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Two Naturally Occurring Insulin Receptor Tyrosine Kinase Domain Mutants Provide Evidence That Phosphoinositide 3-Kinase Activation Alone Is Not Sufficient for the Mediation of Insulin's Metabolic and Mitogenic Effects*

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We have recently reported (1) that two naturally occurring mutants of the insulin receptor tyrosine kinase domain, Arg-1174 → Gln and Pro-1178 → Leu (Gln-1174 and Leu1178, respectively), both found in patients with inherited severe insulin resistance, markedly impaired receptor tyrosine autophosphorylation, with both mutant receptors being unable to mediate the stimulation of glycogen synthesis or mitogenesis by insulin when expressed in Chinese hamster ovary cells. However, these mutations did not fully prevent IRS-1 phosphorylation in response to insulin in these cells, suggesting that IRS-1 alone may not be sufficient to mediate insulin's metabolic and mitogenic effects. In the present study, we have demonstrated that these mutations also impair the ability of the insulin receptor to activate the transcription factor Elk-1 and promote GLUT4 translocation to the plasma membrane. Although at low concentrations of insulin, the mutant receptors were impaired in their ability to stimulate the tyrosine phosphorylation of IRS-1, at higher insulin concentrations we confirmed that the cells expressing the mutant receptors showed significantly increased tyrosine phosphorylation of IRS-1 compared with parental nontransfected cells. In addition, at comparable insulin concentrations, the association of the p85α subunit of phosphoinositide 3-kinase (PI3-kinase) with IRS-1 and the enzymatic activity of IRS-1-associated PI3-kinase were significantly enhanced in cells expressing the mutant receptors. In contrast, no significant stimulation of the tyrosine phosphorylation of Shc, GTP loading of Ras, or mitogen-activated protein kinase phosphorylation was seen in cell lines expressing these mutant receptors. Thus, no activation of any measurable mitogenic or metabolic response was detectable, despite significant insulin-induced phosphorylation of IRS-1 and its association with PI3-kinase in cells stably expressing the mutant insulin receptors. These findings suggest that PI3-kinase activation alone may be insufficient to mediate a wide range of the metabolic and mitogenic effects of insulin. Additionally, the data provide support for the notion that insulin activation of Ras is more closely linked with Shc, and not IRS-1, phosphorylation.

Mutations of the insulin receptor gene have been identified in patients with a wide variety of genetic syndromes of severe insulin resistance (for reviews see Refs. 2 and 3). Studies of the functional properties of these mutant insulin receptors have contributed to the understanding of molecular mechanisms involved in insulin signaling. The insulin receptor is a heterotetrameric receptor tyrosine kinase composed of two extracellular α subunits and two β subunits. Insulin binds to the α subunit of the receptor, an event which activates the tyrosine kinase function of the cytoplasmic domain of the β subunit. This results in autophosphorylation of the β subunit of the receptor itself as well as the phosphorylation of a number of cytoplasmic target proteins that include IRS-1, IRS-2, and Shc (for review see Ref. 4). These tyrosine-phosphorylated molecules are involved in the coupling of the insulin receptor to downstream metabolic and mitogenic events. Thus, the phosphorylation of IRS-1 generates a number of recognition sites for interaction with SH2 domain-containing molecules, including the lipid kinase PI3-kinase, the phosphatase SHP-2, and the adaptor proteins Grb2 and Nck. The phosphorylation of Shc also facilitates its interaction with the SH2 domain of Grb2. Grb2, in tight association with the guanine nucleotide exchange factor Sos, is closely involved with insulin stimulation of Ras activation (5).

A large body of evidence exists to support the hypothesis that the activation of the tyrosine kinase activity of the insulin receptor is an essential first step for most, if not all, of the biological effects of insulin (6, 7). Thus, mutant insulin receptors where the ATP binding site has been altered by site-directed mutagenesis or naturally occurring insulin receptor mutations abolishing receptor tyrosine kinase activity fail to mediate any biological effects of insulin when expressed in transfected cells (8, 9). The importance of IRS-1 tyrosine phosphorylation in the mediation of insulin's downstream effects has also been highlighted by several studies. Insulin receptors in which tyrosine 972 was replaced by phenylalanine were capable of insulin-stimulated autophosphorylation but were markedly impaired in their interaction and phosphorylation of IRS-1 (10–12). Cells expressing this mutant insulin receptor showed severely impaired insulin stimulation of glycogen synthesis, thymidine incorporation, and amino acid uptake, sug-

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gesting that receptor autophosphorylation *per se* might be less relevant to insulin signaling than the ability to phosphorylate downstream substrates (10). Further support for the importance of IRS-1 phosphorylation in mediating the insulin signal comes from studies of a truncated insulin receptor lacking the 82 C-terminal amino acids (Δ82) (13). This receptor displayed severely impaired autophosphorylation, whereas insulin-stimulated tyrosine phosphorylation of IRS-1 was unaffected. Despite this receptor-impaired ligand-induced autophosphorylation, Chinese hamster ovary (CHO)[1] cells expressing this mutant insulin receptor were able to mediate insulin-stimulated thymidine incorporation into DNA as well as insulin-stimulated glucose uptake. Furthermore, in 32D cells, which normally do not contain either the insulin receptor or IRS-1 or -2, insulin-responsive mitogenesis cannot be conferred by transfecting either the insulin receptor or IRS-1 but only by coexpressing both molecules (14). Although the targeted deletion of IRS-1 in mice is not lethal, −/− mice show significant impairment in insulin-stimulated glucose metabolism and reduced size, despite the normal phosphorylation of IRS-2 and Shc (15, 16). These data provide further support for the central importance of IRS-1 phosphorylation in the mediation of insulin action.

We have recently described two insulin receptor mutations, Gln-1174 and Leu-1178, found in patients with inherited forms of severe insulin resistance (1). When stably expressed in CHO cells, these receptors showed severely impaired autophosphorylation and *in vitro* tyrosine kinase activity toward artificial substrates but retained the ability to phosphorylate IRS-1. In contrast to findings with the Δ82 receptor (see above), these receptors were also severely impaired in their ability to mediate both the metabolic (as measured by glycogen synthesis) and mitogenic (as measured by thymidine incorporation) effects of insulin.

We now present further characterization of the signaling properties of these insulin receptors. These new studies provide evidence that insulin-stimulated PI3-kinase activity, although undoubtedly important for insulin action, may not in itself be sufficient to mediate the full metabolic and mitogenic actions of insulin. They also provide supportive evidence that insulin activation of Ras may be more closely related to Shc rather than IRS-1 phosphorylation.

EXPERIMENTAL PROCEDURES

Patient Characteristics—Both mutations were identified in heterozygous form in adolescent females suffering from the Type A syndrome of insulin resistance. Insulin resistance was inherited in a dominant fashion in both families and co-segregated with the mutation. The identification and initial characterization of both mutations has been described previously (1, 17, 18).

Cell Lines—Construction of CHO cell lines expressing Gln-1174 or Leu-1178 insulin receptors has been described previously (1). CHO cells were grown in F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C under an atmosphere of 95% air and 5% CO_2.

Antibodies—Anti-IRS-1 and anti-Shc antibodies (polyclonal) were raised against GST fusion proteins containing IRS-1 amino acids 511–860 and Shc amino acids 366–473, respectively. A monoclonal anti-p85α antibody (U2) was a gift from Dr. Mike Waterfield (University College London, UK). Phospho-specific p44/42 MAP kinase antibody was purchased from New England Biolabs, and anti-Ras antibody Y13-259 was purchased from Oncogene Science. Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (clone 4G10).

Cell Transfections and Reporter Gene Assays—CHO.K1 cells at ~80% confluence in 12-mm dishes were transfected for 2 h with four plasmids: (i) pCMV.hIR, the wild-type IR or mutants thereof, under the control of the nonregulated CMV promoter, (ii) pGL3.G5E4D[Δ]38, the firefly luciferase reporter plasmid, which possesses five GAL4 binding sites upstream of the firefly luciferase gene in the plasmid pGL3 (Promega Corp.), (iii) pRL.CMV, *Renilla* luciferase under the control of the CMV promoter, and (iv) pSG424.Elk1 (83–428), fusion of the GAL4 DNA binding domain and Elk-1 activation domain. Transfections were performed using Tfx-50 (Promega Corp.) transfection reagent (2.2 μg of Tfx-50/μg of DNA in 5% serum-free Ham's F-12). The dishes were further incubated in Ham's F-12 containing 5% fetal calf serum for 4 h, then washed with phosphate-buffered saline and serum-starved for 2 h. The cells were stimulated with 100 nM insulin for 16 h. The cells were then extracted using Stop and Glo^R lysis buffer and assayed sequentially for the firefly and *Renilla* luciferases according to the manufacturer's instructions (Promega Corp.). In brief, 10 μl of crude cell lysate was incubated with 50 μl of luciferin reagent (LARII). After 15 s at room temperature, the luminescence was recorded for 30 s in a Berthold Lumat LB9501 luminometer. 50 μl of Stop and Glo^R reagent was added, and the specific luminescence from the *Renilla* luciferase was recorded for an additional 30 s.

Cell Microinjection and Analysis of GLUT4 Trafficking—CHO.K1 cells on 22-mm-diameter glass coverslips were co-microinjected as described previously (19) with two plasmids; pCMV.hIR (or mutants thereof) at 50 μg/ml and pcDNAneoI possessing a chimera between green fluorescent protein (GFP) fused to the N terminus of GLUT4 at 200 μg/ml. Cells were incubated in growth medium for 16–24 h before serum starvation for 2 h. The cells were subjected to fluorescence analysis in Hepes-buffered Krebs (10 mM Hepes, pH 7.4, 2 mM $NaHCO_3$, 140 mM NaCl, 3.6 mM KCl, 0.6 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose) using a Zeiss Axiovert 100TV microscope with a 40 × oil immersion objective. GFP excitation/emission was achieved with a High Q fluorescein isothiocyanate filter set (Chroma Technology Corp., Brattleboro, VT). Treatment with insulin (100 nM) was for 60 min.

Immunoprecipitation and Western Blotting—Immunoprecipitation and Western blotting was carried out essentially as described previously (1), except blots were probed with ^{125}I-labeled secondary antibodies and proteins were visualized by autoradiography and quantified by phosphoimaging using a Fujix BAS 2000 phosphoimager.

Phosphoinositide 3-Kinase Assay—PI3-kinase activity was assayed using a protocol adapted from Jackson *et al*. (20). Cells were grown until confluency in 3.5-cm (6 well) plates, incubated overnight in serum-free Ham's F-12 media, and then treated with insulin (100 nM) for 5 min. Incubations were terminated by aspirating the medium and rinsing briefly with ice-cold phosphate-buffered saline before the addition of 500 μl of ice-cold freshly prepared PI3-kinase lysis buffer A (20 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 500 μM $NaVO_3$, 1% Nonidet P-40, 10% (w/v) glycerol, 10 μg/ml leupeptin, and 200 μM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation and incubated with anti-IRS-1 antibody (1 in 100 dilution) and 50 μl of protein A-Sepharose (50 mg/ml pre-equilibrated in lysis buffer A) by tumbling end over end at 4 °C for 2 h. The immunoprecipitates were then washed 3 times with lysis buffer A, 2 times in buffer B (500 mM LiCl, 100 mM Tris-HCl, pH 8.0, at 4 °C), once in buffer C (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, at 4 °C), and once in buffer D (20 mM Hepes, 1 mM dithiothreitol, 5 mM $MgCl_2$, pH 7.6, at 4 °C). The beads were then resuspended in 40 μl of buffer E (10 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 30 mM NaCl, 1 mM dithiothreitol, pH 7.2, at 4 °C). 20 μl of phosphatidylinositol/cholate solution (3 mg/ml in 1% (w/v) sodium cholate) was added to each tube, and the reaction was started by the addition of 5 μCi of [γ-^{32}P]ATP in 40 μl of reaction mix (3 μM Na_2ATP, 7.5 mM $MgCl_2$) and incubated at 37 °C for 15 min. Reactions were terminated by the addition of 450 μl of $CHCl_3$:CH_3OH (1:2 v/v). The product was then extracted by the addition of 150 μl of $CHCl_3$ and 150 μl of 0.1 M HCl and then again by the addition of 300 μl of $CHCl_3$ and 300 μl of 0.1 M HCl. Extracted lipid was dried down under vacuum before redissolving in 25 μl of $CHCl_3$, CH_3OH, 0.1 M HCl (200:100:1). Reaction products were separated by thin layer chromatography (run in a pre-equilibrated tank containing methanol:chloroform:ammonia:water, 300:210:45:75) and quantified using a Fujix Bas 2000 phosphoimager.

GTP Loading of Ras—Analysis of the GDP/GTP ratio on Ras was determined as described previously (21). Briefly, cells were grown until confluency in 4-cm dishes, serum-starved overnight, then labeled with [^{32}P]orthophosphate before being stimulated with insulin (100 nM, 5 min). Cells were then lysed in a buffer containing 1% Triton X-114. Lysates were cleared by centrifugation, and the Triton X-114-soluble material was collected by a short temperature shift. The detergent

[1] The abbreviations used are: CHO, Chinese hamster ovary; PI3-kinase, phosphoinositide 3-kinase; CMV, cytomegalovirus; GFP, green fluorescent protein; MAP, mitogen-activated protein; IR, insulin receptor.

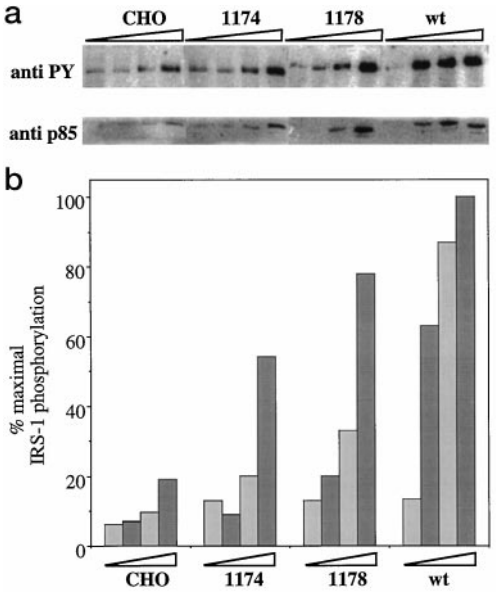

FIG. 1. **CHO cells expressing Gln-1174 and Leu-1178 mutant insulin receptors mediate insulin-stimulated tyrosine phosphorylation of IRS-1 as well as association of p85α with IRS-1.** *a*, cells were grown to confluence, serum starved, and left untreated or treated with insulin (1, 10, or 100 nM) for 2 min. Cells were harvested, and lysates were immunoprecipitated with anti-IRS-1 antibody as described under "Experimental Procedures." After electrophoresis on a 7.5% polyacrylamide gel, proteins were transferred to polyvinylidene difluoride membrane and probed with anti-phosphotyrosine (anti *PY*) (region of blot containing IRS-1) or anti-p85α antibodies, as indicated. *b*, graph showing quantitative analysis of IRS-1 phosphorylation shown in *a*. *wt*, wild type.

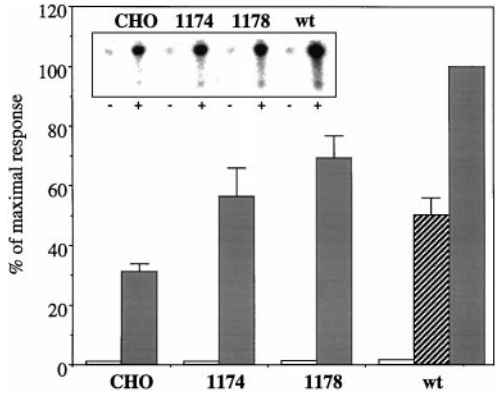

FIG. 2. **CHO cells expressing Gln-1174 and Leu-1178 mutant insulin receptors mediate insulin stimulation of PI3-kinase activity.** Cells were grown to confluence, serum starved, and left untreated (*open bars*) or treated with 100 nM insulin (*shaded bars*) or 1 nM insulin (*hatched bar*) for 5 min. Cells were harvested, lysates were immunoprecipitated with anti-IRS-1 antibody, and immune complexes were assayed for their ability to phosphorylate phosphatidylinositol as described under "Experimental Procedures." Reaction products were resolved by thin layer chromatography, and results were quantitated on a phosphoimager. Results shown are standardized to the maximum response of insulin (in cells expressing wild-type (*wt*) insulin receptors). The data are the mean + S.E. from three independent experiments, each performed in duplicate. The *inset* is taken from a representative experiment showing stimulation at 100 nM for each cell line.

phase was then used to immunoprecipitate Ras. Guanine nucleotides were eluted from the immune complex and resolved by thin layer chromatography. The plates were visualized by autoradiography and quantitated using a Fujix Bas 2000 phosphoimager.

RESULTS

We have previously reported that the mutant insulin receptors Gln-1174 or Leu-1178 are unable to autophosphorylate and that CHO cells stably expressing these receptors are severely defective in insulin-stimulated glycogen synthesis and thymidine incorporation (1). Unexpectedly, significant insulin-stimulated IRS-1 phosphorylation was seen in both cell lines expressing the mutant insulin receptors using anti-phosphotyrosine blotting of total cell lysates and anti-IRS-1 immunoprecipitates (1). These observations suggested to us that IRS-1 phosphorylation *per se* may be insufficient to mediate downstream insulin signaling events. This possibility was further explored by more detailed characterization of the signaling properties of the CHO cell lines stably expressing similar numbers of wild-type, Gln-1174, or Leu-1178 insulin receptors termed wild-type, Gln-1174, or Leu-1178 cell lines, respectively.

To more precisely define the dose response characteristics of IRS-1 tyrosine phosphorylation, all cell lines were treated with various concentrations of insulin for 2 min, and IRS-1 immunoprecipitates were analyzed by anti-phosphotyrosine blotting (Fig. 1). At 100 nM insulin, the Gln-1174 and Leu-1178 cell lines show significant enhancement of insulin-stimulated tyrosine phosphorylation of IRS-1 compared with parental nontransfected CHO cells, although this was somewhat reduced compared with the wild-type cell line. The levels of IRS-1 phosphorylation seen in the Gln-1174 and Leu-1178 cell lines stimulated with 100 nM insulin and the wild-type cell line stimulated with 1 nM insulin were comparable. Thus, while we confirmed the ability of the mutant receptors to promote insulin-stimulated tyrosine phosphorylation of IRS-1, these mutant receptors did so less well than wild-type receptors.

In parallel immunoblotting studies, insulin ability to promote the association of the p85α subunit of PI3-kinase with IRS-1 was examined. Consistent with the studies of IRS-1 phosphorylation, association of p85α with IRS-1 in response to insulin was significantly enhanced in the Gln-1174 and Leu-1178 cell lines compared with parental CHO cells (Fig. 1). Again, similar levels of IRS-1-associated p85α were seen when the Gln-1174 and Leu-1178 cell lines were treated with 100 nM insulin *versus* 1 nM insulin treatment of the wild-type cell line.

To examine whether the insulin-stimulated association of p85α seen in cells expressing the mutant receptors could result in the functional activation of PI3-kinase, IRS-1-associated PI3-kinase activity was measured in all cell lines. Parental CHO cells and Gln-1174 and Leu-1178 cells were incubated with 100 nM insulin for 5 min, whereas wild-type cells were incubated with either 1 or 100 nM insulin for 5 min. PI3-kinase activity in IRS-1 immunoprecipitates from wild-type cells (stimulated with either 1 or 100 nM insulin) or Gln-1174 and Leu-1178 cells showed a stimulation that was significantly greater ($p < 0.03$) than that seen in parental CHO cells (Fig. 2). Also, PI3-kinase activity was comparable in wild-type cells stimulated with 1 nM insulin and Gln-1174 and Leu-1178 cells stimulated with 100 nM insulin.

Thus the Gln-1174 and Leu-1178 mutant receptors, despite showing undetectable autophosphorylation, are capable of phosphorylating IRS-1 such that it can interact with and activate PI3-kinase. Of note, using the identical cell lines, we have previously reported that exposure of the wild-type cell line to 1 nM insulin results in a marked enhancement of glycogen synthesis (~60% maximal), whereas no significant enhancement of glycogen synthesis was seen in the Gln-1174 and Leu-1178 cell lines when studied at concentrations of 100 nM insulin or higher (1). There is therefore a clear discordance between the abilities of these mutant receptors to activate IRS-1-associated PI3-kinase activity and to stimulate glycogen synthesis.

To provide additional confirmation that metabolic signaling through the mutant receptors was markedly impaired, we monitored the translocation of GLUT4 to the plasma membrane in

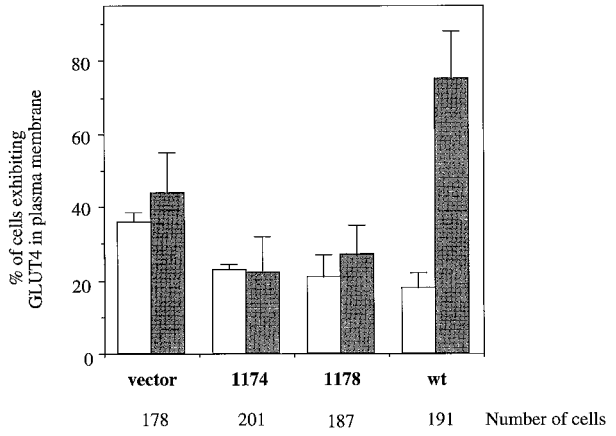

FIG. 3. **CHO cells expressing Gln-1174 and Leu-1178 mutant insulin receptors fail to mediate insulin-stimulated GLUT4 translocation to the plasma membrane.** CHO.K1 cells were microinjected with pGFP.GLUT4 and either empty vector (vector) or vector encoding wild-type insulin receptor cDNA (*wt*), Gln-1174, or Leu-1178 mutant insulin receptors, as indicated. After 16 h, the cells were serum-starved for 2 h. The fraction of cells exhibiting plasma membrane-associated GFP-GLUT4 was scored by fluorescence microscopy (*open bars*). The cells were then treated with insulin (100 nM) for 1 h, and the fraction of cells exhibiting plasma membrane-associated GFP-GLUT4 re-scored (*shaded bars*). The data are mean + S.E. from three independent experiments. The total number of cells imaged for each condition is indicated *below* the *bars*.

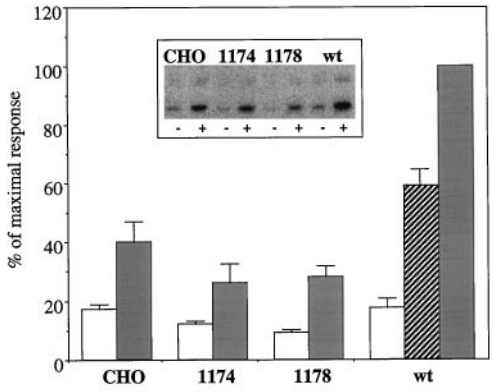

FIG. 4. **CHO cells expressing Gln-1174 and Leu-1178 mutant insulin receptors fail to mediate insulin stimulation of MAP kinase phosphorylation.** Cells were grown to confluence, serum starved, and left untreated (*open bars*) or treated with 100 nM insulin (*shaded bars*) or 1 nM insulin (*hatched bar*) for 5 min. Cells were harvested, and protein lysates electrophoresed on 7.5% polyacrylamide gels followed by transfer to polyvinylidene difluoride membrane. Membranes were probed using phospho-specific p44/42 MAP kinase antibody as described under "Experimental Procedures." Results shown are standardized to the maximum response of insulin (in cells expressing wild-type (*wt*) insulin receptors). The data are the mean + S.E. from three independent experiments, each performed in duplicate. The *inset* is taken from a representative experiment showing stimulation at 100 nM for each cell line.

CHO cells by taking advantage of the recently described fluorescent chimera between GLUT4 and GFP (19). In CHO.T cells that stably overexpress wild-type insulin receptors, we have previously demonstrated that this chimera translocates to the cell surface in response to insulin in approximately 60% of cells (19). We confirmed this observation in CHO.K1 cells using co-microinjection to transiently overexpress wild-type insulin receptors and GFP-GLUT4 (Fig. 3). Cells injected with GFP-GLUT4 and control vector mediated no significant increase in GFP-GLUT4 translocation in response to insulin. In addition, neither the Gln-1174 or Leu-1178 mutant insulin receptors mediated insulin-stimulated GFP-GLUT4 translocation. These results provide additional confirmation that metabolic signaling is severely impaired in the Gln-1174- and Leu-1178-expressing cells. However, as we did not specifically compare wild-type and mutant receptors at insulin concentrations producing comparable degrees of PI3-kinase activation, we cannot use these data to draw any direct conclusions regarding the role of PI3-kinase in the mediation of this particular process.

With respect to the mitogenic effects of insulin, we have previously demonstrated that the Gln-1174 and Leu-1178 mutant receptors are unable to mediate insulin stimulation of thymidine incorporation into DNA or to activate the enzymatic activity of MAP kinase (1). To examine events relating to insulin's mitogenic actions in more detail under conditions where cells expressing mutant and wild-type receptors demonstrated comparable levels of IRS-1 phosphorylation and PI3-kinase activation, the characteristics of MAP kinase phosphorylation were examined in all cell lines. Whereas cells overexpressing wild-type insulin receptors showed a 3-fold stimulation of MAP kinase tyrosine phosphorylation in response to 1 nM insulin and an approximate 5-fold stimulation following 100 nM insulin treatment, no such phosphorylation was seen in the Gln-1174 and Leu-1178 cell lines (Fig. 4). To provide independent confirmation of the inability of these mutant receptors to activate downstream effects related to mitogenesis, we examined the ability of these mutant receptors to activate the ternary complex factor Elk-1. This factor forms a complex with serum response factor at the serum response element within the c-*fos* promoter and is responsible for the observed insulin effect on c-*fos* induction in CHO cells.[2] The activation of this transcription factor is closely associated with the proliferative response of cells to growth stimuli (23). A GAL4-Elk-1 fusion protein was expressed in CHO.K1 cells by transient transfection. The activity of this chimeric transcription factor was monitored with a co-transfected luciferase reporter plasmid, where the luciferase gene is placed under the control of a promoter possessing five GAL4 binding sites. A third plasmid, pRL.CMV, possessing the *Renilla retinformis* luciferase was included in these transfections to monitor cell transfection efficiency. Finally, a fourth plasmid was included to overexpress wild-type, Gln-1174, or Leu-1178 mutant insulin receptors. As shown in Fig. 5, parental CHO.K1 cells exhibited little or no stimulation of Elk-1 in response to insulin, as determined by luciferase activity. When the wild-type insulin receptor was overexpressed, insulin induced an approximate 2-fold increase in luciferase activity after incubation with 1 nM insulin and an approximate 5-fold increase in luciferase activity after incubation with 100 nM insulin. In contrast, neither mutant insulin receptor was capable of activating Elk-1 (Fig. 5).

Thus, at concentrations of insulin that produced comparable levels of IRS-1 phosphorylation and PI3-kinase activation in wild-type- *versus* mutant receptor-expressing cell lines, CHO cells expressing the mutant receptors were markedly impaired in their ability either to phosphorylate MAP kinase or to activate transcription through Elk-1.

To explore whether the capacity of these mutant receptors to phosphorylate IRS-1 was preserved for other substrates of the receptor kinase, the ability of these receptors to phosphorylate Shc was investigated. Cell lines were incubated with 100 nM insulin for 5 min, and Shc immunoprecipitates were analyzed by anti-phosphotyrosine blotting (Fig. 6). Only wild-type cells exhibited insulin-stimulated tyrosine phosphorylation of Shc. Stimulation of wild-type cells with 1 nM insulin invoked an approximate 2-fold increase in Shc phosphorylation and Shc-

[2] M. R. Griffiths, E. J. Black, M. Dickens, P. E. Shaw, D. A. F. Gillespie, and J. M. Tavaré, submitted for publication.

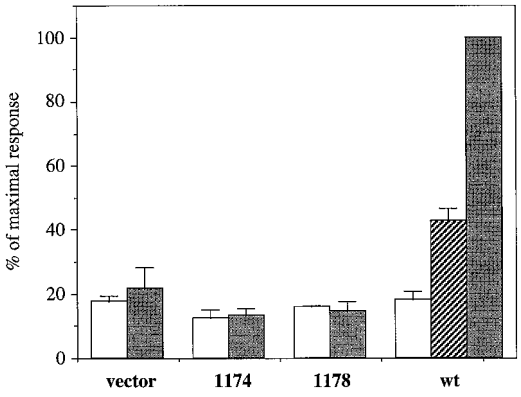

FIG. 5. **CHO cells expressing Gln-1174 and Leu-1178 mutant insulin receptors fail to mediate insulin stimulation of a GAL4-Elk1 transcription factor.** CHO.K1 cells were transiently transfected with pRL.CMV, pSG424.Elk1(83–428), and pGL3.G5E4D[Δ]38. Also included was a fourth plasmid, either empty vector (vector), vector encoding wild-type insulin receptor cDNA (*wt*), or vector encoding either Gln-1174 or Leu-1178 mutant insulin receptors, as indicated. Transfected cells were then treated in the absence (*open bars*) or presence of 100 nM insulin (*shaded bars*) or 1 nM insulin (*hatched bar*). After 16 h, the cells were harvested, and lysates were assayed for firefly and *Renilla* luciferases as described under "Experimental Procedures." Results shown are the ratio of the firefly luciferase/*Renilla* luciferase activities (*i.e.* corrected for transfection efficiency) and are standardized to the maximal response of insulin (in cells expressing wild-type insulin receptors). The data are mean + S.E. from two independent experiments, each performed in duplicate.

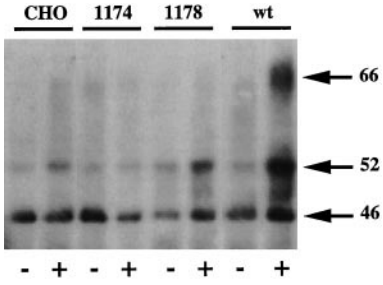

FIG. 6. **CHO cells expressing Gln-1174 or Leu-1178 mutant insulin receptors are unable to mediate insulin-stimulated tyrosine phosphorylation of Shc.** Cells were grown to confluence, serum starved, and untreated or treated with insulin (100 nM) for 5 min as indicated. Cells were harvested, and lysates were immunoprecipitated with anti-Shc antibody as described under "Experimental Procedures." After nonreducing electrophoresis on a 7.5% polyacrylamide gel, proteins were transferred to polyvinylidene difluoride membrane and probed with anti-phosphotyrosine antibodies. *wt*, wild type.

associated Grb2 and an approximate 6-fold increase in Shc phosphorylation and Grb2 association with Shc after 100 nM insulin treatment (data not shown).

At 100 nM insulin, these mutant insulin receptors show a clear discordance in their ability to phosphorylate two downstream targets of the insulin receptor, *i.e.* IRS-1 and Shc. As the relative roles of IRS-1 and Shc in the mediation of insulin activation of Ras has been an area of some debate (21, 24, 25), we utilized the divergent signaling to IRS-1 *versus* Shc seen in the Gln-1174 and Leu-1178 cell lines to explore this issue further. Cells were labeled with [^{32}P]orthophosphate and stimulated with 100 nM insulin, and GTP/GDP association with Ras was determined in Ras immunoprecipitates (Fig. 7). Wild-type cells show a significant insulin-stimulated increase in the GTP loading of Ras. In contrast, no such increase was seen in Gln-1174 and Leu-1178 cells. As, at this concentration of insulin, the mutant receptors phosphorylate IRS-1 but not Shc, these findings provide support for the notion that insulin signaling to GTP loading of Ras is more closely associated with Shc, rather than IRS-1, phosphorylation.

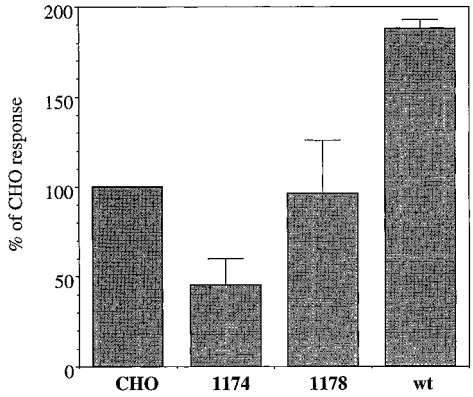

FIG. 7. **CHO cells expressing Gln-1174 or Leu-1178 mutant insulin receptors are unable to mediate insulin-stimulated GTP loading of Ras.** Cells were grown to confluence, serum-starved, labeled with [^{32}P]orthophosphate, and untreated or treated with insulin (100 nM) for 5 min. Cells were harvested, lysates were immunoprecipitated with anti-Ras antibody, and immune complexes were assayed for GDP and GTP content as described under "Experimental Procedures." Products were resolved by thin layer chromatography, and results were quantitated on a phosphoimager. Data shown are standardized against the response seen in parental CHO cells and represent the mean + S.E. from three independent experiments. *wt*, wild type.

DISCUSSION

Two mutant insulin receptors, Gln-1174 and Leu-1178, identified in patients suffering from the inherited Type A syndrome of severe insulin resistance, showed unusual signaling properties when expressed and studied in CHO cells. We have previously reported that despite having severely impaired autophosphorylation and *in vitro* tyrosine kinase activity (1), these receptors are capable of mediating significant insulin-stimulated tyrosine phosphorylation of IRS-1. We have extended our initial observations by characterizing the insulin dose-responsiveness of IRS-1 phosphorylation in cells stably expressing these mutant receptors. Although at 100 nM insulin, the mutant insulin receptors promoted tyrosine phosphorylation of IRS-1 to an extent that is between 60–80% that seen by wild-type receptors, at lower concentrations of insulin the response is more markedly impaired. Thus, even at the very elevated plasma insulin concentrations seen in the patients harboring these mutations, the mutant receptors might be expected to be impaired in their ability to mediate insulin-stimulated IRS-1 phosphorylation *in vivo*. This impairment is likely to be a significant contributor to the insulin resistance seen in the patients.

However, the finding of a dissociation between receptor autophosphorylation and IRS-1 phosphorylation in stably transfected CHO cells expressing the mutant receptors provided the opportunity to dissect insulin post-receptor signaling mechanisms. We have demonstrated significant insulin-stimulated tyrosine phosphorylation of IRS-1 in response to 100 nM insulin in cells expressing the mutant receptors, with the degree of IRS-1 phosphorylation being equivalent to that seen in cells expressing wild-type receptors after stimulation with 1–10 nM insulin. Previously (1), we have shown that stimulation of cells expressing wild-type receptors with 1–10 nM insulin is sufficient to stimulate 60–95% of maximal glycogen synthesis and thymidine incorporation, indicating that such levels of IRS-1 phosphorylation are capable of mediating downstream effects. However, despite comparable IRS-1 phosphorylation after treatment of the Gln-1174 and Leu-1178 cell lines with 100 nM insulin, glycogen synthesis and thymidine incorporation were not significantly stimulated. One possibility for this was that the phosphorylation of IRS-1 by the mutant receptors may be qualitatively abnormal. For example, as anti-phosphotyrosine

blotting detects the sum of all phosphotyrosines, it is conceivable that the mutant receptors do not phosphorylate IRS-1 on the same residues as wild-type receptors. As PI3-kinase is the SH2 domain-containing protein interacting with IRS-1 that has been most closely linked with metabolically relevant insulin signaling, we have concentrated on the examination of this interaction. Insulin (100 nM) did indeed stimulate the association of the p85α subunit of PI3-kinase with IRS-1 in the cells expressing the mutant receptors, and the IRS-1-associated holoenzyme had functional activity, findings that support the notion that functionally important insulin-stimulated tyrosine phophorylation of IRS-1 was occurring in cell lines expressing the mutant receptors. Indeed, the levels of IRS-1-associated PI3-kinase activity were at least equivalent in the Gln-1174 and Leu-1178 cell lines compared with the wild-type receptor-expressing cell line after 100 and 1 nM insulin stimulation, respectively.

We attempted to obtain further insights into the specific pathways leading to downstream metabolic effects of insulin by studying GLUT4 translocation. CHO cells do not contain the specialized transporter GLUT4. This problem was circumvented by microinjecting CHO cells with vectors expressing the wild-type or mutant receptors along with an expression vector encoding a GFP-GLUT4 chimera. We previously demonstrated that insulin induces the translocation of this chimera to the plasma membrane in CHO cells stably expressing the insulin receptor (19), an effect that is completely blocked by wortmannin, suggesting the central importance of PI3-kinase in this event.[3] When transiently expressed in CHO cells, only the wild-type and not the Gln-1174 or Leu-1178 mutant insulin receptors promoted GFP-GLUT4 translocation to the plasma membrane, although at the same insulin concentration a significant increase in IRS-1-associated p85α and PI3-kinase activity was seen in these cells. This may be explained by one or several of the following. The process may be critically dependent on the amount and/or rate of PI3-kinase activation; other events may be involved in the targeting of IRS-1 to the GLUT4-containing vesicle; in addition to PI3-kinase, other signaling pathways may be required to mediate insulin-stimulated GLUT4 translocation, which is consistent with several recent reports suggesting that activation of PI3-kinase *per se* may not be sufficient to mediate GLUT4 translocation. Thus, selective activation of PI3-kinase in 3T3-L1 adipocytes by a thiophosphotyrosine peptide resulting in activation of PI3-kinase to similar levels as those achieved by insulin only increased glucose transport by 20% as compared with insulin-stimulated levels (26). Although transfection studies using constitutively active PI3-kinase in rat adipocytes resulted in cell surface levels of GLUT4 similar to those obtained after insulin treatment (27), adenovirus-mediated expression of constitutively active PI3-kinase in 3T3-L1 adipocytes resulted in activation of PI3-kinase to levels that exceeded the effect of insulin yet produced only a partial stimulation of glucose transport (28). Other ligands such as platelet-derived growth factor, which are able to potently activate PI3-kinase, do not promote glucose uptake in 3T3-L1 cells (29, 30). The subcellular localization of active PI3-kinase is important for its actions on glucose transport (30, 31). Thus, structural features of tyrosine-phosphorylated IRS-1 may be involved in the targeting of IRS-1 and active PI3-kinase to specific cellular compartments. In this regard, however, it is of interest that Isakoff *et al.* (32) recently reported that IL4 was unable to stimulate glucose uptake despite its ability to stimulate IRS-1 phosphorylation and enhance IRS-1-associated PI3-kinase activity in L6 myoblasts transfected with the IL4 receptor. These observations, in combination with those reported here, suggest the possibility that insulin-stimulated association of PI3-kinase with IRS-1 may not be sufficient for insulin actions on metabolic events such as glucose transporter translocation.

To further characterize events involved in insulin's mitogenic actions under conditions where cells expressing the mutant and wild-type receptors demonstrated comparable IRS-1 phosphorylation and PI3-kinase activation, we examined the stimulation of MAP kinase phosphorylation and Elk-1 activation. Unlike the wild-type receptor expressing cells that stimulated mitogen-activated protein kinase phosphorylation and Elk-1 activation, both the Gln-1174 and Leu-1178 cell lines were unable to mediate such insulin-stimulated responses to any extent. These findings suggest that insulin-stimulated association of PI3-kinase with IRS-1 may not be sufficient for insulin's actions on mitogenic events, including MAP kinase and Elk-1 activation.

There are at least three substrates of the insulin receptor tyrosine kinase that may act as intermediates between the receptor and downstream events, namely IRS-1, IRS-2, and Shc. To date, the relative roles of these and other less well-characterized proteins in the mediation of the full repertoire of insulin signaling has not been firmly established. Since these mutant receptors, despite being incapable of autophosphorylation, showed the unexpected property of being able to phosphorylate IRS-1, we also investigated whether they were able to phosphorylate Shc. At concentrations of insulin where clear evidence for IRS-1 phosphorylation was seen, no phosphorylation of Shc was evident in cells expressing the mutant insulin receptors. This dissociation between the ability of the mutant receptors to signal to IRS-1 and Shc provided the opportunity to use these cells as tools with which to attempt to dissect the relative roles of IRS-1 and Shc in insulin signaling to Ras, which is an area of some controversy (21, 24, 25). Insulin promotes the GTP loading of Ras through the guanine nucleotide exchange activity of Grb2·Sos. The insulin-stimulated tyrosine phosphorylation of either IRS-1 or Shc, or indeed both, and their subsequent interaction with Grb2·Sos could be involved in mediation of this effect. Our observation that cells expressing mutant insulin receptors that selectively phosphorylate IRS-1 and not Shc show no significant insulin stimulation of the GTP loading of Ras suggest that this activity may be more closely related to Shc, rather than IRS-1, phosphorylation. In previous investigations, mutant insulin receptors in which two of the three major tyrosine phosphorylation sites had been replaced by phenylalanine showed a marked impairment of IRS-1 phosphorylation but signaled normally to insulin-stimulated Shc phosphorylation and also activated Ras (21). Also, in Rat1 fibroblasts, the time course of insulin-stimulated Grb2 association with Shc paralleled the time course of Shc phosphorylation and Ras-GTP formation, with much more of the cellular Grb2 associated with Shc than IRS-1 (25). These data suggest that the main route from the insulin receptor to Ras is through Shc rather than IRS-1. However, the Δ82 insulin receptor mutant, which showed impaired phosphorylation of Shc, was still able to mediate about 80% of the GTP loading of Ras that was seen in wild-type cells (24). Whereas it remains possible that the relative roles of Shc and IRS-1 in Ras activation may be cell type- specific, this does not explain the differences between our observations and Ouwens *et al.* (21) compared with those of Yonezawa *et al.* (24), as all were made in CHO cells overexpressing mutant insulin receptors.

At a concentration of insulin at which IRS-1 phosphorylation is significantly enhanced, no phosphorylation of Shc, GTP loading of Ras, phosphorylation of MAP kinase, or activation of

[3] S. Dobson and J. M. Tavaré, unpublished observations.

Elk-1 is seen. In combination with our previous observations regarding the severe impairment of insulin-stimulated mitogenesis in these cells, these findings lend support to the notion that Shc, rather than IRS-1, may be more centrally involved in the growth factor-like actions of insulin. However, as it is unlikely that all of the existing insulin receptor substrates have, as yet, been identified and fully characterized, it is possible that molecules such as Grb10 and Grb10/IR-SV1 (33, 34) play central roles in insulin signaling to metabolic and mitogenic events in parallel with IRS-1 and Shc. Indeed, recent evidence suggests that IRS-1 may not be essential for insulin-dependent translocation of GLUT4 in 3T3-L1 cells (22).

In summary, we have studied the signaling properties of two naturally occurring insulin receptor mutations, Gln-1174 and Leu-1178. These mutant receptors display unusual signaling properties that have permitted some insights into the complexity of post-receptor insulin signaling. These studies have revealed that 1) insulin-stimulated IRS-1 phosphorylation can occur in the absence of detectable receptor autophosphorylation, 2) severe defects in a wide range of insulin-stimulated metabolic and mitogenic events can occur despite substantial phosphorylation of IRS-1 and activation of IRS-1 associated PI3-kinase, and 3) insulin stimulation of IRS-1-associated PI3-kinase activity *per se* may not be sufficient to promote GLUT4 translocation to the plasma membrane. Our data are also consistent with the view that Elk-1-mediated activation of the c-*fos* promoter in response to insulin requires the activation of MAP kinase and provide support for the suggestion that the major route involved in insulin activation of Ras is likely to be through Shc rather than IRS-1.

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Two Naturally Occurring Insulin Receptor Tyrosine Kinase Domain Mutants Provide Evidence That Phosphoinositide 3-Kinase Activation Alone Is Not Sufficient for the Mediation of Insulin's Metabolic and Mitogenic Effects

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