

# Identification of Ser-1275 and Ser-1309 as autophosphorylation sites of the insulin receptor

Hadi Al-Hasani<sup>1,a</sup>, Bernd Eisermann<sup>b</sup>, Norbert Tennagels<sup>c</sup>, Claudia Magg<sup>c</sup>,  
Waltraud Paßlack<sup>a</sup>, Marlis Koenen<sup>a</sup>, Dirk Müller-Wieland<sup>d</sup>, Helmut E. Meyer<sup>b</sup>,  
Helmut W. Klein<sup>c,\*</sup>

<sup>a</sup>Diabetes Forschungsinstitut Düsseldorf, Auf'm Hennekamp 65, D-40225 Düsseldorf, Germany

<sup>b</sup>Institut für Physiologische Chemie, Ruhr-Universität, Universitätsstrasse 150, D-44780 Bochum, Germany

<sup>c</sup>Institut für Biochemie, Universität zu Köln, Otto-Fischer-Strasse 12–14, D-50674 Köln, Germany

<sup>d</sup>Klinik II und Poliklinik für Innere Medizin, Universität zu Köln, Joseph Stelzmannstr. 9, D-50924 Köln, Germany

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**Abstract** We have identified Ser-1275 and Ser-1309 as novel serine autophosphorylation sites by direct sequencing of HPLC-purified tryptic phosphopeptides of the histidine-tagged insulin receptor kinase IRKD-HIS. The corresponding peptides (Ser-1275, amino acids 1272–1292; Ser-1309, amino acids 1305–1313) have been detected in the HPLC profiles of both the soluble kinase IRKD, which contains the entire cytoplasmic domain of the insulin receptor  $\beta$ -subunit, and the insulin receptor purified from human placenta. In contrast, a kinase negative mutant, IRKD-K1018A, did not undergo phosphorylation at either the tyrosine or serine residues, strongly suggesting that insulin receptor kinase has an intrinsic activity to autophosphorylate serine residues.

**Key words:** Insulin receptor kinase; Serine autophosphorylation; Baculovirus expression system; Kinase negative mutant

## 1. Introduction

The monomeric enzyme derived from the cytoplasmic portion of the human insulin receptor has been shown to exhibit comparable catalytic properties and identical tyrosine autophosphorylation sites to the native receptor [1–8]. In addition to tyrosine autophosphorylation of the purified insulin receptor, it has been shown that the  $\beta$ -subunit of the insulin receptor (IR) undergoes serine phosphorylation. The attempts of our group and others to separate the serine kinase activity from tyrosine kinase activity [9–11] have led to the conclusion that highly purified insulin receptors contain an intrinsic insulin-dependent serine kinase activity. Moreover, serine phosphorylation was shown to be sensitive to an apparent specific tyrosine kinase inhibitor [10] and occurred independently of the purification state of the receptor [11]. These data have

strengthened the hypothesis that the serine kinase activity is indeed an intrinsic property of the insulin receptor. The aim of our study was to localize the sites of serine autophosphorylation in vitro by direct sequencing of the corresponding peptides of the kinase and to compare these sites with serine phosphorylation sites of the holoreceptor. Therefore, we overexpressed the soluble insulin receptor kinase in insect cells. The construction of two different forms of the kinase enabled us to purify the enzymes using alternative protocols. Furthermore, we examined serine phosphorylation in a kinase deficient mutant, in which tyrosine autophosphorylation was absent.

## 2. Materials and methods

[ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol) was obtained from Amersham. Restriction endonucleases and ATP were from Boehringer Mannheim. Cell culture reagents were from Life Technologies Inc.; poly(L-lysine) ( $M_r$  6000–9000) was from Serva. Tryptic peptides for sequencing reactions were generated with modified trypsin (sequencing grade) from Promega. For HPLC phosphopeptide maps, trypsin (sequencing grade) from Merck was used. Other reagents were obtained from common commercial sources.

### 2.1. Construction of soluble insulin receptor kinases

Construction of the histidine-tagged kinase (IRKD-HIS, 49 kDa) and IRKD was previously described [6,7]. IRKD-K1018A: The amino acid Lys-1018, involved in the ATP-binding site of the insulin receptor [12,13], was substituted by alanine by site-directed mutagenesis of the cDNA using unique site elimination [14]. A mutagenesis primer was constructed (5'-GTGGCGGTGGCGACGGTTAACGAGTCAGC-3') which in addition to the amino acid substitution created a new *HpaI* site in the insulin receptor cDNA. A second primer was used as a selection primer (5'-GAGTGCACCATGGGCGGTGTGAAAT-3'), to eliminate a *NdeI* site in pUC19. The mutagenesis reaction, using pUC-IRKD as a template was performed according to the manufacturer's instructions (Clontech). The mutated region was sequenced to ensure that no other mutation had occurred. The *XhoI/BstXI* fragment, containing the mutation was isolated to replace the corresponding fragment in pUC-IRKD. Construction of the baculovirus transfer vector was performed as described for the IRKD. *Spo-doptera frugiperda* (Sf9) cells were cotransfected with *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA and transfer vectors using the BaculoGold System (PharMingen) according to the manufacturer's instructions. Recombinant viruses were isolated by performing plaque assays [15].

### 2.2. Purification of soluble insulin receptor kinases

Purification of the soluble kinases was accomplished by sequential chromatography using MonoQ and Phenyl-Sepharose (Pharmacia). Alternatively, the histidine-tagged kinase could be purified by immobilized metal chelate affinity chromatography on Ni-NTA agarose (Qiagen) and FPLC phenyl-Sepharose chromatography [7].

\*Corresponding author. Fax: (49) (221) 4705066.

<sup>1</sup>Present address: Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA.

**Abbreviations:** PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; pY, phosphotyrosine; pS, phosphoserine; pT, phosphothreonine.

This paper is dedicated to Prof. Günter Legler on the occasion of his 70th birthday.

### 2.3. Purification of the insulin receptor from human placenta

Purification of the human insulin receptor was performed as described [11]. In brief, the receptors were solubilized with Triton X-100 from placental membranes and affinity purified by successive chromatography on insulin agarose, wheat germ agglutinin Sepharose and hydroxyapatite.

### 2.4. Phosphorylation reactions

All phosphorylation reactions were carried out at room temperature (22°C). For the soluble kinases the reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 μM poly(L-lysine) and 250 μM [ $\gamma$ -<sup>32</sup>P]ATP. Unless otherwise indicated, the concentration of the kinase was 1 μM. Phosphorylation reaction of the purified human insulin receptor were carried out in 30 mM HEPES, pH 7.2, 10 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, 200 μM [ $\gamma$ -<sup>32</sup>P]ATP, 0.1 μM insulin and 0.05% Triton X-100. The receptor was incubated on ice with insulin for 30 min and added to the reaction mixture to a final concentration of ~0.1 μM. The proteins were separated by SDS-PAGE, localized by autoradiography and the radioactivity of the excised bands was determined by measurement of Cerenkov radiation in a Beckman scintillation counter.

### 2.5. Digestion with trypsin

The autophosphorylated soluble kinases and the insulin receptor  $\beta$ -subunit were located on SDS-PAGE by autoradiography of the fixed and dried gels and the corresponding bands were excised. In-gel digestion of the proteins was performed according to [16,17]. The recovery of <sup>32</sup>P typically exceeded 90%. For preparative scale reactions, the autophosphorylated IRKD-HIS was precipitated by addition of trichloroacetic acid to a final concentration of 10%. After centrifugation, the pellet was washed twice with absolute ethanol and resuspended in 100 mM Tris-HCl pH 8.0, 1 mM CaCl<sub>2</sub> to yield a protein concentration of 1 mg/ml. Trypsin was added (1/20, w/w) and the reaction mixture was incubated at 37°C for 16 h.

### 2.6. Separation of tryptic phosphopeptides

Initially, phosphopeptides were separated with a Shimadzu HPLC system using an anion exchange column (Macherey and Nagel, Nucleogel SAX-1000-8/46). The buffers used were (A) 20 mM ammonium acetate, pH 7.0 and (B) 1 M potassium phosphate, pH 4.0. The phosphopeptides were injected onto the column at a flow rate of 0.5 ml/min. Elution of the phosphopeptides was achieved by applying a two-step linear gradient (0–10% B in 40 min; 10–50% B in 80 min) to the column. Fractions of 0.5 ml (1 min) were collected in polypropylene tubes and the radioactivity in each tube was measured as Cerenkov radiation in a Beckman scintillation counter. The recovery of <sup>32</sup>P typically was 75–90%. For rechromatography, an Applied Biosystems HPLC system was used. The phosphopeptides from the

anion exchange chromatography were separated by reversed-phase HPLC using a microbore C<sub>18</sub> column (SGE, Weiterstadt, Germany). The samples were adjusted to 0.1% trifluoroacetic acid (TFA) and applied to the column at a flow rate of 0.1 ml/min. A linear gradient of 0.1% TFA vs. 80% acetonitrile/0.1% TFA in 30 min at a flow rate of 0.1 ml/min was used for elution of the peptides. Elution was monitored by measuring the absorption at 215 and 295 nm in a 32 μl flow-through cuvette and fractions of 25–100 μl were collected by hand. After determination of Cerenkov radiation, the radioactive fractions were subjected to sequence analysis.

### 2.7. Sequence analysis of tryptic phosphopeptides

Phosphoserines were modified to S-ethylcysteines according to the procedure previously described [18,19]. The amino acid sequences were determined by automated Edman degradation in an Applied Biosystems, Inc. (ABI; Foster City, CA) model 470A protein sequencer. Phosphotyrosine-containing peptides were sequenced on an ABI model 473A Sequenator. The presence of PTH-phosphotyrosine was confirmed by capillary electrophoresis of aliquots of each sample from the sequenator [20]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of purified phosphopeptides was performed as described [21].

### 2.8. Phosphoamino acid analysis

Tryptic phosphopeptides were lyophilized in a Speed-Vac, hydrolyzed in 100–200 μl of 6 N HCl at 110°C for 1.5 h, diluted with 500 μl of water and dried. Two dimensional phosphoamino acid analysis was performed by electrophoresis as described by Boyle et al. [22]. Quantification of phosphoamino acids was achieved by using a Phospho-Imager (Fuji BAS1000).

### 2.9. Other procedures

*S. frugiperda* (Sf9) cells were maintained as described [15]. Protein concentrations were determined by a modified method of Bradford [23]. SDS-PAGE was performed according to Laemmli [24].

## 3. Results

### 3.1. Construction, expression and purification of soluble insulin receptor kinases

We have overexpressed two forms of soluble insulin receptor kinases (IRKD) in insect cells with different N-termini: (i) a histidine-tagged kinase (IRKD-HIS) of 49 kDa (Gly-947–Ser-1343 [7]); (ii) a 45 kDa kinase (IRKD) containing the entire cytoplasmic domain of the insulin receptor  $\beta$ -subunit (Arg-941–Ser-1343 [6]). In addition, we have overexpressed a

Table 1  
Sequence analysis results of purified tryptic phosphopeptides derived from the IRKD-HIS

Amino acid sequence of phosphopeptides <sup>a</sup>	Proreceptor residue no. <sup>b</sup>	Phosphorylated residues	Fraction <sup>c</sup>	Domain <sup>d</sup>
APESpEELEMEFEDM(E)NVPLDR	1272–1292	Ser-1275 <sup>e</sup>	m	CT
DGGSSpLGFK	1305–1313	Ser-1309 <sup>e</sup>	b	
DLYpDDDDKDR <sup>e</sup>		Tyr-26 <sup>f</sup>	j	NT
DLYpDDDDK <sup>e</sup>		Tyr-26 <sup>f</sup>	k	
DIYpETDYpYpR	1144–1152	Tyr-1146/1150/1151 (partial)	h	CD
DIYpETDYpYpR		Tyr-1146/1150/1151 (tris)	n	
SYpEEHIPYTHMNGGK	1315–1329	Tyr-1316	f	CT
SYpEE(H)IPY		Tyr-1316	d	
SYEEHIPYpTHMNGGK		Tyr-1322	d	
SYEE(H)(P)YpT(H)MN		Tyr-1322	c	
SYpEEHIPYpTHMNGGK		Tyr-1316/1322 (bis)	g	
SYpEEHIPYp		Tyr-1316/1322 (bis)	e	
		[ $\gamma$ - <sup>32</sup> P]ATP	i	

<sup>a</sup>A 'p' denotes phosphorylation of the preceding amino acid residue. Residues in parentheses yielded no PTH-amino acid signal.

<sup>b</sup>Numbering according to Ullrich et al. [13].

<sup>c</sup>Fractions from HPLC anion exchange chromatography (see Fig. 2A).

<sup>d</sup>Location of the corresponding peptide in the insulin receptor kinase; CT, C-terminal domain; CD, catalytic domain; NT, heterologous N-terminus of the kinase.

<sup>e</sup>Sequence analysis was repeated twice in independent experiments (enzyme purification, autophosphorylation reaction, isolation and separation of the phosphorylated peptides).

<sup>f</sup>Sequence derived from the baculovirus transfer vector.

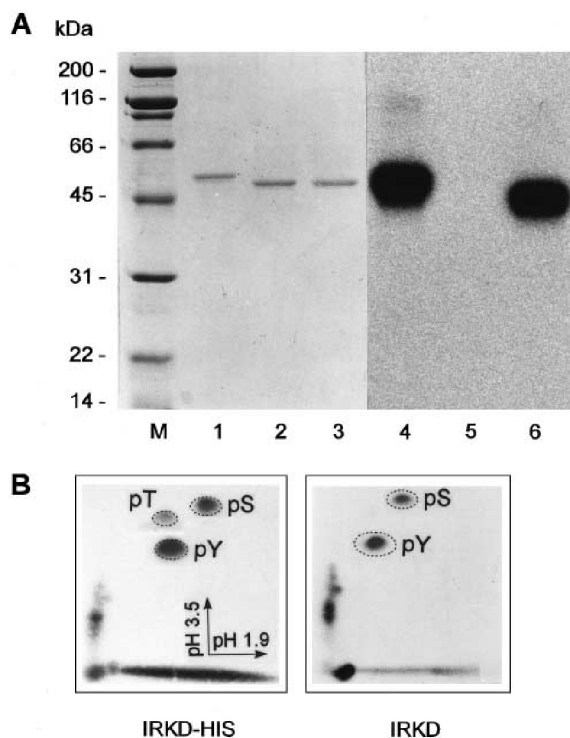


Fig. 1. Autophosphorylation of the soluble insulin receptor kinases and phosphoamino acid analysis. The purified kinases were autophosphorylated for 30 min, separated by SDS-PAGE and analyzed for phosphoamino acid composition. (A) Coomassie stained SDS-PAGE of the purified kinases (1  $\mu$ g each; lanes 1–3) and autoradiography of the autophosphorylated kinases (lanes 4–6). IRKD-HIS (lanes 1,4), IRKD-K1018A (lanes 2,5), IRKD (lanes 3,6). Specific phosphate incorporation was 5 mol/mol for the IRKD-HIS and 4.5 mol/mol for the IRKD. (B) Two-dimensional phosphoamino acid analysis of the kinases. Relative serine/threonine phosphorylation was 17% for the IRKD-HIS and 15% for the IRKD.

45 kDa kinase deficient mutant of the IRKD (IRKD-K1018A). The three proteins were expressed in comparable amounts after infection of Sf9 cells with the recombinant baculoviruses (data not shown). The soluble enzymes were purified by anion exchange chromatography (MonoQ) followed by hydrophobic chromatography (phenyl-Sepharose). Alternatively, the IRKD-HIS could be purified by a protocol that made use of metal chelate affinity chromatography (Ni-NTA agarose) followed by hydrophobic chromatography. The two different purification protocols yielded comparable results for the histidine-tagged kinase. The enzymes were purified to >95% homogeneity based upon density evaluation of Coomassie stained gels with an overall yield of  $\sim 0.75$  mg/ $10^8$  cells, corresponding to  $\sim 5\%$  of the total soluble protein (Fig. 1A, lanes 1–3).

### 3.2. Autophosphorylation of the insulin receptor kinases

In order to characterize the purified IRKD-HIS and IRKD we carried out autophosphorylation reactions [25]. Phosphate incorporation was 4.5–5 and 4–4.5 mol/mol after 30 min of autophosphorylation for the IRKD-HIS and IRKD, respectively (Fig. 1A, lanes 4,6). Although the amount of phosphate incorporated by the two kinases was slightly different, the enzymes exhibited almost identical specific activities for the phosphorylation of exogenous substrates (data not shown).

As expected the kinase deficient mutant, IRKD-K1018A, did not show any phosphate incorporation. This demonstrates that this kinase was both an inactive enzyme and free of any contaminating kinase activities (Fig. 1A, lane 5) whether tyrosine or serine specific. The phosphoamino acid analysis of the autophosphorylation reaction of the active soluble kinases showed a phosphoserine content of  $\sim 15\%$ . In addition, traces ( $\sim 3\%$ ) of phosphothreonine were detected in the IRKD-HIS. However, it should be noted that phosphoserine and phosphotyrosine may exhibit different stabilities during acid hydrolysis of phosphopeptides [26]. The extent of serine/threonine phosphorylation of the IRKD-HIS was independent of the purification protocol (Ni-NTA agarose instead of MonoQ).

### 3.3. Localization of the serine/threonine phosphorylation sites

To identify the serine/threonine phosphorylation sites of the histidine-tagged kinase IRKD-HIS, the autophosphorylated enzyme was digested with trypsin, and the resultant tryptic peptides were resolved by anion exchange HPLC. The tryptic digest was separated into 14 major radioactive peaks with a total recovery of 90% of radioactivity loaded on the column. The radioactive fractions were pooled according to the radioactivity profile (Fig. 2A) and aliquots from each fraction were analyzed for their phosphoamino acid composition by two-dimensional electrophoresis. Two of these fractions (b and m in Fig. 2A) contained peptides which were entirely phosphorylated at serine residues. Both fractions were applied to reversed-phase HPLC chromatography each yielding only one major radiolabeled peptide (Fig. 2B,C). Finally, the resulting peptides were subjected to automated Edman degradation.

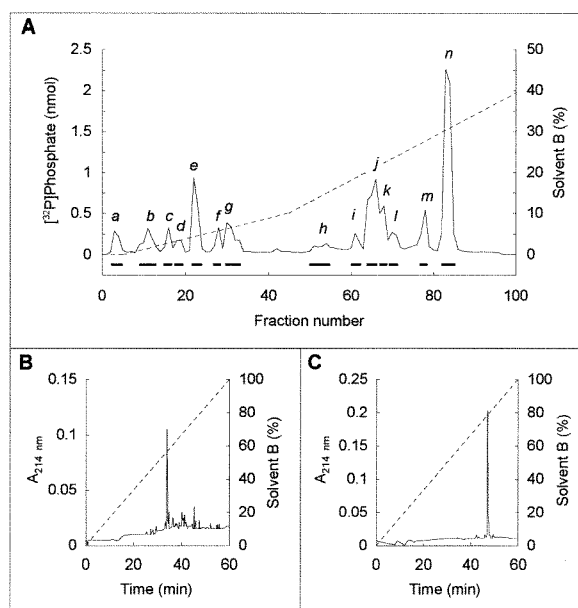


Fig. 2. Separation of tryptic digest and isolation of serine phosphorylated peptides. 8 nmol IRKD-HIS were autophosphorylated for 30 min in the presence of 250  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP and digested with trypsin. (A) HPLC anion exchange chromatography of tryptic digest. Radioactive fractions were pooled (solid bars) and aliquots were analyzed for phosphoamino acid composition (not shown). Fractions b and m contained peptides phosphorylated entirely on serine residues. (B,C) C<sub>18</sub> reversed phase chromatography of phosphoserine-containing fraction b (B) and fraction m (C).

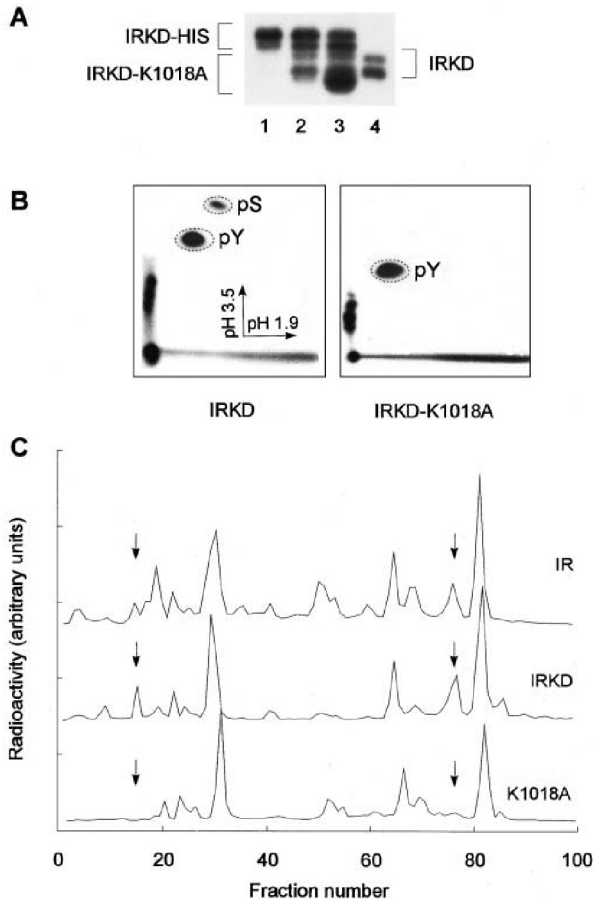


Fig. 3. Comparison of phosphopeptide mapping of the kinase negative mutant IRKD-K1018A and the active kinases. (A) Phosphorylation of the kinase negative mutant (IRKD-K1018A) with IRKD-HIS. Autoradiography of phosphorylated IRKD-HIS (lane 1), 1  $\mu$ M IRKD-K1018A (lane 2), 5  $\mu$ M IRKD-K1018A (lane 3), phosphorylated IRKD (lane 4). Phosphorylation reactions were carried out for 30 min (lanes 1–3) and for 5 min (lane 4) and separated by SDS-PAGE. (B) Two-dimensional phosphoamino acid analysis of IRKD and mutant kinase IRKD-K1018A. (C) HPLC anion exchange chromatography of tryptic digest from phosphorylated IRKD, IR and IRKD-K1018A. The active enzymes (1–2  $\mu$ g) were autophosphorylated for 30 min and separated by SDS-PAGE. Substrate phosphorylation of IRKD-K1018A was obtained as described above (A). The proteins were digested with trypsin and phosphopeptides ( $\sim 10^6$  cpm) were separated by HPLC anion exchange chromatography. Arrows denote the phosphoserine-containing fractions, corresponding to Ser-1275 (right) and Ser-1309 (left).

The phosphoserine-containing peptides were sequenced after transformation of phosphoserine to *S*-ethylcysteine in order to prevent  $\beta$ -elimination during Edman degradation [18,19]. Table 1 summarizes the identified phosphorylation sites of the tryptic phosphopeptides. Two phosphoserine-containing peptides of the IRKD-HIS were identified: (i) peptide of fraction b corresponding to amino acid sequence 1305–1313 (DGGSS\*LGFK) which yielded an *S*-ethylcysteine signal in cycle number 5 (asterisk) and (ii) peptide of fraction m corresponding to amino acid sequence 1272–1292 (APES\*EELEM-EFEDMENVPLDR) where serine in position 4 was converted to *S*-ethylcysteine. Thus, Ser-1275 and Ser-1309 were the phosphorylated residues in the soluble kinase IRKD-HIS. As illustrated in Fig. 2A, each of the two peptides contained

about equal amounts of radioactivity. None of the two serines have been previously described as potential autophosphorylation sites of the insulin receptor kinase. In addition to serine phosphorylation, only the IRKD-HIS is phosphorylated on threonine residues (Fig. 1B). We detected the corresponding threonine phosphorylated peptide in the breakthrough of the anion exchange chromatography. According to sequence analysis and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), this peptide has been derived from the baculovirus transfer vector pBlue-BacHIS (residues 1–23; MRGSHHHHHHGMASMTGGQQ-MGR; Thr-16 phosphorylated).

#### 3.4. Localization of the tyrosine phosphorylation sites

For a comparison of phosphopeptide patterns of the different insulin receptor kinases (IRKD-HIS, IRKD and IRKD-K1018A), as well as of the native insulin receptor purified from human placenta, we identified the major peaks from HPLC-anion exchange chromatography. We sequenced the tyrosine phosphorylated peptides from the IRKD-HIS corresponding to the catalytic domain (Tyr-1146, Tyr-1150, Tyr-1151) and the C-terminal region (Tyr-1316, Tyr-1322; cf. Fig. 2A, Table 1), as described by others [1,27–30].

#### 3.5. Comparison of phosphopeptide mapping of the kinase negative mutant IRKD-K1018A and the active kinases

We have studied the phosphorylation of the negative mutant kinase as a substrate and the histidine-tagged kinase as the catalyst in a substrate phosphorylation reaction. Besides the differences in the catalytic properties, both enzymes are distinct due to the histidine tag in the IRKD-HIS. Taking advantage of the different molecular mass of the histidine-tagged kinase (49 kDa) and the kinase negative mutant (45 kDa), we were able to separate the proteins by SDS-PAGE after the phosphorylation reactions. In addition to the autophosphorylation of the catalyst the substrate was efficiently phosphorylated (Fig. 3A). Multiple bands of the phosphorylated kinase negative mutant most likely arise from different binding of SDS to partially phosphorylated forms of the protein. The IRKD-K1018A incorporated about 3.5 mol phosphate/mol within 30 min of reaction time. However, phosphoamino acid analysis of the mutant kinase revealed only traces of phosphoserine (< 1%) (Fig. 3B). In order to localize the phosphorylation sites of the substrate, tryptic phosphopeptides derived from the IRKD-K1018A were separated by anion exchange HPLC. Fig. 3C shows representative phosphopeptide maps of the mutant kinase and the corresponding active kinases (i.e. IR and IRKD). Obviously, all profiles are very similar. Thus, all tyrosine phosphorylation sites of the kinase-negative mutant were accessible to phosphorylation by the histidine-tagged kinase, although the corresponding serine phosphorylated peptides are absent. Most importantly, serine phosphorylation of the human insulin receptor [11] and the IRKD (Fig. 1B) results in the appearance of two peptides (Fig. 3C; marked by arrows) phosphorylated on serine residues exclusively that correspond to the identified phosphoserine peptides of the IRKD-HIS. Thus, our data strongly suggests, that Ser-1275 (peptide 1272–1292) and Ser-1309 (peptide 1305–1313) are also the major serine autophosphorylation sites *in vitro* of the human insulin receptor as well as of the soluble insulin receptor kinases. No significant serine/threonine phosphorylation was detected in any other peak.

#### 4. Discussion

Recent reports have discussed serine/threonine autophosphorylation of the insulin receptor kinase [9–11]. In this study we have identified two serine phosphorylation sites in the insulin receptor kinase *in vitro*. Additionally, we present strong evidence that these phosphoserines are derived from autophosphorylation reactions due to the intrinsic serine/threonine kinase activity of the protein tyrosine kinase. To ascertain whether the receptor kinase is indeed a dual specificity kinase capable of phosphorylating serine as well as tyrosine residues [31], we made use of two independent strategies (i.e. ion exchange chromatography and immobilized metal chelate affinity chromatography) to purify the two versions of the recombinant protein kinases. In both cases, the purified enzymes undergo autophosphorylation at the tyrosine and serine residues. The major sites of serine autophosphorylation were identified by sequence analysis of HPLC-purified tryptic phosphopeptides of the histidine-tagged kinase, IRKD-HIS. The two phosphopeptides that combined exclusively phosphoserine, were both derived from the carboxy terminus: a highly negatively charged 21 amino acid peptide (residues 1272–1292) and a nonapeptide (residues 1305–1313). Within these peptides Ser-1275 and Ser-1309 were identified by direct sequencing of *S*-ethylcysteine derivatives of the corresponding phosphopeptides. Moreover, our data strongly suggests that Ser-1275 and Ser-1309 constitute the major sites of serine autophosphorylation of the soluble kinase lacking the stretch of histidines, and the purified human insulin receptor. A comparison of the tryptic HPLC maps of the kinases proved this. These peptides are absent in the HPLC map of the kinase negative mutant that has been applied in substrate phosphorylation with IRKD-HIS.

In previous studies, serine residues 1293 and/or 1294 have been proposed as sites of insulin-stimulated receptor phosphorylation [10]. Following their phosphorylation *in vitro* by highly purified insulin receptors, synthetic peptides based on the receptor sequence surrounding these residues (-SSHCQR-) comigrate during HPLC with the <sup>32</sup>P-labeled insulin receptor tryptic peptide phosphorylated in intact cells [4] or *in vitro* phosphorylated after affinity purification [10]. Serine residues 1293 and/or 1294 are potential sites of insulin stimulated receptor phosphorylation; however, this site may represent only a minor fraction of the total serine content of the receptor.

Ellis and his colleagues have reported serine phosphorylation of a partially purified soluble insulin receptor kinase [4]. Two-dimensional phosphopeptide mapping revealed 2 serines that are phosphorylated in considerable amounts in the 48 and 43 kDa soluble kinase derivatives, but not in the 38 kDa kinase, derived by limited tryptic proteolysis of the 48 kDa kinase. The absence of serine phosphorylation within the truncated receptor kinase species (38 kDa) strongly suggests that serine phosphorylation takes place in the carboxy-terminus. Moreover, one of the phosphoserine peptides has a mobility similar to that of the phosphoserine-containing peptide derived from an *in vivo* phosphorylated native receptor  $\beta$ -subunit. In addition, the authors identified a highly negatively charged peptide, which was exclusively phosphorylated on serine. However, the amino acid sequence of this peptide has not been determined. With regard to the highly negative charges it appears similar to one of our sequenced phospho-

serine peptides (peptide m) including Ser-1275 as phosphorylation site. The extent of kinase serine autophosphorylation observed in these studies is between 15 and 25% of the total autophosphorylation of the kinases. Serine phosphorylation reactions are delayed relative to the kinase tyrosine phosphorylation [10,11]. Under our experimental conditions,  $t_{1/2}$  for half-maximal tyrosine incorporation is 0.5 min, whereas  $t_{1/2}$  for half-maximal serine autophosphorylation is in the order of 5 min (data not shown). Studies with a degenerate peptide library [32] indicate that the insulin receptor displays a preference for phosphorylating tyrosines downstream of the acidic residues. Thus, considering the physical and biochemical data, it seems unlikely that the observed serine/threonine phosphorylation results from a direct phosphoryl transfer from ATP to the hydroxyl groups of serine/threonine residues [33]. This is in agreement with the low level of serine phosphorylation that we observed when utilizing the inactive kinase, IRKD-K1018A, as a substrate.

In previous studies, we [7] and others [34] have reported that the insulin receptor kinase is able to auto-dephosphorylate tyrosine autophosphorylation sites. Under pulse-chase conditions, it is evident that the enzyme is capable of transient phosphorylation of certain tyrosine residues [7]. Although highly speculative, it may be proposed that the observed serine/threonine phosphorylation is due to an intramolecular phosphoryl transfer (*cis*-phosphorylation), most probably from transient tyrosine phosphates to serine residues. Further studies on the serine/threonine kinase activity of the insulin receptor kinase are in progress to elucidate the reaction mechanism of the enzyme.

To summarize, our data strongly suggest that the receptor kinase has an intrinsic serine kinase activity by the following observations: (i) The autophosphorylation of the purified active kinases (IRKD-HIS and IRKD) and the purified human insulin receptor [9,11] both resulted in phosphorylation of the serine residues. After 30 min, serine phosphorylation reached a stoichiometry of up to 1.0 mol phosphate per mol of kinase or  $\beta$ -subunit. (ii) Serine autophosphorylation occurred independently of the protocol performed to purify the kinase. (iii) The kinase negative mutant IRKD-K1018A, purified under the same conditions as the active kinases, was not phosphorylated on serine residues. In addition, one could argue that an exogenous serine kinase requires (i) activation by tyrosine phosphorylation and/or (ii) acts only on the tyrosine phosphorylated insulin receptor kinase. However, the most conclusive evidence for the intrinsic serine kinase activity of the insulin receptor kinase results from experiments with the kinase negative mutant. This kinase was phosphorylated as a substrate on tyrosine residues but only traces of serine phosphate were detected (< 1% of labeled phosphate incorporation). Thus, we present direct evidence for the ability of the insulin receptor kinase to autophosphorylate on both tyrosine and serine/threonine residues. In support of our findings, Tauer et al. [35], through the expression of the insulin receptor with a recombinant vaccinia virus, also conclude that the serine kinase activity is a part of the insulin receptor.

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