



## NMR and Circular Dichroism Studies on Human CD99 Transmembrane Domain

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Received February 15, 2003

**Abstract** : Human CD99 is a ubiquitous 32-kDa transmembrane protein encoded by *mic2* gene. Recently it has been reported that expression of a splice variant of CD99 transmembrane protein (*Type I* and *Type II*) increases invasive ability of human breast cancer cells. To understand structural basis for cellular functions of CD99 *Type I*, we have initiated studies on *hCD99*<sup>TMcytol</sup> using circular dichroism (CD) and multi-dimensional NMR spectroscopy. CD spectrum of *hCD99*<sup>TMcytol</sup> in the presence of 200mM DPC and CHAPS displayed an existence  $\alpha$ -helical conformation, showing that it could form an  $\alpha$ -helix under membrane environments. In addition, we have found that the cytoplasmic domain of CD99 would form symmetric dimer in the presence of transmembrane domain. Although it has been rarely figured out the correlation between structure and functional mechanism of *hCD99*<sup>TMcytol</sup>, the dimerization or oligomerization would play an important role in its biological function.

### INTRODUCTION

Human CD99 is a ubiquitous 32-kDa transmembrane protein encoded by *mic2* gene<sup>1-5</sup>. The major cellular functions of CD99 protein are related to homotypic cell adhesion<sup>6</sup>, apoptosis<sup>7</sup>, vesicular protein transport<sup>8</sup>, and differentiation of thymocytes or T cells<sup>9</sup>. From a pathological point, the down-regulation of CD99 is a primary requirement for the generation of Reed-Sternberg (H-RS) cells seen in Hodgkin's disease<sup>11</sup>. Recently several studies suggest some fragmentary information about intracellular signal transduction pathway of

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CD99 with its interacting proteins. For instance, the induction of homotypic aggregation of Jurkat T cells by CD99 activation is mediated by protein kinases including MAP kinases, protein tyrosine kinases, and protein kinase C<sup>12, 13</sup>.

Another special feature of CD99 is that *mic2* gene encodes two types of proteins, which are induced by alternative mRNA splicing of CD99<sup>14</sup>. Two isoforms of CD99 exist as a major form (CD99 *type I*) corresponding to the full-length and a minor form (CD99 *type II*) truncated the intracytoplasmic domain (Fig. 1).

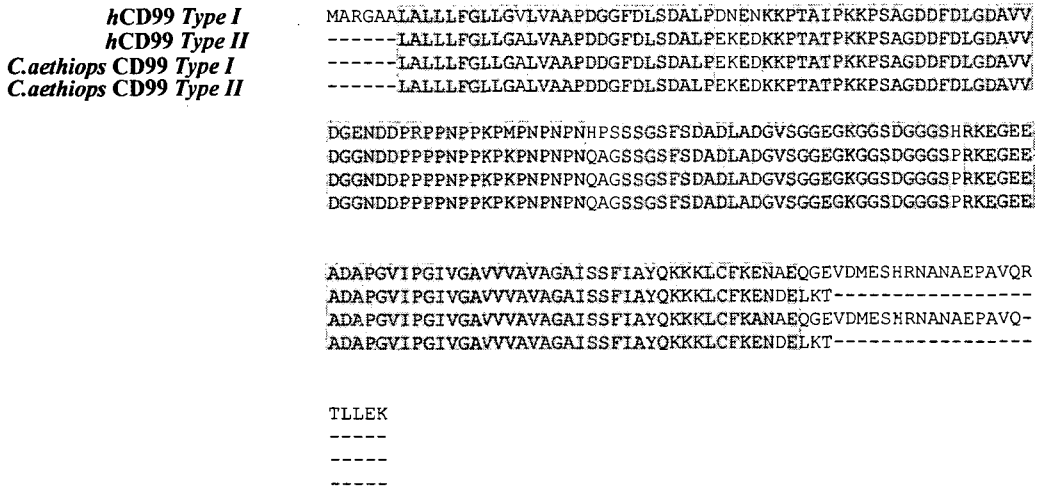


Fig. 1. Alignment of CD99 *Type I* and *Type II*. Identical residues are boxed.

Remarkably, the major form induced homotypic adhesion of the human B lymphoblastoid cell line IM-9, whereas the minor form inhibited the adhesion process. The two forms of CD99 are differentially expressed in most human cells tested and it has been highly conserved in monkey.

These observations suggest that the two forms of CD99 function as positive and negative regulators of LFA-1-mediated adhesion of lymphocytes during an immune response<sup>14</sup> *in vivo*. The latest report shows that expression of the major form in a CD99-deficient Jurkat T cell line is sufficient to promote CD99-induced cell adhesion, whereas co-expression of the two isoforms is required to trigger T-cell death<sup>15</sup>. A lot of previous data have possibly demonstrated the CD99 molecule as a component of signal transduction pathway. To determine the structural and functional relationship, we have initiated structural studies on *hCD99*<sup>TM<sub>cytoI</sub></sup> using circular dichroism (CD) and multi-dimensional NMR spectroscopy.

## MATERIAL AND METHODS

### ***Expression of the GST-fused hCD99<sup>TMcytol</sup>***

The DNA sequence encoding hCD99<sup>TMcytol</sup> (residues from 123 to 185) was cloned into a pGEX 4T-1 (Novagen) plasmid for glutathione-S-transferase (GST)-fused hCD99<sup>TMcytol</sup>. For overexpression, this plasmid was transformed into *Escherichia coli* strain BL21 (DE3) pLysS.

### ***Overexpression, isotope labeling and purification***

A transformed cell was grown in a M9 minimal medium [0.5 % (w/v) D-glucose, 0.1 % NH<sub>4</sub>Cl, 0.6 % Na<sub>2</sub>HPO<sub>4</sub>, 1mM MgSO<sub>4</sub>, pH 7.4] at 37°C. One mM IPTG (isopropyl β-D-thiogalactopyranoside) was added to the growth medium to induce the protein expression after the cell density reached OD<sub>600</sub> of 0.55. The cells were harvested by centrifugation about 20h after induction. Uniformly <sup>15</sup>N- isotopocally labeled protein samples were prepared by growing the cells in a M9 minimal media that contained <sup>15</sup>NH<sub>4</sub>Cl (Cambridge Isotope Laboratory Inc.) as the sole source of nitrogen. The cell pellets were suspended in 1X PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3), 0.1 mM PMSF and 2 mM β-mercaptoethanol] and sonicated. Triton X-100 was then added to 1 % (v/v), and the crude lysate was stirred at 4 °C for 30 min. The lysate was then centrifuged for 30 min at 22500 g. The hCD99<sup>TMcytol</sup> domain fused to GST was purified with Glutathione Sepharose 4B (Amersham Pharmacia Biothech, Uppsala, Sweden). The fusion protein was eluted with 2 bed volume of 50 mM sodium phosphate buffer (pH 6.0), 5 % glycerol, 10 mM glutathion and 1 % (w/v) CHAPS. Repeated elution with 1 bed volume was done until all significant quantity of the protein had eluted as determined by Bradford assay. Fractions were then combined, and the GST was cleaved from the peptide with 300 units of thrombin (Amersham Pharmacia Biothech., Uppsala, Sweden) overnight at 22°C. The thrombin and GST were separated from hCD99<sup>TMcytol</sup> by filtration through 10 kD membrane Centriprep (Amicon, USA). The supernatant was diluted 10-fold in distilled H<sub>2</sub>O and loaded onto a 2 mL SP-Sepharose Fast Flow column at a flow rate of 1.25 mL/min. The column was washed with 30 bed volumes of 0.1× PBS. The peptide was then eluted with a continuous 100 mL linear gradient of ammonium acetate from 0 to 500 mM flowing at a rate of 0.5 mL/min. Fractions of 1.5 mL were collected and analyzed by SDS-PAGE. Pure peptide-containing fractions were combined and lyophilized. Approximately 10 mg of the peptide was obtained from the 1 L of M9 culture.

### ***Circular dichroism***

CD spectra of 80 μM hCD99<sup>TMcytol</sup> were measured in 50 mM sodium phosphate and various concentrations of DPC samples in pH 6.0 at 298 K on a Jasco 810 spectropolarimeter. Far-UV CD spectra were monitored from 190 to 250 nm using quartz

cell having path-length of 0.1 mm. Data were collected at 1 nm-interval and 10 scans were averaged with scan speed rate of 50 nm/min.

### ***NMR spectroscopy***

NMR experiments were carried out on Bruker DRX-500 equipped with a 5 mm triple-resonance probes with shield x, y, z- gradients. Experiments were performed at 310 K, pH 6.0 in both aqueous solution and membrane mimicking environments. The  $^1\text{H}$  chemical shifts were referenced to internal sodium 4, 4-dimethyl-4-silapentane-1-sulfonate (DSS). The  $^{15}\text{N}$  chemical shifts were referenced indirectly using the  $^1\text{H}/\text{X}$  frequency ratios of the zero-point:  $0.101329118 \{^{15}\text{N}-^1\text{H} (^1\text{H DSS})\}$ <sup>17</sup>. Quadrature detection was achieved by either the TPPI or the Stated-TPPI method<sup>18</sup>. The  $^1\text{H}$ - $^{15}\text{N}$  HSQC,  $^{15}\text{N}$ -edited 3D TOCSY-HSQC experiments were acquired for a uniformly  $^{15}\text{N}$ -labeled *hCD99*<sup>TMcytol</sup>. Pulsed-field gradient (PFG) techniques with a WATERGATE sequence<sup>19</sup> were used for all  $\text{H}_2\text{O}$  experiments, resulting in good suppression of the solvent signal. Two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  HSQC<sup>20, 21</sup> spectra were collected using the sensitivity enhanced method.

### ***NMR data Processing***

NMR data were processed with nmrPipe/nmrDraw software (Biosym/Molecular Simulations, Inc.) and XWINNMR (Bruker Instruments) on a Silicon Graphics Indigo<sup>2</sup> workstation. In the acquisition dimension the small residual water resonance was removed by a solvent-suppression time domain filter, apodized by a 60°-shifted squared sine-bell window function, zero-filled to twice the size and Fourier transformed. In the indirectly detected dimension the data were apodized by a 60° shifted squared sine-bell window function, zero-filled twice and Fourier transformed. For the triple resonance experiments, linear prediction was used to extend the data by up to 50 % in the indirectly detected dimension. The superposition of 3D slices necessary for the assignment of resonances was performed using Sparky 3.95.

## **RESULTS AND DISCUSSION**

*hCD99*<sup>TMcytol</sup> was purified after the GST cleavage at a purity of > 95% confirmed by SDS-PAGE (Fig. 2A). Since the peptide contains a hydrophobic transmembrane domain, it is prone to extensive aggregation at the concentrations required for NMR studies. Use of Triton X-100, CHAPS and glycerol mitigated the problem during the protein purification steps.

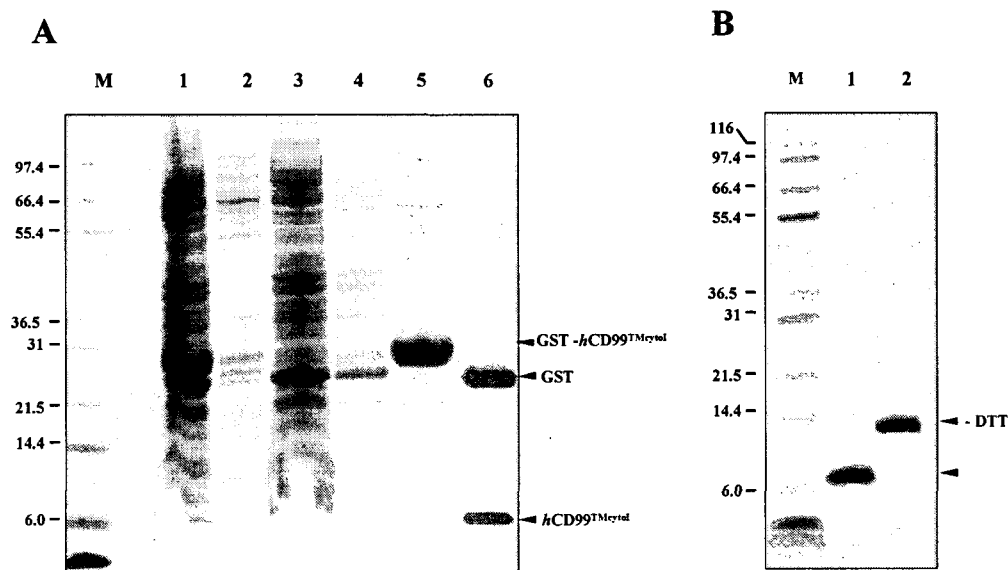


Fig. 2. (A) Purification of the GST-*hCD99*<sup>TMcytol</sup> fusion protein containing the transmembrane domain (GST-*hCD99*<sup>TMcytol</sup>). Lane 1: supernatant; Lane 2: precipitant; Lane 3: flow through; Lane 4: washing; Lane 5: elution with CHAPS 1%; Lane 6: after proteolytic cleavage using thrombin. (B) Dimer formation of *hCD99*<sup>TMcytol</sup>. Lane 1: in the presence of 1 mM DTT; Lane 2: in the absence of DTT. Arrows show dimeric *hCD99*<sup>TMcytol</sup>

Recently it has been reported that CD99 forms a covalent heterodimer or homodimer *in vivo*<sup>15</sup>. In our previous study of *hCD99*<sup>cytol</sup> (the form has only the cytosolic domain), dimerization was not detected in the absence of DTT (data not shown). However, *hCD99*<sup>TMcytol</sup> containing transmembrane domain formed a dimeric structure driven from the supposed covalent disulfide bond between the Cys154 residues at the cytosolic region of its monomer in the absence of DTT (Fig. 2B). To confirm the role of Cys154, the mutation study was performed. The result showed that the protein did not form dimer in the absence of that residue (data not published), proving that the disulfide bond is a key factor to form symmetric dimer of CD99. The transmembrane region is also supposed to play a key role in dimerization.

Data from the circular dichroism suggest that *hCD99*<sup>TMcytol</sup> mainly composed of  $\alpha$ -helical conformation (Fig. 3). In our previous study,  $\alpha$ -helical conformation is also the major secondary structure of *hCD99*<sup>cytol</sup>. The  $\alpha$ -helical structure of *hCD99*<sup>TMcytol</sup> became most stable in 200 mM DPC solution at pH 6.0, and 298 K. However, the spectral change was very small at concentration range of 0 - 200 mM DPC at 208 and 222 nm.

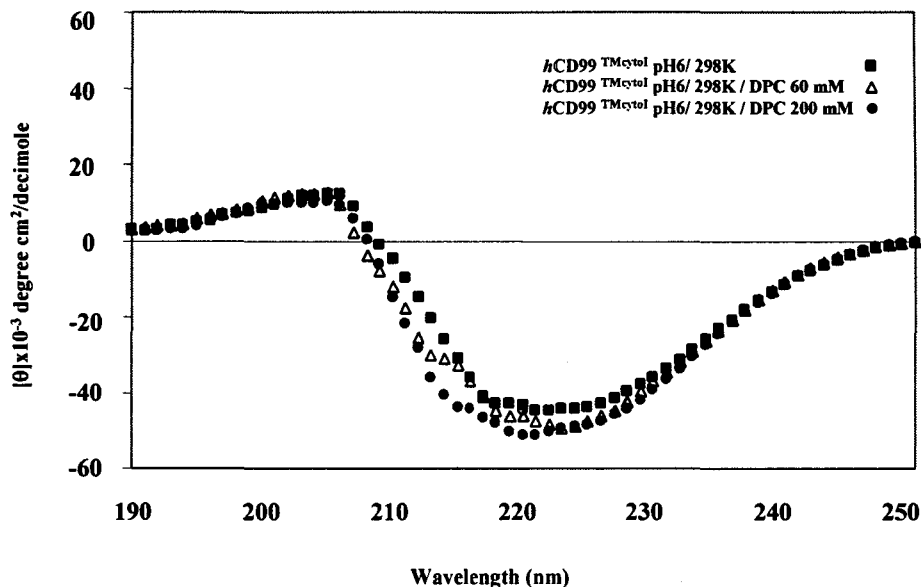


Fig. 3. CD spectrum of  $hCD99^{TM_{cytoI}}$  domain under various conditions: in sodium phosphate buffer contain 1% CHAPS at pH 6.0, 298 K (■), in 60 mM DPC (△), in 200 mM DPC (●).

In order to determine the proper concentration for NMR study under micelle environments, a series of  $^1H$ - $^{15}N$  2D HSQC spectrum have been collected for different DPC concentration (1 %, 4 % and 6 %) (Fig. 4). The increase in the  $^1H$  line width suggests that the  $hCD99^{TM_{cytoI}}$  peptide associates with micelle<sup>22</sup>. The  $^1H$ - $^{15}N$  2D HSQC spectrum that was collected at pH 6.0, in DPC 200 mM (6%) showed conformation change by newly developed and disappeared peaks. This condition was considered to be the state micelle was developed uniformly. The spectrum also confirmed the homogeneity of the NMR sample at this experimental condition (Fig. 4C) and the peptide in this DPC concentration was used further experiments.

Data from the  $^{15}N$ -edited 3D NOESY-HSQC experiment (Fig. 5B) showed that most of the cross peaks in the  $H\alpha$ -HN region were overlapped. The line broadening by detergent<sup>23</sup> observed and the residues involved in transmembrane region- $A^{PG}VI^{PG}IV^GAVVVAVAGAI^{SSF}$ - could affect NMR peak overlap. To resolve these spectral problems for detailed structural work, a selective isotope-labeling technique might be necessary<sup>24</sup>.

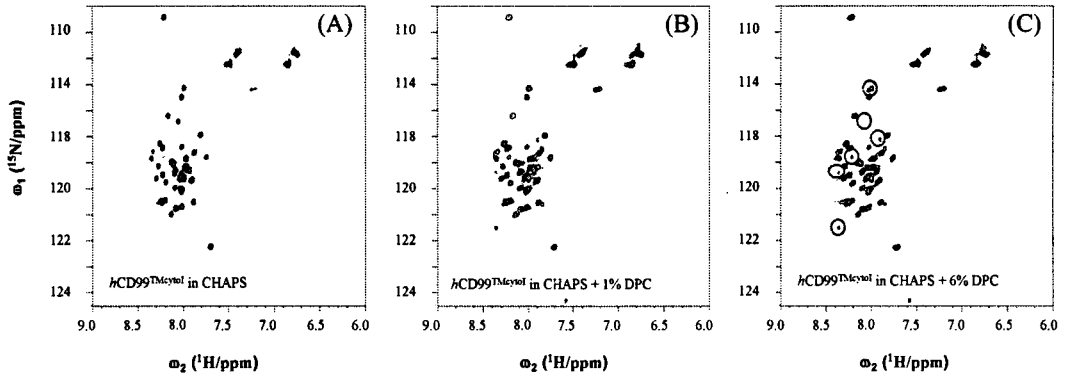


Fig. 4. A series of 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC data acquired by DPC titration. The concentrations were adjusted from (A) 0 %, (B) 1 % and (C) 6 % DPC solution.

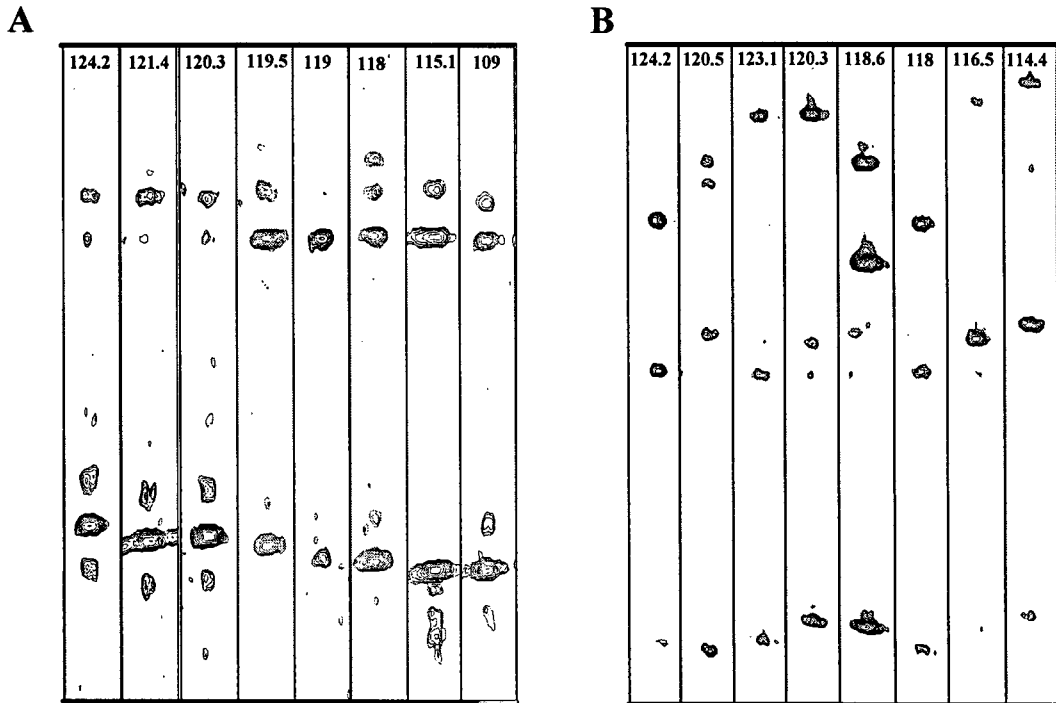


Fig. 5. Three-dimensional NMR spectra collected for  $^{15}\text{N}$ -labeled the  $h\text{CD99}^{\text{Mycytol}}$  domain. (A) Strips plot from  $^{15}\text{N}$  planes of the 3D TOCSY-HSQC spectrum. The  $^{15}\text{N}$ , HN and  $\text{C}_{\alpha\text{H}}$  chemical shifts (ppm) are labeled for each axis. (B) Strips taken from the 3D  $^{15}\text{N}$ -edited NOESY-HSQC spectrum recorded at 310 K.

In the mean time, the putative dimerization form was constructed by combining transmembrane region and cytosolic region *in silico* (Fig. 6). The disulfide bond between Cys154 residues of each monomer was linked and transmembrane regions were paralleled to each other. The final model shows the cytosolic regions stretch in the opposite direction to each other. To determine more detailed conformation of dimer, additional NMR experiments are required.

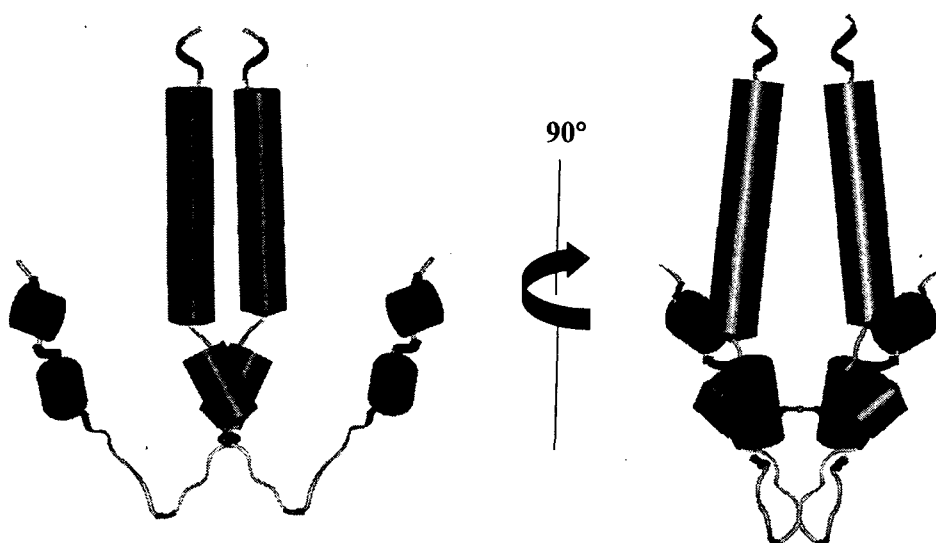


Fig. 6. Model of  $hCD99^{TM_{cyto I}}$  dimerization. Transmembrane region (putative) and cytosolic region (determined solution structure) is combined in InsightII ver.98 (Biosym/Molecular Simulation Inc.). Helix is represented by cylinder.

Consequently our findings from this study will be a good starting point to determine the solution structures of  $hCD99^{TM_{cyto I}}$  and multi-dimensional NMR experiments by the use of newly developed labeling techniques would be mandatory.

#### **Acknowledgements**

Authors would like to thank Prof. Yang-mee Kim for fruitful discussion. This study was supported by the Ministry of Science and Technology of Korea/ the Korea Science and Engineering Foundation through the NRL program of MOST NRDP (M1-0203-00-0020) (W.L.)



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