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Solution structure of the transmembrane domain of the insulin receptor in detergent micelles

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ABSTRACT

The insulin receptor (IR) binds insulin and plays important roles in glucose homeostasis by regulating the tyrosine kinase activity at its C-terminus. Its transmembrane domain (TMD) is shown to be important for transferring conformational changes induced by insulin across the cell membrane to regulate kinase activity. In this study, a construct $IR_{940–988}$ containing the TMD was expressed and purified for structural studies. Its solution structure in dodecylphosphocholine (DPC) micelles was determined. The sequence containing residues L962 to Y976 of the TMD of the IR in micelles adopts a well-defined helical structure with a kink formed by glycine and proline residues present at its N-terminus, which might be important for its function. Paramagnetic relaxation enhancement (PRE) and relaxation experimental results suggest that residues following the TMD are flexible and expose to aqueous solution. Although purified $IR_{940–988}$ in micelles existed mainly as a monomeric form verified by gel filtration and relaxation analysis, cross-linking study suggests that it may form a dimer or oligomers under micelle conditions.

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1. Introduction

The insulin receptor (IR) belongs to receptor tyrosine kinase (RTK) family that contains single-pass transmembrane proteins and comprises 59 members in human [1,2]. RTK family protein interacts with its ligand to play critical roles in cellular signaling through regulating its kinase activity that phosphorylates specific tyrosine residues on other signaling proteins or itself [1,2]. The IR plays a critical role in regulation of glucose homeostasis through interacting with insulin [1,3]. Unlike other RTKs, the IR is a heterodimeric disulfide-linked protein composed of two α-subunits and β-subunits in the absence of its ligand [3]. Both α- and β-subunits are resulting from the cleavage of a same proreceptor. The IR is encoded by its gene and transcripted into IR-A and B isoforms with 12 amino acids difference in the α-subunit [3]. The α-subunits are located outside of the cell membrane and contain two leucine-rich repeats (L1 and L2), a cysteine rich region (CR), and two fibronectin type III domains (FnIII1-2) [3,4]. The FnIII-2 contains a 120 amino acids insert (ID) with a protease cleavage site to generate the α and β subunits [1]. The β-subunits contain two FnIIIs, a transmembrane domain (TMD) and a tyrosine kinase catalytic domain that can be activated through ligand binding to the α subunits [4].

The effect of insulin on the structure of its receptor has been studied for many years. The possible mechanism of insulin induced receptor activation is that insulin binding to the IR induces conformational changes that can be transmitted to the cytoplasmic domain to facilitate its phosphorylation. Phosphorylation on tyrosine residues in the cytoplasmic domains creates docking sites for molecular interaction with downstream signaling proteins or stimulates its catalytic activity [5,6]. Structural studies have been conducted for the domains of the IR using different techniques including X-ray crystallography, NMR spectroscopy and dark-field scanning transmission electron microscopy [4,7,8]. Although structural study of the IR ectodomain is challenging due to its large size, flexibility, glycosylation and existence of disulphide bonds, structural studies have shown the structural basis for its interaction with insulin [4,7,9–12]. Structural basis for the two surfaces of insulin interacting with the IR domains has been provided by several studies [10,13,14]. It is evident that insulin binding to its receptor causes structural changes, but the precise effect of the conformational changes on the kinase domain still remains unresolved [6].

The TMD of the insulin receptor is within the β-subunit and contains 23 amino acids [15]. Earlier studies suggested that modification in the TMD could alter receptor internalization and insulin signaling [16,17]. Mutations in the TMD were shown to have effects on receptor biosynthetic processing and kinase activation [18]. Substitution of the entire TMD of the IR with the TMD of c-neu/erbB2 resulted in constitutive kinase activation in vitro, while replacing the TMD with that of glycophorin A inhibited insulin action [15]. Although the TMD of IR does not interact with insulin directly, studies have suggested that the TM–TM interactions may affect the activation of the kinase domain of the receptor [15].

The structure of the TMD of the IR was predicted to be an α-helix. There is no structure of this domain that has been reported so far. In

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this study, we purified a construct $IR_{940–988}$ containing the TMD and conducted structural studies in detergent micelles using NMR spectroscopy. Our results show that the TMD contains a helix and a kink when it is purified in DPC micelles. The residues 942–948 preceding the TMD have a propensity to be a short helix and may interact with membrane. Our cross-linking study suggested that it may form a dimer or oligomers under micelle conditions.

2. Materials and methods

2.1. Materials

The DNA polymerase, restriction enzyme for molecular cloning was purchased from New England Biolabs. The pET-29b plasmids were purchased from Merck. The SDS-PAGE system, NuPAGE® gels, DH5α competent cells and SDS-PAGE molecular weight standard were purchased from Invitrogen and Bio-rad. The Bl21 (DE3) competent cells for protein expression were purchased from StrataGen. β-D-1-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) and detergents were purchased from Anatrace or Avanti. The $^{15}NH_4Cl$, ^{13}C-glucose and D_2O were purchased from Cambridge Isotope. All other chemicals were purchased from Sigma.

2.2. Expression and purification of TMD of IR

The cDNA encoding residues Thr940 to Leu988 ($IR_{940–988}$) of human IR were synthesized (Genscript). Sequence number is based on the sequence from the protein knowledgebase (www.uniprot.org) with access number P06213. The cDNA was cloned into the NdeI and XhoI sites of pET29b to generate a plasmid-pET29-IRTM encoding a protein sequence with extra residues (EHHHHHH) at its C-terminus to aid protein purification. The resulting plasmid was transformed in *Escherichia coli* (*E. coli*) competent cells and plated onto LB plates containing kanamycin. Two to three colonies were picked up and inoculated in 10 ml of M9 medium and cultured at 37 °C overnight. The overnight culture was then inoculated into 1 l M9 medium with antibiotics. When OD_{600} reaches 0.6–1.0, protein was induced for 12 h at 37 °C by adding IPTG to 1 mM. Isotope-labeled proteins were also induced using the similar method except using labeled carbon and nitrogen sources [19]. The *E. coli* cells were harvested by centrifuging at 11,000 ×g for 10 min at 4 °C, and the cell pellets were re-suspended into a lysis buffer (20 mM Tris-HCl, pH 7.8, 300 mM NaCl, and 2 mM β-mercaptoethanol) and were then broken up by sonication on ice. The cell lysate was cleared by centrifugation at 20,000 ×g for 20 min. The pellet was washed with the lysis buffer one time and collected with centrifugation to obtain inclusion bodies. Inclusion bodies were solubilized in a urea buffer (8 M urea, 300 mM NaCl, 7 mM SDS, 20 mM Tris-HCl, pH7.8) by rotating at room temperature for 2–10 h [20,21]. The solution was then cleared by centrifugation at 48,000 ×g for 20 min at room temperature. The supernatant was loaded in a gravity column containing nitrilotriacetic acid saturated with nickel (Ni^{2+}-NTA) resin. Next, the resin was washed with 10 column volumes of urea buffer. Urea was then removed by washing resin with 10 column volumes of lysis buffer with 7 mM SDS and 20 mM imidazole. Protein was eluted with an elution buffer (300 mM imidazole, pH6.5, 2–20 mM detergent) after washing resin with 10 column volumes of washing buffer (lysis buffer with 2–20 mM detergent). To prepare an NMR sample that is selectively labeled with a ^{15}N-labeled amino acid, the Bl21 (DE3) *E. coli* cells were first inoculated into a M9 medium that contained 0.5 g/l NH_4Cl. When IPTG was added into the medium to induce the protein, the ^{15}N-labeled amino (0.1 g/L) and 19 unlabeled amino acids (0.1 g/l) were added at the same time [19]. Protein was then purified as aforementioned for acquiring a ^1H-^{15}N-HSQC spectrum. To prepare an NMR sample, imidazole was removed by using a PD10 column or gel filtration chromatography. Sample was concentrated and put into an NMR tube for data acquisition.

2.3. Gel filtration experiment

Purified protein using Ni^{2+}-NTA resin was concentrated and loaded on a superdex™ 200 10/300 GL column that was pre-equilibrated with a gel filtration buffer (20 mM sodium phosphate, 8 mM dodecylphosphocholine (DPC) and 1 mM DTT). The flow rate was 0.5 ml/min and experiment was conducted at room temperature. The absorbance at 280 nm was monitored continuously and fractions were collected for analysis.

2.4. NMR experiments

NMR samples (0.3 mM) were prepared in 20 mM sodium phosphate buffer, pH 6.5, 1 mM DTT and (100–200 mM) detergent. All NMR spectra were recorded at 313 K on Bruker Avance 600 and Avance 700 spectrometers with cryogenic triple resonance probes [22]. All the pulse programs were from Topspin 2.1 program library. Data were processed with NMRPipe [23] and analyzed using NMRView [24]. Backbone resonances assignment was obtained using 2D-^1H-^{15}N-HSQC, 3D HNCACB, HNCA, HN(CO)CA, HN(CO)CACB, HNCO and HBHACONH experiments. Secondary structure was identified by analysis of ^{13}C secondary chemical shift [25] and TALOS+[26]. Distance constraints were collected from a 3D ^{15}N-edited NOESY (mixing time = 100 ms) experiment using a ^{13}C and ^{15}N-labeled sample under aforementioned conditions. Paramagnetic relaxation enhancement (PRE) experiment was conducted using ^{15}N-labeled samples. $IR_{940–988}$ was prepared at 0.3 mM concentration in the buffer containing 20 mM sodium phosphate, pH6.5, 1 mM DTT, and 120 mM DPC. The 16-doxyl stearic acid (16DSA) was dissolved in d4-methanol to make a 50 mM stock solution. The ^1H-^{15}N-HSQC spectra of $IR_{940–988}$ in the absence and presence of different concentrations of 16DSA were collected. The peak intensities were obtained using NMRview [24]. The ratio of peak intensities before and after adding 16DSA was plotted against residue number.

2.5. Structure determination

The backbone dihedral angle restraints were generated using TALOS+[26]. The NOEs peaks were picked manually and calibrated using NMRview [24]. The nuclear Overhauser effect (NOEs) peak intensity was converted to distance restraints. H–D exchange experiment was conducted by adding D_2O into lyophilized IR in DPC micelles. ^1H-^{15}N-HSQC was recorded immediately when the sample was dissolved in D_2O. The peaks appeared in the HSQC spectrum were used as hydrogen bond restraints. The upper and lower distances used in hydrogen bond restraints were 2.8 and 1.8 Å, respectively. Structure determination was carried out using XPLORE-NIH [27–29]. Structure determination was carried out using a randomized template. Simulated annealing was performed and energy minimization was carried out as previously described [30]. Simulated annealing was carried out with starting temperature of 3000 K and 50, 000 cooling steps. The structure was energy-minimized with 250 steps of Powell energy minimization. Fifty structures were obtained and twenty of them with lowest energies were selected and deposited in protein data bank with access number 2MFR.

2.6. Relaxation analysis

T_1, T_2 and {^1H}-^{15}N steady-state NOE experiments [31] were measured at 313 K using a ^{15}N-labeled sample on a Bruker Avance II 600 MHz spectrometer. For T_1 measurement, the relaxation delays of 5, 40, 80, 130, 330, 470, 630, 800, 900, 1000, 1200, 1400, 1600 and 1800 ms were recorded. For T_2 measurement, the data were acquired with delays of 16.9, 34, 51, 68, 85, 102, 119, 136 and 153 ms. Steady-state {^1H}-^{15}N NOEs were obtained using two datasets that were collected with and without initial proton saturation for a period of 3 s [32].

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