Two Naturally Occurring Mutant Insulin Receptors Phosphorylate Insulin Receptor Substrate-1 (IRS-1) but Fail to Mediate the Biological Effects of Insulin

EVIDENCE THAT IRS-1 PHOSPHORYLATION IS NOT SUFFICIENT FOR NORMAL INSULIN ACTION*

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Two naturally occurring mutant insulin receptors, Arg-1174 → Gln and Leu-1178 → Pro, found in patients with dominantly inherited Type A insulin resistance, showed unusual signaling properties when stably expressed in Chinese hamster ovary (CHO) cells. Both mutant receptors were expressed on the cell surface and bound insulin normally, but showed markedly impaired autophosphorylation in response to insulin. In addition, the in vitro tyrosine kinase activity of both mutant receptors toward an artificial substrate was also severely impaired. Despite these defects of kinase activity, anti-phosphotyrosine immunoblotting of whole cell lysates and anti-phosphotyrosine immunoprecipitation of 32P-labeled cells showed insulin-stimulated tyrosine phosphorylation of a protein of ~185 kDa to an extent comparable to that seen in CHO cells expressing wild-type human insulin receptors. Anti-insulin receptor substrate-1 (IRS-1) immunoprecipitation followed by anti-phosphotyrosine immunoblotting confirmed that this tyrosine-phosphorylated protein was IRS-1. In contrast, CHO cells expressing an insulin receptor mutated at the ATP binding site (Lys-1030 → Arg) showed no insulin-stimulated autophosphorylation or phosphorylation of IRS-1. Despite exhibiting apparently normal insulin stimulation of IRS-1 tyrosine-phosphorylation, cells expressing the Arg-1174 → Gln or Pro-1178 → Leu receptors showed marked impairment in insulin stimulation of glycogen synthesis, thymidine incorporation, and activation of MAP kinase. The inability of these mutant receptors to signal normally to metabolic and mitogenic responses suggests that insulin-stimulated tyrosine phosphorylation of IRS-1 alone is insufficient to fully mediate insulin action.

The insulin receptor is a heterotetrameric transmembrane tyrosine kinase (Ullrich et al., 1985). Binding of ligand to the extracellular α subunit activates the tyrosine kinase activity of the receptor, the initial substrate of which appears to be the receptor itself (Ullrich and Schlessinger, 1990). Three tyrosine residues in the tyrosine kinase domain of the receptor (1158, 1162, and 1163) are phosphorylated in trans by the reciprocal half-receptor (Lammers et al., 1990). Phosphorylation of all three tyrosines appears to be necessary to allow full activation of the receptor’s tyrosine kinase activity toward other substrates and to allow normal signaling to downstream effectors (Murukami and Rosen, 1991). Unlike many other receptor tyrosine kinases, the phosphorylated tyrosine residues on the receptor itself do not appear to act primarily as docking sites for Src homology 2 domain-containing signal transduction proteins. The insulin and insulin-like growth factor-1 receptors phosphorylate one or more substrate proteins with a molecular mass of approximately 185 kDa. Further characterization of the ~185-kDa substrate(s) resulted in the cloning of insulin receptor substrate-1 (IRS-1)1 (Sun et al., 1991). IRS-1 contains multiple tyrosine residues in YMMX or YXXM motifs suitable for interaction with Src homology 2 domain-containing proteins (Sun et al., 1993; Shoelson et al., 1992). Several specific signaling molecules that interact with tyrosine-phosphorylated IRS-1 have been identified (Backer et al., 1993; Skolnik et al., 1993; Lee et al., 1993; Xiao et al., 1994; Yamauchi et al., 1995). The precise function of each of these signal transduction molecules and their relationship to the spectrum of biological responses to insulin is currently the focus of major attention. Evidence is rapidly emerging to indicate that IRS-1 is not the only protein directly phosphorylated by the insulin receptor tyrosine kinase (Lavan and Lienhard, 1993; Pronk et al., 1993; Tober et al., 1995; Sun et al., 1995; Patti et al. 1995). The establishment of the relative importance of IRS-1 vis à vis other molecular targets to the mediation of insulin’s effects on metabolic and mitogenic responses will be of considerable importance to the fuller understanding of insulin signaling.

Naturally occurring mutations in the insulin receptor have provided considerable insights into the residues and domains of the receptor that are important for function (Taylor et al., 1992). Study of the functional properties of mutations occurring in association with human disease has the advantage that there is often good a priori evidence that altered function caused by the mutation produces a physiologically significant defect in insulin signaling in the whole organism. To date, most studies of both naturally occurring and artificial mutant insu-

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1 The abbreviations used are: IRS-1, insulin receptor substrate-1; MAP, microtubule-associated protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; MBP, myelin basic protein; BSA, bovine serum albumin; PI, phosphatidylinositol.

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insulin receptors have confirmed the important role for IRS-1 phosphorylation in the mediation of insulin signaling. In general, mutant receptors that have impaired autophosphorylation also fail to phosphorylate IRS-1 and show impaired insulin-mediated signaling to downstream metabolic and mitogenic events (Moller et al., 1990; Taylor et al., 1992; Cama et al., 1992). Additional evidence for the importance of IRS-1 phosphorylation for insulin signaling comes from studies of artificial insulin receptor mutants, which show a dissociation between receptor autophosphorylation and IRS-1 phosphorylation (White et al., 1988; Backer et al., 1992; Yamamoto-Honda et al., 1993). Studies of these mutant receptors have supported the concept that the phosphorylation of IRS-1, rather than that of the insulin receptor itself, correlates with insulin’s activation of downstream effects.

We report on the unique functional properties of two naturally occurring mutants in a region of the insulin receptor tyrosine kinase domain distal to the major sites of tyrosine autophosphorylation. These mutations are the cause of dominantly inherited resistance to insulin-mediated glucose disposal in vivo. Both mutations result in the abolition of insulin-stimulated receptor autophosphorylation but retention of insulin-stimulated IRS-1 tyrosine-phosphorylation. Despite mediating apparently normal IRS-1 phosphorylation, neither mutant insulin receptor was capable of stimulating metabolic and mitogenic events. These results lend support to the notion that IRS-1-independent signaling events are necessary for the mediation of the downstream effects of insulin.

### Experimental Procedures

**Site-directed Mutagenesis of the Human Insulin Receptor**—A 4.4-kilobase pair full-length human insulin receptor cDNA (gift of Jonathan White, University of New York, Stonybrook) was subcloned into the pRC.CMV expression vector (Invitrogen) to create pRC.CMV.HIR. A 2.43-kilobase pair cDNA fragment, encoding the β-subunit of the insulin receptor, was then excised from pRC.CMV.HIR using HindIII and BamHI. This fragment was cloned into M13mp18. Mutagenesis was carried out using the Bio-Rad Mutagenex kit, with the primer TGGCATTACACTGACGGG (for the 1174 mutation) or CAGGAGCTCATGCCATCCA (for the 1178 mutation). After mutagenesis, EcoRI M109 were transformed with double-stranded DNA, and several plagues were picked and sequenced around the area of the mutation to confirm the presence of the mutation. The mutated insulin receptor fragment cDNA was then excised with HindIII and BamHI and religated back into the expression vector pRC.CMV.HIR. Religated cloned DNA was then used to transform DH5α, a a rabbit reticulo cytoplasmic system (Promega) to verify that protein products of the appropriate size were synthesized.

**Cell Lines**—CHO cells were grown in F-12 medium supplemented with 10% fetal bovine serum, penicillin (75 μg/ml), streptomycin (50 μg/ml) at 37°C under an atmosphere of 95% air and 5% CO2. For transfection, CHO cells (approximately 30% confluent) in four-well tissue culture plates were incubated at 37°C in OptiMEM medium (Life Technologies, Inc.) with transfection mixture composed of 2 μg of plasmid/DWell and 10 μl of Lipofectin (Life Technologies, Inc.). After 5 h, the medium was changed to F-12 medium supplemented with 10% fetal bovine serum and cells were left for 48 h. Subsequently, the medium was replaced with Geneticin (600 μg/ml) to select neomycin-resistant cells. After 10 days, surviving CHO cells were harvested and subcultured in the presence of Geneticin (600 μg/ml). Single clones were isolated from this cell line by limiting dilution, and cells expressing high levels of human insulin receptors were selected by insulin binding as described previously (Moller et al., 1990; CHO cells expressing human insulin receptors isolated at this site (Lys-1030 → Arg) were generously provided by William J. Rutter (University of California, San Francisco, CA) (Etina et al., 1987).

**Antibodies**—Anti-insulin receptor antibodies were purchased from ICN (PY20) and Upstate Biotechnology Inc. (4G10).

**Insulin Binding**—Transfected CHO cells were grown to confluence in 24-well dishes, washed twice with ice-cold phosphate-buffered saline (PBS) and once with insulin binding buffer (100 mM Hepes, pH 7.4, 10 mM glucose, 1% BSA, 120 mM NaCl, 1 mM EDTA, 15 mM sodium acetate, and 1.2 mM MgSO4). Cells were then incubated in insulin binding buffer at 4°C for 1 h with insulin as indicated, washed with ice-cold PBS, lysed in 500 μl of ice-cold lysis buffer (20 mM Tris, pH 6.8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 500 μM NaVO3, 1% Nonidet P-40, 10% glycerol, and 10 μg/ml leupeptin), and clarified by centrifugation. Supernatants were incubated for 4 h at 4°C with 5 μl of either anti-insulin receptor antibody Ros-1, anti-INS-1 antibody CT, or anti-Erk 2 antibody plus 40 μl of a 50% slurry of protein A-agarose. For anti-phosphotyrosine immunoprecipitation, the monoclonal PY20 antibody was used. Immune complexes were washed four times in ice-cold lysis buffer and once in ice-cold 100 mM Tris, pH 6.8. The beads were resuspended in Laemmli sample buffer (20 μl) containing 100 mM dithiothreitol and boiled for 5 min.

**Western Blotting**—Western immunoprecipitation of Proteins—Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (7.5% slab gels) and transferred by electrophoretic transfer to Immobilon membranes (Millipore Corp.). Membranes were blocked for 2 h in PBS containing 3% BSA and 0.1% Tween 20 (buffer A). Blots were probed for 90 min with either anti-phosphotyrosine antibody 4G10 (1:5000 dilution) in buffer A or with the anti-insulin receptor antibody CT-3 (1:2000 dilution) in PBS containing 1% nonfat milk and 0.1% Tween 20 (buffer B). Blots were washed six times with PBS containing 0.1% Tween 20 and then incubated with a secondary peroxidase-conjugated goat anti-mouse antibody (Dako Ltd.; 1:10,000 dilution) in buffer B. After 1 h, blots were washed as above and proteins were visualized using enhanced chemiluminescence (ECL, Amersham Corp.).

**In Vivo [32P]Phosphate Labeling**—[32P]Phosphosphate-labeled CHO cells were harvested directly in Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 5 min. After sonication and centrifugation at 15,000 × g for 10 min, aliquots of supernatants were resolved by 7.5% SDS-polyacrylamide-gel electrophoresis and Western blot analysis was carried out as described above.

In Vivo [32P]Phosphate Labeling—CHO cells were washed twice with phosphate-free Krebs-Ringer Bicarbonate buffer (20 mM Hepes, pH 7.6, 107 mM NaCl, 5 mM KCl, 3 mM CaCl2, 1 mM MgSO4, 7 mM NaHCO3, 10 mM glucose, and 0.1% BSA) and incubated with 5 ml/mouse Krebb with 1 μCi of carrier-free [32P]orthophosphate (Amersham) for 3 h at 37°C. Insulin was then added to 100 nM final concentration as indicated and incubated for 10 min at 37°C. Cells were then harvested in 400 μl lysis of buffer, and immunoprecipitated with anti-insulin receptor antibody as described above. Immunoprecipitates were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis, and labeled proteins were identified by autoradiography.

In Vivo Tyrosine Kinase Activity—CHO cells were seeded to confluence in 6-cm (four-well) dishes, serum-starved for 16 h, and treated with insulin as indicated, before being harvested into 300 μl of lysis buffer as described above. Lysates were incubated on ice (15 min) and then cleared by centrifugation (15,000 × g, 10 min at 4°C). Insulin receptors were captured by overnight incubation of clarified lysates with either anti-phosphotyrosine antibody 4G10 (1:5000 dilution) in buffer A or with the anti-insulin receptor antibody CT-3 (1:2000 dilution) in PBS containing 1% nonfat milk and 0.1% Tween 20 (buffer B). Blots were washed six times with PBS containing 0.1% Tween 20 and then incubated with a secondary peroxidase-conjugated goat antibody (Dako Ltd.; 1:10,000 dilution) in buffer B. After 1 h, blots were washed as above and proteins were visualized using enhanced chemiluminescence (ECL, Amersham Corp.).

**Glycogen Synthesis**—Transfected CHO cells were grown to confluence in 24-well dishes, serum-starved for 16 h, and treated with insulin as indicated, washed twice with 0.75 mM phosphoric acid, and incorporated [32P]ATP. After 15 min at room temperature, the reaction was stopped by the addition of 5 μl/well of 2 M HCl. [32P]Incorporation into the peptide substrate was measured by spinning the reaction mixture through phosphocellulose columns (Spinzyme™ Pierce). Columns were washed twice with 0.75 mM phosphoric acid, and incorporated [32P]countered in a scintillation counter.
with ice-cold PBS. Insulin was added at the indicated concentrations (10⁻¹⁵ to 10⁻⁶ M) for 1 h, followed by the addition of 1 μCi of [³²P]thymidine/well (Amersham). Plates were placed on ice and washed three times with ice-cold PBS. Insulin was added at the indicated concentrations (10⁻¹⁰ to 10⁻⁶ M) for 16 h, with 1 μCi of [³²P]thymidine/well. CHO cells were then further incubated at 37°C for 90 min. The plates were placed on ice and washed three times with phosphate-buffered saline, and cells were solubilized in 0.03% SDS. 1.5 volumes of chloroform were added, and acid-precipitable radioactivity was then measured.

Thyroid Cell Transfection—Transfected CHO cells were grown to confluence in 24-well dishes, serum-starved for 16 h, and washed two times with ice-cold PBS. Insulin was added at the indicated concentrations (10⁻¹⁰ to 10⁻⁶ M) for 16 h, with 1 μCi of [³²P]thymidine/well. CHO cells were then further incubated at 37°C for 90 min. The plates were placed on ice and washed three times with phosphate-buffered saline, and cells were solubilized in 0.03% SDS. 1.5 volumes of trichloroacetic acid (20%), w/v, was added, and acid-precipitable radioactivity was then determined.

MAP Kinase Activity—MAP kinase immunoprecipitates collected on protein A-Sepharose beads were washed twice in lysis buffer and twice in kinase assay buffer (20 mM Heps, pH 7.4, 0.1 mM NaCl, 5 mM MnCl₂, 5 mM MgCl₂) before being resuspended in 35 μl of kinase assay buffer containing 50 μM ATP, 5 μM of myelin basic protein (MBP) (1.5 mg/ml), and 10 μCi of [³²P]ATP. After 30 min at 30°C with gentle shaking, the reaction was terminated by spinning through a phosphocellulose column (Spinzyme™, Pierce). The columns were washed twice with 0.75 mM phosphoric acid, and [³²P]MBP was counted in a scintillation counter.

RESULTS

Patient Characteristics

The identification of the Arg-1174 → Gln mutation and the Pro-1178 → Leu mutation have been described previously (Moller et al., 1994; Krook et al., 1994). Both mutations were identified in heterozygous form in adolescent females suffering from the Type A syndrome of insulin resistance. In both families insulin resistance was inherited in an autosomal dominant fashion. Both mutations have been described in additional independent kindreds, also in association with Type A insulin resistance (Kim et al. 1993; Moritz et al. 1994).

Studies of Mutant Receptors Expressed in Chinese Hamster Ovary Cells

Insulin Binding—Following transfection and selection with G418, multiple clones with similarly high levels of tracer insulin binding were selected for further study. The displacement of insulin by unlabeled insulin was similar in cells expressing wild-type, Arg-1174 → Gln, and Pro-1178 → Leu receptors. Thus, the mutant insulin receptors were expressed on the cell surface and bound insulin with normal affinity relative to wild-type insulin receptors (data not shown).

In Vitro Insulin Receptor Tyrosine Kinase Activity—The in vitro tyrosine kinase activity of both mutant and wild-type insulin receptors was measured using antibody capture of insulin receptors from insulin-stimulated cells, followed by assaying the kinase activity of the bound insulin receptor toward an artificial peptide substrate. Insulin receptors mutated at the ATP binding site (Arg-1030 → Lys) were studied in parallel. This mutant receptor has previously been shown to have severely impaired insulin stimulated kinase activity (Ebinia et al. 1987). The Arg-1174 → Gln and Pro-1178 → Leu mutated receptors showed markedly impaired tyrosine kinase activity compared to wild-type receptors (Fig. 1). The degree of impairment observed was similar to that seen with the Arg-1030 → Lys insulin receptors.

Tyrosine Phosphorylation of the Insulin Receptor β Subunit and pp185

In order to examine whether the severe defect seen in insulin-stimulated in vitro tyrosine kinase activity was also present in whole cells, cells expressing similar numbers of wild-type, Arg-1174 → Gln, or Pro-1178 → Leu mutant receptors were treated with 100 nM insulin for 2 min, harvested, and immunoprecipitated using an anti-phosphotyrosine antibody. Subsequent anti-insulin receptor immunoblotting of the anti-phosphotyrosine immunoprecipitates revealed that Arg-1174 → Gln and Pro-1178 → Leu receptors failed to undergo insulin-stimulated β-subunit autophosphorylation (Fig. 2A). Defective autophosphorylation of both mutants was confirmed in anti-insulin receptor immunoprecipitates of cells labeled in vivo with [³²P]orthophosphate (Fig. 2B). Anti-phosphotyrosine immunoblotting of total cell lysates also confirmed the absence of normal receptor autophosphorylation (Fig. 3). Surprisingly, however, anti-phosphotyrosine immunoblotting of whole cell lysates obtained from cells expressing the Arg-1174 → Gln and Pro-1178 → Leu mutant receptors revealed insulin-stimulated tyrosine phosphorylation of a protein of approximately 185 kDa. The 185-kDa phosphoprotein appeared to be similar in intensity to that seen in cells expressing wild-type receptor, but it was not seen in the mock-transfected CHO cells. Similarly, anti-phosphotyrosine immunoprecipitation of cells labeled in vivo with [³²P]orthophosphate and stimulated with 1 μM insulin for 5 min again showed absent autophosphorylation of the insulin receptor β-subunit but preservation of tyrosine phosphorylation of pp185 (data not shown).

Tyrosine Phosphorylation of IRS-1 in CHO Cells

To confirm that the ~185-kDa tyrosine-phosphorylated protein seen in Fig. 3 corresponded to (or contained) IRS-1, cells were stimulated with insulin for 2 min, solubilized, and immunoprecipitated with an anti-IRS-1 antibody, separated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 4, this demonstrated that insulin-stimulated tyrosine-phosphorylation of IRS-1 was similar in cells expressing wild-type, Arg-1174 → Gln, and Pro-1178 → Leu receptors. To confirm that this was a specific feature of Arg-1174 → Gln and Pro-1178 → Leu mutant receptors, and not a general property of insulin receptor tyrosine kinase domain mutations, cells overexpressing similar numbers of Lys-1030 → Arg mutated insulin receptors were also studied. In these cells, stimulation of IRS-1 phosphorylation was not in-
creased above the low background seen in mock-transfected CHO cells.

Insulin-stimulated Glycogen Synthesis and Thymidine Incorporation into DNA

Given the discordance between the severely defective receptor autophosphorylation (and in vitro tyrosine kinase activity) and the preservation of insulin-stimulated phosphorylation of IRS-1, experiments were undertaken to determine whether signaling to downstream insulin responses was impaired in cells expressing the Arg-1174 → Gln or Pro-1178 → Leu mutant insulin receptors. As shown in Fig. 5, overexpression of wild-type insulin receptors, as expected, resulted in significantly enhanced insulin sensitivity with respect to [14C]glucose incorporation into glycogen and [3H]thymidine incorporation into DNA. Cells expressing the Arg-1174 → Gln or Pro-1178 → Leu mutant receptors showed marked impairment in both these responses.

At lower (10–100 nM) insulin concentrations, the stimulation of glycogen synthesis through the Arg-1174 → Gln or Pro-1178 → Leu receptors was impaired relative to that seen in mock-transfected CHO cells, suggesting that the mutant receptors were interfering with signaling through endogenous CHO insulin receptors. This dominant-negative effect is consistent with the dominant inheritance of insulin resistance seen in the families with these mutations. Similarly, insulin-stimulated thymidine incorporation was also markedly defective in cells expressing the Arg-1174 → Gln or Pro-1178 → Leu mutant receptors (Fig. 5B). Cells expressing wild-type receptors achieved 65% of maximal response at 1 nM insulin concentration, whereas at that insulin concentration there was no measurable response in the cells expressing either of the mutant receptors. In contrast to insulin-stimulated glycogen synthesis, there was no evidence of dominant-negative interference with signaling through the endogenous CHO insulin receptors with respect to insulin-stimulated thymidine incorporation into DNA.

Insulin-stimulated MAP Kinase Activity

Insulin stimulation of MAP kinase is thought to be important in the mediation of insulin’s biological effects, particularly on mitogenesis (Marshall, 1994). Cells were treated with 10 nM insulin for 10 min, solubilized, and immunoprecipitated with anti-MAP kinase antibody. MAP kinase activity toward myelin basic protein (MBP) was measured in the immunoprecipitates. The insulin-stimulated phosphorylation of MBP by anti-MAP kinase immune complexes derived from insulin-stimulated cells expressing the Arg-1174 → Gln or Pro-1178 → Leu mutants was severely impaired compared to that seen in cells expressing the wild-type receptor (Fig. 6). This result, indicating that the pathway from the insulin receptor to MAP kinase was not activated, correlates with the impaired mitogenic re-
Thus, despite a normal capacity for IRS-1 phosphorylation, Arg-1174 Gln and Pro-1178 Leu mutant receptors fail to mediate normal insulin signaling to glycogen synthesis and mitogenesis. A deletion mutant lacking 12 amino acids in the juxta-membrane region of the receptor similarly displayed normal insulin-stimulated autophosphorylation in CHO cells (expressing wild-type human insulin receptor, Arg-1174→Gln, Pro-1178→Leu, or Arg-1030→Lys mutant receptors that were either untreated or treated with 10 nM insulin for 10 min. The assay was performed as described under "Materials and Methods." The results, from three independent experiments, are presented as the percentage of the insulin-stimulated activity seen in mock-transfected cells (means ± S.E.)

The lack of insulin signaling to downstream effectors in the face of normal IRS-1 tyrosine phosphorylation is in marked contrast to several previous studies of mutant insulin receptors, which have suggested that an insulin receptor's ability to signal to downstream biological effects correlates well with the tyrosine phosphorylation of IRS-1 (Cama et al., 1992; Moller et al., 1990). For example, White et al. demonstrated that a Tyr-972→Phe mutant receptor was capable of normal autophosphorylation but did not phosphorylate IRS-1 (White et al., 1988). Intact cells expressing this receptor show marked impairment of insulin-stimulated glycogen synthesis, thymidine incorporation (White et al., 1988), and phosphatidylinositol (PI) 3-kinase activation (Kapeller et al., 1991). A deletion mutant lacking 12 amino acids in the juxta-membrane region of the receptor may be important in allowing access of IRS-1 to the catalytic domain of the receptor tyrosine kinase, a concept that is further supported by experiments using the yeast two-hybrid system, which demonstrate that residues within this region directly interact with IRS-1 (Gustafson et al., 1995). Conversely, a mutant receptor lacking the 82 C-terminal amino acids did not show any detectable autophosphorylation upon insulin stimulation but tyrosine phosphorylation of IRS-1 was normal (Yamamoto-Honda et al., 1993). CHO cells expressing the Δ82 receptors show normal insulin stimulation of thymidine incorporation and PI 3-kinase activation compared to cells expressing wild-type human insulin receptors. Thus, previous studies of mutant insulin receptors suggest a key role for IRS-1 phosphorylation in the mediation of the biological effects of insulin. Despite exhibiting a pattern of behavior in terms of receptor autophosphorylation versus IRS-1 phosphorylation comparable to that seen with the Δ82 mutant, both the Arg-1174→Gln and Pro-1178→Leu mutant receptors differed markedly from the Δ82 receptor in their failure to mediate insulin-stimulated mitogenic and metabolic effects.

It is possible that we did not detect a more subtle defect in IRS-1 phosphorylation. Thus time points beyond 5 min were not studied, and it is conceivable that mutant receptors might be capable of initiating phosphorylation of IRS-1 yet be defec-
tive in maintaining further rounds of tyrosine phosphorylation. It is also possible that qualitative aspects of IRS-1 phosphorylation, perhaps related to the specific sites of tyrosine phosphorylation, may be defective in cells expressing the mutant receptors. Notwithstanding these caveats, it is of interest that despite apparently normal IRS-1 tyrosine phosphorylation at physiologically relevant concentrations of insulin, downstream signaling remained significantly impaired.

The data obtained from study of both the Arg-1174 → Gln and Pro-1178 → Leu mutant receptors suggests that IRS-1 tyrosine phosphorylation may not be sufficient for full activation of insulin signaling. This is consistent with a growing body of evidence indicating the existence of other signal transduction pathways from the insulin receptor to downstream events. Recent evidence suggests that the main route of insulin-stimulated GTP loading of p21as occurs through the direct phosphorylation by the insulin receptor of Shc (Sasaoka et al., 1994; Yamauchi and Pessin, 1994; Pronk et al., 1994). Thus, insulin receptors in which two of the three major tyrosine autophosphorylation sites have been replaced by phenylalanine show a substantial more severe defect in insulin action than those seen in the IRS-1 knockout experiments (Accili et al., 1995), highlighting the importance of other, non-IRS-1-mediated pathways from the insulin receptor.

Thus, although IRS-1 is apparently required for the full activation of insulin-stimulated biological events, an emerging body of evidence supports the hypothesis that IRS-1 phosphorylation alone is not sufficient for normal insulin signaling. Our findings with the Arg-1174 → Gln and Pro-1178 → Leu insulin receptor mutants indicate that additional signals, other than that of IRS-1 phosphorylation, must be activated to permit normal insulin signaling to downstream events.

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