

Neuroserpin, an axonally secreted serine protease inhibitor

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We have identified and chromatographically purified an axonally secreted glycoprotein of CNS and PNS neurons. Several peptides derived from it were micro-sequenced. Based on these sequences, a fragment of the corresponding cDNA was amplified and used as a probe to isolate a full length cDNA from a chicken brain cDNA library. Because the deduced amino acid sequence qualified the protein as a novel member of the serpin family of serine protease inhibitors, we called it neuroserpin. Analysis of the primary structural features further characterized neuroserpin as a heparin-independent, functional inhibitor of a trypsin-like serine protease. *In situ* hybridization revealed a predominantly neuronal expression during the late stages of neurogenesis and in the adult brain in regions which exhibit synaptic plasticity. Thus, neuroserpin might function as an axonally secreted regulator of the local extracellular proteolysis involved in the reorganization of the synaptic connectivity during development and synapse plasticity in the adult.

Keywords: axonally secreted/neuron-specific/neuroserpin/serine protease inhibitor/synapse formation

Introduction

During the development of the nervous system, neurons form axons which extend along a prespecified path into the target area, where they engage in the formation and the refinement of synaptic connections. These stages depend critically on the capability of the axonal growth cones to interact with a variety of structures which they encounter along their way and at their destination, including cell surfaces, of neuronal and non-neuronal origin, and the extracellular matrix. Along their trajectory and at their target sites, growth cones not only receive and respond to signals from their local environment, but also actively modify it by secreted macromolecules (Sweadner, 1981). In particular, secreted proteases have been implicated in supporting the growth cone advancement through the tissue. More than a decade ago, it was demonstrated that plasminogen activators are produced and axonally secreted by neurons in culture (Krystosek and Seeds, 1981a,b). Recently, their occurrence in the

developing rat nervous system during the period of axon outgrowth has been revealed (Sumi *et al.*, 1992; Dent *et al.*, 1993). Moreover, several pieces of evidence were presented which indicated that serine proteases, such as plasminogen activators or thrombin, are involved in restructuring of the synaptic connectivity during development and regeneration. Such processes include synapse elimination during development (Hantai *et al.*, 1989; Connold and Vrbova, 1994; Liu *et al.*, 1994) and synaptic plasticity associated with learning and memory in the adult (Meiri *et al.*, 1994; Qian *et al.*, 1993).

Here we present the purification, the molecular cloning and the expression pattern of a glycoprotein that is secreted from axons of several neuronal populations of both the PNS and the CNS (Stoeckli *et al.*, 1989). The deduced primary structure of this novel protein was found to be homologous to the members of the serine protease inhibitors of the serpin family (Schapira and Patston, 1991). The expression of this novel serpin is restricted to the nervous system, unlike other members of the serpin family, such as protease nexin 1 (PN-1; Baker *et al.*, 1980; Guenther *et al.*, 1985; Gloor *et al.*, 1986), α 1-antichymotrypsin (AChT; Rayford *et al.*, 1992), plasminogen activator inhibitor 1 (PAI1; Rao *et al.*, 1993) or antithrombin III (ATIII; Deschepper *et al.*, 1991), which are expressed widely outside the nervous system. Based on its structural characteristics and its expression pattern, we have termed this protein neuroserpin. The intriguing temporo-spatial pattern of expression and the fact that it is secreted from axons implicate neuroserpin as a regulator of proteolytic events associated with synapse restructuring functions during neural development, such as the elimination or segregation of synapses and synaptic plasticity in the adult nervous system.

Results

Identification of neuroserpin among the axonally secreted proteins of dorsal root ganglia and ventral spinal cord neurons

Neuroserpin (then denoted axonin-2) was initially characterized as one of two axonally secreted proteins in cultures of chicken embryonic dorsal root ganglion (DRG) neurons using a compartmental cell culture system that provides separate access to somas and neurites (Ruegg *et al.*, 1989; Stoeckli *et al.*, 1989). In that study, neuroserpin was demonstrated to be secreted into the culture medium by dissociated cultures of several other neuronal populations of the CNS and the PNS. In order to investigate also a neuronal population of the CNS with regard to the capability to secrete neuroserpin from the neurites, we have cultured ventral spinal cord (vSC) neurons (Sonderegger *et al.*, 1984) using the same compartmental cell culture system and metabolically labelled the newly synthesized

proteins with [35 S]methionine. The proteins released from the neurites into the side compartment were collected and subjected to two-dimensional SDS-PAGE followed by fluorography (for a detailed description see Stoeckli *et al.*, 1989). As demonstrated in Figure 1, the radioactively labelled proteins of the side compartment medium of the vSC cultures are distinct from those found with DRG neurons except for the 55 kDa protein, which we called neuroserpin, due to the characteristics described below. Based on the *in vitro* results presented in the previous report (Stoeckli *et al.*, 1989) and the *in situ* hybridization studies presented here, neuroserpin is a neuronal protein that is synthesized in and secreted from several neuronal populations. In at least one neuronal population of each of the PNS and the CNS, the neuritic release of neuroserpin has been demonstrated.

Purification and microsequencing of neuroserpin

Quantitative limitations excluded conditioned media of primary cultures as a source of purification. Ocular vitreous fluid (VF) from 14-day-old (E14) chicken embryos was chosen as the source material for the purification of neuroserpin. Although unfractionated VF did not contain detectable amounts of neuroserpin (see also Ruegg *et al.*, 1989), we suspected the presence of small quantities because neuroserpin was previously found to be released from retinal neurons in culture (Stoeckli *et al.*, 1989). In the absence of a functional assay, the fractions of each purification step were tested for the presence of neuroserpin by two-dimensional SDS-PAGE followed by silver staining. The purification procedure, consisting of preparative isoelectric focusing (IEF), hydrophobic interaction chromatography and preparative SDS-PAGE, was devised as a scale-up of the analytical two-dimensional SDS-PAGE, taking advantage of the known isoelectric point (IEP) and molecular mass (M_r) of neuroserpin (Figure 2). With the procedure detailed in Materials and methods we were able to purify ~5–10 μ g neuroserpin from 400 ml VF containing 750 mg protein in total.

An antiserum against the purified neuroserpin was raised in a rabbit. With this antiserum, the axonally secreted 55 kDa protein of the cultured DRG neurons was specifically precipitated (Figure 3). This confirmed the identity of the purified protein from VF with the axonally secreted glycoprotein identified previously in the compartmental cell culture system.

The purified neuroserpin was subjected to partial amino acid microsequencing using an automated sequenator. For the determination of internal sequences, the purified protein was enzymatically cleaved with the endoproteases AspN or trypsin and the generated peptides were isolated by reversed phase liquid chromatography. Contiguous amino acid sequences over 24, 6, 9, 6 and 9 amino acids respectively, were obtained from the amino terminus and four internal peptides of the purified 55 kDa protein (Figure 4).

Molecular cloning and primary structure of the neuroserpin cDNA

The cDNA of neuroserpin was cloned by two independent approaches. First, nested PCR on embryonic chicken retina cDNA was carried out using degenerate oligonucleotide primers corresponding to the amino terminus and two of

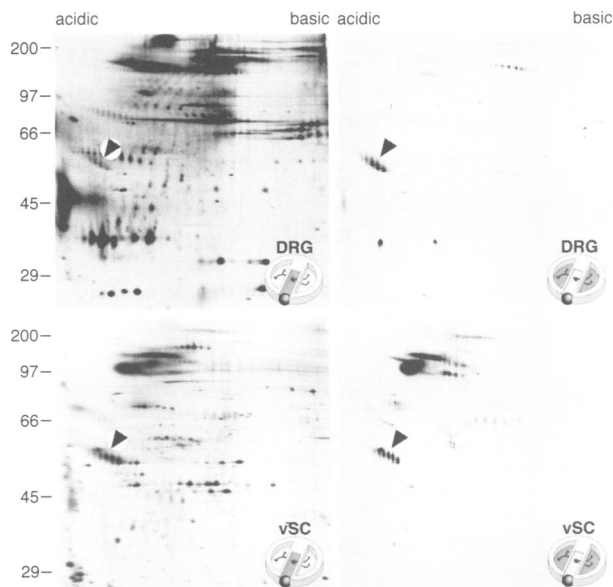


Fig. 1. Characterization of neuroserpin as an axonally secreted protein of DRG and ventral spinal cord neurons in the compartmental cell culture system. Dissociated dorsal root ganglion (DRG) neurons and ventral spinal cord (vSC) neurons respectively, were cultivated in a compartmental cell culture system (Campenot, 1977). In this system, the cell culture surface is subdivided into three chambers by a Teflon divider which is attached to the floor of the culture dish with silicone grease so that a thin film of medium connects the adjacent compartments. If dissociated neurons are plated into the centre compartment, they settle down and remain confined to this area. The axons, however, can grow within the medium film underneath the separating barrier and extend into the side compartments (pictograms at the bottom of each autoradiography). After the neurites had reached the side compartments, the neuronal proteins were metabolically labelled by adding [35 S]methionine to the medium of the centre compartment. Secreted proteins in the conditioned media from both the central and the side compartments, were analysed by two-dimensional SDS-PAGE and fluorography. The origin of the protein sample is indicated as a grey shaded area in the pictogram. Note that neuroserpin (arrow) is secreted into the side compartments (right panels) from the axons of both DRG neurons (top) and vSC neurons (bottom).

the internal peptides. A 700 bp fragment was amplified and subsequently used to screen a chicken embryonic brain cDNA library (Zuellig *et al.*, 1992). Secondly, the same library was screened with the rabbit antiserum against neuroserpin. Several positive clones were identified with each method and were sequenced. In both groups one full length cDNA was found. The two clones were identical except for a different length of the 3' untranslated region (UTR) due to alternative use of polyadenylation sites (Figure 4). The sequence contained one long open reading frame (ORF) of 1230 bp. The nucleotide and the deduced amino acid sequence of neuroserpin are shown in Figure 4. In total, the cDNAs span over 1950 and 1510 nucleotides respectively, subdivided in a short 5' UTR, a 1230 bp ORF and a 0.7 kb or 0.25 kb 3' UTR. Polyadenylation signals (Sheets *et al.*, 1990) are located at positions 1478 and 1920 respectively. The nucleotide sequence adjacent to the putative start methionine at the amino terminus of the only long ORF conforms to the consensus described by Kozak (1987). The amino acid sequence of the ORF starts with a hydrophobic stretch of 16 amino acids conforming to the consensus signal sequences (von Heijne, 1983). The sequence starting with the 17th amino

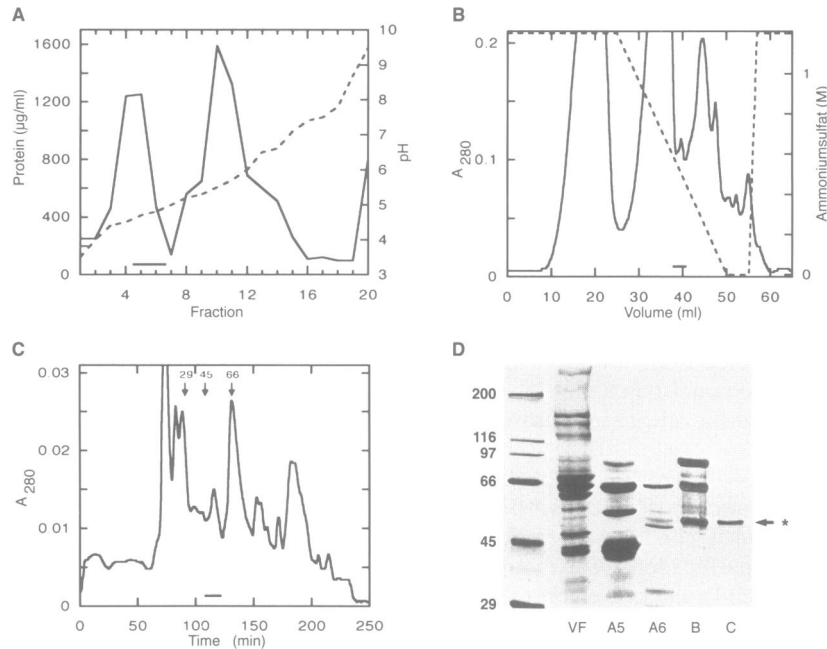


Fig. 2. Purification of neuroserpin from vitreous fluid of chicken embryos. Neuroserpin was purified from the ocular vitreous fluid (VF) of 14-day-old chicken embryos. Fractions of each purification step were tested for the presence of neuroserpin by two-dimensional SDS-PAGE followed by silver staining. The horizontal bar in the elution profile indicates the fractions selected for the following step. (A) Preparative isoelectric focusing of VF using the ROTOFOR chamber. Protein concentration (full line) and pH (broken line) of each fraction were determined. (B) Elution profile of the hydrophobic interaction chromatography using a TSK-Phenyl-5PW column. Elution was achieved with a decreasing gradient of ammonium sulfate (broken line). (C) Preparative SDS-PAGE using the 230 A HPEC system with a 6% polyacrylamide gel. The electrophoretic system was first calibrated with molecular weight standards of 29 kDa, 45 kDa and 66 kDa respectively (elution time indicated by arrows). (D) SDS-PAGE analysis of the fractions of each step that were selected for further processing. Purified neuroserpin after step C appears homogeneous and exhibits a molecular mass of ~55 kDa (lane C, indicated by *).

acid corresponds to the sequence of the amino-terminal peptide. The four internal peptides of purified neuroserpin were found in the deduced amino acid sequence, confirming the authenticity of the cDNA. The deduced amino acid sequence of neuroserpin after cleavage of the presumptive signal peptide adds up to a molecular mass of 45 kDa and an isoelectric point of 4.9. Sequences NHT and NTS representing potential sites for N-linked glycosylation are located at positions 157 and 401. Thus, the 10 kDa difference between the calculated molecular mass and the molecular weight determined by SDS-PAGE (Figure 2D) as well as the isoelectric variants seen on two-dimensional SDS-PAGE (Figures 1 and 3) may be explained by N-linked glycosylation.

Searches in the GenEMBL and SwissProt databases identified neuroserpin as a novel member of the serpin family. With two pairs of nested primers flanking the putative reactive site loop (Figure 4), we amplified a corresponding cDNA fragment of ~450 bp from a commercial human fetal (17–18 weeks) brain cDNA library (Stratagene) and from neonatal mouse mRNA respectively. The deduced amino acid sequences of the human and the mouse cDNA fragments exhibited a sequence identity of 88% and 87% respectively, with chicken neuroserpin. Both the human and the murine fragment were distinct from any of the known mammalian serpins and both had an amino acid identity with the nearest relative of ~35%. Sequence comparisons of neuroserpin with other serpins revealed amino acid sequence identities in the range of 33–42% (Table I). Alignment of the putative reactive site loop of neuroserpin with inhibitory and non-inhibitory

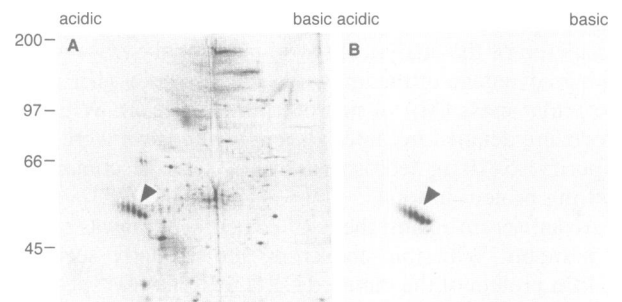


Fig. 3. Immunoprecipitation of neuroserpin from the supernatant of cultured DRG neurons by the polyclonal antiserum raised against the protein purified from vitreous fluid. Total proteins (A) and proteins precipitated by the polyclonal antiserum against purified neuroserpin (B) from the conditioned medium from metabolically labelled DRG neurons were separated by two-dimensional SDS-PAGE and analysed by fluorography. Neuroserpin is recognized by its characteristic spot pattern (arrows). (A) Neuroserpin among other proteins produced and secreted from dissociated cultures of DRG neurons. (B) Neuroserpin specifically precipitated by the rabbit polyclonal antiserum against purified neuroserpin from vitreous fluid.

serpins revealed a high degree of similarity to the inhibitory serpins and a substantially lower resemblance to the non-inhibitory serpins in the functionally important hinge region P₁₇-P₉ (denoted according to Schechter and Berger, 1967) near the carboxy terminus (Figure 5A).

Serpins which are enhanced in their anti-proteolytic function by heparin (for example PN-1) have five to seven basic residues within a short segment in their amino-terminal moiety forming the so-called heparin-binding

1
 GGGGGTGGTGTGAGCTTCTGTGCTGAGCTGGTCTTAATATCTCACAAACATGATATTCCCTGGACTATTGTCTTTGCTCGTTGCGCTAG
 M Y F L G L L S L L V L P S

91
 TAAAGCCTTCAAGACCAATTTCTGATGAACTATTGCTGAGCTTTCTGTGAATGTTACAATCAGCTACGAGCTGCAAGAGAAGATGA
 K A F K T N F P D E T I A E L S V N V Y N O L R A A R E D E

181
 GAATATTCTTTCTGCTCTGAGCATGCAATAGCCATGGGAATGATAGAGCTTGGAGCCCATGGAACCACTTTGAAAGAAATTCGACA
 N I L F C P L S I A I A M G M I E L G A H G T T L K E I R H

271
 TTCATTGGGTTTTCAGACCTTAAAAATGGTGAAGAGTTTACCTTTTGAAGGACCTTTCTGATATGGCAACTACGGAAGAAAGTCATTA
 S L G F D S L K N G E E F T F L K D L S D M A T T E E S H Y

361
 TGTTTTAAATATGGCCAACCTCTCTATGTTTGAAGAGTTTACCTTTTGAAGGACCTTTCTGATATGGCAACTACGGAAGAAAGTCATTA
 V L N M A N S L Y V Q N G F H V S E K F L Q L V K K Y F K A

451
 TGAAGTAGAGAACATAGATTTTCAGCCAGAGTGCAGCTGTAGCTACCCACATCAATAAGTGGGTAGAGAATCATAAACCAATATGATCAA
 E V E N I D F S Q S A A V A T H I N K W V E N H T N N M I K

541
 AGATTTTGTGTCATCAAGAGACTTCAGTCTCACTTGGTTCATCAATGCAATCTACTTTAAAGGCAATTTGGAATCAGTTC
 D F V S S R D F S A L T H L V L I N A I Y F K G N W K S Q F

631
 CAGACCTGAAAATACAAGAACTTTTCTTTCACTAAGGATGATGAACTGAACTGAGTGCAGATTCCTCAATGATGTACCAGCAAGGAGAGTTTAA
 R P E N T R T F S F T K D D E T E V Q I P M M Y Q Q G E F Y

721
 TTATGGAGAGTTCAGTGTGGCTCTAATGAAGCAGGTGGTATCTACCAAGTCTTAGAAAATACCATATGAAGGAGATGAAAATAGTATGAT
 Y G E F S D G S N E A G G I Y Q V L E I P Y E G D E I S M M

811
 GATTGTTCTTTCCAGACAGGAAGTTCGCTGGTCCATGAGCCACTTGTAAAGCCTCATGATTAATGAGTGGGCTAACTCTGTAAA
 I V L S R Q E V P L V T L E P L V K A S L I N E W A N S V K

901
 AAAGCAAAAGTTGAAGTATATTGTCGAAGTTCACAGTAGAGCAAGAAATGATCTGAAAGATGATTTGAAAGGCTTTGGAATTACAGA
 K Q K V E V Y L P R F T V E Q E I D L K D V L K G L G I T E

1081
 AGTGTTCACCGTAGTGTGCTGACCTCACTGCCATGTCTGATAACAAAGAACTTTACCTTGCAAGGCAATTTCAAGGCTTTCTTAGAAGT
 V F S R S A D L T A M S D N K E L Y L A K A F H K A F L E V

1171
 TAATGAGGAAGGATCAGAAGCTGCTGCTGCCTCAGGAATGATTGCCATTAGCAGAATGGCAGTACTGTATCTCTCAAGTTATAGTTGATCA
 N E E G S E A A A A S G M I A I S R M A V L Y P Q V I V D H

1261
 TCCTTTCTTCTTCCGGTCAGAAACAGAAGAACAGGCACTGTGTTTATTCATGGGGAGGTAATGCACCCCTGAAGCAATGAATACAAGTGG
 P F F F L V R N R R T G T V L F M G R V M H P E A M N T S G

1351
 CCATGACTTTGAAGAGCTTTAACTACTGCCAGCTACCAAAATGAGGAAAAAGCACATCAAGTTTCAAGTTAATATATTTAAGGTTTGTCT
 H D F E E L

1441
 GTGTTCTCCCCCAAGATATTGTTTAAAAATGCTTTTAGATCTATGTGCAAGTGAAGTTTCCAGAGGAAAAGTTTCAAGTATTAAAAATAAT
 GGTTCTGTTTMTGTTATTTGTTGATGCTGTGCTCTTAAATAAAATTTGTTGATATGTCACAACTGTTTCTTGTCTAGTGAATTTGGAAG

1531
 TAAAAATCTTGCAATCCATAATCTCGAATGTTTACAGTACTAAGACTTACTGGATCGATGAGACAGGAGCACTATGTTGCTCAGCTCT
 1621
 GAGTAATCAAATGACAAACCATTTTATAGTTGAACTACATCTGGCAAATGTATTCTTGAATAGTAAACAGACTGCAGCATGCTGTCT
 1711
 CAAAGTTGATGGAAGAATCTAAACCTAATGGATTCATCTGAGAAGCTGTGTAGACTGTGTAACCTCCAACGTGTGGTTCTAAAAATGCTG
 1801
 CTGTTATGAATGCTCCTGCATGCAACTCCGCGATTCACCTCCGTAAGTGTGATACTAATAAAATTTGATCGCATGAGTATTTCAGCT
 1891
 AGTCAGTGTAGAATAGCTGGGATATCCAAATTTAAATAGTTGACACTCAAAAAAAAAAAAA

Fig. 4. The cDNA of neuroserpin. Full length cDNA of neuroserpin cloned from a chicken brain E14 cDNA library. A long and a short neuroserpin cDNA with an identical ORF of 410 amino acids were found. They differed only in length at the 3' end due to alternative usage of polyadenylation signals (bold). Sequenced peptides are underlined and the amino-terminal signal sequence is printed in italics. The primers for the initial PCR-amplification are indicated by solid arrows, the primers flanking the reactive site loop by broken horizontal arrows, possible sites for *N*-glycosylation are marked by asterisks (*) and the putative reactive site between R362 and M363 is marked by the scissors pictogram. The GenBank accession number for the neuroserpin sequence is Z71930.

exosite (Evans *et al.*, 1993). When presented as a helical wheel (Figure 5B) the positively charged residues cluster in close proximity. The corresponding segment of neuroserpin (G84–E101) shows a concentration of negative charges on the surface of the corresponding helix. This difference indicates that the anti-proteolytic activity of neuroserpin is heparin-independent.

Expression of neuroserpin mRNA in vivo

The *in vivo* distribution of the neuroserpin mRNA was investigated by Northern blot analysis and *in situ* hybridization. Blot analysis confirmed the existence of two

neuroserpin mRNAs of 1.7 and 2.2 kb respectively. High levels of neuroserpin mRNA were found in embryonic (E14) retina, brain, cerebellum and spinal cord. No transcripts were detected in embryonic lung, liver, kidney, heart or skeletal muscle. In the adult chicken, a high concentration of neuroserpin mRNA was found in the brain. Again, no transcripts were detected in non-neuronal tissue such as liver, kidney, heart or skeletal muscle, except for a small quantity of the 1.7 kb, but not of the 2.2 kb, transcript in the adult lung (Figure 6A).

In situ hybridization revealed strong cellular signals indicative of neuroserpin mRNA in both the PNS and the

Table I. Percentage similarity of serpins

	PN-1(h)	PN-1(r)	PAII(h)	PAII(m)	LEI(h)	ATIII(h)	α 1AT(h)	OA(c)
NS(c)	42	42	39	39	39	38	33	37
PN-1(h)		88	45	43	38	36	28	35
PN-1(r)			46	44	38	36	29	36
PAII(h)				82	35	34	34	32
PAII(m)					36	34	33	32
LEI(h)						47	34	47
ATIII(h)							33	38
α 1AT(h)								35

Seven different serpins and two species homologues of the same serpin were aligned in pairs and the percentage of identical amino acids is indicated. Neuroserpin (NS) was compared with protease nexin 1 (PN-1), plasminogen activator inhibitor 1 (PAII), leucocyte elastase inhibitor (LEI), antithrombin III (ATIII), α 1-anti-protease (α 1AT) and the non-inhibitory ovalbumin (OA). The comparisons between neuroserpin and other serpins are printed in bold. The similarity of species homologues is printed in italics. Species are indicated in parentheses (c, chicken; h, human; r, rat; m, mouse).

CNS. The neuroserpin-expressing cells of the CNS were exclusively located in the grey matter and the majority had large cell somas. During neural development, neuroserpin expression was found in most areas of the nervous system, whereas in the adult, neuroserpin mRNA was restricted to relatively few areas. An overview of the expression of neuroserpin in neural tissue at different stages is shown in Figure 6.

Neuroserpin was first detected at stage 23 (staged according to Hamburger and Hamilton, 1951; corresponding to embryonic day four) in the floor plate and the intermediate zone of the spinal cord (Figure 6B). The expression in the floor plate was found continuously diminished with time and had disappeared at stage 38 whereas a strong signal was still detected in the intermediate zone of the spinal cord at stage 38. At stage 28, medial motoneuron pools started to express neuroserpin mRNA. At stage 33, neuroserpin mRNA appeared in a lateral pool of large motoneurons (marked by asterisks). A few scattered neurons of the ventral horn exhibited strong neuroserpin expression in spinal cord sections of adult chickens.

In the embryonic retina (Figure 6C), the first signals were detectable at stage 36 in the ganglion cell layer and the inner nuclear layer. The strongest expression of neuroserpin mRNA was from stage 43 to 46 (hatching) and transcripts remained detectable until adulthood in the ganglion cell layer and the inner nuclear layer. In contrast, no cells of the plexiform layers or among the photoreceptors were neuroserpin-positive throughout the tested time frame.

In the cerebellum (Figure 6D), a strong signal was seen in Purkinje cells between stages 38 and 43, whereas only a few scattered cells in the granule cell layer expressed neuroserpin at these stages. No cells in the molecular layer were neuroserpin-positive at any time. In the optic tectum, the first neuroserpin transcripts appeared at stage 38 (not shown) in the stratum griseum et fibrosum superficiale (SGFS, according to LaVail and Cowan, 1971), the stratum griseum centrale (SGC) and the stratum griseum periventriculare (SGP), but not in the fibre layers. The strongest signals were detected around stage 43 (Figure 6E) in the large multipolar neurons of the SGC and most of the pyramidal and multipolar cells of laminae g, h and i in the SGFS. Sustained expression was registered in the adult animal in a few isolated neurons of the laminae g

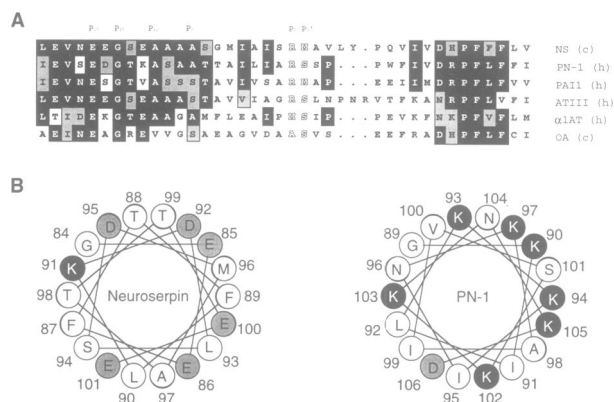


Fig. 5. Alignment of reactive site loops and the heparin-binding exosite of different serpins. (A) The putative reactive site loop of neuroserpin (NS) was compared with reactive site loops of the inhibitory serpins protease nexin 1 (PN-1), plasminogen activator inhibitor 1 (PAII), antithrombin III (ATIII), α 1-anti-protease (α 1AT) and with the non-inhibitory serpin ovalbumin (OA). Sequences were aligned by the program GAP of GCG (according to Needleman and Wunsch, 1970). Identical amino acids are highlighted in black and conservative changes in grey boxes. The putative reactive site amino acids are outlined. Nomenclature of P₁₇ to P₁ and P₁' is according to Schechter and Berger (1967); Species are indicated in parentheses (c, chicken; h, human). (B) A helical wheel comparison of the heparin-binding exosite of PN-1 and the corresponding sequence of neuroserpin shows the markedly different distribution of acidic (grey shaded) and basic (black outlined) amino acids in the putative surface-exposed areas of helix D.

through i in the SGFS (data not shown). A sagittal section through the adult brain (Figure 6F) revealed neuroserpin expression in neurons throughout the hyperstriatum accessorium, the neostriatum and the hippocampus, as well as in particular thalamic nuclei (Kuenzel and Masson, 1988).

Discussion

We purified, microsequenced and cloned neuroserpin, a novel serine protease inhibitor of the serpin family. Neuroserpin is synthesized by neurons of both the CNS and the PNS and it is secreted from axons. Neuroserpin expression starts during the late stages of development and persists in adulthood. The axonal secretion and the temporo-spatial pattern of expression implicate neuroserpin as a local modulator of extracellular proteolytic

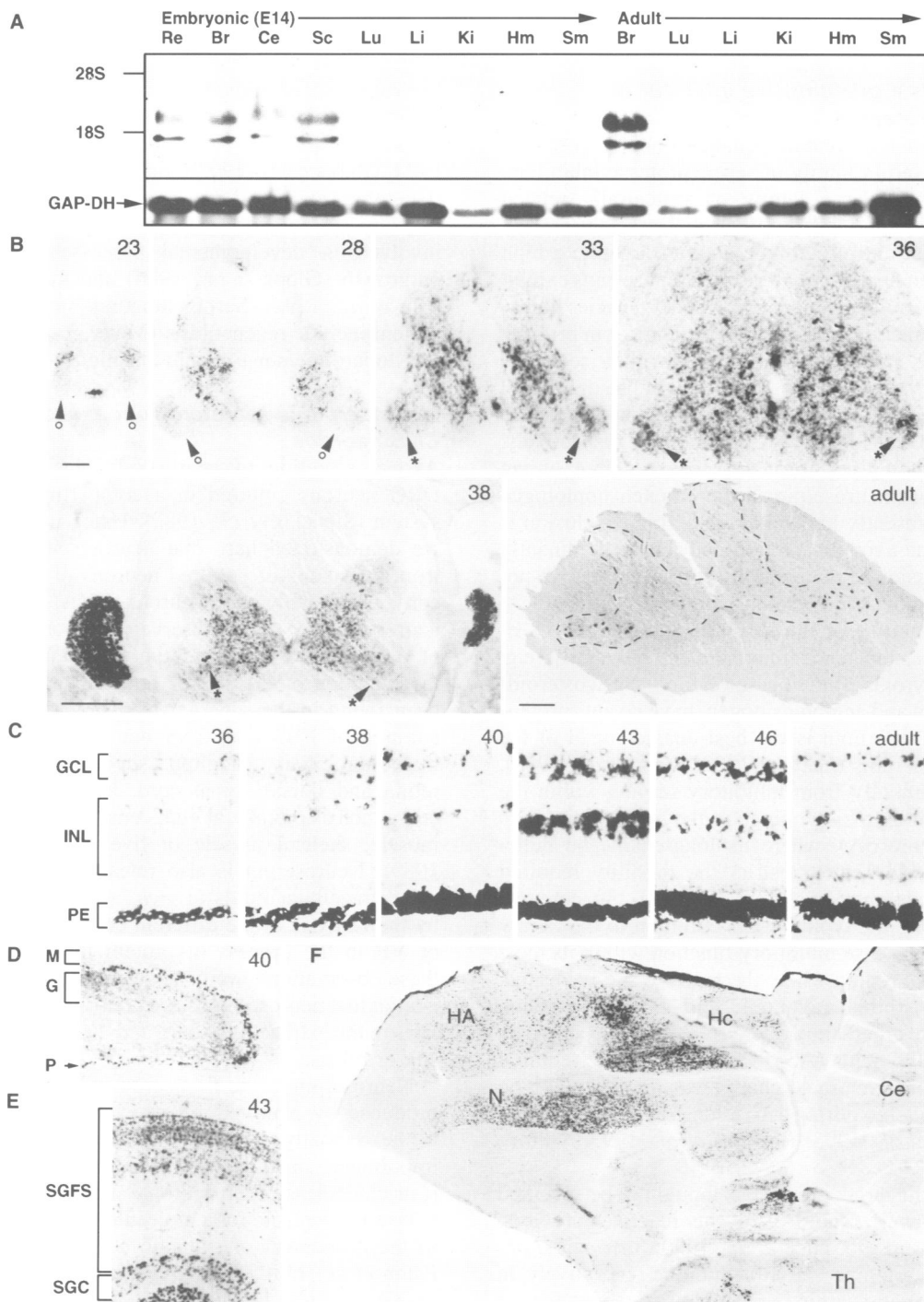


Fig. 6. Expression of neuroserpin mRNA during development and in the adult. (A) Northern blot analysis of neuroserpin mRNA. 7.5 μ g total RNA from embryonic (E14) and adult chicken retina (Re), brain (Br), cerebellum (Ce), spinal cord (Sc), lung (Lu), liver (Li), kidney (Ki), heart (Hm) and skeletal muscle (Sm) were electrophoretically separated, transferred to a PVDF membrane and analysed with DIG-labelled neuroserpin antisense cRNA. Approximately equal loading and integrity of the RNA is demonstrated by hybridization with a glyceraldehyde-3-phosphate dehydrogenase cRNA (GAP-DH). (B–F) *In situ* hybridization of neuronal tissue with DIG-labelled and partially hydrolysed neuroserpin antisense cRNA: (B) Spinal cord and DRGs (stage 23 to adult). Neuroserpin mRNA appears first at stage 23 in the floor plate and in a lateral cell population located in the intermediate zone of the spinal cord. Note that no signal is found in motoneurons at this stage (circles). The expression of neuroserpin in the floor plate disappears completely at stage 38. After stage 28 neuroserpin mRNA is found in more and more cells of the ventral spinal cord including different pools of motoneurons. Highest levels of neuroserpin mRNA are detected during stages 33–36, when motoneurons form functional synapses with their target muscles. For instance, an asterisk indicates the staining of neuroserpin mRNA in a lateral motoneuron pool with highest intensities during stages 33–36. The signal decreases afterwards (stage 38). However, some lateral motoneurons still express neuroserpin in the adult chicken. (Bars: 100 μ m in the upper and 500 μ m in the lower panel; the broken line indicates the boundary between grey and white matter in the adult spinal cord). (C) Retina (stage 36 to adult; GCL, ganglion cell layer; INL, inner nuclear layer; PE, pigment epithelium). (D) The cerebellum (stage 40; M, molecular layer; G, granule cell layer; P, Purkinje cells) and (E) the optic tectum (stage 43; SGFS, stratum griseum et fibrosum superficiale; SGC, stratum griseum centrale). (F) A sagittal section through an adult chicken brain (Ce, cerebellum; Hc, hippocampus; HA, hyperstriatum accessorium; N, neostriatum; Th, thalamus).

processes occurring at the synapse during neural development and in the adult.

Neuroserpin is a presumptive inhibitor of trypsin-like proteases

The primary structural features qualified neuroserpin as a member of the serpin family of serine protease inhibitors. The serpins are a large family of structurally related proteins which are divided into two subclasses, the serpins with and without anti-proteolytic activity. Despite a high similarity of their spatial structure, different serpins exhibit only ~30–50% amino acid identity, whereas species homologues of the same serpin are 80–90% identical in primary structure (Table I). Because no serpin with a sequence identity higher than 42% (PN-1) is known, but the partial amino acid sequences of the human and the mouse neuroserpin show an identity of 88% and 87% respectively, to the corresponding region of the chicken cDNA, it can be ruled out that neuroserpin is the chicken homologue of any of the presently known serpins. This conclusion is supported by the axonal secretion and the predominantly neuronal expression of neuroserpin, features that have not been reported about the presently known serpins.

The great majority of the serpins act as inhibitors of serine proteases, but some members of the family, e.g. ovalbumin, thyroxine-binding globulin, corticosteroid-binding globulin and angiotensinogen, have no anti-proteolytic activity. Ovalbumin is the best characterized of the non-inhibitory serpins (Gettins, 1989; Stein *et al.*, 1990). It differs substantially from inhibitory serpins within the region corresponding to the hinge of the reactive site loop (P₁₇ to P₉; Figure 5A), where its long-chain and bulky amino acid residues may restrict the mobility required for inhibitory action (Wright *et al.*, 1990). In contrast, neuroserpin bears the typical traits common to the family members with protease inhibitory function within its most carboxy-terminal region where the reactive site is located. In particular, it exhibits a glutamic acid (E346) in position P₁₇, a small uncharged amino acid (G 348) at P₁₅ and four short-chain amino acids (A351–A354) at P₁₂–P₉ required for the large conformational changes that anti-proteolytic serpins must undergo during activation (Huber and Carrell, 1989; Gettins *et al.*, 1993; Hopkins *et al.*, 1993; Schulze *et al.*, 1994).

The type of the target protease is thought to be specified by the positions P₁ and P₁' of the reactive site loop (Lawrence *et al.*, 1990; Nick *et al.*, 1990; Sherman *et al.*, 1992). With an arginine and a methionine respectively, at these positions, neuroserpin is most likely targeted against trypsin-like proteases. Neuroserpin does not comprise a sequence rich in basic amino acids in the amino-terminal moiety. Thus, it appears to belong to the heparin-independent group of serpins (Evans *et al.*, 1993). Whereas heparin-dependent serpins, such as ATIII (Blackburn *et al.*, 1984), heparin cofactor (HCOF; Blinder and Tollefsen, 1990) or PN-1 (Stone *et al.*, 1994), bear five to seven basic amino acids at conserved positions in their heparin-binding exosite, neuroserpin carries six acidic residues, but only one lysine at the corresponding positions in this 18-amino-acid stretch (G84–E101; see Figure 5B).

Antiproteolytic serpins have been implicated in a variety of physiological functions, ranging from a rather general protection against proteolytic attack to subtle regulatory

functions within intricate proteolytic cascades (Schulze *et al.*, 1994). Convincing evidence has been accumulated over the past years that extracellular serine proteases play key roles in development, plasticity and regeneration of the vertebrate nervous system. Likewise, protease inhibitors of the serpin family, such as AChT (Rayford *et al.*, 1992), PAI1 (Rao *et al.*, 1993), or—most prominently—PN-1 (Guenther *et al.*, 1985), are found in the developing nervous system and have recently been shown to be involved in developmental processes such as neurite outgrowth (Gloor *et al.*, 1986) and synapse elimination (Liu *et al.*, 1994). Serpin functions in the adult nervous system include regeneration (Meier *et al.*, 1989) and LTP (D.Monard, personal communication).

Neuroserpin is secreted from axons of PNS and CNS neurons

Axonal secretion of neuroserpin was first shown with DRG neurons cultured in a compartmental cell culture system (Stoeckli *et al.*, 1989). Using the same approach, we demonstrated here that neuroserpin is also released from the axons of cultured neurons of the ventral spinal cord, presumably motoneurons. Thus, axonal release of neuroserpin has been observed with one type of neuron of each of the PNS and the CNS. Without a detailed investigation of the site of release, neuroserpin has also been found in the supernatant of a variety of dissociated cultures of PNS and CNS neurons of different origin, including ciliary ganglion, superior cervical ganglion, retina and dorsal spinal cord, but not of non-neuronal cells, such as peripheral glia, central glia, fibroblasts, heart muscle, skeletal muscle or liver cells (Stoeckli *et al.*, 1989). Neuroserpin is also released and accumulates in the extracellular fluids *in vivo*, as we have demonstrated by the analysis of the different chromatographic fractions of VF in the process of protein purification. Based on these observations, we consider as warranted the generalization that neuroserpin is a secretory protein that accumulates in the extracellular fluid and that axons are a preferred site of release.

Neuroserpin is the first serpin which is predominantly produced by neurons and which has been demonstrated to be axonally secreted. The latter fact is of special importance since it opens the possibility of a locally restricted release upon neuronal stimulation, which appears to be a prerequisite for a molecule supposed to be involved in the refinement of synaptic connectivity (Constantine-Paton *et al.*, 1990; Udin and Fawcett, 1988; for a review see Goodman and Shatz, 1993). Recent experiments on the neuromuscular junction have demonstrated that activity-dependent synapse elimination could indeed be prevented both *in vitro* and *in vivo* by addition of the serine protease inhibitors PN-1 (Liu *et al.*, 1994) or leupeptin (Connold and Vrbova, 1994). Intriguingly, spinal motoneurons strongly express neuroserpin mRNA during the period critical for developmental synapse elimination.

Neuroserpin is expressed in neurons during the late stages of neural development and in the adult

The tempo-spatial patterns of neuroserpin expression suggests that neuroserpin is involved in the regulation of extracellular proteolytic events underlying diverse developmental processes. Neuroserpin expression in the

floor plate of the spinal cord coincides well with the crossing of the commissural axons in order to join the contralateral longitudinal tract (Oppenheim *et al.*, 1988; Stoeckli and Landmesser, 1995). Thus, floor plate neuroserpin might be involved in the regulation of axonal pathway decisions at this location. In the defined neuronal populations investigated in this study, the first transcripts appear after the period of axon growth into the target area. For example the spinal motoneurons first express neuroserpin mRNA at stage 28, when they have already extended their axons to the target region and have begun to form synapses. In retinal ganglion cells, neuroserpin expression starts at stage 36 and peaks around hatching (stage 43 to P5). At stage 36, retinal projections have already reached their destination in the optic tectum (Mey and Thanos, 1992) and have begun to form synapses with their target cells. These observations during development implicate neuroserpin as a regulator for synaptogenesis and the subsequent remodelling processes including synapse elimination rather than neurite outgrowth.

The onset of neuroserpin expression after the axons have reached the target area speaks against a role of neuroserpin as a regulator of erase, a trypsin-like protease that has recently been demonstrated to be expressed by certain cells and to regulate the outgrowth of neurites by inducing growth cone collapse upon encounter (Baird and Raper, 1995). However, a discrepancy in the expression of proteins *in vivo* and in cultured cells taken at the same developmental stage is not an uncommon observation and thus, experimental testing will be necessary to address this issue.

The most prominent expression of neuroserpin mRNA in the adult chicken is found in the hyperstriatum accessorium, the neostriatum and the hippocampus. This is intriguing since a subtle balance between proteolytic and anti-proteolytic activity seems to be required for appropriate synaptic function in plastic regions of the adult brain and consequently in processes of learning and memory. Transgenic mice with an enhanced proteolytic activity in the cortex and hippocampus due to overexpression of urokinase plasminogen activator have been found to exhibit impaired spatial, olfactory and taste-aversion learning (Meiri *et al.*, 1994). In line with these observations are recent results with PN-1. An elimination of PN-1 by homologous recombination leads to reduced LTP, whereas PN-1 overexpression results in enhanced LTP of hippocampal neurons.

Conclusion

Neuroserpin is a novel serine protease inhibitor with predominantly neuronal origin which is secreted from the axons of both PNS and CNS neurons. In the developing nervous system it is expressed predominantly during the formation and reorganization of synapses; in the adult CNS it is found in areas in which the synaptic changes associated with learning and memory are thought to occur. These characteristics implicate neuroserpin as a regulator of the subtle balance between proteolytic and anti-proteolytic activities at the synapse, which is thought to be required for an orderly execution of developmental functions such as the formation or reorganization of synaptic connections, as well as for synaptic plasticity in the adult nervous system.

Materials and methods

Cell culture

White Leghorn chicken embryos were used throughout this study. Dorsal root ganglia neurons and ventral spinal cord neurons were dissected from 10- or 6-day-old chicken embryos respectively, and cultured as described by Sonderegger *et al.* (1985). To show axonal secretion, the compartmental cell culture system originally devised by Campenot (1977, 1979) was used. Neurons were cultured 4–5 days prior to metabolic labelling.

Metabolic labelling

Selective labelling of newly synthesized proteins was carried out essentially as described previously (Sonderegger *et al.*, 1983, 1984, 1985). However, the protein content of the medium was reduced to a minimum by omitting horse serum during labelling. The labelling medium consisted of methionine-free growth medium substituted with 0.1–1 mCi/ml [³⁵S]methionine (1000 Ci/mMol, NEN) and 15 μM unlabelled methionine. Incubation time was 40–48 h.

Electrophoretic procedures

SDS–PAGE was carried out according to Laemmli (1970) and two-dimensional SDS–PAGE was according to O'Farrell (1975) as modified by Sonderegger *et al.* (1985). For fluorography, the PhosphorImager (Molecular Dynamics) was used. Silver staining was performed according to the procedure of Switzer *et al.* (1979) as modified by Oakley *et al.* (1980). As molecular weight markers for gels processed by fluorography, [¹⁴C]methylated forms of carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase (97 kDa) and myosin (200 kDa, all NEN) were used. For silver stained gels, we used carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase (97 kDa), β-galactosidase (116 kDa) and myosin (200 kDa, all from Sigma).

Chromatographic procedures

Vitreous fluid of white Leghorn chicken embryos was prepared as described elsewhere (Ruegg *et al.*, 1989). For IEF it was dialysed against water and 2% Ampholines (BioLyte 3–10, Bio-Rad) were added. Preparative IEF was carried out using the ROTOFOR system (Bio-Rad) according to the manufacturer's instructions. The pH of each fraction was determined and fractions with pH 4.8–5.0 were immediately buffered with 50 mM Tris–HCl to pH 7.25 and stored frozen until further use.

For hydrophobic interaction chromatography, ammonium sulfate was added to the selected IEF fractions to a final concentration of 1.2 M prior to loading to the column (TSK Phenyl-5PW, 7.5×75 mm, Bio-Rad) which was equilibrated with loading buffer (50 mM Tris–HCl, 1.2 M ammonium sulfate, pH 7.25). Bound proteins were eluted by a linear gradient of decreasing concentration of ammonium sulfate (1.2–0 M) in 50 mM Tris–HCl, pH 7.25. Fractions from 0.55–0.45 M ammonium sulfate were stored frozen until further use.

For preparative SDS–PAGE, samples from the hydrophobic interaction chromatography were precipitated according to Wessel and Fluegge (1984), redissolved by boiling in sample buffer (3% SDS, 10% glycerol, 62.5 mM Tris–HCl, 0.2% β-mercaptoethanol, pH 6.8) and applied to a 2.5×100 mm 6% polyacrylamide gel on a 230 A preparative electrophoresis system (HPEC, Applied Biosystems). Electrophoresis was carried out according to the manufacturer's instructions and the proteins were eluted with 25 mM Tris–HCl, pH 8.3 in fractions of 50 μl.

Generation of a polyclonal antiserum and immunoprecipitation

Rabbit antibodies against neuroserpin were raised by intramuscular injection of 5–10 μg of purified neuroserpin in complete Freund's adjuvant followed by a booster injection in incomplete Freund's adjuvant 2 months later. The antiserum against neuroserpin obtained 1 week after the booster injection was specific for neuroserpin in conditioned medium of cultured DRG neurons, as demonstrated by immunoprecipitation under native conditions carried out as described elsewhere (Ruegg *et al.*, 1989). Briefly, cultures of dissociated DRG neurons were metabolically labelled (Stoeckli *et al.*, 1989), the conditioned medium was adjusted to precipitation conditions, 10% vitreous fluid was added as a carrier and the solution was cleared by centrifugation. The radiolabelled neuroserpin was incubated with 1/50 (v/v) rabbit antiserum for 12 h at RT and, subsequently, for 2 h at RT with 1/50 of a suspension of glutaraldehyde-fixed *Staphylococcus aureus* in PBS (1 vol/9 vol). Bound antigen-antibody complexes were released by boiling in 2% SDS/5% β-mercaptoethanol followed by centrifugation. Proteins of the supernatant were

precipitated (Wessel and Fluegge, 1984) and processed for two-dimensional SDS-PAGE.

Protein sequence analysis

For the determination of the amino-terminal amino acid sequence of neuroserpin, 10–20 pmol purified protein was directly blotted onto a PVDF-membrane (ProSpin, Applied Biosystems). To obtain internal sequences, enzymatic digestions of neuroserpin were carried out using endoproteinase Asp-N and trypsin respectively. 1/10 (w/w) of the appropriate endoproteinase (both sequence grade, Boehringer) was added to 100–200 pmol neuroserpin in 25 mM Tris-HCl, pH 8.3 and incubated for 5 h (Asp-N) or 12 h (trypsin) at 37°C. The digestion was stopped by adding 200 µg/ml DTT and incubating for 1 h at RT under argon; TFA was added to a final pH of 2. The peptides were separated on a SMART-System (Pharmacia) equipped with a µRPC C2/C18 reversed phase column and a 100 µl DEAE-precolumn (Q-Sepharose fast flow, Pharmacia; see Kawasaki and Suzuki, 1990) using a linear gradient from 0–80% acetonitril in 0.1% TFA. Peptide sequence analysis of the blotted protein or the eluted peptides was carried out on a 477 A protein sequencer on-line with a 120 A HPLC detector (both Applied Biosystems) using standard chemistry.

Cloning and sequencing of the neuroserpin cDNA

Total RNA was prepared from chicken embryonic (E14) retina and neonatal mouse brain by the method of Han *et al.* (1987) and oligo(dT)-primed cDNA was produced using SuperScript RNase H⁻ reverse transcriptase (Gibco) according to the distributor's instructions. As a template for the human neuroserpin fragment, lambda DNA was prepared from a human fetal (17–18 weeks) brain cDNA library (Stratagene) according to Friedman *et al.* (1988). One sense and two antisense primers corresponding to the amino terminus and two of the internal peptides and four primers flanking the reactive site loop and matching the chicken cDNA sequence were synthesized (Microsynth) with inosine at the wobble positions and restriction sites for *EcoRI*, *XbaI* or *BamHI* respectively, at the 5'-end. (sense primer: 5'-GGAATTCACIAA-ITTCICIGATGAAAC-3'; antisense primers: 5'-GGAATTCACIATC-ATIGAIACIATITCATC-3' and 5'-GCTCTAGAAIACITCIGTIATICC-NAG-3'; flanking sense primers: 5'-GCIATITA(C/T)TT(C/T)AA(A/G)-GGIAA(T/C)TGGAA-3' and 5'-GGGGATCCGA(A/G)ACIGA(A/G)-GTICA(A/G)ATICCIATGATG-3'; flanking antisense primers: 5'-CCC-AT(A/G)AAIA(A/G)IACIGTICCNGT-3' and 5'-GGGGATCCGG(A/G)TG(A/G)TCIACIATAC(C/T)TGNGG-3').

Nested PCR was performed using ampliTaQ DNA polymerase (Perkin Elmer) according to the supplier's recommendations. The identity of the amplified fragment was verified by the 15 amino acids following the amino-terminal sequence used for the sense primer. The 700 bp fragment was subcloned in Bluescript SK(-) plasmid DNA (Stratagene).

About 10⁶ plaques of a λZAPII cDNA library of embryonic chicken brain (Zuellig *et al.*, 1992) were screened (i) by hybridization with the radiolabelled PCR fragment under standard high stringency conditions (Sambrook *et al.*, 1989) or (ii) with the rabbit polyclonal antiserum against purified neuroserpin as primary and peroxidase-conjugated anti-rabbit IgG (Bio Science) as secondary antibody. Five positive clones were found by the immunoscreening and 20 positive clones resulted from the hybridization screen. They were subjected to further characterization.

Bluescript plasmids containing the putative neuroserpin inserts were excised *in vivo* from λZAPII phagemids and digested with *EcoRI* endonuclease. Of each screen, the representative with the longest insert was sequenced using the dideoxy-chain-termination method (Sanger *et al.*, 1977) with Sequenase 2.0 (USB). For the sequence analysis we used GCG (Version 7, Unix, Silicon Graphics Inc.).

Northern blot analysis

Total RNA for Northern blots was prepared according to Chomczynski and Sacchi (1987). DIG-labelled riboprobes of neuroserpin and glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) were synthesized using the DIG RNA labelling kit (Boehringer) according to the supplier's recommendations. RNA electrophoresis, transfer to PVDF membranes (Pall), hybridization and detection by alkaline phosphatase-conjugated anti-DIG Fab and CDP-Star (both Boehringer) were performed according to Sambrook *et al.* (1989) and Boehringer's recommendations.

In situ hybridization

In situ hybridization was carried out essentially as described by Schaaeren-Wiemers and Gerfin-Moser (1993). Briefly, 20 µm sections of chicken embryos were mounted on poly-L-lysine coated coverslips and shortly air-dried. DIG labelled sense and antisense cRNA probes corresponding

to the full length cDNA were prepared from neuroserpin cDNA in pBluescript using the DIG labelling kit (Boehringer) in combination with T3 and T7 polymerase respectively. Fragments of 200–300 bp of the cRNA were obtained by alkaline hydrolysis and used at a concentration of 200 ng/ml. Twenty µm sections on poly-L-lysine coated coverslips were hybridized at 52°C and washed in graded concentrations of SSC with a high stringency step of 0.2× SSC at 53°C. Immunological detection of DIG-labelled hybrids was performed using alkaline phosphatase-conjugated anti-DIG Fab and NBT/BCIP (all Boehringer). The colour reaction was carried out 12–24 h at room temperature in the dark.

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