Autoinhibition of the insulin-like growth factor I receptor by the juxtamembrane region

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Abstract The juxtamembrane (JM) regions of several receptor tyrosine kinases are involved in autoinhibitory interactions that maintain the low basal activity of the receptors; mutations can give rise to constitutive kinase activity and signaling. In this report, we show that the JM region of the human insulin-like growth factor I receptor (IGF1R) plays a role in kinase regulation. We mutated JM residues that were conserved in this subfamily of receptor tyrosine kinases, and expressed and purified the cytoplasmic domains using the SF9/baculovirus system. We show that a kinase-proximal mutation (Y957F) and (to a lesser extent) a mutation in the central part of the JM region (N947A) increase the autoposphorylation activity of the kinase. Steady-state kinetic measurements show the mutations cause an increase in $v_{max}$ for phosphorylation of peptide substrates. When the holoreceptors were expressed in fibroblasts derived from IGF1R-deficient mice, the Y957F mutation led to a large increase in basal and in IGF1-stimulated receptor autophosphorylation. Together, these data demonstrate that the JM region of IGF1R plays an important role in limiting the basal activity of the receptor.

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1. Introduction

The human insulin-like growth factor I receptor (IGF1R) plays an important signaling role in many tissues and cell types [1–3]. IGF1R, like the closely related insulin receptor (IR), is a heterotetrameric receptor tyrosine kinase (RTK) consisting of two extracellular $\alpha$ subunits and two transmembrane $\beta$ subunits [4,5]. The kinase catalytic domains are in the cytoplasmic regions of the $\beta$ subunits. IGF1R is activated by binding of the growth factor ligand IGF1 (or the lower-affinity ligand IGF2) to the extracellular domain of the receptor. Ligand binding promotes a conformational change in the $\beta$ subunits, which stimulates the intrinsic tyrosine kinase activity of the receptor. A key step in the activation process is the autophosphorylation of three tyrosines in the activation loop, a flexible segment within the catalytic domain of the kinase [1,5–8]. The activated IGF1R kinase phosphorylates cellular substrates such as IRS-1 and IRS-2, initiating a number of signaling pathways, including the phosphatidylinositol 3-kinase (PI-3K)/Akt and Erk/MAPK pathways.

Autoinhibitory interactions are present in RTKs to prevent enzyme activation in the absence of ligand. For many RTKs, autoregulatory regions lie outside the core kinase domain. The C-terminal portion of the cytoplasmic domains of Tie2 [9,10], ErbB2 [11], PDGF receptor [12], and RON [13] are involved in enzyme regulation. For some RTKs, the juxtamembrane (JM) regions (the portions of the cytoplasmic domains between the transmembrane sequence and the kinase domains) clearly play a negative regulatory role [14]. Tyrosine residues in the JM region of several RTKs function as direct inhibitors of catalytic function, and also serve as recruitment sites for various downstream signaling proteins. These include EphB2 [15], FLT3 (FMS-like tyrosine kinase 3) [16], MuSK [17], and c-Kit [18]. The crystal structures of the autoinhibited forms of these kinases have been solved, and they demonstrate the structural basis for the negative regulatory role of the JM region in RTKs. The JM regions of these RTKs prevent the activation loop from adopting an active conformation, and phosphorylation within the JM region relieves autoinhibition. Mutations in the JM region of c-Kit that give rise to gastrointestinal stromal tumors are predicted to disrupt the autoinhibitory interactions [19].

The juxtamembrane region of IR consists of 35 amino acids and has also been implicated in the control of kinase activity. There are two autophosphorylation sites (Y965 and Y972) within the JM region of IR. In contrast to the RTKs listed above, autophosphorylation at these sites in IR does not appear to affect kinase activity, so autoinhibition is not controlled by phosphorylation in the case of IR [14]. The JM region of IR contains another tyrosine (Y984) which is not a site of auto-phosphorylation, but which has been shown to be important for autoinhibition. A Y984A mutation increases IR kinase activity in vitro and IR activity in cellular assays [20]. The three-dimensional structure of a portion of the IR JM-kinase domain shows that unphosphorylated Y984 interacts with residues in the N-terminal lobe of the kinase domain [20]. This appears to prevent the catalytically important helix $\alpha C$ from pivoting inwards to adopt its productive conformation.

The JM region of IGF1R is ≈65% conserved with IR; tyrosines 972 and 984 are present in IGF1R (IGF1R numbering: Y950 and Y957). There is also a 5-residue deletion in the IGF1R JM region relative to IR (Fig. 1). In this study, we carried out experiments to determine whether the juxtamembrane region of IGF1R plays a role in regulating kinase activity. We demonstrated that a Y957F mutant, and to a lesser extent an N947A mutant, have higher activity than wild-type IGF1R.
in vitro and in intact cells. These data suggest that the kinase-proximal juxtamembrane region has an autoinhibitory role.

2. Materials and methods

2.1. Expression and purification of IGF1R cytoplasmic domain

PCR was used to amplify sequences encoding residues 930–1337 of human insulin like growth receptor (IGF1R). This includes the juxta-membrane (JM) region of the receptor, the kinase domain, and the C-terminal tail. The IGF1R DNA was subsequently cloned as a BamHI–KpnI fragment into plasmid pFastBac HTB (Life Technologies, Inc.). Site-directed mutagenesis was performed using the Strategene QuikChange kit according to the manufacturer’s instructions. Mutations were confirmed by DNA sequencing. Recombinant baculovirus was produced using the Bac-to-Bac system (Life Technologies, Inc.), and this was used to infect Spodoptera frugiperda (Sf9) cells.

For protein production, 600 ml of Sf9 cells were infected with high-titer recombinant IGF1R baculovirus. Cells were harvested after 72 h, at which point maximum IGF1R expression was observed. Cells were lysed in a French pressure cell in a lysis buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM β-mercaptoethanol, 5 mM EDTA, 1% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM PMSF, and 2 mM activated sodium orthovanadate. The lysate was then loaded onto a 5 ml column of Ni-NTA resin (Qiagen) that had been pre-equilibrated in buffer A (20 mM Tris–HCl, pH 8.5, 500 mM KCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 10% v/v glycerol). The column was washed with 10 column volumes of buffer A, followed by 2 column volumes of buffer B (20 mM Tris–HCl, pH 8.5, 1 M KCl, 5 mM β-mercaptoethanol, 10% v/v glycerol), then an additional 5 column volumes of buffer A. IGF1R cytoplasmic domain was eluted with buffer C (20 mM Tris–HCl, pH 8.5, 100 mM KCl, 100 mM imidazole, 5 mM β-mercaptoethanol, 10% v/v glycerol). Fractions containing IGF1R were pooled and stored in aliquots at –80 °C in 40% glycerol.

2.2. Kinetic experiments

The purified proteins were analyzed using a continuous spectropho-tometric assay [21,22]. All experiments were carried out at 30 °C in 50 μl of buffer containing 100 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 1.5 mM phosphoenolpyruvate, 20 U/ml pyruvate kinase, 230 U/ml lactate dehydrogenase, and 0.5 mM NADH. Initial rates were measured, and kinetic parameters were determined by non-linear regression analysis of the rates using the program MacCurveFit.

For determining values of Kₘ for peptide or ATP substrate with phosphorylated IGF1R, enzyme was phosphorylated with 5 mM ATP for 10 min at room temperature prior to being used in the spectrophotometric assay. For determinations of Kₘ for peptide, the synthetic peptide KKEEEEYMMMMG (E4YM4) was used in concentrations of 0–4000 μM. The concentration of ATP in these assays was 1 mM and the concentration of the phosphorylated proteins was 200 nM. For determinations of Kₘ for ATP, the peptide concentration was 1 mM, the enzyme concentration was 100 nM, and the concentrations of ATP were 0–6000 μM. Kinetic parameters were calculated by fitting data to the Michaelis–Menten equation.

For kinetic measurements with dephosphorylated IGF1R, proteins (2 μM) were treated with glutathione-agarose containing GST-YOP for 30 min at room temperature. This treatment was sufficient to completely dephosphorylate the receptors, as detected by anti-pTyr Western blotting (data not shown). The GST-YOP was then inactivated for 5 min with sodium orthovanadate. The beads were removed by centrifugation, and IGF1R was used in the spectrophotometric assay at a concentration of 200 nM. Initial studies on dephosphorylated wild-type and mutant forms of IGF1R with varying concentrations of peptide E4YM4 established that the Michaelis constants were in the millimolar range (≥2 mM). Thus, it was not possible to determine accurate Michaelis constants by using initial rate kinetics. The complete time course for the phosphorylation of peptide was measured by using peptide concentrations less than the Michaelis constant. We analyzed the data graphically as described [23] to determine Kₘ/Kₚ.

To analyze IGF1R autophosphorylation by Western blotting, the proteins were first dephosphorylated as described above. For removal of GST-YOP, the remaining solution was incubated with 100 mM Tris (pH 7.5), 19 mM MgCl₂, and 250 μM ATP in a total volume of 50 μl. Aliquots containing 100 ng protein were removed at various time points and analyzed by 7.5% SDS-PAGE. Proteins were transferred onto PVDF membranes and probed with rabbit polyclonal anti-IGF1R [pYpY1135/1136] antibody (Biosource International, Inc.), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (GE Healthcare). Detection was by ECL Western Blot Detection System (GE Healthcare). All blots were exposed together to a single piece of Kodak MR film to compare time courses of autophosphorylation. To analyze the stoichiometry of autophosphorylation, IGF1R proteins (2 μM) were incubated with 250 μM [γ-32P]-ATP for 15 min under similar conditions. The reactions were stopped by mixing with Laemmli Buffer and boiling, and analyzed by SDS-PAGE followed by staining with Coomassie blue. Bands were excised from the gel and dissolved in H₂O₂, and 32P incorporation was measured using a liquid scintillation counter.

2.3. Receptor activity in R-cells

Wild-type and mutant forms of IGF1R were expressed using plasmid pBPV.IGF1R (a gift of Dr. Derek LeRoith, NIH). R-cells (a gift of Dr. Rennato Baserga, Thomas Jefferson University) were grown to 50% confluency in DMEM plus 4500 mg/l glucose (Fisher/Cellgro), 10% heat inactivated fetal bovine serum (Sigma), 1X antibiotic/antimycotic (Gibco/Invitrogen), 50 μg/ml G418(Sigma). R-cells were transfected using TransIT polyamine transfection reagent (Mirus) according to the manufacturer’s instructions. After 24 h, the media was replaced with starvation media containing DMEM plus 1000 mg/l glucose, 1X antibiotic/antimycotic, 50 μg/ml G418, 0.5% NADH, Initial rates were measured, and kinetic parameters were determined by non-linear regression analysis of the rates using the program MacCurveFit.

For determining values of Kₘ for peptide or ATP substrate with phosphorylated IGF1R, enzyme was phosphorylated with 5 mM ATP for 10 min at room temperature prior to being used in the spectrophotometric assay. For determinations of Kₘ for peptide, the synthetic peptide KKEEEEYMMMMG (E4YM4) was used in concentrations of 0–4000 μM. The concentration of ATP in these assays was 1 mM and the concentration of the phosphorylated proteins was 200 nM. For determinations of Kₘ for ATP, the peptide concentration was 1 mM, the enzyme concentration was 100 nM, and the concentrations of ATP were 0–6000 μM. Kinetic parameters were calculated by fitting data to the Michaelis–Menten equation.

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3. Results

3.1. Mutagenesis of IGF1R juxtamembrane region

As shown in Fig. 1, there is significant homology between the juxtamembrane regions of IR and IGF1R, although the N-terminal portions are divergent and there is a 5-residue gap in IGF1R relative to IR. Of the residues that are conserved between the two receptors, four (N947, Y950, Y957, and D960; IGF1R numbering) are conserved across the entire IR/IGF1R family and in all species [14]. In IR, the residues corresponding to IGF1R Y943 and Y950 are sites of autophosphorylation; however, phosphorylation at these sites does not regulate kinase activity. The IR residue corresponding to Y943 is involved in receptor endocytosis [24], while the IR residue corresponding to Y950 is a docking site for IRS proteins [25], Shc [26], and STAT-5b [27]. A structure of IR has been solved that contains a portion of the JM region [20]. In the structure, residues in the C-terminal half of the JM region of IR (equivalent to V956, Y957, Y958, D960, W962, E963 of IGF1R) participate in interactions that could stabilize the autoinhibited state. The N-terminal half of the JM region was not included in the structural study and has not been explored in IR or IGF1R. There is also biochemical evidence for an autoinhibitory role for Tyr984 in IR (equivalent to Y957 of IGF1R) [20]. To probe the roles of the conserved residues, we produced the following site-directed mutants of IGF1R: N947A, Y950F, and Y957F. To study these mutations in the context of the entire cytoplasmic domain, we used the SP9/baculovirus system to express His-tagged versions of IGF1R containing the juxtamembrane, kinase, and C-terminal domains. To our knowledge, this is the first report on the expression and purification of a full cytoplasmic domain from an IGF1R/IR-family kinase. We purified the wild-type and mutant proteins to near-homogeneity using affinity chromatography (Fig. 2).

3.2. Autophosphorylation of wild-type and mutant IGF1R

As an initial comparison of the activity of the wild-type and mutant receptors, we carried out in vitro autophosphorylation reactions. To ensure that all proteins began the reaction in an equivalent, unphosphorylated state after purification, we treated the purified proteins with phosphatase. After removal of the phosphatase, we incubated the enzymes with ATP and followed the autophosphorylation reactions using a phosphospecific antibody that recognizes pY1135 and pY1136 in the IGF1R activation loop (Fig. 3). These experiments confirmed that all four proteins were capable of in vitro autophosphorylation under the conditions tested. The Y957F mutant showed a more rapid and robust signal for autophosphorylation than wild-type IGF1R or the other mutants (Fig. 3).

To confirm these results, we carried out experiments using a continuous spectrophotometric assay (Fig. 4). In this assay, the production of ADP is coupled to the oxidation of NADH, which is measured as a reduction in absorbance at 340 nm. The enzymes were incubated with ATP in the absence of any exogenous substrate. As we observed previously for the isolated catalytic domain of IGF1R [6], the progress curves were biphasic, because autophosphorylation activates IGF1R. Autophosphorylation of the Y957F and N947A mutants was more rapid than for wild-type IGF1R (Fig. 4). Autophosphorylation of the Y950F mutant was slower than for wild-type. We measured the stoichiometry of autophosphorylation after 15 min of reaction under similar conditions using 32P-labeled ATP. Wild-type IGF1R incorporated 1.5 mol phosphate per mol protein in this experiment, while the N947A mutant incorporated 1.8 mol/mol, Y950F incorporated 1.3 mol/mol, and Y957F incorporated 2.5 mol/mol, consistent with the results from the spectrophotometric assay.

3.3. Kinetic experiments

To test whether the activating effects of the Y957F and N947A mutations would also be manifested toward an exogenous substrate, we carried out steady-state kinetic experiments using receptors that were pre-phosphorylated by ATP. In these studies, we measured phosphorylation of a synthetic peptide substrate using the continuous spectrophotometric assay (Fig. 5). In experiments where the concentration of ATP or peptide was varied, the N947A and Y957F mutants showed higher activity than wild-type, and the Y950F mutant showed lower activity. These differences were primarily due to changes in $V_{\text{max}}$ between the mutants and wild-type; only modest effects were seen on the values of $K_m$ for ATP and peptide (Table 1). The values of $V_{\text{max}}$ for N947A and Y957F were approximately...
twofold higher than for wild-type, while the value for Y950F was approximately twofold lower than wild-type.

We also analyzed the kinetics of the unphosphorylated kinases. We treated the preparations with YOP phosphatase; preliminary experiments confirmed that these conditions were sufficient to completely dephosphorylate the enzymes (data not shown). After removal of YOP, we carried out kinetic experiments using the continuous spectrophotometric assay. As reported previously for the isolated kinase domain of IGF1R [6], the Michaelis constants for peptide and for ATP were much higher for dephosphorylated IGF1R than for phosphorylated IGF1R. Because we were unable to achieve substrate saturation in these experiments, we determined $k_{cat}/K_m$ values from complete enzyme progress curves [23] (Table 2). As expected, these values were $\approx 300$-fold lower than the $k_{cat}/K_m$ values for phosphorylated enzyme (Table 1). These experiments showed that the Y957F mutant had a 3-fold higher $k_{cat}/K_m$ than wild-type IGF1R, whereas the change for N947A was not significant (Table 2).

3.4. Receptor activity in intact cells

We carried out experiments to test the effects of the juxtamembrane mutations in the context of the full-length IGF1R. We conducted these experiments in R-cells, which are murine fibroblasts derived from IGF1R-deficient mice. We transfected R-cells with wild-type, Y950F, N947A, or Y957F IGF1R, starved the cells, then treated them with IGF1. We assayed receptor autophosphorylation by immunoprecipitation and Western blotting with anti-pY1135/pY1136 antibodies (Fig. 6). Both the wild-type and mutant receptors were activated by IGF1 treatment, indicating that the mutations did not disrupt the overall structure of the receptor. The Y957F juxtamembrane mutation resulted in a dramatic increase in both the basal and IGF1-stimulated levels of receptor autophosphorylation. The N947A mutation also increased the basal and IGF1-stimulated level of autophosphorylation, but not as strongly as Y957F. The Y950F mutation resulted in a low level of autophosphorylation in the absence of IGF1, and IGF1 treatment stimulated autophosphorylation to approximately the same degree as wild-type IGF1R. These data demonstrate that Y957 and N947 participate in autoinhibitory interactions in the context of the full-length IGF1 receptor.

4. Discussion

In this report, we present evidence that the juxtamembrane region of IGF1R plays a role in regulating the activity of the enzyme. Our in vitro and cellular data point to a particularly...
In previous studies of IR regulation, biochemical and structural studies on Y984 (the equivalent tyrosine to Y957 of IGF1R) showed that the residue was engaged in autoinhibitory interactions with the kinase catalytic domain [20]. Y984 of IR is not a site of autophosphorylation, and there is no evidence that Y957 of IGF1R is autophosphorylated. Thus, trans-autophosphorylation is not believed to play a role in JM-mediated autoinhibition in this enzyme family. The crystal structure of a JM-catalytic construct showed that Y984 interacts with residues in the N-terminal lobe of the catalytic domain to stabilize a low-activity conformation. Kinetic measurements showed that a Y984A mutant had a fourfold higher $k_{cat}$ than wild-type insulin receptor, and the Y984A mutation increased IR phosphorylation in cells. The increase in basal-state phosphorylation for the Y984 mutant suggested that juxtamembrane region interactions are important for maintaining an arrangement of the two receptor catalytic domains with low-trans-autophosphorylation activity. This arrangement is disrupted upon insulin binding to the extracellular alpha subunits.

The autoinhibitory role for Y957 of IGF1R is supported by the available structural data. Crystal structures of IGF1R have been described in the unphosphorylated (0P), bis-phosphorylated (2P), and triply-phosphorylated (3P) states [6,29,30]. In the IGF1R-0P and -2P structures, Y957 makes similar contacts with hydrophobic residues in the N-terminal lobe of the catalytic domain as seen in the IR juxtamembrane-catalytic structure. As noted previously by Li et al. [20], there is a subtle repositioning of the Y957 sidechain in IGF1R relative to the sidechain of Y984 in IR. Y957 of IGF1R is hydrogen-bonded to K1025 in the N-lobe, whereas Y984 of IR is hydrogen-bonded to E990. Despite this difference, the similarities in the biochemical data for the two mutant proteins (IR Y984A and IGF1R Y957F) suggest that these residues play a fundamentally similar role in the two receptors. The similar roles of IR Y984 and IGF1R Y957 suggest that the two residues might also participate in autoinhibitory interactions in the context of IR/IGF1R hybrid receptors, although this possibility has not yet been investigated. In the structures of the triply-phosphorylated forms of IR [31] and IGF1R [6], the juxtamembrane regions containing Y984/Y957 are disengaged from the kinase catalytic domain, consistent with the high catalytic activity seen in these forms of the receptors.

The juxtamembrane region amino acids beginning at N947 to the kinase catalytic domain. Our data also suggest a role for N947 in IGF1R regulation. The effects of the Y957F and N947A mutations were much more dramatic in cellular studies with the full-length receptor (Fig. 6) than in the in vitro studies on purified cytoplasmic domains (Figs. 3–5). A similar discrepancy was observed for the Y984F mutant form of IR [20]. This discrepancy is probably due to the additional steric constraints imposed on the cytoplasmic domain when it is assembled into the complete $\alpha_2\beta_2$ holoreceptor. For this reason, it is likely that the studies in R-cells (Fig. 6) show more accurately the level to which the JM region is involved in regulating IGF1R activity. In the case of the Y950F mutant, our in vitro experiments showed no evidence for the involvement of Y950 in IGF1R regulation. The cellular data showed a modest degree of basal autophosphorylation for the Y950F mutant, and a normal level of IGF1-stimulated autophosphorylation, as has been reported previously [28].

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autoinhibitory sequences in the JM region of IGFIR include Y957, but also extend distally from the catalytic domain towards the membrane. Broadly speaking, the data show that additional constraints exist (apart from the triple phosphorylation of the activation loop) that limit the basal enzyme activity of this receptor.

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References


