Autophosphorylation of the two C-terminal tyrosine residues Tyr^{1316} and Tyr^{1322} modulates the activity of the insulin receptor kinase in vitro

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Abstract Previously, several studies have demonstrated that autophosphorylation of the C-terminal tyrosine residues (Tyr^{1316} and Tyr^{1322}) affects the signaling properties of the insulin receptor in vivo. To assess the biochemical consequences of the C-terminal phosphorylation in vitro, we have constructed, purified and characterized 45 kDa soluble insulin receptor kinase domains (IRKD), either with (IRKD) or without (IRKD-Y2F) the two C-terminal tyrosine phosphorylation sites, respectively. According to HPLC phosphopeptide mapping, autophosphorylation of the three tyrosines in the activation loop of the IRKD-Y2F kinase (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) was not affected by the mutation. In addition, the Y2F mutation did not significantly change the K_m values for exogenous substrates. However, the mutation in IRKD-Y2F resulted in a decrease in the maximum velocities of the phosphotransferase reaction in substrate phosphorylation reactions. Moreover, the exchange of the tyrosines in IRKD-Y2F led to an increase in the apparent K_m values for ATP, suggesting a cross-talk of the C-terminus and the catalytic domain of the enzyme. In addition, as judged by size exclusion chromatography, conformational changes of the enzyme following autophosphorylation were abolished by the removal of the two C-terminal tyrosines. These data suggest a regulatory role of the two C-terminal phosphorylation sites in the phosphotransferase activity of the insulin receptor.

Key words: Insulin receptor kinase; Regulatory function; Serine autophosphorylation; Baculovirus expression system

1. Introduction

The cytosolic tyrosine kinase domains of the insulin receptor (IR) are activated upon binding of insulin to the extracellular domains of the receptor [1,2]. This insulin-mediated receptor kinase activation leads to receptor autophosphorylation on multiple tyrosine residues in the juxtamembrane domain (Tyr^{960}), the catalytic domain (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}), and the C-terminal domain (Tyr^{1316}, Tyr^{1322}). The autophosphorylation enhances the ability of the receptor to catalyze the phosphorylation of exogenous substrates [3–7]. The C-terminal domain seems to play a distinct role in signal transmission and signal specificity of the receptor. In addition to regulatory sequences [8–10], the C-terminus contains two tyrosine autophosphorylation sites, Tyr^{1316} and Tyr^{1322}. C-terminal deletion mutants of the IR have been shown to be poor mediators of the metabolic effects of insulin, without affecting the mitogenic signalling [11,12]. In contrast, mutant receptors in which the two C-terminal tyrosine autophosphorylation sites have been replaced by phenylalanines, stimulated mitogenic effects more efficiently than wild-type receptors, however, without affecting the metabolic signalling [13–15]. Evidently, the C-terminal tail of the IR is involved in the biological signalling of the receptor. However, the molecular basis of this effect is poorly understood.

To analyze whether the C-terminal tyrosine phosphorylation has an influence on the catalytic properties of the IR kinase domain (IRKD), we have constructed a kinase mutant, in which the two tyrosine residues (Tyr^{1316} and Tyr^{1322}) were replaced by phenylalanine (IRKD-Y2F). In the present study, we have compared the enzymatic properties of the mutant kinase with the wild-type enzyme IRKD, a widely used model for the IR [16–21]. Our data indicate that the phosphorylation of the C-terminal tyrosine residues appears to be play a role in fine-tuning of the kinase activity.

2. Materials and methods

[γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham. Restriction endonucleases were from MBI Fermentas, *Pfu* polymerase was from Stratagene, ATP was from Boehringer Mannheim. Cell culture reagents were from Life Technologies Inc.; poly-L-lysine (M_r 15000–30000) and poly(Glu:Tyr)$_{4:1}$ were from Serva; antibodies (α-phosphotyrosine and GαR-AP) were from Boehringer Mannheim. Other reagents were obtained from common commercial sources.

2.1. Construction and expression of IRKD-Y2F and IRKD

IRKD-Y2F: The amino acids Tyr^{1316} and Tyr^{1322} were substituted by phenylalanine by site-directed mutagenesis of the cDNA using pUC-IRKD as template [21]. Two mutagenesis primer were constructed (5′-CAAGCGGAGCTTCGAGGAACACATTCCTTTCACACACATG-3′; 2: 5′-CATGTGTGTGAAAGGTATGTGTTCCTCGAAGCTCCGCTTG-3′), which, in addition to the amino acid substitution, created a *Xmn*I site in the IR cDNA. The mutagenesis reaction was performed with the Quik-change site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The region of the mutation was sequenced to ensure that no other mutation had occurred. Construction of pVL1393-IRKD-Y2F was performed as described for IRKD [21]. *Spodoptera 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 [22].

2.2. Purification of IRKD and IRKD-Y2F

Purification of the soluble kinases was accomplished by sequential chromatography using MonoQ and phenyl–Sepharose (Pharmacia) as described previously [21].

2.3. Construction and purification of GST-CT and GST-Ex20

For substrate phosphorylation reactions, two IR-derived GST fusion proteins were constructed by polymerase chain reactions (PCR),

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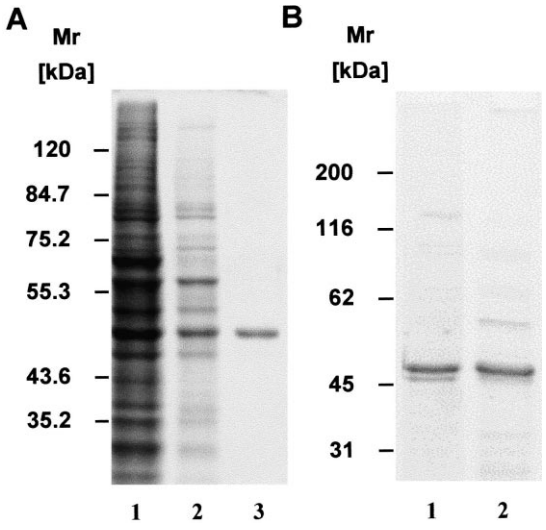

Fig. 1. Purification of IRKD and IRKD-Y2F. A: SDS–PAGE of a representative purification of IRKD. The recombinant kinase was purified by anion exchange chromatography and hydrophobic interaction chromatography. After SDS–PAGE, the proteins were stained with Coomassie blue. Lanes: 1, cell lysate; 2, after anion exchange chromatography; 3, after hydrophobic interaction chromatography. B: Comparison of the purification states of IRKD (lane 1) and IRKD-Y2F (lane 2).

and expressed in *Escherichia coli*. GST-CT contains the C-terminal domain if the IR (Asn^{1249}–Ser^{1343}), includes the two C-terminal tyrosine residues Tyr^{1316} and Tyr^{1322}. GST-Ex20 is derived from IR exon-20, containing the three tyrosine phosphorylation sites of the catalytic domain (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}). The PCR-amplified cDNA fragments were gel-isolated and subcloned into the *Bam*HI–*Eco*RI sites of the expression vector pGEX 3X (Pharmacia). Expression and purification of GST fusion proteins was performed according to the manufacturer's instructions (Pharmacia).

2.4. Size exclusion chromatography

50 μg of purified kinase was subjected to fast protein liquid chromatography on a Superose 12 column (Amersham Pharmacia Biotech) at a flow rate of 0.1 ml min^{-1} in 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM dithiothreitol. Elution was monitored by UV absorption at 280 nm. The column was calibrated with standard proteins (Sigma) of known molecular mass: ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa) and ribonuclease A (13.7 kDa).

2.5. 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 $MnCl_2$, 250 μM [γ^{32}P]ATP and 1 μM poly-L-lysine. For substrate phosphorylation reactions, the kinases were prephosphorylated for 5 min. Phosphorylated 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. Substrate phosphorylations with poly(Glu:Tyr)$_{4:1}$ (1 mg ml^{-1}) were carried out using the phosphocellulose paper assay [23].

2.6. Phosphoamino acid analysis and phosphopeptide mapping

Tryptic digestion of the autophosphorylated kinases was performed as described [24]. The recovery of ^{32}P typically exceeded 90%. 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 [25]. Quantitation of phosphoamino acids was achieved by using a phosphorimager (Bio-Rad Imaging System). HPLC-phosphopeptide mapping was performed as described [21].

2.7. Other procedures

S. frugiperda (Sf9) cells were maintained as described [22]. Protein concentrations were determined by a modified method of Bradford [26]. SDS–PAGE was performed according to Laemmli [27]. Staining of SDS–PAGEs was carried out by a modified Coomassie staining procedure [28].

3. Results and discussion

Several studies have demonstrated that autophosphorylation of the C-terminal tyrosine residues (Tyr^{1316} and Tyr^{1322}) modulates the signaling properties of the IR in vivo [11–15]. However, it was unclear whether phosphorylation of the C-terminal tyrosines affects the physicochemical and catalytic properties of the IR in vitro. Therefore, we have utilized the baculovirus expression system [22] to overexpress and purify the 45 kDa soluble IRKD, and in addition, a mutant IRKD in which both C-terminal tyrosine residues Tyr^{1316} and Tyr^{1322}, have been replaced by phenylalanine (IRKD-Y2F). Both soluble kinases were expressed at similar levels and subsequently purified to $>95\%$ homogeneity (Fig. 1; see Section 2).

In order to characterize the purified kinases, we have carried out autophosphorylation reactions. For the wild-type IRKD, phosphate incorporation after 30 min was 4.4 ± 0.2 mol phosphate per mol protein. Using our experimental conditions (see Section 2), $t_{1/2}$ for this reaction was 0.5 min and the initial velocity was 161 nmol min^{-1} mg^{-1} (Fig. 2A). In contrast, autophosphorylation of IRKD-Y2F resulted in a maximum phosphate incorporation of only 2.9 ± 0.1 mol phosphate/mol protein with an initial velocity of 88 nmol

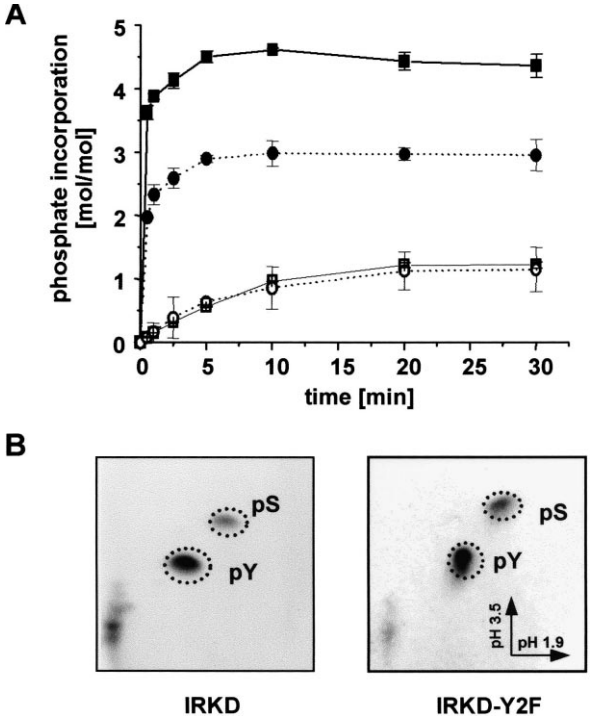

Fig. 2. Time course of the autophosphorylation of IRKD and IRKD-Y2F, and phosphoamino acid analysis. A: Purified IRKD (squares) and IRKD-Y2F (circles) were phosphorylated using standard conditions (see Section 2). At the indicated time points, aliquots were removed and assayed for incorporated phosphate (closed symbols) and serine phosphate content (open symbols). B: Two-dimensional phosphoamino acid analysis of IRKD and IRKD-Y2F after 30 min reaction time.

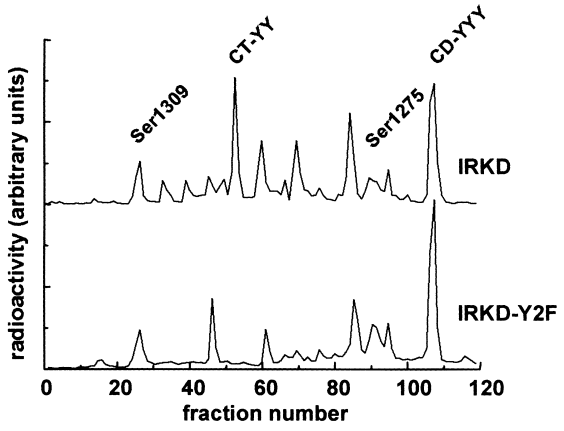

Fig. 3. Phosphopeptide mapping of IRKD and IRKD-Y2F after 30 min reaction time. IRKD and IRKD-Y2F were phosphorylated using standard conditions (see Section 2) and separated by SDS–PAGE. The proteins were digested with trypsin and phosphopeptides were separated by HPLC anion exchange chromatography.

min^{-1} mg^{-1}. Thus, as expected, the C-terminal mutation in the kinase results in a reduction of maximum phosphate incorporation. However, compared to the wild-type enzyme, the half-time of the autophosphorylation reaction is unchanged.

We have shown previously that autophosphorylation of the IRKD in vitro results in the phosphorylation of two serine residues (Ser^{1275} and Ser^{1309}) in the C-terminal domain of the kinase [21]. As shown in Fig. 2, serine autophosphorylation of IRKD and IRKD-Y2 exhibited similar time courses ($t_{1/2} \approx 5$ min; initial velocity $= 3.3 \pm 0.2$ nmol min^{-1} mg^{-1}), resulting in a maximum serine phosphorylation of ≈ 1 mol phosphoserine per mol kinase, respectively. However, it should be noted that phosphoserine and phosphotyrosine may exhibit different stabilities during acid hydrolysis of phosphopeptides [29]. Thus, the observed dual kinase activity of the IRKD is not correlated to the phosphorylation of the C-terminal tyrosine residues, Tyr^{1316} and Tyr^{1322}.

In a previous study, we have identified the major autophosphorylation sites of the soluble IRKD and the human IR by HPLC phosphopeptide mapping and chemical sequencing of tryptic phosphopeptides, respectively [21]. To test whether the mutation of the two phosphorylation sites in the C-terminus affects the autophosphorylation of the tyrosine residues in the catalytic domain, we have performed comparative HPLC phosphopeptide mapping of the two autophosphorylated kinases.

As shown in Fig. 3, the peak containing the phosphorylated C-terminal tyrosine residues of the IRKD (Tyr^{1316}, Tyr^{1322}, CT-YY) was completely absent in the corresponding HPLC profile of the IRKD-Y2F mutant. Also, as expected, the amount of phosphoserine containing peptides, corresponding to Ser^{1275} and Ser^{1309} was similar for both wild-type and IRKD-Y2F mutant. Most importantly, the peak corresponding to the *tris*-phosphorylated catalytic loop of the kinase (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}, CD-YYY) appeared to be similar for both wild-type IRKD and mutant IRKD-Y2F. As indicated in Fig. 3, the total eluted radioactivity of the fractions containing CD-YYY was the same for both wild-type and IRKD-Y2F. Thus, these data demonstrate that the mutation of the two C-terminal autophosphorylation sites has neither an effect on the phosphorylation of the three tyrosines in the activation loop, nor on the phosphorylation of the serine residues in the C-terminus of IR kinase.

We next examined whether the mutation of the C-terminal tyrosine residues affects the phosphorylation of exogenous substrates. As substrates for kinetic studies we used GST fusion proteins containing the major tyrosine phosphorylation sites of the IRKD. GST-Ex20 comprises human IR residues Phe^{1139}–Asp^{1179}, and contains the three tyrosine autophosphorylation sites (Tyr^{1146}, Tyr^{1150} and Tyr^{1151}) of the activation loop of the kinase. GST-CT contains IR residues Asn^{1249}–Ser^{1343}, including the two C-terminal tyrosine autophosphorylation sites Tyr^{1316} and Tyr^{1322}. The phosphorylation reactions were performed in presence of poly-L-lysine (see Section 2), using the pre-phosphorylated enzymes to enable maximum activity of the kinases [30,31]. The resulting kinetic data were fitted according to the Michaelis–Menten model. As indicated by the observed K_m values (Fig. 4A,B and Table 1), both fusion proteins served as high affinity substrates for the

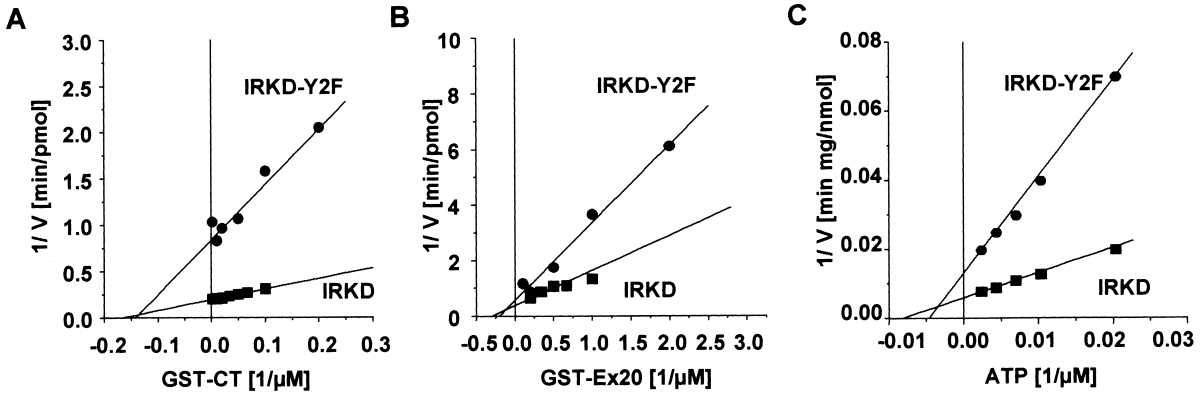

Fig. 4. Kinetic parameters of IRKD and IRKD-Y2F. For substrate phosphorylation reactions the enzymes were pre-phosphorylated for 15 min in the presence of 50 mM Tris–HCl, pH 7.5, 250 µM [γ-^{32}P]ATP, 1 µM poly-L-lysine. Substrate proteins were added at the indicated concentrations and incubated further for 0.5 min. After separation by SDS–PAGE, the amount of incorporated phosphates was determined (see Section 2), and the resulting data were fitted according to the Michaelis–Menten model. Data are the means ± S.E.M. of three separate experiments. A: Lineweaver–Burk plot of GST-CT substrate phosphorylation. B: Lineweaver–Burk plot of GST-Ex20 substrate phosphorylation. C: IRKD and IRKD-Y2F were phosphorylated for 0.5 min with varying ATP concentrations, as indicated. After separation by SDS–PAGE, the amount of incorporated phosphates was determined (see Section 2), and the resulting data were fitted according to the Michaelis–Menten model. Data are the means ± S.E.M. of three separate experiments.

Table 1
Kinetic parameters of IRKD and IRKD-Y2F

	IRKD		IRKD-Y2F	
	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})
GST-CT	6.1 ± 0,5	117.0 ± 3,6	7.4 ± 2.3	26.3 ± 1.9
GST-Ex20	2.9 ± 0,6	53.9 ± 2.1	4.7 ± 0.9	37.7 ± 7.9
ATP	135 ± 28	230 ± 23	299 ± 33	106 ± 6

IRKD. Interestingly, in previous studies the K_m values for short synthetic peptides derived from the autophosphorylation sites of the human IR were reported to be in the upper micro- to millimolar range [32,33]. Accordingly, the ≈5–10-fold lower K_m values for the GST fusion proteins used in the present study indicate that optimal substrate recognition of the enzyme requires an interaction with additional regions in the fusion proteins which were not present in the short synthetic peptides that have been used in previous studies [32,33].

The observed kinetic parameters, K_m and V_{max}, for GST-Ex20 were only slightly different for the wild-type and the mutant IRKD (Table 1). Surprisingly, compared to the wild-type kinase, IRKD-Y2F showed a 4-fold decrease in the maximum velocity when using GST-CT as a substrate, whereas the observed K_m values for GST-CT remained unchanged (Fig. 4A,B and Table 1). Thus, the mutation of the C-terminal tyrosine residues in the kinase alters the transferase activity of the receptor kinase towards GST-CT in a non-competitive fashion. Similarly, the specific phosphotransferase activity of IRKD-Y2F using Poly(Glu:Tyr)$_{4:1}$ as a substrate was also reduced (182 nmol min^{-1} mg^{-1} for IRKD; 100 nmol min^{-1} mg^{-1} for IRKD-Y2F).

Since the latter experiments were all carried out in the presence of poly-L-lysine, which is known to enhance the kinase activity of IRKD [7,17], we have carried out additional phosphorylation reactions with GST-CT in the absence of the basic polycation. Compared to the poly-L-lysine-stimulated reaction, the V_{max} values for both, IRKD and IRKD-Y2F, were about equally reduced (≈20-fold; IRKD: 5 nmol min^{-1} mg^{-1}; IRKD-Y2F: 1.3 nmol min^{-1} mg^{-1}). Likewise, in the absence of poly-L-lysine the observed K_m values for GST-CT were increased ≈2–3-fold for both, IRKD (20 μM) and IRKD-Y2F (11 μM), respectively. As a result, the ≈4-fold reduction in the V_{max} value for IRKD-Y2F was observed even in the absence of poly-L-lysine.

In fact, these data suggest that the mutation of the two C-terminal autophosphorylation sites in the kinase might interfere with the integrity of the active site of the enzyme. Therefore, we have determined the apparent kinetic parameters for ATP in autophosphorylation reactions. As shown in Fig. 4C and Table 1, the observed values for V_{max} and K_m for IRKD agreed reasonable well with previous observations [17,34–36]. In contrast, IRKD-Y2F showed a significant increase in the K_m value whereas V_{max} was decreased. Thus, the mutation of the C-terminal phosphorylation sites of the kinase has a direct influence on the apparent affinity of ATP and furthermore, causes a decrease in the maximum velocity of the reaction. Interestingly, several reports have demonstrated that deletion of the C-terminal domain of the IR kinase affects the affinities for substrates and the maximum velocities (V_{max}) of the kinase reaction in vitro [34,37]. Similar results were obtained using anti-peptide antibodies against regulatory motifs in the C-terminal domain [9,10]. Notably, a recent study in our laboratory revealed that a single point mutation in the catalytic domain of the IRKD (Tyr^{1151} → Phe) led to a decreased autophosphorylation of the C-terminal serine residues, without affecting the autophosphorylation of the two tyrosines, Tyr^{1316} and Tyr^{1322} [38].

To evaluate any interaction between the C-terminal phosphotyrosines and the active site of the kinase we have performed gel filtration chromatography experiments to detect phosphorylation-induced changes in the overall shape of the kinase (Fig. 5). Both, IRKD and IRKD-Y2F were subjected onto a Superose 12 column either in the phosphorylated or non-phosphorylated state, respectively, and the elution of the proteins was measured by UV absorption at 280 nm. The non-phosphorylated IRKD migrated with an apparent molecular mass of ∼60 kDa. However, after autophosphorylation the apparent molecular mass of the IRKD shifted to ∼100 kDa. Since the gel filtration experiments were carried out in the presence of 1 M NaCl (see Section 2), these alteration could rather be explained by changes in the conformation of the molecule, than by the formation of stable kinase dimers. This is in agreement with the observation that phosphorylated and non-phosphorylated IRKD migrated with the same ap-

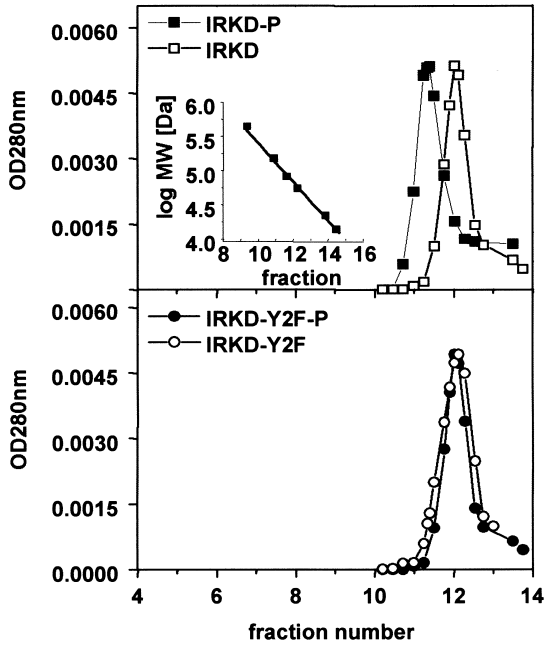

Fig. 5. Size exclusion chromatography. Purified IRKD (upper profile) or IRKD-Y2F (lower profile) were incubated for 30 min with 50 mM Tris–HCl, pH 7.5, 250 μM [γ-^{32}P]ATP in the presence (closed symbols) or absence (open symbols) of 250 μM ATP, respectively, and subjected to size exclusion chromatography. Elution was monitored by UV absorption at 280 nm. The inset depicts the logarithms of the molecular masses of the protein standards, plotted against the respective elution fraction (protein standards: ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin (25 kDa) and ribonuclease A (13,7 kDa)).

parent molecular mass in native polyacrylamide gel electrophoresis (data not shown). In contrast, either in its phosphorylated or non-phosphorylated state, IRKD-Y2F eluted with an apparent molecular mass of 60 kDa from the column. A similar result was obtained for a soluble IR kinase, where 72 amino acids from the C-terminal domain were deleted (E. Bergschneider, unpublished results). Evidently, autophosphorylation of the C-terminal tyrosine residues, Tyr^{1316} and Tyr^{1322}, has a profound effect on the Stoke's radius of the kinase molecule, thereby leading to significant changes in the hydrodynamic properties of IRKD.

In summary, we have shown that mutation of the C-terminal tyrosine autophosphorylation sites (Tyr^{1316} and Tyr^{1322}) in the IRKD apparently does not interfere with the activation of the kinase as judged by the presence of the *tris*-phosphorylated catalytic loop in phosphopeptide maps of IRKD-Y2F. However, the mutation led to a decrease in the V_{max}-value for the IRKD C-terminus in substrate phosphorylation reactions, and, more importantly to a reduced apparent affinity for ATP. Lastly, we have presented evidence that autophosphorylation of the two C-terminal tyrosine residues, induces a large increase in the Stoke's radius of the kinase, indicating conformational changes in the kinase upon phosphorylation of Tyr^{1316} and Tyr^{1322}.

Thus, we propose that phosphorylation of the C-terminal tyrosine residues modulates the kinase activity by phosphorylation-dependent interactions of the C-terminus with the catalytic domain of the kinase. Unfortunately, due to the reported flexibility of the IRKD C-terminus, no crystal structure of the IR kinase containing the C-terminal domain has been made available [40]. However, changes in the conformation of the IR induced by binding of a C-terminal domain to the kinase were also reported by others [39]. Interestingly, Shoelson et al. demonstrated that substrate analogs, containing phenylalanine instead of tyrosine, (i.e. pseudosubstrate sequences) inhibited substrate phosphorylation of the IR in a non-competitive fashion [41]. Most recently, it has been reported that the C-terminal tail of protein kinase A directly interacts with both peptide substrates and the ATP-binding site of the kinase [42]. Hence, one might speculate that the non-phosphorylated C-terminus in IRKD acts in an autoinhibitory fashion by binding to the catalytic site, thereby changing the overall shape of the molecule and modulating the catalytic activity of the enzyme.

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