Tyrosine 984 in the juxtamembrane region of the insulin receptor, between the transmembrane helix and the cytoplasmic tyrosine kinase domain, is conserved among all insulin receptor-like proteins from hydra to humans. Crystalllographic studies of the tyrosine kinase domain and proximal juxtamembrane region reveal that Tyr-984 interacts with several other conserved residues in the N-terminal lobe of the kinase domain, stabilizing a catalytically nonproductive position of α-helix C. Steady-state kinetics measurements on the soluble kinase domain demonstrate that replacement of Tyr-984 with phenylalanine results in a 4-fold increase in $k_{cat}$ in the unphosphorylated (basal state) enzyme. Moreover, mutation of Tyr-984 in the full-length insulin receptor results in significantly elevated receptor phosphorylation levels in cells, both in the absence of insulin and following insulin stimulation. These data demonstrate that Tyr-984 plays an important structural role in maintaining the quiescent, basal state of the insulin receptor. In addition, the structural studies suggest a possible target site for small molecule activators of the insulin receptor, with potential use in the treatment of noninsulin-dependent diabetes mellitus.

The insulin receptor is a member of the receptor tyrosine kinase (RTK)$^1$ family of cell surface receptors. Unlike the majority of RTKs, which are monomeric in the absence of ligand, the insulin receptor is a disulfide-linked heterotetramer comprising two extracellular α subunits and two membrane-spanning β subunits (1, 2). Upon insulin binding to the α subunits, the insulin receptor undergoes a poorly characterized structural rearrangement that facilitates autophosphorylation of specific tyrosine residues in the cytoplasmic portion of the β subunits. Tyrosine autophosphorylation stimulates receptor catalytic (tyrosine kinase) activity (3) and creates recruitment sites for downstream signaling molecules such as the insulin receptor substrate (IRS) proteins (4) and APS (5).

Three tyrosine residues in the cytoplasmic juxtamembrane region of the insulin receptor, Tyr-965, Tyr-972, and Tyr-984, are conserved to various extents in the insulin receptor subfamily of RTKs (Fig. 1). This subfamily includes the insulin receptor, the insulin-like growth factor-1 (IGF1) receptor, the insulin receptor-related receptor (IRR), and insulin receptor-like proteins in invertebrates such as Daf-2 in Caenorhabditis elegans and DIR in Drosophila melanogaster. Tyr-965, an autophosphorylation site that is not conserved in the invertebrate receptors, appears to be involved in receptor endocytosis (6, 7). Tyr-972, invariant in the insulin receptor subfamily, is an essential autophosphorylation site (NPXY motif) that recruits IRS proteins (4), Shc (8), and Stat-5b (9) via a phosphotyrosine-binding domain in these adapter proteins. Tyr-984 is also invariant in this subfamily, yet its role in insulin receptor signaling has not been determined. Biochemical studies indicate that Tyr-984 is not, to any appreciable extent, a site of autophosphorylation (10, 11).

Previous crystallographic studies on the tyrosine kinase domain of the insulin receptor (IRK) have elucidated the mechanism by which trans-autophosphorylation of tyrosines in the kinase activation loop stimulates catalytic activity (12, 13). These studies were performed using a catalytic domain construct comprising the core tyrosine kinase domain (residues 989–1283) and the juxtamembrane region proximal to the kinase domain (residues 978–988). This original construct included two amino acid substitutions in the kinase proximal juxtamembrane region, Cys-983 → Ser and Tyr-984 → Phe. Cys-981, which is not conserved even among mammalian insulin receptors, was substituted with serine to prevent formation of disulfide-linked dimers in vitro. At the time of the initial structural studies, it was unclear whether Tyr-984 was an autophosphorylation site, and to avoid potential autophosphorylation heterogeneity, this tyrosine was substituted with phenylalanine. Use of the Tyr-984 → Phe IRK protein (IRK*984F) in past structural studies, and the disordered state of Phe-984 therein, has left obscure the function of this conserved tyrosine.

To elucidate the role of Tyr-984 in insulin receptor function, we have determined a crystal structure of unphosphorylated IRK containing wild-type Tyr-984. Although crystals of wild-type IRK were obtained, the N-terminal kinase lobe in this crystal form is poorly ordered, and the position of Tyr-984 could not be ascertained. Crystals of a mutant IRK in which conserved Asp-1132 in the catalytic loop was substituted with asparagine (IRKD1132N) diffract to high resolution, and a structure of this protein has been determined at 1.9 Å resolution. In this crystal structure, Tyr-984 is observed in several hydrophobic and hydrogen-bonding interactions with residues in α-helix C (αC) and in the five-stranded β sheet in the N-terminal lobe.
of the kinase. These interactions suggest that Tyr-984 serves a structural role in the kinase activation mechanism. Comparison of the in vitro steady-state kinetics properties of wild-type IRK and IRK<sup>Y984F</sup> shows that the wild-type enzyme has a 4-fold lower <i>k<sub>m</sub></i> than IRK<sup>Y984F</sup>. Moreover, full-length insulin receptors bearing a Tyr-984→Ala mutation are hyperphosphorylated in transiently transfected mammalian cells, either in the basal (without insulin) state or upon insulin stimulation, consistent with an autoinhibitory role for Tyr-984. These data, together with previous structural studies of activated IRK (13), suggest a novel strategy for designing small molecule activators of the insulin receptor with potential application to non-insulin-dependent diabetes mellitus.

**EXPERIMENTAL PROCEDURES**

**IRK Protein Production—**Site-directed mutagenesis was performed using the QuikChange system (Stratagene). Mutant IRK cDNAs were subcloned into the baculovirus vector pFastBac (Invitrogen). All of the constructs were verified by DNA sequencing. Wild-type and mutant IRK proteins (residues 978–1283) were purified from baculovirus-infected insect cells as described by Hubbard et al. (12). Tris-phosphorylated IRK proteins were produced as described by Hubbard (13).

**Crystallographic Studies—**Crystals of IRK<sup>Y984F</sup> were grown at 4°C by vapor diffusion in hanging drops containing 1.5 μl of protein stock solution (15 mg/ml IRK<sup>Y984F</sup>, 50 mM Tris-HCl, pH 7.5, and 170 mM NaCl) plus 1.5 μl of reservoir buffer (20% polyethylene glycol 8000, 100 mM Tris-HCl, pH 7.5, 30 mM NaCl, and 5 mM dithiothreitol). The crystals belong to the orthohombic space group <i>P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub></i>, with unit cell dimensions a = 57.47 Å, b = 69.48 Å, and c = 88.81 Å when frozen. There is one molecule in the asymmetric unit, and the solvent content is 51%. The crystals were transferred stepwise (in ethylene glycol content) into a cryo-soy solution consisting of 20% polyethylene glycol 8000, 100 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 15% ethylene glycol. Data from a single crystal were collected at Beamline X12C at the National Synchrotron Light Source (Brookhaven National Laboratory) and were processed using DENZO and SCALEPACK (14). Because the unit cell of the IRK<sup>Y984F</sup> crystals was very similar to the unit cell of crystals of an Asp-1161→Ala mutant IRK (15) (Protein Data Bank code 1I44), 1I44 was used as the starting model for building and refinement of the IRK<sup>Y984F</sup> structure. Rigid body, positional/β-factor refinement and simulated annealing were carried out with CNS (16), and model building was performed with O (17).

**Steady-state Kinetics Measurements—**Steady-state kinetics values for wild-type IRK and IRK<sup>Y984F</sup>, both the unphosphorylated (0P) and tris-phosphorylated (3P) forms, were derived using a continuous spectrophotometric assay (18, 19). All of the experiments were carried out at 30°C in 75 μl of buffer containing 100 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM phosphoenolpyruvate, 0.5 mM NADH, 11 unit/ml pyruvate kinase, and 156 units/ml lactate dehydrogenase (Sigma). For determinations of <i>K<sub>m</sub></i> for peptide, reactions contained either 500 nM (0P) or 20 nM (3P) enzyme, 5 μM (0P) or 1 μM (3P) ATP, and 0–12 mg/ml poly-Glu-Tyr peptide (Sigma-Aldrich). For determinations of <i>k<sub>v</sub></i> and <i>K<sub>m</sub></i> for ATP, reactions contained either 500 nM (0P) or 20 nM (3P) enzyme, 12 mg/ml poly-Glu-Tyr peptide, and 0–5 mM (0P) or 0–1 mM (3P) ATP. Kinetic parameters were determined by fitting data to the Michaelis-Menten equation. The conditions of the continuous assay, initial rate measurements and no buildup of ADP, limit ADP-dependent autodephosphorylation of the enzyme (20). Rates of ATP consumption for enzyme alone (<10% of rates with peptide) were subtracted before data fitting.

**Transient Transfection of Wild-type and Tyr-984 → Ala Insulin Receptors in HEK 293T Cells—**A mammalian expression vector encoding the full-length insulin receptor (pEF-IR) was kindly provided by Dr. R. Kohanski. Mutations in the insulin receptor were first introduced into the vector pX-CKD, which encodes the cytoplasmic domain of the receptor (21), using the QuikChange system (Stratagene). The mutated pX-CKD insert was cloned into pEF-IR as a 1.6-kb BglII-XhoI fragment as described by Frankel et al. (22). Wild-type and mutant pEF-IR were purified using the Endofree Maxi Kit (Qiagen). HEK 293T cells were seeded in 10-cm tissue culture dishes and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, reaching ~60% confluence on the day of transfection. 60 μl of LipofectAMINE 2000 reagent (Invitrogen) and 10 μg of DNA were used to transfect each 10-cm dish according to the protocol supplied by the manufacturer. The cells were split into two 6-well plates 24 h post-transfection and grown for another 24 h in complete medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics). The cells were then serum-starved for 3 h at 37°C in Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin (RIA grade, Sigma) prior to insulin stimulation. Bovine insulin (Sigma) was diluted with 10 mM HCl to various concentrations and applied to the cells for 10 min at 37°C. The cells were then placed immediately on ice and washed once with ice-cold phosphate-buffered saline.

The cells were lysed by the addition of each well of 200 μl of RIPA buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. The cell lysates were collected and then clarified by centrifugation at 12,000 g at 4°C. Proteins in the lysate were separated on 10% SDS-PAGE gels. Lysates containing wild-type and mutant insulin receptors were run on the same gel to ensure accurate comparison. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (NitroBond, Osmonics) that were blocked overnight in 5% bovine serum albumin in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.5% Tween 20). Polyclonal rabbit antibodies against phosphotyrosine (Upstate Biotechnology, Inc.) and antibodies against phosphotyrosine (Sigma) were used as the secondary antibodies. Goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology) was used as the secondary antibody. Enhanced chemiluminescence (Lightning; Perkin Elmer) was used for signal detection. The phosphorylation content of insulin receptors was determined by scanning the developed films on a Personal Densitometer (Amersham Biosciences) for quantification.

**RESULTS**

**Crystal Structure of Unphosphorylated IRK<sup>D1132N</sup>—**The crystal structure of IRK<sup>D1132N</sup> in its unphosphorylated state was determined by molecular replacement and refined at 1.9 Å.
Autoinhibitory Role for Tyr-984 in the Insulin Receptor

The kinase domain (residues 978–988) and other IRK mutants (23). The superimposed refined structure of IRK\textsuperscript{D1132N} is shown in stick representation, with the proximal juxtamembrane region (residues 978–988) colored orange, residues in αC colored yellow, and residues in the β sheet (and connecting loops) colored cyan. Ordered water molecules are indicated with red spheres. Selected residues are labeled. B, ribbon diagram of the IRK\textsuperscript{D1132N} structure. β strands are colored cyan, and α-helices are colored yellow. The proximal juxtamembrane region including Tyr-984 is colored orange, with the side chain of Tyr-984 shown in ball-and-stick representation, and αC in the N-terminal lobe is labeled. The dashed gray line indicates that the activation loop is disordered (from Met-1153 through Leu-1171). The N and C termini are denoted by N and C.

In the original crystal structure of unphosphorylated IRK (12), the activation loop traverses the cleft between the N- and C-terminal lobes of the kinase, and Tyr-1162 in the activation loop functions as a pseudosubstrate inhibitor, hydrogen-bonded to conserved Asp-1132 and Arg-1136 in the catalytic loop. This loop functions as a pseudosubstrate inhibitor, hydrogen-bonded to invariant Ser-1067. The activation loop conformation, which occludes the active site, is responsible for autoinhibitory role for Tyr-984 in the insulin receptor subfamily. Glu-990 is hydrogen-bonded to invariant Ser-1067.

The conformation of the juxtamembrane region proximal to the kinase domain (residues 978–988) in the IRK\textsuperscript{D1132N} structure differs appreciably from those in IRK structures reported previously. In the IRK\textsuperscript{D1132N} structure, this segment lies along αC in the N-terminal kinase lobe (Figs. 2B and 3A). Tyr-984 is partially buried in a hydrophobic pocket formed at the junction between the five-stranded anti-parallel β sheet and αC in the N-terminal lobe (Fig. 3A). In addition to hydrophobic contacts between Tyr-984 and Leu-1045 and Val-1065, the hydroxyl group of Tyr-984 is hydrogen-bonded to Glu-990, another invariant residue in the insulin receptor subfamily. Glu-990 is also hydrogen-bonded to invariant Ser-1067.

In fact, a cluster of conserved residues in the insulin receptor subfamily is located in the junction between the juxtamembrane region and the kinase domain: Tyr-984, Asp-987, Trp-989, Glu-990, Ala-1048, Lys-1052, and Ser-1067 (Fig. 3C). In the IRK\textsuperscript{D1132N} structure, Lys-1052 (αC) participates in three hydrogen bonds with juxtamembrane residues, two with the side chain of Asp-987 and one with the main chain of Val-985 (Fig. 3, A and C). This conformation of the juxtamembrane region is further stabilized by hydrogen bonds between the side chain of Asn-1046 (αC) and main chain atoms of Ser-981 (Cys-981 in wild-type IRK) and Val-983 in the juxtamembrane region. Asn-1046 is conserved in insulin and IGF1 receptors and is a lysine in IRR.

**Table I**

<table>
<thead>
<tr>
<th>Data collection and refinement statistics</th>
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<tr>
<td>Data collection</td>
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<td>Unique reflections</td>
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<td>Completeness (%)</td>
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<td>( R_{\text{cryst}} ) (%)</td>
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<tr>
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<td>Reflections</td>
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<td>( R_{\text{cryst}}R_{\text{free}} ) (%)</td>
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<td>Bond angles (°)</td>
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</tr>
<tr>
<td>Water</td>
<td>33.6</td>
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</table>

\( R_{\text{cryst}} = 100 \times \Sigma | I_o - I_c | \Sigma I_o \) \( R_{\text{free}} = 100 \times \Sigma | I_o - I_c | \Sigma I_o \). The value in parentheses is for the lowest resolution shell (1.94–1.90 Å).

For bonded protein atoms.
In the recent crystal structures of the IGF1 receptor kinase domain in its unphosphorylated (24) or bis-phosphorylated state (25), the tyrosine equivalent to Tyr-984, Tyr-957, is situated in the same hydrophobic pocket as observed for Tyr-984 in the IRKD1132N structure. Of note, Tyr-957 is hydrogen-bonded to conserved Lys-1025 (Lys-1052 in IRK) in the IGF1 receptor kinase structures, whereas in the IRKD1132N structure, Tyr-984 is hydrogen-bonded to conserved Glu-990. This subtle difference in the positioning of Tyr-984/957 in the insulin/IGF1 receptor subfamily that form the Tyr-984 binding pocket. The residue labels are placed alongside the Ca atoms of the side chains. The backbone nitrogen atoms of Trp-989 and Ser-1067 are included as well as the carbonyl oxygen of nonconserved Val-985 (dark grey). Hydrogen bonds are depicted by dashed lines, and van der Waals’ interactions (<3.8 Å) with Tyr-984 are depicted as concentric half-circles.

In the unphosphorylated IRKY984F structure (12), the proximal juxtamembrane region is disposed along αC in approximately the same manner as in the IRKD1132N structure, but the side chain of Phe-984 is exposed to solvent and disordered, and the hydrophobic pocket between αC and the β sheet is instead filled with Val-985. In this juxtamembrane configuration, conserved Trp-989 and Lys-1052 are poorly ordered, and hydrogen bonding between Lys-1052 and conserved Asp-987 is not observed.

The conformation of the proximal juxtamembrane region in the tris-phosphorylated, activated IRK structure (13) is markedly different from the conformations in the unphosphorylated
centration (100 nM). Standard deviations
between the proximal juxtamembrane region, specifically Tyr-
984, and residues in the N-terminal kinase lobe affect catalytic
dictates the conformation of the proximal juxtamembrane
segment. This juxtamembrane conformation is similar to
that observed in the structure of the tris-phosphorylated form
of the IGF1 receptor kinase domain (11), which contained wild-
type tyrosine at residue 957 (Tyr-984 equivalent) and crystal-
lized in a different space group. These structural observations
established from in vitro kinetics studies that Tyr-984 represses catalytic activity in the soluble kinase domain, we tested whether full-length insulin receptors bearing the substitution Tyr-984→Phen insulin receptors are considerably elevated over the levels in wild-type receptors, both in the basal state and

Fig. 4. Transient transfection of wild-type and Tyr-984→Ala mutant insulin receptors in HEK 293T cells. A, HEK 293T cells were transiently transfected with either wild-type insulin receptors (WT) or insulin receptors bearing the Tyr-984→Ala mutation (Y984A). Serum-starved cells were stimulated with insulin for 10 min, and the lysates were subjected to SDS-PAGE (7.5%) and Western blotting with an anti-phosphotyrosine antibody (top blot) or, from a separate gel, an anti-insulin receptor antibody (bottom blot). Insulin concentrations were 0 nm (lanes 1 and 6), 0.1 nm (lanes 2 and 7), 1.0 nm (lanes 3 and 8), 10 nm (lanes 4 and 9), or 100 nm (lanes 5 and 10). A representative blot is shown. The insulin-stimulated phosphorylation level of endogenous insulin receptors in HEK 293T cells was negligible (data not shown). B, Quantification of the phosphorylation levels of the wild-type (gray) and Tyr-984→Ala (black) insulin receptors as a function of insulin concentration. Anti-phosphotyrosine blots from five independent transient transfection experiments were subjected to densitometry, and the blots were normalized to each other by setting equal to 1.0 the phosphorylation level of the wild-type receptor at the highest insulin concentration (100 nm). Standard deviations are indicated by the error bars (except for the wild-type receptor at 100 nm insulin used for normalization). The fold differences in phosphorylation levels (mutant over wild type) at each insulin concentration were: 0 nm, 29.8; 0.1 nm, 11.6; 1.0 nm, 3.3; 10 nm, 1.6; and 100 nm, 1.9. anti-pY, anti-phosphotyrosine; anti-IR, anti-insulin receptor.

**IRK**WT and IRKY984F structures. In the activated kinase, the juxtamembrane segment is disengaged from the β sheet-αC cleft (Fig. 3B). This juxtamembrane conformation is similar to that observed in the structure of the tris-phosphorylated form of the IGF1 receptor kinase domain (11), which contained wild-type tyrosine at residue 957 (Tyr-984 equivalent) and crystallized in a different space group. These structural observations indicate that the phosphorylation state of the activation loop dictates the conformation of the proximal juxtamembrane segment.

**Steady-state Kinetics Measurements of IRK**WT Versus IRKY984F—To determine whether the observed interactions between the proximal juxtamembrane region, specifically Tyr-984, and residues in the N-terminal kinase lobe affect catalytic activity, the steady-state kinetics parameters ($k_{cat}$, $K_m$ATP, and $K_{m}$peptide) were determined for the purified soluble kinase domains, wild-type IRK (IRK**WT**), and IRKY984F. Kinetics measurements were performed using a continuous spectrophotometric assay (18, 19) on both the unphosphorylated (basal) and tris-phosphorylated (activated) forms of the enzymes. The data presented in Table II demonstrate that IRK**WT** is less active than IRKY984F in the basal state. The $k_{cat}$ value for IRK**WT** is ~4-fold lower than for IRKY984F, with minor differences in the $K_m$ values for ATP and substrate peptide. For tris-phosphorylated IRK**WT** and IRKY984F, the $k_{cat}$ and $K_m$ values are essentially equivalent. The increases in $k_{cat}$ upon phosphorylation of IRK**WT** and IRKY984F are 15- and 4-fold, respectively, accompanied by a 6-fold decrease in $K_m$ATP.

**Steady-state Kinetics Values**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ATP (mM)</th>
<th>$K_m$peptide (mM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRK<strong>WT</strong>-0P</td>
<td>0.80 ± 0.12</td>
<td>3.96 ± 0.32</td>
<td>2.25 ± 0.10</td>
</tr>
<tr>
<td>IRKY984F-0P</td>
<td>1.29 ± 0.11</td>
<td>3.51 ± 0.46</td>
<td>9.19 ± 0.62</td>
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<tr>
<td>IRK<strong>WT</strong>-3P</td>
<td>0.13 ± 0.01</td>
<td>0.62 ± 0.2</td>
<td>35.4 ± 0.7</td>
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<tr>
<td>IRKY984F-3P</td>
<td>0.14 ± 0.01</td>
<td>0.66 ± 0.2</td>
<td>38.2 ± 1.6</td>
</tr>
</tbody>
</table>

Enzyme abbreviations are: IRK**WT**-0P, unphosphorylated wild-type IRK; IRKY984F-0P, unphosphorylated Tyr-984→Phe IRK; IRK**WT**-3P; tris-phosphorylated wild-type IRK; and IRKY984F-3P, tris-phosphorylated Tyr-984→Phe IRK.
upon insulin stimulation. The extent of phosphorylation in the mutant receptors is particularly striking in the absence of insulin, with an average (n = 5) fold increase over wild type of −30. The degree of hyperphosphorylation of mutant versus wild-type receptors diminished as the dose of insulin was increased, yet was still −2-fold at 100 nm insulin (Fig. 4B).

**DISCUSSION**

At the time of the early structural studies of IRK (12), it was not apparent whether Tyr-984, an invariant tyrosine in the juxtamembrane region of insulin receptor subfamily members, was a site of autophosphorylation, and an expression construct was utilized in which Tyr-984 was replaced by phenylalanine. To elucidate the role of conserved Tyr-984 in insulin receptor function, we expressed and purified IRK protein from baculovirus-infected insect cells containing tyrosine at residue 984. From initial *in vitro* experiments, it was evident that wild-type IRK underwent autophosphorylation at a slower rate than IRK<sup>Y984F</sup>. To quantify the catalytic properties of wild-type IRK and IRK<sup>Y984F</sup>, we measured for each enzyme the steady-state kinetics parameters, K<sub>cat</sub> (ATP and substrate peptide) and k<sub>cat</sub>. The results of these experiments (Table II) indicate that k<sub>cat</sub> is 4-fold lower for wild-type IRK than for IRK<sup>Y984F</sup> in the basal (unphosphorylated) state.

To determine the mechanism by which wild-type IRK is catalytically repressed in the basal state relative to IRK<sup>Y984F</sup>, we determined the crystal structure of unphosphorylated IRK containing Tyr-984. In this structure, the juxtamembrane region proximal to the kinase domain (residues 979–988) lies along αC in the N-terminal kinase lobe, with Tyr-984 situated in a hydrophobic pocket between αC and the β sheet in the N-terminal lobe (Fig. 3A).

The interactions of Tyr-984 in the β sheet–αC cleft and a comparison of the αC positions in the unphosphorylated and tris-phosphorylated IRK structures suggest the structural mechanism by which Tyr-984 represses catalytic activity in the basal state. One of the consequences of IRK activation loop autophosphorylation and reconfiguration is the unrestrained rotation of the N-terminal lobe toward the C-terminal lobe (lobe closure) to bind ATP productively (13). In addition to an overall hinge movement of the N-terminal lobe, αC pivots downward toward the C-terminal lobe (Fig. 5A). This independent (from the β sheet) movement of αC facilitates formation of a catalytically important salt bridge between conserved Lys-1020 (β3) and conserved Glu-1047 (αC). Importantly, in the activated IRK structure, the proximal juxtamembrane region is disengaged from the β sheet–αC cleft (Figs. 3B and 5). These observations indicate that, in addition to the activation loop, the proximal juxtamembrane segment, anchored by Tyr-984, provides steric restraints preventing αC from assuming its catalytically competent position.

In many protein kinases, αC is a key structural element in the regulation of catalytic activity. For example, in cyclin-dependent kinase 2, αC (or PSTAIR helix) is not positioned properly to form the conserved lysine-glutamic acid salt bridge (26). Cyclin A binding to cyclin-dependent kinase 2 pushes αC toward the β sheet, resulting in significant stimulation of kinase activity, even in the absence of activation loop phosphorylation (27). In the nonreceptor tyrosine kinase c-Src, intramolecular interactions between the SH3 domain and the SH2-kinase linker stabilize a noncatalytically productive position of αC, contributing to repressed kinase activity (28, 29).

A structural interplay between αC and the activation loop has been documented for c-Src (30). Autophosphorylation of Tyr-416 and concomitant rearrangement of the activation loop disrupts the interactions that stabilize the mispositioning of αC. Conversely, mutations in the β3–αC loop partially activate c-Src irrespective of Tyr-416 autophosphorylation. Similarly, the structural and kinetic data presented here indicate that partial activation of IRK in the basal state results from disengagement of the proximal juxtamembrane region from the β sheet–αC cleft (*via* mutation of Tyr-984). The observation that, once phosphorylated, wild-type IRK and IRK<sup>Y984F</sup> have very similar catalytic efficiencies (Table II) implies that the influence of the activation loop on αC positioning is “dominant” over that of the proximal juxtamembrane region.

To determine whether autoinhibition by Tyr-984 as detailed here for the soluble kinase domain is necessary for maintaining
binding to the extracellular region plays a key role in the mechanism by which insulin/insulin receptor-1 (IRK) and MuSK (36), extended juxtamembrane region (EphB2 (34) and c-Kit (37)), or C-terminal tail (33). Moreover, the constitutive dimeric arrangement of receptors in the insulin receptor subfamily might make receptors in this subfamily particularly sensitive to mutation of conserved Tyr-984, as discussed above.

The structural characterization of the autoinhibitory role of Tyr-984 in the basal state kinase affords a novel strategy for the design of small molecule activators of the insulin receptor. A cell-permeable compound designed to bind in the β sheet-α cleft and displace Tyr-984 should partially activate the receptor in the absence of insulin and sensitize the receptor in the presence of insulin. The specific target site for such a molecule would be the β sheet-α cleft as configured in the activated IRK structure (13) (Fig. 5B).

Two compounds capable of stimulating insulin receptor autophosphorylation by acting on the cytoplasmic domain have been reported (38, 39). One of the compounds (L-783,281) modestly elevates insulin receptor autophosphorylation in the absence of insulin, whereas the other compound (TLK16998) potentiates receptor autophosphorylation in the presence of insulin, suggesting two different mechanisms of action (40). Interestingly, L-783,281 and a derivative (41) are of an appropriate size to bind in the β sheet-α cleft, and L-783,281 was shown to alter the trypsin susceptibility at Lys-1030 (38), not far spatially from Tyr-984 (Fig. 5A). Whether or not the β sheet-α cleft is the site of action of these compounds, the existence of such small molecule activators, together with the data presented here on the autoinhibitory role of Tyr-984, motivates efforts to design novel insulin receptor activators with potential application to noninsulin-dependent diabetes mellitus.

Acknowledgment—We thank R. Kohanski for reagents, helpful discussions, and manuscript comments.

REFERENCES

Autoinhibitory Role for Tyr-984 in the Insulin Receptor