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Impact of Mutations at Different Serine Residues on the Tyrosine Kinase Activity of the Insulin Receptor

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Insulin binding to its receptor activates a cascade of signaling events which are initiated by tyrosine autophosphorylation of the receptor and activation of the tyrosine kinase activity towards the insulin receptor substrates. In addition to phosphorylation at tyrosine residues a serine phosphorylation of the insulin receptor is observed. Neither the functional significance of serine phosphorylation of the receptor nor the location of relevant regulatory sites has been determined exactly so far. We studied potential functions of serine residues in human insulin receptor (HIR) with respect to its ability to undergo insulin stimulated autophosphorylation. Using site directed mutagenesis of HIR we exchanged serine to alanine at 13 different positions in the HIR β-subunit. Sites were chosen according to the criteria of known serine phosphorylation sites (1023/25, 1293/94, 1308/09), conserved positions in hIR, hIGF-1 receptor, hIRR, and dIR (962, 994, 1037, 1055, 1074/78, 1168, 1177/78/82, 1202, 1263, 1267). All HIR mutants were expressed in HEK 293 cells and basal and insulin stimulated autophosphorylation were determined. We found that the exchange of serine to alanine at position 994 and at position 1023/25 increased insulin stimulated receptor autophosphorylation significantly (147 % $\pm$ 12% and 129% $\pm$ 6% of control, $p<0.01$, $n=7$), while all other exchanges did not significantly alter insulin stimulated HIR autophosphorylation. The data suggest that the serine residues at position 994 as well as 1023/25 might be part of inhibitory domains of the insulin receptor.

The initial events of cellular insulin signaling include binding of the hormone to the α-subunit of the receptor, autophosphorylation of the receptor β-subunit at six defined tyrosine residues and activation of the tyrosine kinase activity towards substrates of the receptor [1]. For coupling and phosphorylation of these substrates presently including IRS-1, IRS-2, IRS-3 and Shc the juxtamembrane region of the β-subunit around the tyrosine residue 960 is of major importance. [2-5]. Beside tyrosine phosphorylation of the insulin receptor, phosphorylation on serine residues is observed both in intact cells [6-8] and in vitro [9,10]. Kinetic studies in intact cells have shown that serine phosphorylation of the receptor occurs after tyrosine phosphorylation [11]. The functional significance of serine phosphorylation of the insulin receptor is not understood in all details. It is unclear which serine kinase is responsible for serine phosphorylation of the insulin receptor. Candidates include different PKC isoforms [12,13], PI3-kinase which was shown to phosphorylate IRS-1 on serine residues [14,15] and a serine kinase activity which appears to be intrinsic to the insulin receptor [16,17]. Several positions in the insulin receptor β-subunit were identified as serine phosphorylation sites. These include serine residues in the juxtamembrane region (955/56) [18] as well as the serine residues at positions 1023/25 [19], 1293/94 [20,21] and 1309 [22]. The serines at position 1023/25 were identified as a phosphorylation site for PKCα [19]. We speculated that further positions at the insulin receptor β-subunit might be of functional relevance. To evaluate this hypothesis we choose further candidate serine residues. As outlined in an earlier publication of Danielsen et al. [23] a number of serine residues are found in positions which are conserved in human IR, human IGF-1 receptor, human insulin related receptor and drosophila IR, which all are members of the insulin receptor family. Based on this criterion we prepared mutants of HIR where serine was

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Abbreviations: dIR, drosophila insulin receptor; DMEM, Dulbecco's modified eagle medium; FCS, foetal calf serum; HEK, human embryonic kidney; hIGF-1, human insulin like growth factor-1; HIR, human insulin receptor; hIRR, human insulin related receptor; Hepes, N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid; IRS, insulin receptor substrate; PKC, protein kinase C.

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exchanged to alanine at the following positions: 962, 994, 1037, 1055, 1074 (with the neighboring serine 1078), 1168, 1178/1182 (with the neighboring serine 1177), 1202, 1263, and 1267. Furthermore we prepared HIR mutants of the known phosphorylation sites including 1023/1025, 1293/1294, 1309 (with the neighboring serine 1308). We overexpressed these mutants in HEK 293 cells and studied basal and insulin stimulated autophosphorylation.

MATERIALS AND METHODS

Materials. Cell culture reagents and fetal calf serum were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, phenylmethylsulfonyl fluoride, Na_3VO_4, Triton X-100, and dithiothreitol were from Sigma (Munich, Germany). The reagents for SDS-PAGE and Western blotting were obtained from Roth (Karlsruhe, Germany) and Biorad (Munich, Germany). Nitro-cellulose was from Schleicher & Schuell (Dassel, Germany). All other reagents were of the best grade commercially available. Visualization of immunocomplexes after Western blotting was performed with the non-radioactive enhanced chemiluminescence system (ECL) and the Hyperfilm-ECL from Amersham Buchler (Braunschweig, Germany).

The cDNA for the wild-type receptor (HIR-wt) and the polyclonal rabbit antibody against insulin receptor β-subunit (CT104/anti-β-subunit) were gifts from Axel Ullrich (Max-Planck-Institute, Martinsried, Germany). The monoclonal mouse antibody against phosphorylated tyrosine residues (αPtyr) were from Leinco Technologie Inc. (Ballwin, USA).

Preparation of serine to alanine point mutants of the insulin receptor. All point mutations were prepared by the method of Kunkel [24] in a CMV promoter-based vector pRK-5 [25] containing the cDNA sequence for the wild type human insulin receptor. The mutagenic oligos were purchased from Boehringer Mannheim (Mannheim, Germany). Introduced point mutations were verified by DNA-sequencing using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, USA).

Transient expression of HIR mutants. Human embryonic kidney fibroblast 293 cells (ATCC CRL 1573) were grown in Dulbecco's MEM/Nutrient Mix F12 medium supplemented with 10 % (v/v) fetal calf serum. A total of 4 μg plasmid DNA was transfected per semiconfluent 35-mm diameter dish according to the protocol of Chen and Okayama [26]. Briefly, cells were grown in 6-well dishes at a density of 10^5 cells per well in 2 ml of medium. 4 μl supercoiled plasmid DNA (1 μg/μl) was mixed with 96 μl of 0.25 M $CaCl_2$. To this was added equal amount of 2X transfection buffer (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) 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 DMEM (1000 mg/l glucose) containing 2 mM glutamine.

Stimulation and cell lysis. Serum starved cells were stimulated with or without 10^{-7} M insulin for 5 min at 37°C and lysed in 0.8 ml ice-cold lysis buffer (50 mM Hepes pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin). Cleared crude cell lysates (20 min/12000g) were analyzed on 7.5 % SDS-PAGE (40 μl per lane).

Western blotting. Separated proteins were transferred to nitrocellulose membranes by semidry electroblotting (transfer buffer: 48 mM Tris/HCl pH 7.5; 0,004 % (w/v) SDS; 39 mM glycine; 20 % (v/v) methanol) After transfer, the membranes were blocked with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05 % (v/v) Triton X-100 and 0.25 % (w/v) gelatine, pH 7.4) for 1 h. Subsequently filters were incubated with the first antibody (α-Ptyr or CT104) overnight at 4°C. The membranes were washed four times with NET buffer before incubating with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by enhanced chemiluminescence (ECL).

RESULTS

Because of the high number of different mutants we designed experiments were five to seven mutants were compared with the wild type receptor in each experiment. Figure 1 shows a combination of three representative autoradiograms of HIR-wt and the different HIR-mutants. Receptor autophosphorylation was determined by immunoblots using phosphotyrosine antibodies (α-Ptyr), the amount of HIR expressed was quantified in immunoblots using antibodies against the C-terminus of the insulin receptor (CT104). This figure gives an impression about the expression level of the receptor, the tyrosine phosphorylation and the effect of insulin treatment (10^{-7} M, 5 min).

Basal and insulin stimulated autophosphorylation was quantified by scanning densitometry. The comparison of the different mutants with HIR-wt were performed always within the same experiment setting stimulated HIR-wt as 100 %. The level of tyrosine phosphorylation was normalized to the amount of loaded protein according to the re-blot with the receptor antibody (CT104). Figure 2 shows the result of the quantification (means of phosphorylation level $\pm$ S.E.M.). Serine to alanine exchange at position 994 and at 1023/25 caused a significant difference in insulin stimulated receptor autophosphorylation. (147 % $\pm$ 12 % and 129 % $\pm$ 6% of control, $p<0.01$, $n=7$). While the basal autophosphorylation of HIR-1023/25 was not significantly different from the wild type we observed that in the case of HIR-994 the basal phosphorylation as well was significantly increased compared to wild type (211 % $\pm$ 13 % of basal HIR-wt, $p<0.01$, $n=7$). All other HIR mutants showed no significant difference of basal or insulin stimulated autophosphorylation. The C-terminal mutants 1293/94 and 1308/08 showed a tendency to increased insulin stimulated autophosphorylation, however this effect did not reach statistical significance.

DISCUSSION

Our results support the earlier proposed significance of the serine residues 1023/25 as functionally important serine residues. Liu and Roth [19] have described that serine 1023/25 is a potential phosphorylation site for PKCα. According to our results it seems conceivable that these serine residues are located in a

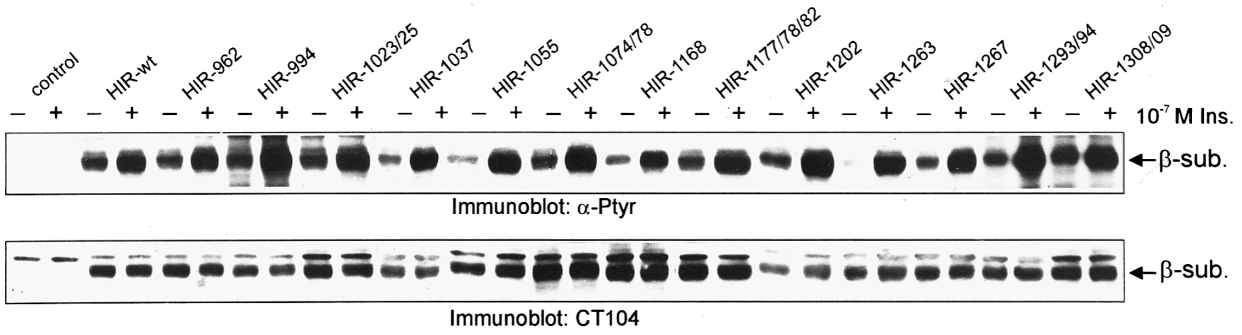

FIG. 1. Insulin-induced receptor autophosphorylation. Whole cell lysates were prepared from HEK 293 fibroblast. Cells overexpressing the wild-type receptor or the mutant receptors were stimulated with 10^{-7} M insulin at 37°C for 5 min. Proteins were separated by SDS-PAGE; Western blots were probed with antibodies against phosphotyrosine (α-Ptyr, upper panel). Equal amounts of insulin receptor were monitored with antibodies against the HIR-β-subunit (CT104, lower panel). Representative immunoblots of parallel experiments are shown (see text for details). The results were reproduced in seven different experiments.

domain which conserves an inhibitory effect as their removal causes an increase of the insulin stimulated tyrosine phosphorylation of this receptor mutant. It is interesting to note that with this mutation the basal autophosphorylation of the insulin receptor is not significantly altered suggesting that this serine residue modulates only a process which is involved in receptor activation by insulin leading to increased tyrosine kinase activity. In contrast to this the serine to alanine exchange at position 994 alters both basal and insulin stimulated tyrosine autophosphorylation. It is known that the insulin receptor is phosphorylated on serine residues already in the basal state i.e. in cells which were not stimulated with insulin. It is possible that serine 994 is an inhibitory site which is relevant already in the basal situation. The mechanism underlying the observed alterations of tyrosine phosphorylation for HIR-994 and HIR-1023/25 might be a phosphorylation of these serine residues. On the other hand a non-specific effect of the point mutation resulting in conformational change of the receptor molecule can not be completely ruled out. However, the observation that all other serine to alanine exchanges did not alter basal or insulin stimulated tyrosine phosphorylation significantly argues against the latter possibility.

The three-dimensional structure of the insulin recep-

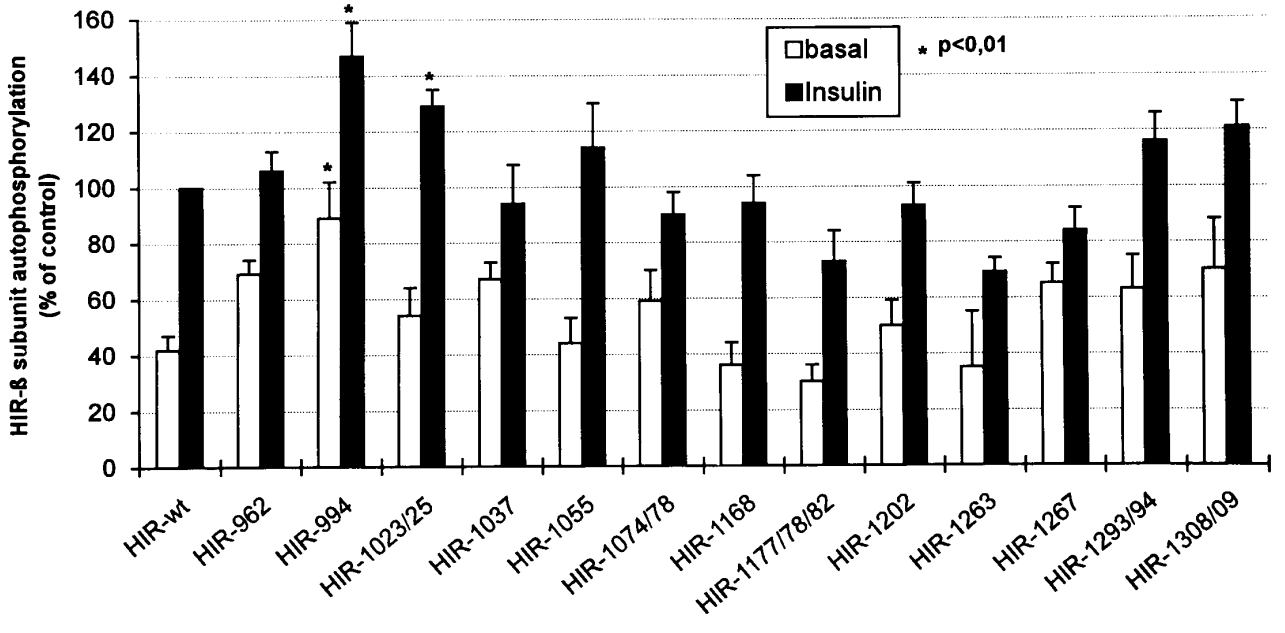

FIG. 2. Statistical analysis of insulin-induced receptor autophosphorylation. Basal and insulin stimulated levels of receptor autophosphorylation were measured by densitometric analysis. The values were compared with the mean value of stimulated wild type receptor taken as 100% (n=7, means ± S.E.M.). Asterisk indicates significance ($p<0.01$, Student's t-test).

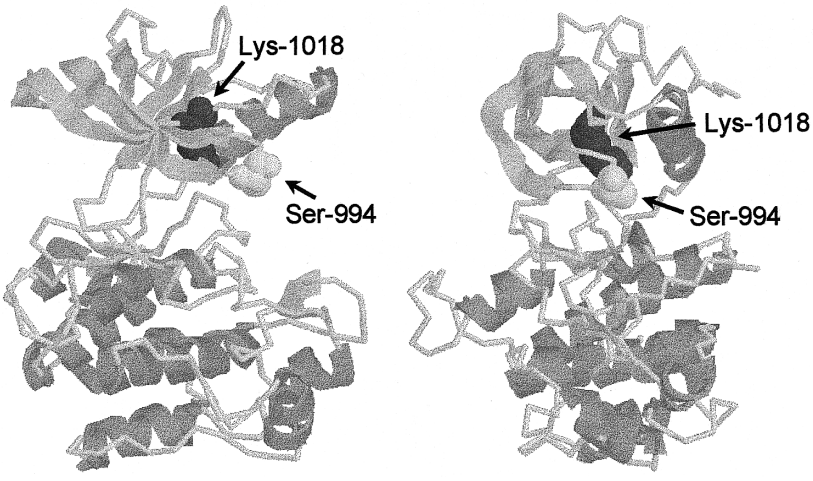

FIG. 3. Localization of serine 994 in the insulin receptor. Three-dimensional representation of the catalytic domain backbone of the IR-β-subunit created with RasMol [30] according to the crystal structure obtained by Hubbard et al. [27]. Serine 994 shown as van der Waals spheres is situated closely to the residue essential for ATP-binding, lysine 1018 [28]. Two different perspectives are shown.

tor [27] suggests that serine 994 is exposed on the surface of the receptor β-subunit (Figure 3) close to the ATP-binding domain around lysine 1018 [28]. Earlier studies have shown decreased ATP-binding capacity of the receptor after phorbol ester treatment, suggesting involvement of PKC-isoforms in the receptor attenuation [29]. Following the concept of receptor down regulation through serine phosphorylation, serine 994 might be involved in this regulatory process as a potential inhibitory site.

The present data therefore suggest that serine 994 is a regulatory serine residue located in an inhibitory domain of the insulin receptor. Further studies are required to evaluate the impact of all these point mutations for the interaction of the insulin receptor with its substrates and to determine whether these serine residues are targets for regulatory serine kinases or whether they are important for interaction of the insulin receptor with other regulatory proteins.

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