The carboxyl-terminal domain of insulin-like growth factor-I receptor interacts with the insulin receptor and activates its protein tyrosine kinase

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Abstract Receptors for insulin and insulin-like growth factor-I (IR and IGFIR) consisting of the $\alpha_2\beta_2$ structure are protein tyrosine kinases (PTKs). Carboxyl-terminal (CT) domains of their β subunits are structurally diverse while the PTK domains share the highest homology. Interactions between CT and PTK domains of IR and IGFIR were studied by means of PTK activity, fluorescence energy transfer or surface plasmon resonance using BIAcore. We present evidence that IGFIR CT directly interacts with both IGFIR and IR. Although binding to both receptors, stimulation of PTK activity only occurs with IR but not IGFIR.

Key words: Insulin-like growth factor-I receptor; Insulin receptor; Protein tyrosine kinase; Carboxyl-terminal domain; BIAcore; Domain interaction

1. Introduction

The insulin-like growth factor-I receptor (IGFIR) and the insulin receptor (IR) are biologically active macromolecules which are responsible for transducing signals of IGF-I and insulin, respectively [1,2]. Both receptors are tetrameric membrane glycoproteins consisting of two α and two β subunits which are disulfide-linked in a β-α-α-β form. The initial event of IGF-I and insulin action is binding to the extracellular α subunits of IGFIR or IR. This interaction activates the intracellular protein tyrosine kinase (PTK) activity of the receptor β subunit by conformational changes and autophosphorylation, and initiates post-receptor signal transduction in the cell that leads to changes in growth and metabolism.

The crystal structure of the IR PTK reported by Hubbard et al. [3] has provided information on the 3D structure of the IR PTK, yet some important structural elements are lacking. The crystal structure of IR PTK contained 306 amino acid residues and lacked the carboxyl-terminal (CT) 72 amino acids of the β subunit as well as 25 amino acids of the juxtamembrane domain. Although the overall homology of human IGFIR and IR CT domains is 44% [4], their structural features are significantly different. IGFIR CT and IR CT consist of 108 and 99 amino acids, respectively. IGFIR CT contains an unusually high content of Pro (12 mol%) and 3 Tyr residues while IR CT contains 7 Pro (7 mol%) and 2 Tyr residues. The different sequences in the CT domains suggest potential differences in their effects on PTK activity as well as signaling pathways that are utilized by the two receptors.

The potential involvement of the CT domains in differential signal transductions for insulin and IGFs has been proposed [5–12]. Initially, a number of studies used CT domain deletion mutants of IR [5–9]. While these results are somewhat controversial, studies utilizing cells expressing chimeric receptors provided more convincing information [10–12]. For example, insulin failed to stimulate pyruvate dehydrogenase (PDH) activity in cells expressing IR carrying IGFIR CT110 amino acids [10]. Replacement of IR CT with IGFIR CT in the IR severely affected insulin-stimulated responses [11]. In cells expressing a chimeric IGFIR having IR CT, stimulation of glycogen synthesis or mitogen-associated protein (MAP) kinase by IGF-I was similar to the level achieved by insulin-stimulated IR [12]. These data suggested that the CT domains of IR and IGFIR are not reciprocally interchangeable.

Amino- and carboxyl-terminal regions of many proteins are often highly susceptible to proteolytic digestion. Previously, we showed that the removal of the CT domain 76 amino acids from IR PTK resulted in much lower PTK activity and enhanced susceptibility to heat inactivation and proteolytic degradation than the intact IR PTK [13]. IR CT stimulated both the intact and CT-truncated forms of IR PTK 2.7- and 2.5-fold, respectively [14]. These results indicate that IR CT plays an important role in the activity and stability of IR PTK. Towards understanding the structure and function of the IGFIR CT, IGFIR CT was purified from a glutatione *S*-transferase (GST) fusion protein. In addition, we expressed and purified $(His)_6$-tag IR CT as well as $(His)_6$-tag IGFIR CT, and investigated whether CT domains of IGFIR and IR can interact with PTK domains of IR and IGFIR. We present evidence that IGFIR CT directly interacts with the PTK domains of both IGFIR and IR, leading to activation of IR PTK without significantly affecting the IGFIR PTK activity.

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Abbreviations: IR, insulin receptor; IGF, insulin-like growth factor; PTK, protein tyrosine kinase; CT, carboxyl-terminal; PDH, pyruvate dehydrogenase; MAP kinase, mitogen-activated protein kinase; PI, phosphatidylinositol; GST, glutathione *S*-transferase; IPTG, isopropylthio-β-D-galactoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; RU, resonance unit; NTA, *N*-(5-amino-1-carboxypentyl) iminodiacetic acid; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide; PDEA, 2-(2-pyridinyldithio)ethaneamine hydrochloride; DMF, *N*,*N*-dimethyl formamide

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2. Materials and methods

2.1. Expression and purification of the IGFIR CT

A DNA fragment corresponding to the CT domain (residues 1231–1337) [4] of IGFIR was amplified by PCR using 3′- and 5′-primers containing *Eco*RI or *Bam*HI restriction enzyme sites, respectively, to permit directional cloning. After digestion with restriction enzymes, the PCR product was inserted into the pGEX3X expression vector (Pharmacia), which allowed production of the inserted polypeptide as a fusion protein with GST. *Escherichia coli* strain JM109 was transformed with the plasmid, and the expression of the GST-CT fusion protein was confirmed by SDS-PAGE. IGFIR CT was then purified from overnight cultures of *E. coli* JM109/pGEX3X CT by the same method previously described [14] except that 30 μg of the restriction protease factor Xa (Boehringer Mannheim, Indianapolis, IN) (enzyme/substrate ratio of ∼1/100) was used for elution of the CT domain. Purified IGFIR CT was analyzed by SDS-PAGE, immunoblotting, amino acid composition, and 1D NMR.

2.2. Expression and purification of IGFIR CT and IR CT with $(His)_6$-tag

Overnight cultures (100 ml NZY medium) of *E. coli* DH52 transformed with pTrcHisB/IGFIR 1229–1337 [4] or pTrc/HisB/IR 1245–1330 [15] were diluted 10 times into 1 liter of fresh LB medium. *E. coli* DH52 transformants were grown for 1 h, and an additional 3 h at 37°C after the addition of isopropylthio-β-D-galactosine (IPTG) (0.1 and 1 mM, respectively), and harvested by centrifugation at 3000 rpm for 10 min at 4°C. Bacteria were then homogenized in 20 mM sodium phosphate, pH 7.8, containing 6 M guanidine-HCl and 0.5 M NaCl at 25°C for 10 min, followed by sonication 3 times for 5 s. The homogenates from 100 ml culture were applied onto a Ni^{2+}-*N*-(5-amino-1-carboxypentyl)-iminodiacetic acid (NTA)-agarose column (1 ml; Qiagen, Valencia, CA). The column was washed with 20 mM sodium phosphate, pH 6.0, containing 8 M urea and 0.5 M NaCl until all proteins were eluted as judged by $A_{280} < 0.01$, and then eluted with 20 mM sodium phosphate, pH 4.0, containing 8 M urea and 0.5 M NaCl. Purified $(His)_6$-tag IGFIR CT and $(His)_6$-tag IR CT were used only for BIAcore experiment 3.

2.3. Fluorescence labeling of IGFIR CT and measurement of emission spectra in the presence of increasing amounts of IR PTK

Purified IGFIR CT was labeled with E-99 (eosin-5-iodoacetamide, Molecular Probes, Eugene, OR) at the unique Cys residue located at the C-terminus. IGFIR CT (∼200 μg) in 0.9 ml of 0.5 M potassium phosphate, pH 8.0, containing 1 mM DTT and 18 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) was mixed with 100 μl of 16 mM E-99 dissolved in *N*,*N*-dimethyl formamide (DMF). Reaction was allowed to continue for 16 h at 4°C, and stopped by dialyzing against 50 mM Tris-HCl, pH 7.4, containing 1 mM DTT at 4°C. To further remove unreacted dye, the dialysate was applied to a Sephadex G-50 column (20 ml) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 1 mM DTT and 0.15 M NaCl at 4°C. The eluates were monitored by absorbance at 280 and 540 nm. Fractions with $A_{280/540} = \sim 6$ were combined. The labeled IGFIR CT (∼3 μM) in 500 μl was titrated with purified and concentrated Triton X-100-free IR PTK or BSA by successively adding 1, 4, 5, 10, and 15 μl of each protein solution (∼2 μg/100 μl). The emission spectra were recorded at 5 min after the addition of IR PTK or BSA by exciting the sample at 305 nM with a Hitachi F-2000 fluorescence spectrometer (Hitachi, Tokyo, Japan).

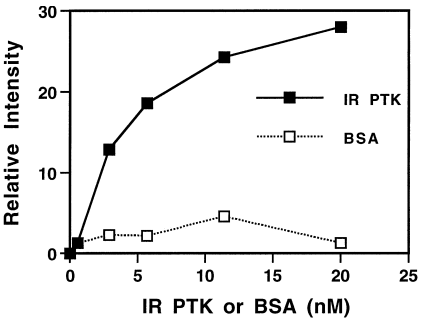

Fig. 1. Emission spectra of the eosin-labeled IGFIR CT in the presence of varying amounts of IR PTK (■) or BSA (□). Emission spectra were recorded each time 5 min after the flurescence-labeled IGFIR CT was mixed with indicated amounts of IR PTK or BSA (∼2 μg/100 μl). Relative fluorescence intensity changes at 540 nm are shown.

2.4. Measurement of binding interaction by surface plasmon resonance

For experiments 1 and 3, IR purified from Rat1HIR cells was immobilized using BIAcore Amine Coupling Kit (Biacore, Piscataway, NJ) with 10 mM sodium acetate, pH 5 [16]. Briefly, sensor chips were activated by 50 mM *N*-hydroxysuccimide (NHS)/200 mM *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide (EDC), coupled with IR (30 μl of 50 μg/ml), and then blocked by ethanol amine-HCl. To the IR-immobilized chips, analytes such as IGFIR CT (50 or 150 μg/ml), insulin (100 μg/ml), BSA (50 μg/ml), $(His)_6$-tag IGFIR CT and $(His)_6$-tag IR CT (50 or 150 μg/ml) were applied and sensorgrams recorded. For experiment 2, IGFIR CT was immobilized on a sensor chip by thiol-disulfide exchange at the unique C-terminal cysteine residue. Briefly, the chip was activated by NHS/EDC as above, and reactive disulfide groups were introduced onto the sensor chip surface by reaction with 80 mM 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) in 100 mM borate buffer, pH 8.5. The chip was then coupled with IGFIR CT (400 μg/ml in 10 mM sodium formate, pH 3.8), and blocked by 50 mM L-cysteine/1 M NaCl deactivating solution. To this chip, IR PTK (10 μg/ml), IR (50 μg/ml), insulin (50 μg/ml), and IGFIR PTK (5 μg/ml) were applied and sensorgrams recorded.

2.5. Other methods

IR PTK, IR PTKΔCT, and IGFIR PTK were expressed in insect cells and purified as previously described [5,6]. IR was purified from Triton X-100 solubilized Rat1HIR cells by sequential affinity chromatography on wheat germ agglutinin (WGA)- and insulin-Sepharose as described [17]. SDS-PAGE and immunoblotting were performed using 15% polyacrylamide gels as described [18]. PTK activity was measured using a poly(Glu,Tyr)(4:1) substrate as described [19].

3. Results

3.1. Purification of CT domains

Approximately 1 mg of IGFIR CT was purified by glutathione-Sepharose chromatography from a 1 liter culture. This IGFIR CT preparation was used for fluorescent-labeling and BIAcore experiments. For determination of the effect of

Table 1
Effects of IR CT and IGFIR CT on IR or IGFIR PTK activity

	Fold stimulation over basal		
	IGFIR CT	IR CT[a]	BSA
IGFIR PTK	0.83 ± 0.24 ($P > 0.1$, $n = 8$)	1.2 ± 0.3 ($P > 0.1$, $n = 11$)	1.03 ± 0.05 ($n = 3$)
IR PTK	1.8 ± 0.6 ($P < 0.05$, $n = 6$)	2.7 ± 1.0 ($P < 0.01$, $n = 7$)	0.98 ± 0.02 ($n = 4$)
IR PTKΔCT	3.7 ± 1.3 ($P = 0.1$, $n = 3$)	2.5 ± 0.4 ($P < 0.05$, $n = 3$)	0.92 ± 0.09 (n=2)

[a]The effects of IR CT on IGFIR PTK, IR PTK, and IR PTKΔCT have been published previously [6]. For the former two PTKs, however, additional PTK assays from this study are included.

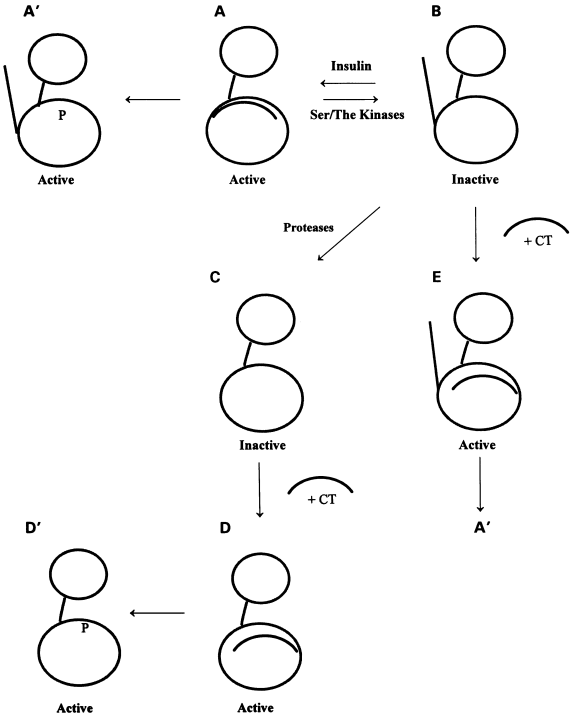

Fig. 2. Schematic presentation of a hypothesis for the structure-function relationship between IR PTK and its CT domain. A and B forms represent CT-bound and CT-open IR PTK, that are in equilibrium. Binding of insulin to the extracellular domain should shift the equilibrium, B→A, whereas Ser/Thr phosphorylation probably shift the equilibrium, A→B. The B form is very susceptible to proteases, and easily degraded to an inactive C form. Both B and C forms can be activated by binding of exogenously added CT domains (IR CT and IGFIR CT) to the IR PTK domain, B→E and C→D. Note that 'inactive' means very low PTK activity such as <10% PTK activity of the active form. A′ and D′ show constitutively activated IR PTK by autophosphorylation whereas A, D, and E are transient forms required for the autophosphorylation reaction.

IGFIR CT on PTK activity, it was further purified by Mono Q chromatography. $(His)_6$-tag IGFIR CT and $(His)_6$-tag IR CT, 360 and 320 μg, respectively, were purified by Ni^{2+}-NTA-agarose chromatography from 33 ml culture. All the purified CT domains were apparently pure as judged by SDS-PAGE and immunoblotting. IGFIR CT isolated from its GST fusion protein contained Gly-Ile at the N-terminal CT domain (1230–1337) due to the introduction of the *Bam*HI site for cloning. $(His)_6$-tag IGFIR CT contained 158 amino acids consisting of IGFIR CT (109 amino acids), $(His)_6$ and a restriction protease processing site at the N-terminus, and 16 amino acids at the C-terminus. $(His)_6$-tag IR CT contained 135 amino acids consisting of IR CT (86 amino acids) and other residues similar to the $(His)_6$-tag IGFIR CT construct. The $(His)_6$-tag IR CT lacked C-terminal 13 amino acids of the IR CT.

3.2. Effect of IGFIR CT on PTK activity

The effect of IGFIR CT on both IR and IGFIR PTK activities was examined together with the effect of IR CT. All the results are expressed as fold stimulation of the maximal effect within the tested range (Table 1). Both IGFIR CT and IR CT stimulated IR PTK and IR PTKΔCT. In contrast, IGFIR CT and IR CT showed slight inhibitory and stimulatory effects on IGFIR PTK, respectively. The observed effects of all tested CT domains on IGFIR PTK were, however, highly variable, and not statistically significant ($P > 0.1$).

3.3. Measurement of the interaction between the IGFIR CT and IR PTK

The purified fluorescence-labeled IGFIR CT was titrated with IR PTK or BSA. The emission spectra relative intensity increased after addition of increasing amounts of IR PTK (∼0.6–20 nM). No increase in the emission spectra was seen when BSA was added. The changes in emission spectra observed at 540 nm after the addition of IR PTK are shown

Table 2
Interactions of CT domains of IGFIR and IR β subunits with their PTKs as measured by surface plasmon resonance: summary of BIAcore experiments

A. Experiment 1		
Ligand on the chip	IR	RU (+2495)
Analyte 1	BSA	0
Analytes 2, 3, and 4	IGFIR CT	7.3 ± 1.2
Analyte 5	Insulin	21
B. Experiment 2		
Ligand on the chip	IGFIR CT	RU (+791)
Analyte 1	IR PTK	51
Analyte 2	IR	16
Analyte 3	Insulin	0
Analyte 4	IGFIR PTK	82
C. Experiment 3		
Ligand on the chip	IR	RU (+1492)
Analytes 1 and 2	IGFIR CT	4.5 ± 1.5
Analytes 3 and 4	$(His)_6$-tag IGFIR CT	18.5 ± 3.5
Analytes 5 and 6	$(His)_6$-tag IR CT	12 ± 3
Analyte 7	BSA	−3
Analyte 8	Insulin	50

Analyte proteins were successively passed though the sensor chips instead of washing the bound analyte off each time. This procedure appeared to be acceptable since even after all the analytes were passed through, only 0.8%, 17%, and 5% of the total available binding sites of immobilized proteins on the chips, for experiments 1, 2, and 3, respectively, were occupied. Increases in RU after the coupling of ligand on the chip are shown in parentheses, 2495, 791, and 1492 for experiments 1, 2, and 3, respectively.

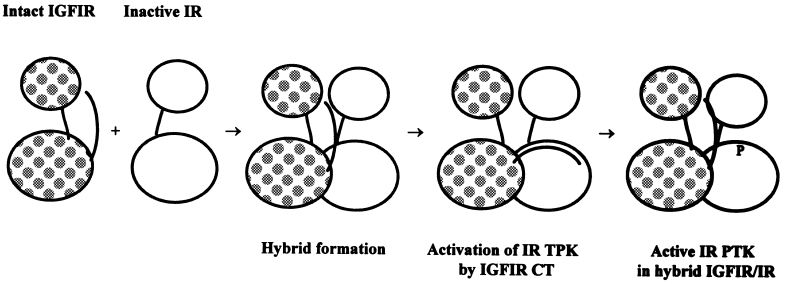

Fig. 3. Schematic presentation of a possible interaction of IGFIR CT with IR TPK in cell. Cytoplasmic domains of an intact IGFIR with its CT domain and an inactive IR without its CT domain are depicted (open and shaded, respectively). When IGFIR and IR hybrids are formed, IGFIR CT may be able to interact with the IR PTK domain, resulting in the activation of the IR PTK.

in Fig. 1. In this particular experiment, up to 12.4% increase in the relative fluorescence intensity at 540 nm was observed. In another experiment, an increase in the relative intensity was 15.9% of the fluorescence intensity measured in the absence of IR PTK. These changes are apparently induced as a result of the IGFIR CT specifically interacting with IR PTK domain. K_d values of 5 and 3.2 nM were obtained from two independent experiments.

3.4. BIAcore experiments

As a more direct method for detecting binding interactions between CT and PTK domains, we used a surface plasmon resonance detector (BIAcore). The results of BIAcore experiments are summarized in Table 2. In experiment 1, purified IR was immobilized to the sensor chip. A resonance unit (RU) increase of 2495 was detected. Analytes as listed were then successively applied. The difference in RU values before and after the addition of an analyte, which indicates a binding interaction, was measured for each analyte. The results, summarized in Table 2A, indicate that control BSA does not bind to IR whereas IGFIR CT and insulin both bind to IR. The RU values observed with IGFIR CT were consistently lower than that of insulin, suggesting low affinity and/or fast dissociation of the interaction between IGFIR CT and IR PTK. In experiment 2, IGFIR CT was immobilized on the chip, and analytes such as IR PTK and IGFIR PTK were passed through. The results show that IR PTK as well as IGFIR PTK bound to IGFIR CT whereas insulin did not bind to it (Table 2B). These results suggest that IGFIR CT binds to both IGFIR and IR PTKs. Experiment 3 was carried out to further confirm our observation that IGFIR CT binds to IR PTK, and also to examine whether IR CT binds to IR PTK. IGFIR CT, $(His)_6$-tag IGFIR CT, and $(His)_6$-tag IR CT were passed over the IR-immobilized chip. The results, shown in Table 2C, not only confirmed that IGFIR CT binds to IR, but also indicated that IR CT binds to IR. Positive and negative controls, insulin and BSA, yielded the expected results. Therefore, BIAcore experiments provided direct evidence that IGFIR CT binds to IR PTK as well as IGFIR PTK, and that IR CT binds to IR PTK as well.

4. Discussion

Our previous and present studies suggest that CT domains of IR and IGFIR modulate PTK activity differently. When the CT domains are exogenously added, IR CT stimulates IR PTK activity, but IGFIR CT does not appear to stimulate IGFIR PTK activity even though the binding interactions between IGFIR CT and PTK domains can be detected by BIAcore. IGFIR PTK was not significantly activated by IR CT, either. Interestingly, however, IGFIR CT stimulates IR PTK activity. BIAcore experiments suggest that IR PTK binds IR CT as well as IGFIR CT. Thus, it is most likely that the binding of the CT domains to the IR PTK domain results in stimulation of IR PTK activity. In contrast, IGFIR PTK activity does not appear to be affected significantly by either IGFIR CT or IR CT. In summary, stimulation of IGFIR PTK activity is distinct from that of IR PTK, and is unaffected by CT domains, suggesting a different mechanism for modulation of PTK activity.

IR and IR PTK contain an intact CT domain whereas IR PTKΔCT lacks the IR CT consisting of 76 amino acids [13]. It is of interest to note that both IR PTK with and without CT were activated by exogenously added IR CT or IGFIR CT, and that both IR PTKs with or without CT bind to the CT domains exogenously added. If the IR CT domain is tightly associated with the IR PTK domain in an intact IR molecule, IR PTK containing the CT domain should not bind exogenously added CT domains. The results of this study thus suggest that the IR CT domain in the IR molecule must be mobile as illustrated in Fig. 2A and B, CT-bound and CT-open forms, respectively, which exist in equilibrium. Only when IR PTK is in the B form (CT-open form), an exogenously added IR CT or IGFIR CT can directly interact with IR PTK domain (Fig. 2B→E). The CT domain has a flexible structure, which is most likely to cause susceptibility to proteolysis (Fig. 2B→C). We showed earlier that the β subunit of IR is extremely susceptible to proteolysis [20]. Consistent with this observation, the 3D structure of IR PTK published did not include the C-terminal 72 amino acids since the N and C termini of the crystallized IR PTK were chosen on the basis of proteolytic studies performed on the entire cytoplasmic domain of the β subunit [3].

Previously, we showed that the deletion of the C-terminal 76 amino acids from IR PTK impairs catalytic efficiency and stability of IR PTK, and suggested that the IR CT domain plays an important role in the activation and stabilization of IR PTK [13]. PTK activity of this CT-truncated form of IR PTK, as illustrated in Fig. 2C, was reduced to ∼10% of the intact IR PTK. This form can be activated by exogenously added CT domains (Fig. 2C→D→D′). The CT-open form can be activated by insulin (Fig. 2B→A→A′) or by exogenously added CT domains (Fig. 2B→E→A′). This model thus predicts that if exogenously added CT domains bind to

the PTK domain, partially inactive IR PTKs, the B and C forms, can be stimulated to an activity approaching that of the A form.

This model also predicts that insulin binding to the extracellular domain may cause a conformational change in the CT domain and therefore shift the equilibrium from B to A form. This accounts for the activation of IR PTK (Fig. 2A → A′). Consistent with this, Baron et al. suggested that insulin binding to IR induces conformational changes in the CT domain [21]. In addition, our model predicts that Ser/Thr phosphorylation may shift the equilibrium from A to B form, which is consistent with previous observations that phorbol ester-stimulated Ser/Thr phosphorylation results in the inhibition of insulin-stimulated PTK activity [22–24]. Excessive Ser/Thr phosphorylation of the IR has been proposed to be involved in the decreased IR PTK activity observed in many insulin-dependent diabetes patients with insulin resistance [25,26].

Finally, it is necessary to extend this in vitro structure-function study into a more physiological realm. Kole et al. very recently reported the role of a synthetic peptide derived from IR CT domain in the regulation of insulin signal transduction [27]. The IR CT peptide corresponding to 1293–1307 stimulated autophosphorylation of the IR β subunit, phosphatidylinositol (PI) kinase activity, and MAP kinase activity in CHO/HIRc cells in the presence of insulin. They also showed that the IR CT peptide was specifically cross-linked to IR and IR lacking 43 amino acids from the carboxyl terminus of the β subunit, but not to IGFIR or EGF receptor. These data suggested that this peptide binds specifically to the IR β subunit in a region other than the carboxyl terminal 43 amino acids. Their results are analogous to our findings including that IR CT binds to PTK domain. In addition, we showed that IR CT stimulates IR PTK activity whereas it does not have an effect on IGFIR PTK (Table 1). It is not known, however, whether the lack of IGFIR PTK stimulation by IR CT is due to the lack of binding between the two molecules or binding without functional stimulation. The study by Kole et al. [27] indicates that the latter may be correct. The major finding of the present study is that IGFIR CT also interacts with IR PTK and thereby stimulates its PTK activity. Although our reconstitution experiments did not directly demonstrate a possible physiological role, one can imagine that this interaction may play a significant role in the IGFIR/IR hybrid molecules which exist in cells expressing both receptors [18,28–30]. As illustrated in Fig. 3, an intact IGFIR could dimerize with an inactive CT-truncated IR. The IGFIR CT domain then could interact with IR PTK, which could result in the activation of IR PTK. Obviously, more experiments will be needed to prove this hypothesis.

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