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Neurotrophin-Dependence Domain

A Domain Required for the Mediation of Apoptosis by the p75 Neurotrophin Receptor

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Abstract

The mechanisms underlying neurotrophin dependence, and cellular-dependent states in general, are unknown. We show that a 29 amino acid region in the intracellular domain of the common neurotrophin receptor, p75^{NTR}, is required for the mediation of apoptosis by p75^{NTR}. Furthermore, contrary to results obtained with Fas, monomeric p75^{NTR} is required for apoptosis induction, whereas multimerization inhibits the pro-apoptotic effect. Within the 29-residue domain required for apoptosis induction by p75^{NTR}, a 14-residue region is sufficient as a peptide inducer of apoptosis. This 14-residue peptide requires the positively charged carboxyterminal residues for its effect on cell death, and these same residues are required by the full-length p75^{NTR}. These studies define a novel type of domain that mediates neurotrophin dependence, and suggest that other cellular-dependent states may be mediated by proteins displaying similar domains.

Index Entries: p75^{NTR}; apoptosis; dependence; neurotrophin; dimerization.

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Introduction

Cellular survival requires specific signals (Raff, 1992): depending on cell type and state of differentiation, cells may require adhesion (Frisch and Francis, 1994), electrical activity (Yan et al., 1994), trophic factors (Levi-Montalcini, 1966), cytokines (Girard et al., 1997), or other stimuli for survival. It has generally been assumed that apoptosis resulting from a lack of such stimuli is owing to the lack of a positive survival signal, such as that which results from tyrosine phosphorylation of the Trk receptors and the associated downstream signaling (Kaplan and Stephens, 1994). Although this undoubtedly explains part of the effect, results obtained over the past few years argue for a distinct and complementary form of signal transduction that is initiated or propagated as a result of the lack of binding of trophic factor or cytokine, or the lack of other required stimuli (Rabizadeh et al., 1993; Bredesen and Rabizadeh, 1997; Bredesen et al., 1998). However, the mechanism(s) by which cells transduce a signal in response to a lack of stimulation is not understood. Nonetheless, the ability of cells to initiate the active process of apoptosis in response to the withdrawal of stimuli such as trophic factors implies the existence of a form of transduction in which the signal is initiated by stimulus withdrawal.

Classic experiments by Levi-Montalcini and Hamburger (Levi-Montalcini, 1966) demonstrated that developing neurons pass through a critical phase, during which typically approx half of the neurons from various subsets die. Death of developing neurons is apoptotic (Martin et al., 1988), and is critically dependent on the availability of neurotrophic factors: supraphysiologic concentrations of neurotrophic factors can block developmental neuronal apoptosis, whereas neurotrophin withdrawal results in enhanced apoptosis.

The common neurotrophin receptor, $p75^{NTR}$, has been shown to mediate neural apoptosis in response to serum or neurotrophin withdrawal (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Rabizadeh and Bredesen, 1994; Bunone et al., 1997), but the mechanism by which this is achieved has not been determined. Interestingly, $p75^{NTR}$ may also mediate nerve growth factor (NGF)-induced cell death (Casaccia-Bonnefil et al., 1996; Frade et al., 1996). NGF-induced neural cell death may play a role in early retinal development (Frade et al., 1996), whereas neurotrophin withdrawal-induced apoptosis plays a critical role during the period of target-cell innervation (Bredesen and Rabizadeh, 1997; Frade et al., 1996).

Studies both in primary neuronal cultures and in vivo have supported the notion that the expression of $p75^{NTR}$ mediates neurotrophin dependence: Barrett and Bartlett (1994) found that the antisense inhibition of $p75^{NTR}$ expression diminished the frequency of apoptosis of dorsal-root ganglion neurons. Studies of $p75^{NTR}$-null mice have shown approx 50% increase in cholinergic neurons of the medial septal region (Van der Zee et al., 1996; Yeo et al., 1997) and diagonal band of Broca (Yeo et al., 1997), as well as neuronal hypertrophy and hyperinnervation of some target fields (Yeo et al., 1997). Furthermore, NGF-deficient mice (NGF+/– heterozygotes), which demonstrate atrophy and loss of cholinergic neurons of the basal forebrain, show rescue of these deficits when crossed with $p75^{NTR}$-null mice, arguing that $p75^{NTR}$ mediates these effects in the presence of a reduced concentration of NGF (Sauer et al., 1996).

Here we show that $p75^{NTR}$ displays a novel type of domain that mediates cell death. This is shown to be distinct from the putative $p75^{NTR}$ death domain predicted by alignment studies with the death domains of tumor necrosis factor receptor (TNFR) I and Fas/Apo-1 (Chapman, 1995; Feinstein et al., 1995); the distinction is demonstrated both by site-directed mutagenesis studies and by virtue of the fact that ligand binding inhibits, rather than induces, the pro-apoptotic effect. Furthermore, multimerization studies utilizing FK binding protein- $p75^{NTR}$ chimeras demonstrate clearly that apoptosis is mediated by the $p75^{NTR}$ monomer, whereas multimerization inhibits the effect just the reverse of the situation for Fas and TNFR-I (Freiberg et al., 1996). Our studies define a domain that mediates $p75^{NTR}$-induced cell death, and suggest that cellular-dependent states may be mediated by proteins displaying similar domains. Because cell death induction by $p75^{NTR}$ is blocked by dimerization and by neurotrophins (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Rabizadeh et al., 1999; Ye et al., 1999) and because it is functionally distinct from the death domains of Fas and TNFR I, we refer to this domain as a neurotrophin-dependence domain (Bredesen et al., 1998).

Materials and Methods

Plasmid Construction and Mutagenesis

Mutations p75E348A and p75W359G were generated using the Altered Sites II in vitro Mutagenesis System (Promega) with single-stranded template of p75^{NTR} cDNA. The primers used were: 5′CCTTTACCCACGCGGCCTGCCCAGT3′ (E348A) and 5′CTGCTGGCCAGCGGGGGTGC CCAG3′ (W359G). Cloning of the mutated p75^{NTR} cDNAs into the pBabepuro mammalian expression vector (Morgenstern and Land, 1990) was performed as described for the cloning of wild-type p75^{NTR} into pBabepuro (Rabizadeh et al., 1993). The carboxyterminal deletion mutants, p75ΔC19 and p75ΔC33, were generated by polymerase chain reaction (PCR) amplification with the enzyme Pfu polymerase (Stratagene). The 5′ primer contains the unique *Bam*HI site located at 700 bp of the rat p75^{NTR} cDNA: 5′ATGGATCCCAAGGTCTAC GCC3′. Both 3′ primers contain a *Sal*I site and introduce a stop codon following Ile 377 or Asp 363, respectively: 5′CGCTGGTCGACTAGATGCGTCG CAG3′ for p75ΔC19 and 5′CGCTGGTCGACTAG TCCTGGGCACC3′ for p75ΔC33. pBabepuro-p75ΔC19 and pBabepuro-p75ΔC33 were constructed by replacing the *Bam*HI-*Sal*I fragment in pBabepuro- p75^{NTR} with the corresponding PCR products. The truncated mutant p75ΔC38 was produced by *Pvu*II partial digestion of p75^{NTR} in a pUC18 construct. The construct was then digested with *Xba*I and filled in with the Klenow fragment of DNA Polymerase I. The resulting 1.3 kb fragment was excised from an agarose gel, religated to create pUC18-p75ΔC38, and then p75ΔC38 was recloned into the pBabepuro vector as described (Rabizadeh et al., 1993). Mutants p75 M1 (p75^{NTR} R375G, R376 G double-mutant) and p75 M2 (p75^{NTR} L370K, L371P double-mutant) were generated from the construct pUC18- p75^{NTR} by excising a *Bam*HI-*Xba*I fragment and replacing it with two fragments, generated by PCR amplification with the enzyme Pfu polymerase; the first fragment spanned from the *Bam*HI site within the p75^{NTR} open reading frame to a new *Hin*dIII site and contained the desired mutation, the second fragment spanned from the same new *Hin*dIII site to the *Xba*I site in the pUC18 vector. The PCR products were digested and ligated into pUC18- p75^{NTR} generating an in-frame mutated protein. The oligonucleotides used to amplify the first fragment were: 5′ATCCCTGGTC GATGGATCCCAA3′, containing the *Bam*HI site, and 5′TCTCTGGATCCCTCCCAGGGCG3′, containing the *Hin*dIII site and the mutation for p75 M1, or 5′CTGGATCCGTCGCAGGGCGGCTGGT TTGG3′, containing the *Hin*dIII site and the mutation for p75 M2. For the second fragment, the oligonucleotides 5′CTGCGACGGATCCAGAGA GCTG3′, containing the *Hin*dIII site, and 5′GCTCT AGAACATCAGTCGTCGGA3′, containing the *Xba*I site, were used. For the deletion mutants p75Δ328–348 and p75Δ328–358, a similar strategy was used: the *Bam*HI/*Xba*I fragment of pUC18-p75^{NTR} was replaced with two fragments generated by the Pfu polymerase amplification of the sequences flanking the desired deletion and either one of the restriction sites *Bam*HI or *Xba*I, and religating them after digestion into pUC18- p75^{NTR}. The mutated cDNAs were subcloned into the pBabepuro mammalian expression vector as described earlier. Chimeric p75^{NTR}-TNFR II expression constructs were a generous gift from E. Shooter, and were made as described (Rovelli et al., 1993), then subcloned into pBabepuro. All constructs were confirmed by DNA sequencing, and protein expression detected by flow cytometry and immunoblotting, using anti- p75^{NTR} antiserum (Promega) for the immunoblots and monoclonal antibody (MAb) 192 for the flow cytometry.

The FKBP12-tagging vector MF1E/MF3E (myristoylated) was a gift from D. Spencer (Spencer et al., 1993), and served as a template for PCR-amplified FK-binding domain, which contained either 1 or 3 repeats of FKBP. Primers were flanked by *Nhe*I (5′ primer) or *Nde*I (3′ primer) sites, and the resulting fragments were subcloned into pcDNA3.1. The intracytoplasmic domain of p75^{NTR} was then digested with *Nde*I and *Bam*HI and ligated at the carboxyterminus of the FKBP sequences within the pcDNA3.1-FKBP construct.

α1-C, α4-C, and α4-5 were constructed via the Quick Change site-directed mutagenesis system (Strategene) as previously described (Mehlen et al., 1998). Initially, the intracytoplasmic domain of p75^{NTR} (residues 245–396) was amplified by PCR and cloned into pcDNA3, producing the expression construct pcDNA3- p75^{NTR}IC. The forward and reverse primers introduced novel *Bam*HI and *Sal*I sites, respectively, which were later used for cloning

into pcDNA3. The sequences of the forward and reverse primers were, respectively: 5′CGGGATCC ATGAAGAGGTGGAACAGC3′ and 5′TGCGGTC GACTCACATTGGGGATGTGGCAGTGGA3′. The construct pcDNA3- p75^{NTR}IC was then used as the template to make α1-C (residues 313–396) and α4-C (residues 350–396) by the QuickChange method. The forward and reverse primers used to make α1-C were, respectively: 5′GAGCTCGGA TCCATGCTGACCAAGCGTGAG3′ and 5′CTC ACGCTTGGTCAGCATGGATCCGAGCTC3′. The forward and reverse primers used to make α4-C were, respectively: 5′GGTACCGAGCTCGG ATCCA TGTGCCCAGTGCGAGCCCTGCTG3′ and 5′CAGCAGGGCT-CGCACTGGGCACATG GATCCGAGCTCGGTACC3′. The template used to construct α4-5 (residues 350–378) was α4-C. The forward and reverse primers used to make α4-5 were, respectively: 5′CGACGCATCCAGTGA GTCGAGCATGCATCT3′ and 5′AGATGCATGCT CGACTCACTGGATGCGTCG3′.

The construct expressing the deletion mutant p75Δ349–358 protein was also made using the QuickChange method. The sequences of the forward and reverse primers were, respectively: 5′TCCTTTACCCACGAGTGGGGCGCCCAGGA CAGTGC3′ and 5′CGACTGTCCTGGGCGCCCC ACTCGTGGGTAAAGGA3′.

Cell Culture and Plasmid Expression

Temperature-sensitive cerebellar R2 neurons and PC12 NRA5 mutant cells, which lack p75^{NTR} expression, were maintained as described previously (Rabizadeh et al., 1993). Cells were transfected with expression constructs using the cationic lipid DOTAP (Roche Molecular Biochemicals) according to the Roche protocol. Stably transfected cells were selected in puromycin (5 μg/mL), and pools of transfectants were compared, in order to avoid the bias inherent in comparing single colonies (Zhong et al., 1993). R2 cells were supplemented with 100 μg/mL NGF every 3 d. Though PC12 NRA5 cells did not require repeated addition of NGF for survival, the number of colonies expressing p75^{NTR} was consistently lower than control transfectants as described earlier (Rabizadeh et al., 1993). Expression of p75^{NTR} mutants was assessed by flow cytometry, using the MAb 192 (Baldwin et al., 1992), as described previously (Rabizadeh et al., 1993). In each transfection, approx 50–70% of PC12 NRA5 cells expressed p75^{NTR}. Cell death was induced, assessed, and quantitated by propidium iodide (PI), as described (Kane et al., 1995; Rabizadeh et al., 1993).

293T cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and plated at a density of 5×10^5 cells into each well of a 6-well plate. 293T cells were then transiently transfected with 5 μg of plasmid DNA using the calcium-phosphate method (Sambrook et al., 1989), in the presence or absence of 2 μ*M* AP1510 or 100 ng/mL neurotrophin-3 (NT-3). After an 18-h incubation, cells were washed with DMEM and placed on DMEM supplemented with 3% FBS and 2 μ*M* AP1510 or 100 ng/mL NT-3 as before. After a further 18-h incubation, transfected cells were placed on DMEM supplemented with 1.5% FBS, 2 μM AP1510 or 100 ng/mL NT-3 as before, and 35 μ*M* tamoxifen was added to induce apoptosis more rapidly (Rabizadeh et al., 1999). Cell death was then quantitated by trypan blue exclusion as previously described (Rabizadeh et al., 1999). 293T cells were transfected with the β-Galactosidase gene and β-Galactosidase activity was assessed to determine the transient transfection efficiency. As described earlier, the transient transfection efficiency was quantitated to be consistently greater than 80% (Rabizadeh et al., 1999).

Cell Culture and Peptide Application

SH-SY5Y neuroblastoma cells were grown and maintained in DMEM supplemented with 10% FBS at 37°C and plated at a density of 1×10^4 cells into each well of an 8-well slide. The peptides were synthesized, purified by high-performance liquid chromatography (HPLC), and analyzed by mass spectrometry by Coast Scientific (San Diego, CA) and Bio Synthesis, Inc. (Lewisville, TX). They were dissolved in water at a concentration of 50 m*M*, diluted in serum-free medium to a concentration of 25 μ*M*, and then added directly onto the cells. After incubation for 6 h, acridine orange (5 μ*M*) and ethidium bromide (5 μ*M*) were added, and then the cells were viewed in a Nikon microscope, as described (Ellerby et al., 1997).

Sympathetic neurons from superior cervical ganglia (SCG) were cultured as previously described

(Garcia et al., 1992). Briefly, SCG from newborn rats were dissociated in dispase for 30 min. Neurons were then mechanically dissociated and plated at low density (10^4/cm^2) in collagen-coated 48-well dishes (Costar). During the first 4 d of culture, neurons were cultured in Leibowitz medium, 5% rat serum, 50 ng/mL 7S NGF (Roche Molecular Biochemicals), and 10^{-5} *M* cytosine arabinoside (AraC). On d 4, the AraC was omitted from the medium. On d 6, SCG neurons were treated with 25 μ*M* peptide in the presence of 2.5% rat serum and antibodies to NGF.

For quantitative apoptosis assays, SH-SY5Y cells were plated onto 96-well plates at a density of 5×10^3 cells/well. Peptides were prepared as noted previously and applied to the cells in 96-well plates. After incubating for 18 h at 37°C, PI (20 μ*M*) was added to the cells and cell death was assessed in a fluorimeter as described previously (Kane et al., 1995).

Cell-Free Apoptosis Studies

Cell-free caspase activation studies were performed as described by Ellerby et al. (1997). Western-blot analyses of caspase-3 were carried out according to Ellerby et al. (1997). For cell-free reconstitution studies (3 h, 37°C), mitochondria were washed and resuspended in cell-free system (CFS) buffer (220 m*M* mannitol, 68 m*M* sucrose, 2 m*M* NaCl, 5 m*M* KH_2PO_4, 2 m*M* $MgCl_2$, 5 m*M* succinate, 10 m*M* HEPES-NaOH, 2 m*M* ATP, 50 μg/mL creatine kinase, 10 m*M* phosphocreatine, 0.75 μg/mL rotenone, pH 7.4) at a concentration of 50–100 mg/mL. The final peptide concentration was 385 μ*M* in these studies.

Results

In order to study the mechanism by which the expression of p75^{NTR} confers neurotrophin dependence (i.e., apoptosis induction that is blocked by neurotrophins) (Rabizadeh et al., 1999), expression constructs were produced that direct the expression of chimeric p75^{NTR}-tumor necrosis factor receptor II (TNFR II) and p75^{NTR}-FK-binding proteins, truncated p75^{NTR} proteins, deletion-bearing p75^{NTR} proteins, or point mutant p75^{NTR} proteins. These constructs were then transfected into both neural and non-neural cells in culture, and their effects on cell death assessed. The rationale for using more than one cell type was simply to ensure that any effect observed was not unique to a specific cell line (Rabizadeh et al., 1993). Chimeric p75^{NTR}-TNFR II expression demonstrated that the extracellular domain of p75^{NTR} is not required for the induction of cell death (Fig. 1): replacement of the extracellular domain of p75^{NTR} with that of TNFR II did not abolish apoptosis induction (although a slight decrease occurred), whereas replacement of the transmembrane and intracytoplasmic domains did (and expression of TNFR II itself did not induce cell death).

For both Fas/Apo-1 and TNFR I, multimerization triggers the assembly of the death-inducing signaling complex (DISC), which results in the activation of caspase-8 (Muzio et al., 1997). In contrast, induction of apoptosis through a monomeric, unbound receptor is unprecedented. In order to distinguish between the possibility that monomeric p75^{NTR} mediates apoptosis, and the alternate, but not mutually exclusive, possibility that multimerization of p75^{NTR} induces apoptosis, chimeras were constructed using FK-binding protein (FKBP) single-copy and triple-copy vectors (Spencer et al., 1993). Cross-linking studies demonstrated that dimerization was induced by the addition of the dimerizing drug AP1510 to the chimeras containing the FKBP (Wang et al., in press). Expression of the p75^{NTR} intracellular domain induced apoptosis only in the monomeric state (Fig. 2); dimerization (using AP1510 with the single-copy FKBP) and higher-order multimerization (using AP1510 with the triple-copy FKBP) both blocked apoptosis induction by the p75^{NTR} intracellular domain (Fig. 2). As expected, the dimerizing agent AP1510 did not inhibit wild-type p75^{NTR}-induced apoptosis (Fig. 2), presumably because this lacked the FKBP domain. Moreover, neurotrophin-3 (NT-3) inhibited p75^{NTR}-induced apoptosis, but did not affect apoptosis induced by the chimeric FKBP- p75^{NTR} intracellular domain protein, which lacks the extracellular domain of p75^{NTR} (Fig. 2). Thus, rescue did not occur as a nonspecific effect of either AP1510 or NT-3.

Au: Define FKBP

Deletion of the carboxyterminal 19 amino acids of p75^{NTR} did not diminish its ability to induce cell death (Fig. 1); on the contrary, there was a slight increase in apoptosis induction, similar to the effect

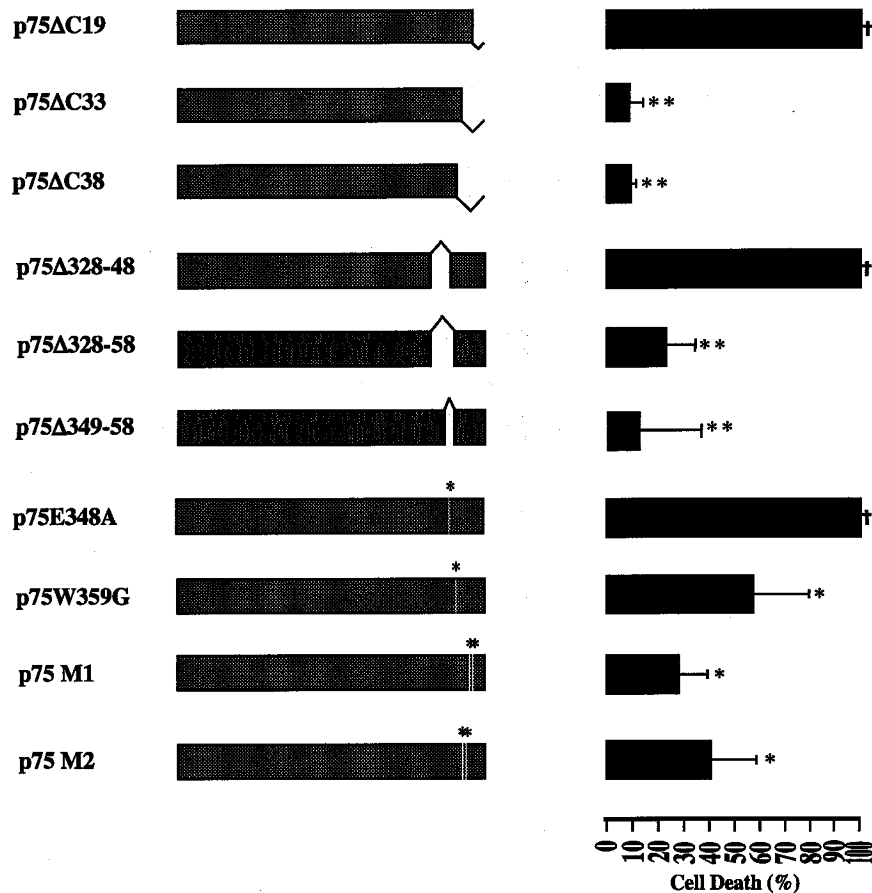


Fig. 1. Induction of apoptosis by mutants of p75^{NTR}. The previously postulated death domain of p75^{NTR} is not required, whereas a much smaller region near the carboxyterminus is required. Cell death was normalized to the effect of wild-type p75^{NTR} (absolute values of cell death induced by p75^{NTR} ranged from 50 to 100%, depending on experiment). Error bars represent standard errors. Each mutant was analyzed in 3–7 separate transfections in both neural (PC12 NRA5) and non-neural (293T) cells. Cell death was assessed 3–9 d after serum withdrawal in PC12 NRA5 cells by quantifying propidium iodide-stained cells using a fluorimeter. Trypan blue exclusion was used to assess the death of transiently transfected 293T cells at 4–12 h after the addition of 35 μM tamoxifen. Statistical significance was assessed by two-tailed *t*-test. $^{*}p < 0.05$. $^{**}p < 0.01$. For ECp75 and ECp70, the gray region represents p75^{NTR}, and the white region represents TNFR II. p75 M1 is the R375 G, R376G double mutant of p75^{NTR}; p75 M2 is the L370K, L371P double-mutant of p75^{NTR}. † labels those mutants that induced cell death at least as effectively as the full-length, wild-type p75^{NTR}. Asterisks (*) over the construct diagrams indicate mutation sites. Cell death induction by TNFR II (p70) was not statistically significantly different from the vector control.

described for a similar manipulation of Fas/Apo-1 (Nagata and Golstein, 1995). However, extending this deletion to include an additional 14 amino acids abolished the ability of p75^{NTR} to induce cell death. Because this critical region, extending from residues 364–377 of the mature polypeptide, lies at the carboxyterminus of a region of similarity to the death domains of Fas and TNFR I (Chapman, 1995; Feinstein et al., 1995), it was conceivable that the putative p75^{NTR} death domain predicted by alignment and modeling studies functions similarly to the death domains of Fas and TNFR I. However, this did not prove to be the case: neither mutation nor deletion of conserved

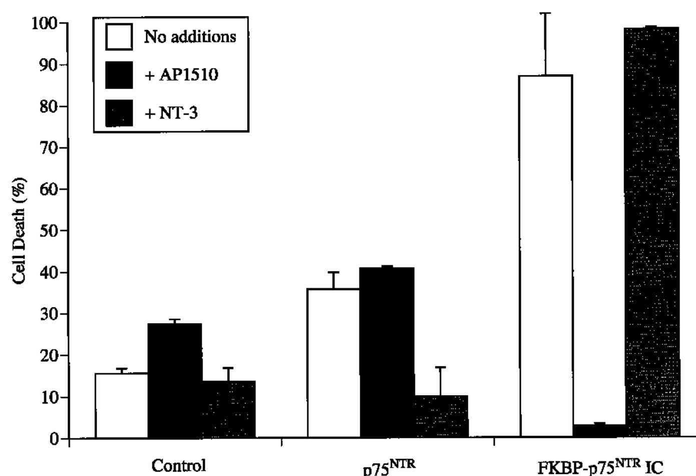


Fig. 2. Cell death is induced by the monomeric p75^{NTR} intracellular domain (FKBP- p75^{NTR}IC, white column). In contrast, both dimerization (using a single-copy FKBP construct) and higher-order multimerization (using a triple-copy FKBP construct) by the dimerizing drug AP1510 blocked cell death induction by the p75^{NTR} intracellular domain (black column) in transiently transfected 293T cells. The differences between cell death in the presence of the monomeric and multimeric groups were highly statistically significant by two-tailed *t*-test. Cell death induced by wild-type p75^{NTR} (white column) was not inhibited by AP1510 (black column). However p75^{NTR}-induced apoptosis was inhibited by NT-3, whereas NT-3 did not inhibit apoptosis induced by the monomeric p75^{NTR} intracellular domain (gray columns). Control experiments expressing the insert-less pcDNA3 vector evaluating the effect of AP1510 and NT-3 on 293T cell survival showed no effect. Trypan blue exclusion was used to quantify cell death 4–12 h after the addition of 35 μ*M* tamoxifen. This experiment was repeated four times. Error bars represent standard error.

amino acids required for death domain function in TNFR I (Tartaglia et al., 1993) blocked the ability of p75^{NTR} to induce apoptosis. Specifically, mutation of glutamate 348 to alanine did not decrease the pro-apoptotic effect of p75^{NTR}, whereas the analogous mutation in TNFR I (Glu369 -> Ala) abolished the death domain effect completely (Tartaglia et al., 1993). Similarly, mutation of Trp359 -> Gly in p75^{NTR} led to only a partial reduction in activity (Fig. 1), despite the fact that the analogous residue in TNFR I (Trp378) is critical for TNFR I pro-apoptotic function (Tartaglia et al., 1993).

Deletion of residues 328–348, a region predicted by alignment studies to be crucial for Fas death induction (Huang et al., 1996) (and for which the similar region of TNFR I lies completely within the death domain), had no effect on apoptosis induction by p75^{NTR} in response to neurotrophin withdrawal (Fig. 1). However, deletion of residues 328–358 and residues 349–358 effectively inhibited p75^{NTR}-induced apoptosis (Fig. 1). These results suggest that the region (or at least one region) within the intracellular domain of p75^{NTR} that is necessary for the induction of apoptosis resides between residues 349 and 377. This region has been determined by Liepinsh et al. (1997) to form the fourth and fifth α-helices in the intracellular domain of p75^{NTR}. Accordingly, expression of constructs coding solely for residues 349–377 (α4–5) resulted in the induction of apoptosis (Fig. 3). Expression of all six helices (α1–C) or the carboxyterminal three helices (α4–C) also induced apoptosis (Fig. 3).

Because the mutation analysis studies demonstrated that the p75^{NTR} neurotrophin-dependence domain includes a region that shares structure similarity with the wasp venom-toxin mastoparan (Chapman, 1995), and because mastoparan has been shown to induce apoptosis (Yan et al., 1995; Ellerby et al., 1997), we next determined whether this region is both necessary (in the context of the protein) and sufficient (as a peptide) to induce apoptosis. Peptides comprising this region were

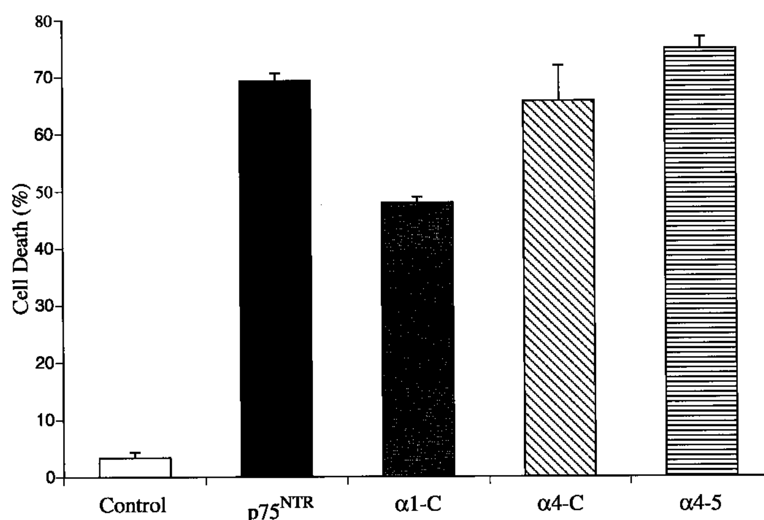
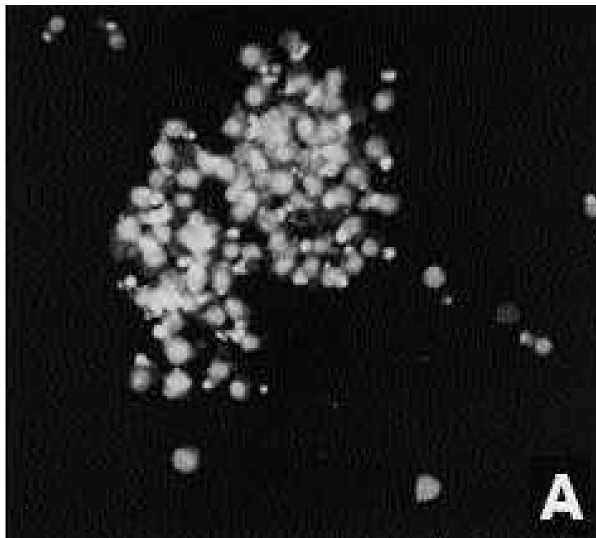


Fig. 3. Induction of apoptosis by fragments of the p75^{NTR} intracellular domain that include helices 4 and 5. Constructs expressing the entire α-helical region (α1-C) or the carboxy three α-helices (α4-C) of the intracellular domain of p75^{NTR} induced apoptosis in 293T cells. A construct solely expressing helices 4 and 5 effectively induced apoptosis relative to the wild-type p75^{NTR}. Control represents the expression of the vector pcDNA3 without any insert. Cell death was quantified by trypan blue exclusion 4–12 h after the addition of 35 μ*M* tamoxifen. This experiment was repeated three times. Error bars represent standard error.

synthesized and their effects on apoptosis assessed (Figs. 4,5). Because of the relatively poor intracellular penetration of many peptides, we utilized an approach in which peptides of interest are coupled to cellular-entry peptides such as those derived from the Antennapedia sequence or the human immunodeficiency virus (HIV) Tat protein sequence (Fawell et al., 1994; Kim et al., 1997, Troy et al., 1997). Control peptides with similar predicted helicity were compared, as well as mutants of the p75^{NTR} peptide and a scrambled p75^{NTR} peptide. When the p75^{NTR} peptide representing residues 364–377 was coupled to the Tat fragment, the resulting peptide-induced apoptosis in cultured neural cells (SH-SY5Y, PC12 NRA5, R2, and NTera2), primary cultures of superior cervical ganglion neurons, and non-neural cells (NIH3T3, PC-3, LNCaP, cos7), whereas the control peptides did not (Figs. 4,5). Apoptosis induction by p75^{NTR}-derived peptides was rapid, with the initial morphological changes being visible approx 1 h after the addition of peptides. Truncation of the peptide after residue 374 (residue 11 of the 14-mer) completely abolished its pro-apoptotic activity (Fig. 5), demonstrating that the carboxyterminal sequence, Arg-Arg-Ile, is required for activity. Similarly, mutation of the cor-

Fig. 4. (*opposite page*) Induction of apoptosis in SH-SY5Y neuroblastoma cells and sympathetic neurons from the superior cervical ganglion (SCG) by a peptide derived from p75^{NTR} residues 364–377. Acridine orange and ethidium bromide staining of SH-SY5Y cells revealed that the wild-type peptide bearing the sequence SATLDALLAALRRI (residues 364–377 of p75^{NTR}) induced morphological alterations characteristic of apoptosis, including nuclear fragmentation and apoptotic bodies after 6-h incubation **(A)**. A scrambled version of the same peptide with the sequence DLSLARLATARLAI was ineffective in inducing cell death **(B)**. It is noteworthy that SH-SY5Y cells treated with the wild-type peptide have shrunken dramatically relative to control cells (Magnification: 200×). Hoffman phase microscopy of 6-d-old SCG neurons from newborn rats treated with the wild-type peptide for 2 h revealed significant somatic and neuritic atrophy **(C)** as compared with SCG neurons treated with the control peptide **(D)**. Hoechst staining revealed nuclear condensation in SCG neurons 6 h after incubation with the wild-type peptide **(E)** whereas the nuclear structure remained unaltered in neurons cultured with the control peptide **(F)**). In each case, 25 μ*M* of each peptide was used. Each peptide also included the sequence GRKKRRQRRRPP from the human immune-deficiency protein Tat at their amino termini to facilitate cellular entry.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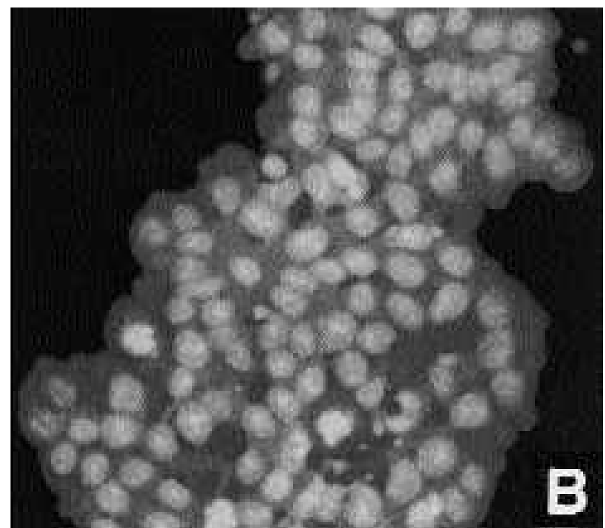
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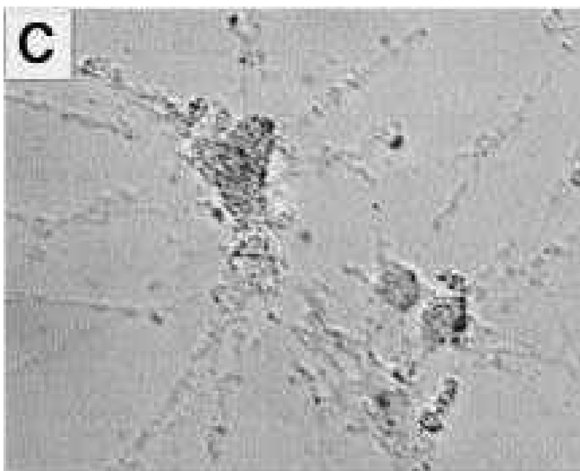
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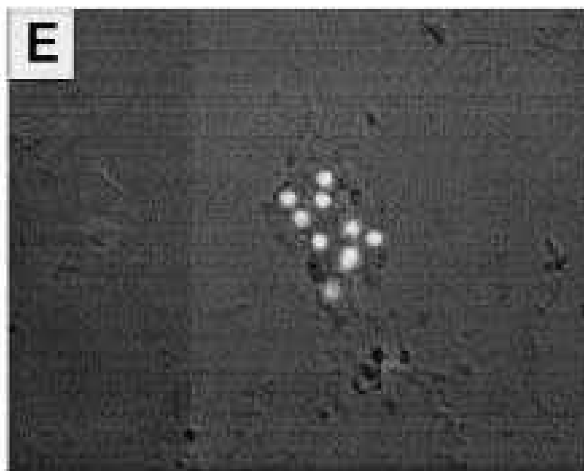
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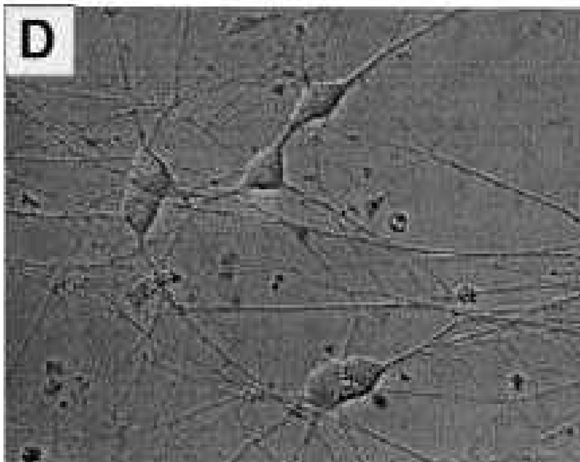
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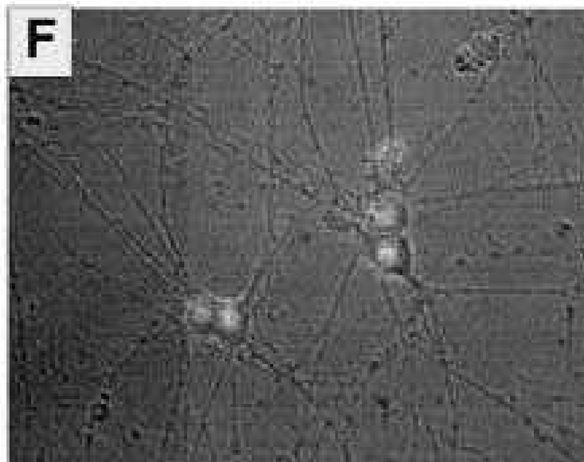
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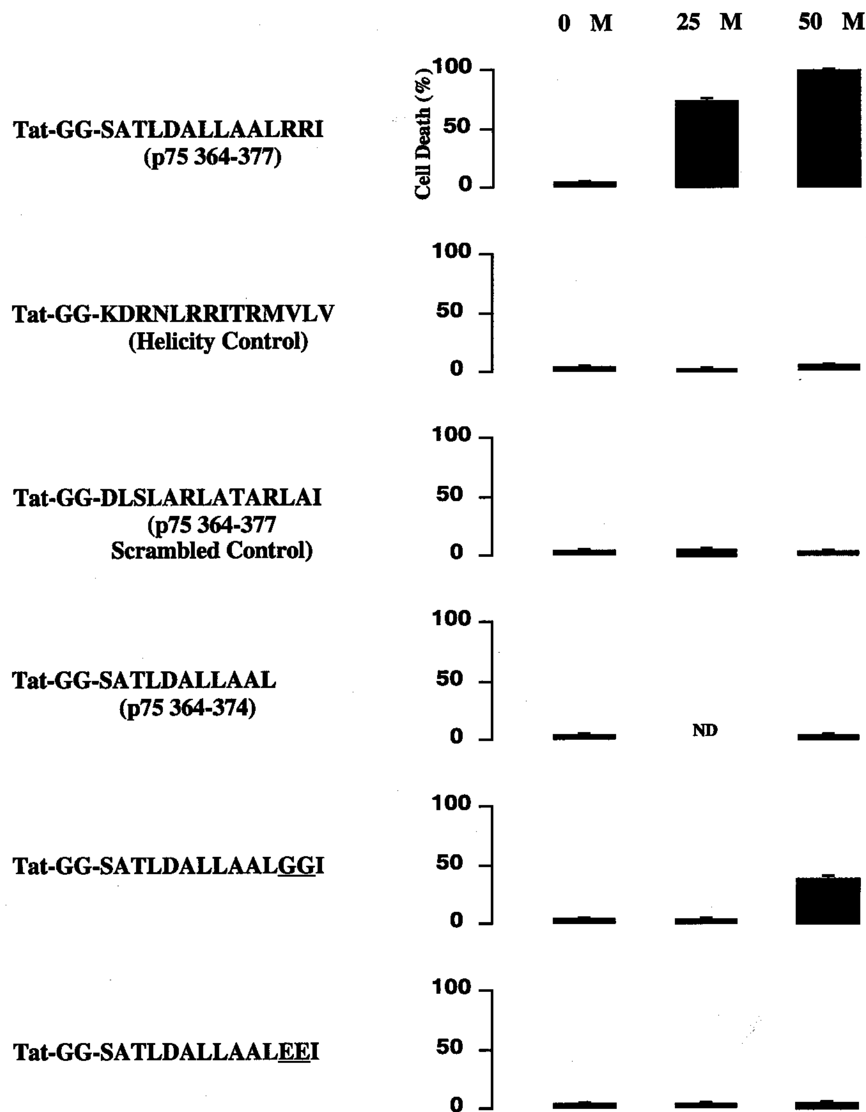

Fig. 5. Cell death induced in SH-SY5Y neuroblastoma cells by peptides derived from $p75^{NTR}$, and related peptides. Control peptides, including a scrambled control, a helicity control, and peptides derived from regions outside the $p75^{NTR}$ dependence domain, did not induce cell death. Propidium iodide was added to SH-SY5Y cells 18 h after incubation with each peptide and cell death was quantified by a fluorimeter. Columns represent percentage cell death, and error bars represent standard errors. Quadruplicate wells were averaged, and the experiment repeated 4–10 times for each peptide. Underlined residues represent mutations.

responding Arg residues, Arg375 and Arg376, to Gly-Gly, within the expression construct, reduced the pro-apoptotic effect of the resultant gene product by approx 70% (Fig. 1); and the corresponding mutation within the peptide (Arg12 and Arg13 to Gly-Gly) led to a similar reduction of its pro-apoptotic effect (Fig. 5), demonstrating that for both the pro-apoptotic peptide and the full-length $p75^{NTR}$, the Arg residues near the carboxyterminus of the mastoparan-like region are critical for apoptosis induction. Other residues within this domain also proved to be critical: mutation of Leu370 and Leu371 to Lys370 and Pro371 also decreased the pro-apoptotic effect of $p75^{NTR}$ (Fig. 1).

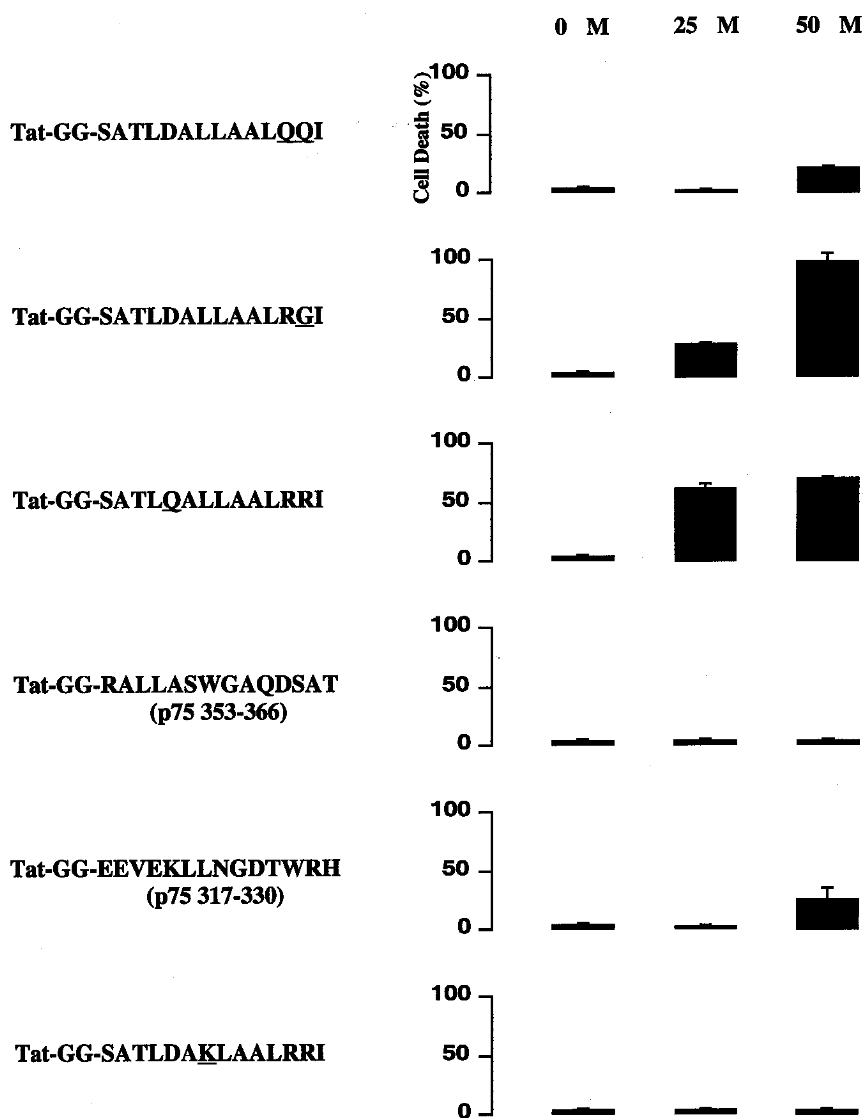

Fig. 5. *continued*

Mutation of the Arg-Arg to Glu-Glu (Fig. 5) resulted in a more complete loss of the pro-apoptotic effect than mutation to Gln-Gln or Gly-Gly, demonstrating that the positive charges at the carboxyterminus are less effectively replaced by negatively charged residues than by uncharged residues. Replacement of the Arg-Arg by Arg-Gly also reduced the pro-apoptotic activity of the peptide, but the effect was modest (Fig. 5). In contrast to the requirement for the carboxyterminal positive charge, mutation of the Asp at position five to Gln had virtually no effect on apoptotis induction (Fig. 5).

The pro-apoptotic effect of the p75^{NTR}-derived peptide was not mimicked by other peptides derived from p75^{NTR}: a peptide representing residues 353–366, which lies immediately upstream from the pro-apoptotic peptide and represents the fourth alpha helix (Liepinsh et al., 1997), had no effect on apoptosis, and a peptide representing residues 317–330 and the first alpha helix had a minimal effect on apoptosis (Fig. 5).

The similarity of the pro-apoptotic p75^{NTR}-derived peptide to mastoparan in primary (Feinstein and Larhammer, 1990) and secondary

(Hileman et al., 1997) structure suggested that it may induce apoptosis in a similar manner. Like mastoparan (Ellerby et al., 1997), the p75^{NTR} 364–377 peptide induced caspase-3 activation in a neural cell-free apoptosis system (Fig. 6).

Discussion

These results demonstrate that the mechanism by which the common neurotrophin receptor, p75^{NTR}, mediates cell death is fundamentally different than the mechanism utilized by Fas and TNFR I. Whereas multimerization of Fas and TNFR I by ligand binding is required for initiation of a pro-apoptotic signal (Freiberg et al., 1996), it is the monomeric p75^{NTR} that mediates cell death in the paradigm used here, with dimerization inhibiting this effect. Furthermore, mediation of cell death by p75^{NTR} requires a novel type of domain that is 29 amino aids in length, stretching from amino acid 349–377, whereas apoptosis induction by Fas and TNFR I requires a death domain that is approx 70 amino acids in length (Nagata and Golstein, 1995).

The structure of the carboxyterminal two-thirds of the intracellular domain of p75^{NTR} has been reported to consist of six α-helices (Liepinsh et al., 1997). The p75^{NTR} dependence domain ranging from residue 349–377 encompasses the fourth (α4) and fifth (α5) α-helices. Deletion of the sixth α-helix (p75ΔC19 construct) or of the second and third helices (p75Δ328–48) did not reduce the receptor's ability to induce apoptosis. However, receptors bearing deletions of α4 (p75Δ349–58) or α5 (p75ΔC33) failed to induce apoptosis. Also, the expression of a p75^{NTR} fragment bearing α4 and α5 (α4–5 construct) induced apoptosis as well as the wild-type receptor. Thus the region that stretches from 349–377 and includes α4 and α5 is necessary and sufficient for apoptosis induction by p75^{NTR}.

A peptide derived from this region—SATLDALLAALRRI—is sufficient to induce apoptosis in several different cell lines, with an EC_{50} of 10–50 μ*M*. This peptide corresponds to α5 plus the three residues aminoterminal to the α5 helix (SerAlaThr) and one residue carboxyterminal to the α5 helix (Ile). It is noteworthy that the human and rat p75^{NTR} sequences are identical in this region. Furthermore, apoptosis induction by both p75^{NTR} and

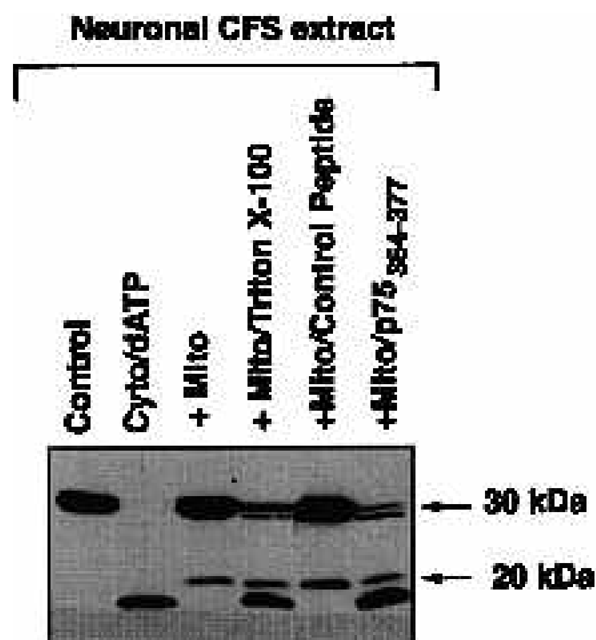

Fig. 6. Activation of caspase-3 cleavage in a cell-free system of neural apoptosis (Ellerby et al., 1997) by the p75^{NTR} 364–377 peptide. Caspase-3 cleavage and activation was induced by the p75^{NTR} 364–377 peptide but not by the control, scrambled peptide in the presence of mitochondria (mito) in a neural cell-free system (CFS). The scrambled control peptide (DLSLARLATARLAI) in the presence of mitochondria effected no change in cleavage relative to the mitochondria alone. The p75^{NTR} 364–377 peptide activated caspase-3 as well as the detergent Triton X-100 (2%), which served as a positive control, in the presence of mitochondria. As shown before (Ellerby et al., 1997), cytochrome c (cyto) (10 μ*M*) effected the activation of caspase-3 in the presence of dATP (1 m*M*).

the peptide requires the arginine residues near their carboxytermini. Substitution of negatively charged residues for the arginine residues effectively inhibited the peptide's apoptotic activity. This finding suggests the possibility that the positive charges may be important in electrostatic interactions with negatively charged lipid or negatively charged amino acids within an interacting protein or proteins. It is noteworthy that mastoparan, a pro-apoptotic wasp toxin, includes a similar region at its carboxyterminus (Lys-Lys-Ile-Leu). Reaper, a larger pro-apoptotic peptide of 65 amino acids, has two Arg-Lys dipeptides near its carboxyterminus.

The p75^{NTR} pro-apoptotic peptide has effects similar to mastoparan on cell survival and on the activation of caspase-3 in a cell-free system.

Contrary to results obtained with the α5 peptide, peptides derived from α4 and α1 did not induce apoptosis. These results suggest that, whereas α4 and α5 are both necessary in the context of the full-length protein, only α5 is sufficient as a peptide to induce apoptosis.

It is not yet known with what this region of p75^{NTR} interacts. However, considering the lack of pro-apoptotic effect of a nonhelical point mutant (Hileman et al., 1997), the requirement for carboxyterminal positive charge, and the structural similarity to mastoparan, one possibility is that direct membrane interaction leads to pore formation or membrane disruptive effects similar to those described for some antibiotic peptides (Sansom, 1991; Jacob and Zasloff; 1994; Javadpour et al., 1996).

Solution structure of a closely related peptide—ATLDALLAALRRIQ—by nuclear magnetic resonance spectroscopy has recently been reported (Hileman et al., 1997). In the presence of lipid micelles, this peptide forms an amphiphilic helix, with the central hydrophobic residues facing the lipid micelles and the carboxyterminal four residues (Arg-Arg-Ile-Gin) opening up the helix. substitution of Lys for Leu at position six destroyed the helix formation, and the same substitution in the p75^{NTR} peptide destroyed the pro-apoptotic effect. However, whether the requirement for amphipathic helix formation will hold up with the testing of additional mutants remains to be determined.

The p75^{NTR} neurotrophin-dependence domain is distinct from previously defined death domains of TNFR I and Fas by the following criteria: 1) Mutations that inactivate the pro-apoptotic effects of TNFR I do not have similar effects for analogous, conserved residues of p75^{NTR}. 2) The similarity of the death domains of TNFR I and Fas to the p75^{NTR} intracytoplasmic domain only encompasses the carboxyterminal one-half of the TNFR I and Fas death domains; the aminoterminal one-half does not show significant similarity (Chapman, 1995). 3) Deletions in the putative p75^{NTR} death domain do not destroy the pro-apoptotic effect of p75^{NTR} unless they include the dependence domain. 4) Ligand binding may inhibit apoptosis induced by p75^{NTR}, whereas the reverse occurs for TNFR I and Fas. 5) For Fas and TNFR I, multimerization initiates pro-apoptotic signaling, whereas for p75^{NTR}, induction of apoptosis requires the monomer.

In theory, it should be possible to create a state of dependence on other receptor-multimerizing ligands, such as epidermal growth factor, by fusing the extracellular sequence of the receptor of interest to the transmembrane and intracytoplasmic domains of p75^{NTR}. Understanding the biochemical mechanisms underlying cellular dependence may find application in developmental biology, suppression of metastatis during carcinogenesis, and neurodegenerative diseases (Bredesen et al., 1998; Mehlen et al., 1998).

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