Biochemical and Biophysical Research Communications **255,** 104–109 (1999)
Article ID bbrc.1999.0101, available online at http://www.idealibrary.com on IDEAL®

Selection of a Peptide Ligand to the p75 Neurotrophin Receptor Death Domain and Determination of Its Binding Sites by NMR

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Received December 14, 1998

The p75 neurotrophin receptor (p75^{NTR}) contains a conserved death domain module similar to that of the cytotoxic receptors Fas and TNFR-1. Here, we describe the selection of peptide ligands from a combinatorial library using a variation of the selectively-infective phage (SIP) method directed to the death domain of p75^{NTR}. The binding sites on the death domain of p75^{NTR} were identified for a 15 amino acid residue peptide by nuclear magnetic resonance (NMR) spectroscopy. The selected peptides may be useful for probing the function of the p75^{NTR} death domain and aid in defining its downstream signalling mechanism.

p75^{NTR} was the first receptor to be isolated for the neurotrophin nerve growth factor (NGF) (1,2). Upon subsequent cloning of other neurotrophins, namely brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), p75^{NTR} was shown to be a common receptor for this family of neurotrophic factors (3–5). Although clearly important for some aspects of normal nervous system development, its actual physiological role remains uncertain (6–17). Recently, the NMR structure determination of the intracellular domain of p75^{NTR} (p75-ICD) revealed a flexible juxtamembrane linker of about 55 amino acid residues followed by a conserved death domain (DD) module at the C-terminal end (18).

The identification of binding partners to the p75 DD is of primary importance to our understanding of the signalling mechanism of this receptor. In this study, we have used a novel technique of phage display called selectively-infective phage (SIP) (19,20) to select peptides from a synthetic random peptide library, which are capable of binding to the p75-ICD. By exploring a small but fully functional sequence space with a trinucleotide-based peptide library, a number of disulfide-constrained peptides could be identified which specifically bound to p75 ICD. NMR spectroscopic analysis of one of these in complex with p75-ICD revealed two different binding sites on the p75 DD. Thus, SIP presents an effective way of generating peptide ligands for protein domains which can be used to identify potential protein-protein interaction sites. Furthermore, peptide ligands to the p75-ICD may prove to be valuable tools to explore the signalling mechanism of this receptor. If a large number of binding peptides can be identified, the sequences of the selected peptides may also aid in the search for motifs found in endogenous binding partners involved in downstream signalling.

MATERIALS AND METHODS

Selection of peptide ligands to p75-ICD with SIP. A DNA cassette encoding a disulfide-constrained peptide library of varying lengths (6-16 amino acid residues; B. Virnekaes, manuscript in preparation), whereby each position was randomized with 19 amino acids excluding Cys was prepared by annealing the primer 5′CTATGGCGCGCCTGTCGACTGT(TRI)$_{6\text{-}16}$TGTGGTGGTGGAGGATCCGAATTCATG3′ (where TRI denotes an equimolar mixture of trinucleotide codons encoding 19 different amino acids excluding the codon for Cys) with primer 5′TGTGGTGGTGGAGGATCCGAATTCATG3′ followed by filling-in with DNA polymerase Klenow fragment. The cassette was purified with a Qiaex (Qiagen) purification kit as described by the manufacturer. The purified cassette was digested with

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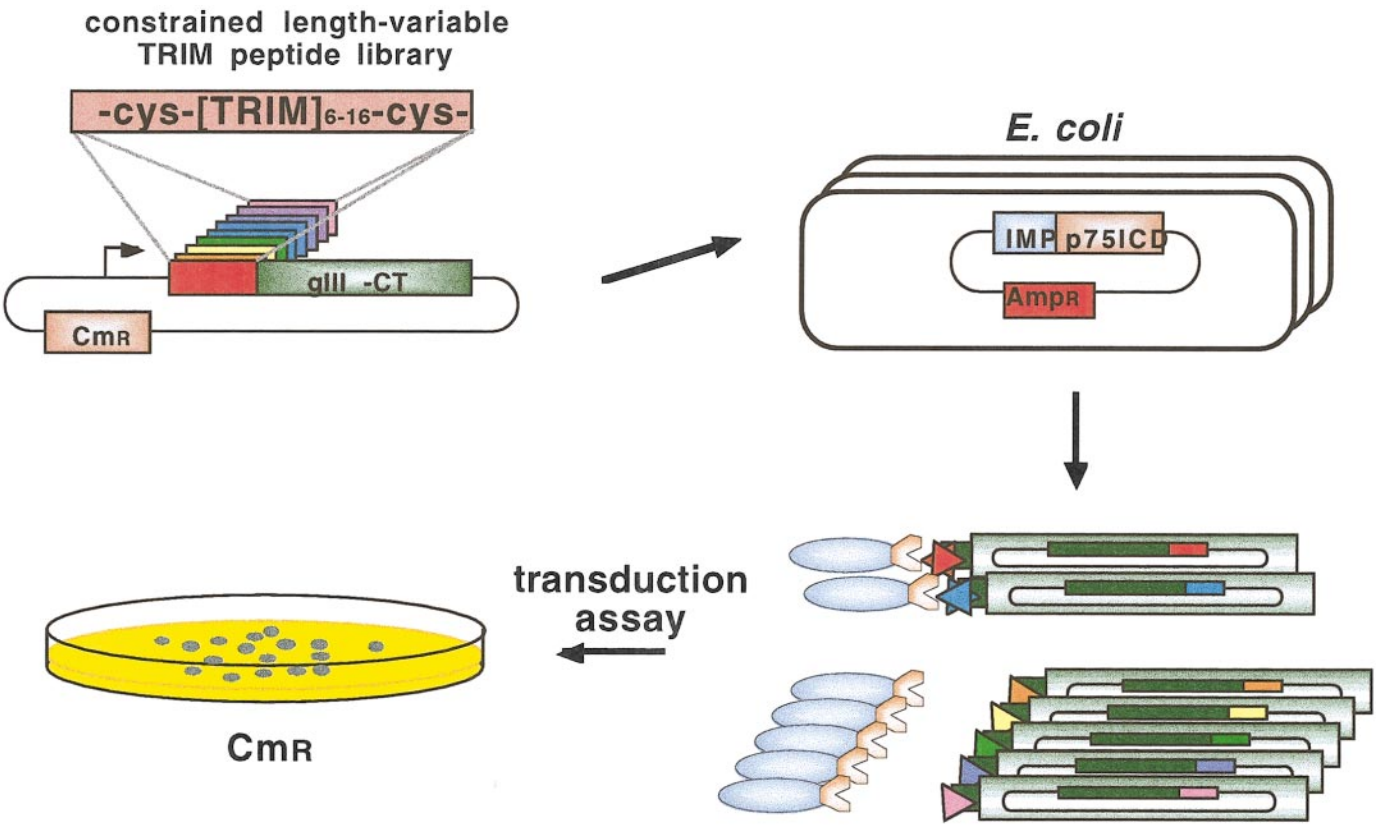

FIG. 1. Outline of the SIP method applied for the screening of peptide ligands to the p75-ICD. Phage DNA encoding a random library of peptides fused to the C-terminal domain of pIII is transformed into *E. coli* cells containing a plasmid encoding the p75-ICD fused to the IMP. In the transduction assay, phages expressing a peptide that is recognized by the p75-ICD-IMP fusion reconstitutes an infective phage allowing for the recovery of the genetic information encoding the peptide ligand. Phages displaying peptides incapable of binding to p75-ICD fail to recruit the IMP domain and are non-infective.

SalI and EcoRI and the smaller fragments were removed by size exclusion with a Nickspin (Pharmacia) column as recommended by the manufacturer. The restricted DNA cassette was cloned into the phage vector fjun_1b (21), pre-digested with SalI and EcoRI. The ligated peptide library was transformed into DH5a cells harboring the plasmid pUC18/IMP-p75-ICD, a pUC18 derivative containing the p75-ICD (18) fused to the N-terminal domain of pIII (amino acids 1–218) by electroporation. A total of 10,000 transformants were scraped and inoculated into 50 ml LB (100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 1 mM IPTG). The suspension was incubated overnight at 30°C. The following day, the cells were separated from the supernatant containing selectively-infective phages by centrifugation. The phages were precipitated twice with 30% PEG6000/3 M NaCl and finally resuspended in 1 ml PBS buffer. The phages were subsequently filtered through a 0.45 μm Sartorius filter and titered with *E. coli* K91 cells for transduction of chloramphenicol resistance. A total of 10^6 transductants were recovered.

DNA sequencing. Twenty-eight independent transductants were picked and their respective DNAs sequenced by the dideoxy chain termination method using an ABI DNA sequencer by Sequiserve (Munich).

Analysis of transductants by SIP. The isolated RF (replicative form) DNA from the selectively-infective phages were co-transformed with pUC18/IMP-p75-ICD (positive control) or pUC18/IMP-HAG (negative control), a nine amino acid peptide derived from the influenza virus hemagglutinin, fused to the N-terminal domain of pIII. As additional control, the c-Jun leucine zipper (30 amino acids) was fused to the C-terminal domain of pIII. Co-transformants were grown in LB (30 μg/ml chloramphenicol, 100 μg/ml ampicillin) overnight. The following day, the supernatant was separated from the cells by centrifugation and filtered through a 0.45 μm filter (Sartorius). The phages were titered with *E. coli* K91 cells for transduction of chloramphenicol resistance.

Analysis of binding by phage ELISA. Duplicate wells were coated with 50 μg of purified p75-ICD or 50 μg of IMP conjugated with fluorescein (IMP-FITC) (22), followed by blocking with 5% low fat milk powder overnight at 4°C. Wells were washed three times with PBS + 0.05% Tween. Phages displaying the peptides were prepared from an overnight culture of cells harboring the phage vector, separated from the cells and filtered through a 0.45 μm Sartorius filter. 100 μl of freshly prepared peptide-displaying phage suspension or wild-type phage (M13K07) was mixed with an equal volume of 5% low fat milk powder, preincubated at 4°C for 30 minutes and loaded into each well. The phages were allowed to bind for one hour at room temperature. The wells were washed extensively with PBS and PBS + 0.05% Tween several times. Sheep anti-M13 phage IgG conjugated to horseradish peroxidase (Pharmacia; diluted 1:5000 in PBS) was added, incubated for 30 minutes, followed by several washing steps with PBS. 200 μl of the substrate, BM Blue POD substrate (Boehringer Mannheim) was subsequently added. Color development was monitored and recorded on an ELISA reader at an absorbance of 630 nm.

NMR. NMR spectra were recorded with a 2 mM solution of uniformly ^{15}N-labeled p75-ICD in 90% H_2O/10% D_2O. The uncomplexed

peptide was measured in a 3 mM solution. The sample of the protein-peptide complex was 0.1 mM in protein and 3 mM in peptide. All NMR measurements were performed at pH 5.8 and 28°C on a Bruker DMX-600 NMR spectrometer. Dithiothreitol (DTT) was added to the samples to prevent oxidation of the cysteine thiol groups and the samples were stored under argon. ^{15}N-HSQC spectra were recorded using t_{1max} = 38 ms, t_{2max} = 162 ms and a spin-lock purge pulse for water suppression (23). Total recording times were about 1 h for the free protein and 60 h for the protein-peptide complex. NOESY spectra were recorded with 100 ms mixing time, using homospoil pulses and a spin-lock purge pulse for water suppression (24). Other experimental parameters were: t_{1max} = 32 (55) ms, t_{2max} =162 (162) ms, experimental time about 6 (12) h for the free (complexed) peptide.

RESULTS AND DISCUSSION

Peptides were selected using a modified version of SIP (19) from a disulfide-constrained peptide library prepared from trinucleotides (25) containing peptides with varying lengths of 6-16 residues of random amino acid sequences. Except for Cys all the other 19 common amino acids were present in the random sequences. The technique is illustrated in Fig. 1. A random peptide library is displayed on filamentous phages as an N-terminal fusion to the C-terminal region of a truncated pIII phage coat protein. The bait, in this case the p75-ICD, was expressed from a separate plasmid as a C-terminal fusion to the N-terminal domain of the pIII protein, also called infectivity mediating particle or IMP. An interaction between the p75-ICD and a member of the library brings together the IMP and the C-terminus of the pIII protein, thereby restoring infectivity of the phage carrying the genetic information of the interacting peptide which can subsequently be recovered.

Twenty-eight independent clones selected by SIP were analysed by DNA sequencing and sixteen unique sequences were observed. Among the 16 unique sequences, two sequences (peptide 1: CFFRGGFFNLWHYC; peptide 2: CFFRGGFFNHNPRYC) were very similar. To determine the specificity of these peptides towards the p75-ICD, independent SIP experiments were performed with the peptides in combination with

TABLE 1

SIP Transduction Assay of Phages Displaying Peptide 1 or Peptide 2 with N-terminal pIII (IMP) Fusion Proteins

pIII C-terminus fusion	IMP fusion	titer (transducing units/ml)
peptide 2	p75-ICD	1.0×10^6
peptide 2	HAG*	<10
peptide 1	p75-ICD	1.1×10^6
peptide 1	HAG*	<10
c-Jun (leu zipper)*	p75-ICD	<10

* Phages displaying the leucine zipper domain of c-Jun and IMP fused to an influenza virus hemagglutinin peptide (HAG) were used as controls.

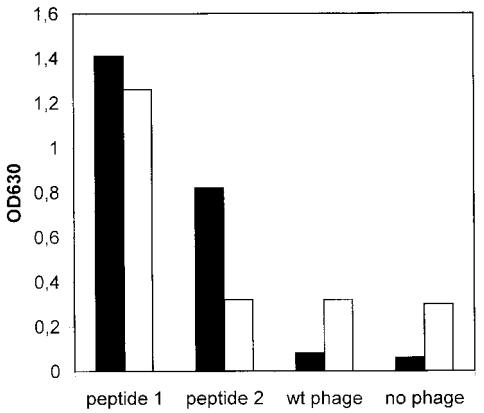

FIG. 2. ELISA of p75-ICD-specific peptides displayed on the surface of filamentous phage particles as a fusion with the C-terminal pIII domain or wild-type phage (M13K07). Wells were coated with either purified p75-ICD (dark bars) or N-terminal pIII conjugated with fluorescein (white bars). The OD_{630} reading is an average of the readings from two wells.

appropriate controls, consisting of IMP without $p75^{NTR}$ sequences and phages displaying irrelevant proteins. Both peptides were specific towards the p75-ICD in this assay with differences of several orders of magnitude over control (Table 1).

As an independent test of peptide specificity, phage ELISA was performed (Fig. 2). Binding of peptides 1 and 2, displayed on the surface of phage, to wells coated with purified p75-ICD or IMP-FITC was detected by anti-M13 polyclonal antisera conjugated with horseradish peroxidase (HRP). Although peptide 2 bound only to p75-ICD coated wells, peptide 1 also bound to wells coated with IMP-FITC or bovine serum albumin (data not shown). This indicated a lower specificity for peptide 1 *in vitro*, compared to the specificity observed *in vivo*, and suggest that the *E. coli* periplasm, where the phage particle is assembled, may provide a suitable environment for fine-tuning binding specificity. Analysis of the sequence of peptide 1 reveals a more hydrophobic character than peptide 2 in the region of divergence, which may explain its non-specific binding *in vitro*.

The deduced cyclic peptide sequences were chemically synthesised and the purified peptides analysed by NMR. Peptide 1 was not soluble and was not used for further studies. Free peptide 2 in solution did not assume any defined conformation, as indicated by the absence of long-range nuclear Overhauser effects (NOEs) and rapid exhange of the amide protons with the solvent (data not shown). To determine binding sites for peptide 2 in p75-ICD by NMR, ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) spectra were recorded for p75-ICD in the absence and presence of peptide 2, monitoring signal intensity changes of the backbone amide resonances (Fig. 3A, B). Eleven amino acids in the p75 DD showed vanishing or significantly

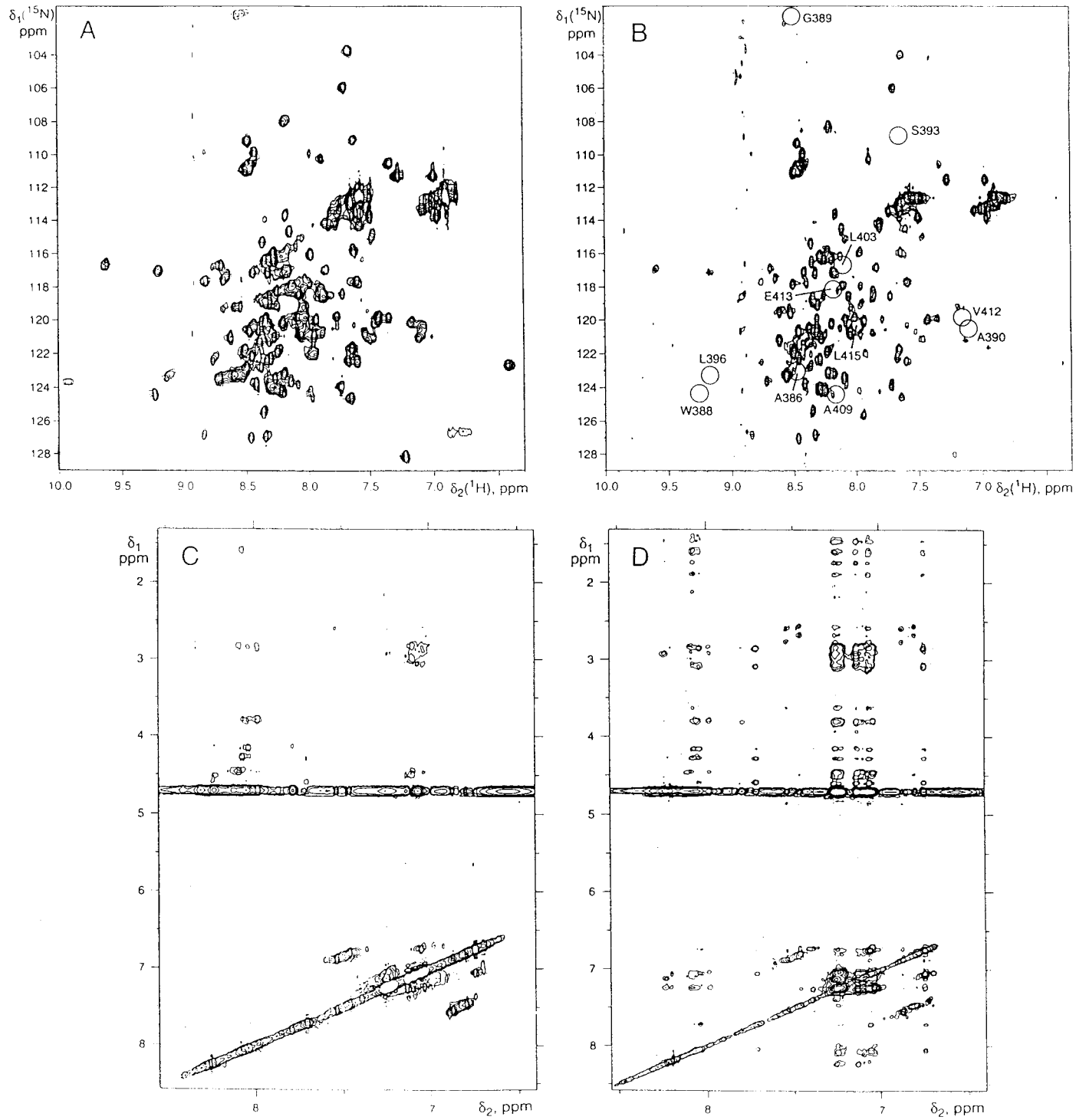

FIG. 3. ^{15}N-^1H correlation spectra of p75-ICD and NOESY spectra of peptide 2. (A) ^{15}N-^1H correlation spectrum recorded with a 2 mM aqueous solution of p75-ICD. (B) ^{15}N-^1H correlation spectrum recorded with a 0.1 mM aqueous solution of p75-ICD in the presence of 3 mM peptide 2. Circles identify amide signals that are clearly present in the free protein but absent or below the plot level in the spectrum of the protein-peptide complex. Assignments are indicated with the one-letter amino acid symbol and the sequence number. (C) Spectral region from NOESY spectra recorded with free peptide 2. (D) Spectral region from NOESY spectra recorded with peptide 2 in the presence of p75-ICD. The protein signals are below the lowest level plotted.

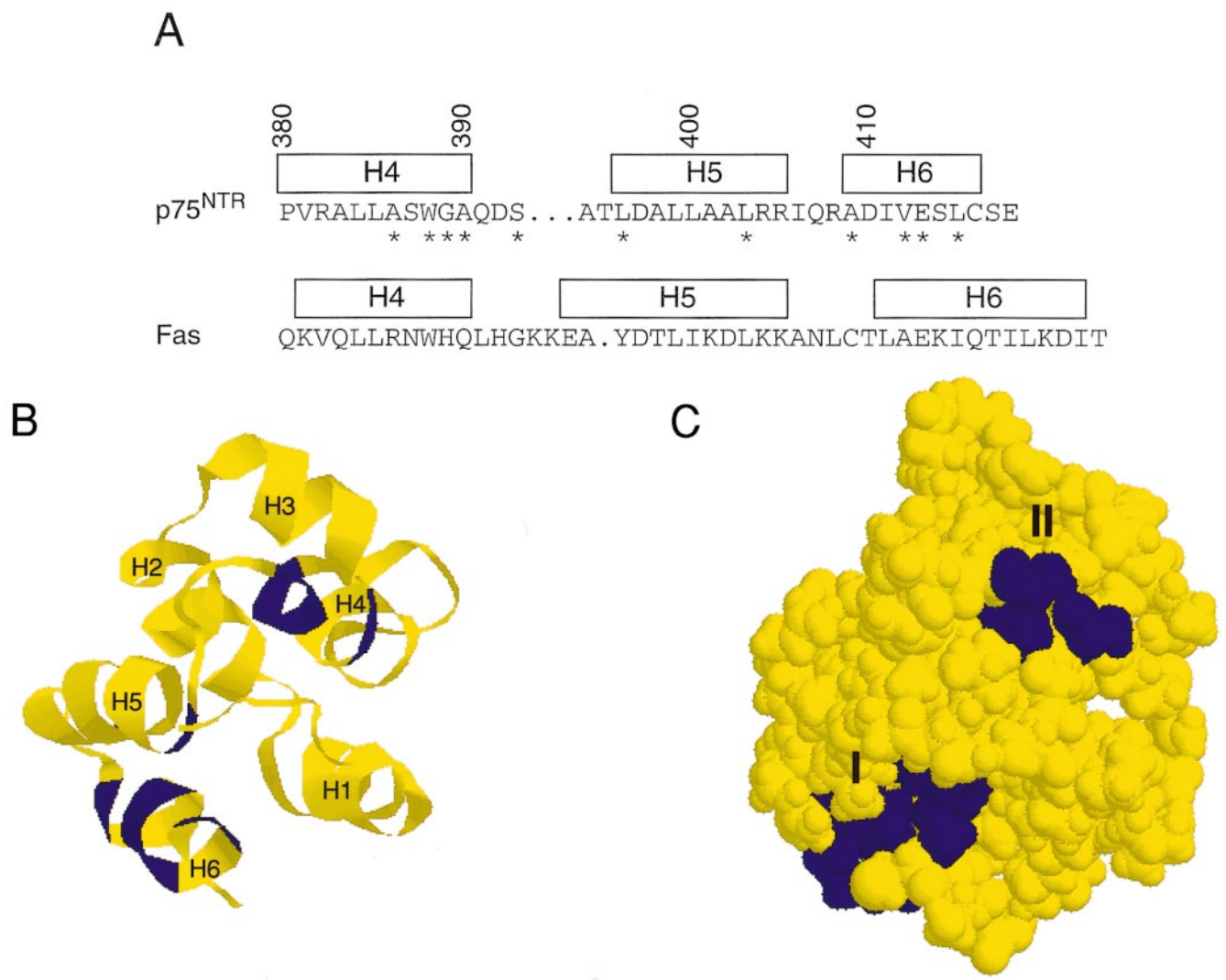

FIG. 4. (A) Partial alignment of the p75 DD sequence with the DD from Fas. Boxes indicate helices and asterisks beneath amino acid residues (in single letter-code) denote residues with perturbed signal intensities upon binding of peptide 2. The two binding sites for peptide 2 on the p75 DD are highlighted (blue regions) in the ribbon (B) and the space filling (C) models. Site 1 (I) is framed by residues of helices H5 and H6 and site 2 (II) is a part of H4.

reduced signal intensities in the presence of peptide 2. A binding site (site 1) was mapped onto a hydrophobic patch framed by helices 5 and 6 (Fig. 4). This region is highly conserved in $p75^{NTR}$ across species. Interestingly, a homologous hydrophobic patch also exists in the Fas DD (26), and most mutations in the TNFR-1 DD critical for the maintenance of cytotoxicity lie in the equivalent region of this DD (27). As structural analyses of Fas and $p75^{NTR}$ show a common global fold for death domains, it is possible that this hydrophobic patch is a general motif important for protein-protein interactions (18,26). A second interaction site (site 2) seems to be affected by peptide binding (Fig. 4A). It is located in a region devoid of charged residues which had previously been identified as a putative interaction surface (18). Since other death domains contain charged residues at this site, it may be specific to $p75^{NTR}$.

Complex formation was further reflected by the NMR spectra of the peptide, where transferred NOEs could be observed between the peptide resonances in the presence of p75-ICD which were absent in the free peptide (Fig. 3C,D). No NOEs between peptide residues separated by more than one residue could be observed, suggesting extended conformations of the bound peptide.

This is the first time that SIP has been used to select for peptide ligands from a random peptide library. Our study demonstrates the efficiency and simplicity of this technique, which neither requires purified components nor tedious bio-panning procedures as in conventional phage display. The peptide described here, as well as

others selected by SIP, should prove valuable tools in probing for the function of the p75 death domain. Unfortunately, there is no robust assay for $p75^{NTR}$ activity.

The p75-ICD binding peptides identified by SIP showed no significant sequence similarity to any known protein which could be involved in down-stream signalling. Possibly, the natural binding partner of p75-ICD could be identified by sequence similarity to a SIP-selected peptide by screening a much larger peptide library than used here.

ACKNOWLEDGMENTS

The authors thank C. F. Ibáñez, A. Plückthun, S. Spada, C. Krebber, B. Virnekäs and L. Ge for critical discussions. This work was supported by a grant from the Biotechnology Programme of the European Commission.

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