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3D Structure of the Juxtamembrane Domain of the Human Epidermal Growth Factor Receptor and Its Interaction with Calmodulin by NMR Spectroscopy

Kiattawee Choowongkomon[1,2]*, Cathleen Carlin[2] and Frank D. Sönnichsen[2]

ABSTRACT

Epidermal growth factor receptor (EGFR) is involved in the regulation of cellular proliferation and differentiation. Its juxtamembrane domain (JX), plays important roles in receptor trafficking since both basolateral sorting in polarized epithelial cells and lysosomal sorting signals are identified in this region. In order to understand the regulation of these signals, we characterized the structural properties of recombinant JX domain in dodecylphosphocholine detergent (DPC) by nuclear magnetic resonance (NMR) spectroscopy. In DPC micelles, structures derived from NMR data showed three amphipathic, helical segments. Furthermore, JX domain showed to interact with calmodulin by NMR techniques. Two binding sites of calmodulin on JX domains were identified. Our data suggested that the activity of sorting signals may be regulated by their membrane association and calmodulin binding which imply the restricted accessibility in the intact receptor.

Key word: epidermal growth factor receptor, juxtamembrane, basolateral, lysosomal calmodulin

INTRODUCTION

The juxtamembrane domain (JX) of EGFR, located between the transmembrane and kinase domains, plays important roles in receptor trafficking. Three important sorting signals are identified in the JX domain. Two sorting signals, a 667-P*XX*P-motif and a 658-LL-motif, are responsible for basolateral sorting in polarized epithelial cells, and a 679-LL-motif targets the ligand activated receptor for lysosomal degradation. The critical residues of a dominant basolateral sorting signal include a positively charged amino terminal residue (Arg662) and a proline-rich core (667-P*XX*P) (He *et al.*, 2002), a motif that bears some similarity with SH3-domain binding motifs. The signal is sufficient to target receptor released at the Trans Golgi network directly to the basolateral plasma membrane, and to recycle endosomal receptor back to basolateral membrane. A second, basolateral sorting motif has been localized to positions 658/659. This signal appears functionally redundant, being active only when the 667-P*XX*P signal is deleted or mutated (He *et al*., 2002). Possibly, as phosphorylation of Thr654 of the activated receptor has been shown to enhance recycling, this feedback mechanism might also invoke the basolateral targeting signals (Bao *et al.*, 2000). A lysosomal sorting signal formed by two leucine residues is present in the

[1] Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[2] Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Ohio, USA
* Corresponding author, e-mail: fsciktc@ku.ac.th

JX domain at residues 679 and 680 (679-LL) (Kil *et al.*, 1999; Kil and Carlin, 2000). As receptors with an inactive 679-LL signal accumulate on the limiting membranes of multivesicular endosomes in ligand-stimulated cells, 679-LL appears to be required for uptake to internal vesicles thereby enabling their delivery to lysosomes (Kil and Carlin, 2000).

Focus of this study is structural characterization of the entire JX domain of EGFR. This region has received less attention so far, partially due to its relatively small extent, the weak secondary structure predictions, and only the recent emergence of functional insight into this domain. Several regulatory protein-protein interaction motifs have now been located to this domain, and our interest focuses initially on the conformations of basolateral and lysosomal sorting signals, and the understanding of how sorting motifs may be regulated and activated. In this study, we characterized properties of the entire JX domain (residues 645-697). Utilizing isotopically labeled protein, heteronuclear NMR spectroscopy provided details on the conformational properties of this region in aqueous, and particularly in DPC-micellar solution, and facilitated the generation of a structural model of the micelle-bound domain.

MATERIALS AND METHODS

EGF receptor sequences encoding amino acid residues Met^{644} to Gly^{697} were expressed in *E. coli* strain BL21 as the $^{15}N/^{13}C$ double-labeled peptide. 1.5 mM peptide with 90 mM DPC detergent was dissolved in 500 µl of H_2O containing 10% D_2O by volume and adjusted to pH 5.0. The assignment of the backbone resonances of EGFR645-697 peptide in DPC micelles was achieved with standard triple-resonance methods and the following set of triple-resonance experiments at both 25 and 35 °C at 500 MHz: HNCO, HN(CA)CO, HNCA, HNCACB, and CBCA(CO)NH. The assignment of the side-chain resonances were accomplished with HCCH-TOCSY, HC-C(CO)NH, and CC(CO)NH experiments at 35 °C on 500 MHz. 3D-$^{15}N/^{13}C$-HSQC-NOESY experiments (80-ms mixing times) were recorded at both 25 °C on 600 MHz and 35 °C on 800 MHz. Residual Dipolar Coupling (RDC) Measurements were done by weakly aligned peptide in a 7% acrylamide gel. All NMR data were processed using software package NMRPipe and transferred into the program NMRView for assignment and data evaluation. For structure calculation, 1,146 distance restraints (396 intraresidue, 453 sequential, and 297 medium ranges), 55 dihedral angle restraints from both TALOS and $^3J_{HN-N}$ coupling constants, and 27 N-H RDC restraints were used as experimental input for simulated annealing calculations using the software package CNS. 46 of 100 calculated structures were taken to represent the NMR structural ensemble, with non-converged structures being identified using final force-field energies and significant violations of covalent restraints as selection criteria. The structures were analyzed by PROCHECK-NMR, visualized with MOLMOL. The DPC-bound and water-accessible residues of EGFR645-697 in DPC micelles were determined by measuring the effects of $MnCl_2$ and 5-doxyl-stearic acid on the intensities of cross-peaks in [^1H-^{15}N]-HSQC spectra.

The CaM gene was cloned from rat brain cDNA and purified as described by Hayashi *et al.* (Hayashi *et al.*, 1998). In the case of labeled CaM, the bacteria were grown in minimal media supplemented with trace elements as described by Oxenoid *et al.* (2001). NMR experiments were carried out on Varian INOVA 500 and 600 MHz NMR spectrometers. Lyophilized peptide samples were dissolved in 500 µl of 50 mM sodium acetate, pH 5.0 containing 10% D_2O by volume to yield a sample concentration of 1.0 to 1.5 mM. The 2D ^1H-^{15}N-HSQC spectra were recorded at 25 and 35°C. Interaction studies were done by titrating ^{15}N-labeled samples with proper amounts of

concentrated unlabeled samples. The titrations were stopped after reaching a 3:1 molar ratio of unlabeled peptide to labeled peptide.

RESULTS AND DISCUSION

Structure of JX domain in DPC micelles

Utilizing isotopically labeled protein, heteronuclear NMR spectroscopy provided details on the conformational properties of this region. In DPC micelles, structures derived from NMR data showed three amphipathic, helical segments: Arg652 to Arg662, Asn676 to Glu685, and Phe688 to Leu694. Models were determined for the average JX conformation using restraints representing the translational restriction due to micelle-surface adsorption, and the helix orientations determined from residual dipolar couplings and spin-label experiments. Two equivalent average structural models were obtained that differed only in the relative orientation between the first and second helices. In these models, the lysosomal sorting motifs were located in the second helices and faced the micelle surface, whereas the basolateral sorting motif was located in a flexible helix-connecting region. This result suggests that the activity of these signals is regulated by their membrane association and restricted accessibility in the intact receptor. EGFR is found as monomers, homodimers, and heterodimers depending on its activation state. The majority of unactivated receptor is monomeric, only slowly internalizing and recycling to the basolateral surface in polarized cells. Upon binding of ligands to extracellular domain, the EGFR dimerizes either into homo- or heterodimers, and is rapidly internalized to early endosome. Since the JX domain in all of our experimental conditions was always a monomer, our model was most likely to represent the structure of JX domain in the un-activated monomer EGFR. The constitutively active recycling of the un-activated EGFR to the basolateral surface correlates with our model, in that only P*XX*P- basolateral signal was accessible whereas lysosomal signal was sterically obstructed from their sorting machineries. This suggested that membrane binding and steric accessibility modulate the activity of the sorting signals.

Interaction with Calmodulin

We identified the CaM-binding residues using NMR chemical shift perturbation. These interaction studies were performed by titrating unlabeled CaM into ^{15}N-labeled EGFR645-697. An overlay of ^{1}H-^{15}N HSQC spectra of EGFR645-697 in the absence and presence of CaM are shown in Figure 3. The differences in peak positions and intensity in both HSQC spectra indicate binding of CaM to EGFR645-697. In the HSQC spectra of EGFR645-697 with CaM, roughly half of the crosspeaks were not be observed as they were broadened beyond detection. The remaining crosspeaks did not largely change in intensity or chemical shifts, which indicate that binding of CaM occurred in regions exhibiting broadened crosspeaks. The broadening of CaM binding residues prevented us from obtaining further structural information of the CaM-binding EGFR645-697; however, the broadened residues could be grouped into two regions of the primary seqeunce: between residues Thr654 and Glu666 and between residues Leu680 and Leu694. The first binding region was the region predicted by Martin-Nieto *et al.* (1998) as CaM-binding region is rich in basic and hydrophobic residues. The second region of CaM binding has never been reported in the literature. It is however predicted to be a putative calmodulin binding site according to the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html). The interaction between the JX domain and CaM was also confirmed by using ^{15}N-labeled CaM being titrated with unlabeled EGFR645-672 or EGFR645-697 (Figure 4). The

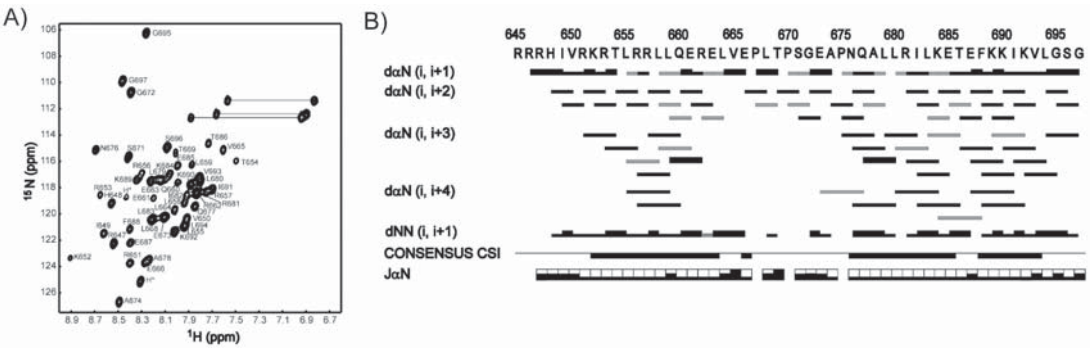

Figure 1 NMR spectra and data summary of the JX domain. A) ^{1}H -^{15}N-HSQC NMR spectra in the presence of DPC, pH 5.0, 35°C. Spectral cross peaks are labeled by residue and sequence number. H* indicates cross-peaks of the C-terminal His-tag residues. B) Summary of the secondary structure indicatives of JX in DPC micelles, pH 5.0, 35°C. Unambiguously assigned distance constraints are represented as black bars with height indicating the strength of nuclear Overhauser Effect (NOE). Grey bars indicate ambiguous sequential constraints. The H_α chemical shift indexes (CSI) in aqueous solution and the consensus (H_α, C_α, C_β, and CO) CSI in DPC micelles are shown. A positive value is indicated by an upward box and a negative index by a downward box. Ja_N coupling constants are presented as one-third filled box represents for J < 6 Hz, as two-third filled box for 6 Hz < J < 8 Hz, and a fully filled box for J > 8 Hz.

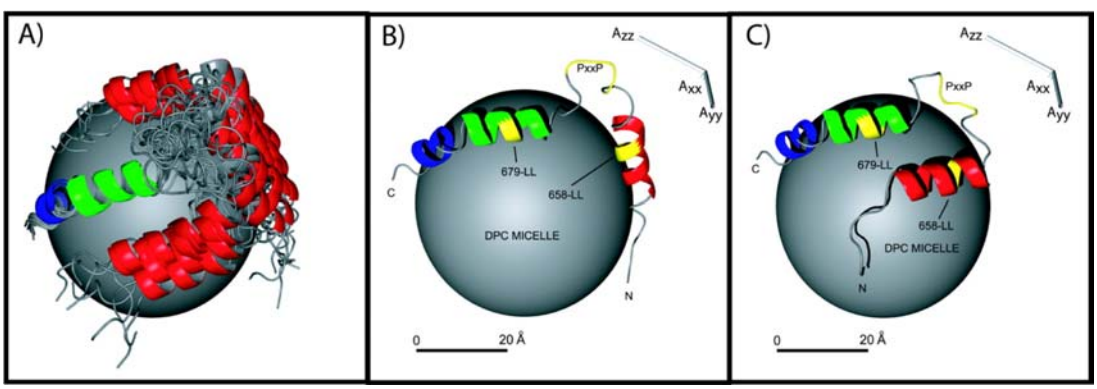

Figure 2 Ribbon presentation of the structural model of the juxtamembrane domain of EGFR adsorbed to the surface of a spherical DPC micelle. *A*, ensemble of 25 calculated JX structures, obtained by including steric restraints for micelle size and helix surface adsorption face in CNS calculations. Structures are superimposed using backbone atoms of helix 2 (*gray*) and helix 3 (*dark*). Selected individual structures representing the two observed groups, in which helix 1 is oriented either nearly perpendicular (*B*) or parallel (*C*) relative to helices 2/3. The common alignment tensor described by the residual dipolar couplings is indicated. The letters *PXXP* highlight the location of the dominant basolateral sorting signal in a flexible loop. The two dileucine signals are located in the hydrophobic, membrane associated faces of helices 1 and 2, with the location of their backbone being highlighted in *white*. (PDB access code 1Z9I)

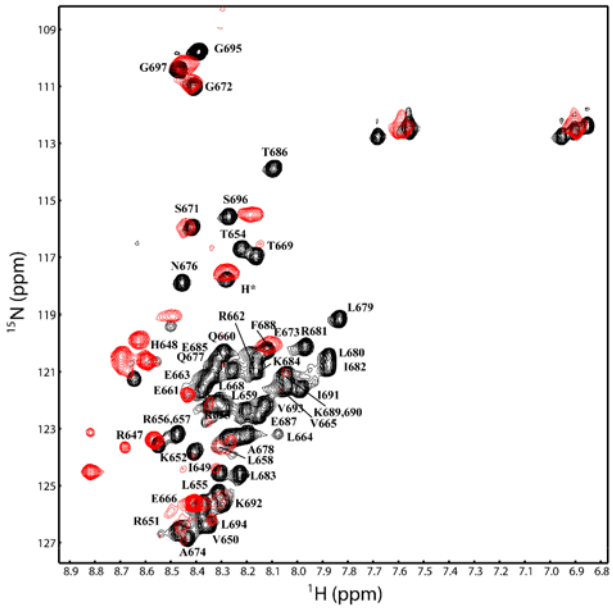

Figure 3 Overlay of ^{1}H-^{15}N-HSQC spectra of ^{15}N-EGFR645-697 without (black) and with (gray) calmodulin (molar ratio is 3:1 = CaM:EGFR) in 50 mM sodium phosphate buffer, pH 5.0. The crosspeaks of EGFR645-697 without CaM are labeled.

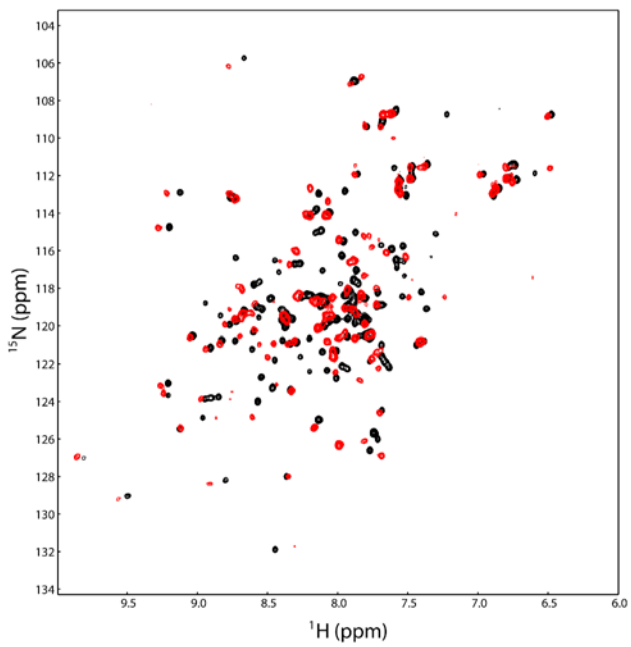

Figure 4 Overlay of ^{1}H-^{15}N-HSQC spectra of ^{15}N-CaM (black) and in presence of EGFR645-672 (blue) and EGFR645-697 (red) (molar ratio is 3:1 = CaM:EGFR) in sodium phosphate buffer, pH 5.0.

differences of intensity and position of crosspeaks in ^{1}H-^{15}N-HSQC spectra of both ^{15}N-CaM with EGFR645-672 and ^{15}N-CaM with EGFR645-697 (in the same molar ratios of labeled and unlabeled proteins, 1:3, and buffer conditions) indicated the different binding modes of both peptides to CaM. Since there are two CaM binding sites in EGFR645-697 while there is only one CaM binding site in EGFR645-672, the different binding modes of both peptides confirmed our two binding sies found in EGFR645-697.

The binding of CaM to the EGFR JX domain has shown to be a weak binding event. It has been determined to have a dissociation rate constant (K_d) of about 0.2-0.3 μM (Li and Villalobo, 2002). Crosspeaks were observed to broaden in the complex between the EGFR645-697 and CaM, which likely originates from intermediate exchanges between free and bound forms on the NMR time scale. Furthermore, conformational heterogeneity of the complex may also play a role in this broadening. Since the JX peptide has two binding motifs for CaM, several possibilities of one-to-one JX/CaM complexes or even larger complexes may result in the broadening of crosspeaks. For structural studies, this heterogeneity may be avoided by separation of two binding motifs into two peptides, each with a single protein binding motif.

CONCLUSION

Our NMR suggested that in inactive monomer form, the dominant basolateral sorting signal RxxxxPxxP of EGFR645-697 peptide in presence of DPC micelles was flexible whereas both dileucine motifs of basolateral sorting and lysosomal sorting were in helical segments. The dominant sorting signal stays accessible whereas dileucine motif may be not accessible. The data suggested that the role of these signals may be regulated by their membrane association and their accessibility in the intact receptor. Furthermore, two binding sites of calmodulin on JX domains of EGFR were identified by NMR techniques. Our data suggested that the activity of sorting signals may be regulated by their membrane association and calmodulin binding which imply the restricted accessibility in the intact receptor.

The atomic coordinates (code 1Z9I) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

Complete chemical shift data and tables of J coupling constants and RDC constants have been deposited in the BioMagResBank databank under accession number 6579 [BMRB].

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