Intrasteric Inhibition of ATP Binding Is Not Required To Prevent Unregulated Autophosphorylation or Signaling by the Insulin Receptor

MARK FRANKEL,¹ ARARAT J. ABLOOGLU,¹ JOSEPH W. LEONE,² ELENA RUSINOVA,¹
J. B. ALEXANDER ROSS,¹ ROBERT L. HEINRIKSON,² AND RONALD A. KOHANSKI^{1*}

*Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029,¹
and Department of Protein Science, Pharmacia and Upjohn, Inc., Kalamazoo, Michigan 49001²*

Received 13 June 2000/Returned for modification 18 July 2000/Accepted 2 April 2001

Receptor tyrosine kinases may use intrasteric inhibition to suppress autophosphorylation prior to growth factor stimulation. To test this hypothesis we made an Asp1161Ala mutant in the activation loop that relieved intrasteric inhibition of the unphosphorylated insulin receptor (IR) and its recombinant cytoplasmic kinase domain (IRKD) without affecting the activated state. Solution studies with the unphosphorylated mutant IRKD demonstrated conformational changes and greater catalytic efficiency from a 10-fold increase in k_{cat} and a 15-fold-lower K_m ATP although K_m peptide was unchanged. Kinetic parameters of the autophosphorylated mutant and wild-type kinase domains were virtually identical. The Asp1161Ala mutation increased the rate of *in vitro* autophosphorylation of the IRKD or IR at low ATP concentrations and in the absence of insulin. However, saturation with ATP (for the IRKD) or the presence of insulin (for the IR) yielded equivalent rates of autophosphorylation for mutant versus wild-type kinases. Despite a biochemically more active kinase domain, the mutant IR expressed in C2C12 myoblasts was not constitutively autophosphorylated. However, it displayed a 2.5-fold-lower 50% effective concentration for insulin stimulation of autophosphorylation and was dephosphorylated more slowly following withdrawal of insulin than wild-type IR. In tests of the regulation of the unphosphorylated basal state, these results demonstrate that neither intrasteric inhibition against ATP binding nor suppression of kinase activity is required to prevent premature autophosphorylation of the IR. Finally, the lower rate of dephosphorylation suggests invariant residues of the activation loop such as Asp1161 may function at multiple junctures in cellular regulation of receptor tyrosine kinases.

Signal transduction through receptor tyrosine kinases (RTKs) is essential throughout the life of an organism. The tight control of RTK signaling required for normal cellular function is dependent on growth factor stimulation of autophosphorylation (50). Autophosphorylation is a unique regulatory motif among protein kinase signaling cascades because no other enzyme precedes or participates in autophosphorylation. Therefore the unphosphorylated RTK must have sufficient catalytic activity to perform the first phosphoryl transfer reaction while (i) avoiding autophosphorylation prior to growth factor binding and (ii) avoiding target phosphorylation prior to autophosphorylation. The first restriction is met by hormone-induced dimerization of monomeric RTKs (50), where the homodimer itself provides the enzyme-substrate pair. The second restriction is met by target or mediator recruitment sites which appear on the RTK as a consequence of autophosphorylation (44, 51). The logic of induced dimerization seems to fail when applied to the insulin receptor (IR). The IR is a heterotetramer with an $\alpha_2\beta_2$ -subunit structure. The extracellular α -subunits are covalently linked to each other and to the membrane-spanning β -subunits by disulfide bonds (36). Nevertheless this dimerized RTK retains an unphosphorylated basal state and does not autophosphorylate or signal until insulin binds.

The IR's recombinant cytoplasmic kinase domain (IRKD)

(residues 954 to 1382, numbered according to Ebina et al. [14]) has been used as a biochemically and biophysically tractable model for kinase activity of the full-length IR. It has kinetic features similar to the basal state of the IR (32, 34), and it can be activated by autophosphorylation (11, 53, 55) so that its structure should reveal aspects of the kinase important for the receptor's function and regulation (24, 26). The crystal structure of the catalytic core (residues 978 to 1283) (26) identified the activation loop (residues D1150 to P1172) as an intrasteric inhibitor which blocked entry of both substrates to the active site. Neither ATP nor peptide can bind in the active site if the activation loop remains in this "gate-closed" conformation, rendering the kinase latent as an enzyme. Also, Y1162—among the first tyrosines to be autophosphorylated (17, 27, 46, 48, 49, 55, 58)—is sterically inaccessible and therefore cannot be phosphorylated by a *trans* autophosphorylation reaction (26). The gate-closed form of the kinase is also latent as a substrate. This dual latency is capable of maintaining an unphosphorylated basal state for the full-length IR because it is not compatible with autophosphorylation.

There is compelling biophysical evidence that a latent form of the kinase accounts for >90% of the IRKD in solution when substrates are absent (3, 18). However, previous studies with the native IR demonstrated that occupancy of the binding site by ATP or nonhydrolyzable analogs promoted *trans* interactions (57) and conformational changes in the receptor (39) and in particular enhanced the autophosphorylation that is responsible for kinase activation (41). Furthermore, kinetic evidence showed that ATP binding predisposes the unphosphorylated receptor for *trans* autophosphorylation (32). Given the appar-

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029. Phone: (212) 241-7288. Fax: (212) 996-7214. E-mail: Ronald.Kohanski@mssm.edu.

ent affinity of the kinase domain for MgATP of ~ 1 mM (1, 7, 18) and the intracellular ATP concentration of ~ 1 mM (possibly as high as 8 mM [37]), a significant fraction of the receptor's kinase domains should have nucleotide bound under physiological conditions and therefore should be nonlatent as an enzyme and nonlatent as a substrate (18). This raises the issue of whether latency is required to maintain the basal state and, conversely, whether the IR with nonlatent kinase domains can be kept from autophosphorylation and signaling. With a single point mutant, we have established a predominantly gate-open state that is relieved of latency. It displays a high turnover number and improved ATP binding, so that it will be saturated at physiological concentrations of ATP and capable of more-rapid autophosphorylation than the native receptor. We used this mutant to test intrasteric inhibition against ATP binding as a potential regulator of the IR's basal state.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT; Sigma Ultra), the disodium salt of ATP (from equine muscle, catalog no. A-5394), ADP, bovine serum albumin (BSA; radioimmunoassay grade), and wheat germ agglutinin (WGA)-agarose were purchased from Sigma; hydrogenated Triton X-100 (protein grade) was from Calbiochem; EDTA was from Fluka; Tris acetate (TrisAc), Tris base, Tris HCl, Triton X-100, and electrophoresis reagents were from Boehringer Mannheim; magnesium acetate (MgAc_2 ; enzyme grade) was from Fisher. Insect and mammalian cell culture media and fetal bovine serum were from Gibco/BRL and Cellgro. The synthetic peptide used for steady-state kinetics has the amino acid sequence REETGSEYMNMDLG (IRS939). It was prepared by 9-fluorenylmethoxy carbonyl chemistry and purified (8). Adenine nucleotides and the synthetic peptide were dissolved in 50 mM TrisAc, pH 7.0, and the pH was readjusted to 7.0. These stocks were kept at -20°C for frequent use or at -80°C for storage. The concentrations of adenine nucleotides and synthetic peptides were determined spectrophotometrically: for ATP and ADP, $\epsilon = 15,300 \text{ cm}^{-1} \text{ M}^{-1}$ at 259 nm; for IRS939, $\epsilon = 1,300 \text{ cm}^{-1} \text{ M}^{-1}$ at 278 nm.

Mutagenesis, IRKD protein expression, and purification. Baculovirus encoding the IRKD (amino acid residues 953 to 1355 of the IR) was a generous gift of the late Ora Rosen (53). The D1161A-IRKD mutant was subcloned in this laboratory using a human IR cDNA originally provided by Jonathan Whittaker (56). The mutation was generated by overlap-extension PCR (22) using pXCKD as the template (6). The mutagenic oligonucleotide primers were 5'-TGAAAC GCGCTACTACCGG-3' and 5'-CCGGTAGTACGCGGTTTCA-3' with the nucleotide changes shown in boldface and the altered coding triplet underlined. The 570-bp PCR product was digested with *Bst*XI and *Sst*I, and the resulting 421-bp fragment was ligated into pXCKD, generating pX-D1161A-IRKD. The mutation was verified by DNA sequencing. A 1,670-bp *Eco*RI-*Pst*I fragment including the coding region of D1161A-IRKD along with 5' and 3' untranslated regions was inserted into the *Eco*RI-*Pst*I sites in the baculovirus expression vector pVL1393 (PharMingen). A 34-kDa form of D1161A-IRKD was made by swapping a 420-bp *Bst*XI/*Sst*I fragment into pX- Δ IRKD (18), which lacks amino-terminal and carboxy-terminal autophosphorylation sites, for use in determining basal state steady-state kinetic parameters (see reference 1). The virus for D1161A-IRKD was generated by cotransfection of Sf9 cells using the Baculogold Kit (PharMingen). Virus was amplified by suspension culture of Sf9 cells grown in spinner flasks, and the titers of the virus were determined with Hi5 cells (Invitrogen) by standard procedures (40). Viral infections to produce protein in Hi5 cells were done for 3 to 4 h at a multiplicity of infection of 10. The infection medium was then replaced with fresh medium. Cells were harvested at 53 h from the start of infection. Cells were resuspended in homogenization buffer (250 mM sucrose, 50 mM Tris base, 20 mM NaCl [pH 7.5]) and stored at -80°C , at least overnight. IRKD purification was done by ion exchange and size exclusion chromatography with a Pharmacia FPLC chromatograph as described in Bishop et al. (3). The purified protein was quantified spectrophotometrically (at 278 nm, $\epsilon = 40,200 \text{ cm}^{-1} \text{ M}^{-1}$) and stored at -20°C in 35% (vol/vol) glycerol, prepared as 38% (wt/wt).

Subcloning D1161A-IR and transient expression in C2C12 myoblasts. The D1161A-IR was generated by ligating the 1.6-kb *Bgl*II-*Xba*I fragment from the pX-D1161A-IRKD plasmid with the 7-kb *Bam*HI/*Xba*I and 1.1-kb *Bam*HI/*Bgl*II fragments from pEF-IR (cDNA of the human IR in a vector with the EF-1 promoter to drive expression; Invitrogen). Transfections in subconfluent mono-

layers of C2C12 cells in 10-cm dishes were done with FuGene (Boehringer Mannheim) and 10 μg of endotoxin-free plasmid [Qiagen Maxiprep kit; pBlue-script SK(+)] was used for the mock transfection. Monolayers were incubated overnight and then passed by trypsinization into replicate culture dishes and regrown overnight.

Limited tryptic cleavage of IRKD. Limited proteolysis of the wild-type IRKD (WT-IRKD) and D1161A-IRKD was done according to Frankel et al. (18). The IRKD was digested at 5 μM in 50 mM TrisAc, 20 mM MgAc_2 , 1 mM DTT, and 2 mM CaCl_2 (pH 7.0), with or without 10 mM ADP. Trypsin was added to a ratio of trypsin to IRKD of 1:20 by mass. The reaction proceeded for 16 min and was then quenched with a one-half volume of threefold-concentrated Laemmli sample buffer (35). The digestion products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fragments were visualized by silver staining, as described (7). The developed slab gels were scanned with an Arcus II scanner (Agfa) with Fotolook and Photoshop software (Adobe).

Fluorescence spectroscopy and iodide quenching. Steady-state fluorescence emission spectra were obtained with an SLM 4800 spectrofluorometer operating in the single-photon counting mode. Magic angle polarization conditions were adopted with the excitation polarizer set at 55° and the emission polarizer set at 0° to minimize polarization-dependent intensity artifacts and to minimize the Wood's anomaly of the emission grating. For intrinsic tryptophan fluorescence spectra, an excitation wavelength of 300 nm was used with excitation and emission slits both set at 8-nm band-pass. Measurements were made at 21°C . Emission spectra were collected over a range of 310 to 420 nm in 1-nm increments. The final spectra were determined from the average of three spectral scans. Time-resolved fluorescence measurements by the time-correlated single-photon-counting method were done with a Coherent laser, according to Hasselbacher et al. (20).

Iodide quenching was done as described previously (3). The IRKD was used at 0.6 μM , and quenching was done at 0.0 to 0.6 M KI in 50 mM TrisAc, 20 mM MgAc_2 , and 0.1 mM sodium thiosulfate, at pH 7.0. The potassium salt concentration was kept constant at 0.6 M by the addition of KCl as needed. Integrated emission intensities were determined from the emission spectra, between 310 and 420 nm, taken as described above. Spectra without IRKD were subtracted for the blank correction, and separate spectra were taken at every iodide concentration, at 0 or 10 mM ADP. The integrated emission intensity, F , was plotted against iodide (quencher) concentration Q , according to the Stern-Volmer equation

$$F_0/F = 1 + K_{sv} \cdot [Q]$$

where F_0 is the integrated fluorescence intensity in the absence of quencher (the average of triplicate determinations), and K_{sv} is the Stern-Volmer quenching constant. The value of K_{sv} was determined from modified Stern-Volmer plots (38) when the initial Stern-Volmer plot was nonlinear, using a plot of $F_0/\Delta F$ versus $1/Q$ according to the equation

$$\frac{F_0}{\Delta F} = \frac{1}{f_a \cdot K_{sv} \cdot [Q]} + \frac{1}{f_a}$$

where f_a is the fraction of intrinsic fluorophores that were subject to quenching and $\Delta F = F_0 - F$.

Endoproteinase Lys-C digestion and peptide mapping. Proteolysis was done for 24 h, with 100 μg of IRKD and 2 μg of endoproteinase Lys-C in 0.1 ml of 50 mM ammonium bicarbonate with a second addition of protease at 16 to 18 h. The digests were lyophilized in a Savant Speed-Vac. Each sample was dissolved in 200 μl of high-performance liquid chromatography (HPLC) buffer A (0.12% trifluoroacetic acid, 1% methanol, 2% acetonitrile, in water [vol/vol]), and 10 μl of a 4 mM solution of TCEP (Molecular Probes) was added to reduce disulfide bonds that had formed during digestion in ammonium bicarbonate. The peptides were resolved by reverse-phase HPLC with a Hewlett-Packard model 1090 liquid chromatograph with a Linear Instruments model 206PHD UV/Vis spectrometer as the detector. The column was an ODS Hypersil (150 by 2 mm) developed at a flow rate of 0.25 ml/min with a gradient from 2% HPLC buffer A (above) to 50% HPLC buffer B (0.1% trifluoroacetic acid, 1% methanol, 2% water, in acetonitrile [vol/vol]). The gradient (with buffer B) was 2 to 19 to 40 to 50% at 0, 16, 140, and 150 min, respectively. Peptides were detected at 230, 278, and 295 nm; the profiles at 230 nm are shown (see Fig. 3). Peptides were recovered and identified by amino acid sequence analysis.

Steady-state kinetics. Peptide phosphorylation was measured with IRS939 and the HPLC-based assay described previously (8). Kinetic studies with WT-IRKD are reported elsewhere (1). The activated D1161A-IRKD was obtained by autophosphorylation for 10 min with 2 μM kinase, 1 mM ATP, 20 mM MgAc_2 , 50 mM TrisAc, 5 mM DTT, and 0.05% hydrogenated Triton X-100 (vol/vol) (pH

7.0) at room temperature. The reaction was quenched with a one-ninth volume of 0.45 M EDTA and kept on ice. Assays of activity were initiated within 2 h. Under these conditions, D1161A-IRKD reaches maximal phosphorylation and maximal activity. Peptide phosphorylation assays with D1161A-IRKD were done with 2 nM phospho-D1161A-IRKD or 20 nM unphosphorylated D1161A-IRKD (34-kDa form) in 50 mM TrisAc (pH 7.0), 1 mM DTT, 0.05% (vol/vol) hydrogenated Triton X-100, 20 mM MgAc₂ with 0.4 to 4.0 mM variable IRS939 (peptide substrate), and 0.01 to 1.0 mM variable ATP. The 34-kDa form lacks the amino-terminal juxtamembrane region and therefore does not undergo partial activation during substrate phosphorylation reactions (6, 7). Rates of substrate phosphorylation were linear with time and kinase concentrations (0.5 to 5 nM activated kinase and 2 to 40 nM unphosphorylated kinase), indicating no changes in the underlying kinetic parameters occurred during the measurements. Steady-state kinetic parameters were obtained by fitting the initial velocity v_i versus the substrate concentrations of ATP and IRS939 according to the equation

$v_i =$

$$\frac{k_{\text{cat}}[\text{ATP}][\text{IRS939}]}{K_m \text{ MgATP} + [\text{ATP}]K_m \text{ IRS939} + [\text{IRS939}]K_m \text{ MgATP} + [\text{IRS939}][\text{ATP}]}$$

where $K_m \text{ MgATP}$ and $K_m \text{ IRS939}$ are the Michaelis constants for nucleotide ATP and peptide IRS939 substrate, respectively, and k_{cat} is the catalytic rate constant. Global data fitting was done by nonlinear regression with SigmaPlot (Jandel Scientific).

Autophosphorylation of WT-IRKD and D1161A-IRKD. Autophosphorylation was done with 2 μM WT-IRKD or D1161A-IRKD, 50 mM TrisAc, 20 mM MgAc₂, 2 mM DTT, 0.05% (vol/vol) hydrogenated Triton X-100 (pH 7.0), and either 10 mM or 0.5 mM ATP (for times, see Fig. 4). The reactions were stopped by the addition of 1.2 volumes of 75 mM TrisAc, 40 mM EDTA, 20 mM DTT (pH 6.65), and 0.01% bromophenol blue. The products were separated by non-denaturing PAGE, and proteins were detected by silver staining, as described previously (7). The developed slab gels were scanned with an Arcus II scanner (Agfa) with Fotolook and Photoshop software (Adobe).

Autophosphorylation of WT-IR and D1161A-IR in vitro. Autophosphorylation was done with D1161A-IR and WT-IR isolated from monolayers expressing D1161A-IR or WT-IR; two 15-cm culture dishes of confluent cells were used for each form of the IR and for the mock-transfected control. The basal state was established by 3 h of serum deprivation in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% radioimmunoassay grade BSA, 25 mM HEPES (pH 7.6), and 2 mM L-glutamine (supplemented DMEM). Monolayers were then washed twice in ice-cold STV buffer (50 mM NaCl, 50 mM Tris, 1 mM Na₃VO₄ [pH 10.5]) and harvested in STV. All subsequent steps leading up to the in vitro reaction were done at 4°C. Cells were disrupted by homogenization, and the membrane fraction was obtained by ultracentrifugation for 60 min at 250,000 $\times g$. Membrane proteins were extracted from the pellets by homogenization in STV (pH 7.4) containing 1.2% (wt/vol) Triton X-100, and insoluble material was cleared by ultracentrifugation for 60 min at 250,000 $\times g$. A fraction enriched for the IR was obtained by adsorption to WGA-agarose and elution in 0.3 M N-acetylglucosamine in STV (pH 7.4) containing 0.12% (wt/vol) Triton X-100. The relative quantities of WT-IR and D1161A-IR were assessed by Western blot analysis of the WGA-agarose eluates with anti-IRKD antibodies which detected the β -subunit of the full-length IR; the rabbit polyclonal antibody raised against WT-IRKD recognizes the mutant and WT proteins equally well, determined from Western blots of D1161A-IRKD and WT-IRKD where the quantities of purified protein loaded in each lane of the gel were known with precision (data not shown). Based on these determinations, the amounts of D1161A-IR and WT-IR loaded per well were the same for all autophosphorylation reactions' time points. The autophosphorylation reactions were carried out with 50 mM TrisAc, 0.12% (wt/vol) Triton X-100, 0.01 mg of BSA/ml, 20 mM MgAc₂ (pH 7.4), and 0.1, 0.5, or 10 mM ATP for 0.5 to 30 min in the absence or presence of 200 nM insulin; reactions were initiated by the addition of ATP and were quenched by the addition of concentrated Laemmli sample buffer for SDS-PAGE. The zero time point was quenched before the addition of ATP. Autophosphorylation was determined by Western blotting with mouse monoclonal antiphosphotyrosine antibodies and enhanced chemiluminescence with Kodak XAR X-ray film as described (54). Western blots were arranged so that a single film was used for WT-IR and D1161A-IR at each ATP concentration. A separate gel and blot were used to compare the extent of phosphorylation at 0.1, 0.5, and 10 mM ATP concentrations with samples containing identical amounts of IR from 30-min reactions of WT-IR and D1161A-IR. Films were scanned as grayscale images with an Arcus II scanner (Agfa) with Fotolook and Photoshop software (Adobe). The grayscale images were analyzed using the Image Quant program (Molecular Dynamics, Sunnyvale, Calif.). The densities were normal-

ized for the maximum autophosphorylation of each kinase in the presence of insulin at each MgATP concentration. A second normalization was done comparing densities from the Western blots of the 30-min time points. Data are the averages of triplicate determinations (three separate reactions and Western blots for each IR at each ATP concentration).

Insulin stimulation in C2C12 myoblasts expressing WT-IR or D1161A-IR. Confluent monolayers of C2C12 cells, transiently expressing D1161A-IR or WT-IR, were prepared as described above with 35-mm culture dishes. The basal state was established by 3 h of serum deprivation in supplemented DMEM. Cells were stimulated at 37°C in supplemented DMEM with bovine insulin for 8 min (for concentrations used, see Fig. 6 through 8). Monolayers were then washed in ice-cold phosphate-buffered saline (PBS) and harvested in 0.5 ml of ice-cold RIPA buffer (200 mM NaCl, 1.2% [wt/vol] Triton X-100, 1% [wt/vol] deoxycholate, 50 mM Tris [pH 7.0], 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄) as described previously (9). Immunoprecipitation from lysates with rabbit polyclonal anti-IRKD antibodies or anti-IR substrate 1 (anti-IRS-1) antibodies was performed by incubating 2 μl of antiserum with 100 μl of cell lysate for 1 h. Immune complexes were bound to protein A-agarose by incubation at 4°C for 1 h, washed three times in ice-cold RIPA buffer, resuspended in an equal volume of 2 $\times$ Laemmli sample buffer, and incubated at 95°C for 5 min. Western blots with enhanced chemiluminescence were done from lysates or immunoprecipitates with mouse monoclonal antiphosphotyrosine antibodies as described (54).

Time courses of IR phosphorylation and dephosphorylation at 37°C were obtained using the protocol described in Kohanski et al. (33) but monitored by antiphosphotyrosine Western blot analysis. Briefly, confluent monolayers of C2C12 cells, transiently expressing the D1161A-IR or WT-IR, were prepared in 35-mm culture dishes and serum deprived as described above. Monolayers were stimulated with 200 nM insulin in supplemented DMEM (for times used, see Fig. 8), washed three times in ice-cold PBS, and lysed in 0.5 ml of ice-cold RIPA buffer. Insulin was removed from cells previously stimulated with 200 nM insulin for 10 min by being washed rapidly five times with 2 ml of supplemented DMEM. Each monolayer was covered with 2 ml of supplemented DMEM without insulin for fixed times (see Fig. 8). Cells were then washed three times in ice-cold PBS and lysed in 0.5 ml of ice-cold RIPA buffer. Western blotting from these lysates with mouse monoclonal antiphosphotyrosine antibodies was performed as described (54).

RESULTS

Characterization of conformational changes induced by mutagenesis. The WT-IRKD and D1161A-IRKD mutant were each purified as intact 46-kDa proteins (Fig. 1, lanes 1 and 2). Using limited proteolysis as a sensitive indicator of activation loop conformation (18), we observed considerable activation loop cleavage in D1161A-IRKD in the absence of ADP compared to the WT-IRKD control (Fig. 1, lane 5 versus lane 3). Bound ADP had an effect on cleavage that was less dramatic in D1161A-IRKD than in WT-IRKD (Fig. 1, lane 6 versus 5, and lane 4 versus 3). The primary sites of cleavage, determined by direct amino acid sequence analysis of the products, were R1164 and K1165, and minor cleavage at R1155 was responsible for the doublet appearance of the 25-kDa amino-terminal fragment and 16-kDa carboxy-terminal fragment. The appearance of the 20-kDa fragment came from direct cleavage of the activation loop in the 46-kDa IRKD, before cleavage in the carboxy terminus which occurred at or near R1304. The appearance of this fragment in both the WT-IRKD control and the D1161A-IRKD mutant protein, which we had not reported before (18), was due to the more aggressive proteolysis conditions employed here.

A second assay for conformational change in the IRKD is iodide quenching. This serves as a reporter of solvent exposure of the active-site cleft, which will increase when intrasteric inhibition is relieved by autophosphorylation (3). As shown previously and reconfirmed here, there was very little iodide quenching in the basal-state WT-IRKD (Fig. 2A). Here we

FIG. 1. Limited tryptic cleavage of IRKD in the activation loop. SDS-PAGE and silver staining give the size distribution of proteins before and after limited proteolysis. The purified WT-IRKD and D1161A-IRKD had molecular masses of 46 kDa (lanes 1 and 2, respectively). Tryptic cleavage of these kinases was done for 10 min in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 10 mM ADP at 20 mM MgAc₂. The IRKD/trypsin ratio was 20:1 (wt/wt), and 2 μ g of IRKD was used in every lane. Cleavage of the 46-kDa kinase domain at or near R1304 in the carboxy terminus gave the 41-kDa tryptic core. Major cleavage in the activation loop at R1164 and K1165 gave the 25-kDa amino-terminal fragment of 25 kDa, and the 16-kDa carboxy-terminal fragment from the T core, as described previously (18). Minor cleavage at R1155 caused the doublet appearance of these fragments. Under the present conditions, a 20-kDa fragment came directly from activation loop cleavage of the 46-kDa kinase domain.

observed increased iodide quenching when the WT-IRKD-MgADP binary complex was formed with saturating ADP (Fig. 2A). In addition, the Stern-Volmer plot became distinctly curved. The iodide quenching observed with the D1161A-IRKD alone was similar to the WT-IRKD-MgADP binary complex. There was very little effect of bound ADP on quenching measured by steady-state fluorescence, and both plots with the D1161A-IRKD showed approximately the same curvature as that with the WT-IRKD-MgADP binary complex. When the data were analyzed using a modified Stern-Volmer plot (Fig. 2B), approximately 60% of the net fluorescence was found accessible to quencher in the WT-IRKD-MgADP binary complex, the D1161A-IRKD alone, and the D1161A-IRKD-MgADP binary complex (f_a) (Table 1). From the slopes and intercepts shown in Fig. 2B, the Stern-Volmer constant K_{sv} was calculated for each kinase (Table 1). On average, the increase was 14-fold higher than the basal value. The bimolecular collisional rate constant k_q was calculated from the intensity-weighted mean fluorescence lifetimes (τ_0) for each kinase determined by time-resolved fluorescence decay measured in the absence and presence of MgADP. As shown in Table 1, bound MgADP caused about a 1-ns decrease in τ_0 for each kinase. The collisional rate constant k_q for WT-IRKD is similar to that observed previously ($0.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (3). The value for k_q for D1161A-IRKD is 13-fold higher than that of WT-IRKD. The value of k_q is the same when comparing the WT-IRKD- and D1161A-IRKD-MgADP binary complexes to each other. Together, the proteolysis and fluorescence studies strongly suggest conformational similarities between the binary complexes of the WT and mutant kinases.

FIG. 2. Iodide quenching of tryptophan fluorescence. Steady-state fluorescence was measured with 50 mM TrisAc, pH 7.0, at constant 0.6 M total potassium salts and 20 mM MgAc₂ with 0.6 μ M unphosphorylated IRKD in the absence or presence of 10 mM ADP. (A) Stern-Volmer plot for WT-IRKD (Δ , $\blacktriangle$) and D1161A-IRKD ($\circ$, $\bullet$) in the absence (Δ , $\circ$) or presence ($\blacktriangle$, $\bullet$) of 10 mM ADP. The fluorescence intensity was determined with $\lambda_{ex} = 300 \text{ nm}$, without (F_0) or with (F) potassium iodide, by integration of the emission spectrum from 310 to 420 nm after correction for a blank spectrum at each iodide concentration (without or with ADP present). (B) The modified Stern-Volmer plot of the experiments shown in panel A; the same symbols are used. The fraction of fluorescence that was quenchable under each condition is obtained by extrapolation to 0 M KI, using equation 2. These solid lines were generated by linear regression.

To be certain that these effects on conformation were due to the mutation and not to undetected phosphorylation, we compared the HPLC elution profiles from endoproteinase Lys-C digests of the two purified proteins (Fig. 3). In this analysis, the only significant difference between these proteins was the elution time of the activation loop peptide, which displayed greater retention in reverse-phase chromatography in accordance with the nature of the carboxylic acid side chain to apolar side-chain mutation. The identity of the peptide in this absorbance peak and the Asp-to-Ala mutation were confirmed

TABLE 1. Fluorescence parameters for WT-IRKD and D1161A-IRKD^a

Parameter (unit)	IRKD			
	WT		D1161A	
	- ADP	+ ADP	- ADP	+ ADP
f_a	NA	0.58 ± 0.06	0.64 ± 0.02	0.62 ± 0.03
$K_{sv} (\text{M}^{-1})$	0.17 ± 0.03	2.91 ± 0.23	1.90 ± 0.08	2.25 ± 0.13
$\tau_0 (\text{ns})$	7.8 ± 0.2	6.8 ± 0.2	6.6 ± 0.2	5.3 ± 0.2
$k_q \times 10^{-8} (\text{M}^{-1} \text{ s}^{-1})$	0.22 ± 0.03	4.3 ± 0.43	2.9 ± 0.29	4.3 ± 0.33

^a NA, not applicable.

FIG. 3. Peptide mapping of WT-IRKD and D1161A-IRKD. Each IRKD was digested using endoproteinase Lys-C, and the resulting peptides were separated by reverse-phase HPLC. The elution profile from a blank injection (lacking only IRKD) was subtracted. The elution profile for WT-IRKD (above the zero absorbance line) and the mirror image of the D1161A-IRKD elution profile (below the zero absorbance line) at 230 nm are shown. The peptides in every peak of absorbance were identified by amino acid sequencing. Peptides containing autophosphorylation sites in the carboxy terminus (CT), activation loop (AL), and juxtamembrane region (JM) are identified. The D1161A mutation causes the activation loop peptide (AL*) to elute later than the corresponding peptide from the WT-IRKD. The elution positions of the activation loop phosphopeptides are marked with bars; these were determined from separate experiments with each IRKD.

by direct amino acid sequence analysis. Furthermore, these elution profiles show that neither WT-IRKD nor D1161A-IRKD contained detectable activation loop phosphotyrosine, shown by the absence of phosphopeptides in the elution profile, whose known elution positions are marked in Fig. 3. A conservative estimate of the sensitivity in this system is 5% conversion of an apo-peptide to a phosphopeptide. Therefore, the properties of D1161-IRKD described so far which report on the major species in solution are those of the unphosphorylated protein.

Steady-state kinetics of D1161A-IRKD. Steady-state kinetic parameters obtained from synthetic peptide phosphorylation are summarized in Table 2. The magnitude of K_m ATP differs from values reported earlier by us and others (6, 11), primarily because Mg^{2+} is used exclusively rather than Mn^{2+} or a Mn^{2+} - Mg^{2+} mixture as the obligatory cation for ATP in the kinase reaction. Mn^{2+} often produces a lower K_m ATP value for protein tyrosine kinases (45). We use Mg^{2+} in this report and other studies (1, 7, 47) for consistency with the IRKD catalytic

core crystal structures where Mg^{2+} has been employed (24, 47) and solution studies of other protein tyrosine kinases (see, for example, reference 43). Comparing unphosphorylated D1161A-IRKD to unphosphorylated WT-IRKD, the data in Table 2 show that k_{cat} was about 10-fold higher and K_m $MgATP$ was 15-fold lower, with K_m $IRS939$ essentially unchanged. The activation that resulted from autophosphorylation was characterized for both kinases. Activation of D1161A-IRKD had almost no effect on k_{cat} , producing only a 2-fold decrease in K_m $MgATP$ but a 25-fold decrease in K_m $IRS939$. There are no significant differences in any of these activated-state kinetic parameters compared to that of activated WT-IRKD. This indicates that the mutation does not perturb the enzymatic activity of the kinase in the activated state. The impact of changing the activation loop conformation on the process of autophosphorylation is considered next.

Autophosphorylation of D1161A-IRKD and WT-IRKD in vitro. To compare the progress of autophosphorylation using kinase domains with a 15-fold difference in K_m $MgATP$ we used 10 mM ATP to saturate both enzymes. Under these conditions WT-IRKD and D1161A-IRKD followed approximately the same time course for autophosphorylation (Fig. 4A). With 0.5 mM $MgATP$, autophosphorylation of the WT-IRKD was slower, but autophosphorylation for the D1161A-IRKD was unaffected (Fig. 4B versus A). Therefore, the gate-open conformation in this mutant IRKD shows a greater biochemical predisposition for *trans* autophosphorylation than WT-IRKD, based on its ability to maintain a high rate of autophosphorylation at the lower ATP concentration. Because previous studies showed that WT-IRKD mimics WT-IR in the absence of insulin (32, 34), it is reasonable to expect that a full-length IR bearing the D1161A mutation also will be in a gate-open conformation and display greater autophosphorylation than WT-IR, at least in the absence of insulin.

Autophosphorylation of D1161A-IR and WT-IR in vitro. WT and mutant IRs were expressed in C2C12 myoblasts, and WGA-agarose eluates enriched for each IR were used to determine the progress of autophosphorylation at different ATP concentrations. The reactions were done in the absence and presence of insulin, and compared by densitometric quantitation of antiphosphotyrosine Western blots (Fig. 5). The rate of autophosphorylation in the absence of insulin was higher for the D1161A-IR than for the WT-IR at all ATP concentrations tested. The difference in rates was most pronounced at 0.1 mM ATP (Fig. 5A), intermediate at 0.5 mM ATP (Fig. 5B), and slight at 10 mM ATP (Fig. 5C). Autophosphorylation in the presence of insulin was faster for D1161A-IR at 0.1 mM ATP

TABLE 2. Steady-state kinetic parameters for WT-IRKD and D1161A-IRKD before and after autophosphorylation

Parameter (unit)	IRKD ^a			
	WT ^b		D1161A	
	-	+	-	+
k_{cat} (s^{-1})	1.2 ± 0.2	8.7 ± 0.3	11.7 ± 0.4	9.0 ± 0.4
K_m $MgATP$ (mM)	0.9 ± 0.1	0.04 ± 0.01	0.06 ± 0.02	0.03 ± 0.01
K_m $IRS939$ (mM)	2.0 ± 0.5	0.05 ± 0.01	2.8 ± 0.5	0.11 ± 0.01

^a -, before autophosphorylation; +, after autophosphorylation.

^b Data for WT-IRKD from Ablooglu and Kohanski (1).

FIG. 4. In vitro autophosphorylation of WT-IRKD (WT) and D1161A-IRKD (DA). The progress of autophosphorylation with 2 μ M IRKD and 10 mM (A) or 0.5 mM (B) ATP was monitored by non-denaturing PAGE and silver staining (7); increased electrophoretic mobility indicates increased stoichiometries of autophosphorylation. The zero time results show the unphosphorylated D1161A-IRKD has lower mobility than the unphosphorylated WT-IRKD because of the anionic state-to-neutral state mutation. At 60 min, the activation loop was 80% tris phosphorylated and 20% bis phosphorylated, and there was extensive autophosphorylation in the carboxy-terminal and juxtamembrane regions, determined by HPLC peptide mapping (not shown). The final stoichiometry of autophosphorylation was 5.5 ± 0.6 mol of phosphate per mol of IRKD.

(Fig. 5A), but the rates were essentially the same at 0.5 and 10 mM ATP (Fig. 5B and C, respectively). These results confirm that the activation loop mutation enhances autophosphorylation of the full-length receptor in the absence of insulin. ATP dependence for the relative differences in autophosphorylation in the absence of insulin was qualitatively similar to the effects observed using the isolated kinase domains, since the difference was more pronounced at the lower ATP concentrations. The results also show that insulin stimulation in vitro will normalize the progress of IR autophosphorylation at 10 and 0.5 mM ATP, which should bracket the range of intracellular ATP concentrations.

FIG. 5. In vitro autophosphorylation of WT-IR and D1161A-IR. Time courses of WT-IR ($\square$, $\blacksquare$) and D1161A-IR ($\circ$, $\bullet$) were done in the absence ($\square$, $\circ$) or presence ($\blacksquare$, $\bullet$) of insulin. Reactions were done at 0.1 (A), 0.5 (B), and 10 (C) mM ATP. The WGA-agarose-enriched fractions from C2C12 cells expressing each IR were used. Densitometric analysis of Western blots with antiphosphotyrosine antibodies, developed by enhanced chemiluminescence, were used to monitor the progress of IR β -subunit autophosphorylation in vitro, with equal amounts of IR loaded in every lane; data are averaged from triplicate determinations. Data for each ATP concentration are normalized to the maximum levels of autophosphorylation observed at 10 mM ATP. Further details are given in Materials and Methods.

Autophosphorylation of D1161A-IR and WT-IR in cells. The D1161A mutation, expressed on the full-length IR, was used to test the importance of a gate-closed versus gate-open conformation in maintaining the cellular unphosphorylated state of the IR prior to insulin stimulation. Both the D1161A-IR and WT-IR were expressed transiently in C2C12 cells. The level of endogenous murine IR was apparently low or not reactive with the polyclonal antibody raised against human IRKD (Fig. 6A, lane 1). The expression and processing of transfected human IR precursor to mature subunits was the same for WT-IR and D1161A-IR (Fig. 6A, lanes 2 and 3), although the rate of expression of D1161A-IR appeared to be slightly lower in this and other transient transfection experiments. Transfected monolayers were stimulated or not stimulated with insulin, and both cell lysates and anti-IR immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine antibodies (Fig. 6B). Insulin-stimulated β -subunit autophosphorylation of the endogenous murine IR was not detected in these blots. There was no constitutive tyrosine phosphorylation of the WT-IR or D1161A-IR β -subunits in these cells (Fig. 6B, lanes 3 and 9, and lanes 5 and 11, respectively). Insulin-stimulated autophosphorylation in the transfected cells was readily observed in the lysates (Fig. 6B, lanes 4 and 6), and the identity of the prominent tyrosine-phosphorylated 95-kDa protein was confirmed by immunoprecipitation with anti-IRKD antibodies (Fig. 6B, lanes 10 and 12). Prior to insulin stimulation there was no increased basal IRS-1 tyrosine phosphorylation in C2C12 cells overexpressing D1161A-IR or WT-IR (Fig. 6C, lanes 1 to 3). Insulin-stimulated tyrosine phosphorylation of IRS-1 in mock-transfected C2C12 cells and cells overexpressing D1161A-IR or WT-IR (Fig. 6C, lanes 4 to 6) indicated that endogenous murine IR was sufficient to provide a maximum level of IRS-1 phosphorylation in these transiently transfected cells; comparable amounts of IRS-1 were present in each immunoprecipitated sample (Fig. 6D). These experiments demonstrate no unregulated signaling despite overexpression of D1161A-IR or WT-IR, shown by the absence of IR autophos-

FIG. 6. Tyrosine phosphorylation of WT and D1161A-IR expressed in C2C12 cells. (A) The Western blot with antikinase domain antibodies shows the relative abundance of IR β -subunit (β) and the glycosylated and uncleaved precursor (pre) from total membranes isolated from mock-transfected (control) cells (lane 1), cells transfected with WT-IR (lane 2) and cells transfected with D1161A-IR (lane 3). Equivalent amounts of membrane protein were loaded in lanes 1 to 3. Lane 4 is a 0.2-ng sample of purified WT-IRKD (KD) as a marker for antibody sensitivity. (B) The Western blot with antiphosphotyrosine antibodies was made from cell lysates (lanes 1 through 6) and anti-IR immunoprecipitates from these lysates (lanes 7 to 12). C2C12 cells were transiently transfected with control vector (M) (lanes 1, 2, 7, and 8), WT-IR (WT) (lanes 3, 4, 9, and 10), or the D1161A-IR mutant (DA) (lanes 5, 6, 11, and 12). C2C12 monolayers were serum deprived for 3 h and then untreated (lanes 1, 3, 5, 7, 9, and 11) or stimulated (lanes 2, 4, 6, 8, 10, and 12) for 8 min with 200 nM insulin. The IR β -subunit (β) and IRS-1 (IRS) are identified. Markers are shown on the left in kilodaltons. (C) Antiphosphotyrosine Western blot of IRS-1. IRS-1 was immunoprecipitated with anti-IRS-1 antibodies from control cells (lanes 1 and 4), cells expressing WT-IR (lanes 2 and 5), or cells expressing D1161A-IR (lanes 3 and 6) without stimulation (lanes 1 to 3) or stimulated for 8 min with 200 nM insulin (lanes 4 to 6). (D) Western blot with anti-IRS-1 antibody of the same immunoprecipitates shown in panel C; the lane assignments are the same. For these last two panels, each immunoprecipitate was divided into equal parts prior to SDS-PAGE.

phorylation and the absence of elevated IRS-1 phosphorylation in the absence of insulin.

It is possible that increased affinity for ATP, associated with the intracellular domain of the mutant full-length receptor, might change the sensitivity to insulin. This would not have been detected in the above experiment, which examined only the endpoints of insulin responsiveness. Therefore, we measured the dependence of IR autophosphorylation on insulin concentration for WT-IR and D1161A-IR expressed transiently in C2C12 myoblasts. The determination of β -subunit tyrosine phosphorylation was done by Western blot analysis with antiphosphotyrosine antibodies (Fig. 7A). With Western blot exposures giving approximately equivalent signals at maximum stimulation (100 nM insulin), quantitative analysis indicated a 2.5-fold shift to a lower insulin concentration needed to produce half-maximal autophosphorylation in D1161A-IR than in WT-IR (Fig. 7B). The 50% effective concentration (EC_{50}) was approximately 1.5 ± 0.4 and 3.7 ± 0.8 nM insulin, respectively.

The level of IR β -subunit autophosphorylation in a cellular context is determined by the relative rates of autophosphorylation and phosphotyrosine phosphatase-dependent dephosphorylation. To determine what impact the D1161A mutation had on these processes, we compared the time courses of insulin-stimulated autophosphorylation and the dephosphorylation that occurs following insulin withdrawal (Fig. 8). Both WT-IR and D1161A-IR achieve steady-state levels of autophosphorylation within 1 min of insulin addition. These levels persist, with continued stimulation, to at least 50 min. Within 2 min after insulin withdrawal from monolayers (insulin stimulated for 10 min), a decrease in IR β -subunit phosphorylation was observed. The rate of this decline was slower for D1161A-IR than for WT-IR, requiring 40 min to reach half the maximal steady-state level versus 10 min for the WT-IR.

DISCUSSION

The functional significance of conformations observed in crystal structures has often been determined through rationally designed mutations. Here we sought to understand whether the intrasteric inhibitory conformation of the activation loop was necessary to maintain the unphosphorylated basal state of the IR. This may be a regulatory feature of the IR family not shared with other RTKs. If it proves to be conserved across species, then there should be an essential role maintained by evolutionary pressures.

The gate-closed conformation was recognized immediately as incompatible with *trans* autophosphorylation of the activation loop (26), an event that is critical for insulin-dependent signaling (17, 48, 58). The gate-open conformation can be promoted by ATP, ADP, or other adenine nucleotide derivative binding in vitro (18). Therefore, to test the function of a gate-open conformation in cellular signaling we needed to relieve intrasteric inhibition against ATP binding while preserving the potential for activation loop autophosphorylation. Two earlier observations led to the specific mutation used here. With full-length IR, Zhang et al. (58) demonstrated elevated autophosphorylation in vitro without constitutive signaling in cells by mutating the two adjacent tyrosines in the activation loop (Y1162F-Y1163F). With IRKD, we had shown this same double mutant (ALY2F) (6) lowered the K_m $MnATP$ for *cis* autophosphorylation, which is in the juxtamembrane region. These effects most likely arose from the same cause, i.e., breaking a subset of noncovalent bonds tethering the activation loop in its gate-closed conformation. The interactions of Y1162 and Y1163 that would contribute to a gate-closed conformation were detected in the crystal structure(s). Another prominent set of H-bond interactions across the peptide binding site was between D1161 in the activation loop and

FIG. 7. Insulin dose-response for tyrosine phosphorylation of WT-IR and D1161A-IR expressed transiently in C2C12 cells. (A) The Western blot with antiphosphotyrosine antibodies, with enhanced chemiluminescence, was made from cell lysates. Regions of the Western blot encompassing the β -subunit are shown. Receptors were expressed after transient transfection of C2C12 cells with WT-IR (WT) or the D1161A-IR mutant (DA). Prior to insulin treatment, monolayers were serum deprived for 3 h and then stimulated with 0 to 100 nM insulin for 10 min, at the concentrations shown above the Western blot. The results are representative of two experiments. (B) Quantitative analysis of the dose-response results for IR autophosphorylation, as shown in panel A, for WT-IR (■) and D1161A-IR (●). Data are averaged from duplicate experiments using films where the densities of β -subunit autophosphorylation at 100 nM insulin were approximately equal, and error bars represent 1 standard deviation from the average. Each line is the best fit of the data for a single EC_{50} for stimulation of each IR: $EC_{50} = 1.5 \pm 0.4$ nM insulin for D1161A-IR and $EC_{50} = 3.7 \pm 0.8$ nM insulin for WT-IR.

R1136, K1085, and Q1208 in the catalytic core, where these interactions also contribute significantly to the P-1 binding site for a peptide substrate (24, 26). We show that loss of these interactions by mutagenesis of D1161 had the desired effects: relieving intrasteric inhibition against ATP, while permitting activation loop autophosphorylation and kinase activation.

Limited proteolysis showed that the activation loop in D1161A-IRKD adopted a conformation different from the WT-IRKD in the absence of adenine nucleotide. The evidence also suggested similar conformations of the activation loop were present in the D1161A-IRKD with and without bound adenine nucleotide, in contrast to the conformational change induced by MgADP bound to WT-IRKD (Fig. 1 and reference 18). In support of this conclusion, we analyzed the intrinsic fluorescence of WT-IRKD, which is dominated by emission of W1175 in the active-site cleft (3). Because the cleft is blocked by the activation loop, this fluorophore is inaccessible to collisional quenching by iodide. Opening of the cleft due to autophosphorylation results in greater exposure. Here we demonstrate, by the increased iodide quenching, opening of the cleft in the WT-IRKD-MgADP binary complex. This implies greater steric accessibility and suggests the term gate-open for

FIG. 8. Time courses of insulin stimulation and withdrawal. (A) Western blots with antiphosphotyrosine antibodies were made with whole-cell lysates of transiently transfected C2C12 myoblasts with WT-IR (WT) or the D1161A-IR mutant (DA). Regions of the Western blots encompassing the β -subunit are shown. Continuous stimulation with 200 nM insulin was done for the times indicated (x axis, bottom). Withdrawal of insulin was preceded by a 10-min stimulation with 200 nM insulin. Incubation in supplemented DMEM without insulin was done the times indicated (x axis, top). (B) Quantitative analysis of these time courses presented as the fraction of maximum autophosphorylation observed. Data presented are means of two independent experiments: D1161A-IR stimulation (●); D1161A-IR withdrawal (○); WT-IR stimulation (■); WT-IR withdrawal (□).

such conformations of the activation loop. Therefore, a gate-open conformation for the D1161A-IRKD, with or without bound adenine nucleotide, was inferred from the iodide-quenching results. In terms of steric access, the bimolecular collisional rate constant of $4.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ is roughly 1 order of magnitude less than the diffusion limit for iodide collisions with a fully exposed indole ring (38). This indicates fairly extensive exposure of W1175 in the active site. There is a twofold difference in k_q for the binary complex versus k_q determined after autophosphorylation ($2.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (3). However, there was also a twofold difference in k_q for the same gate-closed unphosphorylated WT-IRKD between these data sets ($0.22 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ reported here versus $0.43 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ reported previously [3]). Therefore, these values suggest nearly equivalent solute access; taking ATP as the solute, steric access of the nucleotide substrate may be nearly equivalent in the unphosphorylated D1161A-IRKD and the phosphorylated WT-IRKD. However, access of the peptide substrate is still restricted in some way, based on the difference in K_m peptide between the unphosphorylated D1161A-IRKD and the phosphorylated WT-IRKD (Table 2): this kinetic feature of the D1161A-IRKD is more similar to the basal-state un-

phosphorylated WT-IRKD. Additionally, the increase in k_{cat} in the absence of autophosphorylation was unexpected, and it may suggest that activation loop phosphorylation has less of an impact on the orientation of catalytically essential residues than was anticipated from the structure of this (24, 26) or other (5, 12, 25, 28, 29) protein kinases. For the hypothesis tested in this study, the increase in turnover number together with the decrease in $K_m \text{ MgATP}$ shows that intrasteric inhibition was relieved against ATP. Therefore, limited proteolysis, fluorescence, and kinetic studies provide a consistent description of a gate-open conformation for the D1161A-IRKD, in which peptide interaction is more similar to the basal-state WT-IRKD but ATP interaction and turnover number are like the activated kinase. We conclude from these solution studies that the activation loop conformation of the D1161A mutant is different from either the basal or activated states of the WT kinase. The crystal structure of the D1161A-IRKD core (residues 978 to 1283) was determined while the manuscript was under revision (47). That structure and additional solution studies demonstrate a gate-open conformation of the activation loop and thermodynamic properties of the mutant protein that are intermediate between the basal and activated states of the WT-IRKD core.

The mutant D1161A-IRKD is enzymatically active, which is expected, since none of the known catalytically essential residues were mutated. The new conformation of the activation loop still imposes a barrier to peptide substrate binding, which may also decrease the efficiency of intracellular target protein phosphorylation catalyzed by the unphosphorylated mutant IR. However, this feature did not mitigate autophosphorylation by D1161A-IRKD or D1161A-IR. These reactions were rapid at a superphysiological ATP concentration sufficient to saturate WT-IRKD or WT-IR. At lower ATP concentrations, the decrease in apparent rate for the WT-IRKD and WT-IR, compared to the still-rapid autophosphorylation of the mutant, could have resulted from a decreased fraction in a gate-open conformation at the lower ATP concentration in the WT kinase, or it may have been due to the difference in k_{cat} noted above. Which effect predominates cannot be determined here, because a single mutant will not distinguish conformational effects on the substrate kinase domain from conformational and catalytic effects on the enzyme kinase domain. The essential feature is that a biochemically active kinase is more capable of rapid autophosphorylation at a subphysiological ATP concentration than the WT enzyme. The need for intrasteric inhibition against ATP and a low turnover number to maintain the unphosphorylated state of the IR prior to insulin binding was tested with this mutant.

The D1161A mutation in the full-length IR did not cause unregulated (constitutive) autophosphorylation in cells in which this mutant IR was expressed, despite the relief of intrasteric inhibition against ATP binding and the increased ability to autophosphorylate in the absence of insulin. In the physiological range of ATP there may be a heterogeneous population of WT-IR encompassing ATP-bound and -free forms of the kinase, but the D1161A-IR should be saturated. The absence of constitutive autophosphorylation in the D1161A-IR demonstrates that neither intrasteric inhibition against ATP nor low turnover number is essential for maintaining the basal unphosphorylated state in cells. The residual

high $K_m \text{ peptide}$ in the D1161A-IR does not affect receptor autophosphorylation, but it may contribute to the lack of IRS phosphorylation despite the higher k_{cat} and lower $K_m \text{ MgATP}$. However, the absence of constitutive signaling, indicated by the absence of pTyr-IRS without insulin stimulation (Fig. 6C), is most simply explained by the finding that autophosphorylation is a necessary prerequisite for biological signaling through the IR (10, 13) and that the D1161A-IR was not autophosphorylated prior to addition of insulin.

The shifted insulin dose-response curve for autophosphorylation observed as a consequence of the mutation suggests that some linkage is possible between insulin binding to the extracellular domain and ATP binding to the intracellular domain or that the mutation itself directly affects recognition and dephosphorylation by a protein tyrosine phosphatase (PTPase). The recent report of Salmeen et al. (42) showed that the PTPase PTP1B—which is associated with regulation of IR phosphorylation and signaling (15)—might employ Asp1161 as an important determinant for binding the IR activation loop. A potential alteration in binding affinity or rates of insulin release during receptor internalization, as well as the relative contribution of Asp1161 to recognition of the kinase by PTPases, is currently under investigation.

Our finding that intrasteric inhibition and low turnover number are not essential for suppression of IR autophosphorylation, and thus insulin signaling, leaves open the biological importance of the gate-closed conformation. Many protein kinases do not bind ATP because of intrasteric inhibition (e.g., twitchin kinase [23], calcium calmodulin-dependent protein kinases [19], or cyclin-dependent protein kinases [12]). In general, relief from intrasteric inhibition is achieved by allosteric effectors acting through regulatory proteins or subunits (31), and nucleotide binding at the active site is not a typical primary regulator. The basal-state IR appears to be an exception. Its unique feature is not the fact of intrasteric inhibition but that the closed-to-open conformational change can be affected by its substrate at physiological concentrations (18). To the extent that nucleotide binding is necessary for activation loop autophosphorylation in the substrate kinase domain, we speculate that the population of IRs primed by nucleotide binding may vary with intracellular concentrations of adenine nucleotides or derivatives. This may apply under conditions of metabolic stress, since insulin has a fundamental role in regulation of energy homeostasis. Furthermore, because AMP does not promote the gate-open conformation (18) and intracellular AMP changes inversely with respect to ATP and rises under metabolic stress (4, 21), coordinated regulation with the 5'-AMP-activated protein kinase is possible. This may be important because the 5'-AMP-activated kinase is a primary sensor of energy demand (30) whose target pathways are counterregulated by insulin signaling. Therefore, a possible advantage of latency may be variable priming of the IR for responsiveness to insulin under extreme conditions. Whether such an effect would be more important in peripheral or neuronal tissues—where the IR is expressed (2, 16, 52)—will be investigated in future studies.

In summary, we have shown that intrasteric inhibition against ATP binding and low catalytic efficiency are not necessary to maintain the unphosphorylated basal state of the IR. Important for our broader understanding of the regulation of

signal transduction, this finding suggests that suppression of kinase activity is not inherently necessary to prevent premature signaling by an RTK.

ACKNOWLEDGMENTS

This work was supported by NIH grant DK59522 (R.A.K.), and the Coherent laser was obtained through NSF Instrument Development award DBI 9724330 to William R. Laws.

We thank Henry B. Sadowski and his laboratory for guidance on transient transfections and handling C2C12 cells, and Henry B. Sadowski and Mitchell Goldfarb for helpful discussions and critical reading of the manuscript.

REFERENCES

- Ablooglu, A. J., and R. A. Kohanski. 2001. Activation of the insulin receptor's kinase domain changes the rate-determining step of substrate phosphorylation. *Biochemistry* 40:504–513.
- Adamo, M., M. K. Raizada, and D. LeRoith. 1989. Insulin and insulin-like growth factor receptors in the nervous system. *Mol. Neurobiol.* 3:71–100.
- Bishop, S. M., J. B. A. Ross, and R. A. Kohanski. 1999. Autophosphorylation dependent destabilization of the insulin receptor kinase domain: tryptophan-1175 reports changes in the catalytic cleft. *Biochemistry* 38:3079–3089.
- Boiteux, A., B. Hess, and E. E. Sel'kov. 1980. Creative functions of instability and oscillations in metabolic systems. *Curr. Top. Cell. Regul.* 17:171–203.
- Canagarajah, B. J., A. Khokhlatchev, M. H. Cobb, and E. J. Goldsmith. 1997. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* 90:859–869.
- Cann, A. D., and R. A. Kohanski. 1997. Cis-autophosphorylation of juxtamembrane tyrosines in the insulin receptor kinase domain. *Biochemistry* 36:7681–7689.
- Cann, A. D., S. M. Bishop, A. J. Ablooglu, and R. A. Kohanski. 1998. Partial activation of the insulin receptor kinase domain by juxtamembrane autophosphorylation. *Biochemistry* 37:11289–11300.
- Cann, A. D., I. Wolf, and R. A. Kohanski. 1997. A tyrosine kinase assay using reverse-phase high-performance liquid chromatography. *Anal. Biochem.* 247:327–332.
- Chen, J., H. B. Sadowski, R. A. Kohanski, and L.-H. Wang. 1997. Stat5 is a physiological substrate of the insulin receptor. *Proc. Natl. Acad. Sci. USA* 94:2295–2300.
- Chou, C. K., T. J. Dull, D. S. Russell, R. Gherzi, D. Lebowitz, A. Ullrich, and O. M. Rosen. 1987. Human insulin receptor mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J. Biol. Chem.* 268:14553–14556.
- Cobb, M. H., B. C. Sang, R. Gonzalez, E. Goldsmith, and L. Ellis. 1989. Autophosphorylation activates the soluble cytoplasmic domain of the insulin receptor in an intermolecular reaction. *J. Biol. Chem.* 264:18701–18706.
- De Bondt, H. L., J. Rosenblatt, J. Jancarik, H. D. Jones, D. O. Morgan, and S. H. Kim. 1993. Crystal structure of cyclin-dependent kinase 2. *Nature* 363:595–602.
- Ebina, Y., E. Araki, M. Taira, F. Shimada, M. Mori, C. S. Craik, K. Siddle, S. B. Pierce, R. A. Roth, and W. J. Rutter. 1987. Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proc. Natl. Acad. Sci. USA* 84:704–708.
- Ebina, Y., L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J. H. Ou, F. Masiarz, Y. W. Kan, I. D. Goldfine, R. A. Roth, and W. J. Rutter. 1985. The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. *Cell* 40:747–758.
- Elchebly, M., P. Payette, E. Michaliszyn, W. Cromlish, S. Collins, A. L. Loy, D. Normandin, A. Cheng, J. Himms-Hagen, C.-C. Chan, C. Ramachandran, M. J. Gresser, M. L. Tremblay, and B. P. Kennedy. 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283:1544–1548.
- Folli, F., S. Ghidella, L. Bonfanti, C. R. Kahn, and A. Merighi. 1996. The early intracellular signaling pathway for the insulin/insulin-like growth factor receptor family in the mammalian central nervous system. *Mol. Neurobiol.* 13:155–183.
- Flores-Riveros, J. R., E. Sibley, T. Kastelic, and M. D. Lane. 1989. Substrate phosphorylation catalyzed by the insulin receptor tyrosine kinase. Kinetic correlation to autophosphorylation of specific sites in the beta subunit. *J. Biol. Chem.* 264:21557–21572.
- Frankel, M., S. M. Bishop, A. J. Ablooglu, Y.-P. Han, and R. A. Kohanski. 1999. Conformational changes in the activation loop of the insulin receptor's kinase domain. *Protein Sci.* 8:2158–2165.
- Goldberg, J., A. C. Nairn, and J. Kuriyan. 1996. Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. *Cell* 84:875–887.
- Hasselbacher, C. A., E. Waxman, L. T. Galati, P. B. Contino, J. B. A. Ross, and W. R. Laws. 1991. Investigation of hydrogen bonding and proton transfer of aromatic alcohols in nonaqueous solvents by steady-state and time-resolved fluorescence. *J. Phys. Chem.* 95:2995–3005.
- Hess, B., and A. Boiteux. 1971. Oscillatory phenomena in biochemistry. *Annu. Rev. Biochem.* 40:237–258.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
- Hu, S. H., M. W. Parker, J. Y. Lei, M. C. Wilce, G. M. Benian, and B. E. Kemp. 1994. Insights into autoregulation from the crystal structure of twitchin kinase. *Nature* 369:581–584.
- Hubbard, S. R. 1997. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J.* 16:5572–5581.
- Hubbard, S. R., M. Mohammadi, and J. Schlessinger. 1998. Autoregulatory mechanisms in protein-tyrosine kinases. *J. Biol. Chem.* 273:11987–11990.
- Hubbard, S. R., L. Wei, L. Ellis, and W. A. Hendrickson. 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372:746–754.
- Issad, T., J. M. Tavare, and R. M. Denton. 1991. Analysis of insulin receptor phosphorylation sites in intact rat liver cells by two-dimensional phosphopeptide mapping. Predominance of the tris-phosphorylated form of the kinase domain after stimulation by insulin. *Biochem. J.* 275:15–21.
- Johnson, L. N., E. D. Lowe, M. E. Noble, and D. J. Owen. 1998. The eleventh Datta lecture. The structural basis for substrate recognition and control by protein kinases. *FEBS Lett.* 430:1–11.
- Johnson, L. N., M. E. Noble, and D. J. Owen. 1996. Active and inactive protein kinases: structural basis for regulation. *Cell* 85:149–158.
- Kemp, B. E., K. I. Mitchell, D. Stapleton, B. J. Mitchell, Z.-P. Chen, and L. A. Witters. 1999. Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem. Sci.* 24:22–25.
- Kobe, B., J. Heierhorst, and B. E. Kemp. 1997. Intrasteric regulation of protein kinases. *Adv. Second Messenger Phosphoprot. Res.* 31:29–40.
- Kohanski, R. A. 1993. Insulin receptor autophosphorylation. I. Autophosphorylation kinetics of the native receptor and its cytoplasmic kinase domain. *Biochemistry* 32:5766–5772.
- Kohanski, R. A., S. C. Frost, and M. D. Lane. 1986. Insulin dependent phosphorylation of the insulin receptor protein kinase and activation of glucose transport in 3T3-L1 adipocytes. *J. Biol. Chem.* 261:12272–12281.
- Kohanski, R. A., and E. Schenker. 1991. Control of insulin receptor autophosphorylation by polypeptide substrates: inhibition and stimulation by interaction with the catalytic subunit. *Biochemistry* 30:2406–2414.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lane, M. D., G. V. Ronnett, R. A. Kohanski, and T. L. Simpson. 1985. Posttranslational processing of the insulin proreceptor. *Curr. Top. Cell. Regul.* 27:279–292.
- Lehninger, A. L., D. L. Nelson, and M. M. Cox. 1993. Principles of biochemistry, p. 375. Worth, New York, N.Y.
- Lehrer, S. S. 1971. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* 10:3254–3263.
- Maddux, B. A., and I. D. Goldfine. 1991. Evidence that insulin plus ATP may induce a conformational change in the beta subunit of the insulin receptor without inducing receptor autophosphorylation. *J. Biol. Chem.* 266:6731–6736.
- O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1994. Baculovirus expression vectors: a laboratory manual. Oxford University Press, New York, N.Y.
- Ridge, K. D., K. Hofmann, and F. M. Finn. 1988. ATP sensitizes the insulin receptor to insulin. *Proc. Natl. Acad. Sci. USA* 85:9489–9493.
- Salmeen, A., J. N. Andersen, M. P. Myers, N. K. Tonks, and D. Barford. 2000. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol. Cell* 6:1401–1412.
- Saylor, R., C. Wang, T. J. Hirai, and J. A. Adams. 1998. A second magnesium ion is critical for ATP binding in the kinase domain of the oncoprotein, v-Fps. *Biochemistry* 37:12624–12630.
- Songyang, Z., S. E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X. R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, and T. Yi. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* 14:2777–2785.
- Sun, G., and R. J. Budde. 1997. Requirement for an additional divalent metal cation to activate protein tyrosine kinases. *Biochemistry* 36:2139–2146.
- Tavare, J. M., and R. M. Denton. 1988. Studies on the autophosphorylation of the insulin receptor from human placenta. Analysis of the sites phosphorylated by two-dimensional peptide mapping. *Biochem. J.* 252:607–615.
- Till, J. H., A. J. Ablooglu, M. I. Frankel, S. M. Bishop, R. A. Kohanski, and S. R. Hubbard. 2001. Crystallographic and solution studies of an activation loop mutant of the insulin receptor tyrosine kinase. Insights into kinase mechanism. *J. Biol. Chem.* 276:10049–10055.
- Tornqvist, H. E., and J. Avruch. 1988. Relationship of site-specific β -subunit tyrosine autophosphorylation to insulin activation of the insulin receptor

- (tyrosine) protein kinase activity. *J. Biol. Chem.* **263**:4593–4601.
49. **Tornqvist, H. E., J. R. Gunsalus, R. A. Nemenoff, A. R. Frackelton, M. W. Pierce, and J. Avruch.** 1988. Identification of the insulin receptor tyrosine residues undergoing insulin-stimulated phosphorylation in intact rat hepatoma cells. *J. Biol. Chem.* **263**:350–359.
50. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**:203–212.
51. **van der Geer, P., S. Wiley, G. D. Gish, V. K. Lai, R. Stephens, M. F. White, D. Kaplan, and T. Pawson.** 1996. Identification of residues that control specific binding of the Shc phosphotyrosine-binding domain to phosphotyrosine sites. *Proc. Natl. Acad. Sci. USA* **93**:963–968.
52. **van Houten, M., and B. I. Posner.** 1981. Cellular basis of direct insulin action in the central nervous system. *Diabetologia* **20**:255–267.
53. **Villalba, M., S. R. Wente, D. S. Russell, J. C. Ahn, C. F. Reichelderfer, and O. M. Rosen.** 1989. Another version of the human insulin receptor kinase domain: expression, purification, and characterization. *Proc. Natl. Acad. Sci. USA* **86**:7848–7852.
54. **Wang, J. K., and M. Goldfarb.** 1997. Amino acid residues which distinguish the mitogenic potentials of two FGF receptors. *Oncogene* **14**:1767–1778.
55. **Wei, L., S. R. Hubbard, W. A. Hendrickson, and L. Ellis.** 1995. Expression, characterization, and crystallization of the catalytic core of the human insulin receptor protein-tyrosine kinase domain. *J. Biol. Chem.* **270**:8122–8130.
56. **Whittaker, J., A. K. Okamoto, R. Thys, G. I. Bell, D. F. Steiner, C. A. Hofmann.** 1987. High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* **84**:5237–5241.
57. **Wilden, P. A., B. D. Morrison, and J. E. Pessin.** 1989. Relationship between insulin receptor subunit association and protein kinase activation: insulin-dependent covalent and Mn/MgATP-dependent noncovalent association of alpha beta heterodimeric insulin receptors into an $\alpha_2\beta_2$ heterotetrameric state. *Biochemistry* **28**:785–792.
58. **Zhang, B., J. M. Tavaré, L. Ellis, and R. A. Roth.** 1991. The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain. An assessment by replacement with neutral and negatively charged amino acids. *J. Biol. Chem.* **266**:990–996.