Molecular Cloning and NMR Characterization of the Nonreceptor Tyrosine Kinase PTK6 SH3-SH2-Linker Domain

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Received March 11, 2009, Accepted March 26, 2009

Human protein tyrosine kinase-6 (PTK6) is a member of the non-receptor protein tyrosine kinase family and it is found in two-thirds of all breast tumors. Very recently, we proposed that the SH3 domain of PTK6 interacts with the linker region (Linker) between the SH2 and kinase domains, proving that the interaction between SH3 domain and Linker plays an important role in auto-inhibition mechanism. Residues from 1 to 191 corresponding region of SH3-SH2-Linker (SH32L) of PTK6 was cloned into the pET32a expression vector with Tobbaco etch virus (TEV) protease enzyme site by sequence homology and 3D structural model. The purified PTK6-SH32L was determined as a monomer conformation in solution. The amide proton resonances in the ^{15}N-^{1}H 2D-HSQC spectrum suggest that PTK6-SH32L possesses disordered structural region of the flexible/unstructured linker region. In addition, the backbone amide proton chemical shifts of the SH3 domain in the PTK6-SH32L differ from that of the independent domain, indicating that intra-molecular interaction between SH3 and Linker in the PTK6-SH32L is present.

Key Words: PTK6, Molecular cloning, NMR spectroscopy, Domain interaction

Introduction

Protein tyrosine kinases (PTKs) have been known as important molecules in cellular proliferation, differentiation and apoptosis.[1,2] They often involves in the tumorigenesis through mutation, rearrangement, or gene amplification *via* hyper-activation.[3,4] The human PTK6, a non-receptor protein tyrosine kinase is frequently observed in breast carcinomas and colon tumors as an over-expressed form.

PTK6 is composed of Src homology 3 (SH3), Src homology 2 (SH2), and catalytic domains and especially, the SH2 domain is mainly involved in negative regulation of kinase activity.[5-7] Phosphorylation of a highly conserved tyrosine residue in the SH2 domain stabilizes an inactive conformation through its conformational change by interactions between the SH3 domain and a polyproline sequence near the kinase domain. Previously, we have reported that the SH2 domain of human PTK6 contains a consensus α/β-fold with a pTyr peptide binding surface, however, two of the α-helices (αA and αB) are located on opposite faces of the central β-sheet by NMR spectroscopy.[8,9] In addition, we proposed that the SH3 domain of PTK6 interacts with the linker region between the SH2 and kinase domains and a Trp 44 residue in the SH3 domain plays a key role in the intra-molecular interaction with proline-rich linker region by data from site-directed mutagenesis and surface plasmon resonance experiments.[10-13] Since the interaction between SH3 domain and linker region is mainly responsible for both auto-inhibitory and substrate binding mechanism, it is of interest to characterize detailed structural information between SH3-SH2 domain and linker region. Here, we present the molecular cloning and NMR characterization of the SH3-SH2-linker domain (SH32L) for further structural and biochemical analysis related to auto-inhibitory mechanism of the non receptor tyrosine kinase, PTK6.

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Experimental Procedures

Molecular cloning, protein expression and purification. The SH32L is cloned into pET32a vector with Tobbaco etch virus (TEV) protease enzyme site, the Histidine(His)$_6$tag and thioredoxin(Trx) tag(Novagen) on N-terminal region. The transformed vector expressed fusion protein which was induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 16 hr. The harvested cells were lysised in 25 mL of lysis buffer contaning 25 mM sodium phosphate, pH 7.4, 300 mM NaCl, and 10 mM β-mercaptoethanol. The Histidine(His)$_6$tag-fusion protein was purified with immobilized metal affinity chromatography on a Ni-NTA column. The fusion protein was cleaved by purified TEV protease enzyme for 12 hr at 25 °C. Further purification to insure high purity was carried out by gel filtration column chromatography with HiLoad Superdex 75 prep grade column.

Isotope labeling and protein purification. A transformed cell was grown in M9 minimal medium [0.5% (W/V) D-glucose, 0.1% NH_4Cl, 0.05% NaCl, 0.6% Na_2HPO_4, 1 mM $MgSO_4$, pH 7.4] at 37 °C. One mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growth medium to induce protein expression after cell density reached OD_{600} of 0.6. Cells were harvested by centrifugation after induction. Uniformly ^{13}C/^{15}N- or ^{15}N-isotopically labeled protein samples were prepared by growing cells in M9 minimal media containing $^{15}NH_4Cl$, either with or without $^{13}C_6$-D-glucose as the sole source of nitrogen and carbon. The cell pellets were suspended in phosphate-buffered saline (pH 7.0) that contained 7 mM EDTA and 0.01 mM PMSF, and the cells were sonicated. The PTK6 SH3 domain fused to GST was also purified with Glutathion Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) and was subjected to digestion with bovine thrombin (Amersham Pharmacia Biotech, Uppsala, Sweden) for 5 h to remove GST tag. Further

purification was accomplished using fast performance liquid chromatography (FPLC) with Superdex 75 HR 10/30 column in 50 mM potassium phosphate buffer at pH 7.0. The NMR protein samples for NMR measurement were approximately 1 mM concentration in 90% H_2O/10% ^2H_2O and placed in a 5 mm symmetrical micro cell (Shigemi. Tokyo. Japan).

NMR spectroscopy. All NMR experiments were performed at 298 K on a Bruker DRX500 spectrometer equipped with a cryo-probe head and shielded triple-axis gradients coil. The ^1H chemical shifts were referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The ^{15}N chemical shifts were referenced indirectly using the $^1H/X$ frequency ratios of the zero-point: 0.101329118(^{15}N). The ^1H-^{15}N 2D- HSQC experiment was acquired using a uniformly ^{15}N-labeled sample. Pulsed-field gradient (PFG) techniques with a WATERGATE pulse sequence were used for all H_2O experiments.[14,15] The 3D triple resonance experiments. HNCA. HN(CO)CA. HNCACB, CBCA(CO)NH[16] were collected for backbone resonance assignment. All NMR data were processed on a Silicon Graphics Indigo^2 workstation using nmrPipe/nmrDraw software (Biosym/Molecular Simulations, Inc.). Sparky 3.95[17,18] and XEASY programs[19,20] were used for spectral analysis.

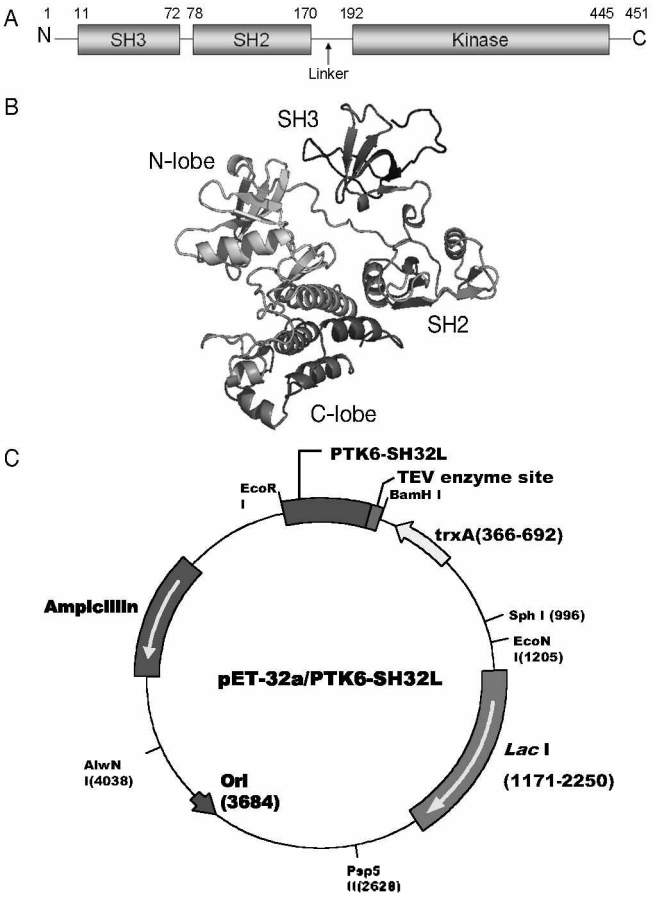

Figure 1. Domain construction, three-dimensional model and vector map of PTK6. (A) Structural domains with sequence information are determined by structural analysis. (B) Three-dimensional model of the PTK6 generated by homology modeling shows domain interactions. (C) The map of *E.coli* expression vectors containing PTK6 SH32L is presented.

Results and Discussion

Molecular cloning and purification of PTK6 SH32L. Figure 1A shows domain organization of PTK6, which consists of SH3. SH2. Linker and kinase domains. Residues from 1 to 191 corresponding region of SH3-SH2-linker (SH32L) of PTK6 for cloning was determined by data from sequence homology and 3D structural model (Fig. 1B). PTK6 SH23L was successfully cloned into the pET32a expression vector with Tobbaco etch virus (TEV) protease enzyme site (Fig. 1C). The PTK6 SH32L was purified as a purity of > 95% determined by SDS-PAGE (Fig. 2A) and NMR spectrum. From our previous studies. we examined that the Linker region could contribute the protein solubility even though it is highly flexible. To determine the oligomeric state of PTK6 SH32L. size exclusion chromatography experiment was performed using Superdex^{TM} 75. Four proteins which are albumin-(66 KDa). carbonic anhydrase (29 KDa), cytochrome C (12 KDa) and aprobinin (6.5 KDa). were used as standard molecular weight and the actual molecular weight of PTK6 SH23L was calculated by program Origin 7.1 (Fig. 2B). The purified PTK6 SH32L was determined as a monomer conformation in solution (Fig. 2B).

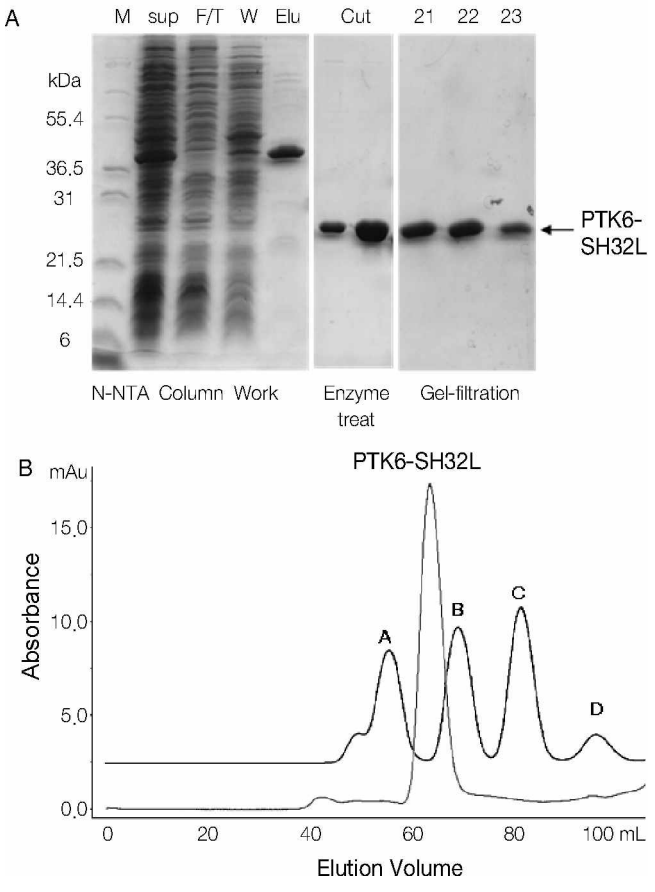

Figure 2. The SDS pages showing solubility and purification of the PTK6 SH32L. (A) SH32L proteins were identified by 15% SDS-PAGE and the molecular weight was measured using molecular size marker, Mark 12 (Invitrogen Co.) and gel filtration chromatography. (B) Elution profile of the size exclusion chromatography. The standard marker proteins were loaded and analyzed by Origin 7.01 (Origin Lab.).

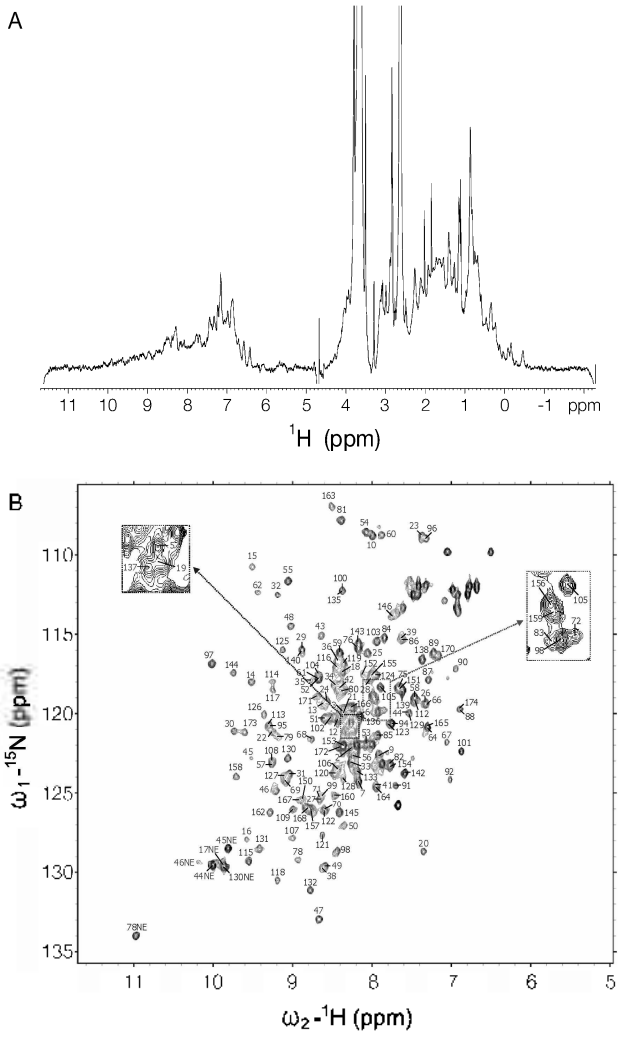

Figure 3. One-dimensional proton and ^{1}H-^{15}N 2D HSQC spectra of PTK6 SH32L. (A) One-dimensional ^{1}H NMR spectrum of the PTK6 SH32L in 90% H_2O/10% ^2H_2O solution at pH 7.0, 25 °C. Presaturation of water was applied to minimize water signal. (B) ^{1}H-^{15}N 2D-HSQC spectra of the PTK6 SH32L are displayed. The backbone resonance assignment of PTK6-SH32L is presented. The resonances of the SH3 and SH2 domain are indicated by navy blue and brown colors, respectively. NMR experiments were performed in Bruker DRX 500MHz spectrometer equipped with Cryoprobe™.

NMR characterization of the PTK6 SH23L. Figure 3A shows one-dimensional ^{1}H NMR spectrum of PTK6 SH3 in 90% H_2O/10% ^2H_2O solution at pH 7.0. A number of high-field shifted methyl proton resonances from 0.5 to 1.0 ppm and amide proton resonances (7.5-9.5 ppm) strongly suggest that the PTK6-SH32L has a stable tertiary structure in our experimental condition. ^{15}N-^{1}H 2D-HSQC spectrum collected at pH 7.0 also implies that PTK6 SH32L has a unique folded conformation based on a number of cross peaks at low field region. The backbone resonance assignments of the SH32L has been performed by data from HNCACB, CBCA(CO)NH, HNCO and HCACO spectra. The results from backbone assignment are summarized in Fig. 3B. The resonances of the SH3 and SH2 domain are indicated by navy blue and brown colors, respectively.

Structural and functional implication. The line widths of the amide proton resonances near 8.3 ppm in the ^{15}N-^{1}H 2D-HSQC spectrum suggest that PTK6 SH32L might possess unstructured region at this experimental condition (Fig. 3B). This might be due to flexible/unstructured structure of the Linker region. In addition, a number of backbone amide proton resonances of both SH3 and SH2 domain in the SH32L differ from those of the free forms.[10,11] This could be easily explainable from intra-molecular interaction between each domain in the SH32L. For instance, we have previously shown that the residues in the SH3 domain interact with those in the Linker region, resulting auto-inhibition of the PTK6 activity.[12,13] Since the biological role of the SH32L of PTK6 is still unclear despite the structural homology with those of other family proteins, three-dimensional structural information together with biological data would be of importance in understanding the molecular function of PTK6. Therefore, our report from this study will contribute for detailed structure-functional investigation of the SH32L domain and multi-dimensional NMR experiments to determine the detailed three-dimensional structure of the SH32L are currently in progress.

Conclusion

Human protein tyrosine kinase-6 (PTK6) found in two-thirds of all breast tumors has been an excellent target for drug development. Recently, we have shown that the interaction between SH3 domain and linker region plays an important role in auto-inhibition mechanism. In this report, residues from 1 to 191 corresponding region of SH3-SH2-linker (SH32L) was cloned and purified as a monomer conformation in solution. NMR data reveals that the PTK6-SH32L possesses unstructured region due to the flexible/unstructured region of the Linker. The intra-molecular interaction between SH3 domain and Linker region is clearly observed in the ^{15}N-^{1}H 2D-HSQC spectra of PTK6-SH32L.

Acknowledgments. This work was supported by a grant (No. 070292) from National R&D program for Cancer Control from the Ministry of Health & Welfare. This work was also supported in part by the Brain Korea 21 Project.

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