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Structural Mapping of the C-terminal Domain of Human P73

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INTRODUCTION

Human P73 is a recently-discovered homologue of the tumor suppressor P73. The location of its gene in chromosome 1 is deleted in many cancers, including colon and breast cancers, melanoma and neuroblastoma.[1] Its gene encodes two splice-variants, P73-α and β, both exhibiting high sequence and structural similarity to P53, with an N-terminal transactivation domain, a central DNA-binding domain, and an oligomerization domain.[1] However P73-α contains 136 extra residues in its C-terminus compared to the β variant, and 213 additional residues when compared to P53. Since this domain is not present in P53 or the P73β variant, it may be important in defining a unique function for P73, or may function in modulating P53-like functions of P73 through alternative oligomerization arrangements or interactions.

Towards understanding the functional and/or structural roles of this C-domain of P73α, we have begun NMR spectroscopic studies of several constructs of the C-terminus, chosen using sequence alignments with two other proteins which also exhibit an extended C-terminus: rat KET protein,[2] and squid(*Loligo forbesi*) P53.[1,2] We report here the NMR characterization of several constructs of C-terminus of P73 and the identification of a small folded globular domain formed by residues 487-554.

EXPERIMENTAL PROCEDURES

Protein expression and purification

The sequences encoding residues 345-636, 487-636, 487-600, 487-565, and 487-554 of human P73 were subcloned into the PET-15b vector using PCR techniques.[3] Gel-purified digests of PCR products were inserted into the PET-15b expression vector following cleavage with the appropriate restriction enzymes. All the plasmids express the 21 residue his-tag in the N-terminus.

E. coli BL21(DE3)[pLysS], harboring the PET-15b plasmid clone of the constructs was pre-cultured in 20 ml LB medium to stationary phase. This pre-culture was added to 2L of M9 medium with 240mg/L $MgSO_4$, 15mg/L $CaCl_2$, 20mg/L thiamine, 1g/L $^{15}NH_4Cl$, and/or 2.5g/L $^{13}C_6$-D-glucose. As the sole nitrogen and carbon source, $^{15}NH_4Cl$ and $^{13}C_6$-D-glucose were used for uniform ^{15}N-labeling and $^{15}N/^{13}C$-labeling. Cells were grown at 37°C to an A_{600} of 0.7 before induction with 1mM IPTG. Cultivation was continued after the induction for 5 hrs, and the cells were harvested by centrifugation. Following cell lysis by sonication in lysis buffer (5mM imidazole, 500mM NaCl, 20mM tris, pH 8, 1mM benzamidine, 1mM PMSF, 0.1% Triton X-100), and centrifugation to remove cell debris, the supernatent was loaded onto a Ni^{2+}-NTA affinity column. The proteins were eluted with a stepwise gradient of 5-500mM imidazole. Next, the proteins were concentrated by using the ultrafiltration units, and finally, the proteins were dialyzed into the final NMR buffer (25mM sodium phosphate, pH 6, 150mM NaCl, 1mM DTT, 1mM benzamidine). Each preparation yielded 10 to 20 mg protein per two liter culture. The final concentrations of the proteins were 1-1.2 mM.

NMR spectroscopy

All the NMR data were acquired at 25℃ on a Varian Unity600 or Varian Unity+500 spectrometer equipped with an actively shielded triple resonance probe. All experiments in H_2O solution were performed using Pulse-Field Gradient (PFG) techniques for water suppression. 2D ^{15}N-^1H HSQC spectra[4] were collected using the enhanced sensitivity method.[5] NOE connectivities were identified from the 3D ^{15}N-NOESY-HMQC (150ms)[6] and $^{13}C/^{15}N$ NOESY-HSQC spectra (150ms) [7] in 90% H_2O/10% D_2O. All data were processed on Sun Sparc2 or Sparc10 workstations using the NMRpipe software system and analyzed with program NMRview.

RESULTS AND DISCUSSION

Sequence alignment

It has recently revealed that squid P53 protein and rat KET protein have very strong similarity to human P73, exhibiting an extra domain in the C-terminus as with human P73. The alignment of the primary sequences of squid P53, rat KET protein, and P73 was carries out to identify the possible constructs for the expression of the C-terminus domain. The alignment shows significant homology among between the 3 proteins for residues 489-598 of P73 C-terminus (Figure 1). Initially, three candidate constructs (345-636 includes the P73 oligomerization domain, 487-636, and 487-600) were selected, subcloned and expressed. These constructs were not very soluble and therefore were purified under denaturing conditions. The constructs were subsequently refolded, retaining the N-terminus His-tag for improved solubility.

P73(487-600)

Among the 3 candidate constructs, P73(487-600) was most soluble (up to 0.7mM). 2D ^{15}N-^{1}H HSQC and 3D ^{15}N-NOESY-HMQC experiments on P73(487-600) were performed. The 2D ^{15}N-^{1}H HSQC spectrum of this domain is shown Figure 2. Most of peaks are shown to be well resolved except the overlapped central region.

However, this construct showed low stability against proteolytic digestion and aggregation during the acquisition of 3D ^{15}N-NOESY-HMQC spectra. Some peaks in ^{15}N-^{1}H HSQC spectrum shifted their positions during the acquisition. After 3-day acquisition, two protease-digested fragments were detected by SDS gel electrophoresis. Addition of more protease inhibitors such as aprotinin and leupeptin stabilized the domain against proteolysis but it caused increased aggregation, leading to the elimination of many peaks. Higher temperatures (up to 37℃) during the acquisition did not improve the NMR spectra obtained.

P73(487-554)

From the biochemical analysis of the P73(487-600), two more constructs of the P73 C-terminus were designed and subcloned. The MW of the shortest proteolytic fragment of P73(487-600) was found to be 10 KDa from the mass spectrum. N-terminal sequencing of this proteolytic fragment showed that the N-terminal residues were intact, indicating that the C-terminal region of the protein was digested by proteases during the NMR acquisition.

Therefore, two shorter, more stable constructs against proteolytic digestion were designed: residues 487-554 and 487-565. The maximum achievable concentration of P73(487-565) was 0.3 mM. On the other hand, P73(487-554) was highly soluble (up to 1.8 mM), enabling purification of the domain under native conditions. This construct was also very stable against proteolytic digestion and aggregation in contrast to the P73(487-600). Therefore, P73(487-554) was selected as the most optimal construct for NMR study.

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p73    ADPSLVSFLT GLGCPNCIEY FTSQGLQSIY HLQNLTIEDL GALKIPEQYR MTIWRGLQDL 548
KET    TDCSIVSFLA RLGCSSCLDY FTTQGLTTIY QIEHYSMDDL ASLKIPEQFR HAIWKGILDH 558
p53aq tENTIAQWLT KLGLQAYIDN FQQKGLHNMF QLDEFTLEDL QSMRIGTGHR NKIWKSLLDY 514

PHD    ...HHHHHHHH...HHHHHHHHH..HHHHHH.....HHHHHH....HHHHHHHHHHHHHHHHH..

p73    KQGHDYSTAQ QLLRSSNAAT ISIGGSGELQ RQRVMEAVHF RVRHTITIPN 598
KET    RQLHDFSSPP HLLrpSGAST VSVGSS.ETR GERVIDAVRF TLRQTISFPP 608
p53aq RRLLSSGTES QALqaSNAST LSVGSQNSY. CPGFYEVTRY TYKHTIS... 562

PHD    .............EEEE..........EEEEEEEEEEEEE............
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Fig. 1. The C-terminal sequence of human P73 and comparison with those of the rat KET and squid P53(p53aq)proteins The secondary structure prediction of the human P73 was performed by using the PHD(Profile network prediction Heidelberg)program in the PredictProtein server of EMBL(European Molecular Biology Laboratory).[8] "H" and "E" indicate α-helix and β-sheet, respectively while "."indicates loop.

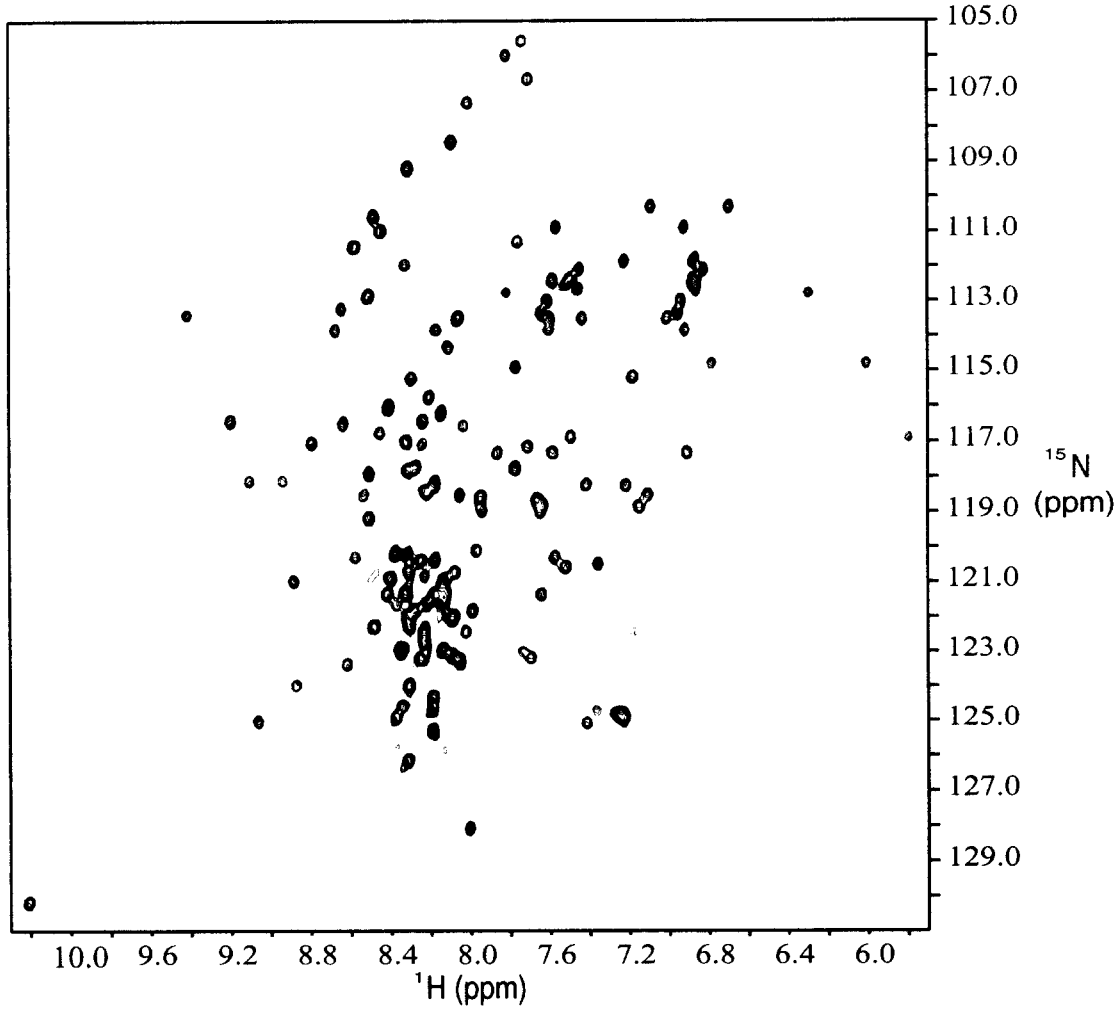

Fig. 2. The ^{15}N-^{1}H HSQC spectrum of P73(487-600)

The domain of P73(487-554) shows much better-resolved ^{15}N-^{1}H HSQC spectrum in Figure 3 than that of P73(487-600).

Two conformers of the P73(487-554)

From the ^{15}N-^{1}H HSQC spectra, two sets of peaks with different intensities were observed. It is likely that two sets of peaks correspond to 2 different species. The 3D ^{15}N-NOESY-HMQC spectrum showed that they have the same chemical shift in aliphatic region but with the different chemical shift in the amide region, indicating that the two sets of peaks might come from the same residues which have slightly different backbone conforma-

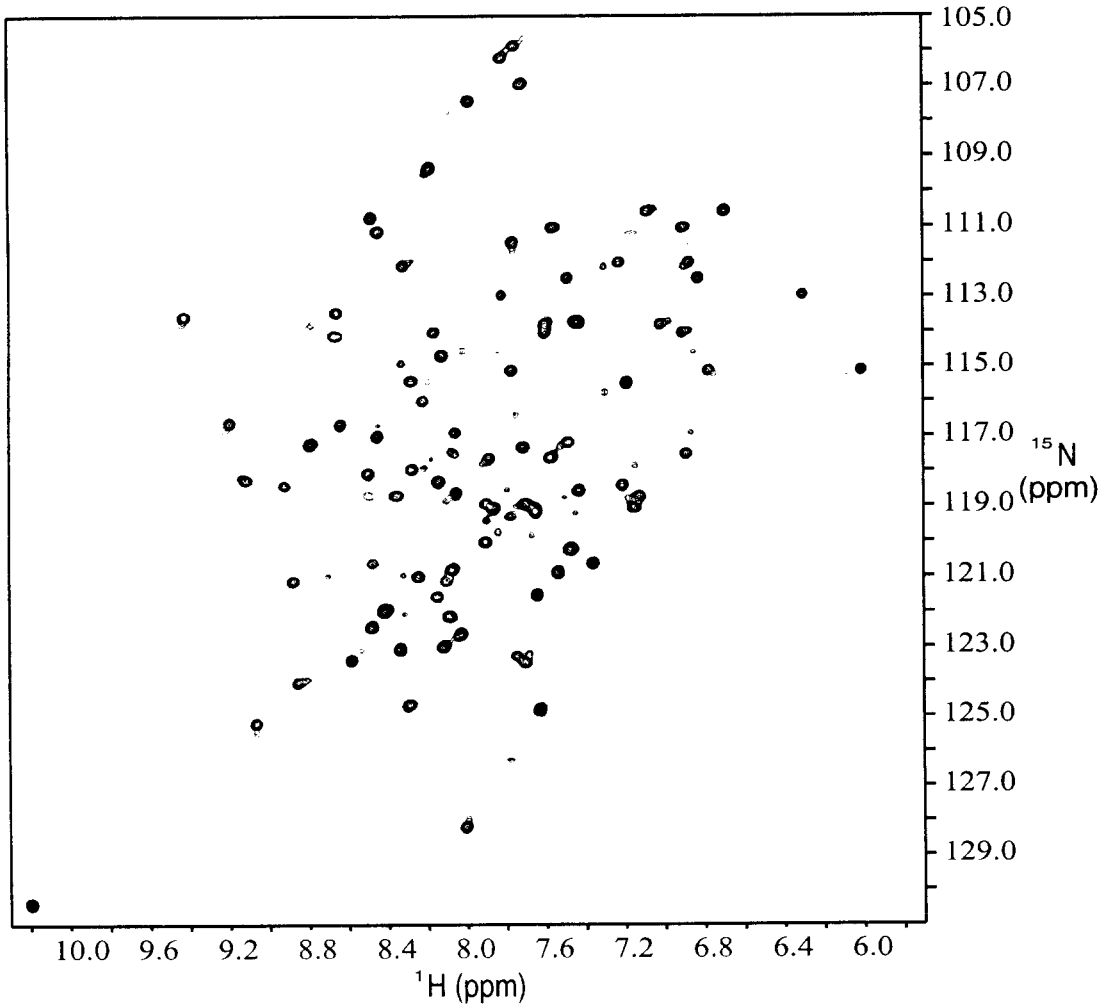

Fig. 3. The ^{15}N-^{1}H HSQC spectrum of P73(487-554)

tions. No exchange peaks between diagonal peaks in ^{15}N-NOESY-HMQC were observed, indicating that the two species are in slow exchange on the NMR timescale. A slight change of relative intensities between the two conformers was observed during the acquisition. The intensities of weaker peaks became weakened after a 3 day acquisition of an ^{15}N-NOESY-HMQC spectrum. This indicates the possibility that the two conformers are dynamically interconvertible.

Assuming the presence of 2 dynamically interconvertible species, we tried to shift the equilibrium between the two different populations to get only one stabilized conformer by changing the temperature and the salt in the buffer. The temperature was changed from 25℃ to 37℃, but no effects were observed. The salt was also changed from NaCl toNa_2SO_4; Sulphate ion is known to be a chaotropic agent leading to increased hydration around protein surface, which was expected to increase the thermal stability of the protein.

Contrary to our expectation, the intensities of the weaker set of peaks increased.

Comparison between P73(487-600) and P73(487-554)

From the comparison of 2D ^{15}N-^{1}H HSQC spectra, it was found that the set of peaks with higher intensities in P73(487-554)have chemical shifts similar to those of P73(487-600). The NOEs in the ^{15}N-NOESY-HSQC spectra of both constructs were analyzed and compared. Few difference between them could be detected when the positions of cross peaks in both ^{15}N-^{1}H HSQC spectra matched. This agreement seems to be shown particularly in the structured region as most of the matched peaks have inter-residue NH to NH NOEs. It appears therefore that there are no significant structural differences between the both constructs. The overlapping peaks in the central region of the ^{15}N-^{1}H HSQC of P73(487-600)have no match with those of P73(487-554) and have no inter-residue NH to NH NOEs in ^{15}N-NOESY-HMQC spectra. They are presumed to be from the unstructured C-terminal portion of P73(487-600), which is not present in P73(487-554). Circular Dichroism spectra of both the constructs showed that they have substantial α-helical content. The α-helical content of P73(487-554)is 14% higher than that of P73(487-600). Thus it may be concluded that P73(487-554)retains a structure similar to that adopted by P73(487-600), and therefore we will concentrate our NMR analysis on the shorter construct.

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