Postbinding Characterization of Five Naturally Occurring Mutations in the Human Insulin Receptor Gene: Impaired Insulin-Stimulated c-jun Expression and Thymidine Incorporation despite Normal Receptor Autophosphorylation

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ABSTRACT: Some patients with extreme insulin resistance have mutations in their insulin receptor gene. We previously identified five such mutations located in the extracellular domain of the insulin receptor (Asn→Lys^{15}, His→Arg^{209}, Phe→Val^{382}, Lys→Glu^{460}, and Asn→Ser^{462}) and studied the effects of these mutations upon posttranslational processing, insulin binding, and tyrosine autophosphorylation. We now characterize the ability of these mutant receptors to mediate biological actions of insulin in transfected NIH-3T3 fibroblasts. All cell lines expressing mutant receptors showed marked impairment in insulin-stimulated c-jun expression and thymidine incorporation when compared with cells expressing wild-type human insulin receptors. The most severe impairment was seen in cells expressing the Val^{382} mutant (a mutation which causes an intrinsic defect in receptor autophosphorylation). These cells had insulin responses similar to the untransfected cells (used as a negative control). In contrast, cells expressing the Lys^{15} mutant have the ability to achieve a normal level of maximal autophosphorylation but require an abnormally high concentration of insulin to do so (as the result of decreased insulin binding affinity). These cells show a higher basal rate and much lower insulin stimulation of both c-jun expression and thymidine incorporation when compared with the cells expressing the wild-type human insulin receptors. This pattern is also seen in the cells expressing the other mutants with normal autophosphorylation (Arg^{209}, Glu^{460}, and Ser^{462}). Although the most severe defects in insulin action are seen with the mutation which has an intrinsic defect in receptor autophosphorylation, the ability to undergo normal autophosphorylation does not seem to preclude mutations from impairing the ability of receptors to mediate some of the actions of insulin.

The insulin receptor is a cell-surface transmembrane glycoprotein consisting of a heterotetramer of two α- and two β-subunits linked by disulfide bonds. The α-subunit is extracellular and contains the insulin binding domain while the β-subunit contains the intracellular tyrosine kinase domain. The binding of insulin to its receptor activates the receptor tyrosine kinase activity. In addition to phosphorylation of cellular substrates, phosphorylation of tyrosine residues in the β-subunit also occurs. This autophosphorylation is thought to be important if not essential for the insulin signal transduction pathway (Rosen, 1987; Kahn & White, 1988).

The insulin receptor mediates diverse processes involved in cell growth, differentiation, and metabolism. The precise molecular mechanisms of insulin's multiple actions are not well understood. Mutations in the human insulin receptor gene have been discovered in patients with syndromes of extreme insulin resistance (Taylor, 1990; Moller & Flier, 1991). The most prominent physiological defect in these patients is the markedly impaired ability of insulin to lower plasma glucose. We previously described five missense mutations in the α-subunit of insulin receptors from patients with extreme insulin resistance (Asn→Lys^{15}, His→Arg^{209}, Phe→Val^{382}, Lys→Glu^{460}, and Asn→Ser^{462}). Table I summarizes the previous characterization of these mutant receptors with respect to binding kinetics, autophosphorylation, posttranslational processing and transport, and receptor life-cycle (Kadowaki et al., 1988, 1990a,b,c, 1991; Accili et al., 1989, 1991). All of these mutations cause a decrease in the number of receptors on the cell surface in vivo. This is caused by either impairing transport of receptors to the cell surface or by accelerating receptor degradation. It is possible that this decrease in receptor number completely explains the patients' insulin resistance. Nevertheless, it is also possible that mutations impair the intrinsic activity of the receptors to mediate the biological actions of insulin. In this study, we examine insulin-stimulated receptor autophosphorylation, c-jun expression, thymidine incorporation, and 2-deoxyglucose uptake in NIH-3T3 fibroblasts overexpressing wild-type and mutant human insulin receptors in order to gain additional insight into the molecular mechanisms of insulin action. We show that all five mutations studied cause significant impairment in the ability of insulin to stimulate c-jun expression and thymidine incorporation. These defects appear to be independent of the ability of the mutant receptors to autophosphorylate. The most severe impairment is seen with the

Table I: Previously Characterized Properties of Five Mutant Receptors[a]

	binding affinity	autophosphorylation	intracellular processing and transport	degradation of cell-surface receptors
Lys^{15}	↓↓ (5×)	↓ sensitivity	↓	normal
Arg^{209}	normal	normal	↓↓↓	normal
Val^{382}	normal	↓	↓↓	normal
Glu^{460}	↑ (2×)	normal	normal	↑
Ser^{462}	normal	normal	normal	↑

[a] This is a summary of previously published properties of the five mutant receptors studied in this work: Lys^{15} (Kadowaki et al., 1990c), Arg^{209} (Kadowaki et al., 1991), Val^{382} (Accili et al., 1989, 1991), Glu^{460} (Kadowaki et al., 1988, 1990a), and Ser^{462} (Kadowaki et al., 1990b; McElduff et al., 1984).

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Val^{382} mutant which has decreased autophosphorylation. This is consistent with the hypothesis that receptor autophosphorylation is necessary to mediate insulin action. However, significant abnormalities (with a characteristic pattern) are seen in the other mutants that have normal autophosphorylation. This suggests that mutations in the insulin receptor that do not affect autophosphorylation may nevertheless impair the mitogenic action of insulin.

EXPERIMENTAL PROCEDURES

Cell Lines. Previously established NIH-3T3 fibroblast cell lines were grown in DMEM with 10% fetal calf serum and 10% CO_2. Details of the methods used to transfect the cell lines overexpressing mutant or wild-type insulin receptors (using a bovine papilloma virus expression vector, pRSVNeo plasmid, and neomycin resistance selection) are described elsewhere (Kadowaki et al., 1988). Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, and Ser^{462} mutants were all constructed by site-directed mutagenesis as previously described (Kadowaki et al., 1988, 1989, 1990a,b,c, 1991; Accili et al., 1989). An untransfected cell line was maintained for use as a negative control.

Insulin Binding. Binding studies were done as previously described (Kadowaki et al., 1990a). Briefly, transfected NIH-3T3 cells were grown to confluence in six-well plates. On the day of the experiment, the cells were washed twice with PBS. The cells were then incubated overnight at 4 °C in 1 mL of binding buffer (NaCl, 120 mM; $MgSO_4$, 1.2 mM; KCl, 2.5 mM; sodium acetate, 15 mM; glucose, 10 mM; EDTA, 1 mM; HEPES, 50 mM, pH 7.8; bovine serum albumin, 10 mg/mL) containing 0.1 ng/mL [^{125}I]insulin (receptor grade, 360 Ci/g; Dupont–NEN; Wilmington, DE) in the presence or absence of unlabeled insulin (10 μg/mL). Thereafter, the cells were washed twice with ice-cold PBS to remove unbound insulin, the cells were solubilized in 1 N NaOH for 2 h, and cell-associated radioactivity was quantitated in a γ counter.

Autophosphorylation of Receptors in Intact Cells. Transfected NIH-3T3 cells were grown to confluence in 6-cm tissue culture dishes. The cells were then incubated for 24 h in 3 mL of DMEM without fetal calf serum. After serum starvation, the cells were incubated with varying concentrations of insulin (0–10^{-6} M) for 1 min at 37 °C. The incubation media were aspirated and the cell monolayers frozen in liquid nitrogen. The monolayers were solubilized on ice with 0.4 mL of Laemmli's sample buffer and boiled for 2 min. The Val^{382}, Arg^{209}, and Lys^{15} cell lines were solubilized in 0.2 mL of Laemmli buffer and then concentrated by evaporation to a final volume of 0.1 mL to achieve receptor concentrations which were comparable to the cells overexpressing human wild-type receptor. The insoluble material was removed by centrifuging the samples at 15000*g* for 30 min. β-Mercaptoethanol was added to the samples (final concentration 10%, v/v) prior to analysis by 6.5% $NaDodSO_4$–polyacrylamide gel electrophoresis. The contents of the gels were transferred by electroblotting onto nitrocellulose membranes, and the phosphotyrosine-containing proteins were detected with monoclonal anti-phosphotyrosine antibody (UBI; Lake Placid, NY) plus ^{125}I-labeled anti-mouse γ-globulin. Relative receptor amounts were determined by probing the blot with anti-insulin receptor antibody Ab-50 plus ^{125}I-labeled anti-rabbit γ-globulin. Results were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Induction of c-jun by Insulin. Transfected NIH-3T3 cells were grown in 10-cm tissue culture dishes. Before the cells reached confluence, they were serum-starved for 24 h. The cells were then incubated in the presence or absence of insulin (10 μg/mL) for 45 min at 37 °C. Total cellular RNA was obtained using RNAzol B (Cinna/Biotecx, Friendswood, TX). Northern blot analysis was performed using a random-primed ^{32}P-labelled cDNA probe for c-jun (RSV c-jun from Michael Karin). Results were quantified with a PhosphorImager (Molecular Dynamics).

[^{3}H]Thymidine Incorporation. Transfected NIH-3T3 cells were seeded in six-well plates at a density of 10^5 cells per well (in 2 mL of DMEM containing 10% fetal calf serum). The cells were incubated for 24 h at 37 °C and then serum-starved for an additional 24 h by replacing the media with DMEM containing 0.1% (w/v) bovine serum albumin. The media were then replaced either with DMEM containing fetal calf serum (10%, v/v) or with DMEM containing 0.1% bovine serum albumin plus varying concentrations of insulin (0–10^{-7} M). After the incubation was continued for another 16 h, the media were replaced by the same media supplemented with HEPES (25 mM, pH 7.4) and [^{3}H]thymidine (500 nCi/mL; Dupont–NEN). After 1-h incubation with [^{3}H]thymidine, the media were removed, and the cells were washed 3 times with ice-cold PBS. The cells were solubilized in 1 mL of $NaDodSO_4$ (0.1%, w/v) for 2 h. An equal volume (1 mL) of trichloroacetic acid (20%, w/v) was added to the solubilized cells. This mixture was centrifuged at 1800*g* for 30 min and the supernatant discarded. The pellet was solubilized in 0.3 mL of NaOH (1 N) and neutralized with 0.1 mL of HCl (3 N). Radioactivity in the trichloroacetic acid precipitate was measured (in a 50-μL aliquot) by liquid scintillation counting.

2-Deoxyglucose Transport. Transfected NIH-3T3 cells were grown to confluence in six-well plates. The cells were then serum-starved for 24 h in DMEM without fetal calf serum. The cells were washed twice with Krebs–Ringer phosphate buffer containing HEPES (10 mM, pH 7.4) plus bovine serum albumin (0.5%, w/v) at 24 °C. The cells were then incubated for 20 min at 24 °C with 1 mL of the same buffer containing varying concentrations of insulin (0–10^{-7} M). The media were then replaced with fresh media (1 mL) of the same composition supplemented with 2-[^{3}H]deoxyglucose (3.2 μCi/mL in 20 μM 2-deoxyglucose; Dupont–NEN). Nonspecific 2-deoxyglucose uptake was estimated by adding an excess of unlabeled 2-deoxyglucose (10 mM). The incubation with 2-[^{3}H]deoxyglucose was stopped after 2 min by washing with ice-cold PBS with Ca^{2+} and Mg^{2+}. The cells were washed 5 times to remove untransported 2-deoxyglucose and then solubilized in 1 mL of $NaDodSO_4$ (0.1%, w/v) for 2 h. Cell-associated radioactivity was measured in a 100-μL aliquot by liquid scintillation counting.

RESULTS

Insulin Binding. Insulin binding experiments were used to estimate the number of insulin receptors present on the cell surface of each of the cell lines studied (Table II). The cell line transfected with wild-type insulin receptor cDNA expresses approximately 2×10^6 receptors per cell while the untransfected NIH-3T3 cells display approximately 1×10^4 receptors per cell. Cells expressing mutant receptors that are transported normally to the cell surface (Glu^{460} and Ser^{462}) have approximately the same number of receptors per cell as the cells expressing wild-type receptors (2×10^6 and 1×10^6 receptors per cell, respectively). Cells expressing mutant receptors that are impaired in their ability to be transported to the cell surface (Lys^{15}, Arg^{209}, and Val^{382}) show fewer cell-surface receptors in proportion to the severity of the transport defect.

Autophosphorylation of Insulin Receptors in Intact Cells. Insulin-stimulated autophosphorylation of the insulin receptor

Table II: Quantitation of Cell-Surface Receptors[a]

cell line	estimated no. of receptors/cell	cell line	estimated no. of receptors/cell
WT	2×10^6	Val^{382}	3×10^5
Glu^{460}	2×10^6	Arg^{209}	9×10^4
Lys^{15}	1×10^6	NIH-3T3	1×10^4
Ser^{462}	1×10^6		

[a] Untransfected NIH-3T3 cells and NIH-3T3 cells expressing either wild-type (WT) or mutant (Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, Ser^{462}) receptors were incubated for 20 h at 4 °C with tracer amounts of ^{125}I-labeled insulin (0.1 ng/mL) in the presence or absence of a large excess of unlabeled insulin (10 μg/mL). The percentage of specific insulin binding was calculated by subtracting the nonspecific binding (binding in the presence of a large excess of unlabeled insulin) from the binding at tracer concentrations of labeled insulin. To estimate the number of receptors on each cell line, the value of bound/free insulin [measured in the presence of ^{125}I-labeled insulin (0.1 ng/mL)] was divided by the apparent affinity constant of the unoccupied receptor, K_e (DeMeyts et al., 1976), as determined in previous studies (for references, see legend to Table I). In general, there was close agreement between the estimates of receptor number obtained in the present study and those reported previously.

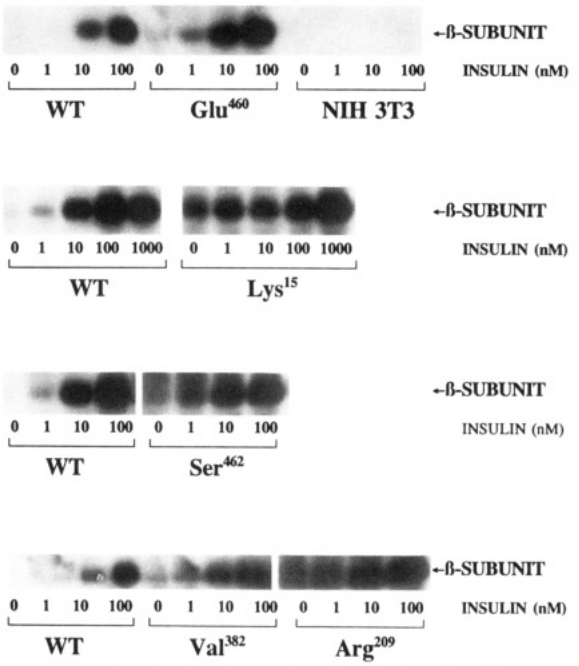


FIGURE 1: Insulin-stimulated autophosphorylation of insulin receptors in intact cells. Untransfected NIH-3T3 cells and NIH-3T3 cells expressing either wild-type (WT) or mutant (Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, Ser^{462}) receptors were serum-starved for 24 h and then incubated with insulin (0–10^{-7} M) for 1 min. Thereafter, the cells were solubilized and samples analyzed by $NaDodSO_4$–polyacrylamide gel (6.5%) electrophoresis. Comparable amounts of receptor were loaded onto each lane of the gel (as estimated from binding data). Phosphotyrosine-containing proteins were detected with monoclonal anti-phosphotyrosine antibody followed by autoradiography. The β-subunit of the insulin receptor runs at a molecular weight of 95K. These blots were obtained under identical conditions and are representative of the results obtained in a total of at least three similar experiments. The blots shown for the Glu^{460}, NIH-3T3, Ser^{462}, and corresponding WT cell lines represent experiments done on the same day. Similarly, the blots shown for the Lys^{15}, Val^{382}, Arg^{209}, and corresponding WT cell lines represent experiments done on the same day.

was assessed because this is one of the first events to occur after the binding of insulin to its receptor. Furthermore, it is thought to be important, if not essential, for mediating many of the actions of insulin. Western blots of insulin-stimulated receptor autophosphorylation agree with our previous results (Figure 1) (Kadowaki et al., 1990a,b,c, 1991; Accili et al., 1991). Insulin dose–response experiments from cell lines

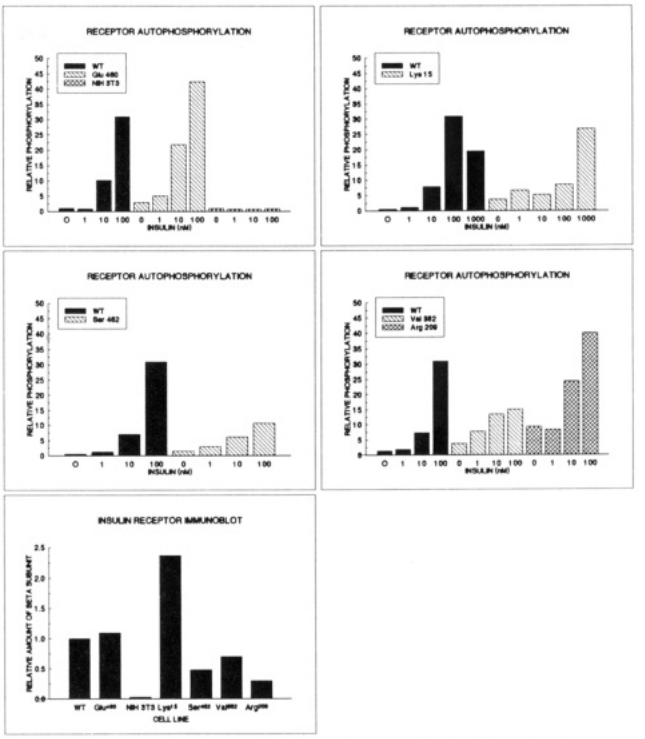


FIGURE 2: Quantitation of Western blot results by PhosphorImager. Western blots shown in Figure 1 were analyzed using a PhosphorImager and the results expressed in arbitrary units. The four blots of the autophosphorylated experiments were normalized to give equal maximal insulin stimulation for cells expressing the wild-type receptor. Also shown is the quantitation of an insulin receptor immunoblot (using anti-β-subunit antibody Ab-50) indicating the relative amounts of insulin receptor from each cell line loaded onto the gels in Figure 1. For the Arg^{209} and NIH-3T3 cell lines, the signal generated by the small receptor numbers on these blots is only minimally above background and is at the limit of detection by the PhosphorImager.

expressing the various receptor mutants, the untransfected cell line, and the cell line expressing human wild-type receptor are compared. An attempt was made to load comparable amounts of receptor for each cell line in a particular blot (estimated from the binding data). In the cases of the untransfected NIH-3T3 cells and Arg^{209} cell lines, this was not possible because the number of cell-surface receptors is very small when compared with the wild-type cell line. Western blots were also done for the β-subunit of the insulin receptor for each cell line as an independent estimate of the relative number of receptors loaded on each blot. These data were quantitated using a PhosphorImager and are represented in graphic form in Figure 2. The Glu^{460} and Ser^{462} mutants appear to have normal autophosphorylation in response to insulin (when normalized for receptor number) while the Lys^{15} mutant shows a decrease in sensitivity to insulin reflecting the 5-fold decrease in insulin binding affinity (normal maximal stimulation achieved at higher insulin concentration). The Val^{382} mutant appears to have impaired autophosphorylation in response to insulin. Cells expressing Arg^{209} mutant receptors, and the untransfected NIH-3T3 cells are difficult to evaluate because quantitation of the receptor number for these cell lines is uncertain. Nevertheless it appears that the insulin-stimulated autophosphorylation in the untransfected NIH-3T3 cells is absent (confirming that these cells possess a negligible number of insulin receptors) while the Arg^{209} mutant shows a measurable response. As has been reported previously, the Arg^{209} response is near normal if corrected for receptor number (Kadowaki et al., 1991).

Induction of the c-jun Protooncogene by Insulin. The effect of insulin on c-jun expression was measured because the effects

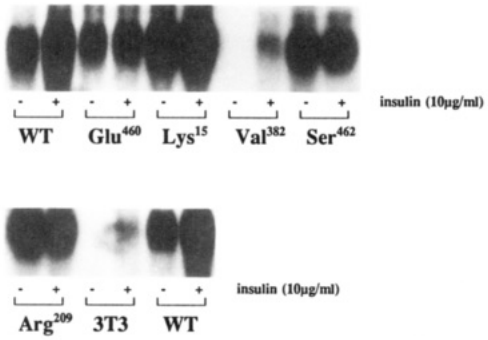


FIGURE 3: Insulin-stimulated c-jun expression. Untransfected NIH-3T3 cells and NIH-3T3 cells expressing either wild-type (WT) or mutant (Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, Ser^{462}) insulin receptors were serum-starved for 24 h. Thereafter, the cells were incubated in the presence or absence of insulin (10 μg/mL) for 45 min. This was followed by extraction of total RNA and Northern blot analysis using a cDNA probe for c-jun. In this blot, the lanes containing samples from cells expressing the Lys^{15} mutant receptor had 40 μg of RNA while all the other lanes had approximately 20 μg of RNA. The blot is representative of the results obtained in a total of three similar experiments.

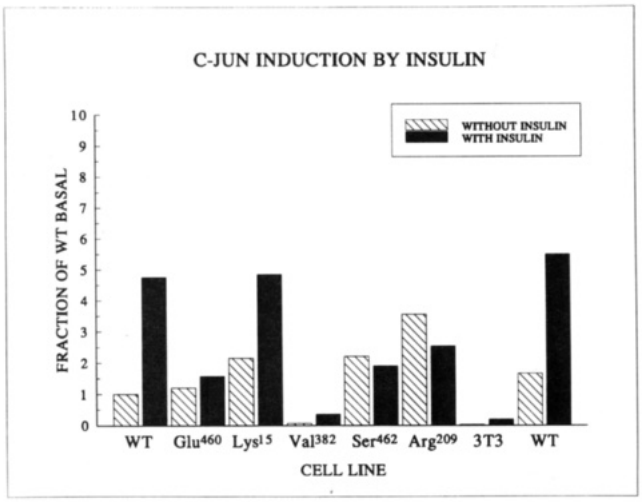
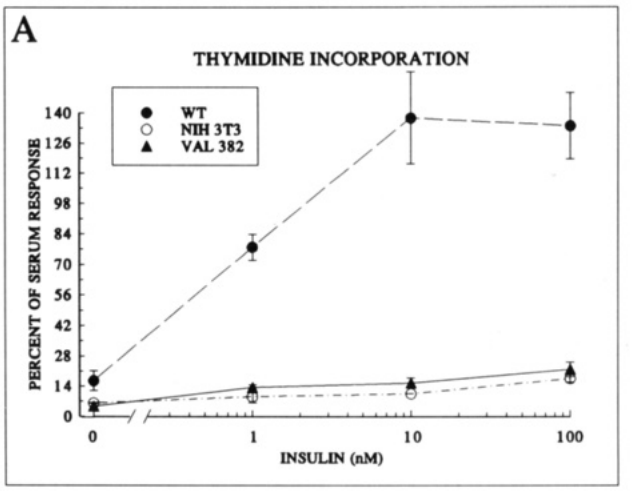


FIGURE 4: Quantitation of Northern blot results by PhosphorImager. The Northern blot in Figure 3 was quantitated using a PhosphorImager and the results expressed as arbitrary units of hybridization intensity per microgram of RNA applied to the gel.

of insulin on growth and differentiation may be mediated by c-jun and other related protooncogenes (Burgering et al., 1989, 1991; Stumpo & Blackshear, 1986; Stumpo et al., 1988; Benito et al., 1991). c-jun expression was assessed by Northern blot analysis of total cellular RNA obtained from cells incubated in the presence or absence of insulin (10 μg/mL) for 45 min (Figure 3). Data were quantitated with a PhosphorImager and are represented in graphic form in Figure 4. Cells expressing the wild-type receptor show a 4–5-fold induction of c-jun in response to insulin. All of the cell lines expressing mutant receptors show an impairment in the ability of insulin to stimulate c-jun expression on a percentage basis (independent of their ability to autophosphorylate the insulin receptor). Interestingly, cells expressing insulin receptors with mutations at Lys^{15}, Arg^{209}, and Ser^{462} appear to express c-jun at a higher level in the basal state than the cells expressing the wild-type receptor while cells expressing the Val^{382} mutant and the untransfected NIH-3T3 cells have virtually undetectable basal and insulin-stimulated c-jun expression.

Insulin-Stimulated Thymidine Incorporation. To determine if the abnormalities in insulin-stimulated c-jun expression seen in the cells expressing mutant receptors correlate with the ability of the cells to respond to the mitogenic effect of



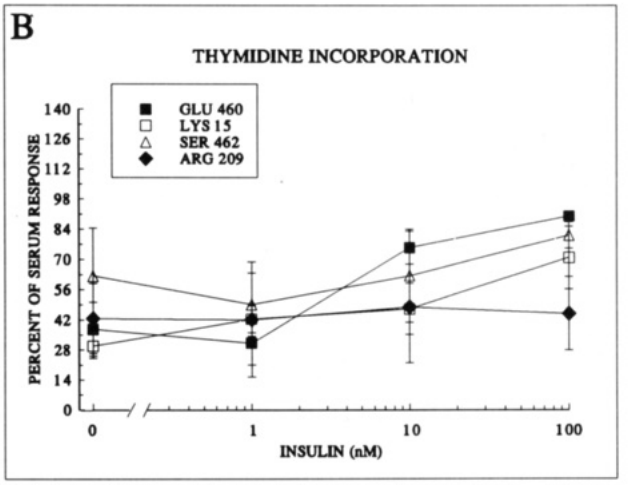


FIGURE 5: Insulin-stimulated thymidine incorporation. Untransfected NIH-3T3 cells and NIH-3T3 cells expressing either wild-type (WT) or mutant (Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, Ser^{462}) insulin receptors were serum-starved for 24 h. The cells were then incubated with insulin (0–10^{-7} M) or 10% fetal calf serum for 16 h. Finally, the cells were incubated with the same media plus [^{3}H]thymidine for 1 h. The cells were solubilized, and trichloroacetic acid-precipitable radioactivity was measured by liquid scintillation counting. Results shown are the average of three experiments (mean ± SEM). Each of the three experiments was done in triplicate. Results are shown in two panels for clarity although each experiment tested all cell lines at the same time. (A) WT, NIH-3T3, Val^{382}. (B) Glu^{460}, Lys^{15}, Ser^{462}, Arg^{209}.

insulin, we measured the effect of insulin upon thymidine incorporation. The dose–response to insulin was measured and plotted as a percentage of the response to 10% serum (Figure 5). In cells expressing wild-type insulin receptors, insulin causes a 9-fold increase in thymidine incorporation. Cells expressing the Val^{382} mutant and the untransfected NIH-3T3 cells show low basal thymidine incorporation and very little insulin stimulation, paralleling the pattern of insulin-stimulated c-jun expression shown above. There is a similar concordance when the results of insulin-stimulated c-jun expression and thymidine incorporation are compared for cells expressing the other mutations. Cells expressing the Glu^{460}, Lys^{15}, and Arg^{209} mutants have basal thymidine incorporation levels which are approximately twice that of the cells expressing wild-type receptors. The cells expressing the Ser^{462} mutant have a basal level of approximately 4 times that of the cells expressing the wild-type receptors. Maximal insulin stimulation is approximately 2-fold in the cells expressing Glu^{460} and Lys^{15}, while there is almost no insulin response seen in cells expressing the Ser^{462} and Arg^{209} mutants.

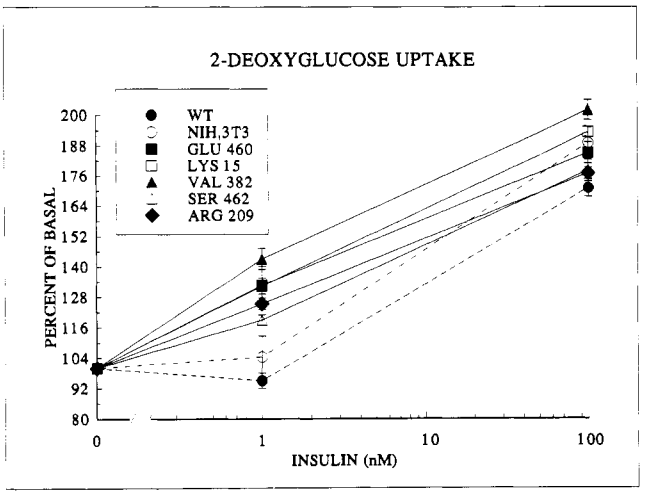


FIGURE 6: Insulin-stimulated 2-deoxyglucose uptake. Untransfected NIH-3T3 cells and NIH-3T3 cells expressing either wild-type (WT) or mutant (Lys^{15}, Arg^{209}, Val^{382}, Glu^{460}, Ser^{462}) insulin receptors were serum-starved for 24 h. The cells were then incubated for 20 min in the presence of insulin (0–10^{-7} M) followed by incubation for 2 min with insulin plus 2-[^{3}H]deoxyglucose. Nonspecific 2-deoxyglucose uptake was assessed by adding an excess (10 mM) of unlabeled 2-deoxyglucose. Cell-associated radioactivity was measured by liquid scintillation counting. Results shown are the average of three triplicates (mean ± SEM) of insulin-stimulated 2-deoxyglucose uptake minus the nonspecific uptake.

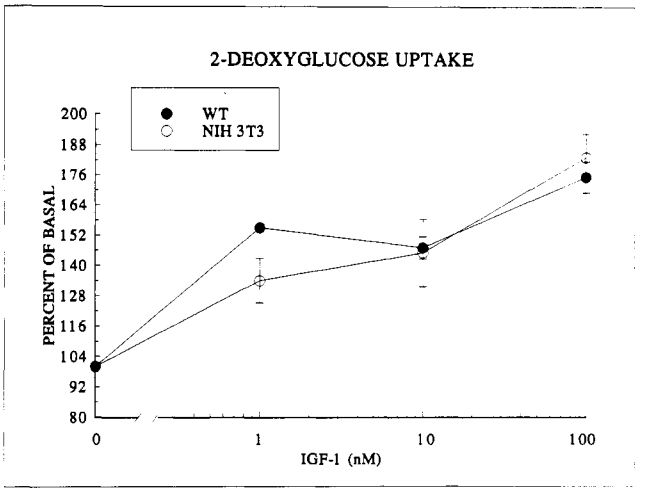


FIGURE 7: IGF-1-stimulated 2-deoxyglucose uptake. These experiments were carried out as described in Figure 6 except that IGF-1 (0–10^{-7} M) was used in place of insulin. Results shown are the average of three triplicates (mean ± SEM) of IGF-1-stimulated 2-deoxyglucose uptake minus the nonspecific uptake.

2-Deoxyglucose Uptake. All of the mutations studied here were originally discovered in patients with syndromes of extreme insulin resistance. Despite having marked elevations in the level of plasma insulin, the patients did not become hypoglycemic. Thus, they must be resistant to the effects of insulin upon glucose metabolism. Therefore, we attempted to assess the ability of the mutant receptors to mediate 2-deoxyglucose uptake in transfected NIH-3T3 fibroblasts. Insulin dose–response effects were measured in each of the cells lines and plotted as a percentage of basal uptake versus insulin concentration (Figure 6). The range of maximal insulin stimulation was between 1.6 and 2 times the basal uptake for all cell lines. The untransfected cells and the cells expressing wild-type receptors had responses to insulin which were indistinguishable, and low concentrations of insulin (10^{-9} M) did not seem to stimulate the cells at all. Therefore, we inquired whether the small effect of insulin on 2-deoxyglucose uptake in NIH-3T3 fibroblasts might be mediated through the insulin-like growth factor 1 (IGF-1) receptor which would be expected to be present on all the cell lines in equal numbers. To address this issue, we measured the ability of IGF-1 to stimulate 2-deoxyglucose uptake in a dose-dependent manner (Figure 7). The IGF-1 dose–response curves for the untransfected cells and cells expressing the wild-type receptor are virtually identical. In addition, the maximal effect elicited by 10^{-7} M IGF-1 is identical to that for insulin stimulation (approximately 1.8 times basal uptake). Furthermore, a low concentration of IGF-1 (10^{-9} M) stimulated 2-deoxyglucose uptake by 25% in both cell lines whereas insulin at the same concentration had no effect on either cell line.

DISCUSSION

The five missense mutations studied here were discovered in the insulin receptor genes of patients with extreme insulin resistance. Defects in insulin action in these patients are presumably related to their mutations. Properties intrinsic to these mutant receptors (i.e., binding kinetics, autophosphorylation, posttranslational processing, receptor life-cycle) were characterized previously (Kadowaki et al., 1988, 1990a,b,c, 1991; Accili et al., 1989, 1991). In this work, we have studied the ability of these mutant receptors to mediate postbinding events related to some of the actions of insulin.

Untransfected NIH-3T3 fibroblasts express relatively few insulin receptors. In these cells, insulin does not induce protooncogenes such as c-jun (thought to be important for cell growth and differentiation). However, insulin can specifically increase the expression of protooncogenes in transfected fibroblasts overexpressing insulin receptors. This demonstrates the importance of insulin receptors to this signal transduction pathway (Burgering et al., 1991). Similarly, the effect of insulin on thymidine incorporation (used as a measure of mitogenic effect) is small in untransfected fibroblasts while a large effect can be seen in fibroblasts overexpressing normal insulin receptors (Hofmann et al., 1989). Thus, fibroblasts transfected with mutant insulin receptors may be useful for understanding the effects of these mutations on postbinding events (using wild-type transfectants and untransfected cells as positive and negative controls, respectively).

Expression of Insulin Receptors in Transfected Cells. Insulin binding studies are consistent with the idea that the number of receptors on the cell surface of the transfectants is inversely proportional to the severity of the defect previously observed in receptor transport (Kadowaki et al., 1990c, 1991; Accili et al., 1989, 1992). The ratio of transfected human receptors to endogenous murine receptors is ≈200 in the case of the wild-type receptors and ≈10 in the case of the Arg^{209} mutant (which has the most severe defect in receptor transport). Therefore, effects mediated through the insulin receptor in these cells most likely reflect the influence of the transfected receptors rather than the small number of endogenous receptors. This conclusion is supported by the observation that insulin has a negligible effect upon receptor autophosphorylation (Figures 1 and 2), c-jun expression (Figures 3 and 4), and thymidine incorporation (Figure 5) in untransfected NIH-3T3 cells.

Relationship between Receptor Autophosphorylation and Insulin Action. As previously demonstrated, the intrinsic ability of the mutant receptors to be autophosphorylated (when normalized for receptor number and binding affinity) appears normal in all cases (Kadowaki et al., 1990a,c, 1991) except for the Val^{382} mutant which is impaired (Accili et al., 1991).

None of the mutants appeared to have high basal autophosphorylation. If all insulin actions are mediated exclusively through receptor autophosphorylation, then the insulin resistance seen in patients with receptor mutations that exhibit normal autophosphorylation must be explained on some other basis. For example, the binding affinity of the Lys^{15} mutant is 5 times lower than normal. This decreases insulin sensitivity even though maximal autophosphorylation can be achieved with sufficiently high concentrations of insulin. Similarly, the Glu^{460}, Ser^{462}, and Arg^{209} mutations decrease the number of insulin receptors on the surface of the patients' cells either by accelerating receptor degradation (Kadowaki et al., 1990a; McElduff et al., 1984) or by impairing receptor transport (Kadowaki et al., 1991; Accili et al., 1992). This may alter the sensitivity to insulin even though no intrinsic autophosphorylation defect exists.

Alternatively, receptor mutations may affect insulin signal transduction pathways which are independent of autophosphorylation. Although there is much evidence that suggests autophosphorylation is necessary for many of the actions of insulin [for reviews, see Kahn and White (1988), Rosen (1989), and Becker and Roth (1990)], this area remains the subject of intense controversy. For example, there are also reports of insulin-mediated actions which occur normally in the absence of autophosphorylation (Gottschalk, 1991; Wilden et al., 1989; Luttrell et al., 1990; White et al., 1988; Backer et al., 1989; Moller et al., 1991). Furthermore, a quantitative dissociation between epidermal growth factor (EGF) receptor autophosphorylation and protooncogene induction and thymidine incorporation has been reported (Hauguel-de Mouzon et al., 1991). This supports the idea that insulin signal transduction pathways exist which may be partially or completely independent of autophosphorylation.

Our data demonstrating significant defects in insulin-stimulated c-jun expression and thymidine incorporation in cells expressing mutant receptors (despite normal autophosphorylation with four of the mutants) suggest that autophosphorylation is not sufficient to trigger all of the biological actions of insulin. The cell lines expressing the mutant receptors with normal autophosphorylation have a pattern of higher basal c-jun expression and thymidine incorporation, but markedly decreased response to insulin when compared with cells expressing the wild-type receptor. The data demonstrating increased basal activities are consistent with the idea that the extracellular domain of the receptor normally exerts tonic inhibitory control over insulin's actions (Ellis et al., 1987; Shoelson et al., 1988). Mutations in the α-subunit may impair the normal inhibitory influence of the extracellular domain upon receptor signaling so that the mutant receptor exerts an insulin-like effect even in the absence of ligand. Nevertheless, these cells still show a larger response to insulin than the untransfected cells, indicating that the mutant receptors are still able to transduce some signal. The cells expressing the Val^{382} mutant (in which autophosphorylation is impaired) behave instead like the untransfected cells, that is, extremely low basal c-jun expression and thymidine incorporation and almost no response to insulin.

Our results are compatible with at least three different mechanistic schemes (Figure 8). One possibility is that two or more independent insulin signal transduction pathways (one involving receptor autophosphorylation) converge at some point to mediate the mitogenic effect of insulin (Figure 8A). Since the contribution of these pathways may not be equal, disabling one pathway might not impair the mitogenic effect of insulin to the same degree as disabling another (or disabling several pathways simultaneously). A second possibility is that different insulin actions may be mediated by distinct phosphorylated substrates (Figure 8B). For example, a receptor mutation may alter the tyrosine kinase activity of the insulin receptor so that certain substrates involved in mediating the mitogenic effect of insulin may not undergo phosphorylation normally while other substrates may be phosphorylated normally. For example, the Tyr→Phe^{960} mutation has been reported to selectively impair phosphorylation of pp185 (IRS-1) without impairing receptor autophosphorylation (White et al., 1988). Receptor mutations causing a more global defect in phosphorylation (i.e., neither the receptor nor other substrates are phosphorylated normally) might cause an even more severe defect in insulin action. A third possibility is the existence of two or more completely independent pathways (one involving tyrosine phosphorylation) with each pathway mediating distinct insulin actions (Figure 8C). While this possibility has not been completely ruled out by our experiments, we consider such a scheme unlikely with respect to the effect of insulin on mitogenesis. The fact that mutations that abolish receptor autophosphorylation also abolish insulin's effect on thymidine incorporation and protooncogene induction suggests that receptor tyrosine kinase activity is necessary (although probably not sufficient) for the mitogenic activity of insulin (Cama et al., 1992; Stumpo & Blackshear, 1991).

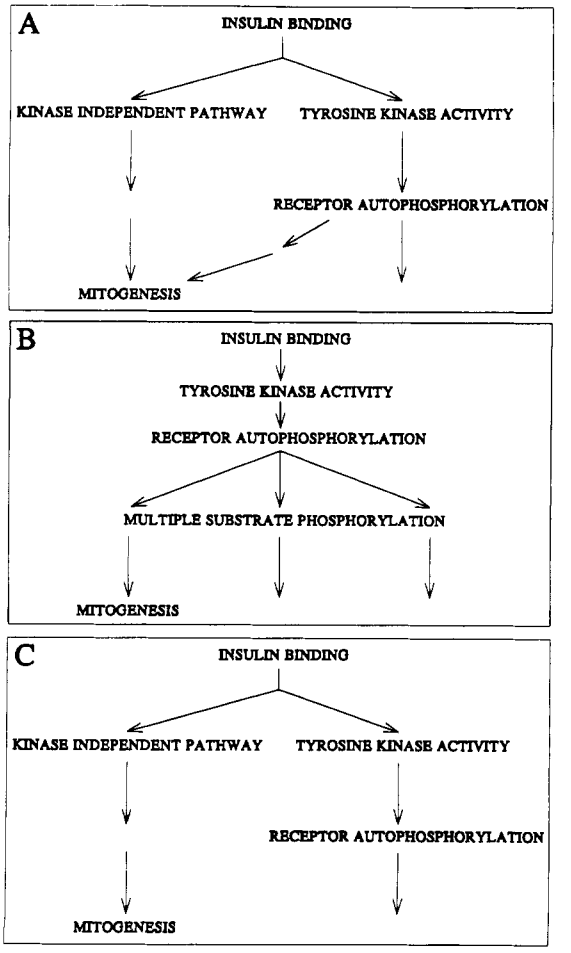


FIGURE 8: Possible insulin signal transduction pathways. (A) Pathways dependent and independent of receptor tyrosine kinase activity converge to mediate c-jun expression and mitogenesis. (B) Receptor tyrosine kinase phosphorylates several substrates, each of which mediates distinct biological actions. (C) Pathways independent of autophosphorylation mediate c-jun expression and mitogenesis while pathways dependent on autophosphorylation mediate other insulin actions.

Effect of Insulin on 2-Deoxyglucose Uptake in Transfected NIH-3T3 Cells. We wanted to examine the ability of mutant receptors to mediate 2-deoxyglucose uptake because patients with these mutations are resistant to the effects of insulin on glucose metabolism. Unfortunately, our experiments did not allow us to draw any conclusions about the effect of mutant insulin receptors on 2-deoxyglucose uptake. The magnitude of the insulin-stimulated response was small (2 times basal at most) in all the cell lines. In addition, untransfected NIH-3T3 cells showed the same insulin response as cells expressing wild-type receptors. Furthermore, both untransfected cells and cells expressing wild-type receptors showed the same magnitude of effect (and higher sensitivity) to IGF-1 stimulation. This suggests that the small effect of insulin on 2-deoxyglucose uptake in transfected NIH-3T3 cells is probably mediated through the IGF-1 receptor. Therefore, NIH-3T3 cells (under our experimental conditions) were not useful for assessing the effect of insulin receptor mutations on this particular measure of insulin action. Furthermore, NIH-3T3 fibroblasts do not contain all of the postreceptor machinery necessary to mediate the responses seen in classical target tissues such as muscle and fat. Indeed, the predominant glucose transporter in fibroblasts is GLUT1 while the predominant insulin-responsive glucose transporter in muscle and fat is GLUT4 (Bell et al., 1990).

Although insulin-stimulated 2-deoxyglucose uptake is often studied in the context of fibroblasts transfected with mutant insulin receptors, the presentation of these data can be confusing. Effects with magnitudes of 1.5–2 times basal are often represented as "percent of maximal effect" when the data shown actually represent the percent of the *difference* between maximal and basal uptake [e.g., see McClain et al. (1987), Maegawa et al. (1988), and Lammers et al. (1989)]. That is, the difference between basal and maximal uptake is scaled to 100%. This is confusing because small effects appear on the graph as large effects. Furthermore, "100%" may represent a small effect of insulin in one cell line but a large effect of insulin in another cell line. Thus, comparison of small unequal effects rescaled in this fashion may be difficult to interpret.

Conclusions. We characterized the effects of five mutations in the human insulin receptor gene on insulin-stimulated c-jun expression and thymidine incorporation. We demonstrated evidence for insulin signal transduction pathways that may be partially independent of the ability of the receptor to autophosphorylate. That is, four of the mutations in the α-subunit of the insulin receptor (Glu^{460}, Lys^{15}, Ser^{462}, and Arg^{209}) appear to impair the ability of insulin to promote mitogenesis even though these receptors autophosphorylate normally. These mutant receptors also mediate higher basal rates of thymidine incorporation and c-jun induction than the wild-type receptor. This increase in basal activity is even more apparent when differences in receptor binding affinity and cell-surface expression are considered. Although some pathways may exist that are partially independent of autophosphorylation, the Val^{382} mutant (which has impaired autophosphorylation) showed the most profound defect in insulin-stimulated c-jun expression and thymidine incorporation.

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