kinase domain, and all known sites of tyrosine phosphorylation except for the tyrosines found in the juxtamembrane region of the insulin receptor (Tyr^{965}, Tyr^{972}, and Tyr^{984}). To assess *in vivo* autophosphorylation of the insulin receptor at these tyrosine residues and to examine the subsequent phosphorylation of endogenous substrates, cells were incubated with various concentrations of insulin (10^{-8}–10^{-6} M) for 1 min at 37 °C. Cell lysates were then prepared and subjected to Western blot analysis using anti-phosphotyrosine (Figure 5A) and anti-insulin receptor antibodies (Figure 5B). As shown in Figure 5A (lanes 1–4), insulin stimulated phosphorylation of two prominent bands that can be visualized in cells expressing the WT receptors. The 95-kDa band corresponds to the β-subunit of the insulin receptor and the 185-kDa band probably corresponds to pp185/IRS-1 (Sun et al., 1991). Insulin rapidly stimulated autophosphorylation of the β-subunit in WT cells, whereas no β-subunit phosphorylation was detectable in the parental cell line (lanes 9–12) or in cells expressing the truncated receptor (lanes 5–8). Furthermore, phosphorylation of IRS-1 was seen only in cells expressing the WT receptor. Western blot analysis using anti-insulin receptor antibodies confirmed that detectable amounts of the truncated receptor were present in the samples used in the phosphorylation studies (Figure 5B). These data are consistent with the hypothesis that insulin receptor autophosphorylation plays an important role in mediating insulin-stimulated phosphorylation of IRS-1.

Insulin-Stimulated Biologic Effects. Although several lines of evidence support the hypothesis that insulin receptor autophosphorylation is necessary for transducing the mitogenic effect of insulin on target cells, this point remains controversial (Becker & Roth, 1990; Gottschalk, 1991; Chou et al., 1987; McClain et al., 1987). To determine whether the Δ1000 mutant receptor was capable of mediating the mitogenic action of insulin, we measured the insulin-stimulated uptake of [^{3}H]-thymidine in nontransfected cells and cells expressing either WT or truncated receptors. The dose–response was measured and plotted as a percentage of the response to 10% serum (Figure 6). In cells expressing the WT insulin receptor, insulin causes a 4-fold increase in thymidine incorporation. In contrast, untransfected cells and cells expressing the truncated receptor underwent very little, if any, insulin-stimulated thymidine incorporation. Similar effects were observed when c-*jun* and c-*fos* expression were examined by Northern blot analyses (Figure 7). Insulin stimulated a 2.5–5-fold increase in c-*jun* and c-*fos* expression in cells expressing the WT receptor, whereas the nontransfected cells and the Δ1000 mutant receptors showed no significant increase in either message level compared to that seen in the unstimulated state.

DISCUSSION

Several naturally occurring insulin receptor mutations have been described that lead to severe truncations of the insulin receptor's cytoplasmic domain and result in genetic syndromes associated with insulin resistance. In one study, a truncation at codon 1109 was described. These mutant receptors were shown to lack insulin-activated tyrosine kinase activity and to suppress the function of normal insulin receptors produced from the other insulin receptor allele (Taira et al., 1989). Likewise, two unrelated families have been described that

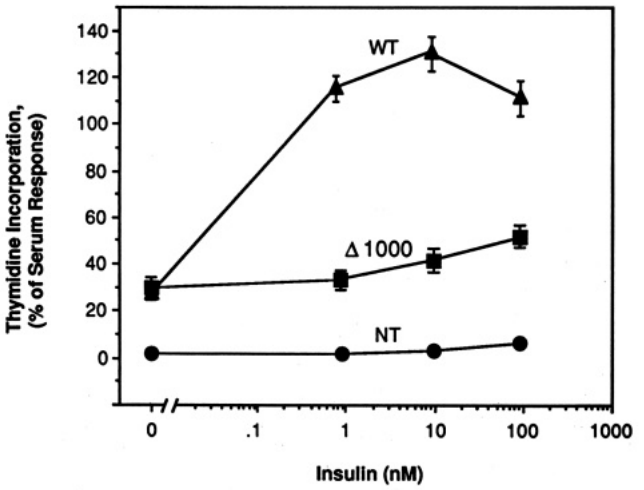

FIGURE 6: Insulin-stimulated thymidine incorporation. Untransfected NIH-3T3 cells (NT) and NIH-3T3 cells expressing either wild-type (WT) or truncated (Δ1000) insulin receptors were serum-starved for 24 h. The cells were then incubated with insulin (0–10^{-7} M) or 10% fetal calf serum for 16 h, followed by [^{3}H]thymidine for 1 h. The cells were solubilized and trichloroacetic acid-precipitable radioactivity was measured by liquid scintillation counting. Results are expressed as a percentage of the serum-stimulated values, which were set to 100%. Absolute counts following serum stimulation were as follows: NT, 16 707 ± 390 dpm; WT, 18 315 ± 810 dpm; Δ1000, 20 457 ± 842 dpm. A typical experiment (mean ± SEM, n = 3) is shown.

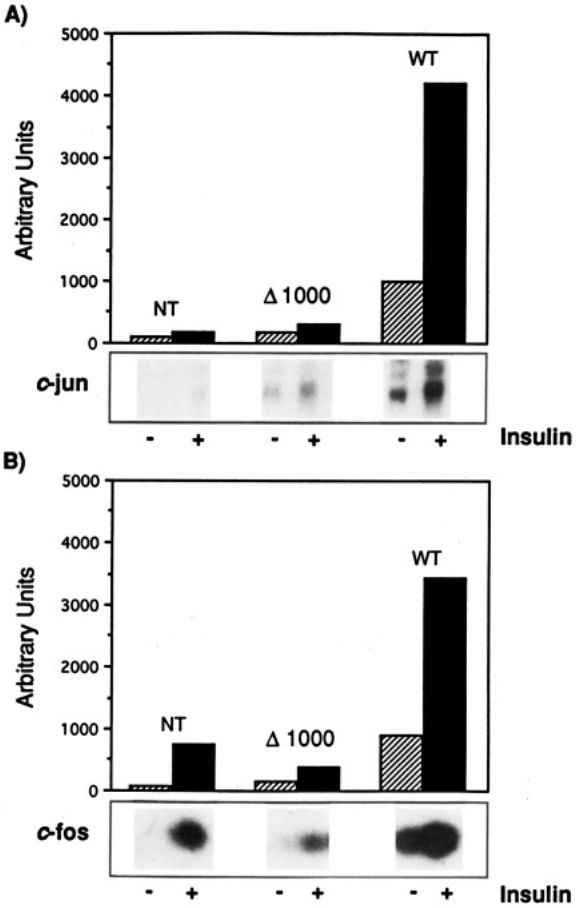

FIGURE 7: Insulin-stimulated c-*jun* and c-*fos* expression. Untransfected NIH-3T3 cells (NT) and NIH-3T3 cells expressing either wild-type (WT) or truncated (Δ1000) insulin receptors were serum-starved for 24 h. The cells were incubated in the presence or absence of insulin (10^{-7} M) for 45 min. The cells were then chilled, and total RNA was isolated and Northern blot analyses were performed as described in Experimental Procedures. Ten-microgram aliquots of total RNA were probed with (A) c-*jun* and (B) c-*fos* cDNAs and the blots were quantified using a phosphorimager.

possess a stop codon at position 1000 of one of their insulin receptor alleles (Kusari et al., 1991). In one family, simple heterozygotes were shown to display a degree of insulin resistance that was greater than would be predicted on the basis of having only one defective insulin receptor allele (Moncada et al., 1986; Kadowaki et al., 1990; Kusari et al., 1991). In contrast, a receptor truncated at codon 990[2] has recently been studied following transfection into Rat-1 cells (Sasaoka et al., 1993). Rather than displaying a dominant negative phenotype as would be predicted from the patient data, the Δ990 mutant receptors instead showed a dominant stimulatory effect on wild-type receptors. To try to understand these paradoxical data, we have studied the biochemical properties of the naturally occurring Δ1000 mutant receptor following its expression in NIH-3T3 cells.

Defect in Receptor Endocytosis. When expressed in NIH-3T3 cells, the Δ1000 mutant receptor is deficient in insulin-stimulated endocytosis. Rather than utilizing a rapid, saturable pathway that delivers the ligand–receptor complex into endosomes, the truncated receptor internalizes slowly via a ligand-independent pathway. A number of determinants appear critical for rapid coated pit internalization. Two critical determinants for rapid insulin receptor endocytosis are receptor autophosphorylation and kinase activation. These events are thought to free the receptor from local restraints maintaining it on microvilli, allowing it to then interact with the coated pit machinery (Carpentier et al., 1992, 1993). The presence of intracellular tyrosine-containing β-turns in the receptor molecule are also needed for rapid internalization. They are thought to be important for interacting with the coated pit machinery. Our findings with the Δ1000 mutant receptors, which possess these potential sites for β-turn formation in their juxtamembrane domain but lack C-terminal sequences (Backer et al., 1991, 1992), support the idea that these β-turns are not sufficient to signal entry into coated pits (Carpentier et al., 1993). By deleting sequences C-terminal to the juxtamembrane tyrosine residues, we may have altered the conformation of these β-turns, resulting in an internalization defect. Similarly, we may have removed important downstream determinants that are required for direct multivalent interactions of the insulin receptor tail with coated pit-associated proteins involved in ligand-stimulated endocytosis (Pearse, 1988; Beltzer & Spiess, 1991). Additional mutagenesis studies will be needed to resolve these questions.

Normal Turnover of Truncated Receptors. A number of deletions have been introduced into the carboxyl terminus of the insulin receptor in order to investigate the role of this region in mediating insulin action [reviewed by Tavare and Siddle (1993) and Yan et al. (1993)]. Removal of the C-terminus by deletion of 112 (Ellis et al., 1986) or 113 (Levy-Toledano et al., 1993) amino acids produces unstable receptors. In contrast, deletion of 69 (Chin et al., 1991; Dickens et al., 1992) and 43 (Mclain et al., 1988; Meyers et al., 1991) C-terminal amino acids yields stable receptor molecules. In this study, we found that when 343 carboxyl-terminal amino acids are deleted, thereby removing the C-terminal and most of the tyrosine kinase domains, the resulting truncated receptors are processed normally and exhibit a half-life similar

[2] For clarity, the numbering system used throughout the text for describing the amino acid positions in the insulin receptor are based on the cDNA that includes exon 11 (Ebina et al., 1985). Therefore, position 990 (Ebina et al., 1985) is equivalent to position 978 using the numbering for the insulin receptor cDNA lacking exon 11 (Ullrich et al., 1985).

to that seen for the wild-type receptor. Taken together, these findings suggest that C-terminal sequences may be important for stabilizing the receptor molecule or that regions of the tyrosine kinase domain, when exposed by truncation of downstream regions, destablize the molecule. Construction of a receptor chimera containing the C-terminal and juxtamembrane domains, but lacking the tyrosine kinase domain, may help to shed further insight into the sequence and/or structural requirements important for degradation of the insulin receptor following ligand addition.

Defect in Receptor Autophosphorylation and IRS-1 Phosphorylation. In vivo phosphorylation of the juxtamembrane region of the wild-type insulin receptor accounts for approximately a third of the receptor's phosphorylation, and it has been suggested that phosphorylation of Tyr^{972} plays an important role in signal transduction (Feener et al., 1993; White et al., 1988; Murakami & Rosen, 1991; Kohanski, 1993). Since cellular proteins that undergo tyrosine phosphorylation are thought to be important components of the insulin signal transduction cascade, they have been the focus of a number of recent studies. At present, the structural features of the insulin receptor molecule that mediate the interactions with cellular substrates, such as Shc and IRS-1, remain poorly defined. To address these issues, we looked at the ability of the Δ1000 mutant receptor to undergo insulin-stimulated phosphorylation and to regulate tyrosine phosphorylation of endogenous substrates.

The Δ1000 mutant receptor studied here lacked intrinsic tyrosine kinase activity and was expected to be deficient at autophosphorylation. Nevertheless, the possibility remained that the truncated receptor might become phosphorylated via another mechanism—perhaps via an intramolecular mechanism involving the formation of hybrid molecules between endogenous rodent insulin receptors and transfected truncated receptors (Lammers et al., 1990) or via an intermolecular mechanism, utilizing another insulin-stimulated kinase such as the IGF-1 receptor (Levy-Toledano et al., 1994) or a yet-uncharacterized cytosolic kinase. However, we found that the truncated receptor did not undergo insulin-stimulated phosphorylation when expressed in NIH-3T3 cells. No phosphorylation of the truncated cytoplasmic domain containing juxtamembrane tyrosine residues 965, 972, and 984 was seen. Furthermore, we detected no IRS-1 phosphorylation. The lack of IRS-1 phosphorylation could result from the inability of our truncated receptor molecule to bind IRS-1 (White et al., 1988; Sun et al., 1991; Backer et al., 1993). Perhaps the formation of a specific binding site that mediates the interactions between IRS-1 and the insulin receptor requires phosphorylation of the insulin receptor in the juxtamembrane region or requires a conformational change triggered by phosphorylation.

Events downstream of IRS-1 were also affected. The Δ1000 mutant receptor did not mediate the effect of insulin to stimulate [^{3}H]thymidine incorporation or c-*fos* and c-*jun* expression. These inhibitory effects of the Δ1000 truncation are consistent with the results obtained previously for several insulin-resistant patients expressing truncated insulin receptors. They also suggest that our *in vitro* system accurately mimics the *whole body* system. The evidence that kinase-deficient receptors exert a dominant negative effect *in vivo* derives primarily from comparisons between individuals who are heterozygous for mutations in the kinase domain and individuals who are heterozygous for a null allele of the insulin receptor gene. All of the published mutations in the kinase domain caused clinically recongnizable insulin resistance in the heterozygous state (Odawara et al., 1989; Taylor et al., 1992). In contrast, most of the null alleles initially came to medical attention only because they were present in individuals with two mutant alleles of the insulin receptor gene (Kadowaki et al., 1988, 1990a; Taylor et al., 1992). Subsequently, null alleles have been identified in the heterozygous state in family members. In some of these individuals, heterozygosity for null alleles was associated with subtle biochemical evidence of insulin resistance (e.g., elevations in plasma insulin levels). However, if both types of mutant alleles are compared in the heterozygous state, the mutations in the kinase domain caused more severe insulin resistance and more severe clinical disease than was observed in patients who are heterozygous for null alleles. The data presented here for the truncated receptor support the clinical findings that receptors with inactive kinase activities cannot mediate insulin action.

Our data, however, contrast with the findings recently presented by Sasaoka et al. (1993). These investigators studied Rat-1 cells transfected with a mutant receptor truncated at amino acid 990. They found that fibroblasts expressing the Δ990 mutant receptor had levels of IRS-1 phosphorylation very similar to those found in cells expressing the wild-type receptor and significantly higher levels than those found in the parental cell line. Also, despite the absence of an intact β-subunit, the authors concluded that the cells expressing the Δ990 mutant receptor displayed enhanced insulin sensitivity for glycogen synthesis, c-*fos* expression, and thymidine incorporation when compared to the parental Rat-1 cell line. However, in this study, the biologic effects of insulin were of small magnitude, ranging from 2.1-fold in untransfected Rat-1 cells to 1.7-fold in cells expressing WT receptors. The small effects seen in the Rat-1 cells highlights the problem of interpreting the effects of insulin receptor mutations on downstream events encountered in some tissue culture cells transfected with insulin receptors (Kato et al., 1993; Quon et al., 1992). These limitations, however, cannot explain the differences in IRS-1 phosphorylation found in the two studies.

How can we explain these differences? The differences in IRS-1 phosphorylation might be due to differences in the composition of Rat-1 and NIH-3T3 fibroblasts. Rat-1 fibroblasts might contain an insulin-stimulated kinase not found in NIH-3T3 cells that can be recruited and activated by the truncated receptor, resulting in an increase in IRS-1 phosphorylation. Alternatively, the somewhat larger deletion present in the Δ990 mutant receptor might expose a cryptic motif not exposed in the Δ1000 mutant receptor that then recruits an insulin-stimulated kinase from the cytoplasm of Rat-1 cells. Whether this interaction would be physiologic importance is unknown. In any event, it appears not to be very important in our tissue culture system or in the major target cells for insulin action *in vivo* in the insulin-resistant patients.

Conclusion. The ability of insulin to stimulate the intrinsic tyrosine kinase activity of its receptor provides an attractive mechanism for transmembrane signaling. Historically, the role of insulin receptor phosphorylation in insulin action has been controversial. However, the weight of evidence, including the findings reported here for the Δ1000 kinase-defective receptor, now supports the critical role of insulin receptor tyrosine kinase activity in insulin signaling. Continued progress in elucidating how tyrosine phosphorylation is coupled to later events, such as activation of *ras* (Skolnik et al., 1993; Baltensperger et al., 1993) and MAP kinase (Tobe et al., 1992), through use of *in vitro* systems, should prove invaluable in understanding pleotropic diseases such as diabetes.

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