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Purification and Spectroscopic Characterization of the Human Protein Tyrosine Kinase-6 SH3 Domain

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The human protein tyrosine kinase-6 (PTK6) polypeptide that is deduced from the cDNA sequence contains a Src homology (SH) 3 domain, SH2 domain, and catalytic domain of tyrosine kinase. We initiated biochemical and NMR characterization of PTK6 SH3 domain in order to correlate the structural role of the PTK6 using circular dichroism and heteronuclear NMR techniques. The circular dichroism data suggested that the secondary structural elements of the SH3 domain are mainly composed of β-sheet conformations. It is most stable when the pH is neutral based on the pH titration data. In addition, a number of cross peaks at the low-field area of the proton chemical shift of the NMR spectra indicated that the PTK6 SH3 domain retains a unique and folded conformation at the neutral pH condition. For other pH conditions, the SH3 domain became unstable and aggregated during NMR measurements, indicating that the structural stability is very sensitive to pH environments. Both the NMR and circular dichroism data indicate that the PTK6 SH3 domain experiences a conformational instability, even in an aqueous solution.

Keywords: PTK6, SH3, Circular dichroism, NMR

Introduction

Non-receptor protein tyrosine kinases play an essential role in important intracellular functions, such as cell proliferation and differentiation, by relaying signals from the cell-surface receptors to intracellular targets (Bolen, 1993; Neet and Hunter, 1996). The human full-length cDNA of PTK6 was cloned from breast carcinoma cells (Mitchell *et al*., 1994). The cDNA of its mouse homolog, Sik, which has a 80% identity to PTK6 in the amino acid sequence, was cloned from mouse intestinal crypt cells (Vasioukhin *et al*., 1995). PTK6 and Sik are expressed at high levels in a normal gastrointestinal tract (Vasioukhin *et al*., 1995; Lee *et al*., 1998; Llor *et al*., 1999). Although the expression of PTK6 was not detected in normal mammary epithelial cells and normal melanocytes (Lee *et al*., 1993; Barker *et al*., 1997), its elevated expression has been reported in breast carcinomas (Mitchell *et al*., 1994; Barker *et al*., 1997), melanomas (Easty *et al*., 1997), and colon carcinomas (Chen *et al*., 1999; Llor *et al*., 1999).

The PTK6 polypeptide that was deduced from the cDNA sequence contains a Src homology (SH) 3 domain, an SH2 domain, and a catalytic domain of tyrosine kinase (Mitchell *et al*., 1994). PTK6 shows the strongest homology to Src family members, such as Src, Yes, and Fyn (43-41% identity) (Lee *et al*., 1998). However, for several reasons, PTK6 was not believed to be another Src family member. First, homology among the Src family members is significantly higher (55-75% identity) than those between PTK6 and the Src family members (Lee *et al*., 1998). Secondly, PTK6 lacks a consensus sequence for myristoylation in the N-terminus (Mitchell *et al*., 1994). Thirdly, the exon-intron boundaries of the PTK6 gene, which was sublocalized to human chromosome 20q13.3 by fluorescence *in situ* hybridization (Park *et al*., 1997), were quite different from those of the Src family genes, which are evolutionarily conserved (Lee *et al*., 1998). The SH3 domains are small protein modules with a β-barrel type structure that contain approximately 50-70 amino acid residues. They are found in a wide variety of intracellular or membrane-associated proteins, including a variety of proteins with effector proteins (Morton and Campbell, 1994). Considering the primary structure and expression profile, PTK6 that contains a SH3 domain is thought to play a role in signal transduction for the regeneration or differentiation of gastrointestinal epithelium and enzymatic activity, adaptor proteins that lack catalytic activity, and cytoskeletal proteins (Musacchio *et al*., 1992; Pawson, 1995). The SH3 domains mediate the assembly of specific protein complexes via binding proline-rich peptides. They are involved in linking the

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signals that are transmitted from the cell surface by protein tyrosine kinases to the downstream development of epithelial carcinomas (Vasioukhin and Tyner, 1997; Lee *et al*., 1998; Llor *et al*., 1999). To understand the structural role of the PTK6 SH3 domain, we initiated biochemical and NMR characterization of the PTK6 SH3 domain using circular dichroism and heteronuclear NMR techniques.

Materials and Methods

Expression of the GST-fused PTK6 SH3 The cDNA fragment that encodes the PTK6 SH3 domain (residues 3-75) was amplified using a primer pair (5'-TGGTCCTGCCGCTGCGCCCG-3' and 5'-GAACCACGGaTCCGACTCCA-3'; a lowercase letter was introduced for the *Bam*HI site) by PCR from the full-length PTK6 cDNA, pBS-PTK6-MR (Lee *et al*., 1998). The PCR product that was digested with *Nco*I and *Bam*HI was cloned into the *Nco*I-*Bam*HI sites of pET-32c(+) (Novagen). The resultant plasmid (pET-32c-PTK6-SH3) was cleaved with *Sma*I and *Not*I. The 252-bp *Sma*I-*Not*I fragment that encodes the PTK6-SH3 domain (residues 3-75) and 13 amino acid residues (DPNSSSVDKLAAA) that are derived from pET-32c was cloned into the *Sma*I-*Not*I sites of pGEX-4T-3 (Amersham Pharmacia Biotech. Uppsala, Sweden). The resultant plasmid (pGEX-4T-3-PTK6-SH3) was transformed to the *E. coli* BL21(DE3) for glutathione-S-transferase (GST)-fused PTK6-SH3. The PTK6-SH3 domain that was purified after thrombin cleavage contained the following: GSPN residues that were introduced for thrombin cleavage, a PTK6-SH3 domain (residues 3-75), DPNSSSVDKLAAA residues that were derived from pET-32c, and a S residue that was derived from pGEX-4T-3.

Overexpression, isotope labeling, and purification A transformed cell was grown in a 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 the cell density reached OD_{600} of 0.6. The cells were harvested by centrifugation about 20 h after induction. Uniformly $^{13}C/^{15}N$- or ^{15}N-isotopically labeled protein samples were prepared by growing the cells in a M9 minimal media that contained $^{15}NH_4Cl$, 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 sonicated. The PTK6 SH3 domain that was fused to GST was purified with Glutathion Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). It was then subjected to digestion with bovine thrombin (Amersham Pharmacia Biotech. Uppsala, Sweden) for 5 h in order to remove the GST tag. Further purification of the PTK6 SH3 domain was accomplished using fast performance liquid chromatography (FPLC) with Superdex 75 HR 10/30 column in a 50 mM potassium phosphate buffer at pH 7.0. The concentration of the NMR sample was approximately 1 mM in 90% H_2O/10% ^2H_2O. It was placed in a 5 mm symmetrical micro cell (Shigemi. Tokyo, Japan).

Circular dichroism CD spectra were measured in 50 mM of a potassium phosphate buffer for pH values of 6.0, 6.5, 7.0, and 7.5 at 25°C on a Jasco 720 spectropolarimeter. Far-UV CD spectra were monitored from 190 to 250 nm using a protein concentration of 30 μM with a path length of 0.1 mm, 20 mdeg sensitivity, response time of 1 s, and scan speed of 50 nm/min. The spectra were recorded as a 6 scan average value.

NMR experiments All of the NMR experiments were preformed at 298 K on either a Bruker DRX-500 MHz or a Varian Unity INOVA 500 MHz spectrometer that was equipped with triple resonance probes with shielded x, y, z-gradients. In the 2D and 3D experiments, 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) (Wishart *et al*., 1995). States-TPPI quadrature detection and spectrum aliasing were employed in indirect dimensions for all of the experiments. Pulsed-field gradient (PFG) techniques with a WATERGATE sequence (Piotto *et al*., 1992) were used for all of the H_2O experiments, resulting in a good suppression of the solvent signal. Two-dimensional ^1H-^{15}N HSQC (Kay *et al*., 1992) spectra for different pH values were collected using the enhanced sensitivity method. ^{15}N-edited NOESY-HSQC (Marion *et al*., 1989) with a mixing time of 150 ms and ^{15}N-edited TOCSY-HSQC (Krishnamurthy, 1995) with a mixing time of 77.7515 ms spectra for the uniformly ^{15}N-labelled SH3 domain of PTK6 were recorded. The ^1H spectra were referenced to the water resonance at 4.76 ppm.

NMR Data Processing The NMR data were processed using the nmrPipe/nmrDraw (Delaglio *et al*., 1995) software packages, and analyzed by the Sparky 3.95 software and XEASY program (Bartels *et al*., 1995). In the acquisition dimension, the small residual water resonance was removed by a solvent-suppression time domain filter, apodized by a 65°-shifted squared sine-bell window function, zero-filled to twice the size, and Fourier-transformed. In the indirectly-detected dimensions, the experimental data were extended by linear prediction and zero-filled to give 2048×512 data matrices. It was then processed using gaussian multiplication and a shifted ($\pi/3$) sine bell function prior to Fourier transformation.

Results and Discussion

Fig 1A shows the structural domain of PTK6. The segment of PTK6 from 11 to 69 is considered a SH3 domain. It was included in the expression vector for this study. The primary sequence of the PTK6 SH3 domain was aligned with other SH3 domains of Src, Fyn, and Hck (Fig. 1B). Based on the sequence alignment data, PTK6 SH3 shows relatively low homology to other SH3 domains in these regions.

The PTK6 SH3 domain was purified after the GST cleavage at a purity of >95% by SDS-PAGE (Fig. 2A). The monomer conformation of the purified PTK6 SH3 domain was confirmed by a gel filtration FPLC elution profile by comparing the molecules of the standard molecular weights (data not shown). Data from the circular dichroism suggested that the secondary structural elements of the SH3 domain are mainly composed of β-sheet conformation. They are most

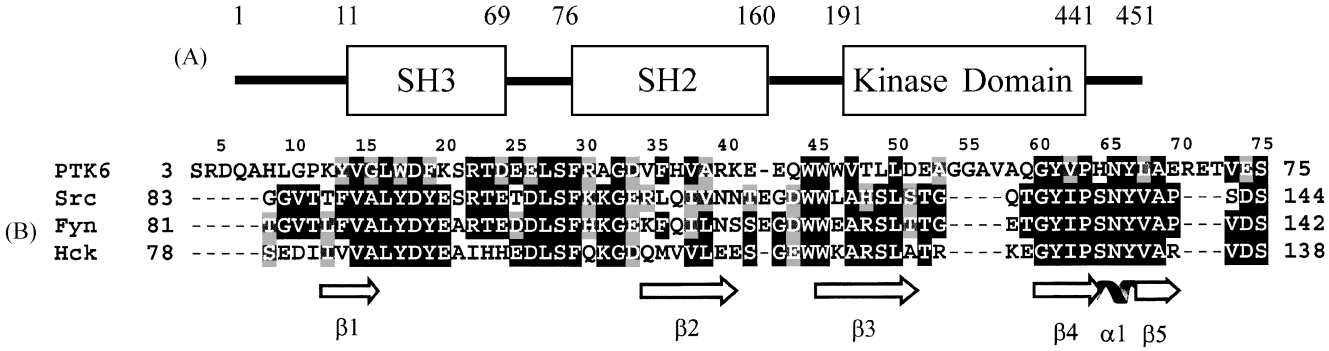

Fig. 1. (A) Domain structure of PTK6. (B) The sequence alignment of PTK6 SH3 domain with other SH3 domains with highest homology. The alignment is made by CLUSTALW.

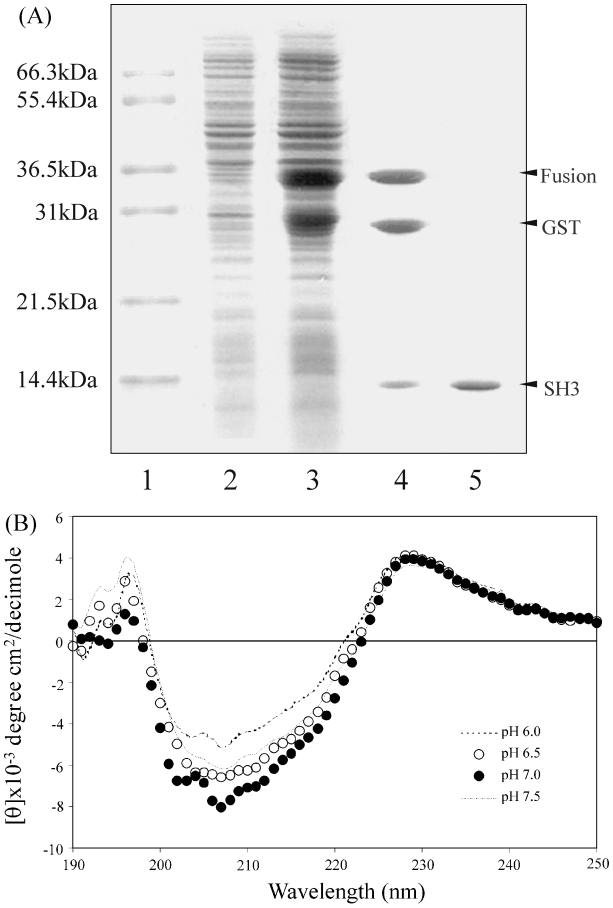

Fig. 2. (A) Purification of PTK6 SH3 domain. Lane 1: molecular weight standard; Lane 2: before induction; Lane 3: after induction; Lane 4: after proteolytic cleavage using thrombin; Lane 5: purified SH3 domain using glutathione sepharose 4B (B) CD spectrum of PTK6 SH3 domain at different pH values. Baseline was corrected by the buffer solution for each pH value.

stable at neutral pH (Fig. 2B). The CD spectra changed only slightly from pH 6.5 to 7.5. However, the spectrum did change at pH values below 6.0, based on increased ellipticity about 20% at 208 nm.

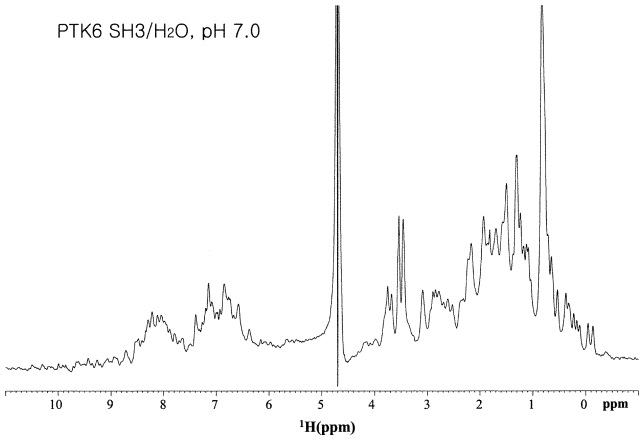

Fig. 3. One-dimensional ^{1}H NMR spectrum of soluble PTK6 SH3 domain in 90% H_2O/10% $^{2}H_2O$ solution collected on a Bruker DRX500 spectrometer at pH 7.0, 25°C. Presaturation of water was applied to minimize water signal.

Fig. 3 shows the one-dimensional ^{1}H NMR spectrum of PTK6 SH3 in a 90% H_2O/10% $^{2}H_2O$ solution at pH 7.0. A number of high-field shifted methyl proton resonances from the 0.5 to 1.0 ppm range strongly suggest that the SH3 domain has a stable tertiary structure at this experimental condition. The ^{15}N-correlated 2D HSQC spectra that was collected at pH 7.0 confirmed the homogeneity of the NMR sample at this experimental condition (Fig. 4A). In addition, a number of cross peaks at the low-field area of the proton chemical shift supported the supposition that the PTK6 SH3 domain retains a unique and folded conformation at the neutral pH condition. In order to monitor pH dependence, a series of ^{15}N-correlated 2D HSQC spectra (Fig. 4) were also collected for the pH ranges of 5.0-7.0. They showed that the SH3 domain retains a stable folded conformation at the pH 7.0 condition. For other pH conditions, the protein became unstable and aggregated during NMR measurements. This result confirms that the structural stability of the PTK6 SH3 domain is very sensitive to its pH environment.

Data from the ^{15}N-edited 3D NOESY-HSQC experiment

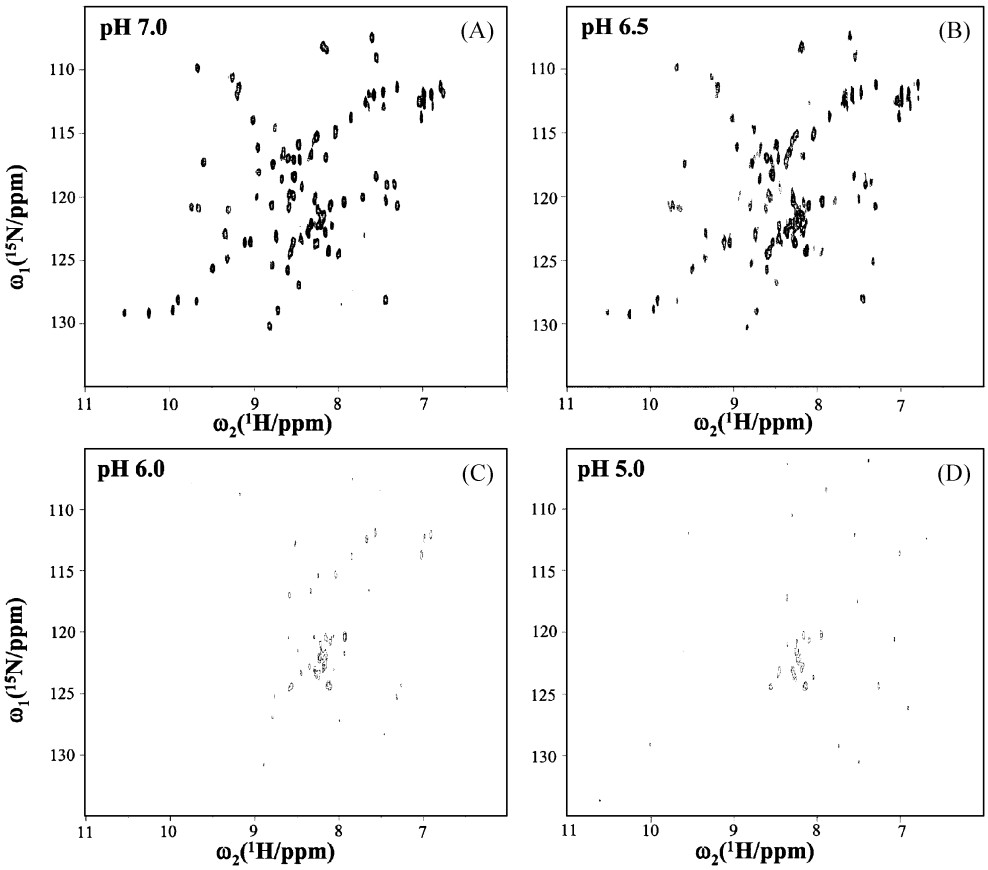

Fig. 4. A series of 2D ^{1}H-^{15}N HSQC data acquired at pH values of 5.0-7.0. The pH values were adjusted by adding 0.1 N HCl solution.

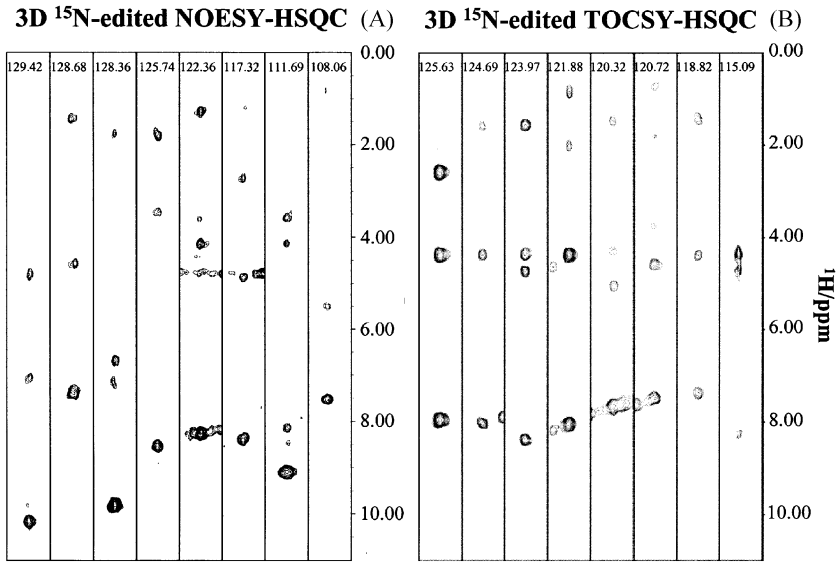

Fig. 5. Three-dimensional NMR spectra collected for ^{15}N-labeled the PTK6 SH3 domain. (A) Strips taken from the 3D ^{15}N-edited NOESY-HSQC spectrum recorded at 25°C. (B) Strips plot from ^{15}N planes of the 3D TOCSY-HSQC spectrum. The ^{15}N, HN and $C_{\alpha H}$ chemical shifts (ppm) are labeled for each axis.

suggest that even though the SH3 domain has a defined tertiary structure with a high population of β-sheets, the NMR sensitivity decreased dramatically during 3D NMR measurements (for three days) (Fig. 5). Zhang and Forman-

Kay reported that the N-terminal SH3 domain of *Drosophila* drk existed in a 1 : 1 ratio of the equilibrium state between the folded and unfolded conformations, even at a neutral pH condition (Zhang and Forman-Kay, 1995). However, our NMR data indicate that the PTK6 SH3 domain experiences a severe conformational instability in different pH environments. It was suggested that the PTK6 SH3 domain may be involved in signal transduction for the regeneration or differentiation of gastrointestinal epithelium, as well as the development of epithelial carcinomas (Lee *et al*., 1998; Llor *et al*., 1999). In particular, a recent report proposed that PTK6 could bind to the proline-rich region of Sam68, thereby phosphorylating Sam68 (Derry *et al*., 2000). Since the biological role of the SH3 domain of PTK6 is still unclear, despite the structural homology with those of other SH3 domains, the three-dimensional structural information together with the protein stability data would still be important in understanding the molecular function of PTK6. Therefore, our findings from this study will be a good starting point to determine the detailed structural information of the SH3 domain. Multi-dimensional NMR experiments are underway to determine the detailed three-dimensional structure.

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