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Neuroserpin is an axonally secreted serine proteinase inhibitor that is expressed in neurons during embryogenesis and in the adult nervous system. To identify target proteinases, we used a eucaryotic expression system based on the mouse myeloma cell line J558L and vectors including a promoter from an Ig-kappa-variable region, an Ig-kappa enhancer, and the exon encoding the Ig-kappa constant region (C kappa) and produced recombinant neuroserpin as a wild-type protein or as a fusion protein with C kappa. We investigated the capability of recombinant neuroserpin to form SDS-stable complexes with, and to reduce the amidolytic activity of, a variety of serine proteinases in vitro. Consistent with its primary structure at the reactive site, neuroserpin exhibited inhibitory activity against trypsin-like proteinases. Although neuroserpin bound and inactivated plasminogen activators and plasmin, no interaction was observed with thrombin. A reactive site mutant of neuroserpin neither formed complexes with nor inhibited the amidolytic activity of any of the tested proteinases. Kinetic analysis of the inhibitory activity revealed neuroserpin to be a slow binding inhibitor of plasminogen activators and plasmin. Thus, we postulate that neuroserpin could represent a regulatory element of extracellular proteolytic events in the nervous system mediated by plasminogen activators or plasmin.

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The Axonally Secreted Serine Proteinase Inhibitor, Neuroserpin, Inhibits Plasminogen Activators and Plasmin but Not Thrombin*

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Neuroserpin is an axonally secreted serine proteinase inhibitor that is expressed in neurons during embryogenesis and in the adult nervous system. To identify target proteinases, we used a eucaryotic expression system based on the mouse myeloma cell line J558L and vectors including a promoter from an Ig-κ-variable region, an Ig-κ enhancer, and the exon encoding the Ig-κ constant region (Cκ) and produced recombinant neuroserpin as a wild-type protein or as a fusion protein with Cκ. We investigated the capability of recombinant neuroserpin to form SDS-stable complexes with, and to reduce the amidolytic activity of, a variety of serine proteinases *in vitro*. Consistent with its primary structure at the reactive site, neuroserpin exhibited inhibitory activity against trypsin-like proteinases. Although neuroserpin bound and inactivated plasminogen activators and plasmin, no interaction was observed with thrombin. A reactive site mutant of neuroserpin neither formed complexes with nor inhibited the amidolytic activity of any of the tested proteinases. Kinetic analysis of the inhibitory activity revealed neuroserpin to be a slow binding inhibitor of plasminogen activators and plasmin. Thus, we postulate that neuroserpin could represent a regulatory element of extracellular proteolytic events in the nervous system mediated by plasminogen activators or plasmin.

Extracellular proteolysis exerted by serine proteinases has been implicated in a variety of processes in the nervous system during development and in adulthood. Among the serine proteinases recently reported to play a role in neural development and function, there are several well known proteins that had previously been found and characterized in nonneuronal functions, in particular blood coagulation and fibrinolysis. For example, tissue-type plasminogen activator (tPA)[1] and urokinase plasminogen activator (uPA) were found to be expressed in the nervous system (1, 2), and they have been demonstrated to be engaged in developmental processes such as cerebellar granule cell migration (3, 4), Schwann cell migration, and wrapping of axons (5), or neuromuscular synapse elimination (6). In the period of neurite outgrowth, plasminogen activators (PAs) have been found to be secreted at the growth cones of cultured neurons or neuronal cell lines (7, 8), and they were demonstrated to modify the molecular composition of the neurites' substrata *in vitro* (9). In the adult nervous system, tPA is induced in the hippocampus after seizure, kindling, and long term potentiation (LTP) (10) and in the cerebellum after motor learning tasks (11), and mice lacking the gene for tPA (12) show a different form of hippocampal LTP (13, 14). Furthermore, tPA has been demonstrated to be involved in excitotoxin-induced neuronal cell death in the murine hippocampus by converting locally secreted plasminogen to active plasmin (15, 16). Thrombin, which has been extensively characterized due to its important function in the blood clotting system, has been reported to be expressed in the nervous system (17). It has been found to negatively affect neurite outgrowth *in vitro* (18) by inducing growth cone collapse via proteolytic activation of the thrombin receptor (19). Recently, four novel extracellular proteinases have been reported in the nervous system. A serine proteinase termed "erase" was proposed to be associated with membranes of neurons from the peripheral but not from the central nervous system (20), and the cDNAs of three serine proteinases called "neuropsin," "neurosin," and "neurotrypsin," respectively, which are preferentially expressed in the nervous system, have been cloned and characterized (21–23).

In analogy to processes of tissue remodeling, blood coagulation, and fibrinolysis, one would expect specific inhibitors of serine proteinases belonging to the structural class of the serpins (serine proteinase inhibitors; for a review, see Ref. 24) to act as regulators of proteolytic activity in the nervous system. Guenther *et al.* (25) and Gloor *et al.* (26) have found a glial cell line-derived activity, which promotes neurite outgrowth *in vitro* and which is identical to protease nexin-1 (PN-1), a member of the serpin family. PN-1 is directed against thrombin; it is widely distributed in the nervous system (27) and might serve as a physiological regulator of thrombin (28). Recently, a novel member of the serpin family has been purified from bovine brain (29). It has been shown to be expressed in neurons and in

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[1] The abbreviations used are: tPA, tissue-type plasminogen activator; BSA, bovine serum albumin; Cκ, constant region of Ig-κ; cNS, recombinant chicken neuroserpin; cNS-Cκ, chicken neuroserpin fusion protein with Cκ; cNS_{EP}-Cκ, chicken neuroserpin reactive site mutant fusion protein with Cκ; DRG, dorsal root ganglion; FCS, fetal calf serum; $hNS-H_6$, recombinant human neuroserpin fusion protein tagged with 6 C-terminal histidines; LTP, long term potentiation; PA, plasminogen activator; PAGE, polyacrylamide gel electrophoresis; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PN-1, protease nexin-1; SI, stoichiometric index; uPA, urokinase plasminogen activator; VF, vitreous fluid.

glial cells and to interact *in vitro* with several serine proteinases (*e.g.* thrombin) (30, 31). In contrast to thrombin, the PAs in the brain have not been associated with a regulatory serpin. The plasminogen activator inhibitors of nonneuronal tissues (PAI-1 and PAI-2) were found in the nervous system (*e.g.* Refs. 32 and 33), but they are not coexpressed with PAs in a pattern suggestive for a role of their physiological regulators in the brain.

We have recently purified a neuronal serpin, neuroserpin, from ocular vitreous fluid (VF) of chicken embryos and have cloned the cDNA (34). The amino acid sequence of neuroserpin is highly conserved between chick, rodents, and man (35),[2] especially in the region of the reactive site loop (between P17 and P5′; following the standard nomenclature introduced by Schechter and Berger (36)) (Fig. 1). A considerable amount of work over the past decade revealed the unusually flexible reactive site loop to be essential for the activity and the specificity of serpins (*e.g.* Refs. 37–40). Based on its amino acid sequence within the reactive site loop, neuroserpin belongs to the inhibitory Arg serpins and might therefore be an inhibitor of trypsin-like serine proteinases, and the high similarity of neuroserpin sequences of chicken, mice, and men within this region (Fig. 1) suggests a high conservation of target specificity. However, the speculations about inhibitory activity and possible target proteinases were solely based on amino acid sequence comparisons, and no antiproteolytic activity of neuroserpin has been demonstrated so far. The purpose of the presented study was to determine experimentally whether neuroserpin is an inhibitory serpin and whether it is targeted against serine proteinases expressed in the nervous system. We used complex formation assays and inhibition assays to investigate the interaction between recombinant neuroserpin and the neural serine proteinases tPA, uPA, plasmin, and thrombin. We found that the inhibitory activity of neuroserpin is directed specifically against PAs and plasmin, whereas no activity *versus* thrombin was observed.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors pcNS, pcNS-Cκ, and pcNS$_{EP}$-Cκ—The cDNA fragments termed cNS-wt and cNS-fus were amplified with polymerase chain reaction (PCR), using the full-length cDNA clone Sc3a4 as a template and the oligonucleotide primer pairs cNS-for/cNS-wt-back and cNS-for/cNS-fus-back, respectively. For site-directed mutagenesis of the reactive site, the mutagenic backward primer cNS-mt-back was combined with cNS-int-for to amplify the *Xba*I-*Sca*I fragment of neuroserpin with a mutated reactive site (Fig. 2*A*). (cNS-for, 5′-GTC TTA AGA GCT CAC AAC ATG TAT TTC C-3′; cNS-int-for, 5′-GTA TCT ACC AAG TTC TAG AAA TAC C-3′; cNS-wt-back, 5′-GGG AAG CTT ACT TAC CTA AAG CTC TTC AAA GTC ATG GCC-3′; cNS-fus-back, 5′-GGG AAG CTT ACT TAC CTA GCT CTT CAA AGT CAT GGC C-3′; cNS-mt-back, 5′-GGA TAC AGT ACT GCA GGT TCG CTA ATG GC-3′.) PCR amplification was carried out in a reaction mixture containing 0.025 units/ml AmpliTaq DNA Polymerase (Perkin-Elmer), 50 μM each of dATP, dCTP, dGTP, and dTTP, 1.3 mM Mg^{2+} (3 mM for cNS-mt), 200 nM amounts of each primer, and 0.25 μg of template. Sc3a4 was linearized by *Kpn*I digestion, and cNS-wt or cNS-fus, respectively, were amplified in a 50 μl volume in a 16-cycle amplification (hot start with denaturation for 5 min at 95 °C; cycles 1–5, annealing 1 min at 60 °C; elongation 1.5 min at 72 °C; Denaturation 1 min at 95 °C; Cycles 6–16, annealing and elongation 2 min at 72 °C; denaturation 1 min at 95 °C; 7 min completing at 72 °C). For the amplification of cNS-mt, *Kpn*I-linearized pBluescript containing cNS-fus was used as template, and the PCR conditions were as above, except the annealing temperature (59 °C for cycles 1–5 and 60 °C for cycles 6–16) and an elongation time of 1 min in all cycles. cNS-wt and cNS-fus were digested with *Sac*I and *Hin*dIII (all restriction endonucleases from New England Biolabs, Beverly, MA; recognition sites underlined in the primers) and ligated into the identically digested vector pCD4-FvCD3-Cκ (kindly provided by Dr. K. Karjalainen), resulting in the expression vectors pcNS for wild-type and pcNS-Cκ for the fusion protein, respectively (Fig. 2*B*). For mutant neuroserpin, the *Sac*I-*Hin*dIII fragment of pcNS-Cκ was excised and subcloned in pBluescript SK(+) (Stratagene, La Jolla, CA), and the wild-type *Xba*I-*Sca*I fragment covering the reactive site loop was replaced by the mutated fragment cNS-mt amplified by PCR. Replacing the *Sac*I-*Hin*dIII fragment of pcNS-Cκ with the mutated neuroserpin yielded the expression vector pcNS$_{EP}$-Cκ for mutant neuroserpin as a fusion protein with Cκ. The integrity of the resulting constructs was confirmed by double-strand sequencing using the dideoxy chain termination method (41) with Sequenase 2.0 (U. S. Biochemical Corp.) or with the SequiTherm Long Read Cycle Sequencing Kit-LC (Epicentre Technologies, Madison, WI).

Protoplast Fusion, Selection, and Screening for Positive Clones—Stable transfectants were obtained by transfecting pcNS, pcNS-Cκ, or pcNS$_{EP}$-Cκ, respectively, into the mouse myeloma cell line J558L by protoplast fusion (42) as described by Oi and colleagues (43). To achieve independent transfectants, the cells were diluted in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS; both from Life Technologies, Basel, Switzerland) and cultivated in 96-well microtiter plates at 37 °C in 10% CO_2. After 2 days, selection of transfectants was started by adding 5 mM L-histidinol (Sigma). Two weeks later, histidinol-resistant colonies were expanded and cultivated for 3–4 days in 24-well plates containing 600 μl of selection medium (5 mM L-histidinol in Dulbecco's modified Eagle's medium, 10% FCS) per well. To screen for recombinant wild-type neuroserpin (cNS) and for neuroserpin fusion protein with Cκ (cNS-Cκ), 50 μCi/ml [^{35}S]methionine (1000 Ci/mmol, NEN Life Science Products) was then added, and the expanding colonies were metabolically labeled for 20 h. Afterward, the relative expression levels of cNS and cNS-Cκ of different clones were compared by immunoprecipitation of the recombinant protein from the supernatants using the polyclonal antiserum R35 as described below, followed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For mutant neuroserpin fusion protein (cNS$_{EP}$-Cκ), supernatants of the surviving colonies were incubated with a sheep antibody against mouse Cκ (The Binding Site, Birmingham, UK) dotted on nitrocellulose, and the expression levels of the tested cell lines were compared by incubating the dot blot with a peroxidase-conjugated sheep antibody against mouse IgG (H + L; from Kirkegaard & Perry Laboratories, Gaithersburg, MD) and by developing with 0.5 mg/ml 4-chlor-1-naphthol (Merck, Dietikon, Switzerland) in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 200 mM NaCl). The clones with the highest expression were subcloned and adapted to low serum conditions (Dulbecco's modified Eagle's medium with 2% FCS).

Purification of cNS, cNS-Cκ, and cNS$_{EP}$-Cκ—Recombinant cNS was partially purified from supernatants of transfected myeloma cells by a two-step chromatographic procedure. The supernatants were first passed through a column containing Blue Sepharose (CL6B, Pharmacia, Dübendorf, Switzerland). The flow-through was dialyzed against ion exchange chromatography loading buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl), and proteins were fractionated using an anion exchange column (MonoQ HR5/5, Pharmacia, Dübendorf, Switzerland) and a linear gradient from 200 to 500 mM NaCl. The fractions from 350 to 400 mM NaCl were highly enriched with cNS; they were dialyzed against phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.2, 140 mM NaCl, 4 mM KCl) and stored frozen until further use. For the isolation of cNS-Cκ and cNS$_{EP}$-Cκ, respectively, supernatants of the transfected myeloma cells were passed through an immunoaffinity column with the immobilized monoclonal antibody 187.1 (Rat anti-mouse-Cκ IgG, ATCC). Bound antigen was eluted with 50 mM diethylamine, pH 11.5. The eluate was immediately neutralized by adding 230 mM Tris-HCl, pH 6.5, and then dialyzed against PBS. All procedures, except ion exchange chromatography, were carried out at 4 °C.

Procaryotic Expression and Purification of Recombinant Human Neuroserpin (hNS-H$_6$)—Human neuroserpin (PI12) was cytoplasmically expressed in *Escherichia coli* with a stretch of six histidines fused to the C terminus of the protein. Briefly, a fragment of the human neuroserpin cDNA encoding amino acids 1–394 of human neuroserpin (according to the numbering of Schrimpf *et al.* (35)) was amplified in a PCR using the oligodeoxynucleotide primers 5′-AAT TTC TAG AGA AAG GAG ATA CAT ATG ACA GGG GCC ACT TTC CCT-3′ and 5′-GGG AAG CTT CTA GTG GTG ATG GTG GTG GTG AAG TTC TTC GAA ATC ATG GTC C-3′. The cDNA fragment was cloned into the vector pAK400 (44) via the *Xba*I and *Hin*dIII sites of the vector, allowing expression of the cDNA from the *lac* operator/promoter located immediately upstream. For expression, a colony of *E. coli* strain BL21DE3 harboring the expression plasmid was precultured overnight

[2] Krueger, S. R., Ghisu, G. P., Cinelli, P., Gschwend, T. P., Osterwalder, T., Wolfer, D. P., and Sonderegger, P. (1997) *J. Neurosci.* **17,** 8984–8996.

at 37 °C in 100 ml of LB medium containing 30 μg/ml chloramphenicol. After inoculation of the same medium with the preculture, bacteria were grown at 25 °C and induced with 1 mM isopropyl-1-thio-β-D-galactosidase at an A_{600} of 0.5. The bacteria were harvested by centrifugation 6 h after induction, resuspended in Ni-NTA-binding buffer (1 M NaCl, 50 mM Tris-Cl, pH 8.0), and disrupted in a French press. The soluble protein extract was incubated overnight at 4 °C with 0.4 ml of Ni-NTA resin (Qiagen, Chatsworth, CA). Following extensive washing with Ni-NTA binding buffer, bound proteins were eluted with Ni-NTA binding buffer containing 200 mM imidazole. The eluted protein was dialyzed against PBS and immediately frozen at −80 °C.

Generation of Polyclonal Antisera Against Neuroserpin—Generation and specificity of the rabbit antiserum R35 against neuroserpin purified from VF has been described earlier (34). The rabbit antiserum R61 against recombinant neuroserpin was generated by intramuscular injection of 10–20 μg of cNS-Cκ in complete Freund's adjuvant followed by two booster injections of cNS-Cκ in incomplete Freund's adjuvant 2 and 4 months later. As shown in Fig. 2*D*, the antiserum R61 against cNS-Cκ obtained 1 week after the second booster injection was specific for neuroserpin of both conditioned medium of cultured dorsal root ganglion (DRG) neurons and VF.

Cell Culture, Metabolic Labeling, Immunoprecipitation, and Ocular Vitreous Fluid—VF of 14-day-old chicken embryos was prepared as described earlier (45). DRG neurons were dissected from 10-day-old chicken embryos and cultured essentially as described by Sonderegger and co-workers (46). Selective labeling of newly synthesized proteins was carried out essentially as described by Stoeckli and co-workers (47). The labeling medium consisted of growth medium containing 50 μCi/ml [^{35}S]methionine (1000 Ci/mmol, NEN Life Science Products), and the incubation time was 24–48 h. Immunoprecipitation of neuroserpin using the polyclonal antiserum R61 was carried out as described previously (45).

Enzymes, Inhibitors, and Substrates—Human tPA (Activase®, recombinant Alteplase, 580,000 IU/mg; kindly provided by Genentech, South San Francisco, CA) was dissolved in water to a final concentration of 1 mg/ml and stored in aliquots at −80 °C; human uPA (100,000–300,000 Plough units/mg, Sigma U-8627) was delivered in a concentration of 1 mg/ml and stored at 4 °C; porcine plasmin (3–5 units/mg, Sigma, P-8644) and human thrombin (50–100 NIH units/mg, Sigma, T-4648) were dissolved in water to final concentrations of 2 and 1 mg/ml, respectively. The enzyme substrate S-2288 (*H*-D-Ile-Pro-Arg-*para*-nitroanilide) was purchased from Chromogenix (Mölndal, Sweden), dissolved in water to a concentration of 25 mg/ml, and stored frozen until use. Active enzyme concentrations were determined by measuring the amidolytic activity of the proteinases in the presence of 1 mM S-2288, using values for substrate turnover of ΔA_{405} = 0.275 min^{-1} cm^{-1}, 0.031 min^{-1} cm^{-1}, 0.030 min^{-1} cm^{-1}, and 0.042 min^{-1} cm^{-1}, for 4 nM thrombin, uPA, single chain tPA, and plasmin, respectively, as indicated by the substrates' supplier. Recombinant neuroserpin was prepared as detailed above, stored frozen, and thawed immediately before use if not indicated otherwise. The concentrations of cNS-Cκ and cNS_{EP}-Cκ were determined by amino acid analysis on an Aminoquant II equipped with the fluorescence detector 1046A (Hewlett-Packard, Palo Alto, CA) using standard procedures. The concentration of cNS was estimated by SDS-PAGE and silver staining. The concentration of hNS-H_6 was estimated using the Bradford protein assay (Bio-Rad, Glattbrugg, Switzerland) in combination with densitometric analysis of SDS-polyacrylamide gels stained with Coomassie Brilliant Blue. Recombinant PN-1 (active concentration 1.2 mg/ml) was kindly provided by Dr. D. Monard and was stored frozen until use.

Reaction Buffers—Chemicals for reaction buffers were purchased from Sigma, unless indicated otherwise. For complex formation assays, a complexation buffer containing 67 mM Tris-HCl, pH 8.0, 133 mM NaCl, and 0.13% PEG 8000 was used. Inhibition buffer contained 10 mM phosphate buffer, pH 7.2, 140 mM NaCl, 4 mM KCl, 0.1% PEG 8000, and 0.2 mg/ml bovine serum albumin (BSA; from Serva, Heidelberg, Germany). Coating solution contained 1% BSA, 0.5% w/v PEG 8000, and 0.01% v/v Triton X-100.

Complex Formation Assays—In an Eppendorf reaction tube, proteinases and inhibitors were mixed in 30 μl of complexation buffer and incubated for 30 min at 37 °C if not indicated otherwise. For experiments with hNS-H_6, incubation was 15 min at 4 °C. The final amounts of enzymes and inhibitors are indicated in Figs. 3–5. The reaction was stopped by adding an equal volume of 2-fold concentrated sample buffer for SDS-PAGE (containing 6% SDS, 10% β-mercaptoethanol, 30% glycerol, 31.3 mM Tris-HCl, pH 6.8) and by immediately boiling the sample for 5 min.

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out according to Laemmli (48), and 2-dimensional SDS-PAGE was according to O'Farrell (49) as modified by Sonderegger *et al.* (46). For autoradiography, the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used. Silver staining was performed according to the procedure of Switzer *et al.* (50) as modified by Oakley *et al.* (51). Carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa, all from Sigma) were used as molecular mass markers throughout the study. Electrotransfer of resolved proteins onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) was carried out according to Towbin *et al.* (52) at 30 V for 16 h or at 100 V for 1–2 h at 4 °C. Molecular mass markers were visualized by 3 min incubation of the membranes in 0.1% Ponceau S (Sigma P-3504) in 1% acetic acid followed by destaining with distilled water. Immunodetection of neuroserpin was performed using the polyclonal antisera R35 or R61 and the BM Chemiluminescence Western blotting kit (Boehringer, Mannheim, Germany) according to the supplier's recommendations, or goat anti-rabbit IgG conjugated to peroxidase (Bio-Science products, Emmenbrücke, Switzerland) at a dilution of 1/1,000.

Amidolytic Assays—Enzyme inhibition was determined by mixing enzyme and inhibitor in a 96-well plate in 98 μl of inhibition buffer. The final concentrations of enzymes were as follows: uPA, 19.1 nM (9.1 nM for experiments with hNS-H_6 shown in Fig. 5); tPA, 7.9 nM; thrombin, 18.8 nM; plasmin, 12.7 nM. Concentrations of inhibitor were 5 and 25 nM (cNS-Cκ) or 30 and 150 nM (hNS-H_6). cNS-Cκ was preincubated for 20 min at 37 °C, while hNS-H_6 was 10 min at room temperature. After preincubation, the amidolytic reactions were started simultaneously by adding 2 μl of substrate solution (25 mg/ml S-2288) to each well. Residual amidolytic activity was determined by measuring the hydrolysis over time (velocity) using an enzyme-linked immunosorbent assay reader (Dynatech, Denkendorf, Germany).

Determination of Kinetic Parameters—The kinetics of the interaction between cNS-Cκ and tPA, uPA, or plasmin, was determined by the progress curve method (53). Polystyrene cuvettes were coated for 1–4 h at room temperature with coating solution. Reactions were started by adding a constant, catalytic amount of enzyme (tPA, 1.6 nM; uPA, 3.6 nM; plasmin, 1.4 nM) to inhibition buffer containing a fixed substrate concentration (1.08 mM S-2288) and variable inhibitor concentrations (ranging from 4.6 to 46.8 nM), preincubated at 37 ± 1 °C. Tight-binding conditions were avoided by using sufficiently high substrate and inhibitor concentrations. Since the interaction between serpins and serine proteinases is assumed to follow slow binding kinetics, product formation was described with Equation 1.

$$[P] - v_s t + (v_z - v_s)(1 - e^{-k't})/k' + d \qquad \text{(Eq. 1)}$$

v_s and v_z represent the velocities at steady state and at zero time, respectively; k' represents the apparent first-order rate constant for approach to the steady state, and d is a displacement factor compensating for small uncertainties in absorbance at the start of the reaction. For each of several inhibitor concentrations, v_s, v_z, k', and d were determined by fitting Equation 1 to the data sampled from progress curves. The association and dissociation constants were determined from the relationship (53) shown in Equation 2.

$$k' = k_d + \frac{k_a}{1 + [S]/K_m}[I] \qquad \text{(Eq. 2)}$$

K_m of S-2288 was 3×10^{-6} M, 2×10^{-4} M, 1×10^{-3} M, and 9×10^{-3} M for thrombin, uPA, single chain tPA, and plasmin, respectively, as indicated by the supplier. An absorption coefficient ϵ_{405} = 10,500 M^{-1} cm^{-1} for the released *para*-nitroaniline was used to determine the product concentrations.

RESULTS

Heterologous Expression of cNS, cNS-Cκ, and cNS_{EP}-Cκ—Only small amounts of denatured neuroserpin could be purified from chicken embryonic VF by the three-step purification strategy detailed earlier (34). Therefore, we decided to recombinantly express neuroserpin in a heterologous system. Since neuroserpin contains two potential sites for *N*-glycosylation, of which at least one is used, we have chosen a eucaryotic expression system based on myeloma cells (54, 55) that are able to produce large amounts of glycosylated, neuronally secreted proteins (56). We amplified three different forms of the chicken neuroserpin cDNA by PCR, which all cover the entire open reading frame (Fig. 2*A*); cNS-wt was amplified using the back-

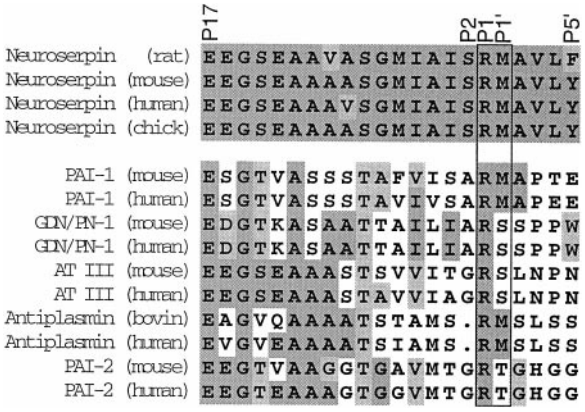

FIG. 1. **Alignment of the reactive site loops of different serpins.** Reactive site loops of serpins directed against plasminogen activators, plasmin, or thrombin are aligned to the corresponding region of neuroserpin from four different species. *Dark shading* indicates amino acids identical to chicken neuroserpin, and *light shading* indicates conservative changes. A *frame* is drawn around the putative P1 and P1′ amino acids at the reactive site. Rat, human, and murine neuroserpin show an identity of 91, 95, and 100%, respectively, to the chicken sequence in the region from P17 to P5′ but diverge strongly from plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2), glia-derived nexin/protease nexin-1 (GDN/PN-1), antithrombin III (AT III), and antiplasmin.

ward primer cNS-wt-back with the naturally occurring stop codon TAA mutated to TAG to generate the splice consensus donor site (AGGTAAGT) immediately downstream of the coding region; cNS-fus was amplified with the backward primer cNS-fus-back designed to replace the stop codon TAA with a G which, after splicing, generates a continuous open reading frame with the sequence of the constant region of the κ light chain (Cκ). Either of these fragments were cloned via *Sac*I and *Hin*dIII into the eucaryotic expression vector pCD4-FvCD3-Cκ (54, 55), replacing the region coding for CD4 and FvCD3, and giving rise to the neuroserpin expression vectors pcNS and pcNS-Cκ, respectively (Fig. 2*B*). The mutant form of neuroserpin was generated using the mutagenic primer cNS-mt-back, in which the putative reactive site positions P1 and P1′ (see Fig. 1) were mutated (P1[R362E] and P1′[M363P]) to generate an inactive form of neuroserpin. The *Xba*I-*Sca*I fragment carrying this mutation was introduced into pcNS-Cκ replacing the wild-type reactive site loop and thereby yielding $pcNS_{EP}$-Cκ. The myeloma cell line J558L was transfected with either of the vectors by protoplast fusion (42), and the expression levels of histidinol-resistant clones were tested by analysis of supernatants by immunoprecipitation of metabolically labeled, recombinant protein with the antibody R35 against purified chicken neuroserpin (34) or with a sandwich dot-blot test using two different antibodies against mouse IgG. The clones DG3 for cNS, 3B6 for cNS-Cκ, and F2 for cNS_{EP}-Cκ, respectively, were subcloned, adapted to low serum conditions, and expanded. The fusion proteins were purified from supernatants by affinity chromatography using a monoclonal antibody against Cκ, whereas cNS was only enriched after removing most of the albumin by passing through a Blue Sepharose column and by fractionation over an anion exchange column. As shown in Fig. 2*C*, all recombinant proteins had the expected size (65 and 54 kDa, respectively) and were recognized by the polyclonal antiserum R35 (*Blot*). Interestingly, cNS-Cκ always appeared as a double band. This effect was observed earlier with recombinant Cκ fusion proteins[3] but could not be explained so far. Although the immunoaffinity purification yielded electrophoretically pure cNS-Cκ and cNS_{EP}-Cκ, fractions of cNS still contained 30–50% serum proteins, which were not recognized by R35 (Fig. 2*C*, *lanes 1* and *2*). With cNS-Cκ as immunogen, the polyclonal antiserum R61 was raised in rabbit; it precipitated neuroserpin under native conditions from medium conditioned by primary DRG neurons (DRG CM), and specifically recognized neuroserpin among the proteins in embryonic chicken VF on Western blots (Fig. 2*D*). Different isoelectric variants of the recombinant proteins, most likely due to glycosylation, were observed. N-terminal sequencing of cNS-Cκ revealed an identical N terminus as found in chicken neuroserpin purified from VF, indicating correct signal peptide cleavage (data not shown).

Complex Formation of cNS with Neural Serine Proteinases—Upon binding of their target proteinases, serpins form highly stable complexes that resist dissociation by SDS in the presence of reducing agents (57). Since neuroserpin is predominantly expressed in the nervous system, we tested the serine proteinases tPA, uPA, plasmin, and thrombin, which exhibit trypsin-like substrate specificity and are expressed in the nervous system (1, 2, 16, 17), for their ability to form SDS-stable complexes with neuroserpin. cNS was incubated with different concentrations of the respective proteinases, and the samples were analyzed by SDS-PAGE and Western blotting (Fig. 3). The estimated concentrations of inhibitors and proteinases are indicated. cNS formed high molecular mass complexes of approximately 80, 86, and 112 kDa with uPA and tPA, respectively, that matched the expected sums of the N-terminal part of native neuroserpin (Phe^{17}–Arg^{362}, approximately 49 kDa) plus the catalytic subunits of the proteinases (uPA, approximately 30 kDa; two-chain tPA, approximately 35 kDa, single-chain tPA, approximately 65 kDa). cNS behaved more substrate-like with plasmin, as this enzyme cleaved cNS into one major fragment with the expected size of native neuroserpin minus the C-terminal part (Met^{363}–Leu^{410}). Only a small amount of cNS appeared to form SDS-stable complexes of the expected size (approximately 75 kDa) with plasmin. No complex formation and only very marginal proteolytic cleavage of cNS were observed with thrombin. PN-1, which was used as a control, readily formed complexes of approximately 65 and 54 kDa, with thrombin.

Complex Formation and Inhibitory Activity of cNS-Cκ and cNS_{EP}-Cκ—The question, whether the observed complex formation was accompanied by an inhibition of the target proteinases, was studied using the fusion protein cNS-Cκ. Although partially purified cNS still contained 30–50% serum proteins (see Fig. 2*C*, *lane 2*), the fusion proteins could be purified to apparent homogeneity by a single step affinity chromatography (see Fig. 2*C, lanes 4* and *6*). The ability of cNS-Cκ to form complexes was tested in the same assay as used previously for cNS. We found that cNS-Cκ formed complexes of the same apparent molecular masses as the recombinant wild-type neuroserpin (Fig. 4*A*). In particular, cNS-Cκ readily formed complexes with tPA and uPA, but a substrate-like reaction with only a small portion of stable complexes was observed with plasmin, and no complex formation and marginal proteolytic cleavage was seen with thrombin. This suggested that the presence of the Ig domain Cκ at the C terminus did not interfere with the ability of recombinant neuroserpin to form SDS-stable complexes with its target proteinases. To test whether the complex formation reflected an antiproteolytic activity of neuroserpin, we measured the residual amidolytic activities of the proteinases after complex formation using an appropriate chromogenic enzyme substrate (Fig. 4*C*). In accordance with the results of the complex formation assays, we found that tPA and uPA were inhibited by cNS-Cκ in a dose-dependent manner, but the proteolytic activity of thrombin was not affected. Interestingly, cNS-Cκ also inhibited plasmin, although the re-

[3] C. Rader, personal communication.

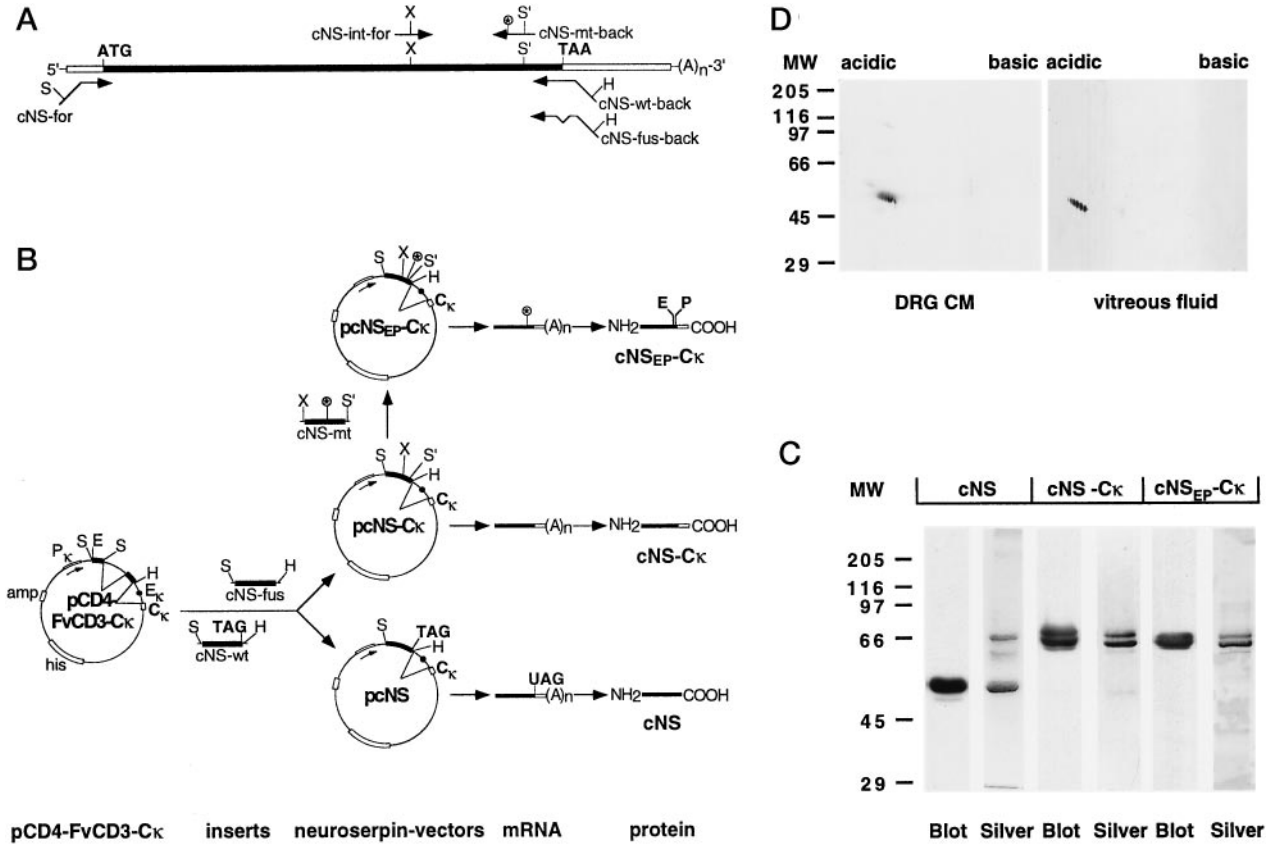

FIG. 2. **Recombinant expression of neuroserpin.** *A*, two cDNA fragments were amplified by PCR from the neuroserpin cDNA Sc3a4 using the two primer pairs cNS-for/cNS-wt-back and cNS-for/cNS-fus-back, respectively. The primers were designed to introduce a *Sac*I restriction site upstream of the translation start signal (ATG) at the 5′ end (cNS-for) and a splice donor site followed by a *Hin*dIII restriction site at the 3′ end (cNS-wt-back and cNS-fus-back) of neuroserpin. In the primer cNS-fus-back, the wild-type stop signal (TAA) was deleted. Using the primer cNS-int-for and the mutagenic primer cNS-mt-back, an *Xba*I-*Sca*I fragment carrying the mutated reactive site (R362E, M363P, indicated by a *star*) was amplified using the chicken cDNA as a template. *B*, PCR fragments encoding neuroserpin (cNS-wt and cNS-fus, respectively) were ligated into the parental expression vector pCD4-FvCD3-Cκ (55) substituting for CD4 and FvCD3 and are therefore put under the control of an Ig Vκ promoter (Pκ) and an Ig κ enhancer (Eκ) and forced to splice onto an Ig Cκ exon (Cκ). The novel expression vectors pcNS and pcNS-Cκ gave rise to the wild-type neuroserpin cNS and the fusion protein cNS-Cκ (with the deleted neuroserpin translation stop signal), respectively. To generate mutant neuroserpin fusion protein, the mutated fragment (cNS-mt) was introduced into pcNS-Cκ using the parental *Xba*I and *Sca*I restriction sites, yielding $pcNS_{EP}$-Cκ, which gave rise to the mutant fusion protein cNS_{EP}-Cκ with P1 and P1′ mutated (*amp*, ampicillin resistance gene for procaryotic selection; *his*, histidinol resistance gene for eucaryotic selection; *S*, *E*, *H*, *X*, and *S*′ indicate recognition sites for the restriction endonucleases *Sac*I, *Eco*RI, *Hin*dIII, *Xba*I, and *Sca*I, respectively). *C*, SDS-PAGE followed by silver staining (*Silver*) or Western blot (*Blot*) using rabbit anti-neuroserpin, R35, of partially purified wild-type (cNS) and affinity purified neuroserpin fusion proteins (cNS-Cκ, cNS_{EP}-Cκ). *D*, two-dimensional SDS-PAGE of immunoprecipitated, metabolically labeled neuroserpin from conditioned medium of primary DRG neurons (DRG CM) and Western blot of neuroserpin in the ocular vitreous fluid (VF) of chicken embryos, using the rabbit antiserum R61 raised against the recombinant fusion protein cNS-Cκ. *Numbers* on the *left* indicate the molecular masses of marker proteins in kDa, and the direction of the isoelectric focusing is indicated at the *top*.

sults of the complex formation assays with neuroserpin and plasmin pointed toward a more substrate-like interaction (see Fig. 3 and Fig. 4*A*). Together, these results suggested that neuroserpin inhibits the PAs via the formation of a tight, stoichiometric complex, whereas it interacts with plasmin in a serpin-like mechanism with a higher partition ratio.

It had been shown previously that the inhibitory activity and specificity of serpins critically depend on their amino acid composition at the reactive site (37, 38). In particular, PAI-1, which is a close relative of neuroserpin according to its amino acid sequence, had been studied in detail by site-directed mutagenesis. Although PAI-1 must carry an arginine or a lysine at the P1 position, P1′ is more promiscuous, allowing every residue except proline (37). To test whether a mutation found to be "lethal" in PAI-1 abolishes the inhibitory activity of neuroserpin, we produced a fusion protein carrying a mutation of both P1 and P1′ (cNS_{EP}-Cκ, R362E, M363P). As expected, the ability to form stable complexes with any of the tested proteinases was completely lost in the mutant neuroserpin (Fig. 4*B*). In line with this observation, cNS_{EP}-Cκ was unable to reduce the

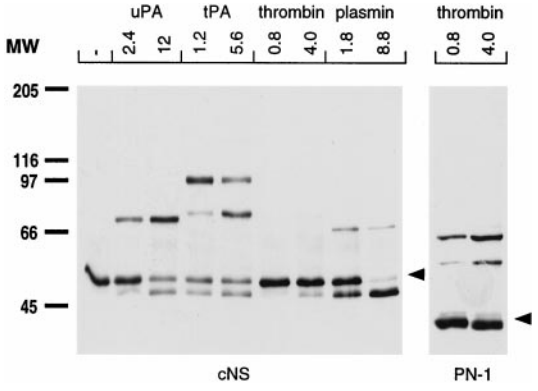

FIG. 3. **Complex formation between neuroserpin and different serine proteinases.** Approximately 2 pmol of recombinant wild-type neuroserpin (cNS) or 2.8 pmol of protease nexin-1 (PN-1) were incubated either alone (−), or with uPA, tPA, thrombin, or plasmin and analyzed by SDS-PAGE and Western blot. *Numbers* on the *top* indicate amounts of proteinases in picomoles, and *numbers* on the *left* indicate the molecular masses of marker proteins in kDa. *Arrowhead* on the *right* indicates the molecular masses of the free inhibitors.

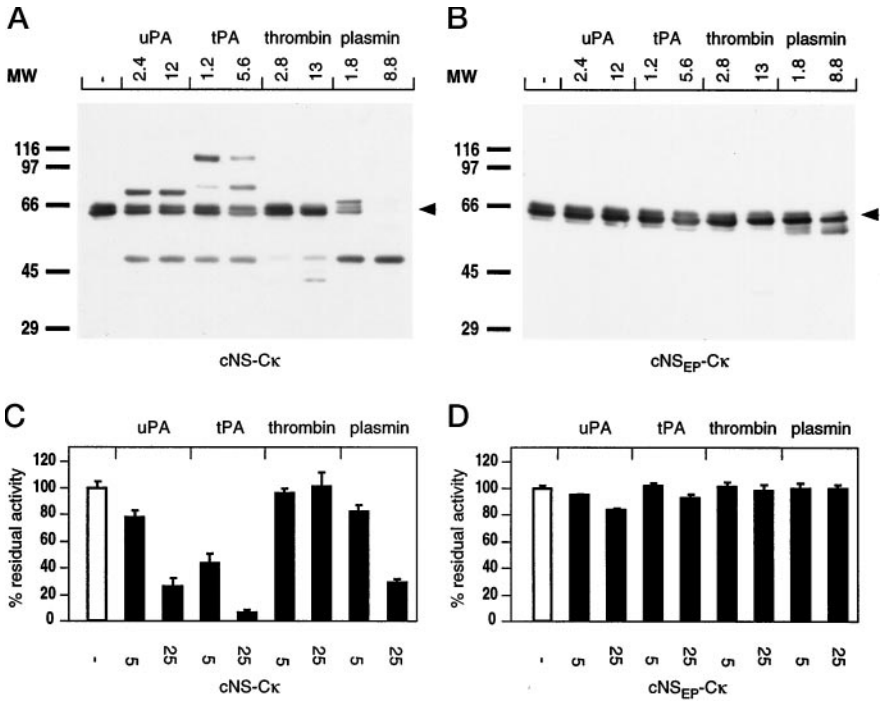

FIG. 4. **Complex formation and inhibitory activity of neuroserpin fusion proteins.** *A* and *B*, complex formation between 0.9 pmol of cNS-Cκ or 0.9 pmol of cNS$_{EP}$-Cκ, respectively, and the proteinases uPA, tPA, thrombin, or plasmin are monitored using SDS-PAGE followed by Western blotting. *Numbers* on the *top* indicate amounts of proteinases in picomoles, and *numbers* on the *left* indicate the molecular masses of marker proteins in kDa. *Arrowheads* on the *right* indicate the molecular masses of the free inhibitors. *C* and *D*, inhibitory activity of the fusion proteins is shown by plotting the residual amidolytic activity of several proteinases after preincubation with two different concentrations of cNS-Cκ or cNS$_{EP}$-Cκ, respectively. The final concentrations of enzymes were as follows: uPA, 19.1 nM; tPA, 7.9 nM; thrombin, 18.8 nM; plasmin, 12.7 nM. *Numbers* at the *bottom* indicate the final concentrations (in nM) of cNS-Cκ and cNS$_{EP}$-Cκ, respectively, and *numbers* on the *left* indicate the residual amidolytic activity of the proteinases in % of the samples without inhibitor (*white column*, 100% per definition).

proteolytic activity of either of the tested proteinases more than 15% (Fig. 4*D*).

Complex Formation and Inhibitory Activity of hNS-H$_6$—The extremely high conservation of the primary structure within the reactive site loop of neuroserpin from different species (Fig. 1) suggested a conserved target specificity from birds to men. To test this hypothesis experimentally, we examined the capacity of human neuroserpin to form complexes with, and its inhibitory activity toward, uPA, tPA, thrombin, and plasmin. We found that hNS-H$_6$ formed SDS-stable complexes with uPA, tPA, and plasmin, but no reaction with thrombin could be observed (Fig. 5*A*). Moreover, a significant amount of neuroserpin was proteolytically cleaved by the target proteinases, but the two shorter forms of neuroserpin (Fig. 5*A, open arrowheads*) appeared to be inactive and are most probably produced by alternative usage of translation start signals or N-terminal proteolytic degradation of hNS-H$_6$ by the bacterial host strain. Amidolytic assays revealed a dose-dependent inhibitory activity of hNS-H$_6$ against uPA, tPA, and plasmin, whereas thrombin was not affected (Fig. 5*B*). These results are qualitatively in accordance with the results obtained for chicken neuroserpin.

Stability of Complexes and Latency of Neuroserpin—Complexes between serpins and serine proteinases exhibit various degrees of stability, depending on the nature of the serpin and the cognate proteinase as well as on the reaction conditions (58). Moreover, the well characterized serpin PAI-1 had been demonstrated to become inactive after a relatively short incubation by assuming a so-called "latent form" (59). We have therefore investigated the complexes between neuroserpin and the PAs with regard to their stability, and we have also included tests of the stability of free neuroserpin in the absence of a proteinase. cNS and cNS-Cκ were incubated at various temperatures and over different times in the presence or absence of uPA or tPA. As shown in Fig. 6, the results were similar for uPA (*upper panels*) and tPA (*lower panels*), and no obvious difference was found between cNS (*left panels*) and cNS-Cκ (*right panels*). At low temperature, no evidence for a transition into a latent form was observed. The reactivity of neuroserpin remained the same whether it was thawed and immediately used for the complexation test (*lanes 1*) or whether it was preincubated for 5 h on ice prior to mixing with the proteinases (*lanes 2*). A slightly reduced reactivity after preincubation for 5 h at 37 °C was observed for both cNS and cNS-Cκ, as indicated by the higher intensity of the bands representing free cNS (I_w) or cNS-Cκ (I_f) at 54 or 65 kDa, respectively (*lane 3*). Once formed, a large proportion of the complexes remained stable for at least 5 h on ice (*lane 5*), whereas incubation at 37 °C lead to a slightly increased decay (*lane 4*), as indicated by the higher intensity of the band I_{cl} representing the proteolytically cleaved form of neuroserpin at 49 kDa.

Kinetics of the Interaction between cNS-Cκ and Trypsin-like Proteinases—Second-order rate constants, k_a, for the interaction of cNS-Cκ with uPA, tPA, and plasmin, were determined under pseudo first-order conditions using the progress curve method (53). This also permitted a direct comparison between the constants obtained for neuroserpin and previously published second-order rate constants for PN-1 (60). Plots of k' values *versus* the corresponding inhibitor concentrations were linear in all cases. We performed a statistical test for a departure from linearity. The slope of the k' *versus* [*I*] plot was significantly different from 0, and there was no indication that a hyperbola fit the data better than a straight line. Similarly, the dependence of v_z upon [*I*], in all cases, revealed a slope not significantly different from 0, indicating the independence of v_z from [*I*]. The profiles of v_s *versus* [*I*] were hyperbolic and fit well to a classical, fully competitive inhibition mechanism, whereas fitting to a tight binding model gave considerably worse results.

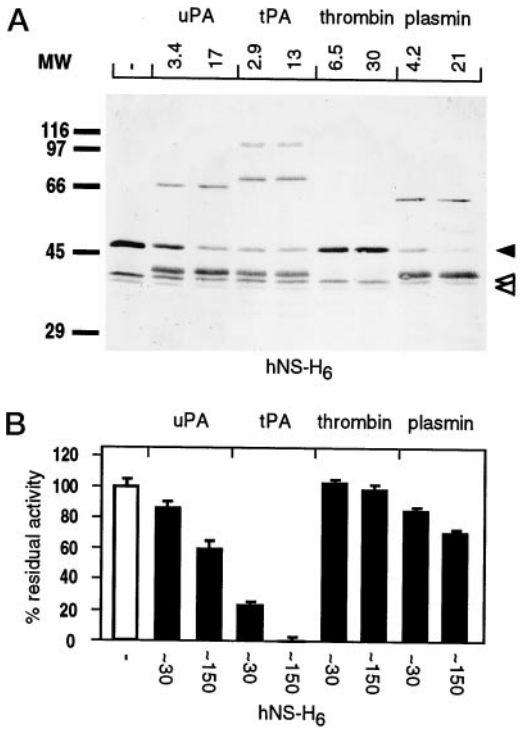

FIG. 5. **Complex formation and inhibitory activity of human neuroserpin.** *A*, approximately 2 pmol of procaryotically expressed hNS-H_6 were incubated either alone (−), or with uPA, tPA, thrombin, or plasmin and analyzed by SDS-PAGE followed by Western blotting using the polyclonal serum R35. *Numbers* on the *top* indicate amounts of proteinases in picomoles, and *numbers* on the *left* indicate the molecular masses of marker proteins in kDa. *Filled arrowhead* on the *right* indicates the molecular mass of the free inhibitor; *open arrowheads* indicate N-terminally truncated forms of recombinant neuroserpin that most probably arise from alternative usage of translation start signals or from proteolytic cleavage by the bacterial host strain. *B*, the inhibitory activity of hNS-H_6 is shown by plotting the residual amidolytic activity of the indicated proteinases after 10 min preincubation with two different concentrations of hNS-H_6 (indicated at the *bottom* in nM). The final concentrations of protease were as follows: uPA, 9.1 nM; tPA, 7.9 nM, thrombin, 18.8 nM; plasmin, 12.7 mM. *Numbers* on the *left* indicate the residual amidolytic activity of the proteinases in % of the samples without inhibitor (*white column*, 100% per definition).

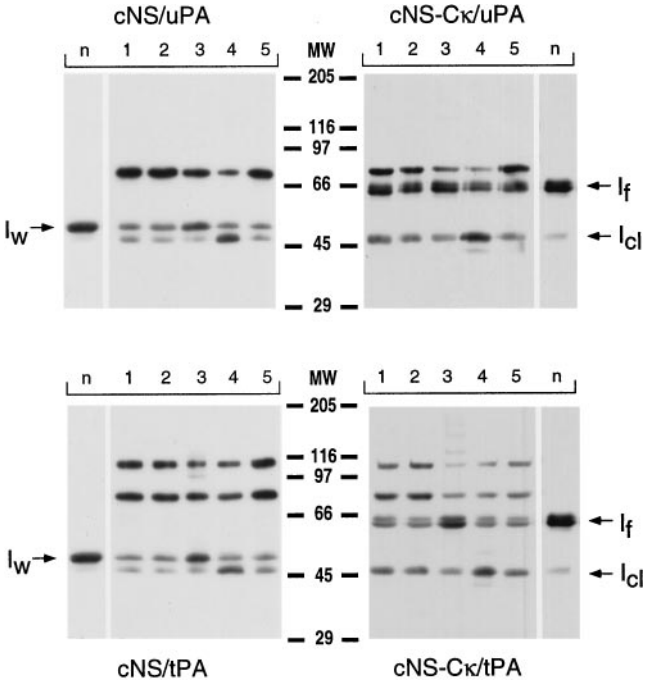

FIG. 6. **Latency of neuroserpin and decay of complexes with tPA or uPA.** An antiserum against neuroserpin recognizes cleaved neuroserpin (I_{cl}, 49 kDa) and free cNS (I_w, 54 kDa) and cNS-Cκ (I_f, 65 kDa) or complexes with uPA (80 kDa) or tPA (86 kDa and 112 kDa) after SDS-PAGE and Western blotting. *Numbers* in the *middle* indicate the relative molecular masses of marker proteins in kDa. Samples were treated as follows: *n*, no proteinase; *1*, no preincubation of neuroserpin; *2*, 5 h preincubation on ice; *3*, 5 h preincubation at 37 °C before addition of proteinases; *4*, 5 h incubation at 37 °C; *5*, 5 h incubation on ice after addition of proteinases. Reaction time with proteinases was 15 min in all cases. The reactions were stopped by addition of SDS-PAGE sample buffer and boiling.

These properties justified the calculation of the association and dissociation constants according to Equation 2 (53). Fig. 7 exemplifies the case of tPA, and the results are summarized in Table I (the primary data for uPA and plasmin are not shown). The behavior was typical for a slow, tight binding mechanism. Although k_a can be considered sufficiently precise, k_d values for uPA and tPA were too small for experimental evaluation. The estimated range in which their actual value may lie is shown for qualitative purposes as the 95% confidence interval of the intercept in the *k′ versus* [*I*] plot. An estimate of k_d for uPA and tPA can be calculated from the K_i values determined from the dependence of v_s upon [*I*], namely 4.2×10^{-5} s^{-1} and 4.5×10^{-5} s^{-1} for uPA and tPA, respectively. No inhibition of thrombin was detected, even at an 80-fold cNS-Cκ concentration over enzyme (data not shown).

DISCUSSION

A detailed analysis of the amino acid composition within the putative reactive site loop led us to predict that neuroserpin could serve as an inhibitor of serine proteinases with trypsin-like substrate specificity, and the high conservation of this region further suggested a conservation of the target proteinases between species ranging from birds to men (Fig. 1). Among the serine proteinases with trypsin-like substrate specificity, we focused on tPA, uPA, thrombin, and plasmin, due to a colocalization with neuroserpin in the nervous system (1, 2, 16, 17). Since the published purification procedure for chicken neuroserpin from VF allowed the purification of only very small amounts of SDS-denatured protein (34), we established a heterologous system for the eucaryotic expression of neuroserpin. The recombinantly expressed proteins were of the expected size, and the co- and posttranslational modifications, such as signal peptide cleavage and glycosylation, were in agreement with the characteristics found for purified neuroserpin. Furthermore, the antiserum raised against recombinant neuroserpin was cross-reactive with chicken neuroserpin, and the antiserum against purified neuroserpin recognized cNS, cNS-Cκ, and cNS_{EP}-Cκ, both under native and denatured conditions. Therefore, it seemed reasonable to base the functional study of neuroserpin on recombinantly expressed protein.

Neuroserpin Is a Typical Serpin-like Inhibitor of Serine Proteinases—The presented results characterized neuroserpin as a mechanism-based (suicide substrate) inhibitor of tPA, uPA, and plasmin. Neuroserpin formed SDS-stable complexes with the PAs and plasmin, although no interaction with thrombin was found. The two different complexes found with tPA most probably represented complexes with the single chain form of tPA and with the catalytic subunit in the proteolytically cleaved two chain form. Both forms of tPA are proteolytically active (61) and interact with PAI-1, their physiological inhibitor in the blood (62). Since we used the recombinant single chain form of tPA, the two chain form appears to be generated by autoproteolytic cleavage. Whether this cleavage occurred before or after the interaction with neuroserpin cannot be distinguished by our experiments. Interestingly, only a small proportion of neuroserpin was found in an SDS-stable complex

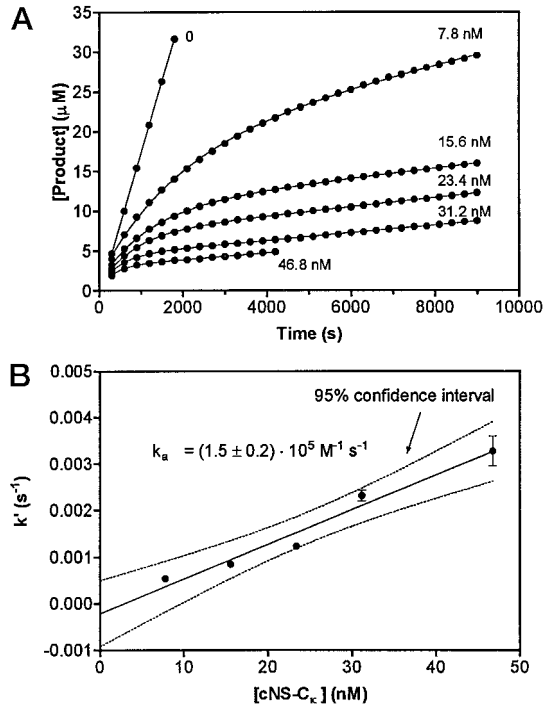

FIG. 7. **Inhibition of tPA by cNS-Cκ under pseudo first-order conditions.** The interaction of tPA and cNS-Cκ was measured under pseudo first-order conditions using the progress curve method. Inhibition of tPA at different concentrations of cNS-Cκ in inhibition buffer was followed by measuring the product concentration every 300 s. *A*, tPA progress curves were measured with 1.6 nM tPA, 1.08 mM substrate, and cNS-Cκ as indicated by *numbers* near the *curves*. The first-order rate constants (k') were calculated for each inhibitor concentration by a nonlinear regression fit using Equation 1. Best fit curves are shown as *solid lines*. *B*, dependence of the first-order rate constant (k') on the concentration of inhibitor. A second-order rate constant was obtained from the slope of this line and corrected with K_m using Equation 2.

with plasmin, where most of the inhibitor was found in a modified form of approximately 49 kDa (43 kDa for hNS-H_6), most probably representing the thermodynamically stable serpin core I^* after cleavage between P1 and P1′ (63, 64). This suggested that neuroserpin interacts with plasmin by a serpin-like mechanism with a higher partition ratio. Calculation of the stoichiometric index (SI) from the residual activity of plasmin after 30 min of preincubation with cNS-Cκ yielded an SI of approximately 3 for plasmin, whereas the values for PAs are close to 1, which is in agreement with the results of the complex formation assays.

The size of the complexes with the proteinases as well as the size of the cleaved form of neuroserpin were undistinguishable for cNS or cNS-Cκ, and the patterns of complex formation were identical for the wild-type and the fusion protein. Moreover, the stability of both free neuroserpin and the complexes of neuroserpin with tPA and uPA were very similar for cNS and cNS-Cκ. Due to the close resemblance with respect to selectivity and stability, it seemed reasonable to base the determination of the inhibitory activity of neuroserpin on the fusion protein cNS-Cκ. Using cNS-Cκ was advantageous, because it could be obtained as an apparently homogeneous protein without disturbing contaminations of serum proteins by a one-step immunoaffinity purification.

The results of the inhibition assays confirmed the specificity of neuroserpin determined by complex formation assays. Neuroserpin inhibited uPA, tPA, and plasmin in a dose-dependent manner, although inhibition of plasmin had a higher SI. No inhibition of thrombin was observed. The large, C-terminal Ig domain did not interfere with the activity nor with the specificity of neuroserpin. The experiments with mutant neuroserpin provided strong evidence for the putative reactive site P1-P1′ (Arg^{362} and Met^{363}) being involved in the interaction with all target proteinases, since the reactive site mutant cNS_{EP}-Cκ formed no complexes with either of the proteinases, and the antiproteolytic activity was strongly reduced. The weak inhibition of uPA by an excess of cNS_{EP}-Cκ might represent a competitive effect that does not lead to the formation of stable complexes. Altogether, the results characterized neuroserpin as a typical serpin-type inhibitor of tPA, uPA, and plasmin, with the amino acids Arg^{362} and Met^{363} forming the reactive site P1-P1′.

Chicken and Human Neuroserpin Exhibit the Same Target Specificity—Complex formation tests and inhibition assays revealed the same target proteinase preference for the human and the chicken form of neuroserpin. Both hNS-H_6 and cNS-Cκ readily formed SDS-stable complexes with plasminogen activators and with plasmin but did not react with thrombin. Inhibitory assays with hNS-H_6 and with cNS-Cκ resulted in qualitatively the same target preference pattern. Both hNS-H_6 and cNS-Cκ exhibit the strongest inhibitory activity against tPA, although hNS-H_6 in general shows a lower specific activity. The higher stoichiometric indices observed for hNS-H_6 as compared with cNS-Cκ could reflect a lower stability of the complexes formed by human neuroserpin. Alternatively, this observation could be explained by the fact that the hNS-H_6 used for these experiments was of procaryotic origin and, thus, less stable due to a lack of glycosylation (65).

Neuroserpin Is a Slow Binding Inhibitor of tPA, uPA, and Plasmin—A considerable amount of work has been done over the last years to uncover the mechanism by which serpins inhibit their target serine proteinases (64, 66, 67, for a recent review, see Ref. 68). In accordance with a general mechanism of serpins, we observed the generation of a modified inhibitor species under particular experimental conditions, namely at relatively high enzyme and inhibitor concentrations, with enzyme and inhibitor concentration of the same order of magnitude, and in the absence of substrate. Conversely, progress curves of amidolytic activity were obtained with catalytic amounts of enzyme, in the presence of a relatively high concentration of a synthetic substrate and at inhibitor concentrations greatly exceeding those of the enzymes. Therefore, kinetic parameters could be determined under fully competitive conditions. Furthermore, the rate constants obtained under pseudo first-order conditions showed a linear dependence upon the inhibitor concentration, which allowed the usage of the progress curve method (53) to calculate second-order rate constants k_a for the association of neuroserpin with uPA, tPA, and plasmin. These data could be compared with analogous data previously determined for PN-1 by Scott *et al.* (60), although obtained under different experimental conditions. Neuroserpin interacted relatively fast with tPA and plasmin and slightly slower with uPA. However, the high stability of the complex with tPA, in comparison to plasmin, as well as the pronounced cleavage of neuroserpin by plasmin pointed toward tPA as the most likely physiological target of neuroserpin. Interestingly, the association of tPA with neuroserpin occurred about 2 orders of magnitude faster than with PN-1, which is the closest relative of neuroserpin in the nervous system.

Is Neuroserpin the Physiological Inhibitor of tPA in the Nervous System?—There is growing evidence for tPA and thrombin playing an important role in the nervous system. Secretion of tPA by neurons *in vitro* was interpreted to reflect its role in facilitating neurite growth (7, 8) and neuronal migration (4). The strong expression of tPA in particular regions of the adult

TABLE I
Rate constants for endopeptidase inhibition by cNS-Cκ and PN-1

Reaction conditions were as follows: PBS, 0.1% PEG 8000, 0.2 mg/ml BSA, pH = 7.2; temperature = 37 ± 1 °C. k_a and k_d were calculated by the progress curve method, and k_a values for PN-1 were taken from Ref. 60.

Enzyme	cNS-Cκ			PN-1 k_a	cNS k_a/PN-1 k_a
	k_a	k_d	K_i		
	$M^{-1}\ s^{-1}$	s^{-1}	nM	$M^{-1}\ s^{-1}$	
uPA	$(4.7 \pm 0.8) \times 10^4$	$(-0.2–2) \times 10^{-4}$[a]	0.9[b]	1.5×10^5	0.31
tPA (single chain)	$(1.5 \pm 0.2) \times 10^5$	$(-1–5) \times 10^{-4}$[a]	0.3[b]	1.5×10^3	100
Plasmin	$(1.1 \pm 0.1) \times 10^5$	$(5.2 \pm 1.4) \times 10^{-4}$	4.7 ± 1.3[c]	1.3×10^5	0.85
Thrombin	No inhibition			6.0×10^5	0

[a] 95% confidence interval of the intercept of the k' versus [I] plot.
[b] Calculated by nonlinear regression analysis of v_s versus [I] for a fully competitive inhibition mechanism.
[c] Calculated as k_d/k_a.

brain (1) and the observation of tPA mRNA being up-regulated after motor learning or experimental seizures, kindling, or LTP (10, 11) led to the speculation that tPA could also be involved in synaptic plasticity subserving learning and memory. In recent studies of $tPA^{-/-}$ mice, indeed a retardation in cerebellar granule cell migration (74) as well as a different form of hippocampal LTP (13, 14) were found. On the other hand, several lines of evidence point toward a role of thrombin during neural development and establishment of neuromuscular connectivity as follows: prothrombin mRNA is expressed in the nervous system and in muscles (17, 69); neurite retraction is induced by proteolytic activation of the thrombin receptor *in vitro* (19); and the proteolytic activity of thrombin is required for neuromuscular synapse elimination *in vitro* and *in vivo* (28, 69). The inhibitory activity of neuroserpin is directed against tPA but not against thrombin. This specificity is remarkable, since it is complementary to the inhibitory activity of PN-1. PN-1 was initially found to promote neurite outgrowth (25, 26). It now seems clear that this activity is due to its fast and strong inhibition of thrombin, which induces neurite retraction *in vitro* (for a review, see Ref. 70). PN-1 only slowly interacts with tPA, in particular when the latter is present in the single chain form (71). Although tPA is converted into a two chain form by several proteinases *in vitro* (61, 72, 73), the single chain form is proteolytically active (62), and it is not certain in which form tPA is present in the nervous system. Neuroserpin forms stable complexes with both forms of tPA, and it interacts with single chain tPA approximately 2 orders of magnitude faster than PN-1. Together with the colocalization of tPA and neuroserpin in the nervous system of the mouse,[2] these results make neuroserpin an interesting candidate for a physiological, local regulator of tPA in the nervous system. Based on results indicating a discrepancy between tPA expression and its proteolytic activity in the hippocampus and in the cerebellum of mice, Sappino *et al.* (1) recently proposed an inhibitor of tPA different from PAI-1, PAI-2, or PN-1 to exist in the murine brain. It will be of particular interest to clarify whether neuroserpin is responsible for the inhibition of tPA observed in their assay.

Despite indications for an interaction between neuroserpin and tPA in the developing and in the adult nervous system, there is good reason not to exclude other serine proteinases as potential targets of neuroserpin. Since only one plasminogen activator (namely uPA) is thought to exist in chicken, and so far all attempts to find a chicken tPA failed, uPA might replace tPA in its functions in the chicken nervous system. Therefore, inhibition of uPA *in vitro* might reflect a regulatory function of neuroserpin toward PA-mediated processes in birds. Moreover, recently discovered serine proteinases such as neuropsin (21), neurosin (23), or neurotrypsin (22) fulfill the prerequisites for a target of neuroserpin (namely extracellular location, temporal and spatial coexpression). They have not yet been available for tests with recombinant neuroserpin. It will therefore be important to test biochemically the interaction between neuroserpin and new neuronal proteinases, to identify the physiological pathways of proteolysis in the developing and the adult nervous system. In conclusion, the data presented here make tPA a likely candidate for a physiological target of neuroserpin. The striking differences in target specificity between the two neural serpins, neuroserpin and PN-1, would allow the selective regulation of different proteolytic cascades in the extracellular space of the developing and the adult nervous system.

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