Neurotrophin p75 Receptor Is Involved in Neuronal Damage by Prion Peptide-(106–126)*

In this work we have investigated the molecular basis of the neuronal damage induced by the prion peptide by searching for a surface receptor whose activation could be the first step of a cascade of events responsible for cell death. By using a human neuroblastoma cell line lacking all the neurotrophin receptors and derived clones expressing the full-length or truncated forms of the low affinity neurotrophin receptor (p75NTR), we have been able to demonstrate that the neuronal damage induced by the prion protein fragment PrP-(106–126) is an active process mediated by a) the binding of the peptide to the extracellular region of p75NTR; b) the signaling function of the intracytoplasmic region of the receptor; and c) the activation of caspase-8 and the production of oxidant species.

The aetiological agent of the prion diseases, a set of sporadic, genetic, and infectious neurodegenerative disorders affecting humans and animals, has been proposed to be an aberrant isoform of the cell surface glycoprotein (PrPSc) named Scrapie prion protein (PrPC), which arises posttranslationally via conformational changes converting PrPC into PrPSc (1, 2). Therefore, the mechanisms by which the extracellular deposition of PrPSc, a protein characterized by its high content of β-pleated sheet and ability to form amyloid fibrils, elicits the neuropathological changes of the prion diseases, i.e., spongiform degeneration, neuronal loss, and appearance of reactive astrocytes and microglial cells (1), has remained unknown. In recent years, it has been demonstrated that a prion protein fragment, PrP-(106–126), could induce neuronal death by apoptosis in rat hippocampal cultures (3). This peptide mimics the physico-chemical properties of PrPSc by exhibiting a prevalent β-pleated structure and forms fibrillar aggregates that are partially resistant to proteolysis (4). The ability of PrP-(106–126) to elicit a neurotoxic effect has been widely confirmed (5–8) and, hence, it is considered a valid model for studies into the mechanisms of neuronal damage by prions. So far, the knowledge of such mechanisms had remained rather limited despite a series of recent findings, the most significant of which are: a) the binding of the prion peptide to cellular prion protein PrPSc and the consequent inhibition of PrPSc function (9); b) the perturbation of ion (especially calcium) homeostasis (6, 10, 11); c) the induction of oxidative stress (12); and d) the cooperative pathogenetic role of the activation of microglia with production of oxidant species (5).

In the search for a prion receptor we focused our attention on the p75 low affinity NGF receptor, which belongs to the family of death receptors (13) and can trigger apoptosis (14–16). The rationale of this investigation was 2-fold. First, it has been observed that PrP-(106–126) exhibits a prevalent β-sheet structure and forms fibrillar aggregates similarly to β-amyloid peptides (4). Second, the β-amyloid peptides of Alzheimer’s disease may exert their neurotoxic action via an interaction with p75NTR (17–19). The data herein presented support the view that the prion peptide-mediated cell damage occurs through its interaction with the p75 neurotrophin receptor.

Experimental Procedures

Materials—The peptide PrP-(106–126), synthesized as previously described (3), was a generous gift of Prof. S. Salvadori (Department of Pharmaceutical Sciences, University of Ferrara, Italy) or was purchased from Bachem (Budendorf). The PrP-(106–126) was prepared by Amersham Pharmacia Biotech. Scrambled PrP-(106–126) was a kind gift of Dr. M. Salona (Istituto Mario Negri, Milano). Peptides were dissolved in 200 mM phosphate buffer, pH 5.0, and aged for 2–3 days at 37 °C to increase fibrillogenesis, which was measured with the Thioflavin test. Goat polyclonal antibody sc-6189 against a peptide mapping at the amino terminus of human p75NTR was purchased from Santa Cruz Biotechnology. All the chemicals, when not indicated, were from Sigma.

p75NTR Constructs—All p75NTR constructs were generated via different strategies. The wild type p75NTR construct was produced by cloning the p75NTR cDNA into the pCEP4 vector within the PvuII site. The deletion mutants p75ΔCD and p75ΔECD were obtained by polymerase chain reaction utilizing specific primers that introduce either a deletion of the carboxyl-terminal domain or specific internal deletions within the extracellular cysteine-rich domains or the intracellular domains. The scheme of full-length p75NTR and of the truncated forms of the receptor is reported in Fig. 1A.

Cell Clones and Detection of p75NTR mRNA and Proteins—The parental neuroblastoma SK-N-BE cells (20) (BENR-free) were grown in RPMI 1640 medium containing fetal bovine serum (15% v/v) and antibiotics. The cells were transfected with the different pCEP4β-p75NTR constructs utilizing the polyethylene-immune method. After transfection, the cells were split and grown in RPMI medium containing hygromycin (150 μg/ml) to allow for the selection of positive clones. Surviving clones were picked, expanded, and characterized for expression of both wild type and mutated p75NTR proteins. All clones were tested for expression of mRNA by Northern blot analysis (Fig. 1C) as previously described (20). Briefly, total RNA was extracted from cell clones by Tri-Reagent (Sigma) following manufacturer protocol. 15 μg of total RNA have been
loaded per lane and run on a 3% formaldehyde, 1.5% agarose gel, transferred to a nylon membrane (Hybond N/H11001, Amersham Pharmacia Biotech) and probed with the p75 NTR full-length cDNA labeled with 32P using MegaPrime kit (Amersham Pharmacia Biotech) following the manufacturer protocol. For the Western blot analysis cell lysates were processed as previously described (20), and the expression of p75NTR protein was tested using 9992 rabbit polyclonal antiserum (a gift from Dr. M. V. Chao), which recognizes the intracellular region of the receptor (Fig. 1D). For each p75NTR construct, a battery of cell clones expressing comparable levels of either full-length or truncated proteins was chosen for the experimental work. The correct localization of the p75NTR proteins in the plasmamembrane was detected immunohistochemically (Fig. 1B). Cells were permeabilized with 0.1% Triton X-100 and then stained with the rabbit polyclonal antiserum (9992), or a mouse monoclonal antibody (ME20.4) (a gift from Dr. M. V. Chao) raised against the p75NTR extracellular region (20).

Cytotoxicity of PrP-(106–126)—Cells were plated (10,000/cm²) in RPMI medium supplemented with fetal bovine serum (15% v/v), glutamine (2.0 mM), gentamicine (50 μg/ml) and hygromycine (150 μg/ml) in the case of the transfected cells) and maintained at 37 °C in humidified atmosphere of air with 5% (v/v) CO2 added. Two days later, the growth medium was replaced with fresh RPMI medium containing fetal bovine serum (1% v/v) and PrP-(106–126) or staurosporine. When required the inhibitors of caspases Z-Val-Ala-DL-Asp-fluoromethyl-ketone (Z-VAD-FMK) or Z-Ile-Glu-Thr-Asp-fluoromethyl-ketone (Z-IETD-FMK) (Calbiochem) or of NADPH oxidase diphenyleneiodonium were added 2 h before prion peptide or staurosporine. Specimens were sampled at various intervals thereafter. The cell damage was assessed by means of epifluorescence microscopy after staining the plated cells with a solution 1:1 (v/v) of acridine orange (0.1 mg/ml in phosphate-buffered saline; filter setting for fluorescein isothiocyanate) and ethidium bromide (0.1 mg/ml in phosphate-buffered saline; filter setting for rhodamine) according to Spector et al. (21).

Binding Assay—Cells plated as for cytotoxic assays were incubated...
RESULTS AND DISCUSSION

We have studied the effects of PrP-(106–126) on both the human neuroblastoma SK-N-BE cells (BENTR-free), which express neither p75NTR nor any of the high affinity neurotrophin receptors (Trks) (20), and SK-N-BE cell clones expressing the full-length p75NTR (BEp75) (Fig. 1). The cell damage was analyzed by double staining of the specimens with both the cell-permeable acridine orange and the cell-impermeable ethidium bromide (21) as shown in Fig. 2. The results of these analyses, summarized in Fig. 3, A and B, demonstrate that PrP-(106–126) could induce cell death in BEp75 cell clones, while being totally harmless for BENTR-free parental cells and for SK-N-BE cells transfected with an empty pCEP4 vector (not shown). Importantly, the neurotoxic action of PrP-(106–126) on BEp75 cell clones was detectable already at very low concentrations (Fig. 3C). Scrambled PrP-(106–126) was unable to induce cell death in either cell clones (not shown). To our knowledge, this is the first evidence that p75NTR is involved in the neurotoxic activity of prion peptide.

Two mechanisms could be responsible for the function of p75NTR in the cell damage by the prion peptide. This receptor might be merely permissive for the cytotoxic action of PrP-(106–126) elicited via a mechanism independent of the actual binding of the peptide as in the case of excitotoxicity (22). Alternatively, the binding of prion peptide to p75NTR might be directly involved in cell death. A series of experimental findings shows that the latter mechanism is the most likely one.

First, we have treated SK-N-BE-derived cell clones expressing a truncated p75NTR (BEp75/ECD) (Fig. 1) lacking the four cysteine-rich repeats of the extracellular region either with PrP-(106–126) or with staurosporine. The results (Fig. 4) show that, while being still susceptible to staurosporine-elicited apoptosis, BEp75/ECD cells were insensitive to the cytotoxic effects of PrP-(106–126), a finding indicating the requirement of the extracellular region of p75NTR for the cytotoxic effect of PrP-(106–126).

Second, we have shown that PrP-(106–126) binds to p75NTR. In fact, the results reported in Fig. 5A show that 125I-PrP-(106–126) binds to BEp75 cell clones but not to BENTR-free cells or to BEp75/ECD cells lacking the extracellular region of the recep-
tor. The specificity of the binding was shown by its competitive inhibition by an excess of cold aggregated PrP-(106–126). Scatchard analysis (Fig. 5C) showed that the binding of the aggregated peptide to p75NTR occurs with a $k_d$ of $4.6 \pm 0.7 \text{ nm} (n = 4)$ and is saturable (Fig. 5B). The specificity of the binding was also shown by the finding that it was inhibited by the antibody sc-6189 raised against the amino terminus of p75NTR (Fig. 5D) and by NGF (Fig. 5E). Since NGF binds to p75NTR with a $k_d$ of $4–7 \text{ nm}$ (18), which is similar to that of PrP-(106–126), the high concentration of NGF required for inhibiting the binding of the prion peptide may indicate that the two ligands interact with different sites of p75NTR.

The results so far presented, showing that PrP-(106–126) is cytotoxic by binding to p75NTR, raise the problem of the role of this receptor in cell death induced by the peptide. Two mechanisms could be hypothesized. The binding of the prion peptide to p75NTR might be able to activate the receptor and trigger the signals for cell death via the receptor’s intracellular region or it could serve solely as an anchorage allowing for PrP-(106–126)-induced cell damage by other mechanisms. We demonstrated the validity of the first hypothesis by investigating the effect of PrP-(106–126) on cell clones expressing a truncated p75NTR devoid of the entire intracellular region (BEp75ICD) (Fig. 1). The results reported in Fig. 4 show that these cells, despite normally binding the peptide (Fig. 5A), were insensitive to its toxic effect indicating that the intracellular region of the receptor is necessary for signaling cell death.

We have also investigated some of the biochemical events usually involved in active cell death. The results (Fig. 6) demonstrate that p75NTR-mediated cell death induced by PrP-(106–126) is associated with the activation of caspases as it was fully suppressed by either Z-VAD-FMK (100 μM), a nonspecific inhibitor of caspases, or Z-IETD-FMK (20 μM), an inhibitor of receptor-activable caspase-8. These findings are consistent with the concept that prion peptide-elicited neuronal cell death via p75NTR is an active process that, by involving caspase-8, is reminiscent of the cell death induced by other members of the death receptors family (13). The data of Fig. 6 show that the cytotoxic effect of 50 nM staurosporine, a receptor-independent apoptogenic drug, was sensitive to Z-VAD-FMK but could not be suppressed by Z-IETD-FMK. The results of Fig. 6 also show that diphenyleneiodonium (100 nM), an inhibitor of NADPH oxidase, the enzyme forming reactive oxygen intermediates, and other flavo-protein dehydrogenases (23), could fully suppress the cell death by PrP-(106–126) mediated by p75NTR. This finding agrees with the notion that an oxidative stress is involved in the pathogenesis of cell death by prion peptide (12).

Our results, showing that PrP-(106–126) damages neuronal cells by activating p75NTR, raise a series of problems to be investigated. These problems include the types of interaction such as the type of interaction of the prion peptide with the extracellular region of p75NTR, the signaling function of the different domains of the intracellular region, the identification of the events occurring down-stream the intracellular region (24, 25), and the comparison of the role of p75NTR with that of other cell surface proteins recently found to interact with prion peptide as the receptor for advanced glycation end-products (RAGE) (26), a 66-kDa protein with unknown function (27, 28),

**Fig. 5.** Specific binding of $^{125}$I-PrP-(106–126) to p75NTR-expressing clones. A, binding of labeled peptide to whole cells of different clones in the presence or absence of unlabeled aggregated peptide. Data are reported as means ± S.D. of 18 experiments with BENTR-free and BEp75 clones and of six experiments with BEp75ECD and BEp75ICD clones. B, saturation curve of the peptide binding to cell clone expressing full-length p75NTR (BEp75), and C, the same data expressed as Scatchard plot. Data are of one experiment representative of four. D and E, concentration-dependent inhibition of the binding of peptide to cells expressing full-length p75NTR (BEp75) by antibody sc-6189 and by NGF, respectively. Data are of one experiment representative of three.

**Fig. 6.** Effect of inhibitors on the cell damage by PrP-(106–126) and by staurosporine. Effect of the unspecific inhibitor of caspases (Z-VAD-FMK, 100 μM), of the specific inhibitor of caspase-8 (Z-IETD-FMK, 20 μM), and of an inhibitor of reactive oxygen intermediates-forming NADPH oxidase and other flavoprotein dehydrogenases (diphenyleneiodonium, 100 nM) on the neurotoxic action of PrP-(106–126) (20 μM) or staurosporine (50 nM) in BEp75 cells. Data are reported as means ± S.D. of at least four experiments.
a 37-kDa laminin receptor precursor (29), the formyl peptide receptor-like 1 (30), and the PrP\(^{Sc}\) itself (9).

The therapeutic and/or preventive strategies concerning the prion diseases are related to the mechanisms of formation and propagation of PrP\(^{Sc}\) and to the pathogenesis of the neuronal damage by PrP\(^{Sc}\). It is likely that the results presented herein obtained by using the model of PrP-(106–126)-neurotoxic effect open new avenues for effective therapeutic strategies. Further studies will clarify if the neurotoxicity of purified infective PrP\(^{Sc}\) protein involves mechanisms similar to those of the PrP-(106–126) peptide.

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