A Single Substitution of the Insulin Receptor Kinase Inhibits Serine Autophosphorylation in Vitro: Evidence for an Interaction between the C-Terminus and the Activation Loop†

Volker Noelle,‡ Norbert Tennagels,§ and Helmut W. Klein*

Institute of Biochemistry, University of Cologne, Otto-Fischer-Strasse 12-14, D-50674 Cologne, Germany

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ABSTRACT: We examined the effects of mutations of tyrosine and serine autophosphorylation sites on the dual specificity of the insulin receptor kinase (IRKD) in vitro using autophosphorylation and substrate phosphorylation and phosphopeptide mapping. For comparable studies, the recombinant kinases were overexpressed in the baculovirus system, purified, and analyzed. The phosphate incorporation into the enzymes was in the range of 3−4.5 mol/mol, and initial velocities of autophosphorylation were reduced up to 2-fold. However, the mutation Y1151F in the activation loop inhibited phosphate incorporation in the C-terminal serine residues 1275 and 1309, due to a 10-fold decrease of the initial velocity of serine autophosphorylation. Although the K_M and V_{MAX} values of this mutant were only slightly altered in substrate phosphorylation reactions using a recombinant C-terminal insulin receptor peptide (K_M: Y1151F, 9.9 ± 0.4 μM; IRKD, 6.1 ± 0.2 μM; V_{MAX}: Y1151F, 72 ± 4 nmol min^{-1} mg^{-1}; IRKD, 117 ± 6 nmol min^{-1} mg^{-1}), diminished phosphate incorporation into serine residues of the peptide was observed. In contrast, the phosphorylation of a recombinant IRS-1 fragment, which was shown to be phosphorylated markedly on serine residues by IRKD, was not affected by any kinase mutation. These results underline that IRKD is a kinase with dual specificity. The substrate specificity toward C-terminal serine phosphorylation sites can be modified by a single amino acid substitution in the activation loop, whereas the specificity toward IRS-1 is not affected, suggesting that the C-terminus and the activation loop interact.

The insulin receptor kinase (IRKD)[1] is a 45 kDa soluble protein that contains the cytosolic part of the insulin receptor [R941−S1343, according to Ullrich et al. (*1*)]. IRKD has comparable catalytic properties and identical phosphorylation sites to the native holoreceptor (*2*−*9*). Therefore, IRKD is an established model for the human insulin receptor (IR), especially for studies of the phosphorylation mechanism in vitro. While tyrosine autophosphorylation and phosphorylation of insulin receptor substrates are well studied, less is known about the mechanism of the serine kinase activity of IRKD. In vivo, serine/threonine phosphorylation of the IR is thought to be caused by exogenous serine kinases, mainly PKC (*10*−*16*). However, a serine kinase activity coupled to the IR and the IRKD in vitro has been observed by several groups (*17*−*19*). Attempts to isolate a copurified exogenous serine kinase failed, and the activity was described to be intrinsic to the insulin receptor itself (*20*, *21*). Recently we have shown that IRKD produced by baculovirus expression autophosphorylates in vitro on two serine residues in the C-terminus, S1275 and S1309, whereas a kinase-inactive mutant was not phosphorylated at all (*8*). Serine autophosphorylation reached 25% of total phosphate incorporation, corresponding to 1−1.2 mol of serine phosphate/mol of enzyme.

Tyrosine autophosphorylation sites have been reported to affect the serine/threonine phosphorylation of the IR. Substitution of Y1150 abolished threonine phosphorylation in vivo, while serine phosphorylation was still apparent in vivo and in vitro after the replacement (*22*). Feener et al. (*23*) observed serine phosphorylation outside the juxtamembrane region which was dependent on Y1151 in vitro. Although tyrosine residues may play an important role in the activation and the dual specificity of the kinase, the crystal structure of the unphosphorylated and the trisphosphorylated form of the insulin receptor kinase (*24*, *25*) did not explain the mechanism by which serine phosphorylation might occur. In the structure of the phosphorylated and activated kinase, Y1150 and Y1151 form hydrogen bonds (Y1150: to R1152; Y1151: to R1143 and to the backbone amide nitrogen of G1154), thus stabilizing the activation loop.

Generally, the role of serine/threonine phosphorylation in the cell is to control enzyme activities. It is not clear whether this is also the case for the IR. Serine phosphorylation of insulin receptor substrates seems to inhibit tyrosine phosphorylation of the substrate and therefore results in a desensitization of the insulin signal (*26*, *27*). This was affirmed by the mutation of four serine residues in the middle

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* To whom correspondence should be addressed. Tel.: (49)-221-4703208; Fax: (49)-221-4705066; Email: h-w.klein@uni-koeln.de.

‡ Present address: Miltenyi Biotec GmbH, Friedrich-Ebert-Str. 68, 51429 Bergisch Gladbach, Germany.

§ Present address: Aventis Pharma Deutschland GmbH, DG Metabolic Diseases, Bldg. H825, 65926 Frankfurt, Germany.

[1] Abbreviations: IRKD, soluble insulin receptor kinase; WT, wild-type IRKD; pT, phosphorylated threonine residue; pY, phosphorylated tyrosine residue.

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domain of IRS-1, which directly follow a YxxM sequence, resulting in a modulation of phosphatidylinositol-3′ kinase activity (*28*). In addition, it has been reported that Akt/PKB, MAP kinases, or glycogen synthase kinase-3 (GSK-3) phosphorylate IRS-1 on serine residues (*29*−*32*). Serine phosphorylation of the IR itself may also modulate signaling. However, the substitution of SS1308/1309AA did not alter IR autophosphorylation or 2-deoxyglucose-mediated inhibition of IR autophosphorylation in HEK 293 cells (*33, 34*).

To analyze the effects of autophosphorylated tyrosine residues on the serine kinase activity of IRKD, mutants with monosubstitutions in the kinase domain (Y1146, Y1150, and Y1151) and a triple mutant with substitutions in the C-terminus (Y1316 and Y1322) and the juxtamembrane domain (Y960) were characterized. For the function of serine phosphorylation with respect to autophosphorylation and transferase activity, an insulin receptor kinase with imitated phosphorylated residues, S1275 and S1309, was constructed by replacing these residues by aspartic acid residues. After expression in Sf9 cells in a baculovirus system, the kinases were purified by anion exchange and hydrophobic interaction chromatography. The recombinant enzymes were characterized in autophosphorylation and substrate phosphorylation reactions, and further investigated by HPLC phosphopeptide mapping and phosphoamino acid analysis.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham. Restriction endonucleases were from MBI Fermentas and Boehringer Mannheim, Pfu polymerase was from Stratagene, and ATP was from Boehringer Mannheim. Cell culture reagents were from Life Technologies Inc.; poly-L-lysine (M_r 15 000−30 000) was from Serva. For HPLC phosphopeptide maps, trypsin (sequencing grade) from Merck was used. Oligonucleotide primers were from Pharmacia Biotech. Other reagents were obtained from common commercial sources.

Construction of the Soluble Receptor Kinases. Construction of the IRKD was previously described (*8, 31*). *IRKD-Y46F, -Y50F, -Y51F, -Y3F, -S2D*: Mutations were achieved by using the QuikChange Site-directed Mutagenesis kit (Stratagene). Two mutagenesis primers each (Y3F: four) were used according to manufacturer's instructions (Y46F: GACCAGAGATATCTTTGAAACGGATTACTACC and GGTAGTAATCCGTTTCAAAGATATCTCTGGTC; Y50F: GACCAGAGATATCTATGAAACGGATTTCTACCGGAAAG and CTTTCCGGTAGAAATCCGTTTCATAGATATCTCTGGTC; Y51F: GACCAGAGATATCTATGAAACGGATTACTTCCGGAAAGG and CCTTTCCGGAAGTAATCCGTTTCATAGATATCTCTGGTC; Y3F: ACTGGCACTGAGAAACTCAGGGTTTGAAGAAGC and CAAACCCTGAGTTTCTCAGTGCCAGTGATG for Y960F, CAAGCGGAGCTTCGAGGAACACATTCCTTTCACACACATG and CATGTGTGTGAAAGGTATGTGTTCCTCGAAGCTCCGCTTG for YY1316/1322FF; S2D: GGCTCCCGAGGATGAGGAGCTCGAGATGGAGTTTG and ACTCCATCTCGAGCTCCTCATCCTCGGGAGCCTTG). The mutations were verified by sequencing.

Cotransfection of *Spodoptera frugiperda* (Sf9) cells and isolation of recombinant viruses were performed as described (*8, 35*).

Purification of Soluble Receptor Kinases. Purification of the soluble kinases was accomplished by sequential chromatography using ResourceQ and Phenyl-Sepharose (Pharmacia) as described (*8, 35*). Purified kinases were washed 3 times in 50 mM Tris/HCl, pH 7.5, 1 mM DTT, concentrated in Centricon C-30, and stored at −80 °C.

Construction and Expression of GST Fusion Proteins. (A) GST-CT. The cDNA encoding N1249−S1343 of the insulin receptor with the mutation C1296S was cloned into the GST expression vector pGEX-3X (Pharmacia). The fusion protein was expressed in *E. coli* DH5α for 3 h at 37 °C and solubilized in PBS buffer, 1 mM PMSF, 10 μg/mL leupeptin (*36*). After removal of cell debris by centrifugation, GST-CT was purified by affinity chromatography with glutathione−Sepharose (Pharmacia). The eluate was washed in 50 mM Tris/HCl, pH 7.5, concentrated in Centricon C-30, and stored at −80 °C.

(B) GST-CT-YY1316/1322FF and GST-CT-SS1275/1309DD. The mutations were achieved by using the QuikChange Site-directed Mutagenesis kit (Stratagene). Mutagenesis primers (CAAGCGGAGCTTCGAGGAACACATTCCTTTCACACACATG and CATGTGTGTGAAAGGTATGTGTTCCTCGAAGCTCCGCTTG for YY1316/1322FF; GGCTCCCGAGGATGAGGAGCTCGAGATGGAGTTTG and ACTCCATCTCGAGCTCCTCATCCTCGGGAGCCTTG for S1275D, along with CCGGGATGGAGGATCCGATCTGGGTTTCAAGC and CTTGAAACCCAGATCGGATCCTCCATCCCGG for S1309D) were used according to manufacturer's instructions. The mutations were verified by sequencing. Expression was the same as for GST-CT.

(C) GST-IRSp30. Construction of hIRSp30 was previously described (*37*). The cDNA encoding D516−P777 from hIRSp30 was cloned into pGEX-3X and expressed as above. The pGEX-3X plasmid containing hIRSp30 was a gift from D. Müller-Wieland, Cologne, Germany.

Phosphorylation Reactions. All phosphorylation reactions were carried out at room temperature (22 °C). The reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 μM poly-L-lysine, and 250 μM [γ-^{32}P]ATP. Unless otherwise indicated, the concentration of the kinase was 1 μM. The substrates were applied in concentrations of 2.5 μM (GST-IRSp30) and 5 μM (C-terminal substrates), respectively. The proteins were separated by SDS−PAGE and localized by autoradiography, and the radioactivity of the excised bands was determined by measurement of Cerenkov radiation in a Beckman scintillation counter.

Digestion with Trypsin. The autophosphorylated soluble kinases were eluted after SDS−PAGE as described (*8*). The recovery of ^{32}P typically exceeded 90%.

Separation of Tryptic Phosphopeptides (Phosphopeptide Mapping). Phosphopeptides were separated on a Beckman Gold HPLC system using an anion exchange column (Macherey-Nagel, Nucleogel SAX 1000-8/46) as described (*8*). Monitoring was followed by a HPLC radiometer 505TR from Packard with a 0.5 mL Cerenkov cell.

Phosphoamino Acid Analysis. Tryptic phosphopeptides were lyophilized in a Speed-Vac, hydrolyzed in 250 μL of 6 N HCl at 110 °C for 2 h, washed twice with water, and dried. Two-dimensional phosphoamino acid analysis was performed by electrophoresis as described by Boyle et al. (*38*). Quantification of phosphoamino acids was achieved by using a Phospho-Imager GS-363 (Bio-Rad).

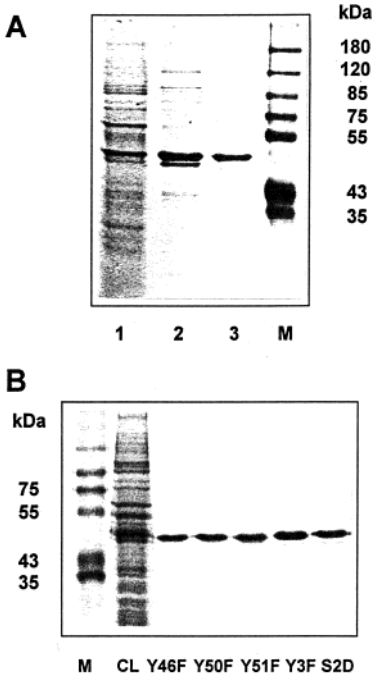

FIGURE 1: Purification of insulin receptor kinases. (A) Representative course of the purification of IRKD. The recombinant kinase was purified by anion exchange chromatography and hydrophobic interaction chromatography. After SDS−PAGE, the proteins were stained with a modified Coomassie stain. Lanes: 1, cell lysate; 2, after anion exchange chromatography; 3, after hydrophobic interaction chromatography; M, marker. (B) Overview of highly purified insulin receptor kinases by anion exchange chromatography and hydrophobic interaction chromatography. After SDS−PAGE, the kinases were stained with a modified Coomassie stain. CL, cell lysate; M, marker.

Determination of K_M Values and V_{MAX} Values. K_M values and V_{MAX} values were determined by Lineweaver−Burk, Hanes, Eadie−Hofstee, and parameter space plots using the computer program Hyper 1.1s (*39, 40*).

Other Procedures. *Spodoptera frugiperda* (Sf9) cells were maintained as described (*8, 35*). Protein concentrations were determined by a modified method of Bradford (*41*). SDS−PAGE was performed according to Laemmli (*42*). Protein staining after SDS−PAGE was carried out by a modified Coomassie stain according to Blakesley and Boezi (*43*).

RESULTS

Expression and Purification of IRKD Mutants. The soluble kinases of the insulin receptor were expressed in Sf9 cells using the baculovirus system. Immunological detection was carried out with antibodies against the C-terminus of the cytosolic parts of the IR (data not shown). The purification of mutated kinases was achieved by a two-step protocol using anion exchange chromatography and hydrophobic interaction chromatography (*8, 35*). Figure 1A shows the course of purification after SDS−PAGE for IRKD resulting in a highly purified enzyme (lane 3). The same purification protocol was applied to obtain the following kinase mutants: Y46F, Y50F, Y51F, monosubstitutions of tyrosine to phenylalanine residues in the kinase domain; Y3F, triple substitution of Y960, Y1316, and Y1322 to phenylalanine; and S2D, double mutation of S1275 and S1309 to aspartic acid residues (Figure 1B).

Effects of Mutations on IRKD Autophosphorylation. IRKD autophosphorylation occurs on tyrosine residues in three clusters: Y1146, Y1150, and Y1151 in the activation loop of the kinase domain; Y1316 and Y1322 in the C-terminus; and Y960 in the juxtamembrane domain. In the C-terminal domain, two serine autophosphorylation sites, S1275 and S1309, are located. To investigate the effect of the substitutions at these residues, the mutant insulin receptor kinases were autophosphorylated in the presence of [γ-^{32}P]ATP and separated by SDS−PAGE (Figure 2A). The result demonstrates that all mutant enzymes showed kinase activity. Phosphate incorporation after 30 min reaction time was measured (Figure 2B). The autophosphorylated IRKD was found to reach a stoichiometry of 4.6 ± 0.2 mol of phosphates/mol of enzyme, and due to the loss of tyrosine (and serine) phosphorylation sites, the mutations led to partial reductions in phosphate incorporation. Indeed, Y46F, Y50F, and S2D incorporated 1−2 mol of phosphate/mol of enzyme less than the wild-type kinase, as expected due to the loss of tyrosine or serine autophosphorylation site(s). The phosphate content of the triple mutant Y3F was comparable to Y46F and Y50F, although a further reduction was expected for Y3F. However, the substitution of Y1151 reduced the maximal phosphorylation only slightly (4.1 ± 0.3 mol/mol). In a comparison of the initial velocities of autophosphorylation, the velocities of Y46F, Y50F, and Y3F were in the range of 200−250 nmol min^{-1} mg^{-1} and therefore 2-fold lower than wild-type IRKD (WT: 430 ± 48 nmol min^{-1} mg^{-1}, measured after 10 s reaction time). The imitation of serine phosphorylation by aspartic acid residues (S2D) did not inhibit the initial autophosphorylation reaction of the enzyme (455 ± 43 nmol min^{-1} mg^{-1}). In contrast to Y46F and Y50F, Y51F showed only a slight reduction (371 ± 30 nmol min^{-1} mg^{-1}), suggesting that the tyrosine kinase activity was almost unaffected by this substitution.

For the analysis of the serine kinase activity, the serine phosphate content of the autophosphorylated enzymes was examined. It should be noted that phosphoserine and phosphotyrosine may exhibit different stabilities during acid hydrolysis of phosphopeptides (*44*). For the wild-type enzyme, 1.3 ± 0.2 mol of serine phosphate/mol of enzyme were detected. Similar values were obtained for Y46F, Y50F, and Y3F. Although two serine autophosphorylation sites in S2D were absent, the phosphate incorporation into serine residues was not abolished completely, suggesting that additional serine phosphorylation must be present. The initial velocity of serine autophosphorylation in S2D was reduced 10-fold to about 0.3−0.4 nmol min^{-1} mg^{-1} (Figure 2C), indicating that the remaining serine phosphorylation occurred on minor sites. Interestingly, the phosphate incorporation into serine residues of Y51F was also decreased about 3-fold, while the stoichiometry of tyrosine phosphorylation remained nearly unchanged. The decrease of the serine phosphate content was caused by a reduction of the initial velocity of serine phosphorylation in Y51F to a similar level to that observed for the S2D kinase. To analyze specific phosphorylation sites, HPLC phosphopeptide mapping was used as previously described [(*8*); Figure 2D]. For wild-type IRKD, the two phospho-signals of S1275 and S1309 were present. As expected, these signals were absent in the chromatographic profile of S2D; in addition, no specific peaks representing other serine phosphorylation sites could be

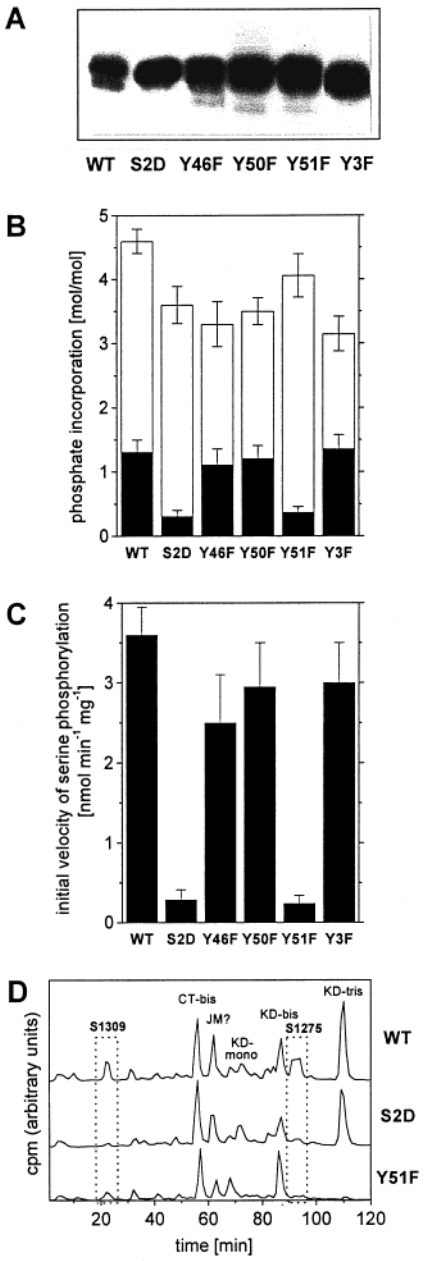

FIGURE 2: Effects on autophosphorylation of insulin receptor kinases by mutations. (A) Insulin receptor kinases were phosphorylated for 10 s to 30 min and separated by SDS−PAGE. The autoradiographies show the phosphate incorporation after 30 min (an analysis of individual phosphorylation reactions is shown). (B) The autophosphorylated kinases were characterized by phosphoamino acid analysis. Phosphate incorporation is expressed as moles of phosphate per mole of enzyme. White bars, tyrosine phosphate; black bars, serine phosphate. (C) Initial velocities of serine phosphate incorporation. Velocities were determined in the linear part of serine autophosphorylation. (D) Phosphopeptide map of autophosphorylated wild-type kinase, S2D, and Y51F. KD-mono, -bis, and -tris, mono-, bis-, and trisphosphorylated form of the kinase domain (DIYETDYYR); CT-bis, bisphosphorylated form of the C-terminus (Y1316/Y1322); JM, potential tyrosine phosphorylation in the juxtamembrane region; S1275, S1309, phosphopeptides containing S1275 and S1309.

detected. For Y51F, a similar decrease of serine phosphate containing peptides (S1275 and S1309) was observed. Therefore, the diminution in serine autophosphorylation of Y51F was mainly due to an almost complete loss of the phosphorylation of the C-terminal residues S1275 and S1309.

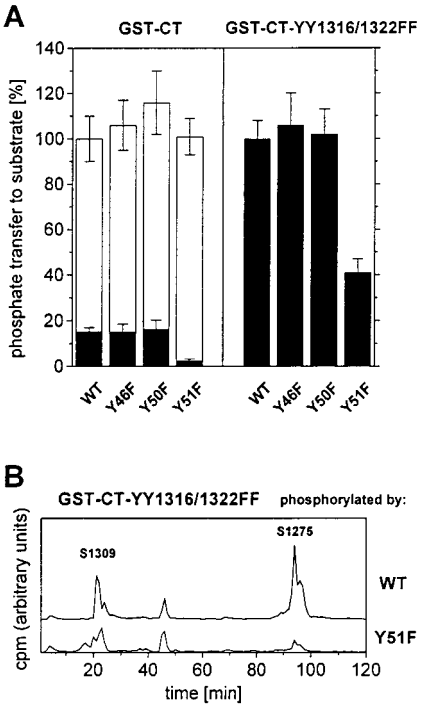

FIGURE 3: Phosphorylation of C-terminal domains by insulin receptor kinases. (A) Phosphate incorporation into the substrates. 5 μM GST-CT and GST-CT-YY1316/1322FF were phosphorylated for 20 min and separated by SDS−PAGE. GST-CT was analyzed for phosphoamino acid composition.[2] Phosphorylation by wild-type kinase was set to 100%. White bars, tyrosine phosphate; black bars, serine phosphate. (B) Phosphopeptide map of GST-CT-YY1316/1322FF, phosphorylated by either wild-type kinase or Y51F. S1275, S1309: Phosphopeptides containing S1275 or S1309, respectively.

Phosphorylation of the C-Terminal Domain of the IR as a Peptide Substrate. To further analyze the effect of monosubstitutions in the kinase domain, particularly Y1151F, on the serine phosphorylation activity of the insulin receptor kinase toward the C-terminal domain, the recombinant C-terminus of the IR was phosphorylated as an exogenous substrate. The C-terminal domain (N1249−S1343) was expressed as a GST fusion protein in *E. coli* (GST-CT). To suppress a potential effect of substrate tyrosine residues on GST-CT serine phosphorylation, a mutant peptide with substituted residues Y1316 and Y1322 was also used (GST-CT-YY1316/1322FF); this peptide still contains the two serine phosphorylation sites 1275 and 1309. Both substrates were phosphorylated by the insulin receptor kinases for 20 min and separated by SDS−PAGE. No significant difference in total phosphate incorporation in GST-CT was observed (Figure 3A, left panel; phosphorylation by WT was set to 100%). On analysis of the phosphoamino acid content, GST-CT phosphorylated by Y46F and Y50F showed 15−16% phosphorylation on serine residues, which was in the same range as GST-CT phosphorylated by the wild-type IRKD. However, a striking reduction of the phosphoserine content was detected in GST-CT phosphorylated by Y51F (3% of total phosphate incorporation). Consistent results were

[2] To account for the different stabilities of phosphotyrosine and phosphoserine, we compared the C-terminal peptides GST-CT and GST-CT-YY1316/1322FF with the mutants GST-CT-SS1275/1309AA and GST-CT-SS1275/1309DD. The recovery rates of phosphotyrosine and phosphoserine were nearly identical (data not shown).

Table 1: Kinetic Parameters of GST-CT and GST-CT-YY1316/1322FF as Substrates for Monosubstituted Kinases[a]

	GST-CT		GST-CT-YY1316/1322FF	
	K_M (μM)	V_{MAX} (nmol min^{-1} mg^{-1})	K_M (μM)	V_{MAX} (nmol min^{-1} mg^{-1})
wild type	6.1 ± 0.2	117 ± 6	5.2 ± 1.7	3.0 ± 0.8
Y46F	7.2 ± 0.2	114 ± 4	6.2 ± 2.3	2.8 ± 1.2
Y50F	6.8 ± 0.3	109 ± 7	5.9 ± 2.4	2.5 ± 1.2
Y51F	9.9 ± 0.4	72 ± 4	7.0 ± 4.1	0.27 ± 0.19

[a] 3.6−24 μM GST peptides were phosphorylated for 30 s by insulin receptor kinases and separated by SDS−PAGE, except for GST-CT-YY1316/1322FF which was phosphorylated for 5 min to obtain a significant signal. V_{MAX} is expressed as nmol of phosphate min^{-1} (mg of kinase)$^{-1}$.

obtained by phosphorylating GST-CT-YY1316/1322FF. While the phosphotransferase activities of Y46F and Y50F were comparable to IRKD, this mutated CT peptide was phosphorylated only with about 40% efficiency by Y51F, as compared to wild-type IRKD (Figure 3A, right panel, phosphorylation by WT was set to 100%). Using phosphopeptide mapping, it was verified that wild-type IRKD was able to phosphorylate GST-CT-YY1316/1322FF on both S1275 and S1309 (Figure 3B). In contrast, this substrate was phosphorylated poorly on the serine residues by Y51F. Taken together, these results indicate that the mutation Y1151F diminished the phosphorylation of S1275 and S1309, regardless of whether the C-terminus was intrinsic to the kinase or served as an exogenous substrate.

Apparent Affinity of IRKD to Its C-Terminus. The effect of the mutation Y1151F might be caused by an altered affinity of this mutant kinase to the C-terminus. To explore this possibility, different concentrations of GST-CT (3.6−24 μM) were phosphorylated for 30 s by monosubstituted insulin receptor kinases and separated by SDS−PAGE. Wild-type IRKD, Y46F, and Y50F had comparable apparent K_M values for GST-CT (6.1−7.2 μM), but Y51F showed an increase to about 10 μM (Table 1). Moreover, the maximal velocity of phosphate transfer to tyrosine residues was reduced for Y51F to 60% of the value for the wild type, while Y46F and Y50F did not alter V_{MAX}.

Additionally, kinetic constants of the mutated peptide GST-CT-YY1316/1322FF as a substrate of insulin receptor kinases were examined (Table 1). While the apparent K_M values of 5−7 μM were similar to those obtained with GST-CT, the maximal velocity of serine phosphorylation of GST-CT-YY1316/1322FF (measured after 5 min) was slow compared to tyrosine phosphorylation of GST-CT. Interestingly, the V_{MAX} of GST-CT-YY1316/1322FF was 10-fold slower for Y51F than for the other kinases. The mutant peptide GST-CT-SS1275/1309DD, which lacks serine phosphorylation sites, showed almost identical K_M and V_{MAX} values to GST-CT (data not shown).

Phosphorylation of the Recombinant IRS-1 Fragment. IRS-1 is the main substrate of the IR which is phosphorylated on tyrosine and serine residues in a insulin-dependent manner in vivo (*27*, *45*). We have found that IRKD itself is capable of phosphorylating IRS-1 on serine and threonine residues. To examine the ability of the mutated insulin receptor kinases to phosphorylate IRS-1, we used a recombinant fragment of it (hIRSp30, D516-P777), including 12 potential tyrosine phosphorylation sites (5 in YxxM motifs) and 54 serine and threonine residues. This part of IRS-1 is a high-affinity substrate in vitro [K_M < 12 μM (*37*)]. The IRS-1 fragment, which was expressed as a GST fusion protein (GST-IRSp30), was phosphorylated for 20 min and separated by SDS−PAGE. Serving as a substrate for wild-type IRKD, a phosphate incorporation of about 3 mol of phosphate/mol of GST-IRSp30 (=100%) was observed (Figure 4). The serine/threonine phosphate content of GST-IRSp30 reached 25% of total phosphate incorporation under the conditions of a kinase:substrate stoichiometry of 1:2.5. Surprisingly, none of the mutations inhibited tyrosine and serine/threonine phosphorylation of the substrate. In summary, these experiments showed that (1) imitation of serine autophosphorylation in IRKD (S2D) did not alter the phosphotransferase activity toward GST-IRSp30, and (2) in contrast to the phosphorylation of the C-terminus, the mutation Y1151F did not lead to a decrease in the serine/threonine phosphate content of GST-IRSp30. Obviously, no autophosphorylation site of the insulin receptor kinase analyzed here is directly involved in the serine phosphorylation of IRS-1.

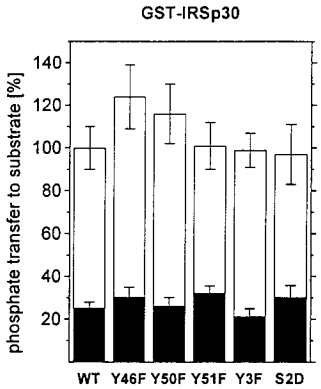

FIGURE 4: Phosphorylation of GST-IRSp30. 2.5 μM GST-IRSp30 was phosphorylated for 20 min and separated by SDS−PAGE. Phosphate incorporation into GST-IRSp30 was analyzed for phosphoamino acid composition. Phosphorylation by wild-type kinase was set to 100%. White bars, tyrosine phosphate; black bars, serine/threonine phosphate.

DISCUSSION

Mutations of tyrosine residues of the IR and the insulin receptor kinase have been already described in the literature (reviewed in ref *46*). However, the effects of these mutations are difficult to compare since different cell systems and purification protocols as well as various constructs for in vivo and in vitro assays were applied. In addition, transmembrane enzymes such as the insulin holoreceptor are difficult to analyze kinetically in vitro because of heterogeneity and contaminating detergents. In the study presented here, the recombinant insulin receptor kinase constructs were expressed and purified to homogeneity using an identical purification protocol. Thus, the activity of these highly purified enzymes can be compared in in vitro phosphorylation reactions. Moreover, the serine kinase activity of the IR is less well characterized; therefore, the IRKD kinases were systematically investigated for an alteration of this activity as a result of substitutions in tyrosine and serine autophosphorylation sites.

The monosubstitutions Y46F and Y50F showed reductions in total phosphate incorporation and a decrease in the initial

velocity of tyrosine autophosphorylation (≤2-fold). Interestingly, the triple-substituted Y3F mutant displayed a phosphate content comparable to the monosubstituted mutants. Previously, a similar observation has been reported for a 35 kDa insulin receptor kinase lacking the C-terminal domain and juxtamembrane tyrosine phosphorylation sites, which incorporated up to 3 mol of phosphates/mol of enzyme (*9*). This could be due to a partial phosphorylation of the wild-type enzyme and might indicate a potential inhibitory function of the C-terminal tyrosines upon the autophosphorylation reaction. However, Y46F, Y50F, and Y3F showed a serine autophosphorylation similar to wild-type enzyme. Although the initial velocity of serine autophosphorylation is slow compared to tyrosine autophosphorylation [$t_{1/2}$ for half-maximal phosphate incorporation: less than 30 s for tyrosine residues vs about 5 min for serine residues (*8*)], up to 1.3 mol of phosphate was found in serine residues after a 30 min reaction. It was noticed that the serine kinase activity of IRKD strongly depended on a highly active enzyme; long-term storage at −80 °C led to a reduction or a loss of this activity. The serine kinase activity of Y46F, Y50F, and Y3F toward serine residues in the C-terminal peptide and the IRS-1 fragment was also unaltered. In agreement with these data, apparent K_M and V_{MAX} values of Y46F and Y50F for the C-terminal peptide substrate were similar to the values of the wild type, suggesting that an interaction between the C-terminal domain and the activation loop is not affected.

Two mutations caused major alterations: the exchange of serine resides 1275 and 1309 for aspartic acid residues (S2D) and the monosubstitution Y1151F. The mutations in S2D diminished the phosphate incorporation into serine residues to 0.3−0.4 mol of phosphate/mol of enzyme. In addition, this phosphorylation of unknown serine residues was found to occur 10-fold slower and is consistent with our previous findings that S1275 and S1309 are the major serine autophosphorylation sites (*8*). Using phosphopeptide mapping, no other specific serine phosphorylation sites were detected. However, serine phosphorylation has been reported on residues S1293/S1294 (*17*) and in the juxtamembrane region (*23*). Under the conditions of phosphopeptide mapping applied here, a monophosphorylated peptide comprising S1293/S1294 (SSHCQR) would elute in the flow-through. This might apply to the unsequenced flow-through fraction of S2D [Figure 2D; (*8*)] which contained serine phosphate (data not shown). This fraction comprised less than 3% of the total HPLC eluate, indicating a potential but minor phosphorylation of S1293/S1294.

Serine phosphorylation of the IR is reported to function as an inhibitor of receptor tyrosine phosphorylation. The results presented here argue against a homologue desensitization of the IR, caused by the phosphorylation of S1275 and S1309. Assuming that substitution with aspartic acid residues imitates serine phosphorylation correctly, serine autophosphorylation (S2D) inhibited neither tyrosine autophosphorylation of the insulin receptor kinase nor phosphate transfer to substrates (CT peptide, IRS-1). Hence, the role of IR serine autophosphorylation remains to be elucidated.

A special case was the mutation Y1151F in the activation loop of the IRKD, which only slightly affected the initial velocity of tyrosine autophosphorylation and the amount of phosphate incorporation into tyrosine residues. This result is in accordance with the observation that monosubstitution of Y1151 had smaller effects on the kinase activity than Y1146 or Y1150 (*47*). Interestingly, however, the substitution of Y1151 markedly reduced the phosphorylation of S1275 and S1309. The inhibition of serine kinase activity of the Y51F mutant toward the C-terminal serine residues could also be detected in a C-terminal peptide of the IR when used as a substrate of the insulin receptor kinase. These results confirm that the serine kinase activity of the IRKD is an intrinsic property of the wild-type enzyme. More evidence for this intrinsic characteristic was given by the observation that highly purified insulin receptors showed the same phosphorylation sites in vitro, while a kinase-inactive IRKD was not phosphorylated at all (*8*). In the latter case, one could argue that the C-terminal serine phosphorylation sites might be hidden in the inactive enzyme, and therefore an exogenous serine kinase is not able to phosphorylate them. However, copurification of a serine kinase whose activity is dependent on the phosphorylation of Y1151 is unlikely.

Using GST-CT as a substrate, the K_M and V_{MAX} values of Y51F were only slightly changed. However, Y51F effected an alteration of the serine phosphorylation state of this peptide and also of the mutant peptide GST-CT-YY1316/1322FF, which might indicate a conformational change of the phosphorylated activation loop. This hypothesis is supported by the crystal structure of the phosphorylated insulin receptor kinase. pY1151 has been shown to form hydrogen bonds, thereby stabilizing the conformation of the phosphorylated activation loop. Although studies of Tyr→Phe substitutions in the activation loop have not demonstrated a predominant role for a particular tyrosine (*46*), the tyrosine corresponding to Y1151 has been shown to be the critical autophosphorylation site in the FGF receptor (*48*) and in MET (*49*). Moreover, pY1151 is structurally related to pT197 in the serine/threonine kinase cyclic AMP-dependent protein kinase, which underlines the predominant role of Y1151 in the activation loop. It has been reported that segments of the activation loop of the unphosphorylated insulin receptor kinase are relatively mobile, suggesting that they play an autoinhibitory role (*50*). However, full activation of the insulin receptor kinase is achieved only upon autophosphorylation of Y1151 (*25*). Therefore, one might expect that the mutation Y1151F should result in a decreased stability of the activation loop.

How do available crystal structures explain the dual specificity of IRKD? One possibility is that sequence conservation of Thr (for serine kinases) versus Pro (for tyrosine kinases) at the P0 binding site on the enzyme contributes to serine versus tyrosine specificity. The conserved proline (P1160 in IRKD) kinks the activation segment such that the substrate is held further from the active site (*24*, *25*). No crystal structures of the insulin receptor kinase with its C-terminal domain or of dual specificity kinases such as MEK are available. However, there are structural data for dual specific phosphatases which might point to a potential mechanism. Dual specific phosphatases seem to act similarly to protein tyrosine phosphatases, but the active sites of the dual specific enzymes are shallower, permitting entry on the shorter phosphoserine/threonine residue (*51*). To explain our data, we would like to speculate that the active site of IRKD is flexible enough to widen while interacting with the C-terminus, so that serine residues may fit (although not perfectly) and therefore be in position to enable phosphate

transfer. Simultaneously, the C-terminal domain may modulate the conformation of the activation loop. The suboptimal fitting of serine residues would be in agreement with the observed relative slow rate of serine phosphorylation. The recognition of serine substrates probably relies on an extensive protein−protein interface, as discussed for the dual specific phosphatase Cdc25A (*52*). Although highly speculative, mutation of Y1151 may affect the widening of the catalytic center by destabilizing the activation loop and/or the interacting with the C-terminal domain, resulting in the inhibition of C-terminal serine phosphorylation.

For the fully active serine kinase activity of IRKD toward S1275 and S1309, Y1151 has to be present. This result confirms and improves the prior observation that serine phosphorylation outside the juxtamembrane region of the IR is dependent on Y1151 (*23*). In contrast, the serine kinase activity of IRKD toward the IRS-1 fragment was independent of the presence of Y1151. This may account for local differences in the residues around the corresponding serine phosphorylation sites. The serine phosphorylation sites of the IRS-1 fragment as a substrate of IRKD are still unidentified, but it was speculated that serine residues in the neighborhood of YxxM motifs could be the target of serine kinases (*28*). However, the sequences around S1275 and S1309 of IRKD are KAPES^{1275}EELE and DGGSS^{1309}LGFK, respectively, showing no common motif; moreover, comparable sequences are absent in IRSp30. Short peptides (∼14 aa) as substrates of IRKD are not phosphorylated on serine residues (unpublished results); therefore, structural features may be mainly important for the recognition of serine phosphorylation sites.

Taken together, these results demonstrate that the specificity of IRKD toward serine phosphorylation sites in the C-terminus can be modified by a single amino acid substitution in the activation loop, whereas the substrate specificity toward IRS-1 is not affected by the replacement, suggesting that interactions between the activation loop and the C-terminal domain occur. The Y1151F mutation is the first observation that the dual specificity of IRKD still acts on IRS-1, but is inhibited in the autophosphorylation of serine residues S1275 and 1309.

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