

# NMR structure of the death domain of the p75 neurotrophin receptor

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**The intracellular domain of the p75 neurotrophin receptor (p75ICD) lacks catalytic activity but contains a motif similar to death domains found in the cytoplasmic regions of members of the tumor necrosis factor receptor family and their downstream targets. Although some aspects of the signaling pathways downstream of p75 have been elucidated recently, mechanisms of receptor activation and proximal signaling events are unknown. Here we report the nuclear magnetic resonance (NMR) structure of the 145 residue long p75ICD. The death domain of p75ICD consists of two perpendicular sets of three helices packed into a globular structure. The polypeptide segment connecting the transmembrane and death domains as well as the serine/threonine-rich C-terminal end are highly flexible in p75ICD. Unlike the death domains involved in signaling by the TNF receptor and Fas, p75ICD does not self-associate in solution. A surface area devoid of charged residues in the p75ICD death domain may indicate a potential site of interaction with downstream targets.**

**Keywords:** death domain/NMR/p75 neurotrophin receptor

## Introduction

The low-affinity p75 neurotrophin receptor (p75) was identified 10 years ago as a membrane protein with the ability to bind nerve growth factor (NGF) (Chao *et al.*, 1986; Radeke *et al.*, 1987). Later works established that p75 was capable of binding all members of the NGF family (Rodríguez-Tébar *et al.*, 1990, 1992; Rydén *et al.*, 1995), also known as the neurotrophins, a group of structurally and functionally related polypeptides that regulate neuronal survival, differentiation and repair (Ibáñez *et al.*, 1995; Lewin and Barde, 1996). Because the short intracellular domain of p75 (p75ICD) lacked features found in other receptors with known catalytic activity, the subsequent discovery of the Trk family of receptor tyrosine kinases as ligand-specific signaling receptors for the neurotrophins cast doubts on the intrinsic signaling capabilities of p75 (Kaplan *et al.*, 1991; Klein *et al.*, 1991). Various models involving the ligand-binding domain of p75 have been presented to account for the documented ability of p75 to aid in the formation of high

affinity binding sites (Hempstead *et al.*, 1991; Mahadeo *et al.*, 1994) and in Trk signaling (Barker and Shooter, 1994; Hantzopoulos *et al.*, 1994; Verdi *et al.*, 1994), including models of ligand presentation, ligand concentration and changes in receptor conformation.

The extracellular domain of p75 has structural similarities to the tumor necrosis factor p55<sup>TNFR</sup> receptor and to the Fas antigen (Meakin and Shooter, 1992), which, upon ligand binding, signal through a cascade of protein–protein interactions triggered in part by a 90 residue long motif in the cytoplasmic tail of these receptors. Because of the cytotoxic activities of p55<sup>TNFR</sup> and Fas, this motif has been known as the death domain (Tartaglia *et al.*, 1993). Several downstream targets of the p55<sup>TNFR</sup> and Fas death domains have been identified and these also contain death domain sequences (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995; Stanger *et al.*, 1995; Duan and Dixit, 1997) indicating a signaling mechanism triggered by the association of death domain-containing proteins (Varfolomeev *et al.*, 1996). The similarity of the ligand-binding domain of p75 to those of p55<sup>TNFR</sup> and Fas has been reinforced by the discovery of a somewhat divergent death domain in p75ICD (Chapman, 1995; Feinstein *et al.*, 1995). This second subtype of death domain has also been found in several other proteins, including p100 NF- $\kappa$ B, p105 NF- $\kappa$ B, DAP kinase and myD88 (Feinstein *et al.*, 1995). The functional role of the death domain in any of these proteins is, however, unknown. Recently, evidence has begun to accumulate supporting the intrinsic signaling capacities of p75. It has been shown that neurotrophin binding to p75, in a cell devoid of cognate Trk receptors, stimulates sphingomyelin hydrolysis and ceramide production (Dobrowsky *et al.*, 1994, 1995). In addition, only NGF among the neurotrophins has been shown to trigger the nuclear translocation of NF- $\kappa$ B in a p75-dependent fashion in Schwann cells and p75-expressing fibroblasts (Carter *et al.*, 1996b). Finally, using the yeast two-hybrid system, at least one novel protein has been isolated that is capable of interacting with p75ICD (Carter *et al.*, 1996a). A link between p75 and cell death has also been established, although there are discrepancies as to whether the death induced by p75 is ligand-dependent or ligand-independent (Rabizadeh *et al.*, 1993; Barrett and Bartlett, 1994; Casaccia-Bonnet *et al.*, 1996; Frade *et al.*, 1996).

The importance of the death domain as a signaling module, and of p75 in the control of cell death and neurotrophin signaling, prompted us to elucidate the three-dimensional structure of p75ICD, comprising the p75 death domain and most of the juxtamembrane region. Recently, the nuclear magnetic resonance (NMR) structure of the Fas death domain was reported (Huang *et al.*, 1996). We present here the NMR structure of the p75 death domain which, unexpectedly, shows significant differences to the death domain of Fas. Our structural data

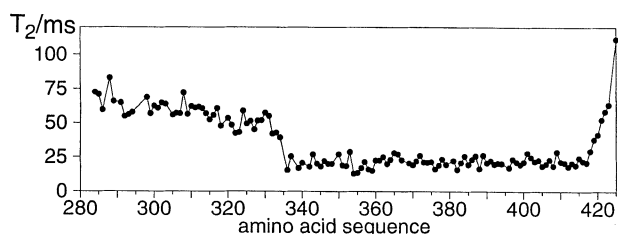
on the full-length p75ICD suggest novel mechanisms of signaling for proteins containing a subtype 2 death domain as in p75.

## Results and discussion

### Solution structure of p75ICD

Different linewidths were observed for the  $^1\text{H}$  NMR signals of p75ICD, indicating substantially increased mobility on a subnanosecond timescale for much of the polypeptide chain. A quantitative measurement of the transverse relaxation of the amide protons showed that residues 336–418 were structurally rigid, whereas the first 55 and the last six amino acid residues of p75ICD were highly flexible (Figure 1). The flexible N- and C-terminal residues of p75ICD retained their mobility also in the presence of high osmolyte concentration (2 M glycine) which, in other examples, protects protein conformations against denaturation (Matthews and Leatherbarrow, 1993). The conformationally structured segment comprising residues 336–416 represents the death domain of p75ICD (Feinstein *et al.*, 1995).

The NMR structure of the death domain of p75ICD (Table I) contains six helices (Figure 2). Their approximate lengths are from residues 343 to 351, 356 to 363, 368 to 375, 380 to 390, 396 to 406 and 409 to 416. The helices are arranged in two perpendicular sets of three helices each, with approximately parallel helix axes within each set. The N- and C-terminal polypeptide segments of the death domain are close together. A search of the Brookhaven protein data bank (Bernstein *et al.*, 1977) with the program DALI (Holm and Sander, 1993) did not reveal any other protein or protein domain of similar three-dimensional structure. Thus, the only known structure of similar topology is represented by the recently described death domain of the Fas antigen (see below), the coordinates of which are not yet accessible in the database. The



**Fig. 1.** Transverse relaxation time  $T_2$  of the amide protons versus the amino acid sequence of p75ICD.

death domain of p75 is a small globular structure of ~25–30 Å diameter in all dimensions. The side chains of the charged residues are more or less uniformly distributed over most of its surface, with the exception of a surface area facing the viewer in the orientation shown in Figure 2. This part of the domain is largely devoid of charged residues and therefore is the most plausible contact area for putative hydrophobic interactions with as yet unknown downstream signaling proteins.

The conformation of the death domain of p75ICD was found to be not very stable. Only a few amide protons of helix 5 were significantly protected from exchange with the solvent in experiments in which lyophilized p75ICD was dissolved in  $\text{D}_2\text{O}$  and the  $^1\text{H}$  NMR signals of the amide protons monitored (data not shown). The limited stability of the death domain is also indicated by the onset of denaturation at pH 5 and below.

### Structural comparison with other death domains

The NMR structure of the death domain of p75 is the first example of a subtype 2 death domain (Feinstein *et al.*, 1995). Compared with the NMR structure recently described for the subtype 1 death domain of the Fas antigen (Huang *et al.*, 1996), the death domain of p75 shows a number of important similarities and differences which allow the delineation of the conserved structural features of death domains.

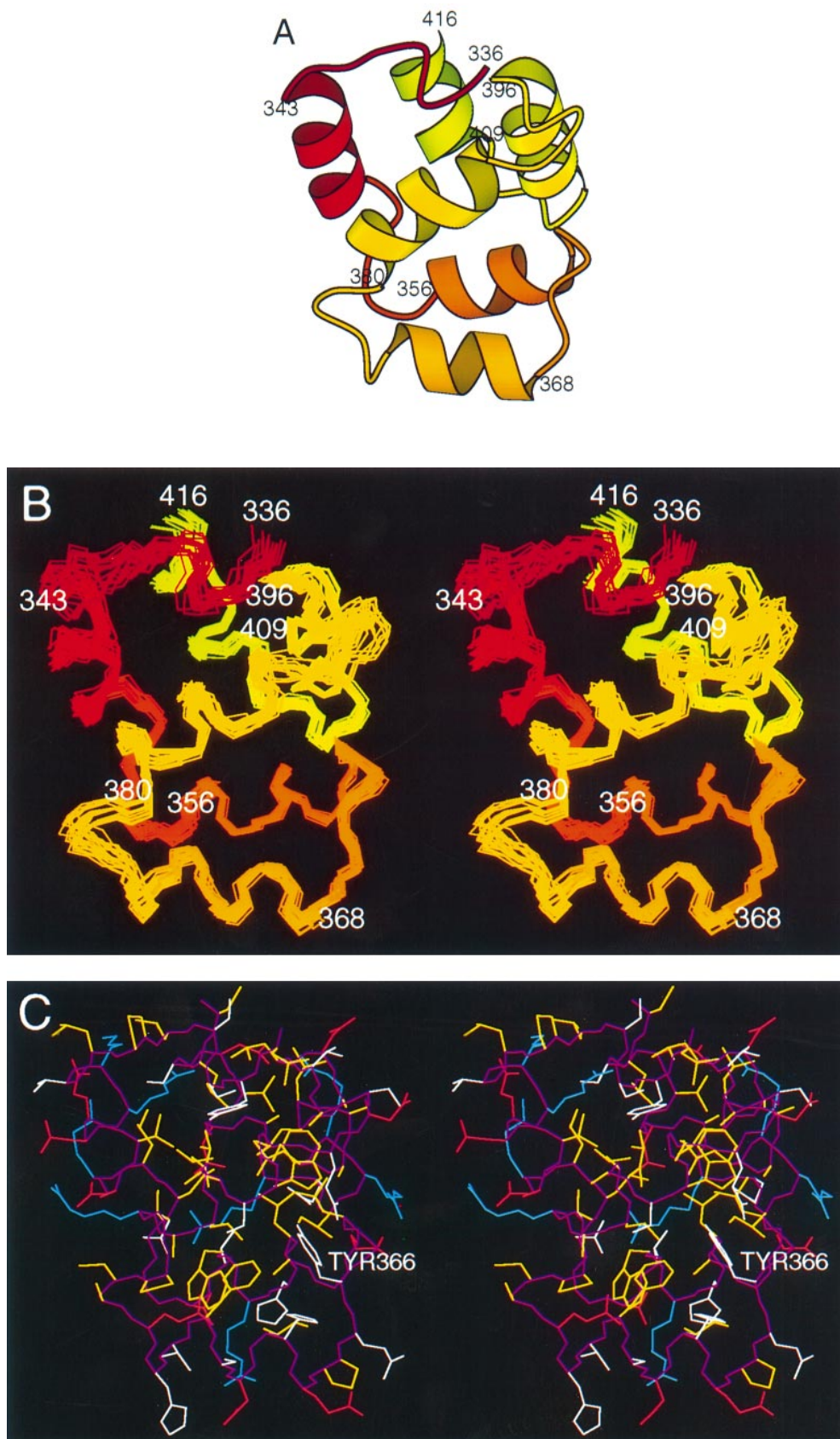
The total number of helices (six) and the overall fold of the death domain is similar in p75 and in Fas. Yet, while the relative orientations of helices 2–6 are conserved, helix 1 in p75ICD is almost perpendicular to the orientation of helix 1 in the Fas death domain. This helix lies about parallel to helices 2, 3 and 4 in Fas, but is more closely aligned to helices 5 and 6 in p75ICD (Figure 2). The extent of this structural rearrangement is unusually large for a conserved protein domain. On the other hand, there is little sequence similarity in helix 1 between p75 and Fas (Figure 3) (Feinstein *et al.*, 1995), and the death domain of Reaper appears to lack helix 1 altogether (Golstein *et al.*, 1995). The amino acid sequence of helix 1 is better conserved among subtype 2 than subtype 1 death domains (Feinstein *et al.*, 1995), suggesting that the structures of subtype 2 death domains will in general be close to the death domain structure of p75ICD.

With the exception of helices 2, 5 and 6, the helical segments are of similar lengths in the death domains of p75 and Fas (Figure 3). Helix 5 in p75ICD starts with a classical N-cap residue (Thr395) (Richardson and

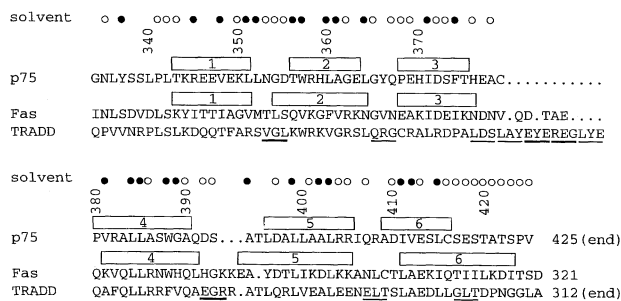
**Table I.** Parameters characterizing the NMR structure of p75ICD

Parameter	Value
Number of distance constraints (residues 334–418)	914
Number of dihedral angle constraints (residues 334–418)	210
Distance constraint violations $>0.1$ Å (per conformer)	$0.55 \pm 0.67$
Dihedral angle constraint violations $>2.5^\circ$ (per conformer)	$0.40 \pm 0.58$
Intra-protein AMBER energy (kcal/mol)	$-3910 \pm 59$
R.m.s.d. to the mean for N, $\text{C}^\alpha$ and $\text{C}'$ of residues 336–416	$0.6 \pm 0.1$
R.m.s.d. to the mean for all heavy atoms of residues 336–416	$1.0 \pm 0.1$

The NMR structure of p75ICD is represented by 20 conformers calculated with the program DIANA (Güntert *et al.*, 1991) starting from random structures. The structures were calculated closely following the protocol of Liepinsh *et al.* (1994). The conformers were energy minimized with the program OPAL (Luginbühl *et al.*, 1996).



**Fig. 2.** Solution structure of the death domain of rat p75ICD. (A) Ribbon representation of residues 336–416. The positions of the N-terminal residues of the helices are labeled. (B) Stereo view showing a superposition of the backbone atoms of residues 336–416 in the 20 conformers representing the NMR structure (Table I). Same orientation and labeling as in (A). (C) Stereo view of the conformer closest to the mean structure of the 20 conformers shown in (B). Residues 336–416 are shown in a heavy atom representation in the same orientation as in (A) and (B). The polypeptide backbone is drawn in purple. The following colors were used for the side chains: blue, Arg and Lys; red, Glu and Asp; yellow, Ala, Cys, Ile, Leu, Phe, Pro, Trp, Val; white, Asn, Gln, Ser, Thr, His. Tyr366 at the site of the *lpr*<sup>cs</sup> mutation is labeled.



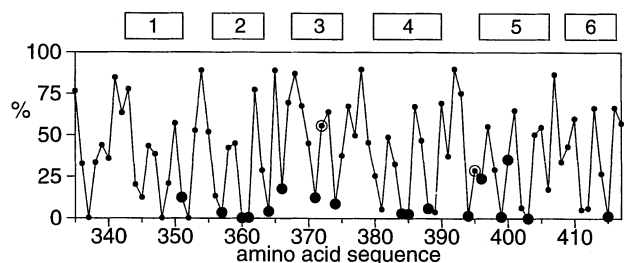
**Fig. 3.** Amino acid sequence alignment of the death domains of p75, Fas and TRADD. The top panel shows the solvent accessibility of the amino acid side chains in p75ICD (Figure 4). Filled and open circles: <15% and >50% side chain solvent accessibility, respectively. The sequence numbering above the amino acid sequences is that of rat p75. The helix locations in p75 and Fas are indicated above the respective amino acid sequences. Bars underneath the amino acid sequence of TRADD identify the tripeptide segments which were mutated to alanine in the study of Park and Baichwal (1996) and for which the side chains in the wild-type protein are presumably highly solvent exposed at the same time (compare top panel). Narrow and wide bars identify, respectively, little and significant reduction of self-association upon mutation (Park and Baichwal, 1996). Note that the sequence alignment used for helix 1 differs from that of Feinstein *et al.* (1995) for optimum alignment of the helical secondary structure. Furthermore, rat p75 has a deletion of three amino acid residues between helices 1 and 2 compared with human p75.

Richardson, 1988) which is highly conserved in most other death domains except that of Fas (Feinstein *et al.*, 1995). The N-terminal extension of helix 5 may thus be specific to the Fas receptor. Similarly, helix 6 extends much further towards the C-terminus in Fas than in p75ICD, which has instead a flexible C-terminal segment rich in serine and threonine (Figures 1 and 3).

Despite the structural variability observed between death domains of different subtypes, the hydrophobic core appears to be built by homologous residues. Using the sequence alignment of Feinstein *et al.* (1995), 14 of the 19 core residues conserved in different death domains are buried in the interior of the death domain of p75ICD with side chain solvent accessibilities below 15% (Figure 4). Of the two conserved residues with the highest solvent accessibility, one is charged (Asp372 in p75ICD) and one is a classical N-cap residue for helix 5 (Thr395 in p75ICD) in almost all other death domains. About 85% of the residues with <15% side chain solvent accessibility in p75ICD are hydrophobic. Most of these residues are also hydrophobic in the other death domains (Feinstein *et al.*, 1995).

### Functional implications

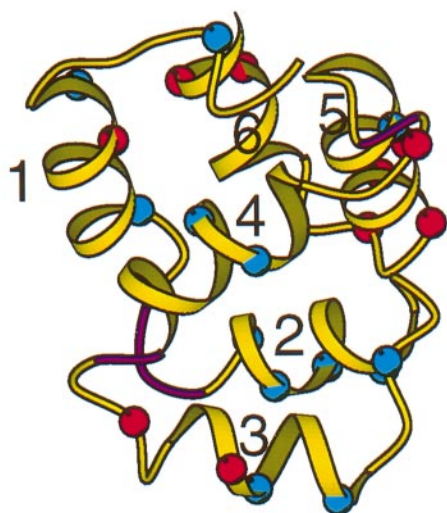
While most of the subtype 1 death domains associate with each other and with other death domains of the same subtype, none of these death domains have been found to associate with p75ICD (Varfolomeev *et al.*, 1996). Furthermore, we found no sign of self-association for p75ICD. Thus, the mechanism of activation of p75 appears to be different from that of Fas and p55<sup>TNFR</sup>, in which the direct association between intracellular death domains is crucial for signaling after ligand binding (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Interestingly, the only protein isolated so far that is capable of specifically interacting with the p75ICD does not seem to contain a death domain



**Fig. 4.** Solvent accessibility of the amino acid side chains in the death domain of p75. The solvent accessibilities are expressed as a percentage of their accessibilities calculated for the situation where the respective amino acid residues are located in a hypothetical poly-Gly  $\alpha$ -helix with a fully extended side chain (Sevilla-Sierra *et al.*, 1994). The values were averaged over the 20 NMR conformers. Hydrophobic and hydrophilic core conserved residues (Feinstein *et al.*, 1995) are identified by large filled and open circles, respectively. The helix locations in p75ICD are indicated at the top.

(Carter *et al.*, 1996a). The recruitment of p75 death domains in signaling therefore appears to involve different mechanisms, perhaps via phosphorylation of its serine/threonine-rich C-terminal segment, or involvement of the juxtamembrane domain. Interestingly, high flexibility was observed for this N-terminal segment of our p75ICD construct which comprised all but the first seven amino acid residues of the polypeptide linker between the transmembrane and death domains. Fully extended, this linker would span >200 Å. Flexibility in this long linker region *in vivo* would make it difficult to explain the apparent capacity of p75 to be activated selectively by different ligands. It has been noted previously that p75 can signal elevated cytoplasmic levels of ceramide in cultured oligodendrocytes as well as activation of NF- $\kappa$ B in cultured Schwann cells only after binding to NGF, but not to other neurotrophins such as BDNF or NT-3 (Carter *et al.*, 1996b; Casaccia-Bonnel *et al.*, 1996), despite the ability of all these ligands to bind p75 with comparable affinity. Clearly, the flexible juxtamembrane linker of p75ICD would effectively prevent the propagation to the death domain of any conformational differences in the extracellular domain which might be expected from the binding of different ligands. The association of intracellular factors with the flexible N-terminal domain of p75ICD could conceivably stabilize the juxtamembrane linker and allow the recruitment of the p75 death domains for downstream signaling in a ligand-specific manner. For example, the p75 death domain might be constitutively associated with intracellular factors; a first step in receptor activation after ligand binding could then involve the dissociation of these proteins. At this point, no structural information for this linker region is available for any of the receptors containing subtype 1 death domains (Huang *et al.*, 1996). It has been noted previously, however, that deletion of most of the linker between the transmembrane and death domains did not affect signaling in p55<sup>TNFR</sup> (Tartaglia *et al.*, 1993), indicating that this region is not critical for activation of receptors with death domains capable of self-association.

With death domain structures of subtypes 1 and 2 in hand, the effects of mutations in death domains can be rationalized. A systematic mutational analysis has been performed with the TRADD death domain (Park and Baichwal, 1996). Sequence alignment of p75, Fas and TRADD death domains (Figure 3) shows insertions in



**Fig. 5.** Mapping of the amino acid sequence of TRADD onto the death domain of p75. The death domain of p75 is drawn as a ribbon highlighting the C $\alpha$  positions of those residues which are positively (blue) or negatively (red) charged in TRADD. Loop regions in p75ICD corresponding to sites in TRADD where mutations to alanine suppressed the self-association of the TRADD death domain (Park and Baichwal, 1996) are shown in magenta (see also Figure 3).

TRADD, compared with p75, in the loops between helices 3 and 4, and helices 4 and 5. The sequence of TRADD appears to be compatible with the p75 fold, in that the pattern of buried hydrophobic and solvent-accessible charged residues is conserved. The only charged residue predicted to be buried in TRADD is located in helix 1 (Asp218 in TRADD), illustrating the ambiguities involved in aligning sequences corresponding to this helix. The charged amino acid side chains of TRADD appear to be relatively uniformly distributed over the surface of the molecule, and there is no evidence for the presence of an extended hydrophobic surface area as in p75ICD (Figure 5). The same applies to the charged residues in the death domains of p55<sup>TNFR</sup> and MORT1/FADD when mapped onto the structure of p75ICD (data not shown). The mutational analysis of TRADD involved the systematic exchange of consecutive stretches of three to four residues into alanine (Park and Baichwal, 1996). Most mutations affected buried residues, which in p75ICD correspond to side chains with <15% solvent accessibility, and may therefore have disrupted the structure of the molecule. However, some of the mutations that affected the self-association of TRADD occurred in residues of presumably high solvent accessibility, in particular the loop regions between helices 1 and 2, and on either side of helix 4 (Figures 3 and 5). Because these sites are too far apart to be buried simultaneously in the interface of a homodimer, self-association of TRADD should be expected not to stop at dimerization, but to lead to multimers.

The inactivating *lpr<sup>cg</sup>* mutation in the Fas receptor (Val238Asn, corresponding to Tyr366 in p75) has also been shown to interfere with homo- and hetero-association of the death domains of p55<sup>TNFR</sup> and MORT1/FADD (Varfolomeev *et al.*, 1996). This residue defines the spacing between helices 2/3 and 4/5 in the death domain (Figure 2C) (Huang *et al.*, 1996). Since different death domains have different residues at this position, this spacing is

likely to be different even between death domains of the same subtype. Interestingly, the signaling capacity of TRADD was hardly affected when this residue was mutated to alanine (Park and Baichwal, 1996).

In conclusion, the NMR structure determination of p75ICD revealed a globular death domain flanked by flexible regions and allowed the delineation of the conserved structural features of different types of death domains. In addition, our data point to possible mechanisms of p75 activation which appear to be distinct from those previously described in receptors containing subtype 1 death domains such as p55<sup>TNFR</sup> and Fas. The p75ICD structure will now facilitate the design of decisive mutational studies to unravel mechanisms of p75 activation and proximal signaling events.

## Materials and methods

### Preparation of p75ICD

A DNA fragment corresponding to the intracellular domain of the neurotrophin low affinity receptor p75 was amplified by PCR from a plasmid containing the rat p75 cDNA, using primers that had *Sa*I restriction sites to aid subcloning, and an N-terminal methionine for cyanogen bromide (CNBr) cleavage. This fragment was inserted into a pQE31 vector (Qiagen) which added on a histidine tail (His-tag) to facilitate purification. This construct produced a fusion protein containing 20 extra amino acids due to the linker sequence and the His-tag. The fusion protein was expressed in the *Escherichia coli* strain M15-(pREP4) (Qiagen) grown in 20 l of modified M9 minimal medium (containing <sup>15</sup>NH<sub>4</sub>Cl as the sole nitrogen source) at 37°C. Expression was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at OD<sub>600</sub> = 0.8, and cells were harvested 5 h after induction. The cells were sonicated and the fusion protein was purified using Ni<sub>2</sub><sup>+</sup>-NTA column chromatography (Quiagen). During the course of purification, we observed the presence of a shorter p75 fragment generated by cleavage between Lys280 and Glu281, i.e. between the sixth and seventh N-terminal residue of the intracellular domain. Because of its reduced size (145 residues), this species was more suitable for NMR. It was purified subsequently by ion exchange chromatography on an S-Sepharose column (Pharmacia) followed by reverse phase HPLC using a C8 column (Vydac) with a water/acetonitrile gradient. The protein was soluble and monomeric as judged by gel filtration on a Superose 12 column (Pharmacia). The identity and mass of the protein were verified by N-terminal protein sequencing and by electrospray mass spectrometry. The final yield was ~1 mg of pure <sup>15</sup>N-labeled p75ICD protein per liter of culture.

### NMR spectroscopy and collection of structure constraints

NMR spectra were recorded using an ~2.5 mM solution of p75ICD in 90% H<sub>2</sub>O/10% D<sub>2</sub>O at 28°C at 600 and 800 MHz <sup>1</sup>H NMR frequency on Bruker DMX 600 and DMX 800 NMR spectrometers. The sample was prepared under argon. Complete sequential resonance assignments of the structured part of the protein were obtained at pH 7 from three-dimensional (NOESY-<sup>15</sup>N-HSQC, TOCSY-<sup>15</sup>N-HSQC) and two-dimensional (NOESY, TOCSY, DQF-COSY) NMR spectra (Ernst *et al.*, 1987; Cavanagh *et al.*, 1996). The flexible residues were assigned at pH 6. Dihedral angle constraints were derived from <sup>3</sup>J<sub>H $\alpha$ H $\beta$ , <sup>3</sup>J<sub>H $\beta$ H $\gamma$  and <sup>3</sup>J<sub>H $\beta$ H $\delta$  coupling constants measured in DQF-COSY, <sup>15</sup>N-HSQC (Szyperski *et al.*, 1992) and HNHB (Archer *et al.*, 1991) spectra, respectively. Stereospecific assignments were obtained for 63 pairs of diastereotopic methylene protons and methyl groups. Distance constraints for the structure calculation were derived from a two-dimensional NOESY spectrum recorded at 800 MHz with a mixing time of 40 ms at pH 7. Amide proton T<sub>2</sub> relaxation times were measured from a <sup>15</sup>N-HSQC experiment preceded by a spin-echo delay with selective decoupling of the H $\alpha$  resonances. The relaxation times were obtained by fitting a single exponential decay to the cross peak intensities of six experiments recorded with spin-echo delays of 10, 20, 40, 80, 160 and 320 ms. Slowly exchanging amide protons were identified in a <sup>15</sup>N-HSQC spectrum recorded at 28°C within 20 min after dissolving the lyophilized protein in D<sub>2</sub>O. The experiment was performed at pH 6 and 7. Table I gives an overview of the structure calculation. The program MOLSCRIPT (Kraulis, 1991) was used to draw Figures 2 and 5.</sub></sub></sub>

**Coordinates**

The coordinates of the 20 NMR conformers, the chemical shifts and the NMR constraints used as input in the structure calculation are available from the Brookhaven protein data bank, accession codes INGR and RINGRMR, respectively.

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