Role of the Activation Loop Tyrosines in Regulation of the Insulin-like Growth Factor I Receptor-tyrosine Kinase*

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The tyrosine kinase activity of insulin-like growth factor I receptor (IGF1R) is under tight control. Ligand binding to the extracellular portion of IGF1R stimulates autophosphorylation at three sites (Tyr\(^{1131}\), Tyr\(^{1135}\), and Tyr\(^{1136}\)) in the activation loop within the tyrosine kinase catalytic domain. Autophosphorylation at all three sites is required for maximum enzyme activity, and for IGF1-stimulated cellular activity of the receptor. Previous studies have not clarified the contributions of the individual tyrosines to enzymatic activation. Here, we produced single Tyr-to-Phe mutations at these positions, and compared activities of the purified kinase domains (unphosphorylated and phosphorylated) with wild-type IGF1R. Rates of autophosphorylation of the three mutants were more rapid than for wild-type IGF1R; this was most apparent for the Y1135F mutant. Substrate phosphorylation studies on the unphosphorylated forms of IGF1R confirmed that the value of \(V_{\text{max}}\) for Y1135F was elevated relative to wild-type IGF1R, consistent with a disruption of an autoinhibitory interaction. In contrast, activity measurements on the fully phosphorylated enzymes indicated that \(k_{\text{cat}}/K_m\) values were lowered relative to wild-type IGF1R; this effect was most dramatic for Y1136F. We confirmed these findings using limited proteolysis and tryptophan fluorescence experiments. The results demonstrate that Tyr\(^{1135}\) plays a particularly important role in stabilizing the autoinhibited conformation of the activation loop, while Tyr\(^{1136}\) plays the key role in stabilizing the open, activated conformation of IGF1R.

Insulin-like growth factor 1 receptor (IGF1R)\(^2\) is a transmembrane tyrosine kinase that is essential for fetal and postnatal growth and development (1–5). It is expressed in most human tissues and cell types (the only known exceptions are hepatocytes and mature B cells). The receptor is activated by binding of the secreted growth factor ligand IGF1 (or the lower affinity ligand IGF2) to the extracellular domain of IGF1R.

Ligand binding stimulates the intrinsic tyrosine kinase activity of the cytoplasmic domain. This in turn triggers a number of cellular signaling pathways, including the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway (the main mechanism by which IGF1R protects cells from apoptosis), and the Erk/MAPK pathway leading to mitogenesis. Aberrant IGF1R signaling has been implicated in malignant transformation of cells, and the enzyme has emerged as a target for anticancer drug design (6–10).

IGF1R, insulin receptor (IR) and insulin-related receptor (IRR) belong to the same receptor-tyrosine kinase (RTK) family (11, 12). These receptors are composed of two extracellular \(\alpha\) subunits containing the ligand binding domain and two transmembrane \(\beta\) subunits possessing tyrosine kinase activity. IGF1R and IR share 70% sequence identity overall, and 84% identity within the tyrosine kinase catalytic domains. The mechanism for receptor activation is thought to be similar for IR and IGF1R (2–4,13).

The structures of the IGF1R and IR catalytic domains display the typical two-lobe protein kinase fold (14–18). ATP binding and catalysis takes place in a deep cleft between the two lobes. These RTKs contain a segment known as the activation loop (A-loop), which contains the three principal autophosphorylation sites (Tyr\(^{1131}\), Tyr\(^{1135}\), and Tyr\(^{1136}\)) in IGF1R (Fig. 1). Ligand binding to the extracellular \(\alpha\) subunit promotes a conformational change that leads to autophosphorylation of these sites. The first site of autophosphorylation is predominantly Tyr\(^{1135}\), followed by Tyr\(^{1131}\) and then by Tyr\(^{1136}\) (16). The unphosphorylated, monophosphorylated, bisphosphorylated, and trisphosphorylated forms of IGF1R (IGF1R-0P, -1P, -2P, and -3P, respectively) have increasing levels of enzyme activity (16).

The three-dimensional structures of IGF1R-0P, -2P, and -3P have been solved (16–18). In IGF1R-0P, the A-loop inhibits access to substrates (17). Phosphorylation of Tyr\(^{1131}\), Tyr\(^{1135}\), and Tyr\(^{1136}\) triggers a large change in the A-loop conformation, which allows access of ATP and peptide substrate to the catalytic site. Thus, autophosphorylation of IGF1R is the crucial regulator of the catalytic activity. The details of how autophosphorylation controls enzymatic activity, however, are not completely understood. Steady state kinetic studies show that a significant decrease in the substrate \(K_m\) occurs in the transition from the 1P state (mainly Tyr\(^{1135}\)) to the 2P state (Tyr\(^{1135}\) and Tyr\(^{1131}\)) (16). The conformation of the trisphosphorylated activation loop in IGF1R-3P is stabilized by numerous interactions between the activation loop and other segments of the kinase. The purpose of the present

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*This work was supported by National Institutes of Health Grant CA28146 (to W. T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: IGF1R, insulin-like growth factor I receptor; RTK, receptor-tyrosine kinase; FPLC, fast protein liquid chromatography; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; pY, phosphotyrosine.
A study was to define the roles of Tyr\textsuperscript{1131}, Tyr\textsuperscript{1135}, and Tyr\textsuperscript{1136} in regulation of autophosphorylation. When expressed in fibroblasts, a mutant lacking all three A-loop tyrosines showed dramatically reduced signaling in response to IGF-1 (19, 20). A Y1135F/Y1131F double mutant was incapable of further autophosphorylation in NIH3T3 cells, and did not promote downstream Shc and IRS-1 signaling (21). Mutation of Tyr\textsuperscript{1131} alone significantly reduced the level of IGF1-induced receptor autophosphorylation, whereas it did not have an impact on cell proliferation or phosphorylation of the substrate IRS-1 (22). In a separate study, a Y1136F mutant showed a marked decrease in IGF1-stimulated receptor autophosphorylation, whereas it did not have an impact on cell proliferation or phosphorylation of the substrate IRS-1 (22). In a separate study, a Y1136F mutant showed a marked decrease in IGF1-stimulated receptor autophosphorylation, whereas it did not have an impact on cell proliferation or phosphorylation of the substrate IRS-1 (22).

We previously reported a method to separately purify each of the phosphorylated forms of IGF1R (IGF1R-0P, -1P, -2P, and -3P) (16). Steady-state kinetic analyses of the isolated phosphorylated forms of the IGF1R kinase domain have not previously been published.

We previously reported a method to separately purify each of the phosphorylated forms of IGF1R (IGF1R-0P, -1P, -2P, and -3P) (16). We analyzed the conformation of the A-loop and the level of enzymatic activity in these forms of IGF1R. Our results indicate that each tyrosine is important for full activation of the IGF1R kinase domain. Furthermore, our results shed light on the individual roles of the three tyrosines. Autophosphorylation of Tyr\textsuperscript{1135} and Tyr\textsuperscript{1131} appears to destabilize the autoinhibitory conformation of the activation loop, whereas Tyr\textsuperscript{1136} phosphorylation plays the key role in structural stabilization of the A-loop.

**EXPERIMENTAL PROCEDURES**

**Materials**—10% Tris-HCl gels were from Bio-Rad. Mass spectrometry grade trypsin was obtained from Promega. ADP and potassium iodide were obtained from Sigma.

**Mutagenesis and Purification of IGF1R Kinase Domain**—IGF1R mutants were produced with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocols. The mutants were made in a pFastBac-1 baculovirus transfer vector (Invitrogen) as described previously (16). Sf9 cells were cultured in Excel 410 (JRH Biosciences) with 5% heat-inactivated serum (Sigma) and 1% penicillin/streptomycin/amphotericin at 27 °C. The cells were harvested after 72 h of infection.

The purification of the mutants was accomplished using similar procedures as for wild-type IGF1RK (16). Unphosphorylated forms of the mutants were purified in three chromatographic steps on an FPLC system (GE Healthcare): 1) a Source Q-15 column, 2) a Superdex-75 gel filtration column, and 3) a Mono-Q HR10/10 column. To produce the completely phosphorylated forms of IGF1RK (i.e. IGF1RK-3P for wild-type enzyme, or IGF1RK-2P for the single site mutants), enzymes were incubated with 10 mM ATP and 30 mM MgCl\textsubscript{2} for 5 min at room temperature. The autophosphorylation reactions were terminated by addition of 100 mM EDTA. Native gel analysis confirmed that these reactions contained predominantly the completely phosphorylated forms. Sodium orthovanadate (200 \(\mu\)M) was added to prevent dephosphorylation. The reactions were then passed over Superdex 75 to remove nucleotides, and

In this study, we used similar methods to purify unphosphorylated and phosphorylated forms of IGF1R containing single mutations at Tyr\textsuperscript{1131}, Tyr\textsuperscript{1135}, and Tyr\textsuperscript{1136}. We analyzed the conformation of the A-loop and the level of enzymatic activity in these forms of IGF1R. Our results indicate that each tyrosine is important for full activation of the IGF1R kinase domain. Furthermore, our results shed light on the individual roles of the three tyrosines. Autophosphorylation of Tyr\textsuperscript{1135} and Tyr\textsuperscript{1131} appears to destabilize the autoinhibitory conformation of the activation loop, whereas Tyr\textsuperscript{1136} phosphorylation plays the key role in structural stabilization of the A-loop.
the phosphorylated forms were separated by Mono-Q chromatography (16). The pooled fractions were concentrated and stored at −80 °C in 20 mM Tris-HCl, pH 7.5. All final protein concentrations were determined by the Bradford method (Bio-Rad).

Kinase Assays—The kinetics of IGF1R autophosphorylation and substrate peptide phosphorylation were measured by a continuous spectrophotometric assay (16, 23). In this assay, the consumption of ATP is coupled to the oxidation of NADH, which is monitored as a reduction in NADH absorption at 340 nm. Reactions (50 μl) contained 100 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM phosphoenolpyruvate, 1.2 mg/ml NADH, 111 units/ml pyruvate kinase, 156 units/ml lactate dehydrogenase, and substrate peptide (KKEEEEYMMMMG). Autophosphorylation reactions contained 6 μM enzyme and 1 mM ATP. Peptide phosphorylation reactions contained 100 nM enzyme. For determinations of $K_m$ (ATP), reactions contained a peptide concentration of 2 mM and varying concentrations of ATP (10–4000 μM). For determinations of $K_m$ (peptide), reactions contained an ATP concentration of 1 mM and varying concentrations of peptide (10–5000 μM). Data were recorded every 6 s. The kinetic parameters were determined by fitting to the Michaelis-Menten equation.

Native-PAGE autophosphorylation assays were carried out using 6 μM enzyme, 10 mM ATP, and 30 mM MgCl₂ at room temperature (24). The reactions were stopped by addition of 100 mM EDTA at various time points. Autophosphorylation reactions were analyzed by 10% Tris-HCl native PAGE and visualized by Coomassie Blue staining.

Limited Proteolysis of IGF1RK—Limited proteolysis of wild-type and mutant forms of IGF1RK was carried out according to previous methods established for IRK (25). Proteins were diluted to 9 μM in 50 mM Tris-HCl, pH 7.0, 30 mM MgCl₂, and 2 mM dithiothreitol, with or without 10 mM ADP. Trypsin was added at a ratio of trypsin to IGF1RK of 1:25–30 by mass. The reactions proceeded at room temperature for 15 min and were stopped by addition of 5× SDS-PAGE buffer. The digestion products were resolved by SDS-PAGE, and the fragments were visualized by Coomassie Blue staining.

Fluorescence Spectra and Iodide Quenching—Tryptophan fluorescence experiments were carried out in 100 mM Tris-HCl and 1 mM dithiothreitol using methods described for IRK (26). Experiments were carried out in volumes of 500 μl using 0.5 μM IGF1RK (wild-type or mutants). Potassium iodide was included at varying concentrations to quench emission of tryptophan residues. Changes in $K_m$ for ATP were modest; the largest change was a 2-fold reduction for the Y1135F mutant. The activation was most apparent in the case of the Y1135F and Y1131F mutants; the final autophosphorylation rates for these mutants were 63.1 and 31.8 mOD/min, respectively, compared with 6.2 mOD/min for wild-type IGF1RK. The Y1136F mutant had a rate that was closer to wild-type IGF1RK (12.2 mOD/min).

Substrate Phosphorylation Assays—We carried out steady-state kinetic measurements of phosphorylation of a synthetic peptide substrate by wild-type and single site mutants of IGF1RK. We first tested the unphosphorylated forms of the proteins. To ensure that the enzymes remained in their unphosphorylated states during the initial rate measurements, we analyzed samples from the reactions by native PAGE; there was no detectable conversion to 1P, 2P, or 3P forms in the peptide substrate by wild-type and single site mutants of IGF1RK. We expressed mutant forms of IGF1RK in which each tyrosine was changed to phenylalanine (Y1135F, Y1131F, and Y1136F). We expressed wild-type and mutant IGF1RKs using the Sf9/baculovirus system, and purified the unphosphorylated forms of the enzymes. In wild-type IGF1R, the first A-loop tyrosine to be phosphorylated is Tyr¹¹³⁵, followed by Tyr¹¹³¹, and then Tyr¹¹³⁶ (16). To determine whether elimination of single A-loop tyrosines blocked the autophosphorylation of the remaining two sites, we incubated the enzymes with Mg-ATP and followed the time course of autophosphorylation by native PAGE analysis (Fig. 2A). The various phosphoforms of IGF1R are readily resolved by native PAGE (Fig. 2A and Ref. 16). All three mutants were capable of autophosphorylation at the remaining two sites, indicating that no one site is obligatory for full phosphorylation (Fig. 2A).

To compare the rates of autophosphorylation for wild-type and mutant forms of IGF1RK, we used a continuous spectrophotometric assay. We incubated the enzymes with ATP in the absence of any exogenous substrate. As we observed previously (16), the enzyme progress curves were biphasic, because autophosphorylation activates IGF1RK. Autophosphorylation of all three single-site mutants was more rapid than for wild-type IGF1R (Fig. 2B), suggesting that to a certain extent all three tyrosines participate in autoinhibitory interactions. Activation was most apparent in the case of the Y1135F and Y1131F mutants; the final autophosphorylation rates for these mutants were 63.1 and 31.8 mOD/min, respectively, compared with 6.2 mOD/min for wild-type IGF1RK. The Y1136F mutant had a rate that was closer to wild-type IGF1RK (12.2 mOD/min).

where $F_0$ is the fluorescence intensity in the absence of quencher.

RESULTS

Autophosphorylation of Single Site Mutants—To analyze the importance of the individual A-loop tyrosines, we produced mutant forms of IGF1RK in which each tyrosine was changed to phenylalanine (Y1135F, Y1131F, and Y1136F). We expressed wild-type and mutant IGF1RKs using the Sf9/baculovirus system, and purified the unphosphorylated forms of the enzymes.
Kinetic characterization of unphosphorylated IGF1RK and mutants

| Enzyme          | Peptide | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | $K_m$/μmol/min/mg | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$) |
|-----------------|---------|------------|-------------------------|-------------------|------------|-------------------------|-------------------------------------|
| WT (IGF1RK-0P)  | Y1131F | 1.4 ± 0.3  | 1.1 ± 0.2               | 2.9 ± 0.2         | 23         | 64 ± 0.6                | 1.1 × 10$^6$                         |
|                 | Y1135F | 2.3 ± 0.1  | 0.76 ± 0.1              | 9.0 ± 0.3         | 139        | 70 ± 0.6                | 8.4 × 10$^5$                         |
|                 | Y1136F | 2.8 ± 0.3  | 0.54 ± 0.04             | 17 ± 1.1          | 211        | 2.5 ± 0.1               | 1.0 × 10$^5$                         |
|                 | Y1135F | 4.5 ± 0.9  | 0.96 ± 0.1              | 6.4 ± 0.8         | 50         | 0.02 ± 0.01             | 4.0 × 10$^4$                         |

Kinetic characterization of fully phosphorylated IGF1RK and mutants

| Enzyme          | Peptide | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$) |
|-----------------|---------|------------|-------------------------|-------------------------------------|
| WT (IGF1RK-3P)  | Y1135F | 0.15 ± 0.02| 0.12 ± 0.01             | 1.3 × 10$^4$                         |
|                 | Y1135F | 0.9 ± 0.12 | 0.24 ± 0.02             | 1.4 × 10$^5$                         |
|                 | Y1135F | 3.3 ± 0.5  | 0.27 ± 0.02             | 7.0 × 10$^5$                         |

These differences were due for the most part to increases in peptide substrate $K_m$, especially for the Y1136F mutant (Table 2). These results indicate that Y1136 plays a particularly important role in stabilizing a conformation of IGF1R that is catalytically competent for phosphorylation of exogenous substrates.

Conformational Changes in the A-loop—To confirm that the single site mutations destabilized the autoinhibited conformation of the A-loop, we carried out limited proteolysis experiments. This approach was developed by Kohanski and coworkers (25) to analyze the conformation of the IRK activation loop; the rates of limited trypsin proteolysis were used to estimate the differences in global conformation. While wild-type, unphosphorylated IRK was relatively stable to trypsin, addition of adenine nucleotide (to generate autophosphorylated IRK) gave rise to fragments of ~25 kDa and ~16 kDa. These fragments were generated by cleavage at an Arg-Lys sequence within the activation loop (25). The Arg-Lys sequence is conserved in IGF1R (Fig. 1). We applied the limited proteolysis strategy to purified preparations of unphosphorylated and phosphorylated wild-type and mutant IGF1R. First, we carried out limited proteolysis experiments on purified IGF1RK-0P, Y1135F-0P, Y1131F-0P, and Y1136F-0P (Fig. 3A). After quenching the reactions with SDS-PAGE loading buffer, we analyzed the products on SDS-PAGE. As was the case for IRK, IGF1RK-0P was cleaved into fragments of ~24 kDa and ~14 kDa only in the presence of ADP. The Y1131F and Y1136F mutants behaved similarly to wild-type IGF1R. In contrast, the Y1135F mutant showed significant A-loop cleavage in the absence of ADP (Fig. 3A), suggesting that this mutation promotes the “open” conformation of the A-loop.

We carried out similar experiments on the fully phosphorylated forms of the proteins (IGF1RK-3P, Y1135F-2P, Y1131F-2P, and Y1136F-2P) by MonoQ-FPLC. We then used the spectrophotometric assay to measure $V_{max}$ and $K_m$ for both ATP and peptide substrate. Each mutant showed a lowered value of $k_{cat}/K_m$ relative to wild-type IGF1RK (Table 2); values for Y1135F, Y1131F, and Y1136F were decreased by 9.3-fold, 2.2-fold, and 18.6-fold, respectively. These differences were due for the most part to increases in peptide substrate $K_m$, especially for the Y1136F mutant (Table 2). These results indicate that Y1136 plays a particularly important role in stabilizing a conformation of IGF1R that is catalytically competent for phosphorylation of exogenous substrates.
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FIGURE 3. Limited proteolysis of wild type and mutant forms of IGF1R. A, purified unphosphorylated enzymes were incubated with or without 10 mM ADP. Trypsin (1:30, w/w) was added, and the cleavage reactions were stopped by addition of SDS-PAGE sample buffer after 15 min. The digestion products were resolved by SDS-PAGE and visualized by Coomassie Blue staining. Molecular weight markers were run in the first and last lanes of the gel. The arrowheads indicate the positions of the IGF1RK cleavage products. B, similar experiments were carried out on the purified fully phosphorylated enzymes. In this case, 1:25 (w/w) trypsin was used.

As a complementary approach to study conformational changes in the IGF1RK A-loop, we measured tryptophan fluorescence. This method has been previously applied to IR; the solvent accessibility of Trp1175 differs between IRK-0P and IRK-3P, as detected by solute quenching (26). The catalytic domains of both IR and IGF1R have five tryptophan residues. One is on the surface of the small lobe, and the other four are in the interior of the large lobe. Mutagenic analysis demonstrated that the fluorescence emission spectrum of IRK was dominated by excitation at 295 nm and emission spectra collected from 310–420 nm. Experiments were carried out at constant ionic strength using 0.5 μM IGF1RK.

FIGURE 4. Iodide quenching of IGF1RK-0P and IGF1RK-3P fluorescence. Stern-Volmer plot of fluorescence quenching of IGF1RK-0P (circles) and IGF1RK-3P (squares) by potassium iodide. The excitation wavelength was 295 nm and emission spectra were collected from 310–420 nm. Experiments were carried out at constant ionic strength using 0.5 μM IGF1RK.

TABLE 3
Fluorescence quenching for wild-type and mutant forms of IGF1RK
Quenching of tryptophan 1148 was carried out with varying concentrations of potassium iodide, and Stern-Volmer constants (Ksv) were determined as described under “Experimental Procedures.”

| Enzyme | Ksv for unphosphorylated enzyme | Ksv for fully unphosphorylated enzyme |
|--------|----------------------------------|--------------------------------------|
| WT     | 0.29 ± 0.03                      | 1.72 ± 0.05                          |
| Y1131F | 0.6 ± 0.03                       | 1.35 ± 0.06                          |
| Y1135F | 1.17 ± 0.03                      | 1.46 ± 0.04                          |
| Y1136F | 0.43 ± 0.02                      | 1.20 ± 0.04                          |

DISCUSSION
The autophosphorylation of IGF1R at three sites within the activation loop is crucial for full catalytic activity and important for the physiological roles of the receptor in normal and transformed cells (13, 19, 20). Autophosphorylation of the A-loop tyrosines serves two functions: it disrupts the autoinhibitory contacts in the closed form of the receptor, and it introduces phosphate groups that stabilize the open conformation, in which ATP and protein substrates gain access to the active site (14, 16). Previous studies have shown that there is a preferred sequence of A-loop phosphorylations (Tyr1135, followed by Tyr1131, then Tyr1136), and that each phosphorylation increases...
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enzyme activity (16). It was not clear, however, to what extent each phosphorylation contributed to the two functions listed above. Here, we carried out mutagenesis experiments to address the individual roles of the A-loop tyrosines.

Our autophosphorylation assays (Fig. 2) showed that removal of individual A-loop tyrosines did not block the auto-

phosphorylation of the remaining sites. Furthermore, for each mutant, rates of autophosphorylation at the remaining two sites exceeded the rate for wild-type IGF1RK (Fig. 2B). This effect was most pronounced for the Y1135F mutant. The Y1131F mutant also showed a significantly higher autophosphorylation rate than wild-type, while the Y1136F mutant was only slightly increased. The data suggest that the three A-loop tyrosines are involved in IGF1R autoinhibition in the following order of importance: Tyr1135 → Tyr1131 → Tyr1136. This possibility was borne out by steady-state kinetic measurements using the unphosphorylated forms of the enzymes (Table 1). For the mutants (particularly for Y1135F), values of $V_{max}$ for phosphorylation of a synthetic peptide substrate were elevated as compared with wild-type IGF1R kinase.

We also investigated how these three autophosphorylation sites contribute to the activity of the fully phosphorylated forms of the enzyme toward an exogenous substrate. If destabilization of the autoinhibited conformation is sufficient for full enzyme activity, we would expect the fully phosphorylated forms of the mutants to reach a level of activity that is comparable to wild-type IGF1RK. Our kinetic studies on the purified phosphorylated forms of the mutants (Table 2) showed that this is not the case. The $k_{cat}/K_m$ values for Y1135F, Y1131F, and Y136F were decreased by 9.3-fold, 2.2-fold, and 18.6-fold, respectively. For Y1136F, the $K_m$ for peptide substrate was raised by over 20-fold. These results indicate that the phosphorylated A-loop tyrosines are required for full enzyme activity, in the order of Tyr1135 > Tyr1131 > Tyr1136.

We confirmed the enzyme activity results using two probes of the A-loop conformation: limited trypsin proteolysis and fluorescence quenching. Unphosphorylated IGF1RK behaved similarly to insulin receptor in these studies (the Stern-Volmer constant for unphosphorylated IR was 0.3 M$^{-1}$, compared with 0.29 M$^{-1}$ for IGF1RK). In the proteolysis experiments on unphosphorylated forms of IGF1RK, we found that only the Y1135F mutant showed significant cleavage in the absence of ADP (Fig. 3A). Similarly, fluorescence experiments showed the largest degree of iodide quenching for Y1135F (Table 3). Y1131F and Y1136F also gave greater fluorescence quenching than wild-type, with the same rank order as was observed for the enzyme activity assays. The data suggest that removal of these tyrosine residues (particularly Tyr1135) results in a more flexible conformation of the A-loop.

Limited proteolysis experiments on purified IGF1RK-3P showed that the A-loop was inaccessible to trypsin cleavage under these conditions, even in the presence of ADP (Fig. 3A). Phosphorylation appears to anchor the A-loop in such a way as to inhibit trypsin cleavage. In the structure of IGF1RK-3P, the phosphate group of Tyr1135 is salt bridged to Arg1137 within the trypsin cleavage site and the phosphate group of pY1136 is salt-bridged to Lys1138 (16). This might explain, at least in part, why trypsin can access the Arg-Lys site only when the A-loop has a flexible conformation. The phosphorylated forms of the mutants all showed enhanced cleavage relative to wild-type IGF1R, suggesting that the open form of the A-loop was destabilized in the mutants. The effect was most pronounced for Y1136F. Iodide quenching experiments showed the expected increase in quenching for IGF1RK-3P as compared with IGF1RK-0P, due to a change in solvent accessibility in the open conformation (26). The phosphorylated forms of the Y1135F, Y1131F, and Y1136F mutants showed progressively larger decreases in iodide quenching relative to IGF1RK-3P (Table 3). The results suggest that the A-loop in the single-site mutants adopts a conformation that is intermediate between IGF1RK-0P and IGF1RK-3P. The results are consistent with enzyme activity assays and point to a key role for Tyr1136 in stabilizing the activated conformation.

Our results on the roles of the A-loop tyrosines are consistent with the available structural data. In the unphosphorylated forms of IGF1RK (17) or IRK (15), the A-loop adopts an auto-

inhibitory conformation with Tyr1135 (IRK residue 1162) bound in the active site. Tyr1135 of IGF1R interacts with Asp1105, Arg1109, and Pro1145 (17). The side chain of Tyr1135 is hydrogen-bonded to Ser1059. Phosphorylation of Tyr1135 and Tyr1131 would be expected to incrementally destabilize the autoinhibited conformation of the A-loop. In the structure of IGF1RK-3P (Fig. 1), the phosphotyrosine side chains interact with numerous residues in the A-loop and in other regions of the enzyme (16). The phosphate group of Tyr1135 interacts with Arg1137, whereas the phosphate group of Tyr1136 is salt-bridged to Lys1138 and Arg1128. The latter interaction is conserved in the structure of the tris-phosphorylated form of insulin receptor. In the crystal structure of IGF1R-2P, in which residues Tyr1131 and Tyr1135 are phosphorylated but Tyr1136 is not, the central part of the activation loop is disordered (18). Tyr1136 appears to trigger the final lobe closure in which ATP and substrate are brought close together, facilitating catalysis. Our data indicate that Tyr1136 autophosphorylation is necessary to stabilize the activated state of IGF1R.

Our results are relevant for the development of conformationally selective inhibitors of IGF1R. Most of the clinically tested inhibitors of tyrosine kinases are compounds that target the ATP-binding site. There were initial concerns that the conserved architecture of kinase ATP binding sites would preclude the development of specific inhibitors. A large body of evidence now demonstrates that different ATP-binding sites have distinct features (shape of the pockets, composition of amino acids) that can serve as unique drug targets. In particular, it has been argued that the inactive conformations of protein kinases may differ more dramatically than the fully active conformations (27). Gleevec is an example of a tyrosine kinase inhibitor that targets the unphosphorylated, inactive conformation (28). Thus, it is possible to design inhibitors that not only discrimi-

nate among related tyrosine kinases, but also between confor-
mational states of a given tyrosine kinase. Among other advan-
tages of targeting the unphosphorylated form of IGF1R, there are greater differences in the ATP-binding pocket between IGF1R-0P and IR-0P than between their triply phosphorylated counterparts (17). Furthermore, the active site of IGF1R-2P is not as tightly closed as that of IGF1R-3P, suggesting that it
could accommodate inhibitors that are too large to fit in the relatively small, closed IGF1R-3P active site (18). The variety of conformational states of IGF1R present potential targets for inhibitor design. For example, the cyclolignan PPP was recently shown to block the phosphorylation of Tyr1136, but not the other two A-loop tyrosines (29). A full understanding of the enzymatic and conformational changes that occur with each A-loop phosphorylation is an important prelude to the development of conformation-specific inhibitors.

Acknowledgments—We thank Aftabul Haque and Hongling Zhao for performing site-directed mutagenesis, and Dr. Stevan Hubbard for critical review of the manuscript.

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