Replacement of the Conserved Tyrosine 1210 by Phenylalanine in the Insulin Receptor Affects Insulin-Induced Dephosphorylation of Focal Adhesion Kinase but Leaves Other Responses Intact†

Gerard C. M. Van der Zon, D. Margriet Ouwens, Jannette Dorrestijn, and J. Antonie Maassen*

Department of Medical Biochemistry, Sylvius Laboratory, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

Received February 13, 1996; Revised Manuscript Received May 22, 1996⊗

ABSTRACT: The families of tyrosine and serine/threonine kinases exhibit shared clusters of conserved amino acid residues. Some conserved residues are confined to the family of tyrosine kinases (TKs), like Tyr at position 1210 in the insulin receptor. Nearly all TKs have at this position Tyr, whereas Ser/Thr kinases generally have Phe at this site. The three-dimensional structure of the insulin receptor TK domain shows Tyr1210 to be located in the cleft, below bound ATP, in a region which potentially contributes to substrate binding. We have examined whether this specific Tyr residue contributes to the generation of TK-specific responses, such as Tyr phosphorylation of Shc, activation of Ras and Erk1,2, and stimulation of DNA synthesis. In addition, we have examined the contribution of Tyr1210 to insulin receptor-specific responses as Tyr phosphorylation of IRS1, stimulation of glycogen synthesis, and dephosphorylation of focal adhesion kinase (FAK). Wild-type and a mutant insulin receptor, in which Tyr1210 was replaced by Phe, were stably expressed in CHO cells, and clones expressing similar numbers of insulin receptors were selected. It was found that replacement of Tyr1210 by Phe resulted in a receptor which was nearly inactive in inducing dephosphorylation of FAK. The mutant receptor was able to induce RasGTP formation, glycogen synthesis, and activation of phosphatidylinositol 3-kinase, though the magnitude of stimulation of some responses was decreased. These findings indicate that Tyr1210 is not essential for the induction of tyrosine kinase-specific responses, such as activation of the Shc/Ras/Erk1,2 pathway and mitogenicity. On the other hand, the abrogation of insulin-induced FAK dephosphorylation indicates that Tyr1210 is involved in coupling of the activated receptor to some downstream targets. Thus, Tyr1210 may fine tune the signal generated by the activated insulin receptor.

The insulin receptor is a member of the tyrosine kinase superfamily (Ullrich et al., 1985; Ebina et al., 1985; Hanks et al., 1988). Members of this family are generally involved in the induction of cell proliferation and differentiation via a signaling pathway involving Shc phosphorylation, RasGTP formation, and activation of the kinase cascade involving Erk1,2 (Yarden & Ullrich, 1988; Pazin & Williams, 1992). A characteristic of the insulin receptor is the stimulation of additional cellular responses as Tyr phosphorylation of IRS1, which is involved in activation of phosphatidylinositol 3-kinase (PI3kinase), stimulation of glucose uptake, and glycogen synthesis (Myers & White, 1993). Another recently identified insulin response is the dephosphorylation of focal adhesion kinase (FAK) by activating a phosphotyrosine phosphatase (Pillay et al., 1995; Ouwens et al., 1995). Mutations in the insulin receptor are associated with a variety of insulin resistance syndromes (Taylor et al., 1992).

The three-dimensional structure of the cytoplasmic domain of the insulin receptor has recently been resolved and shows a strong similarity with the structure of protein kinase A, a member of the family of Ser/Thr kinases (Hubbard et al., 1994; Knighton et al., 1991). Both structures show two lobes with a single connection between them. The ATP binding site is located within the cleft between the lobes. In the case of protein kinase A (PKA), an inhibitory peptide which prevents interaction of PKA with substrates binds to the surface of the lower lobe at a region in the vicinity of Phe238 (Knighton et al., 1991b). This residue corresponds to Tyr1210 in the insulin receptor. The overall folding of the insulin receptor and PKA are quite similar in this region, and it is feasible that the region around position 1210 in the insulin receptor is involved in substrate recognition. Nearly all Tyr kinases have conserved a Tyr at the position corresponding to 1210 in the insulin receptor, whereas most Ser/Thr kinases have other amino acids at this site, frequently a Phe (Hanks et al., 1988).

Receptor tyrosine kinases activate various postreceptor processes, leading to a mitogenic response (Marshall, 1995). PKA and other Ser/Thr kinases mostly are involved in nonmitogenic responses and phosphorylate a number of substrates on Ser/Thr residues in particular consensus sequences (Kemp & Pearson, 1990). It is plausible that the conserved Tyr residue in tyrosine kinases, corresponding to Tyr1210 in the insulin receptor, contributes to the selection of specific substrates and to generation of tyrosine kinase-specific signals.

† This work was supported by grants from the Netherlands Organization for the Advancement of Pure Research through the area of Medical Sciences (NWO-Medische Wetenschappen) and the Diabetes Fonds Nederland.

* Address correspondence to this author at the Department of Medical Biochemistry, Sylvius Laboratory, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. Telephone: +31 71 5276127. Fax: +31 71 5276125.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

S0006-2960(96)00350-9 CCC: $12.00

We have examined the contribution of Tyr1210 in the insulin receptor to the induction of tyrosine kinase-specific responses like activation of the Shc/Ras/Erk1,2 pathway and mitogenic response. In addition, its contribution to the induction of insulin-specific responses was examined, like IRS1 phosphorylation, dephosphorylation of FAK, and stimulation of glycogen synthesis. For those purposes, we have replaced in the WT-insulin receptor (WT-IR) the residue Tyr1210 by Phe (F1210-IR). The mutant receptor was expressed in CHO cells, and the effect on insulin-induced responses was examined.

EXPERIMENTAL PROCEDURES

Materials and Methods. All chemicals were analytical grade. ^{32}P-labeled inorganic phosphate and A14 mono[^{125}I]-iodoinsulin (2000 Ci/mmol) were from Amersham, U.K. Polyclonal anti-phosphotyrosine (anti-PY) and anti-IRS1 antibodies, raised in rabbits, and determinations of phosphatidylinositol 3-kinase activity in immunoprecipitates have been described previously (Ouwens et al., 1994); anti-FAK and anti-Erk1,2 were obtained from UBI, and anti-Shc was a kind gift from Dr. J. L. Bos. Monoclonal anti-PY was from Oncogene (U.S.A.). Cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) (Ouwens et al., 1994). Isolation of glycoproteins and receptor autophosphorylation was performed according to Maassen at al. (1991).

Construction of the Expression Vector for F1210-IR. A WT-IR expression vector was used as a template to generate by recombinant PCR a cDNA fragment of the insulin receptor. Two sets of primers, for fragments A and B, respectively, were used. Fragment A was amplified with primer 2937 (TTATGAATTCCTGAAAGAGGCAGCC) and the mutant primer 3745 (CCTGTTCATTAGACAGGCCT-TGGAAAGGCTGTTCTGCC). Amplification of fragment B was with the complementary mutant primer 3708 (GGCA-GAACAGCCTTTCCAAGGCCTGTCTAATGAACAGG) and primer 4350 (CATGGGGTGCACAGCAAGTTG). The mutant primers introduce the Phe codon TTC at nucleotide 3722. Fragments A and B were purified by electrophoresis through low-melting agarose, mixed in equimolar amounts, and denatured by incubation at 97 °C. After renaturation, the mixture was amplified using primers 2937 and 4350. The fragment of 1413 bp was digested with *Bst*X1 and *Spe*I, and the resulting fragment was purified by electrophoresis on low-melting agarose. This fragment was ligated in the WT-insulin receptor cDNA in a pSP65 vector after digestion with *Bst*XI and *Spe*I. The ligation mixture was transfected into *Escherichia coli* strain C600. Clones containing the ligated insert were selected by hybridization. DNA sequencing was performed to confirm the correct sequence of the exchanged fragment and the presence of the TTC codon for Phe. Subsequently, the total cDNA insert was replaced in the SV40-driven expression vector (Riedel et al., 1986).

CHO Cell Lines Expressing WT-IR and F1210-IR. The expression vectors encoding WT-IR and IR1210F were transfected into CHO 9 cells using the calcium phosphate precipitation procedure, as described previously (Ouwens et al., 1994). After selection on G418 (600 μg/mL), clones were picked and cloned by limiting dilution and clones were examined for receptor expression by insulin binding experiments (Maassen et al., 1991).

Determination of Erk1,2, Shc, IRS1, and FAK Phosphorylation. These analyses have been described previously (Ouwens et al., 1994, 1996). In brief, confluent cells in 5 cm dishes were made quiescent by incubation for 24 h in DMEM containing 1% bovine serum albumin. Subsequently, cells were either stimulated by insulin for the indicated time periods or kept unstimulated and lysed in 300 μL of SDS[1] sample buffer [0.1 M TRIS-phosphate (pH 6.8), 3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue]. Approximately 25 μL (50 μg of protein) was used for Western blotting. Tyr phosphorylation was visualized using anti-PY antibodies. Erk1,2 phosphorylation was analyzed using antibodies for Erk1,2. Phosphorylation induces a mobility shift of the 4 and 44 kDa bands. In addition, cells were lysed in 300 μL of lysis buffer [20 mM TRIS-HCl (pH 7.6) 20 mM NaF, 0.1 mM EDTA, 0.1 mM PMSF, 1 μg/mL leupeptin, 100 μM sodium vanadate, and 1% Triton X100] and IRS1, Shc, or FAK was immunoprecipitated by appropriate antibodies and the immunoprecipitate collected by protein A Sepharose beads. The immunoprecipitate was analyzed by Western blotting using monoclonal anti-PY antibodies (PY20).

Alternatively, cells were labeled with ^{32}Pi (see under RasGTP Formation), stimulated with insulin, or kept unstimulated, proteins were immunoprecipitated by appropriate antibodies, as mentioned in the legends of the figures, and the immunoprecipitates were analyzed by SDS−PAGE. Incorporation of radioactivity into proteins was visualized by autoradiography.

RasGTP Formation. This assay has been described previously (Burgering et al., 1992) and involves culturing the cells for 16 h on phosphate free DMEM medium, supplemented with ^{32}Pi. Subsequently, cells were insulin stimulated or kept unstimulated, as indicated in the legends of the figures, and lysed. Ras was collected by immunoprecipitation, and coprecipitating guanine nucleotides were analyzed by thin layer chromatography. Radioactivity in the GDP and GTP spots was quantitated on a phosphoimager.

[^{3}H]Thymidine Incorporation. The details of the procedure have been described previously (Ouwens et al., 1994). Briefly, cells were serum starved on DMEM with 1% bovine serum albumin for 24 h, followed by the addition of various concentrations of insulin or by addition of 10% FCS. Incubation was continued for another 16 h, and [^{3}H]-thymidine (0.5 μCi, 81 Ci/mmol) was added. Two hours later, cells were washed and lysed, and incorporated radioactivity was determined by liquid scintillation counting.

Incorporation of Glucose into Glycogen. Cells were grown to maximal confluency and were made quiescent by incubation for 24 h in DMEM with 1% bovine serum albumin. Subsequently, cells were washed with PBS and incubated for 3 h at 37 °C in MEM containing 2.5 mM glucose, 0.1% BSA, and 10 mM HEPES (pH 7.4). Three hours later, new medium was added, supplemented with [^{14}C]-glucose (0.5 μCi/mL, 283 mCi/mmol). Incubation was continued for 90 min at 37 °C. Subsequently, cells were washed three times with ice-cold PBS, lysed in 20% KOH for 30 min at 37 °C. The lysates were transferred to Eppendorf vials, carrier glycogen was added to 1 mg/mL,

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TRIS, tris(hydroxymethyl)aminomethane.

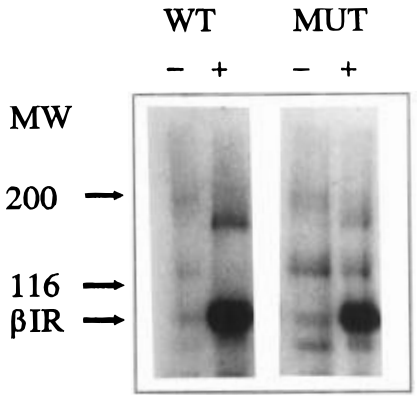

FIGURE 1: Insulin-induced Tyr phosphorylation of proteins. CHO cells expressing WT- or F1210-IRs (MUT) were labeled with ^{32}Pi. Cells were stimulated (+) with insulin for 5 min or kept unstimulated (−), followed by lysis of the cells and immune precipitation of Tyr-phosphorylated proteins with polyclonal phosphotyrosine antibodies. The immunoprecipitate was analyzed by SDS−PAGE and autoradiography. Molecular weights are indicated as is the position of the insulin receptor β-chain.

and vials were incubated at 100 °C for 30 min. Glycogen was precipitated by adding ethanol to a 70% final concentration followed by incubation for 1 h at 0 °C. Precipitate was collected by centrifugation. The glycogen precipitate was washed three times by 1 mL of ice-cold 70% ethanol and dissolved in 1 mL of water. Radioactivity was quantitated by liquid scintillation counting.

RESULTS

Insulin Binding Characteristics and Insulin-Induced Autophosphorylation, and Phosphorylation of IRS1 and Shc. Clonal CHO cell lines expressing WT-IR and mutant F1210-IR were selected, and receptor expression was examined by insulin binding experiments. Two clones each with similar levels of expression were selected. Scatchard plots showed the presence of approximately 1.3×10^6 receptors of high-affinity wild-type and mutant receptors. The K_D for both the WT-IR and F1210-IR was approximately 2.5 nM. Western blot data showed that protein levels of IRS1, Shc, and Erk1,2 were similar in these cell lines [not shown; see, however, also Figure 5 and Ouwens et al. (1994)].

Insulin-induced receptor autophosphorylation was examined in two sets of experiments. In one set, the cells were labeled with ^{32}Pi. Subsequently, cells were made quiescent or stimulated with 1 μM insulin. Insulin receptors and other Tyr-phosphorylated proteins were immunoprecipitated by a polyclonal anti-PY antibody, and the immunoprecipitate was examined by SDS gel electrophoresis and autoradiography. Figure 1 shows an example of such an experiment on CHO WT-IR cells and CHO F1210-IR cells. No major differences in induction of β-chain phosphorylation are present, judging from the autoradiogram. When the data on the various cell lines were quantitated on a phosphorimager, no significant difference in the level of phosphorylation of the WT- and F1210-IR β-chain was detected.

Subsequently, we have examined the stimulation of Tyr phosphorylation of IRS1 by insulin in the cell lines expressing WT and F1210 mutant receptors. Cells were made quiescent and stimulated with insulin, and Tyr phosphorylation of IRS1 was examined by Western blotting of cell lysates and probing the blot with anti-phosphotyrosine antibodies (anti-PY). These data are shown in Figure 2. Both

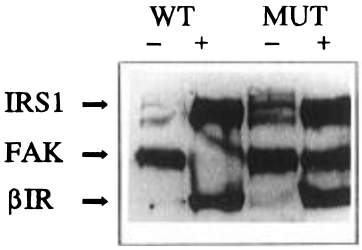

FIGURE 2: Analysis of Tyr-phosphorylated proteins in transfected cell lines by Western blotting. CHO cells expressing WT- and F1210-IRs were stimulated with insulin (+) or kept unstimulated (−). Cells were lysed, and cell lysate (50 μg of protein) was analyzed by Western blotting using a monoclonal anti-PY antibody.

WT- and F1210-IRs were active in inducing Tyr phosphorylation of IRS1. When experiments on three different clonal cell lines each were evaluated, it was found that the maximal level of phosphorylation induced by mutant receptors was approximately 70% of the level reached by WT receptors.

Tyr phosphorylation of Shc was determined by immunoprecipitating Shc from cells metabolically labeled with ^{32}Pi. It was found that WT and mutant receptors were similarly active in inducing Tyr phosphorylation of Shc (not shown).

Insulin-Induced Dephosphorylation of FAK. A specific response of insulin and not of most other tyrosine kinase receptors is the dephosphorylation of phosphotyrosine residues on FAK. When CHO WT-IR and CHO F1210-IR cells were labeled with ^{32}Pi and stimulated with insulin or kept unstimulated, followed by immunoprecipitation of Tyr-phosphorylated proteins by antibodies against PY, we noticed in the immunoprecipitates the disappearance of a band with an apparent molecular mass of 120 kDa in the case of CHO WT-IR cells but not in CHO F1210-IR cells (Figure 1). Tyr dephosphorylation of the 120 kDa band in the case of CHO WT cells is also seen in the experiments involving Western blot analysis of total cell lysates by PY antibodies (Figure 2). Immune precipitation with anti-FAK antibodies and analysis of the immunoprecipitate by Western blot analysis with monoclonal anti-PY confirmed the identity of the 120 kDa band to be FAK (not shown). The experiment was repeated on three different clonal cell lines each of cells expressing WT and mutant receptors with similar results.

Activation of IRS1-Associated PI3Kinase. Insulin-induced activation of PI3kinase is predominantly mediated by Tyr phosphorylation of IRS1 and subsequent binding of the p85/p110 PI3kinase complex to IRS1 (Myers et al., 1992). We have examined the ability of WT- and F1210-IRs to activate PI3kinase in anti-IRS1 immunoprecipitates. CHO cell lines, expressing WT-IR and F1210-IRs, respectively, were made quiescent and stimulated with insulin or kept unstimulated. Cells were lysed, IRS1 was immunoprecipitated, and PI3kinase activity was determined in the immunoprecipitate. Figure 3 shows the quantitated data. F1210-IRs did activate PI3kinase, though to a lower level than WT-IRs.

Insulin-Induced RasGTP Formation, Phosphorylation of Erk1,2, and Mitogenicity. Insulin-induced RasGTP formation proceeds largely by interaction of Tyr-phosphorylated Shc with a complex between the adaptor protein Grb2 and the guanine nucleotide exchange factor SOS (Li et al., 1993). We have examined the increase in RasGTP levels in response to insulin stimulation in CHO WT-IR cells and CHO F1210-IR cells. Quiescent cells, metabolically labeled with ^{32}Pi, were kept unstimulated or stimulated with 1 μM insulin for

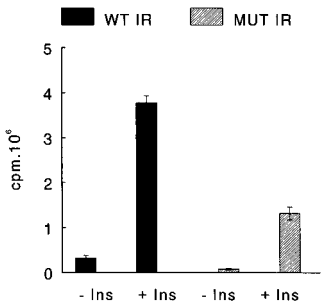

FIGURE 3: Insulin-induced stimulation of phosphatidylinositol 3-kinase activity. Cells expressing WT- or F1210-IRs were stimulated with insulin or kept unstimulated. IRS1 was immunoprecipitated, and coprecipitating PI3 kinase activity was assessed by a PI3 kinase activity assay using [^{32}P]ATP and phosphatidylinositol as substrate. Formation of 3-phosphophosphatidylinositol was analyzed by thin layer chromatography, followed by quantitation of the amount of radioactivity in the 3-phosphophosphatidylinositol spots by phosphoimager. Standard deviations are indicated.

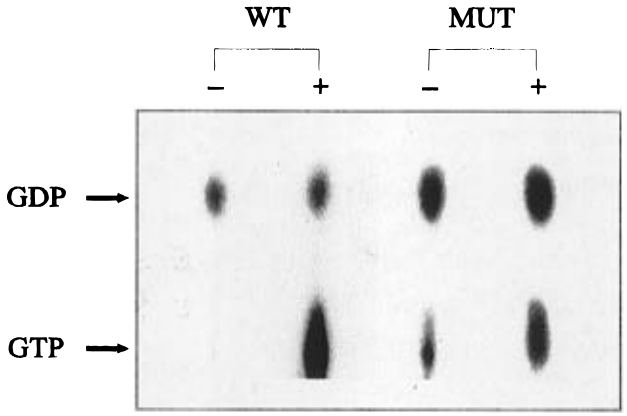

FIGURE 4: Insulin-induced formation of RasGTP. CHO cells expressing WT- or F1210-IRs (MUT) were labeled with ^{32}Pi. Cells were stimulated with insulin (+) or kept unstimulated (−), followed by lysis of the cells and immune precipitation of Ras. Coprecipitating guanine nucleotides were analyzed by thin layer chromatography and autoradiography. Positions of GDP and GTP are indicated. The quantitated data on three clonal cell lines are as follows: (standard deviation): WT-basal (6 ± 3%), WT-stimulated (66 ± 9%), mutant-basal (20 ± 5%), and mutant-stimulated (47 ± 4%).

5 min. Subsequently, cells were lysed, Ras was immunoprecipitated, and the amount of coprecipitating GDP and GTP was analyzed by thin layer chromatography and autoradiography. Figure 4 shows the autoradiogram and quantitated data. Cells expressing the F1210 mutant showed higher basal levels of RasGTP and a somewhat lower induction of RasGTP formation.

The dose−response relation of insulin-induced phosphorylation of Erk1 and Erk2 was examined by stimulating the CHO WT-IR and CHO F1210-IR cells with increasing concentrations of insulin for 10 min, followed by analysis of Western blots with antibodies against Erk1,2. Phosphorylation leads to a shift of Erk1,2 to a lower mobility. The results, given in Figure 5, indicate that both receptors induce a marked phosphorylation of Erk1,2.

CHO WT-IR and CHO F1210-IR cells were also examined for the ability of insulin to stimulate [^{3}H]thymidine incorporation. The dose−response relation is presented in Figure 6. CHO WT-IR cells show the characteristic bell-shaped dose−response relation, as reported previously (Osterop et al., 1994). Cells expressing the mutant receptor showed a

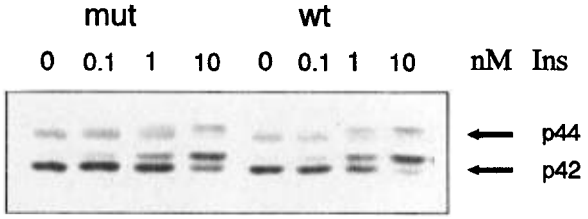

FIGURE 5: Insulin-induced phosphorylation of Erk1 and Erk2. CHO cells expressing WT- or F1210-IRs were stimulated with increasing concentrations of insulin for 10 min or kept unstimulated. Cells were lysed, and phosphorylation of Erk1 and Erk2 was analyzed by Western blot using an antibody recognizing Erk1 and Erk2. These positions are indicated.

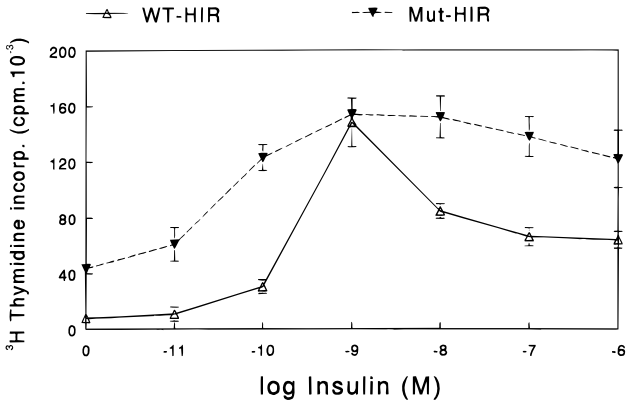

FIGURE 6: Insulin-stimulated incorporation of [^{3}H]thymidine into DNA. CHO cells (10^6 cells) expressing WT- or F1210-IRs were made quiescent by incubation for 16 h in medium containing bovine serum albumin. Subsequently, cells were kept unstimulated or stimulated with increasing concentrations of insulin and [^{3}H]-thymidine was added. Incorporation of radioactivity in DNA was determined by liquid scintillation counting. The level of radioactivity incorporated in the presence of 10% FCS was approximately 200 000 cpm. Experiments were performed in triplicate on two clonal cell lines each. SDs are indicated.

marked stimulation of [^{3}H]thymidine incorporation, though the fold stimulation of basal uptake was decreased and also the bell shape was less pronounced.

Insulin-Stimulated Synthesis of Glycogen. Unlike most other tyrosine kinases, the activated insulin receptor triggers a series of events which leads to an increased conversion of glucose into glycogen. We have examined the stimulation of glycogen synthesis by insulin at various insulin concentrations in CHO WT-IR and CHO F1210-IR cells. Cells were made quiescent, insulin was added, and the incorporation of [^{14}C]glucose into glycogen was subsequently assayed. The dose−response relation of insulin-stimulated incorporation of glucose into glycogen in these cell lines was similar. A maximum of 2-fold stimulation was reached at 100 nM insulin, the ED_{50} being 0.5 nM (data not shown). The results show that the F1210- and WT-IRs have a similar activity in stimulating glycogen synthesis. This result was also seen in other clonal cell lines.

Temperature Stability of WT- and F1210-IR. We have subsequently examined whether the mutation affects the temperature stability of the insulin receptor tyrosine kinase domain. For that, WT and mutant receptors were partially purified by wheat germ lectin chromatography of lysates of CHO WT-IR and CHO F1210-IR cells. The receptor preparations were incubated for 1 h with 1 mM insulin at 37 °C and at higher temperatures and subsequently subjected to receptor autophosphorylation conditions by addition of

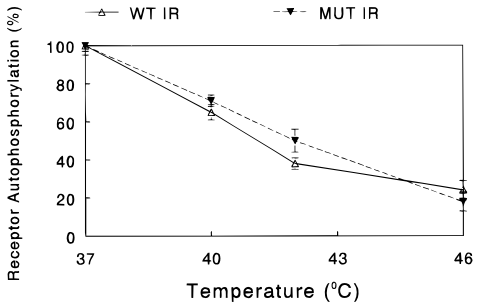

FIGURE 7: Temperature-induced inactivation of insulin-induced receptor autophosphorylation. Insulin receptors were partially purified from CHO cells expressing WT- or F1210-IRs. Receptors were incubated for 1 h at 37 °C or at higher temperatures in the presence of 1 μM insulin. Receptor autophosphorylation was subsequently determined by adding [^{32}P]ATP. Radioactivity incorporated in the β-chain was quantitated by immune precipitation of the IR, followed by phosphoimager analysis of β-chain labeling. The incorporated radioactivity is expressed as the percentage of the radioactivity incorporated at 37 °C.

manganese ions and ^{32}P-labeled ATP. Subsequently, receptors were immunoprecipitated and subjected to gel electrophoresis, and β-chain labeling was determined by phosphorimager analysis. The quantitated data are presented in Figure 7. Both WT- and F1210-IR showed the same pattern of receptor inactivation with increasing temperatures. Incubation of insulin alone for 1 h at 46 °C had no effect on the activity of the hormone to induce receptor autophosphorylation, indicating that the decreased autophosphorylation activity is due to inactivation of the insulin receptor.

DISCUSSION

Signal transduction by receptor tyrosine kinases is in general triggered by receptor autophosphorylation and Tyr phoshorylation of substrate proteins, such as Shc and IRS1. Phosphorylated Tyr residues can serve as docking sites for proteins containing matching SH2 domains (Cantley et al., 1991). This interaction can activate enzymes, like in the case of PI3kinase by binding to Tyr-phosphorylated IRS1, or it can induce translocation of proteins to a particular compartment in the cell, e.g. in the case of binding of the Grb2−SOS complex to Tyr-phosphorylated Shc (Myers et al., 1992; Li et al., 1993). Alternatively, Tyr phosphorylation itself is sufficient to alter certain enzymatic properties of the protein, reflected by activation of phospholipase C by Tyr phosphorylation (Kim et al., 1991). In the case of insulin receptor signal transduction, early postreceptor processes involve Tyr phosphorylation of IRS1 and of Shc and dephosphorylation of Tyr-phosphorylated FAK. IRS1 phosphorylation and FAK dephosphorylation are specific responses of the insulin receptor and are not induced by most other Tyr kinases (Myers & White, 1993; Pillay et al., 1995).

Nearly all receptor Tyr kinases contain a conserved Tyr residue, corresponding to Tyr1210 in the insulin receptor. Ser/Thr kinases lack this particular Tyr residue (Hanks et al., 1988), despite the overall conservation of many other conserved residues through the families of Tyr and Ser/Thr kinases. In the three-dimensional structure of the insulin receptor, Tyr1210 is located at the surface on the lower lobe, in the cleft where ATP binds. It is plausible that this part of the surface on the lower lobe is involved in substrate recognition. We have examined to what extent the conserved Tyr1210 contributes to the main characteristic response of all Tyr kinases, i.e. stimulation of a mitogenic response through Shc phosphorylation, and activation of Ras and Erk1/2. In addition, the contribution to insulin-specific responses such as IRS1 phosphorylation, FAK dephosphorylation, and induction of glycogen synthesis was studied.

We observed that replacement of the conserved Tyr residue by Phe had no major effect on the magnitude of the mitogenic response and, in accordance, on insulin-stimulated Shc phosphorylation, RasGTP formation, and phosphorylation of Erk1,2. Though the mutant receptor did show some minor alterations in the dose−response relations or the magnitude of the responses. This finding indicates that the conserved Tyr1210 is not an essential amino acid residue in recognition of the substrate leading to Shc phosphorylation, nor is it required in conferring the kinase activity specific to Tyr residues. Tyr1210 only seems to modulate slightly the coupling of the receptor with downstream targets, e.g. as reflected by the higher basal RasGTP levels in cells expressing the mutant receptor. The mutant receptor is also able to induce Tyr phosphorylation of IRS1, being a specific response for the insulin receptor and not of most other Tyr kinases, and concomitant activation of PI3kinase. The mutant receptor shows a reduced ability for these responses, suggesting a contribution of Tyr1210 in recognition of the substrate leading to IRS1 phosphorylation.

The findings indicate that this particular Tyr residue which is conserved in nearly all tyrosine kinases is not essential for action of the insulin receptor tyrosine kinase.

Another possibility is that the Tyr residue is involved in stabilizing the three-dimensional structure of the TK domain to protect the protein against thermal inactivation in a living organism. This possibility is suggested by the three-dimensional structure as the hydroxyphenyl moiety of Tyr1210 is inward-bound and hydrogen bonded to two well-ordered, internal water molecules (S. R. Hubbard, personal communication). We have examined whether replacement of Tyr1210 by Phe leads to an increased sensitivity to temperature-induced destabilization of the tyrosine kinase activity. For that, we performed insulin-induced receptor autophosphorylation experiments using both WT and mutant receptors after incubating the receptor at increasing temperatures. That approach showed a parallel temperature-induced loss of receptor autophosphorylation of the mutant and WT-IRs, arguing against a contribution of Tyr1210 to the stability of the tyrosine kinase domain.

Another recently observed response which is specific for the insulin receptor is the dephosphorylation of focal adhesion kinase and paxillin (Pillay et al., 1995; Ouwens et al., 1995). This response is quite specific for the insulin receptor as activation of most other tyrosine kinases is without effect on FAK phosphorylation or even stimulates phosphorylation. Recently, we provided evidence that insulin-induced FAK dephosphorylation involves the phosphotyrosine phosphatase PTP-1D or SYP (Ouwens et al., 1996). To our surprise, we observed that the F1210-IR exhibits a severely reduced activity in mediating insulin-induced dephosphorylation of FAK. Unfortunately, the precise mechanism by which insulin receptors induce the dephosphorylation of FAK is not yet known, and this situation hampers the characterization of the substrate(s) whose activity is modulated by replacement of Tyr1210 by Phe. The finding, however, that replacement of Tyr1210 by Phe severely affects the response of FAK dephosphory-

lation indicates that this conserved Tyr residue somehow contributes to signaling by the activated insulin receptor, the substrate, or substrates involved requiring further elucidation. A contribution of Tyr1210 in fine tuning postreceptor signaling pathways is also reflected by the altered pattern of insulin stimulation of glycogen synthesis and [^{3}H]-thymidine incorporation, upon replacement of this Tyr by Phe. These fine-tuning processes may generate the constraint which has led to the conservation of this particular tyrosine residue in most tyrosine kinases during evolution.

ACKNOWLEDGMENT

We thank Dr. A. Ullrich for providing the WT-IR cDNA expression vector and Dr. S. R. Hubbard for helpful suggestions.

REFERENCES

Burgering, B. M. Th., Medema, R. H., Maassen, J. A., Van de Wetering, M. L., Van der Eb, A. J., McCormick, F., & Bos, J. L. (1992) *EMBO J. 10*, 1103−1109.

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281−302.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clausser, E., Ou, J. H., Masiarz, F., Kan, Y. W., & Rutter, W. J. (1985) *Cell 40*, 747−758.

Hanks, S., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42−51.

Hubbard, S. R., Wei, L., Ellis, L., & Hendrickson, W. A. (1994) *Nature 372*, 746−754.

Kemp, B. E., & Pearson, R. B. (1990) *Trends Biochem. Sci. 15*, 342−346.

Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., & Rhee, S. G. (1991) *Cell 65*, 435−441.

Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991a) *Science 253*, 407−414.

Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991b) *Science 253*, 414−420.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., & Schlessinger, J. (1993) *Nature 363*, 85−88.

Maassen, J. A., Van der Vorm, E. R., Van der Zon, G. C. M., Klinkhamer, M. P., Krans, H. M. J., & Möller, W. (1991) *Biochemistry 30*, 10778−10783.

Marshall, C. J. (1995) *Cell 80*, 179−185.

Myers, M. G., & White, M. F. (1992) *Diabetes 42*, 643−650.

Myers, M. G., Backer, J. M., Sun, X. J., Shoelson, S. E., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., & White, M. F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10350−10354.

Osterop, A. P. R. M., Medema, R. H., Van der Zon, G. C. M., Möller, W., & Maassen, J. A. (1994) *Biochemistry 33*, 7453−7459.

Ouwens, D. M., Van der Zon, G. C. M., Pronk, G. J., Bos, J. L., Moller, W., Cheatham, B., Kahn, R. C., & Maassen, J. A. (1994) *J. Biol. Chem. 269*, 33116−33122.

Ouwens, D. M., Mikkers, H. H. M., Van der Zon, G. C. M., & Maassen, J. A. (1995) *Diabetologia 38* (Suppl. 1), A64.

Ouwens, D. M., Mikkers, H. M. M., Van der Zon, G. C. M., Stein-Gerlach, M., Ullrich, A., & Maassen, J. A. (1996) *Biochem. J.* (in press).

Pazin, M. J., & Williams, L. T. (1992) *Trends Biochem. Sci. 17*, 374−378.

Pillay, T. S., Sasaoka, T., & Olefsky, J. M. (1995) *J. Biol. Chem. 270*, 991−994.

Riedel, H., Dull, T. J., Schlessinger, J., & Ullrich, A. (1986) *Nature 324*, 68−70.

Taylor, S. I., Cama, A., Accili, A., Barbetti, F., Quon, M. J., Luz Sierra, M., Suzuki, Y., Koller, E., Levy-Toledano, R., Wertheimer, E., Moncada, V. Y., Kadowaki, H., & Kadowaki, T. (1992) *Endocr. Rev. 13*, 566−595.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature 313*, 756−761.

Yarden, Y., & Ullrich, A. (1988) *Annu. Rev. Biochem. 57*, 443−478.

BI960350R