Original Articles

Serine Residues 1177/78/82 of the Insulin Receptor Are Required for Substrate Phosphorylation but Not Autophosphorylation

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Serine residues of the human insulin receptor (HIR) may be phosphorylated and negatively regulate the insulin signal. We studied the impact of 16 serine residues in HIR by mutation to alanine and co-overexpression in human embryonic kidney (HEK) 293 cells together with the docking proteins insulin receptor substrate (IRS)-1, IRS-2, or (SHC) Src homologous and collagen-like. As a control, IRS-1 was also cotransfected with an HIR with a juxtamembrane deletion (HIRΔJM) and therefore not containing the domain required for interaction with IRS-1. Coexpression of HIR with IRS-1, IRS-2, and SHC strongly enhanced tyrosine phosphorylation of these proteins. A similar increase in tyrosine phosphorylation was observed in cells overexpressing IRS-1, IRS-2, or SHC together with all HIR mutants except HIRΔJM and a mutant carrying exchanges of serines 1177, 1178, and 1182 to alanine (HIR1177/78/82), although this mutant showed normal autophosphorylation. Analysis of total cell lysates with anti-phosphotyrosine antibodies showed that in addition to the overexpressed substrates, other cellular proteins displayed reduced levels of tyrosine phosphorylation in these cells. To study consequences for phosphatidylinositol 3-kinase (PI 3-kinase) activation, we established stable NIH3T3 fibroblast cell lines overexpressing wild-type HIR, HIR1177/78/82, and other HIR mutants as the control. Again, HIR1177/78/82 showed normal autophosphorylation but showed a clear decrease in tyrosine phosphorylation of endogenous IRS-1 and activation of PI 3-kinase. This decrease in kinase activity also occurred in an in vitro kinase assay towards recombinant IRS-1. Finally, we performed a separation of the phosphopeptides by high-performance liquid chromatography and could not detect any differences in the profiles of HIR and HIR1177/78/82. In conclusion, we have defined a region in HIR that is important for substrate phosphorylation but not autophosphorylation. Therefore, this mutant may provide new insights into the mechanism of kinase activation and substrate phosphorylation. *Diabetes* 49:889–895, 2000

Binding of insulin to the α-subunit of the human insulin receptor (HIR) induces rapid tyrosine phosphorylation of the insulin receptor β-subunit. Insulin-stimulated tyrosine phosphorylation occurs at 6 tyrosine residues, the tyrosines 1146/50/51 in the kinase domain, the tyrosines 1316 and 1322 in the COOH-terminal tail of the receptor β-subunit, and tyrosine 960 in the juxtamembrane region. Numerous studies have demonstrated that, in particular, the phosphorylation of the 3 tyrosines in the kinase domain is essential for further signal transduction on the postreceptor level (1). Recently, it was shown that these tyrosines are also important for the interaction with the kinase receptor ligand binding domain of insulin receptor substrate (IRS)-2 (2). The phosphorylation of tyrosine in the juxtamembrane region appears to be important for docking protein binding through their phosphotyrosine-binding domains (3). The rapid insulin-stimulated tyrosine phosphorylation of the receptor β-subunit is followed by a somewhat slower increase of serine and threonine phosphorylation of the receptor β-subunit (1). Even though insulin-induced serine/threonine phosphorylation was described for the first time in 1982 (4), the significance of serine/threonine phosphorylation of the receptor is still not understood. A number of serine/threonine phosphorylation sites in the β-subunit were identified. Sites proposed to be phosphorylated upon stimulation by protein kinase C (PKC) include the following: threonine 1336 (5), serine 1293/94 (6), serine 1315 (7), serine 1309 (8), and serine 1023/25 (9). Insulin-stimulated serine phosphorylation appears to occur on serines 955/56 in the juxtamembrane region (10,11). The functional significance of these serine-phosphorylated domains is not clear. One potential function is the inhibition of insulin receptor tyrosine kinase activity, which leads to impaired insulin signaling. This hypothesis is based on studies in which it was shown that PKC stimulation by phorbolesters or hyperglycemic conditions (12) and transfection of cells with PKC isoforms α, β, δ, ε, and θ may reduce insulin receptor tyrosine autophosphorylation or insulin-dependent tyrosine phosphorylation of downstream elements of the insulin-signaling chain (12–14). IRS-1 phosphorylation at serine residues also seems to be involved in the inhibitory effect

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CK-2, casein kinase 2; FCS, fetal calf serum; HEK, human embryonic kidney; HIR, human insulin receptor; HIRΔJM, human insulin receptor with juxtamembrane deletion; HPLC, high-performance liquid chromatography; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; SHC, Src homologous and collagen-like.

of some PKC isoforms (α, δ, and θ) on the insulin receptor. Whereas serine phosphorylation of IRS-1 by PKC leads to an apparent molecular weight shift of IRS-1, an analogous shift for the insulin receptor has not been demonstrated (14). It is therefore still unknown whether serine phosphorylation of the receptor by PKC occurs in relevant quantities and whether it is required for inhibition of the receptor. We have used 16 serine mutants of HIR as well as a COOH-terminal deletion to study the importance of kinase inhibition after treatment of cells by 2-deoxyglucose (15). None of the serine sites seemed to be required for the 2-deoxyglucose–dependent inhibition of the receptor (16). We also have used these constructs to define whether any of the serine residues are required for PKC-dependent receptor inhibition in a cotransfection system in human embryonic kidney (HEK) 293 cells. In this study, HIR994 and HIR1023/25 showed an impaired inhibition by PKC-β_2 and PKC-θ, suggesting that these domains are important for the inhibitory effect of PKC on the insulin receptor (16a). It remains to be determined whether this effect involves phosphorylation of these domains by PKC or whether these domains are important for the inhibitory interaction with serine-phosphorylated IRS-1.

In addition to a potential function of serine phosphorylation for negative receptor modulation, a role in downstream signaling is another possible function. The aim of the present study was to determine the significance of the serine phosphorylation sites for downstream signaling. Our data show that the HIR1177/78/82 mutant exhibits an altered interaction with the substrates IRS-1, IRS-2, and (SHC) Src homologous and collagen-like, but is not impaired in its autophosphorylation capacity.

RESEARCH DESIGN AND METHODS

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Egenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, phenylmethylsulfonyl fluoride, Na_3VO_4, Triton X-100, and dithiotreitol were from Sigma (Munich). The reagents for SDS-PAGE and Western blotting were from Roth (Karlsruhe, Germany) and Bio-Rad (Munich). Protein A sepharose CL 4B was from Pharmacia (Uppsala, Sweden). Nitrocellulose was from Schleicher & Schuell (Dessel, Germany). All other reagents were from the best grade commercially available. A polyclonal antibody recognizing the insulin receptor β-subunit was raised against the COOH-terminal 15 amino acids of the receptor. Monoclonal anti-phosphotyrosine antibody (PY20) was purchased from Leinco Technologies (St. Louis, MO), and a polyclonal antibody directed against the extracellular domain of the insulin receptor and suitable for blotting was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody 83-14 directed against the extracellular domain of HIR was a generous gift from Ken Siddle (Cambridge, U.K.). The polyclonal rabbit antibodies against IRS-1 and IRS-2 and the cDNA for IRS-1, IRS-2, and SHC were a generous gift from M.F. White (Joslin Diabetes Center, Boston, MA). Visualization of proteins after Western blotting was performed using the nonradioactive enhanced chemiluminescence system and Hyperfilm from Amersham-Pharmacia Biotech (Freiburg, Germany). [γ-^{32}P]ATP and [^{32}P]orthophosphate were obtained from NEN-DuPont (Bad Homburg, Germany). Recombinant IRS-1 was from Upstate Biotechnology (Lake Placid, NY).

Preparation of serine-to-alanine point mutants of the insulin receptor. All point mutations were prepared by the method of Kunkel et al. (17) in the cytomegalovirus immediate early promoter-based vector pRK5 (18) containing the cDNA sequence for the wild-type human insulin receptor. The mutagenic oligos are as follows: serine 1023/25: 5′-TCCGCTCTCGGAGAgcGGCTG cCTCGTTGACCGTC-3′; serine 1074/78: 5′-CTGGCCGCAGAGcACGGAGGTAG gcCTTCAGGTCTCC-3′; serine 1177/78/82**:** 5′-CCACGCCAAAGGcCCACATGTC AGcAGcAGTGGTGAAGACC-3′; serine 1293/94: 5′-CCCTCTGACAGTGCGcGGc ACGGTCCAGGGGC-3′; serine 1308/09: 5′-CTTGAAACCCAGCGcGGcCCCTCC ATCCCGG-3′. Point mutations were verified by DNA sequencing using the Sequenase version 2.0 kit (Amersham-Pharmacia Biotech).

Transient expression in HEK 293 cells. HEK 293 fibroblast cells (ATCC CRL 1573) were grown in Dulbecco's modified Eagle's medium/Nutrient Mix F12 medium supplemented with 10% FCS. Cells were transfected according to the protocol of Chen and Okayama (19). Briefly, cells were grown in 6-well dishes at a density of 3×10^5 cells per well in 2 ml of medium. A total of 4 µg supercoiled plasmid DNA was mixed with 0.25 mol/l $CaCl_2$ in a final volume of 0.1 ml. To this, an equal amount of 2× transfection buffer (50 mmol/l N,N-bis[2-hydroxyethyl]-2-aminothansulfonic acid, pH 6.95, 280 mmol/l NaCl, 1.5 mmol/l Na_2HPO_4) was added, and after incubation for 10 min at room temperature, the mixture was given dropwise to the cells. After incubation for 16 h at 37°C and 3% CO_2, the cells were serum starved for 24 h in Dulbecco's modified Eagle's medium (1 g/l glucose) containing 0.5% FCS.

Generation of recombinant retroviruses. The cDNAs for HIR and the different mutants were cloned into the ***Hpa***I site of the retroviral expression vector pLXSN (20). To prepare retroviral stocks by transient transfection (21), HEK 293 cells were cotransfected with each of these constructs and a packaging Ψ$^-$ retroviral vector (a gift from Dr. Dan R. Littman). The titer of the recombinant retroviruses was determined after infection of NIH3T3 cells with serial dilutions of retrovirus-containing cell-free supernatants. Titers were in the range of $1–3 \times 10^5$ infectious units/ml.

Retrovirus-mediated gene transfer. One million NIH3T3 cells per 10-cm dish were incubated with supernatants of HEK 293 cells containing the recombinant retroviruses for 12 h at 37°C, 5% CO_2, and in the presence of 8 µg/ml polybrene. Virus-containing medium was then removed, and cells were grown in medium containing 750 µg/ml geneticin. After 2–3 weeks of selection, at least 12 single clones were picked per infection and analyzed for the expression level of the transduced gene.

Stimulation with insulin, cell lysis, and immunoprecipitation. Serum-starved cells were stimulated with or without 10^{-7} mol/l insulin for 5 min at 37°C and lysed in 0.2 ml per 10 cm^2 dish of ice-cold lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 100 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, and 2 mmol/l Na_3VO_4). Crude lysates were cleared by centrifugation (20 min/12,000***g***) and analyzed on SDS-PAGE (40 µg protein per lane) or used for immunoprecipitations. For immunoprecipitations, 200 µl crude lysates were diluted with the same volume of immunoprecipitation buffer (50 mmol/l HEPES, pH 7.5, 0.1% Triton X-100, 150 mmol/l NaCl, 10% glycerol, and 2 mmol/l Na_3VO_4), combined with 30 µl protein A sepharose and antiserum, and rotated overnight at 4°C. Immunoprecipitates were washed 3 times with 1 ml wash buffer, boiled for 5 min in 1× Laemmli buffer, and separated by 7.5% SDS-PAGE.

Western blotting. After SDS-PAGE, proteins were transferred to nitrocellulose membranes by semidry blotting (transfer buffer: 20 mmol/l NaH_2PO_4, 20 mmol/l Na_2HPO_4, pH 8.8). After transfer, filters were blocked with NET-G buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l TRIS, 0.05% Triton X-100, and 0.25% gelatine, pH 7.4) for 1 h. Subsequently, filters were incubated with the first antibody overnight at 4°C. The membranes were washed 3 times with NET-G buffer before they were incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by enhanced chemiluminescence.

Phosphatidylinositol 3-kinase assay. Phosphatidylinositol 3-kinase (PI 3-kinase) assays were performed as described by Morgan et al. (22), with some modifications. Briefly, IRS-1 was immunoprecipitated from NIH3T3 cell lysates as described above. Immunoprecipitates were washed twice in phosphate-buffered saline, 1% Nonidet-P40, and 100 µmol/l Na_3VO_4; twice in 500 mmol/l LiCl, 100mmol/l Tris/Cl (pH 7.5), and 100mmol/l Na_3VO_4; and twice with 10mmol/l Tris/Cl (pH 7.5), 100mmol/l NaCl, 1mmol/l EDTA, and 100µmol/l Na_3VO_4. The beads were resuspended in 25 µl of 60 mmol/l Tris/Cl (pH 7.5), 300 mmol/l NaCl, 12mmol/l $MgCl_2$ and 12.5 µl phosphatidylinositol (1mg/ml). The kinase reaction was started by adding 12.5 µl of 40 µmol/l ATP containing 10 µCi [γ-^{32}P]ATP, and samples were incubated for 20 min at room temperature. The reaction was stopped by addition of 150 µl 1N HCl. Lipids were extracted once with 450 µl chloroform:methanol (1:1, vol/vol), and the phospholipid-containing organic phase was resolved on thin-layer chromatography plates (Silica Gel 60; Merck, Darmstadt, Germany) and developed in chloroform:methanol:aceton:glacial acetic acid:water (40:13:15:12:7, vol/vol). Thin-layer chromatography plates were dried and exposed on Kodak (Rochester, NY) X-AR 5 film.

Insulin receptor kinase activity. HEK 293 fibroblasts were stimulated with insulin, and cell lysates were prepared and added to microwells coated with anti-HIR antibody. After the receptor had bound to the antibody, wells were washed, and kinase activity was measured in the presence of 0.3 µmol/l [γ-^{32}P]ATP (100–200 Ci/mmol) and 2.4 µg/ml recombinant IRS-1. Wells were then washed again, and insulin binding activity (defined as the amount of insulin specifically bound at 8.7 nmol/l) was analyzed as previously described (23).

In vivo ^{32}P-labeling, isolation, tryptic digestion, and high-performance liquid chromatography phosphopeptide analysis of the insulin receptor. HEK 293 cells were plated in 100-mm dishes, transfected with the cDNA of HIR or HIR1177/78/82 as described above, and serum starved for 15 h. For [^{32}P]-labeling, cells were incubated for 2 h in phosphate- and serum-free RPMI 1640 medium and then maintained in phosphate-free RPMI 1640 containing 0.5 mCi/ml [^{32}P]orthophosphate for 2 h. After insulin stimulation (5 min,

100 nmol/l), incubation was stopped by placing the cells on ice followed by 1 wash with ice-cold phosphate-buffered saline. Cells were lysed, and the insulin receptors were isolated by immunoprecipitation with 83-14 antibody and separated by SDS-PAGE as outlined above. After visualization of the phosphorylated β-subunit by autoradiography, the band was cut from the gel, reduced (dithiothreitol), and alkylated (4-vinylpyridine) before tryptic digestion.

The following high-performance liquid chromatography separation of the phosphopeptides was carried out with a SunChrom high-performance liquid chromatography (HPLC) system (SunChrom, Friedrichsdorf, Germany) using a TSK Gel Super-ODS 120 × 0.3 mm capillary column (Grom, Herrenberg, Germany) at a flow rate of 5 µl/min. The mobile phase consisted of water with 0.05% trifluoroacetic acid (solvent A) and acetonitrile:water:trifluoroacetic acid (80:10:0.075, solvent B). The phosphopeptides were eluted by a stepwise acetonitrile gradient from 5% solvent B (20 min) to 45% solvent B within 90 min, to 95% solvent B within 50 min and again 95% solvent B for 10 min. Fractions were collected at 2-min intervals in 0.2 ml polymerase chain reaction tubes, and the radioactivity was measured as Cerenkov radiation using an Amersham-Pharmacia Biotech scintillation counter.

RESULTS

We have previously reported that a mutation of several serine residues to alanine in HIR does not lead to a significant change in receptor autophosphorylation. This is in contrast to the mutation of serine residues 994 and 1023/25, which increased autophosphorylation or a 12–amino acid deletion in the juxtamembrane that showed a reduced autophosphorylation capacity. To investigate the potential of the different mutants to tyrosine phosphorylate HIR substrates, we used a transient expression system and transiently overexpressed the wild-type HIR or its mutants together with IRS-1 in 293 cells. After the cells were made quiescent, they were stimulated with insulin as indicated in Fig. 1 and then lysed. Aliquots of the total cell lysates were run on SDS-PAGE, and proteins were transferred to nitrocellulose and blotted with phosphotyrosine antibodies. Cells transfected with IRS-1 alone revealed a weak autophosphorylation of the endogenous HIR β-subunit after insulin stimulation (second lane in Fig. 1) and a strong phosphorylation of a 180-kDa protein. This protein does not represent the overexpressed IRS-1, since it has a lower apparent molecular weight on the gel and is also found in cells not overexpressing IRS-1 (second lane in Fig. 4). It may therefore represent IRS-4, which is abundant in these cells. Co-overexpression of HIR and IRS-1 yielded a β-subunit autophosphorylation that was partly insulin independent. Tyrosine phosphorylation of IRS-1 was very strong and mostly insulin independent, indicating that the low insulin-independent activity of HIR was sufficient for full phosphorylation of IRS-1. At around 200 kDa, a tyrosine-phosphorylated protein was found that represents an unprocessed HIR precursor that also is autophosphorylated constitutively. Co-overexpression of the HIRΔJM or the HIR1177/78/82 mutant and IRS-1 gave a similar result but showed overall reduced amounts of substrate phosphorylation, whereas the other serine-to-alanine mutations and COOH-terminal deletion mutant did not lead to a reduced tyrosine phosphorylation of IRS-1. The presence of similar amounts of HIR and IRS-1 was verified by stripping the antibodies from the filters and reprobing with the appropriate antisera.

To investigate more specifically the phosphorylation on IRS-1, the residual lysates of some of the transfections shown in Fig. 1 were used for immunoprecipitation. The immunoprecipitates were analyzed as above, and the results are presented in Fig. 2. Again, HIR mutants with the juxtamembrane deletion or the serine mutations 1177/78/82 were not or were only partly capable of phosphorylating IRS-1.

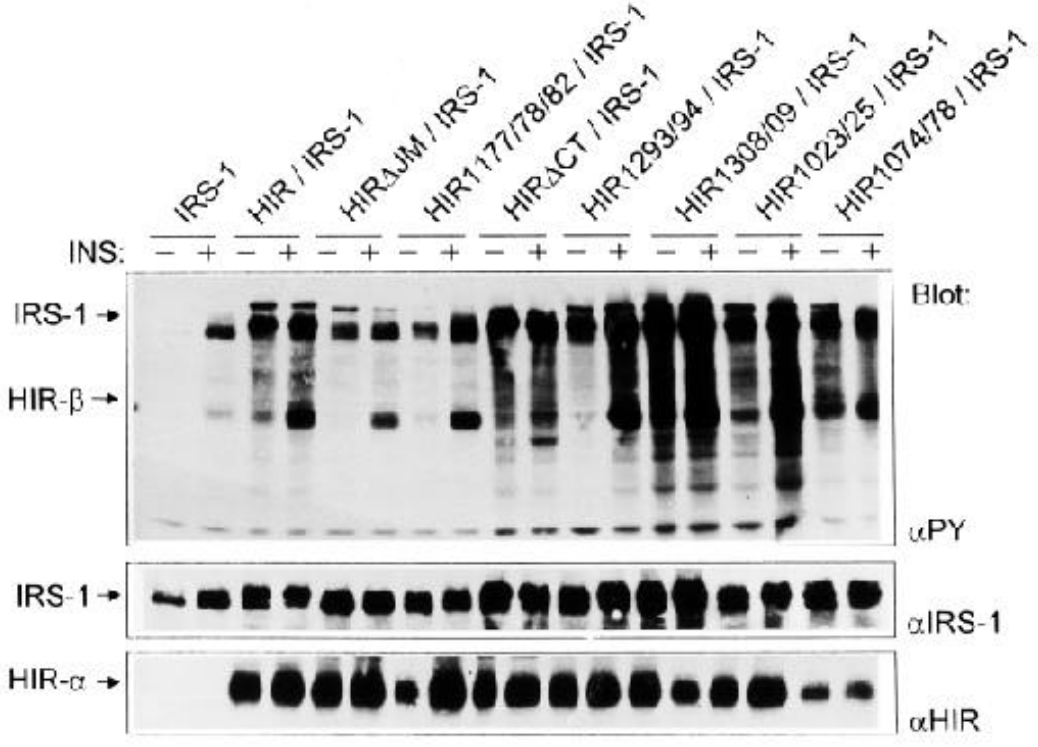

FIG. 1. Insulin-induced tyrosine phosphorylation pattern in IRS-1 cotransfections. HEK 293 fibroblast cells overexpressing IRS-1 alone or together with the wild-type receptor (HIR) or the mutant receptors were stimulated with 10^{-7} mol/l insulin at 37°C for 5 min. Whole-cell lysates were prepared, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against phosphotyrosine (αPY, upper panel). To prove equal amounts of overexpressed proteins, the filters were reprobed with antibodies against IRS-1 and the HIR-β–subunit (lower panels). HIRΔCT, HIR with COOH-terminal deletion; INS, insulin.

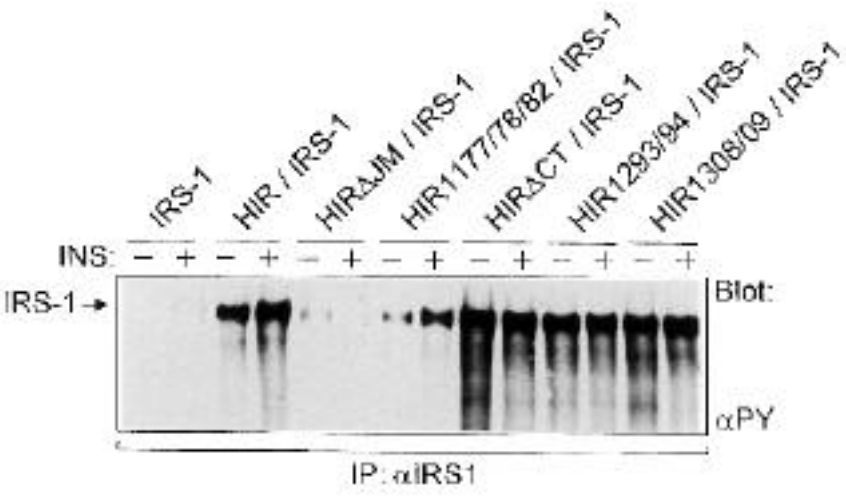

FIG. 2. IRS-1 tyrosine phosphorylation in anti–IRS-1 immunoprecipitates. Cell lysates from the cotransfection experiment shown in Fig. 1 were immunoprecipitated with anti–IRS-1 antibodies, and immunoprecipitates were analyzed by SDS-PAGE, transferred to nitrocellulose, and blotted with anti-phosphotyrosine antibodies (αPY). HIRΔCT, HIR with COOH-terminal deletion; INS, insulin.

The capability of the HIR1177/78/82 mutant to phosphorylate IRS-2 is demonstrated in Fig. 3. Similar to the previous experiments, 293 cells transiently overexpressed either HIR or HIR1177/78/82 together with IRS-1 or IRS-2. Cells were stimulated with insulin as indicated, lysed, and immunoprecipitated with the appropriate antibodies. As shown in Fig. 3***A***, the mutant HIR had a reduced phosphorylation capability not only for IRS-1, but also for IRS-2. The presence of similar amounts of overexpressed IRS-1 or IRS-2 is demonstrated in the lower panel of Fig. 3***A***, while Fig. 3***B*** shows the autophosphorylation and amount of HIR and the mutant.

Another important substrate of HIR is the adapter protein SHC, which was also analyzed for tyrosine phosphorylation in this system (Fig. 4). When overexpressed in 293 cells, SHC was not phosphorylated by the endogenous HIR but was strongly and constitutively tyrosine phosphorylated when co-overexpressed with HIR. Furthermore, SHC was also phosphorylated by the COOH-terminal deletion mutant and most of the serine-to-alanine mutants of HIR but not the juxtamembrane deletion or the HIR1177/78/82 mutant. The overexpression of the proteins encoded by the transfected plasmids was verified by reblotting the filter with antibodies against SHC and HIR. We conclude that in addition to IRS-1 and IRS-2, SHC is also phosphorylated to a lesser extent by the HIR1177/78/82 mutant in the transient overexpression system.

Because transient overexpression systems may pose problems in the specificity of protein–protein interactions, we next tried to repeat our results in established cell lines. We transfected NIH3T3 cells with expression plasmids encoding either the wild-type HIR or the mutants with a juxtamembrane deletion or serine exchanges. Cell colonies were isolated and tested for the expression of the transfected HIR type, and clones with similar expression levels were selected. In addition, a pool of HIR1177/78/82 overexpressing 3T3 cells was also established. The different cell lines were grown to confluence, stimulated with insulin or left untreated, lysed, and endogenous IRS-1 immunoprecipitated and analyzed for tyrosine phosphorylation, as described previously. The filter was then tested for the presence of similar amounts of IRS-1 (Fig. 5, lower panel). As shown in the upper panel, in established cell lines, the phosphorylation of IRS-1 was strictly dependent on insulin stimulation and was much reduced in cell lines overexpressing the juxtamembrane deletion mutant or HIR1177/78/82 but not wild-type HIR or other serine mutants.

Because a reduced phosphotyrosine content of IRS-1 should lead to a reduced association of the regulatory subunit of the PI 3-kinase, we evaluated the associated amount of PI 3-kinase in our cell lines. Again, cells were grown to confluence and stimulated with insulin, lysed, and IRS-1 immunoprecipitated. The PI 3-kinase activity was determined in the

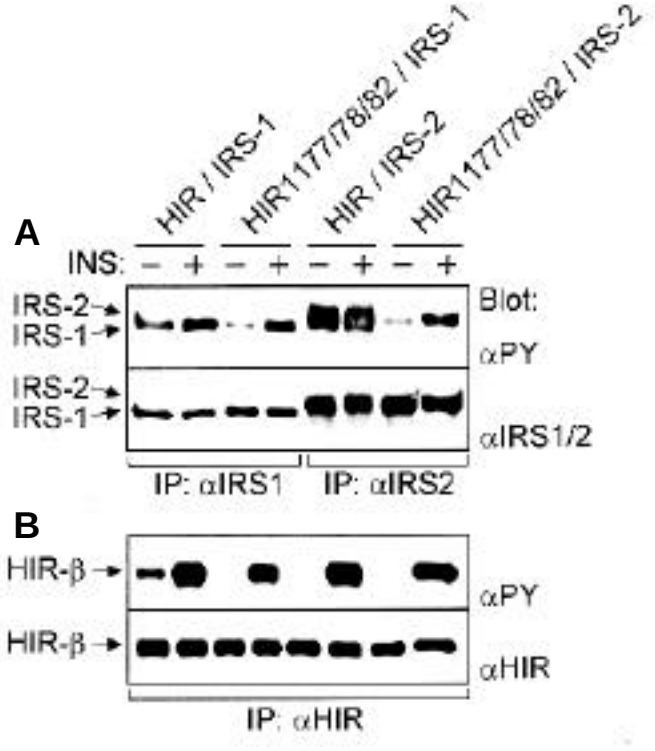

FIG. 3. Comparison of tyrosine phosphorylation in IRS-1 or IRS-2 cotransfections. Cells overexpressing IRS-1 or IRS-2 together with HIR or the mutant receptor HIR1177/78/82 were stimulated with 10^{-7} mol/l insulin at 37°C for 5 min. *A*: Cells were lysed, and IRS-1/IRS-2 proteins were immunoprecipitated. After separation by SDS-PAGE and protein transfer to nitrocellulose, filters were blotted with anti-phosphotyrosine antibodies (αPY) (upper panel) and reprobed with αIRS-1 or αIRS-2 antibodies (lower panel). *B*: Lysates were immunoprecipitated with antibody 83-14 directed against the α-subunit of the insulin receptor. After separation of immunoprecipitates by SDS-PAGE, proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine (upper panel) and reprobed with anti-HIR antibodies (lower panel). INS, insulin.

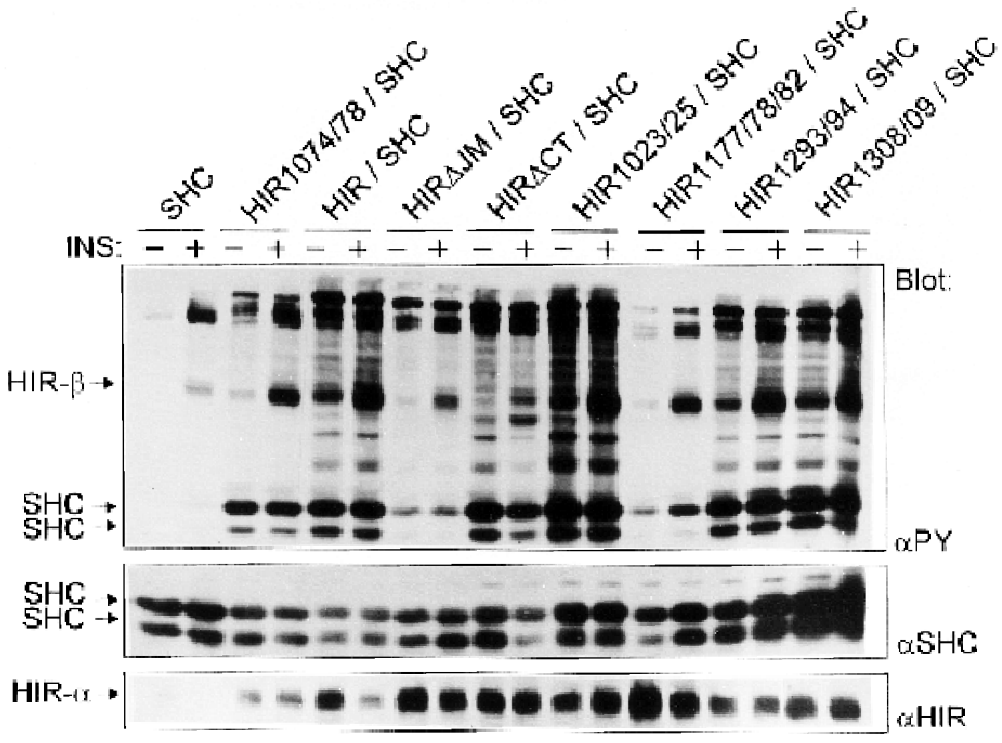

FIG. 4. Insulin-induced tyrosine phosphorylation pattern in SHC cotransfections. HEK 293 cells overexpressing SHC alone or together with the HIR or mutant receptors were stimulated with insulin, cells were lysed, and proteins were separated by SDS-PAGE. After protein transfer to nitrocellulose, the filters were blotted with antibodies against phosphotyrosine (αPY) (upper panel). Similar amounts of overexpressed proteins were monitored with antibodies against SHC and HIR (lower panels). HIRΔCT, HIR with COOH-terminal deletion; INS, insulin.

immunoprecipitate in an in vitro assay (Fig. 6***A***). Clearly, the insulin signal through the wild-type HIR produced a much stronger response than the juxtamembrane deletion or the HIR1177/78/82 mutants. To quantify this observation, we analyzed 4 independent experiments by scanning densitometry and set the PI 3-kinase activity in insulin-stimulated wild-type HIR transfected cells as 100% (Fig. 6***B***). Stimulation of the mutated receptor HIR1177/78/82 led to a significantly reduced PI 3-kinase activity that associates with IRS-1 (60% of HIR wild-type, $P = 0.003$). These results indicate that the insulin receptors behaved similarly in the transient expression system and in established cell lines and that the HIR1177/78/82 mutant was not capable of mediating a full insulin response.

Although the HIR1177/78/82 mutant could autophosphorylate to a similar extent as the wild-type receptor, the phosphorylation of 3 different substrates was impaired. We therefore asked whether low substrate kinase activity is a general feature of the mutation and performed an in vitro kinase assay. Wild-type HIR and HIR1177/78/82 mutant were transiently overexpressed in 293 cells, and the cells were stimulated with insulin or left untreated, lysed, and subjected to the assay using recombinant IRS-1. As shown in Fig. 7, the mutant receptor was unable to phosphorylate IRS-1 to a significant extent. Therefore, the kinase of HIR1177/78/82 is capable of producing a normal signal for autophosphorylation but cannot transmit the insulin signal efficiently.

Finally, we addressed the question of whether the phosphorylation pattern of the mutated receptor is different from the wild-type insulin receptor. Therefore, we performed in vivo labeling studies in which the metabolically [^{32}P]-labeled insulin receptor was isolated from transfected HEK 293 cells by immunoprecipitation. Subsequent to SDS-PAGE, the β-subunit was isolated and digested with trypsin, and the resulting phosphopeptides were analyzed by reversed-phase HPLC. When comparing insulin-stimulated autophosphorylation with basal phosphorylation of the wild-type insulin receptor β-subunit (Fig. 8***A***), an increased phosphorylation after insulin stimulation was found in several fractions (fraction 7, 11, 14, 31, 35, and 40). Analogous to results from White et al. (24), the interval between fractions 5 and 22 of this phosphopeptide pattern corresponds to the phosphopeptides of the regulatory loop

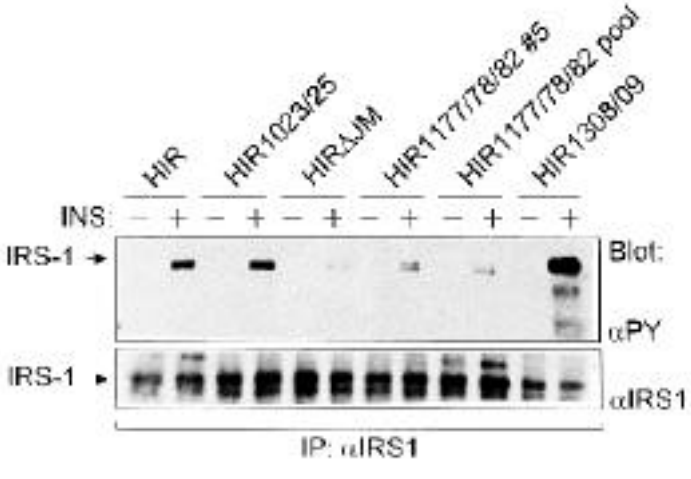

FIG. 5. Insulin-induced tyrosine phosphorylation of IRS-1 in NIH3T3 cells. Cell lysates were prepared from insulin-stimulated NIH3T3 cells overexpressing the different insulin receptor constructs. Endogenous IRS-1 was immunoprecipitated and analyzed for tyrosine phosphorylation (αPY) (upper panel). The presence of similar amounts of IRS-1 was verified by reprobing the filter with anti–IRS-1 antibodies (lower panel). INS, insulin.

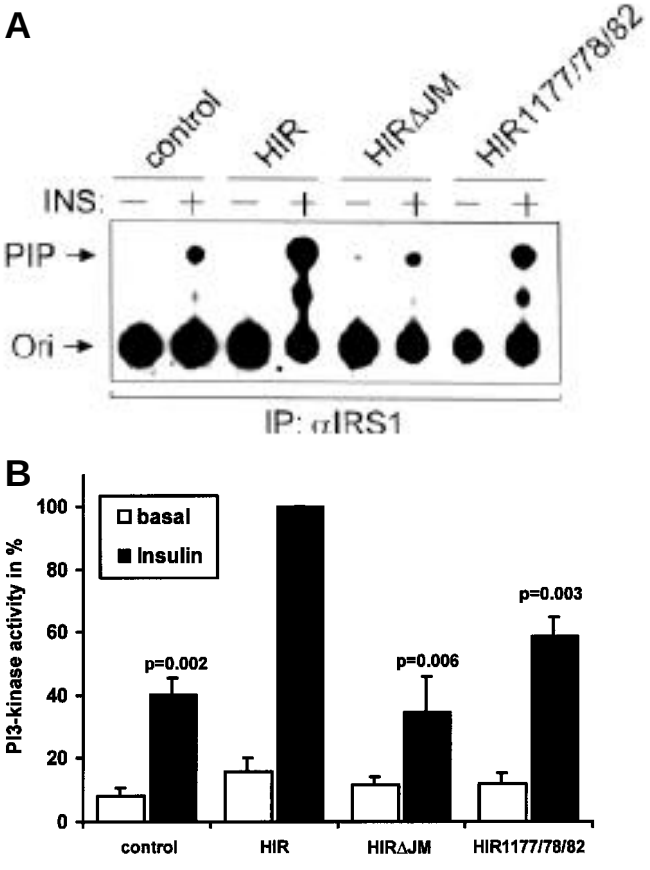

FIG. 6. PI 3-kinase activity in anti–IRS-1 immunoprecipitates from NIH3T3 cells. NIH3T3 cells overexpressing HIR, HIRΔJM, or HIR1177/78/82 were stimulated with insulin or left untreated, lysed, and incubated with anti–IRS-1 antibodies. PI 3-kinase activity was assayed in the immunoprecipitates, and [^{32}P]phosphate incorporation into phosphatidylinositol was visualized by separation of the labeled lipids on thin-layer chromatography plates and autoradiography (*A*). *B*: Densitometric scanning of 4 independent experiments. The insulin-stimulated PI 3-kinase activity from HIR wild-type overexpressing cells was set as 100%. Statistical analysis was performed with a Student's *t* test, and *P* values for significance level are shown. INS, insulin; IP, immunoprecipitation; Ori, the origin; PIP, phosphatidylinositol 3-phosphate.

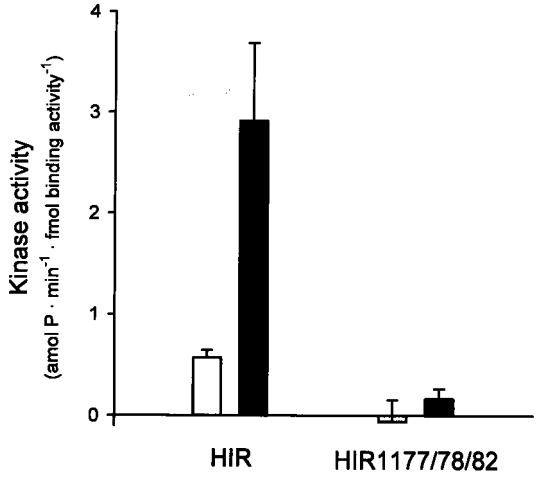

FIG. 7. In vitro kinase activity of the insulin receptor mutant. Cells overexpressing the wild-type or mutant insulin receptor were stimulated with insulin (□, control; ■, 10^{-7} mol/l) and lysed, and cell lysates were added to microwells coated with anti-HIR antibody. After insulin receptor immobilization, kinase reactions with recombinant IRS-1 were carried out. Kinase activity was normalized for insulin binding activity measured in the same well. The means of 2 different cell preparations analyzed in duplicate in 1 experiment are shown. P, phosphate.

where the major insulin-stimulated phosphate incorporation occurs. In Fig. 8*B*, the HPLC profiles after insulin stimulation of the wild-type HIR and the HIR1177/78/82 mutant are compared. Insulin stimulation led to an identical phosphopeptide pattern of both receptor proteins. Thus, we conclude that substitution of serines did not change the ^{32}P incorporation into any of the peptides of the regulatory loop.

DISCUSSION

In the present study, we have investigated the effects of serine to alanine mutations on the signaling of HIR. We have identified serine residues 1177/78/82 as dispensable for receptor autophosphorylation but required for substrate phosphorylation. In 3 different experimental systems, i.e., transient expression, established cell line, and in vitro, the mutant receptor was not capable of phosphorylation of HIR substrates or initiating events further downstream but was responsive to insulin and fully autophosphorylated. Furthermore, the in vivo labeling studies suggest that the autophosphorylation cascade of the mutated receptor is unchanged.

Several mutations in the intracellular part of the β-subunit of HIR have been described that were found in diabetic patients (25). Most of these mutations affect both autophosphorylation and the substrate phosphorylation and thus have a general effect on the kinase activity. Cocozza et al. (26) have described the exchange of Arg 1152 to Gln, which is still capable of autophosphorylation but not of in vitro substrate phosphorylation. In other studies, however, this mutant receptor was described to be impaired in autophosphorylation and to have a high constitutive kinase activity (27). Therefore, it is not clear whether this mutant is similar to HIR1177/78/82.

Like the mutations with a general effect on kinase, the exchanged Arg 1152 residue lies in a region that is important for catalytic activity, whereas the serine mutations used in this study are found COOH-terminally of the activation loop (28). Nevertheless, serine 1178 is conserved in tyrosine kinases and supposed to stabilize the kinase structure (28). It is therefore conceivable that the mutation of serine 1178 is the one that is causal for the impaired substrate phosphorylation and that this mutation interferes with substrate phosphorylation. A computer search for possible consensus sites for serine/threonine phosphorylation revealed that serine 1177 is a putative target for casein kinase 2 (CK-2). This finding creates the possibility that phosphorylation of serine 1177 could interfere with the suggested hydrogen binding of serine 1178 to threonine 1175 and thus also destabilize the kinase. Experiments are ongoing to investigate whether CK-2 can phosphorylate this residue of HIR and whether this phosphorylation has an effect on substrate phosphorylation.

From our in vivo labeling experiments, we conclude that the phosphorylation of all major phosphopeptides is identical and that the serine substitutions have no influence on the autophosphorylation cascade. Furthermore, we suggest that 1177, 1178, and 1182 are not major phosphorylation sites, since we could not detect any phosphopeptide in wild-type HIR lost in the HIR1177/78/82 preparations. However, it is still possible that these sites are minor phosphorylation sites that

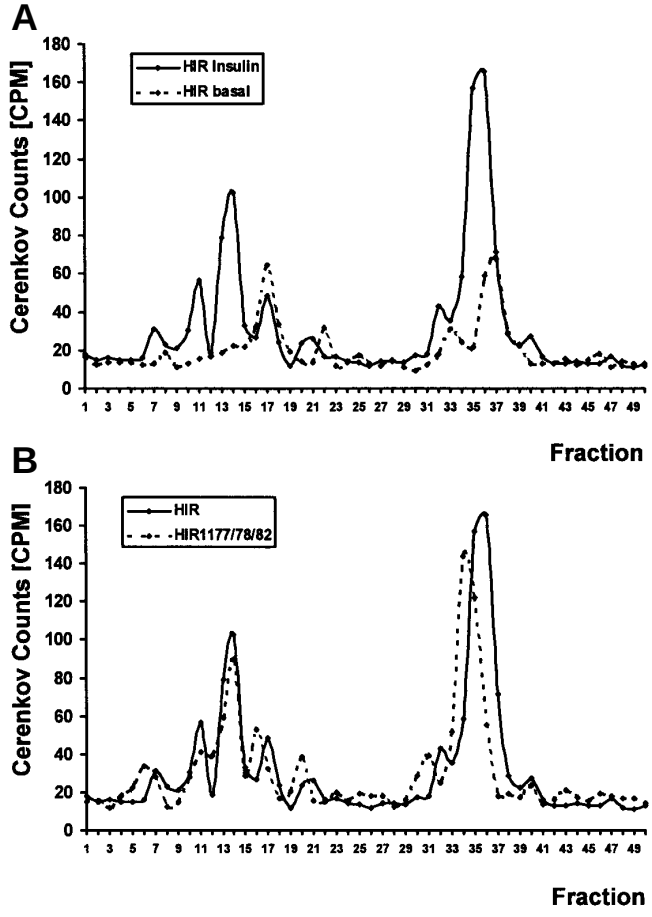

FIG. 8. Comparison of the in vivo phosphorylation pattern of HIR wild-type and HIR1177/78/82. The insulin receptor was isolated from transfected ^{32}P metabolically labeled HEK 293 cells by immunoprecipitation. Subsequent to SDS-PAGE, the β-subunit was isolated and digested with trypsin, and the resulting phosphopeptides were analyzed by reversed-phase HPLC. *A* represents the phosphopeptide pattern of the basal and insulin-stimulated wild-type HIR; *B* shows identical insulin-stimulated profiles of HIR versus mutated HIR1177/78/82.

were not detectable by this experimental approach. The decreased signal capacity of HIR1177/78/82 might therefore be due to an impaired phosphate transfer to its substrate.

In conclusion, we have identified a new type of mutation in HIR that permits autophosphorylation but interferes with substrate phosphorylation.

REFERENCES

1. White MF, Haring HU, Kasuga M, Kahn CR: Kinetic properties and sites of autophosphorylation of the partially purified insulin receptor from hepatoma cells. ***J Biol Chem*** 259:255–264, 1984
2. Miele C, Caruso M, Calleja V, Auricchio R, Oriente F, Formisano P, Condorelli G, Cafieri A, Sawka-Verhelle D, VanObberghen E, Beguinot F: Differential role of insulin receptor substrate (IRS)-1 and IRS-2 in L6 skeletal muscle cells expressing the Arg1152 → Gln insulin receptor. ***J Biol Chem*** 274:3094–3102, 1999
3. Wolf G, Trub T, Ottinger E, Groninga L, Lynch A, White MF, Miyazaki M, Lee J, Shoelson SE: PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. ***J Biol Chem*** 270:27407–27410, 1995
4. Kasuga M, Zick Y, Blith DL, Karlsson FA, Haring HU, Kahn CR: Insulin stimulation of phosphorylation of the beta subunit of the insulin receptor: formation of both phosphoserine and phosphotyrosine. ***J Biol Chem*** 257:9891–9894, 1982
5. Lewis RE, Cao L, Perregaux D, Czech MP: Threonine 1336 of the human insulin receptor is a major target for phosphorylation by protein kinase C. ***Biochemistry*** 29:1807–1813, 1990
6. Lewis RE, Wu GP, MacDonald RG, Czech MP: Insulin-sensitive phosphorylation of serine 1293/1294 on the human insulin receptor by a tightly associated serine kinase. ***J Biol Chem*** 265:947–954, 1990
7. Coghlan MP, Pillay TS, Tavare JM, Siddle K: Site-specific anti-phosphopeptide antibodies: use in assessing insulin receptor serine/threonine phosphorylation state and identification of serine-1327 as a novel site of phorbol ester-induced phosphorylation. ***Biochem J*** 303:893–899, 1994
8. Al-Hasani H, Eisermann B, Tennagels N, Magg C, Passlack W, Koenen M, Muller WD, Meyer HE, Klein HW: Identification of Ser-1275 and Ser-1309 as autophosphorylation sites of the insulin receptor. ***FEBS Lett*** 400:65–70, 1997
9. Liu F, Roth RA: Identification of serines-1035/1037 in the kinase domain of the insulin receptor as protein kinase C alpha mediated phosphorylation sites. ***FEBS Lett*** 352:389–392, 1994
10. Liu F, Roth RA: Identification of serines-967/968 in the juxtamembrane region of the insulin receptor as insulin-stimulated phosphorylation sites. ***Biochem J*** 298:471–477, 1994
11. Feener EP, Backer JM, King GL, Wilden PA, Sun XJ, Kahn CR, White MF: Insulin stimulates serine and tyrosine phosphorylation in the juxtamembrane region of the insulin receptor. ***J Biol Chem*** 268:11256–11264, 1993
12. Berti L, Mosthaf L, Kroder G, Kellerer M, Tippmer S, Mushack J, Seffer E, Seedorf K, Haring HU: Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. ***J Biol Chem*** 269:3381–3386, 1994
13. Bossenmaier B, Mosthaf L, Mischak H, Ullrich A, Haring HU: Protein kinase C isoforms beta 1 and beta 2 inhibit the tyrosine kinase activity of the insulin receptor. ***Diabetologia*** 40:863–866, 1997
14. Kellerer M, Mushack J, Seffer E, Mischak H, Ullrich A, Haring HU: Protein kinase C isoforms alpha, delta and theta require insulin receptor substrate-1 to inhibit the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (HEK 293 cells). ***Diabetologia*** 41:833–838, 1998
15. Mosthaf L, Berti L, Kellerer M, Mushack J, Seffer E, Bossenmaier B, Coghlan M, Siddle K, Ullrich A, Haring HU: C-terminus or juxtamembrane deletions in the insulin receptor do not affect the glucose-dependent inhibition of the tyrosine kinase activity. ***Eur J Biochem*** 227:787–791, 1995
16. Strack V, Bossenmaier B, Stoyanov B, Mosthaf L, Kellerer M, Lammers R, Häring HU: The inhibitory effect of 2-deoxyglucose on insulin receptor autophosphorylation does not depend on known serine phosphorylation sites or other conserved serine residues of the receptor β-subunit. ***FEBS Lett*** 449:111–114, 1999

16a. Strack V, Hennige AM, Krützfeldt J, Bossenmaier B, Klein HH, Kellerer M, Lammers R, Häring HU: Serine residues 994 and 1023/25 are important for insulin receptor kinase inhibition by protein kinase C isoforms β2 and θ. ***Diabetologia*** 43:443–449, 2000

17. Kunkel TA, Roberts JD, Zakour RA: Rapid and efficient site-specific mutagenesis without phenotypic selection. ***Methods Enzymol*** 154:367–382, 1987
18. Lammers R, Bossenmaier B, Cool DE, Tonks NK, Schlessinger J, Fischer EH, Ullrich A: Differential activities of protein tyrosine phosphatases in intact cells. ***J Biol Chem*** 268:22456–22462, 1993
19. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. ***Mol Cell Biol*** 7:2745–2752, 1987
20. Miller AD, Rosman GJ: Improved retroviral vectors for gene transfer and expression. ***Biotechniques*** 7:980–990, 1989
21. Landau NR, Littman DR: Packaging system for rapid production of murine leukemia virus vectors with variable tropism. ***J Virol*** 66:5110–5113, 1992
22. Morgan SJ, Smith AD, Parker PJ: Purification and characterization of bovine brain type I phosphatidylinositol kinase. ***Eur J Biochem*** 191:761–767, 1990
23. Klein HH, Kowalewski B, Drenckhan M, Fehm HL: Insulin stimulation of human adipocytes activates the kinase of only a fraction of the insulin receptors. ***Am J Physiol*** 272:E576–E583, 1997
24. White MF, Shoelson SE, Keutmann H, Kahn CR: A cascade of tyrosine autophosphorylation in the β-subunit activates the phosphotransferase of the insulin receptor. ***J Biol Chem*** 263:2969–2980, 1988
25. Krook A, O'Rahilly S: Mutant insulin receptors in syndromes of insulin resistance. ***Baillieres Clin Endocrinol Metab*** 10:97–122, 1996
26. Cocozza S, Porcellini A, Riccardi G, Monticelli A, Condorelli G, Ferrara A, Pianese L, Miele C, Capaldo B, Beguinot F: NIDDM associated with mutation in tyrosine kinase domain of insulin receptor gene. ***Diabetes*** 41:521–526, 1992
27. Formisano P, DeNovellis G, Miele C, Tripodi F, Caruso M, Palumbo G, Beguinot L, Beguinot F: Internalization of the constitutively active arginine 1152 → glutamine insulin receptor occurs independently of insulin at an accelerated rate. ***J Biol Chem*** 269:16242–16246, 1994
28. Hubbard SR, Wei L, Ellis L, Hendrickson WA: Crystal structure of the tyrosine kinase domain of the human insulin receptor. ***Nature*** 372:746–754, 1994