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Tyrosine kinase-deficient mutant human insulin receptors (Met^{1153} → Ile) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4

(insulin action/HA1 epitope/glucose transporters)

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ABSTRACT **Insulin regulates essential pathways for growth, differentiation, and metabolism *in vivo*. We report a physiologically relevant system for dissecting the molecular mechanisms of insulin signal transduction related to glucose transport. This is an extension of our recently reported method for transfection of DNA into rat adipose cells in primary culture. In the present work, cDNA coding for GLUT4 with an epitope tag (HA1) in the first exofacial loop is used as a reporter gene so that GLUT4 translocation can be studied exclusively in transfected cells. Insulin stimulates a 4.3-fold recruitment of transfected epitope-tagged GLUT4 to the cell surface. Cells cotransfected with the reporter gene and the human insulin receptor gene show an increase in cell surface GLUT4 in the basal state (no insulin) to levels comparable to those seen with maximal insulin stimulation of cells transfected with the reporter gene alone. In contrast, cells overexpressing a naturally occurring tyrosine kinase-deficient mutant insulin receptor (Met^{1153} → Ile) show no increase in the basal cell surface GLUT4 and no shift in the insulin dose–response curve relative to cells transfected with the reporter gene alone. These results demonstrate that insulin receptor tyrosine kinase activity is essential in insulin-stimulated glucose transport in adipose cells.**

Mutations in the human insulin receptor gene have been identified in patients with syndromes of extreme insulin resistance (1). Although these patients have clear abnormalities in glucose homeostasis, it is difficult to study the effects of their receptor mutations on the molecular mechanisms of insulin-stimulated glucose metabolism in a physiologically relevant cell type. In transfection studies of mutant insulin receptors, cell lines with very few endogenous insulin receptors (e.g., NIH 3T3 fibroblasts or CHO cells) are often chosen as the recipient cells in which to express recombinant insulin receptors. This is advantageous because it is easier to study the properties of transfected insulin receptors when they are overexpressed in vast excess relative to endogenous receptors. However, these cells lack the cellular machinery necessary to mediate the metabolic effects of insulin seen in classical target tissues (e.g., muscle and adipose tissue). For example, fibroblasts do not express the major insulin-regulatable glucose transporter GLUT4 (2). Furthermore, even if GLUT4 is cotransfected into these cells along with insulin receptors, the cells are much less responsive to insulin than adipose cells (3). Thus, interpretation of data from transfected fibroblast cell lines regarding the mechanisms of insulin-mediated glucose transport is problematic.

Recently, we reported a method for transfecting DNA into rat adipose cells in primary culture (4). We now extend our method to use a GLUT4 reporter gene containing an epitope tag. This reporter gene allows exclusive study of the transfected cells without interference from nontransfected cells. We have cotransfected rat adipose cells with our reporter gene and an expression vector for either normal or mutant insulin receptors. Thus we can examine the functional consequences of mutations in the human insulin receptor on insulin-stimulated glucose transport in a bona fide insulin target tissue. Our results demonstrate that insulin receptor tyrosine kinase activity is essential for insulin-stimulated glucose transport in adipose cells.

MATERIALS AND METHODS

DNA Vector Constructions. *pCIS2.* pCIS2 is an expression vector that contains a cytomegalovirus promoter and enhancer with a generic intron located upstream from the multiple cloning site (obtained from Cornelia Gorman) (5).

pCIS-GLUT4-HA. A *Sal* I fragment containing the cDNA for human GLUT4 [obtained from Graeme Bell (6)] was ligated into the *Xho* I cloning site of pCIS2. This plasmid was digested with *Sau* I and a double-stranded DNA oligonucleotide (made by annealing two single-stranded oligonucleotides) coding for the influenza hemagglutinin epitope (HA1) was ligated in. The sense oligonucleotide was 5′-TGA GAT CGA TTA TCC TTA TGA TGT TCC TGA TTA TGC-3′. The antisense oligonucleotide was 5′-TCA GCA TAA TCA GGA ACA TCA TAA GGA TAA TCG ATC-3′. This codes for a peptide, IDYPYDVPDYAE, which is inserted between amino acids 67 and 68 in the first exofacial loop of GLUT4. The construction was verified by direct sequencing of the regions surrounding and including the oligonucleotide insert.

pCIS-hIR. A *Sal* I fragment containing the cDNA for the human insulin receptor [obtained from Axel Ullrich (7)] was ligated into the *Xho* I cloning site of pCIS2.

pCIS-Ile. A *Sal* I fragment containing the cDNA for the mutant insulin receptor Ile^{1153} (described in ref. 8) was ligated into the *Xho* I cloning site of pCIS2.

Plasmid DNA was obtained by using a Magic Megaprep kit (Promega). The concentration of plasmid DNA was determined by comparison to known DNA markers using ethidium bromide staining of restriction-digested plasmids run on an agarose gel.

Isolated Rat Adipose Cell Preparation. Isolated adipose cells were prepared from the epididymal fat pads of male rats (170–200 g; CD strain; Charles River Breeding Laboratories) by collagenase digestion as described (4, 9).

Electroporation. Electroporation was performed as described (4). The cells were then transferred to 6-cm polysty-

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Abbreviations: HA, hemagglutinin; PM, plasma membrane; LDM, low density microsomal membrane.

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rene tissue culture dishes (10 cuvettes per dish) and incubated at 37°C in 5% CO_2/95% air. Two hours after electroporation, 4.5 ml of Dulbecco's modified Eagle's medium (pH 7.4) [containing 7% albumin, 2 mM glutamine, 200 nM (*R*)-N^6-(1-methyl-2-phenylethyl) adenosine, gentamicin (100 μg/ml), and 25 mM Hepes] was added to each dish. The cells were incubated for 16–24 hr at 37°C in 5% CO_2/95% air prior to further studies.

Adipose Cell Counting. Aliquots of cells were fixed with osmium tetroxide as described (10). The cell number in each aliquot was determined with a Coulter Counter.

Insulin Binding. Insulin binding studies were performed at room temperature (25°C) on cells that had been electroporated and cultured for 16–24 hr as described (4).

Subcellular Fractionation of Adipose Cells and GLUT4 Immunodetection. Plasma membrane (PM) and low density microsomal membrane (LDM) fractions were prepared by differential ultracentrifugation as described (11, 12). PM and LDM proteins were subjected to SDS/PAGE using 10% polyacrylamide resolving gels (Novex, San Diego). The contents of the gels were transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell). Immunological detection of GLUT4 was carried out with a rabbit polyclonal antiserum prepared against a 20-amino acid peptide corresponding to the C-terminal sequence of GLUT4 (kindly provided by Hoffmann–La Roche) in conjunction with ^{125}I-labeled protein A. The immunolabeled bands were visualized by autoradiography and quantitated by PhosphorImager analysis (Molecular Dynamics). The percentage distribution of GLUT4 between PM and LDM was calculated by multiplying the GLUT4 concentrations per mg of protein times the total membrane recoveries (in mg), assuming similar PM and LDM yields from the starting homogenates (12).

Assay for Cell Surface Epitope-Tagged GLUT4. At 16–24 hr after electroporation, adipose cells were transferred to 15-ml polystyrene tubes (Corning no. 25311) and washed with 13 ml of Krebs–Ringer bicarbonate Hepes medium (KRBH) (pH 7.4) at 37°C containing 10 mM $NaHCO_3$, 30 mM Hepes, 200 nM adenosine, and 1% (wt/vol) bovine serum albumin. Cells were then resuspended to a cytocrit of 15% using KRBH with bacitracin at 0.5 mg/ml. Aliquots (2 ml) of the cell suspension were placed in 20-ml polyethylene vials (Wheaton Scientific). Insulin (5 μl) in KRBH was added to each vial to give final insulin concentrations of 0, 0.02, 0.04, 0.12, or 7 nM and the cells were incubated in a water bath at 37°C for 30 min. To prevent redistribution of GLUT4 after insulin stimulation, KCN (final concentration, 2 mM) was added after the insulin incubation and the cells were incubated for an additional 5 min. The cells were then incubated for 1 hr at 24°C with the anti-HA1 mouse monoclonal antibody 12CA5 (1 mg/ml) (Berkeley Antibody, Berkeley, CA) at a final dilution of 1:400. After incubation with 12CA5, the cells were washed twice with 13 ml of KRBH. The cells were resuspended in a final vol of 0.7 ml and transferred to 7-ml polyethylene vials (Apple Scientific, Chesterland, OH). A 200-μl aliquot was removed from each vial for cell counting before the final incubation with ^{125}I-labeled sheep anti-mouse IgG (10 μCi/μg; 1 Ci = 37 GBq) (final dilution, 1:500) (Amersham) for 1 hr at 24°C. Aliquots (300 μl) were then placed in polypropylene microcentrifuge tubes (4 × 45 mm) containing 100 μl of dinonylphthalate oil. The cells were rapidly separated from the medium by centrifugation at 10,000 × *g* for 30 sec. Cell-associated radioactivity was counted in a γ-counter.

Immunofluorescence. Cells were processed exactly as described above except that a fluorescein-conjugated horse anti-mouse IgG antibody (final dilution, 1:1000) (Vector Laboratories) was used instead of the ^{125}I-labeled antibody. Cells were then incubated for 2 min with a Hoechst stain (bisbenzimide; Hoechst no. 33342) before visualization under a fluorescence microscope.

RESULTS

Subcellular Fractionation of Transfected Adipose Cells. Electroporated rat adipose cells were treated with or without insulin (7 nM) after 1 day in culture and membrane fractions were analyzed by immunoblotting with an antibody directed against GLUT4 (Fig. 1). In the basal state, ≈95% of the GLUT4 is in the LDM fraction with ≈5% in the PM fraction. With insulin stimulation, we observe a 4.4-fold increase in PM GLUT4 and a 2.2-fold decrease in the amount of GLUT4 in the LDMs. Thus, adipose cells retain the ability to respond appropriately to insulin even after they have undergone electroporation and incubation *in vitro* for 24 hr.

Detection of Epitope-Tagged GLUT4 on the Cell Surface of Transfected Adipose Cells. Adipose cells transfected with pCIS-GLUT4-HA or pCIS2 were treated acutely with or without insulin (7 nM) after 1 day in culture. The cells were then incubated with the anti-HA1 monoclonal antibody 12CA5. In intact cells, antibody can bind to epitope-tagged GLUT4 only when the transporter is located in the PM since the HA1 epitope is located in an exofacial loop of the GLUT4 reporter gene. As shown in Fig. 2, cells transfected with pCIS2 show low levels of nonspecific antibody binding, which do not change significantly with insulin stimulation. In contrast, cells transfected with pCIS-GLUT4-HA have basal levels of antibody binding ≈57% above the nonspecific levels seen with the pCIS2 transfected cells and show a significant increase in antibody binding with insulin stimulation. When data from seven independent experiments are expressed as percentage of maximal counts for each experiment (Fig. 2), the cells transfected with pCIS2 had mean basal and insulin-stimulated values of 27.2% ± 3.4% and 30.8% ± 3.5%, respectively. Cells transfected with pCIS-GLUT4-HA had mean basal and insulin-stimulated values of 45.6% ± 2.8% and 100%, respectively. When the data are corrected for nonspecific binding, a 4.3-fold increase in cell surface epitope-tagged GLUT4 is observed with insulin stimulation. The basal level of cell surface epitope-tagged GLUT4 is proportional to the amount of DNA transfected (over the range of 2–10 μg of plasmid DNA per cuvette). As the amount of transfected DNA is increased, the basal levels of cell surface epitope-tagged GLUT4 also increase with a concomitant decrease in the -fold stimulation by insulin (data not shown).

When a fluorescein-conjugated secondary antibody is used, the cell surface epitope-tagged GLUT4 can be directly visualized by immunofluorescence (Fig. 3). In the insulin-stimulated state, cells transfected with pCIS-GLUT4-HA display fluorescence at the cell surface. In contrast, little fluorescence is observed in the basal state. Untransfected cells and cells transfected with pCIS2 display extremely low levels of background fluorescence in both the basal and insulin-stimulated states. Using Hoechst stain no. 33342 to visualize the cell nuclei, we estimate the efficiency of our transfection protocol to be ≈5%.

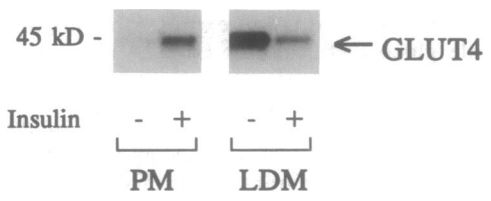

FIG. 1. GLUT4 immunoblot of subcellular membrane fractions from electroporated rat adipose cells. An antibody against the C terminus of GLUT4 was used. After electroporation and incubation for 20 hr, cells were treated with or without insulin (7 nM) for 30 min prior to membrane fractionation. Twenty micrograms of membrane protein was loaded on each lane.

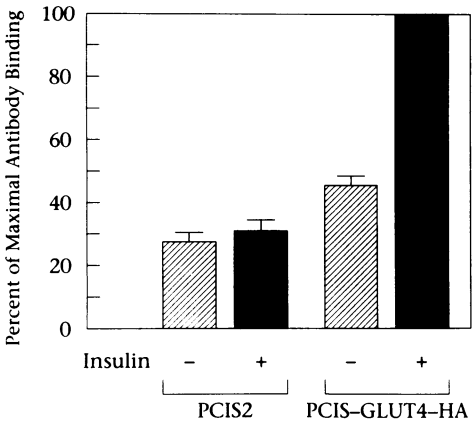

FIG. 2. Cell surface HA1 binding assay for epitope-tagged GLUT4. After electroporation in the presence of 3 μg of plasmid DNA per cuvette (pCIS2 or pCIS-GLUT4-HA), cells were incubated for 20 hr and then treated with or without insulin (7 nM) for 30 min followed by KCN (2 mM) for 5 min. Cells were then incubated for 1 hr with an anti-HA1 mouse monoclonal antibody (12CA5). After washing, cells were incubated with a secondary ^{125}I-labeled sheep anti-mouse IgG antibody for 1 hr. Cell-associated radioactivity was measured in a γ-counter. Results from each experiment were normalized for cell number. Results shown are means ± SEM of seven independent experiments expressed as percentage of maximal counts for each experiment.

Cotransfection of Normal and Mutant Human Insulin Receptors with Epitope-Tagged GLUT4. We cotransfected adipose cells with the pCIS-GLUT4-HA reporter and the expression vector pCIS-hIR, pCIS-Ile, or pCIS2. Insulin binding studies show an increase of ≈80% in cell surface insulin binding sites in cells electroporated with pCIS-hIR or pCIS-Ile when compared with pCIS2 (data not shown). Assuming a 5% transfection efficiency (estimated from immunofluorescence data), this translates to a 16-fold overexpression of human insulin receptors relative to endogenous rat insulin receptors in the cells that are transfected. The increase in insulin binding observed in electroporated cells is proportional to the amount of insulin receptor DNA present (over the range of 2–10 μg of plasmid DNA per cuvette).

The insulin dose–response of cotransfected cells was measured using 12CA5 anti-HA1 antibody in conjunction with a ^{125}I-labeled second antibody (Fig. 4). Cells transfected with pCIS2 alone were used to determine nonspecific binding. When nonspecific binding is subtracted, cells cotransfected with pCIS2 and pCIS-GLUT4-HA show a 4-fold increase in cell surface epitope-tagged GLUT4 in response to maximal insulin stimulation. The half-maximal insulin dose is ≈50 pM. Cells cotransfected with pCIS-hIR and pCIS-GLUT4-HA show an elevation in the basal cell surface epitope-tagged GLUT4 to a level near that seen with maximal insulin stimulation. The magnitude of elevation in basal epitope-tagged GLUT4 is proportional to the amount of pCIS-hIR DNA transfected (data not shown). Cells cotransfected with pCIS-Ile and pCIS-GLUT4-HA have an insulin dose–response similar to that of pCIS2/pCIS-GLUT4-HA transfected cells. Although the Ile^{1153} receptors are unable to signal GLUT4 recruitment, they do not appear to have a dominant negative interaction with the endogenous rat receptors. In contrast to pCIS-hIR, increasing the amount of pCIS-Ile DNA transfected does not affect the basal levels of epitope-tagged GLUT4 (data not shown).

DISCUSSION

Since the seminal discovery of Levine *et al.* (13) that insulin promotes glucose transport *in vivo*, understanding the molecular mechanisms of insulin action has been of major interest. We developed a transfection system to study the molecular mechanisms of insulin-stimulated glucose uptake in a physiologically relevant insulin target tissue. In this report, we study the functional consequences of an insulin receptor mutation on GLUT4 translocation. However, our approach can be extended to study other genes involved in insulin action.

Characterization of Transfected Adipose Cells and Epitope-Tagged GLUT4. One of the distinguishing characteristics of insulin target tissues (e.g., muscle and adipose tissue) is their ability to increase glucose transport dramatically in response to insulin via translocation of GLUT4 to the cell surface. Previously, we showed that electroporated rat adipose cells maintained in primary culture for 1 day remain highly re-

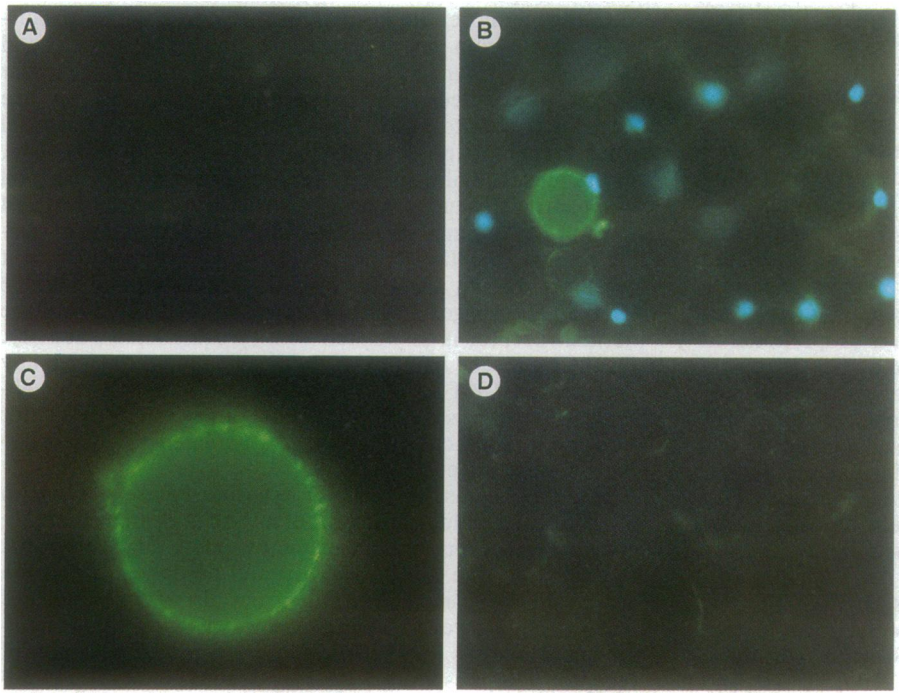

FIG. 3. Immunofluorescence studies of transfected rat adipose cells. Cells transfected with pCIS-GLUT4-HA (6 μg per cuvette) or pCIS2 (6 μg per cuvette) were processed as described in Fig. 2 except that a fluorescein-conjugated horse anti-mouse IgG antibody was used as the secondary antibody. (*A*) View of unstimulated cells transfected with pCIS-GLUT4-HA. (×12.) (*B*) View of insulin-stimulated cells transfected with pCIS-GLUT4-HA. Green immunofluorescence is seen on the cell surface of insulin-stimulated cells expressing epitope-tagged GLUT4. Cell nuclei are visualized in blue using a Hoechst stain in conjunction with UV illumination. (×12.) (*C*) View of an insulin-stimulated cell expressing epitope-tagged GLUT4. (×40.) (*D*) View of insulin-stimulated cells transfected with pCIS2. (×12.)

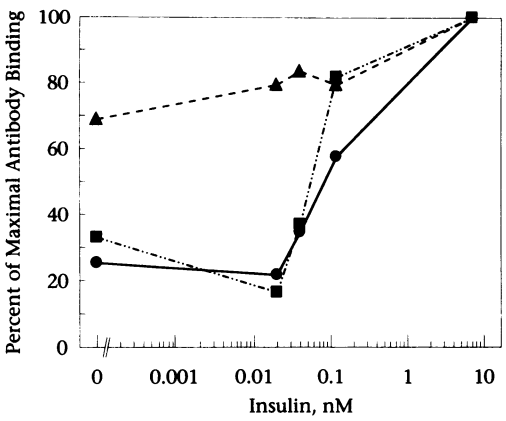

FIG. 4. Insulin dose–response in cotransfected cells (composite of two representative experiments). Cells transfected with pCIS2 (9 μg per cuvette) or cotransfected with pCIS2/pCIS-GLUT4-HA (●) (6 and 3 μg per cuvette), pCIS-hIR/pCIS-GLUT4-HA (▲) (6 and 3 μg per cuvette), and pCIS-Ile/pCIS-GLUT4-HA (■) (6 and 3 μg per cuvette) were processed as described in Fig. 2. In one experiment, insulin doses of 0, 0.02, 0.04, and 7 nM were used while another experiment used insulin doses of 0, 0.04, 0.12, and 7 nM. Mean values are shown for insulin doses of 0, 0.04, and 7 nM. Results obtained in cells transfected with pCIS2 alone represent nonspecific binding (cf. Fig. 2) and were used to calculate specific binding in the other samples. Results are expressed as percentage maximal specific antibody binding. Absolute counts measured with maximal insulin stimulation were similar for all three conditions in which cells were transfected with pCIS-GLUT4-HA: 737, 644, and 810 cpm in cells cotransfected with pCIS-hIR, pCIS-Ile, and pCIS2, respectively.

sponsive to insulin as assessed by 3-*O*-methylglucose transport (4). In this study, we demonstrate that this increase in glucose transport is mediated by the normal mechanisms of GLUT4 translocation. The increase we observe in plasma membrane GLUT4 (4.4-fold) is likely to be an underestimate as has been extensively discussed elsewhere (11, 12, 14).

One advantage of expressing GLUT4 with an epitope tag inserted in an exofacial loop (GLUT4-HA) is that we are able to use a double-antibody binding assay to follow cell surface GLUT4 in transfected adipose cells. GLUT4-HA appears to function identically to endogenous GLUT4 with respect to insulin-stimulated translocation. The 4.3-fold increase in cell surface GLUT4-HA seen with maximal insulin stimulation correlates well with the GLUT4 immunoblot data. Again, this increase is likely to be an underestimate because of the low signal/noise ratio observed in the basal state using either the ^{125}I binding assay or immunofluorescence. In addition, there are several reasons transfected adipose cells might not have as large an insulin response as that reported in freshly isolated adipose cells (14). Electroporation and culturing results in an elevation of basal glucose transport and a decrease in the -fold insulin response when compared with freshly isolated adipose cells (4). Furthermore, overexpression of epitope-tagged GLUT4 in cells containing endogenous GLUT4 results in an increased basal GLUT4, which is proportional to the amount of GLUT4-HA DNA transfected. This is consistent with previous observations that overexpression of GLUT4 in COS-7 cells results in an increase in basal cell surface GLUT4 (15). Therefore, one disadvantage of our transient transfection system is the diminished insulin response in cells expressing the epitope-tagged GLUT4 as compared with freshly isolated adipose cells. Nevertheless, the magnitude of the insulin response is still significantly greater than that seen in other transfected cell systems [e.g., transfected NIH 3T3, CHO, or COS cells (3, 16, 17)].

Others have used GLUT4 with c-myc or HA1 epitope tags placed in the first exofacial loop (3, 15, 18). However, these studies used tissue culture cell lines (e.g., CHO, 3T3-L1, and COS cells), which do not behave like classical insulin target tissues. For example, CHO cells cotransfected with epitope-tagged GLUT4 and human insulin receptors are much less sensitive and responsive to insulin than transfected rat adipose cells (3). Whereas insulin exerts a half-maximal effect on GLUT4 recruitment at a concentration of 50 pM in transfected adipose cells, the half-maximal dose observed in CHO cells expressing GLUT4 is ≈400 pM. Furthermore, dose–response curves for insulin-stimulated recruitment of epitope-tagged GLUT4 in cotransfected CHO cells overexpressing either mutant (Phe^{972}) or normal human insulin receptors are indistinguishable when results are expressed as a percentage of maximal recruitment for each group (reanalysis of data presented in ref. 3). These results underscore the importance of studying insulin-stimulated GLUT4 translocation in a physiologically relevant cell.

Another advantage of our GLUT4-HA construct is the ability to use it as a reporter gene. As the immunofluorescence studies show, expression of GLUT4-HA occurs in only 5% of the adipose cells subject to electroporation. Using GLUT4-HA as a reporter allows one to overcome the potential difficulty of studying such a small population of cells. That is, the transiently transfected cells can be studied exclusively without interference from nontransfected cells.

Coexpression of Insulin Receptors with GLUT4-HA Reporter Gene. In the cotransfection experiments, we used twice as much insulin receptor cDNA as GLUT4-HA cDNA to increase the likelihood that cells transfected with the GLUT4-HA reporter gene would also be transfected with the insulin receptor. The fact that cells cotransfected with the normal human insulin receptor and GLUT4-HA have a strikingly different insulin dose–response curve from cells cotransfected with pCIS2 and GLUT4-HA strongly suggests that coexpression is occurring.

Role of Insulin Receptor Tyrosine Kinase in Glucose Transport. In the absence of insulin, adipose cells overexpressing the normal human insulin receptor have an amount of GLUT4-HA present on the cell surface almost comparable to that of cells cotransfected with pCIS2/GLUT4-HA and stimulated with maximally effective concentrations of insulin. The elevation in basal cell surface GLUT4-HA is proportional to the amount of insulin receptor cDNA transfected. In contrast, adipose cells overexpressing the Ile^{1153} mutant insulin receptor do not have an elevation in the basal GLUT4-HA. Furthermore, the insulin dose–response curve is indistinguishable from that of cells cotransfected with pCIS2 and GLUT4-HA. Changing the amount of mutant insulin receptor cDNA transfected does not alter the basal cell surface GLUT4. Taken together, these results suggest that normal insulin receptors possess intrinsic activity in the basal state. In the presence of a sufficient excess of receptors, the total basal activity induces near-maximal recruitment of GLUT4 even in the absence of insulin. The fact that cells cotransfected with the Ile^{1153} mutant (a tyrosine kinase-deficient receptor) do not have an elevation in basal cell surface GLUT4-HA suggests that the intrinsic basal activity of the normal receptor is associated with tyrosine kinase activity. Furthermore, the inability of the Ile^{1153} mutant to signal any detectable GLUT4-HA recruitment (in the presence or absence of insulin) strongly suggests a direct role for receptor tyrosine kinase activity in insulin-stimulated glucose transport in adipose cells.

The role of insulin receptor tyrosine kinase activity in mediating the biological actions of insulin is an area of intense controversy. Considerable evidence exists suggesting that receptor tyrosine kinase activity and autophosphorylation are necessary to mediate most, if not all, of the actions of insulin (19). However, several reports also describe insulin-mediated actions that occur normally in the absence of receptor autophosphorylation (e.g., see refs. 20–24). In ad-

dition, naturally occurring mutant insulin receptors have been described that impair some of the actions of insulin despite normal receptor autophosphorylation (17). The relationship between receptor tyrosine kinase activity and insulin-stimulated glucose transport has been particularly controversial because it has been difficult to study these signaling pathways by molecular biological techniques in physiologically relevant tissues such as muscle and fat. Previous studies in rat adipose cells using anti-phosphotyrosine antibodies and specific inhibitors of tyrosine kinase activity suggested a role for tyrosine kinase activation in insulin-stimulated glucose transport (25, 26). However, our transfection system for adipose cells has provided the strongest evidence to date for the essential role of the insulin receptor tyrosine kinase in mediating recruitment of GLUT4 in a physiologically relevant target tissue.

The Ile^{1153} insulin receptor is a naturally occurring mutation discovered in an insulin-resistant patient (8). Therefore, it was expected that this mutation would impair insulin-stimulated glucose transport. However, since the patient is heterozygous for the mutant allele, it was hypothesized that the mutation exerted a dominant negative effect. Indeed, when we studied this mutant in transfected NIH 3T3 cells, in addition to being kinase deficient, this mutant appeared to exert a dominant negative effect on the mitogenic and metabolic effects of insulin (16). In the present study, the Ile^{1153} mutant is unable to transduce any signal for GLUT4 recruitment in transfected adipose cells under conditions where we estimate a 16- to 20-fold overexpression of mutant insulin receptors relative to endogenous rat receptors. Therefore, this defective receptor does not interfere with the signaling of the endogenous rat insulin receptors. If the Ile^{1153} receptor were functioning in a dominant negative fashion, a rightward shift in the insulin dose–response curve would be expected. Thus, the Ile^{1153} mutation appears to impair insulin-stimulated GLUT4 translocation in a nondominant fashion. It is possible that species differences might account for the absence of a dominant negative effect of mutant human insulin receptors on endogenous rat receptors. However, we consider this unlikely since others have shown both hybrid formation and dominant negative interactions between rodent and human insulin receptors (27, 28). After transfection, the cells were incubated for 2–3 half-lives of the insulin receptor. However, we do not know the length of the lag time between transfection and initiation of biosynthesis of the recombinant human insulin receptors. If this lag time is long (relative to the incubation time), one might expect a decrease in the number of hybrid receptors formed over the time course of the experiment. As a result, a dominant negative effect may be less apparent under these circumstances.

It is noteworthy that the patient in whom the Ile^{1153} mutation was discovered has had a fluctuating clinical course with respect to her degree of insulin resistance (8). Because the Ile^{1153} mutation has a defect in receptor internalization relative to normal receptors (16), it is possible that the ratio of normal receptors to mutant receptors on the surface of the patient's cells may change depending on factors that promote or inhibit receptor endocytosis (e.g., hyperinsulinemia, obesity, etc.). Thus, if the mutant receptor behaves in a nondominant fashion, the number of normal receptors on the cell surface may impact on the degree of insulin resistance observed in the patient.

In conclusion, we have developed a transfection system that has provided strong evidence for an essential role of insulin receptor tyrosine kinase activity in insulin-stimulated glucose transport. This system will be helpful for elucidating the insulin signal transduction pathways involved in enhancing glucose transport in classical insulin target tissues.

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