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Heteronuclear NMR studies on 44 kDa dimer, syndesmos

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Abstract Syndesmos, which is co-localized with syndecan-4 cytoplasmic domain ($Syn4^{cyto}$) in focal contacts, interacts with various cell adhesion adaptor proteins including $Syn4^{cyto}$ to control cell signaling. Syndesmos consists of 211 amino acids and it exists as a dimer (44kDa) in solution. Recently, we have determined the structure of syndesmos by x-ray crystallography, however, dynamics related to syndecan binding still remain elusive. In this report, we performed NMR experiments to acquire biochemical and structural information of syndesmos. Based on a series of three-dimensional triple resonance experiments on a $^{13}C/^{15}N/^{2}H$ labeled protein, NMR spectra were obtained with well dispersed and homogeneous NMR data. We present the sequence specific backbone assignment of syndesmos and assigned NMR data with combination structural information can be directly used for the studies on interaction with $Syn4^{cyto}$ and other binding molecules.

Keywords syndesmos, $Syn4^{cyto}$, NMR spectroscopy, triple resonance, backbone NMR assignment

Introduction

Syndesmos, nucleoside diphosphate linked moiety X (nudix)-type motif 16-like 1 (Nudt16l1), is a member of the nudix hydrolase family that possesses pyrophosphate hydrolysis activity.[1-4] However, syndesmos is epecially expressed in several organs and participates in focal adhesion formation upon binding to the cytoplasmic domain of syndecan-4 ($Syn4^{cyto}$) or focal adhesion proteins unlike Nudt16 proteins.[5-7] When syndesmos is overexpressed in cells, cell spreading and cytoskeleton organization are improved, which is confirmed by enhanced formation of filipodia.[5] These results were not affected by serum or media type, but only by cell adhesion complex formation, suggesting that the cellular features caused by syndesmos overexpression are closely related to the interaction cell adhesion complex including $syn4^{cyto}$.[5,8-9] Recently, the structure of syndesmos has been determined by x-ray crystallography[7] and molecular interaction information was already identified on the side of $Syn4^{cyto}$. However, it is still deficient in molecular interaction mechanism on the side of syndesmos.[7] Thus we initiate the NMR studies on syndesmos to investigate the structural characteristics through NMR experiments and by extension, to identify interaction regions or binding module between syndesmos and focal adhesion adaptor proteins including $syn4^{cyto}$.

Syndesmos is a large protein which exists as a dimer

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(44kDa) in solution. Therefore, deuterium labeling method was carried out to overcome low signal-to-noise and resolution by increasing relaxation rate.[10-14] Based on a series of triple resonance spectra measured on a triple isotope-[^{2}H/^{13}C/^{15}N]-labeled protein, TROSY-based triple resonance spectra {HNCACB, HNCA and HNCO}[15-17] were obtained showing well dispersed and homogeneous signals. Here, we report the sequence-specific backbone resonance assignments of syndesmos partially, but assigned NMR data in combination with structural information can be used for the studies on interaction with cell adhesion adaptor protein including syn4^{cyto} in the future.

Experimental Methods

Cloning, protein expression and purification- cDNA encoding Syndesmos was amplified by PCR using synthetic oligonucleotides as primers and were subcloned into the *Escherichia coli* expression vector pET32a (Amersham Biosciences). Protein was expressed in *E. coli* strain BL21(DE3). Cells were grown in either Luria-Bertani (LB) or M9 minimal media at 37°C until OD_{600} of 0.6. After 1 mM isopropyl β-D-thiogalactoside (IPTG) was added, Cells were cultured further for 15 h at 25°C. Subsequently, the cells were harvested by centrifugation and stored at -80°C. Harvested TRX-$(His)_6$ tag-fusion proteins were purified by immobilized metal affinity chromatography (IMAC) using a Ni-NTA column (Amersham Pharmacia), and the TRX-$(His)_6$ tag was cleaved by incubation with tobacco etch virus protease for 2–3 hours at 25°C. The purified proteins were then subjected to size exclusion chromatography using a HiLoad Superdex 200 prep grade column (Amersham Pharmacia).

Preparation of ^{2}H/ ^{13}C/ ^{15}N labeled proteins- To label the protein in ^{2}H, uniformly [^{13}C,^{15}N], Protein overexpressed in *E.coli* grown in an <90% D_2O media supplemented with U-$^{13}C_6$-glucose and $^{15}NH_4Cl$ (Cambridge Isotope Laboratories Inc.). Plasmids were freshly transformed into *E. coli* strain BL21(DE3). Cells were grown in M9 minimal media and incubated at 37°C for 12hrs. Grown cells were inoculated in M9 media added 30% D_2O. Cells grown in 30% D_2O media at 37°C for 12hrs were again inoculated in 50% D_2O media. Cell were grown in D_2O media gradually increased until 100% D_2O every 12hrs (e.g. 30%, 50%, 70% and 100% D_2O). After D_2O cell training, Cells were grown in 100% D_2O medium containing U-$^{13}C_6$-glucose and $^{15}NH_4Cl$ (Cambridge Isotope Laboratories Inc.) at 37°C until OD_{600} of 0.6. After 1 mM isopropyl β-D-thiogalactoside (IPTG) was added, Cells were cultured further for 15 h at 25°C. For NMR experiments, Cells cultivated with labeling ^{2}H, ^{13}C and 15N were purified by using purification protocols described above. The final buffer contained 10 mM HEPES (pH 7.0), 150 mM NaCl, and 0.01% NaN_3.

NMR Spectroscopy- All NMR experiments were performed on a Bruker DRX- spectrometer equipped with a triple resonance probes with shielded x, y, z-gradients. NMR data of syndesmos were obtained at 37°C. The 2D and 3D triple resonance experiments, TROSY-^{15}N-HSQC, HNCA, HNCACB, HNCO were conducted. All data were processed with NMRPipe and analyzed with the Sparky program.[16-17]

Results and Discussion

Expression and purification of syndesmos- Syndesmos gene was amplified by PCR and subcloned into pET32a (Amersham Biosciences). This construct contains a thioredoxin(TRX), hexahistidine(His_6) affinity tag, and tobacco etch virus (TEV) protease recognition site in the N-terminus (Figure 1). Syndesmos proteins were successfully expressed in 250 mL M9 media containing 100% D_2O using 1 mM IPTG at 25°C. Fusion proteins purified by IMAC and TRX-His_6 of fusion protein was removed by TEV. The target protein, Syndesmos was eluted on buffer added 20 mM imidazole (Figure 2A). The molecular size of the purified fusion proteins was determined about 36

kDa and the final molecular weight after tag cleavage was about 22 kDa, respectively. To confirm the oligomeric state of syndesmos in solution, size exclusion chromatography has been performed using superdex TM200 column (Figure 2B). The peak eluted at a volume of 89.8 ml, consistent with a protein dimer in solution (~ 36.4 kDa). Molecular weight of syndesmos was calculated by the equation (log Y = 1.6319X+7.6534, R^2 = 0.9927, x = elution volume). Four proteins which are albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12 kDa), aprotinin (6.5 kDa) were used as a standard molecular marker (data not shown). The final yields of syndesmos were 2 mg ~ 3 mg for 250 mL culture.

Assignment of backbone resonance- The 2D [^{2}H-^{15}N] HSQC spectrum of syndesmos with assignment showed that most of the backbone NH resonances are spread uniformly over the spectrum, indicative of well- folded protein (Figure 3A). Backbone resonances were assigned using data from TROSY-HNCACB, HNCA, and HNCO experiments with triple labeled protein. In these experiments the ^{13}C-, ^{15}N-labeled protein was fractionally deuterated. The number of cross-peaks visible in the spectrum of the 75 % fractionally deuterated sample is much higher than in the spectrum recorded on fully protonated syndesmos (data not shown). Figure 3B shows strip plot taken from the HNCACB spectrum (left panel) and HNCA (right panel) displaying the C_α or/and C_β sequential connectivity of residues Ser194 through Ala189 in the ^{2}H/^{13}C/^{15}N labeled syndesmos. The assignment of remaining resonances was not possible due to spectral overlap and ambiguity. The excellent sensitivity observed validates the labeling strategy.

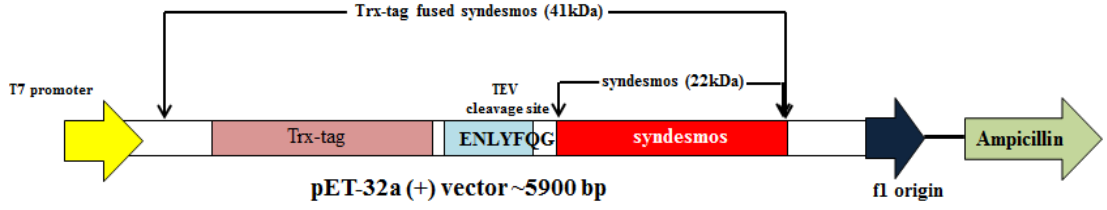

Figure 1. Schematic representation of structural domain of syndesmos. The vector consists of a Thioredoxin, $(His)_6$ tag and TEV enzyme cleavage site (ENLYFQG).

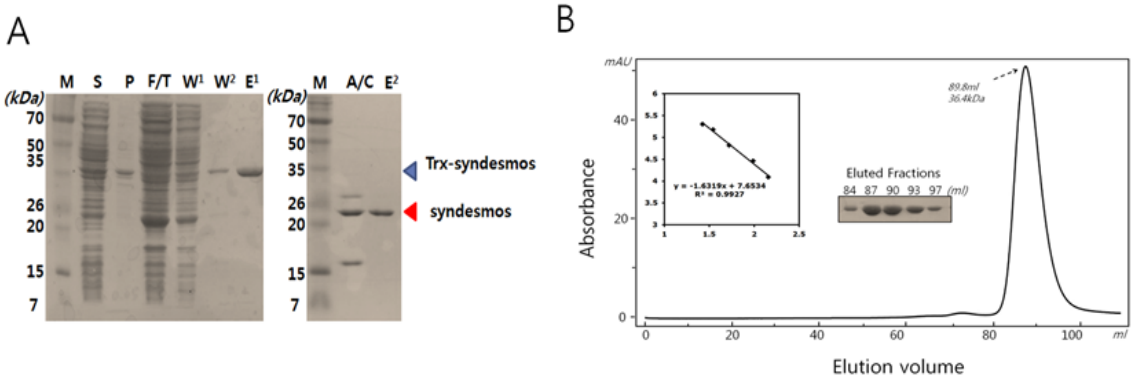

Figure 2. The SDS-PAGE for purification of syndesmos and elution profile of the size exculusion chromatography (Superdex 200). (A) SDS-PAGE analyses of syndesmos. The molecular weight syndesmos is determined as 36kDa and 21kDa after cleavage. The molecular weight marker is indicative of the protein size in each lane. (B) Elution profile of the size exclusion chromatography. The peak eluted at a volume of 89.8 ml, consistent with a protein dimer (~ 36.4 kDa). Molecular weight of syndesmos was calculated by the equation (log Y = –1.6319X+7.6534, R^2 = 0.9927, x = elution volume).

Conclusion

Focal adhesion protein, syndesmos has been cloned and purified. We utilized the labeling protocols for deuterium labeling of large protein and successfully performed ^{15}NMR experiments (TROSY-HNCACB, HNCA, and HNCO) for sequence-specific backbone resonance assignments. Although we carried out the backbone resonance assignments of syndesmos in part, assigned NMR data will be useful for verifying exact cell adhesion adaptor proteins including syn4^{cyto} binding site of syndesmos.

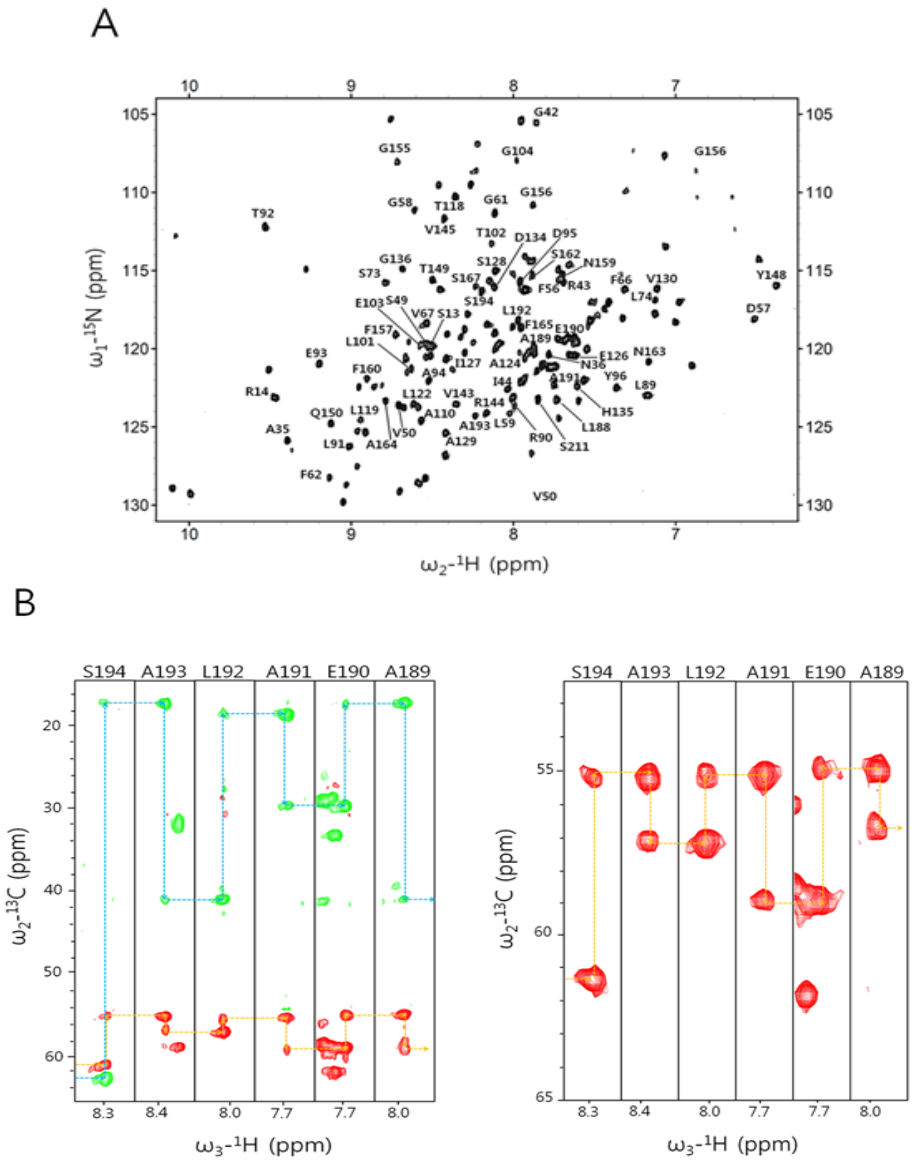

Figure 3. Two-dimensional HSQC spectrum and Strip plots from the HNCACB, HNCA spectra record syndesmos. (A) ^{2}H-^{15}N HSQC spectrum of syndesmos with resonance assignments. The spectrum was collected at pH 7.0 using a Bruker 900 MHz spectrometer. (B) Strip plots from ^{15}N-plans of the HNCACB(left panel) and HNCA (right panel) spectra of the ^{2}H, uniformly [^{15}N, ^{13}C]-labeled syndesmos illustrating through-bond sequential assignments for residues Ser^{194}-Ala^{189} (Helix-7 region).

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