The N-terminal 26-residue fragment of human programmed cell death 5 protein can form a stable α-helix having unique electrostatic potential character

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PDCD5-(1–26) is a N-terminal 26-residue fragment of human PDCD5 (programmed cell death 5) protein. PDCD5 is an important novel protein that regulates both apoptotic and non-apoptotic programmed cell death. The conformation of PDCD5 protein is a stable helical core consisting of a triple-helix bundle and two dissociated terminal regions. The N-terminal region is ordered and contains abundant secondary structure. Overexpression and purification of the N-terminal 26-residue fragment, PDCD5-(1–26), was performed in this study to better understand its tertiary structure. The spectroscopic studies using CD and hetero- and homo-nuclear NMR methods determine a stable α-helix formed by Asp³–Ala¹⁹ of PDCD5-(1–26). The N-terminal residues Asp³–Ala¹⁹ of PDCD5 were then affirmed to have the capacity to form a stable α-helix independently of the core of the protein. Analysis of the helical peptide of PDCD5-(1–26) indicates that the surface of this well-formed α-helix has a unique electrostatic potential character. This may provide an environment for the N-terminal α-helix of PDCD5 to serve as an independent functional entity of the protein. The apoptosis activity assay shows that the deletion of the N-terminal α-helix of PDCD5 significantly attenuates the apoptosis-promoting effects on HL-60 cells induced by serum withdrawal.

Key words: apoptosis, electrostatic potential surface, α-helix, NMR, programmed cell death 5 (PDCD5), solution structure.

INTRODUCTION

PDCD5 (programmed cell death 5) is a protein that is involved in the regulation of cell apoptosis [1]. Studies of the biological activities of the PDCD5 protein have found that PDCD5 is expressed in tumour cells during apoptosis, and the expressed level of PDCD5 is significantly higher than that in normal cells. The expressed PDCD5 protein has been shown to traverse rapidly from the cytoplasm to the nucleus of cells and distribute uniformly in cells that undergo apoptosis [2]. The enhancement of TAJ [toxicity and JNK (c-Jun N-terminal kinase) inducer]/TROY-triggered paraptosis-like cell death by PDCD5 overexpression suggested the cytoplasm to the nucleus of cells and distribute uniformly in cells that undergo apoptosis [3].

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The N-terminal sequence Ser¹–Gly²² of PDCD5 has eight amino acids identical with residues in the sequence Met¹–Gln²⁰ of MTH1615 (Figure 1). Thus it is interesting to determine experimentally whether the N-terminal residues of PDCD5 form an α-helix independently of the core of the protein. In the present study, we have successfully expressed and purified PDCD5-(1–26), a fragment containing residues 1–26 of PDCD5. The three-dimensional conformation of PDCD5-(1–26) was studied by CD and multidimensional NMR methods. Analysis of the structural features of PDCD5-(1–26) suggests that the N-terminal peptide corresponding to the first 20 residues of PDCD5 can form a stable α-helix independently of the core of the protein. For an investigation of the functional entity of the N-terminal α-helix in PDCD5, an N-terminal-truncated PDCD5 consisting of residues Met¹–Tyr¹⁵, PDCD5-(39–125), was purified. The apoptosis activity assay of PDCD5-(39–125) and PDCD5 was carried out.

MATERIALS AND METHODS

Expression and purification of PDCD5-(1–26) and PDCD5-(39–125)

For expression of PDCD5-(1–26), the fusion expression system used for expression of the intact human PDCD5 protein [6] was adopted. PDCD5-(1–26) was produced by mutation of Ala²⁷ of PDCD5 to a terminator in the expression plasmid pET-3d-HR52-PDCD5 by the QuikChange (Stratagene) method [7]. Two primers, primer 1 (5′-CGCCGATCCCTGTTAGATTAACCCACACAGGAAACCAAACG-3′) and primer 2 (5′-GCTTGGTCTCC-TGTTGGTCTAACCCAGGATCCCGG-3′) were used as the

Abbreviations used: HMOC, heteronuclear multiple-quantum correlation; HSQC, heteronuclear single-quantum correlation; MTH1615, Methanobacterium thermoautotrophicum 1615; NOE, nuclear Overhauser effect; PDCD5, programmed cell death 5; PS, phosphatidylserine; R.M.S.D., root mean square deviation.

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3 The atomic co-ordinates for PDCD5-(1–26) will appear in the Protein Data Bank under the accession code 1YYB.

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The solution was freeze-dried and stored at and has good solubility in water, since it is a small fragment. dissolved in 5 ml of water. Only PDCD5-(1–26) can refold well.

Far-UV CD measurement
The far-UV CD spectrum for PDCD5-(1–26) at concentration of 99.996 % 2H2O containing 50 mM phosphate buffer (pH 6.5) and 200 mM NaCl. The two-dimensional homonuclear 1H NOESY and TOCSY experiments were also carried out for naturally abundant PDCD5-(1–26) in aqueous solution.

Structure calculation
Based on the main-chain and side-chain resonance assignments, the NOE connectivity was identified for PDCD5-(1–26). The torsion angle restraints for PDCD5-(1–26) were generated with the program TALOS [11]. The structure calculations were carried out on Silicon Graphics station Onyx2 employing the program CNS1.1 [12]. A family of 200 structures was calculated, and the final family consisting of 20 conformers with the lowest target function was selected for structural analysis.

Induction and detection of cell apoptosis
HL-60 cells were cultured in complete medium consisting of RPMI 1640 medium supplemented with 15 % foetal bovine serum. For induction of apoptosis, HL-60 cells were washed twice in medium without serum. After the second wash, cells were resuspended and adjusted to 2 × 105/ml. Cell suspensions were added into 24-well plates (0.5 ml for each well), and then both intact PDCD5 and PDCD5-(39–125) with different amounts (20 and 30 µg) were added to each well. BSA (20 µg) and etoposide (20 µg) were used as negative and positive controls respectively. The cells were harvested after treatment for 20 h and washed twice with PBS, followed by resuspending in 100 µl of annexin-V-binding buffer (10 mM Hepes, 140 mM NaCl, 2 mM MgCl2, 5 mM KCl and 2.5 mM CaCl2, pH 7.4). FITC-conjugated annexin V (10 µl) (Beijing Biosea Biotechnology Co.) was added according to the manufacturer’s protocol. After incubation for 20 min at room temperature in the dark, another 400 µl of binding buffer was added, and samples were immediately analysed on

Figure 1 Sequence alignment of PDCD5-(1–26) with the N-terminal 29 residues of MTH1615 protein from a thermophilic archaeon [4]

<table>
<thead>
<tr>
<th>Human PDCD5(1–26)</th>
<th>GSADEELBAILRQRQRAELGAKHG ⋯ DPGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTH1615(1–29)</td>
<td>⋯ MTDLEIRRRKMLTEQKAQQAMEAADAQ</td>
</tr>
</tbody>
</table>

Samples of uniformly 15N-labelled and unlabelled PDCD5-(1–26) were prepared for NMR studies. PDCD5-(1–26) (2.0–4.0 mM) was dissolved in H2O/H2O (9:1, v/v) containing 50 mM phosphate buffer (pH 6.5), 200 mM NaCl and 0.01 mM NaN3.

All NMR spectra were acquired on a Bruker DMX 600 spectrometer equipped with a z-gradient triple-resonance cryo-probe. The three-dimensional heteronuclear NMR experiments, 1H–15N NOESY–HSQC (heteronuclear single-quantum correlation) and TOCSY–HSQC, were performed on 4.0 mM 15N-labelled PDCD5-(1–26) in aqueous solution with mixing times of 200 ms and 70 ms respectively. The two-dimensional 1H–15N HSQC experiments were recorded on 2.0 mM 15N-labelled PDCD5-(1–26) in aqueous solution. The two-dimensional 1H–13C HMQC (heteronuclear multiple-quantum correlation), HMBC–NOESY, and HMBC–TOCSY spectra were acquired for 3.0 mM naturally abundant PDCD5-(1–26) in 99.996 % 2H2O containing 50 mM phosphate buffer (pH 6.5) and 200 mM NaCl. The two-dimensional homonuclear 1H NOESY and TOCSY experiments were also carried out for naturally abundant PDCD5-(1–26) in aqueous solution.

The two-dimensional 1H–15N R2, R1, and NOE (nuclear Overhauser effect) spectra were obtained using standard pulse sequences with a water flip-back scheme [8]. 15NR2 rates were measured by a series of experiments performed with T2 relaxation periods of 0.02, 0.06, 0.14, 0.24, 0.36, 0.53, 0.76, 1.15 and 1.50 s. 15NR1 rates were recorded with a CPMG (Carr–Purcell–Meiboom–Gill) spin-echo period of 500 µs, and with T2 relaxation periods of 17.0, 33.9, 55.9, 67.8, 84.8, 101.8, 118.7, 135.7, 169.6 and 203.5 ms. The 1H–15N NOE values were collected by two 1H–15N HSQC experiments. In the NOE experiment, a delay of 2.0 s was followed by 1H saturation for 3.0 s. In the control experiment, the saturation duration was replaced by a delay of equivalent duration (3.0 s). The two experiments were performed in an interleaved manner.

All NMR data were processed and analysed with FELIX98 (MSc/Accentrics). The data points in each indirect dimension were usually doubled by linear prediction before zero filling to the appropriate size [9]. A 70–90° shifted square sine bell apodization was used for all dimensions before Fourier transformation. Chemical shifts were referenced to internal DSS (2.2-dimethyl-2-silapentane-5-sulphoxinate). 15N and 13C chemical shifts were referenced indirectly [10]. Experimental R1, R2, and NOE relaxation values, and the uncertainties associated with these parameters, were obtained by the same procedures as described in [4].
a FACSCalibur. Cells (1 x 10^6) were collected and analysed with CELLQuest software (BD Bioscience). Apoptotic cells are expressed as a percentage of total cells.

RESULTS

Expression and purification of PDCD5-(1–26) and PDCD5-(39–125)

For structural analysis of PDCD5-(1–26), a better signal separation of NMR spectra is necessary for resonance assignments. The most important advantage of the multinuclear and multidimensional NMR experiments is to reduce signal degeneracy in NMR spectra. Stable-isotope-labelled PDCD5-(1–26) is then required for this study. For fulfilling this requirement, the expression plasmid pET-3d-HR52-PDCD5-(1–26) with a small fusion partner His6-tagged HR52 was obtained. Expression and purification of PDCD5-(1–26) in Escherichia coli was analysed by 16.5 % tricine SDS/PAGE. The desired recombinant His6-tagged HR52–PDCD5-(1–26) was then purified by following similar procedures as described above. For investigation of the functional entity of the N-terminal α-helix in PDCD5, the N-terminal-truncated PDCD5 is required. PDCD5-(39–125) is a fragment of PDCD5 which was obtained by deletion of the N-terminal α-helix and a segment connecting the α-helix and triple-helix bundle of the protein. PDCD5-(39–125) was purified successfully, and the purification was analysed by following similar procedures as described above.

Secondary structure of PDCD5-(1–26) inferred from the CD spectrum

The far-UV CD spectrum of PDCD5-(1–26) is shown in Figure 2. Two apparent negative minima at 208 and 222 nm of the spectrum in the far-UV region show the presence of a stable helical secondary structure in PDCD5-(1–26). Analysis of the molar ellipticity values for PDCD5-(1–26) using the CONTIN program in the software package CDPro (http://lamar.colostate.edu/~sreeram/CDPro) provided the estimations of the secondary-structural components: 54.4% of α-helix, 28.4% of random coil, 12.6% of turn and 4.6% of β-sheet contents in PDCD5-(1–26).

Secondary chemical shifts of PDCD5-(1–26)

Analysis of the acquired heteronuclear NMR spectra provide the 1Hα and 15N resonance assignments for PDCD5-(1–26). Figure 3(A) shows the two-dimensional 1H–15N HSQC spectrum of PDCD5-(1–26). The dispersion of 1Hα–15N cross-peaks for residues of PDCD5-(1–26) in Figure 3(A) is highly similar to the distribution of cross-peaks for corresponding residues in PDCD5-(1–26). The majority of the cross-peaks appeared in the range 7.8–8.4 p.p.m. for 1Hα resonances and 108–123 p.p.m. for 15N resonances in both cases. Most cross-peaks for PDCD5-(1–26) have chemical shifts identical with corresponding cross-peaks for PDCD5, except for the two terminal residues Gly25 and Asp26. Cross-peak His21 showed slightly different chemical shifts in two spectra. The minor cross-peaks were observed for many residues of PDCD5-(1–26) in the two-dimensional 1H–15N HSQC spectrum (Figure 3A). The X-prolyl bond of Asp23–Pro24 may result in the appearance of major and minor cross-peaks of remote residues in the sequence of PDCD5-(1–26). Side-chain resonance assignments for PDCD5-(1–26) were identified by comprehensive analysis of all homonuclear and heteronuclear multidimensional NMR data collected for PDCD5-(1–26).

The CSI (chemical shift index) derived from 13Cα, and 1Hα chemical shifts for PDCD5-(1–26) is shown in Figure 4. A ‘dense’ group of positive secondary chemical shifts ΔαCα was observed for residues Ser1–Leu17. Secondary chemical shifts ΔαHα provided negative values for all residues in sequence region Asp3–Pro24 of PDCD5-(1–26). As has been indicated, 13Cα resonances of residues in α-helix experience a downfield shift of 2.0–4.0 p.p.m. compared with those in random coil, and 1Hα resonances shift upfield 0.15–0.60 p.p.m. [13,14]. Therefore the segment composed of residues Asp3–Leu17 of PDCD5-(1–26) is supposed to form an α-helical structure.

Determination of α-helix structure of PDCD5-(1–26)

Sequential and medium-range NOE connectivities were identified for the sequence of PDCD5-(1–26) through δαN, δαN, δαN, and δαN NOEs (Figure 5). PDCD5-(1–26) did not show any long-range NOE (more than five residues apart). The observed NOEs...
Figure 4 Secondary chemical shifts for $^{13}\text{C}_\alpha$ and $^{1}\text{H}_\alpha$ resonances of PDCD5-(1–26)

Secondary chemical shifts of PDCD5-(1–26) as a function of residue number (right panel). For comparison, the secondary chemical shifts for corresponding residues of human PDCD5 protein [4] are provided (left panel). The $\Delta^{13}\text{C}_\alpha$ and $\Delta^{1}\text{H}_\alpha$ values represent the characteristic NOE connectivity features of an $\alpha$-helix structure. The NOE connectivity together with the chemical shift index obtained for PDCD5-(1–26) provided a preliminary identification of the $\alpha$-helix structure in the fragment.

Figure 5 Summary of NOE connectivities obtained for PDCD5-(1–26)

The relative NOE intensities are indicated by the thickness of the bars, and the medium-range NOEs are indicated by lines connecting the two coupled residues.

represent the characteristic NOE connectivity features of an $\alpha$-helix structure. The NOE connectivity together with the chemical shift index obtained for PDCD5-(1–26) provided a preliminary identification of the $\alpha$-helix structure in the fragment.

Calculation of the structures was carried out using a total of 223 distance and 19 torsion angle restraints. Distance and dihedral angle restraints for structure determination and structure statistics of the 20 lowest energy structures from the family of 200 calculated structures are listed in Table 1. The superposition of 20 structures is shown in Figure 6(A). Validation of geometries of the 20 structures was performed using the program PROCHECK-NMR [15]. Ramachandran map analysis showed that the majority of the dihedral angles were in the most favored regions (85.7%) and none were in the disallowed regions (Table 1). The averaged R.M.S.D. (root mean square deviation) values for the segment involved in the $\alpha$-helix structure were $0.23 \pm 0.07$ Å ($1 \text{ Å} = 0.1$ nm) and $3.09 \pm 0.96$ Å for the backbone and all heavy atoms respectively, showing the well defined $\alpha$-helix structure (Figures 6B and 6C).

Backbone dynamics of PDCD5-(1–26)

The values of the $^{1}\text{H}$–$^{15}\text{N}$ relaxation parameters, $R_1$, $R_2$, and NOE are shown in Figure 7. The overall pattern of variations in the $R_1$, $R_2$, and NOE values as a function of residue number are similar to each other. The sequence dependence of the relaxation parameters for residues in sequence region Asp$^3$–Ala$^{19}$ are fairly constant; in particular, the $R_2$ and NOE values are generally smoother than $R_1$ values. The average values of $R_1$ and $R_2$ in this region are $3.34 \pm 0.43$ Hz and $5.36 \pm 0.36$ Hz respectively. The NOE values do not exceed the theoretical maximum, and the average value for residues Asp$^3$–Ala$^{19}$ is $0.58 \pm 0.14$. The residues at the C-terminal region Lys$^{20}$–Asp$^{26}$ have different relaxation features. A general decrease in the relaxation parameters occurs from residue Lys$^{20}$. Therefore residues Asp$^3$–Ala$^{19}$ form a stable ordered $\alpha$-helix structure, and residues in the tail region remain highly mobile.

Apoptosis triggered by PDCD5-(39–125) and intact PDCD5

A biochemical hallmark of apoptotic cell death is the translocation of PS (phosphatidylserine) from the cytoplasmic surface of the cell membrane to the external cell surface [16]. Exposure of PS at the surface of apoptotic cells is easily determined by flow cytometry using fluorescence-labelled annexin V, which specifically binds to PS (phosphatidylserine) from the cytoplasmic surface of the cell [16]. Exposure of PS at the surface of HL-60 cells induced by serum withdrawal. Since residues Lys$^{20}$–Glu$^{26}$ of PDCD5 is a segment that connects the N-terminal $\alpha$-helix and triple-helix bundle of PDCD5, weakening the effect on apoptosis-promoting cell apoptosis by PDCD5-(39–125) can be attributed to the deletion of the N-terminal $\alpha$-helix in PDCD5.

DISCUSSION

Usually, peptides are shown to be flexible and have no ordered structure in solution owing to the lack of long-range interactions.
Figure 6  Solution structure of PDCD5-(1–26)

(A) A stereo view of superimposed 20 accepted solution structures of PDCD5-(1–26). (B) Stereo view shows the side-chains of residues Asp²–Ala¹⁹ of PDCD5-(1–26). The heavy atoms of the side-chains are presented as sticks. (C) Ribbon presentation of α-helix structure of PDCD5-(1–26).
Figure 7  Relaxation rate constants $R_1$ and $R_2$ and steady-state NOE values for PDCD5-(1–26)

Errors were derived from the uncertainties of the least-squares fit to the exponential decay function (for $R_1$ and $R_2$) or from the signal-to-noise ratios (for NOE).

The N-terminal residues of PDCD5 do not provide any long-range NOE contacts with the residues in the main part of the protein [4]. However, a stable well-formed $\alpha$-helix was determined for residues Asp$^3$–Ala$^{19}$ of fragment PDCD5-(1–26), and the tail region consisting of residues Lys$^{20}$–Asp$^{26}$ is flexible and unstructured. The similar secondary chemical shifts and distribution of cross-peaks in the two-dimensional $^1$H–$^{15}$N HSQC spectra obtained for residues of PDCD5-(1–26) to those obtained for corresponding residues of PDCD5 (Figures 3 and 4) suggest that the N-terminal residues of PDCD5 can form a stable $\alpha$-helix structure independently of the core of the protein.

The sequence region Asp$^3$–Ala$^{19}$ of PDCD5-(1–26) contains four leucine residues, three alanine residues, and eight charged and two polar amino acid residues. Figure 9(A) shows a helical wheel construction of residues Asp$^3$–Ala$^{19}$ in the $\alpha$-helix. The helical wheel model reveals a symmetric distribution of the majority of residues for this $\alpha$-helix structure. Paired residues Leu$^{17}$/Leu$^6$, Arg$^{10}$/Arg$^{13}$, Leu$^{14}$/Leu$^9$, Glu$^7$/Glu$^{16}$, and Ala$^{15}$/Ala$^8$, having identical amino acid type, are located symmetrically on the left and the right of the helical wheel in Figure 9(A). If we spread up the front and the back sides of the helix, as shown in Figure 9(B), we notice an interesting arrangement of the residues along the $z$-axis of the helix. In the sketched map for the front side of the helix, residues Glu$^7$, Arg$^{10}$, Arg$^{13}$ and Glu$^{16}$ of two paired residues Glu$^7$/Glu$^{16}$ and Arg$^{10}$/Arg$^{13}$ aligned along an oblique line downwards with the parallel alignment of four leucine residues, Leu$^6$, Leu$^9$, Leu$^{14}$ and Leu$^{17}$, on both sides (left-hand

Figure 8  Flow cytometric analysis of HL-60 cells apoptosis induced by serum withdrawal

HL-60 cells were treated with a different amount of both PDCD5-(39–125) and intact PDCD5. Percentages of cell apoptosis were determined by flow cytometry with annexin-V–FITC. Numbers of M2 indicate the corresponding proportions of apoptotic cells that display positive annexin-V–FITC labelling. BSA and etoposide were used as negative and positive controls respectively. Experiments were performed in triplicate, with similar results.
Figure 9 Surface character of helix formed by PDCD5-(1–26)

(A) Helical wheel model of residues Asp3–Ala19 of PDCD5-(1–26) with NMR results taken into account. (B) Sketch maps showing the spread front (left-hand panel) and back (right-hand panel) sides of the helix. (C) Electrostatic potential surface of the helix calculated using MOLMOL [22]. The left-hand representation is the front face and the right-hand one is the back face of potential surface. The surface ionic groups are coloured from blue to red in accordance with the electrostatic charge from most positive to most negative.

Panel, Figure 9B). The arrangement of Leu6/Arg10/Leu14 and Leu9/Arg13/Leu17 generates a characteristic hydrophobic/hydrophilic/hydrophobic potential surface of the front side of the helix (Figure 6B). On the spread back side of the helix, Ala8 and Ala15 aligned nearly parallel to the z-axis of the helix, with the hydrophilic residues, Glu5, Glu12 and Glu16 on one side, and Glu4, Glu7, Arg11 and Gln18 on the other side (right-hand panel, Figure 9B). The back side of the helix provides a potential surface having hydrophilic/hydrophobic/hydrophilic character. In general, the back side of the helix has a surface that is more hydrophilic than the front side of the helix. Figure 9(C) shows the electrostatic potential surface on the front and back faces of the helix. Therefore the helix formed by PDCD5-(1–26) has a special character, which is different from the amphiphilic helical peptide. As is well known, the amphipathic character of amphiphilic helical peptide, defined as an α-helical domain containing opposite polar and non-polar ‘face’ [18], is found in many helices that are associated with plasma membrane. The plasma-membrane-associated helices may be either the trans-membrane or in-plane helices in the membrane bilayer [19].

Observation of the expression and localization of PDCD5 in the cell apoptosis process demonstrated the rapid translocation of PDCD5 to the nucleus of target cells undergoing apoptosis. The accumulation of PDCD5 in the nucleus precedes the chromosome DNA fragmentation and PS externalization [2]. However, how PDCD5 crosses the nuclear envelope and functions in the nucleus is still unclear. The N-terminal α-helix of PDCD5 has free orientation relating to the main part of the protein [4]. This may provide an environment for the N-terminal α-helix to serve as a functional entity independent of the PDCD5 protein. The apoptosis activity assay indicates that the deletion of the N-terminal α-helix of PDCD5 attenuates the apoptosis-promoting effects on HL-60 cells induced by serum withdrawal. This may correlate with different accumulations of PDCD5-(39–125) and intact PDCD5 in the nucleus. Thus it may be that the features of translocation of PDCD5-(39–125) and intact PDCD5 to the nucleus of cells are different. The N-terminal α-helix may play some role in the translocation of PDCD5 to the nucleus of target cells.

This research was supported by the National Natural Science Foundation of China (NNSFC 30170201), the 863 Special Program of China (Grant No. 2002BA711A13) and the Key Important Project from Chinese Academy of Sciences (Grant No. KSCX1-SW-17).

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Received 27 April 2005; accepted 5 August 2005
Published as BJ Immediate Publication 5 August 2005, doi:10.1042/BJ20050688

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