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Phosphoryl exchange is involved in the mechanism of the insulin receptor kinase

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Abstract

The cytoplasmic kinase domain of the human insulin receptor (IRKD; M_r 49 kDa) has been over-expressed in insect cells using the baculovirus expression system. To investigate the kinase mechanism, we have compared the stoichiometry of ADP formation and phosphoryl transfer. After an initial phase of autophosphorylation, ATP is consumed without a stoichiometric increase in incorporated phosphate. During substrate phosphorylation using poly(Glu:Tyr) (4:1) phosphoryl transfer comes close to ATP turnover, which is independent of the presence of the substrate, indicating an increased efficiency (i.e. ATP turnover/phosphate incorporation) of phosphoryl transfer. Autophosphorylation under pulse-chase conditions suggests the existence of a phosphoenzyme intermediate.

Key words: Insulin receptor kinase; Phosphoenzyme intermediate; Baculovirus expression system

1. Introduction

The soluble insulin receptor kinase domain (IRKD) derived from the cytoplasmic portion of humans can be used as a model enzyme for studying the kinase mechanism of the insulin receptor. The monomeric enzyme exhibits comparable substrate specificities, kinetic constants and autophosphorylation sites as the human insulin receptor $(\alpha\beta)_2$ heterotetramer [1–4]. However, the enzyme lacks hormone-mediated activation. It is constitutively active, like other extracellular truncated growth factor receptors [5]. Several attempts have been made to elucidate the mechanism of insulin receptor autophosphorylation and phosphorylation of exogenous substrates. Most of the studies have focused on the phosphoryl acceptor of the kinase reaction, whereas ATP consumption of the bisubstrate reaction has been neglected. In order to get a more detailed analysis of the kinase reaction we asked the question of whether ATP consumption equals phosphate incorporation. Using a mixture of α- and γ-labeled ATP we were able to distinguish between ADP formation (equal to ATP consumption) and phosphoryl transfer to the kinase itself (autophosphorylation) or an exogenous substrate, respectively.

2. Materials and methods

Restriction enzymes, ATP, AMP-PNP, histone 2b, fetal bovine serum and staurosporine were obtained from Boehringer, Mannheim. Grace's insect medium, gentamycine sulfate and amphotericin B were from Life Technologies Inc. Poly(L-lysine) (M_r 6000–9000) was from Serva: Poly(Glu:Tyr) (4:1) was from Sigma. DEAE-cellulose, phenyl-Sepharose and peptide M_r markers were from Pharmacia. [α-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham; [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from New England Nuclear. The cDNA of the human insulin receptor precursor was kindly provided by Dr. Axel Ullrich, Max Planck Institute, Martinsried, Germany. Other reagents were obtained from common commercial sources.

2.1. Construction and isolation of recombinant baculovirus

The baculovirus transfer vector pBlueBacHis-C (Invitrogen) was linearized with *Bam*HI. After generation of blunt ends using Klenow polymerase [6] followed by digestion with *Pst*I, the plasmid was gel-purified. A *Bgl*I/*Eco*RI fragment of the human insulin receptor cDNA was treated with T4 polymerase in the presence of all deoxynucleotides to produce blunt ends. After digestion with *Pst*I, the fragment encoding the human insulin receptor amino acid sequence 947–1343 [7] was isolated from a gel and ligated into the transfer vector. Sf9 cells were co-transfected with AcNPV DNA and the resulting transfer vector using the BaculoGold System (Pharmingen) according to the manufacturer's instructions. Recombinant viruses were isolated by performing sequential rounds of plaque assays [8].

2.2. Purification of IRKD

Sf9 cells (4×10^8) growing in suspension were infected with a multiplicity of infection of > 10. At 72 h post-infection, cells were harvested by low-speed centrifugation and homogenized in 40 ml of 50 mM Tris-HCl, pH 7.6, containing 0.25 M sucrose, 1 mM PMSF and 10 μg/ml leupeptin. The lysate was centrifuged for 5 min at $1000 \times g$ and the supernatant was re-sedimented at $120{,}000 \times g$ for 1 h. The high-speed supernatant was applied to a DEAE-cellulose column, equilibrated with starting buffer (50 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl, 1 mM DTT, 1 mM PMSF and 10 μg/ml leupeptin). After washing the column with starting buffer, proteins were eluted with a linear gradient of increasing salt concentration, using starting buffer with 0.4 M NaCl. IRKD-containing DEAE fractions were pooled and adjusted to contain 20% $(NH_4)_2SO_4$, stirred for 30 min on ice, and centrifugated for 20 min at $20{,}000 \times g$. The supernatant was loaded onto a HR 5/5 phenyl-Sepharose column, equilibrated with starting buffer (50 mM Tris-HCl, pH 7.6, containing 0.25 M NaCl, 20% $(NH_4)_2SO_4$, 1 mM DTT, 1 mM PMSF and 10 μg/ml leupeptin). After washing the column with a stepwise gradient of decreasing salt concentration, elution of the kinase was achieved by washing the column with 50 mM Tris-HCl, Ph 7.6, 1 mM DTT, 1 mM PMSF and 10 μg/ml leupeptin. Finally, fractions containing IRKD were pooled, concentrated 5-fold (Amicon concentrator, C-30, Amicon) and stored at −70°C.

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; AcNPV, *Autographa californica* nuclear polyhedrosis virus; DEAE, diethylaminoethyl.

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2.3. Protein kinase reactions

All reactions were carried out at 22°C. The reaction mixtures contained 30 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 μM poly(lysine) and ATP as indicated. Substrate phosphorylations were performed using poly(Glu:Tyr) at 1 mg/ml. For the determination of incorporated phosphate and formation of ADP, [γ-^{32}P]ATP and [α-^{32}P]ATP were included (1–2.5 μCi/assay), respectively. Reactions were initiated by adding 2.5 pmol IRKD to the reaction mixture and terminated at indicated times by the addition of EDTA to a final concentration of 20 mM. Aliquots were spotted onto P81 paper (Whatman) and incorporated ^{32}P was determined as previously described [9]. Nucleotides were separated by thin layer chromatography [10] on HPTLC NH_2 F_{254}s plates (Merck) and analyzed with a PhosphoImager (Fuji BAS1000).

2.4. Other procedures

Sf9 cells were maintained as described [8]. Protein concentrations were determined by the method of Bradford [11]. SDS-PAGE was performed according to Laemmli [12]. The K_m and V_{max} values of ATP were determined using the Enzfitter program (Elsevier Biosoft). Phosphopeptides were analyzed by Tricine-SDS-PAGE [13] after BrCN cleavage [14] of ^{32}P-labelled IRKD.

3. Results

3.1. Purification of the insulin receptor kinase domain

The cytoplasmic kinase domain of the human insulin receptor (IRKD; M_r 49 kDa) was over-expressed in insect cells using the baculovirus expression system. Purification of the protein was accomplished by sequential chromatography on DEAE-cellulose and phenyl-Sepharose with an overall yield of 1–2 mg/l cell culture. The purity of IRKD typically exceeded 90% based upon density evaluation of Coomassie blue-stained gels (Fig. 1).

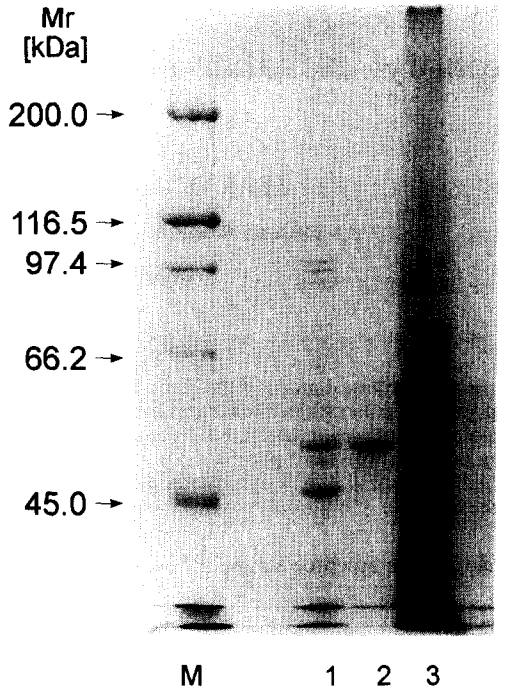

Fig. 1. SDS-PAGE analysis of IRKD during purification. Aliquots from the purification steps were analyzed by Coomassie blue staining after 10% SDS-PAGE. Lanes: M, M_r markers; 1, DEAE-cellulose pool; 2, phenyl-Sepharose pool; 3, crude extract.

3.2. Kinetic constants for the autophosphorylation

For the autophosphorylation reaction we determined the initial velocities of phosphate incorporation into IRKD and concomitant ADP formation using different ATP concentrations. As illustrated in Fig. 2, the kinetic constants of both reactions are almost identical, reflecting indicating that phosphate incorporation and formation of ADP in the initial phase of autophosphorylation are approximately equal. The K_m values of metal ATP for phosphate incorporation into IRKD and ADP formation are 52 ± 6.2 μM and 52.7 ± 3.4 μM; the corresponding V_{max} values are 171.2 ± 8 nmol·mg^{-1}·min^{-1} and 168.6 ± 4.2 nmol·mg^{-1}·min^{-1}, respectively. These values are in agreement with previously reported kinetic constants [2].

3.3. Time–course of phosphate incorporation and formation of ADP during autophosphorylation

To characterize phosphoryl transfer of the prephosphorylated kinase we have performed autophosphorylation reactions using 250 μM ATP. Fig. 3A shows a representative time–course of the autophosphorylation of the IRKD. Within 30 min, a maximum of 4–5 mol of phosphates were incorporated per mol of enzyme. Surprisingly, as the reaction proceeded further, phosphate incorporation into IRKD reached saturation, while the concentration of ADP continued to increase. Prolonged incubation of the kinase resulted in a decreased phosphate content of the enzyme accompanied by a depletion of ATP (cf. [15]). The degree of dephosphorylation at a given time appears to be influenced by the ratio of kinase-to-ATP in the assay (Fig. 3B). After 90 min of IRKD autophosphorylation at an enzyme-to-ATP ratio of 1:125, 1:250 and 1:500, we observed a loss of 48, 38 and 22% of incorporated phosphate, respectively. An analysis of the first 10 min of the autophosphorylation reaction showed that only the initial phosphate incorporation (≈ 3 mol/mol enzyme within 1 min) was accompanied by an equimolar formation of ADP (Fig. 3C). After 10 min, the ratio of ADP to incorporated phosphate was about 1:10. If poly(lysine) was omitted, both activities, phosphate incorporation and formation of ADP, decreased 15-fold, leaving the ratio unchanged (data not shown).

3.4. Effects of kinase inhibitors on the autophosphorylation

The observation that ATP is consumed after the initial phase of autophosphorylation without a stoichiometric increase in phosphate content of the enzyme could be either the result of a phosphatase activity or an ATPase activity, resulting in a depletion of ATP after prolonged reactions. Common phosphatase inhibitors (e.g. vana-

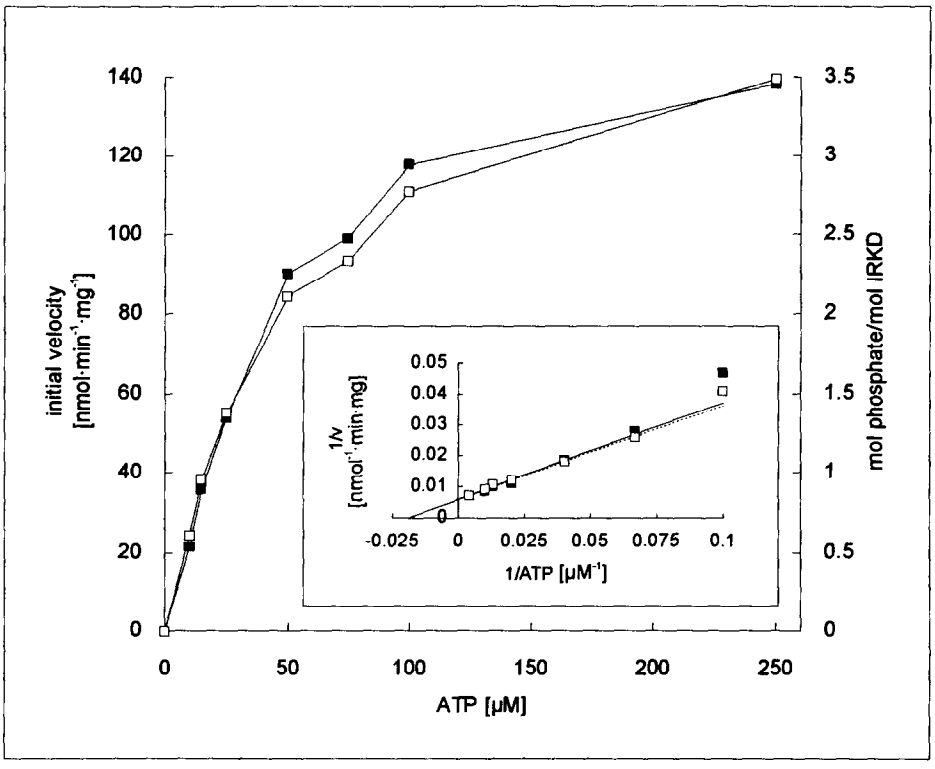

Fig. 2. Initial velocities of phosphate incorporation and formation of ADP during IRKD autophosphorylation using different ATP concentrations. The reactions were initiated by the addition of 2.5 pmol enzyme. After 30 s the reaction was stopped by the addition of 20 mM EDTA. Determination of incorporated phosphate (□) and ADP (■) were performed as described in section 2. Values reported are the means of 3–4 independent experiments. Inset: Lineweaver–Burk plot of IRKD autophosphorylation.

date) had no effect on either ATP turnover or phosphate incorporation with respect to autophosphorylation (data not shown). Nevertheless, both activities can be clearly distinguished by the use of kinase inhibitors: (i) if the formation of ADP is due to removal of phosphate by a phosphatase followed by subsequent rephosphorylation, the prephosphorylated enzyme should exhibit loss of phosphate in the presence of a kinase inhibitor; (ii) if ATP is hydrolyzed by a contaminating. ATPase, consumption of ATP should continue even though kinase inhibitors are present.

After 5 min of autophosphorylation in the presence of [γ-^{32}P]ATP followed by addition of staurosporine or the non-hydrolyzable ATP analogue AMP-PNP, no dephosphorylation of the IRKD was observed after 60 min of incubation, in contrast to the reaction where the inhibitors were omitted (Fig. 4A). The experimental conditions for the experiment were chosen such that a significant loss of incorporated phosphate was detectable after 60 minutes. If the reaction was initiated in the presence of the inhibitors (using EC_{50} concentrations of staurosporine (3 μM) or AMP-PNP (0.5 mM)) both autophosphorylation and formation of ADP were inhibited to the same extent (Fig. 4B). In addition, both autophosphorylation and ATP consumption are dependent on Me^{2+}-ATP, since EDTA prevented both phosphate incorporation and ADP formation (data not shown). Therefore, these data are not consistent with contaminating phosphatases or ATPases and suggest that the observed ATPase-like activity of the phosphorylated enzyme is intrinsic to the kinase.

3.5. *Phosphoryl transfer and ATP consumption during substrate phosphorylation*

To test whether the formation of ADP is influenced by the presence of a substrate for the kinase, we used the model substrate poly(Glu:Tyr) (Fig. 3D). In contrast to the rate of the autophosphorylation reaction (Fig. 3C), that of phosphate incorporation into the substrate paralleled the rate of ADP formation for a longer period of time. However, the ATP turnover remained almost unchanged compared to the autophosphorylation reaction. Thus, in the presence of an appropriate phosphoryl acceptor, only the efficiency of phosphoryl transfer is increased.

3.6. *Autophosphorylation under pulse-chase conditions*

On account of our results showing that after an initial autophosphorylation ATP consumption seems to be independent of the phosphorylation state of the enzyme, the question arises of the possible existence of a transitory phosphorylated enzyme. To prove this hypothesis

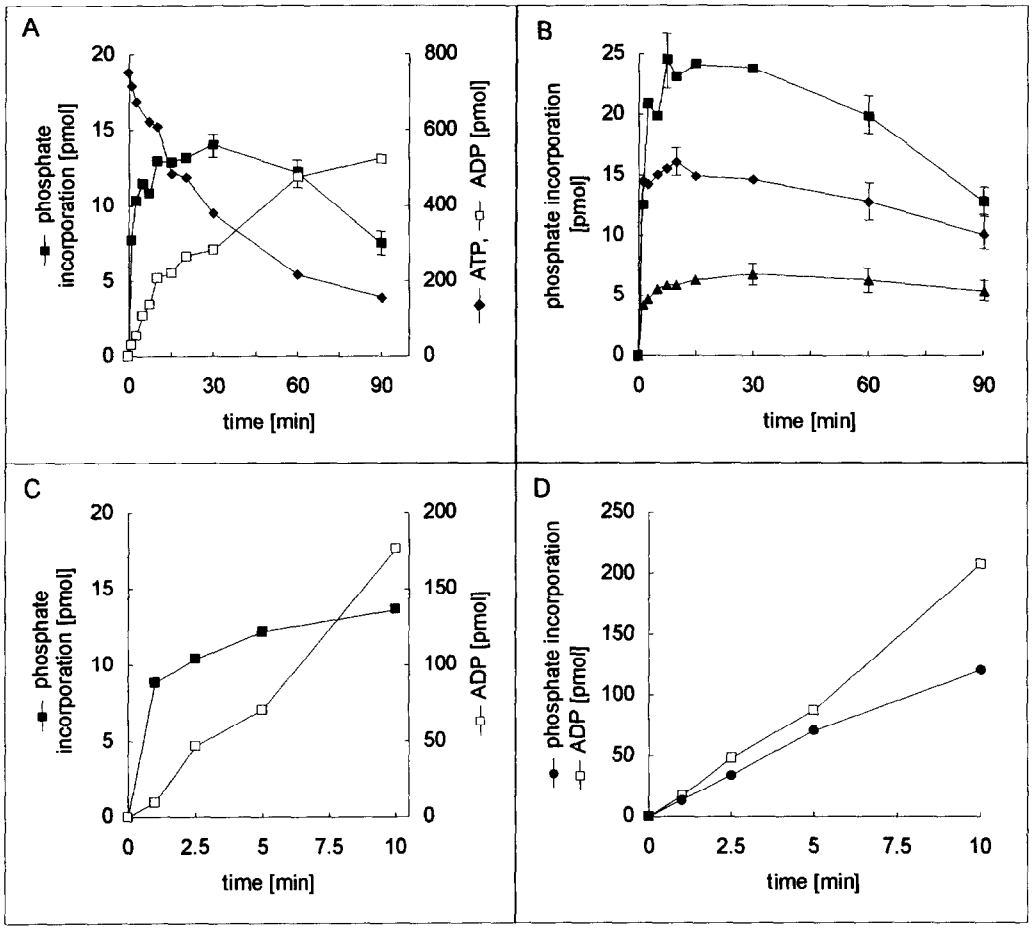

Fig. 3. Time-course of phosphate incorporation and formation of ADP during IRKD autophosphorylation and phosphorylation of poly(Glu:Tyr). An ATP concentration of 250 μM was used. (A) Autophosphorylation was initiated by addition of IRKD. At indicated times aliquots (3.8 pmol IRKD) were removed and analyzed for radioactive phosphate content of IRKD (■), ATP (◆) and ADP (□). (B) Autophosphorylation of IRKD at different concentrations of enzyme (■ 2 μM, ◆ 1 μM, and ▲ 0.5 μM). At given times, aliquots (2.5 μl) corresponding to 5, 2.5 and 1.25 pmol were removed and analyzed for incorporated phosphate. (C) Phosphate incorporation into IRKD (■) and formation of ADP (□). (D) Phosphate incorporation into poly(Glu:Tyr) (●) and formation of ADP (□). Values reported are the means of 2–3 independent experiments.

we have performed autophosphorylation reactions under pulse-chase conditions. Prephosphorylation of the enzyme in the presence of 200 μM unlabeled ATP was followed by adding [γ-^{32}P]ATP to the reaction mixtures. As shown in Fig. 5A the almost completely phosphorylated enzyme shows incorporation of ^{32}P, indicating a constant turnover of enzyme-bound phosphates. In a similar experiment where autophosphorylation was initiated in the presence of [γ-^{32}P]ATP followed by the addition of excess unlabeled ATP, we observed a decrease in incorporated radioactivity (data not shown).

4. Discussion

A 49 kDa cytoplasmic kinase domain of the human insulin receptor has been cloned, expressed and purified to near homogeneity. Determination of the ATP turnover using [α-^{32}P]ATP and [γ-^{32}P]ATP as tracers suggests that IRKD autophosphorylation is a biphasic reaction [4]. In the initial phase of the autophosphorylation reaction, at saturating ATP concentrations, the kinase incorporates $\approx$3 mol phosphates generating an equal amount of ADP (Fig. 3C). Once the enzyme has been partially phosphorylated, the ATP turnover remains constant with almost initial velocity. Furthermore, the ATP turnover appears to be independent of the presence of the substrate poly(Glu:Tyr) (Fig. 3D). However, the efficiency of the phosphoryl transfer in the presence of the substrate is increased. The observation that the turnover number of the enzyme is independent of the presence of the exogenous phosphoryl acceptor indicates a different mechanism of substrate recognition to that reported for other kinases (e.g. hexokinase) involving an 'induced fit' [16]. Moreover, the rate of ADP formation in the initial autophosphorylation seems to be the maximum velocity of the phosphotransferase reaction to the artificial substrate poly(Glu:Tyr). Autophosphorylation reactions

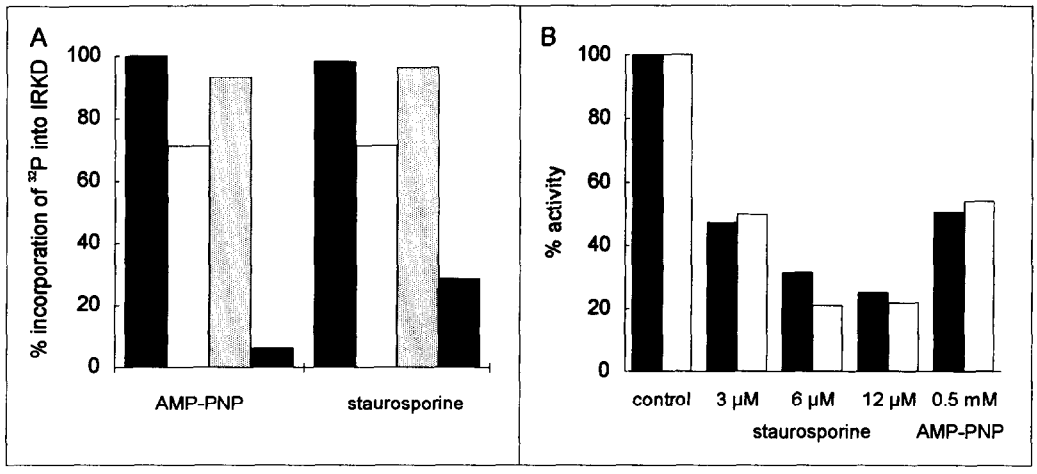

Fig. 4. Inhibition of phosphotransferase activity by staurosporine and AMP-PNP. (A) 2.5 pmol IRKD was prephosphorylated using 200 μM [γ-^{32}P]ATP. After 5 min (filled bars) and 60 min (open bars) the reactions were assayed for incorporated phosphate. In parallel reactions, inhibitors (15 μM staurosporine; 10 mM AMP-PNP) were added after 5 min of autophosphorylation. After an additional 55 min the reactions were assayed for incorporated phosphate (lightly shaded bars). To prove the effectiveness of the inhibitors, autophosphorylation was initiated in presence of inhibitors. After 5 min the reactions were assayed for incorporated phosphate (dark shaded bars). (B) IRKD autophosphorylation was initiated with 100 μM ATP in the absence (control) and presence of staurosporine or AMP-PNP. After 10 min aliquots were assayed for incorporated phosphate (open bars) and ADP (filled bars). In the controls, 100% incorporated phosphate and ADP formation were equivalent to 8 and 110 pmol, respectively. Values reported are the means of 3 independent experiments.

under pulse-chase conditions revealed a constant turnover of incorporated phosphates (Fig. 5A). These data are best explained by a phosphorylated enzyme cycling between phosphorylated and dephosphorylated states. Thus, continuous formation of ADP by the completely phosphorylated kinase is consistent with a phosphorylated enzyme intermediate that is capable of phosphoryl exchange. Since both reactions – dephosphorylation of the kinase and utilisation of ATP – are inhibited by the kinase inhibitors staurosporine and

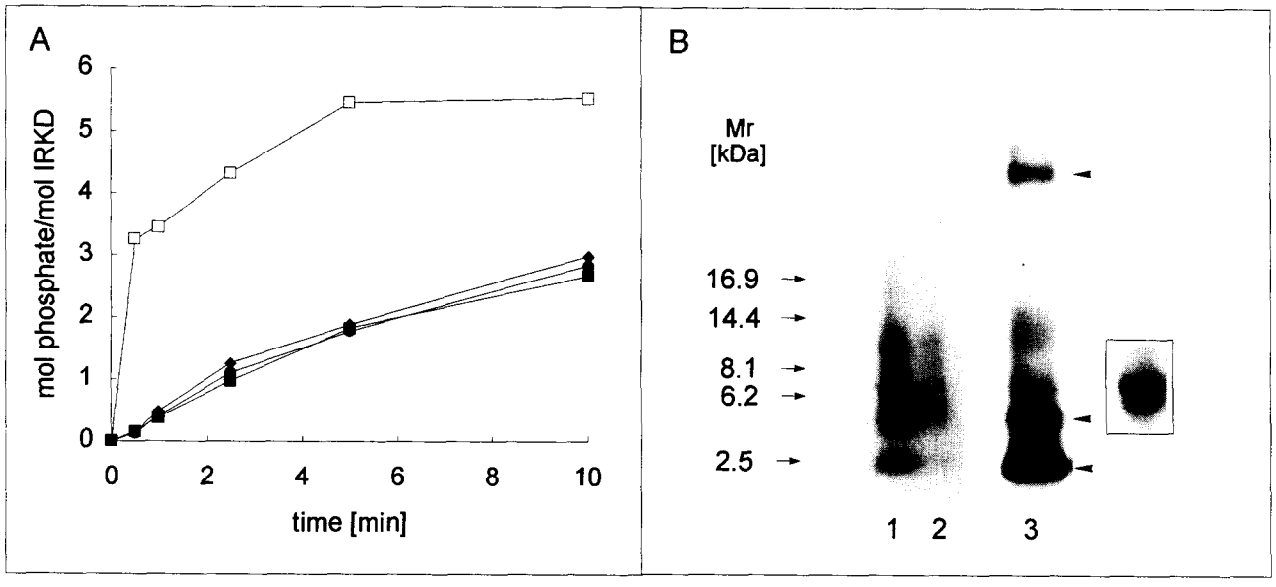

Fig. 5. Autophosphorylation of IRKD under pulse-chase conditions. (A) The enzyme was phosphorylated under standard conditions in the presence of 200 μM [γ-^{32}P]ATP (□; pulse reaction). In parallel reactions the enzyme was prephosphorylated using unlabeled 200 μM ATP. 2 μCi [γ-^{32}P]ATP was added ('hot chase') after 10 min (◆), 20 min (●) and 30 min (■). At indicated times aliquots were removed and assayed for incorporated phosphate. Values reported are means of two independent experiments. (B) Autoradiograph of cyanogen bromide-derived phosphopeptides resolved by Tricine-SDS-PAGE. Lane 1, pulse reaction (8 min; 17400 cpm incorporated); inset, region between 6 and 8 kDa, derived from a partial digest; lane 2, hot chase reaction (8 min; 8030 cpm incorporated) after 2 min of prephosphorylation in presence of unlabelled ATP; lane 3 (reference), phosphopeptides of the human insulin receptor (IR) after 10 min pulse reaction in the presence of 50 μM [γ-^{32}P]ATP. The arrows denote the position of the 31 kDa juxtamembrane peptide, the 6 kDa C-terminal peptide and the 2.5 kDa phosphopeptide of the central domain.

AMP-PNP, an involvement of contaminating phosphatases or ATPases seems unlikely. Moreover, evidence against the presence of phosphatases in the assays also comes from phosphorylation studies of substrates. The substrates tested were recombinant peptides representing insulin receptor autophosphorylation sites and the model substrate histone 2b. No reversal of substrate phosphorylation following lengthy incubations was seen (data not shown).

A reversal of the kinase reaction involving ADP and phosphorylated enzyme [17–19] does not account for the phosphoryl exchange we discuss here. The reverse reaction would result in a steady-state equilibrium where the concentrations of the phosphorylated enzyme, ATP and ADP reach constant values. Instead, the generation of inorganic phosphate at a constant rate of ATP consumption started when the ratio of ADP to ATP was about 0.01 and can not be compared to the dephosphorylation reaction observed when the concentration of ATP is significantly decreased.

However, the role of exchangeable phosphates of the IRKD remains to be elucidated. As the data suggest, only a fraction of phosphorylation sites of the IRKD is involved in phosphoryl exchange (Fig. 5A). A comparison of the phosphopeptides of the IRKD shows that exchangable phosphates are present in all of the observed phosphorylation sites, although to a different extent (Fig. 5B). Two phosphopeptides (~2.5, ~6.2 kDa) of the IRKD and the human insulin receptor correspond to each other and are most probably derived from the central and the C-terminal domain of the kinases. As expected, the cyanogen bromide fragments of the juxtamembrane domain of the human insulin receptor and the IRKD are not identical. Whereas the insulin receptor fragment migrates as an ~31 kDa peptide (U. Mathey, unpublished results), the corresponding IRKD fragment (~7.5 kDa) is hardly resolved. However, the position of the exchangeable tyrosine phosphates in the primary sequence have still to be elucidated. Recently, the insulin receptor has been characterized as a protein kinase with dual specificity [20,21]. Therefore, a phosphoenzyme intermediate could play a role in catalyzing phosphoryl transfer during the autophosphorylation reaction to tyrosine as well as to serine. The question currently under investigation is whether dephosphorylation functions as a switch for mediating association with receptor-interacting proteins like insulin receptor substrate 1 (IRS-1) [22].

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