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Purification and enzymological characterization of murine neurotrypsin

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ABSTRACT

An increasing number of studies indicate that serine proteases play an important role in structural plasticity associated with learning and memory formation. Neurotrypsin is a multidomain serine protease located at the presynaptic terminal of neurons. It is thought to be crucial for cognitive brain functions. A deletion in the neurotrypsin gene causes severe mental retardation in humans. For a biochemical characterization, we produced murine neurotrypsin recombinantly in a eukaryotic expression system using myeloma cells. From the culture medium we purified neurotrypsin using heparin-, hydrophobic interaction- and immobilized metal affinity chromatography. For an enzymological characterization two fragments of agrin containing the natural cleavages sites of neurotrypsin were used as substrates. The highest catalytic activity of neurotrypsin was observed in the pH range between 7.0 and 8.5. Calcium ions were required for neurotrypsin activity and an ionic strength exceeding 500 mM decreased substrate cleavage. Site-specific mutations of the amino acids flanking the scissile bonds showed that cleavage is highly specific and requires a basic amino acid preceded by a glutamate residue on the N-terminal side of the scissile bond. This sequence requirement argues for a unique substrate binding pocket of neurotrypsin. This observation was further substantiated by the fact that almost all tested serine protease inhibitors except dichloroisocoumarin and PMSF did not affect neurotrypsin activity.

Neurotrypsin is a member of the serine protease family S1A. The murine ortholog of neurotrypsin consists of 761 amino acids with a molecular mass of 84 kDa and a theoretical pI of 8.6. It consists of a signalpeptide (SP),[1] a proline-rich basic (PrB) region, a kringle (Kri) domain, three scavenger receptor cysteine rich (SRCR) domains, and a serine protease (Prot) domain. The protease domain comprises 244 amino acids and exhibits a 40% amino acid identity with trypsin. A zymogen activation site at the N-terminus of the protease domain bears the furin recognition sequence RRQKR. Murine neurotrypsin has three *N*-glycosylation site motives, one in the proline-rich basic domain and two in the protease domain [1]. In contrast to the rodent form of neurotrypsin, the human form exhibits an additional SRCR domain, which is conserved over all primates. The protease domain of murine and human neurotrypsin exhibit an amino acid identity of 89.9%. The overall amino acid sequence identity between murine and human neurotrypsin is 82.5% [2].

In mice, expression of neurotrypsin is predominantly found in the brain [1,3,4], the kidney [3], and the lung [1,3]. *In situ* mRNA hybridization on adult mouse brain sections revealed most prominent expression of neurotrypsin mRNA in the cerebral cortex, the subicular complex, the Ammon's horn, the dentate gyrus, and the lateral amygdala [4]. Upregulation of neurotrypsin RNA is found in many brain regions especially in the hippocampus, neocortex, and the midbrain during pre- and postnatal development of the central nervous system [4]. Immunoelectron microscopic studies showed an association of neurotrypsin with the presynaptic membrane and the presynaptic active zone [5].

Neurotrypsin plays an indispensible role for adaptive synaptic processes that are required for cognitive functions. A 4 base-pair deletion in the human neurotrypsin gene, which results in a truncated form of neurotrypsin lacking the protease domain, was identified as the cause of a severe form of mental retardation [6]. Recent studies identified the proteoglycan agrin as the so far unique proteolytic target of neurotrypsin, and demonstrated that neurotrypsin-dependent cleavage of agrin occurs at two homologous and highly conserved cleavage sites [3]. Live imaging studies demonstrated that both translocation of vesicular neurotrypsin to presynaptic nerve terminals and synaptic exocytosis of neurotrypsin are stimulated by neuronal activity [7]. Preparation of synaptosomes by subcellular fractionation and differential centrifugation and comparison of agrin and neurotrypsin-dependent agrin fragments in neurotrypsin-overexpressing and neurotrypsin-deficient

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[1] *Abbreviations used:* SP, signalpeptide; PrB, proline-rich basic; Kri, kringle; SRCR, scavenger receptor cysteine rich.

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mice indicated neurotrypsin-dependent cleavage of agrin as a process that is located at the synapse [7]. By local cleavage of the synapse-regulating proteoglycan agrin, neurotrypsin may be crucial for adaptive synaptic functions, such as the reorganization of synapses and neuronal circuits, which are required to establish and/or maintain higher cognitive functions.

This study describes the expression of full-length murine neurotrypsin in myeloma cells and its purification. It further provides an initial characterization of murine neurotrypsin that allowed to set-up an *in vitro* activity assay.

Materials and methods

Materials

The chromatography resins were all purchased from GE Healthcare. The secondary antibodies HRP-conjugated anti-rabbit and anti-goat were from Sigma–Aldrich. The 4–12% NuPAGE gels were from Invitrogen. SYPRO Ruby was from Molecular Probes, USA.

Generation of neurotrypsin-specific antibodies

Goat antiserum G87 was raised against the recombinant protease domain of human neurotrypsin (CAA04816), produced in *Escherichia coli*. The goat antiserum G86 was generated against the SRCR domains 1–4 of human neurotrypsin antigen, produced in *E. coli*. Goat antiserum G93 was raised against full-length murine neurotrypsin (CAA73646) produced in stably transfected mouse myeloma J558L cells. Rabbit antiserum SZ177 was raised against two synthetic peptides of the proline-rich basic segment corresponding to amino acids 22–40 and 45–57 of mouse neurotrypsin. Rabbit antiserum R89 was raised against the kringle domain of human and rat neurotrypsin (CAC35028), produced in *E. coli*.

Cloning of full-lengh neurotrypsin for expression in myeloma cells

For expression of neurotrypsin in myeloma cells, the coding region of full-length murine neurotrypsin (CAA73646) ranging from Met_1 to Ser_{761}, was inserted via the restriction sites SacI and HindIII into the pCD4-FvCD3-c vector [8]. This mammalian expression vector comprises an immunoglobuline V_κ promotor and an immunoglobuline κ enhancer. For positive selection the vector included a histidinol resistance gene [9].

Large-scale production of recombinant murine neurotrypsin in myeloma cells

The protoplast fusion of the neurotrypsin expression vector and the murine myeloma cells was done as described previously [10,11]. To achieve individual transfectants, the cells were diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Germany) and plated in 96-well microtiter plates (PTT, Switzerland). Two days after transfection, cells with an insertion of neurotrypsin were selected by additional 5 mM L-histidinol to the growth medium (Sigma–Aldrich). Supernatants of surviving clones were analyzed for expression by Western blotting using the anti-neurotrypsin antibody SZ177. Clones with neurotrypsin expression were subcloned by limiting dilution for three rounds of single cell dilution. Briefly, cells were diluted and transferred into a 96-well microtiter plate to a calculated density of 0.5 cells per well. After 10–14 days in culture in presence of 5 mM L-histidinol the supernatants were again tested for neurotrypsin expression. Finally the best expressing clone was chosen for the large-scale production of murine neurotrypsin.

Adaptation of the neurotrypsin-expressing clones to protein-free medium

Starting from a medium composed of DMEM (Sigma–Aldrich) containing 4 mM L-glutamine and 10% FCS, the cells were adapted stepwise to grow in DMEM medium supplemented with 1% FCS. Adaptation was performed in 24-well plates (Nunc, Denmark) and the medium was exchanged every second day. When a density of about 1×10^6 cells/ml was reached, cells were transferred into a new well with a density of 3×10^5 cells/ml. Adapted cells growing in DMEM containing 1% FCS with a cell-doubling time of around 18 h were then transferred to the serum-free but protein-containing medium HL-1 (BioWhittaker Inc., USA) supplemented with 0.5% FCS. In HL-1 medium, the cells were then stepwise adapted to grow in HL-1 medium without FCS. Finally the HL-1 medium was stepwise exchanged from 0% to 50%, to 90%, to 98%, to 99.5% and finally to 100% by the protein-free medium TP-6 (Cell Culture Technology, Switzerland). The cells were maintained for neurotrypsin expression in roller bottles (Greiner Bio-One, Belgium) rotating at 2 rpm in humidified incubaters at 37 °C and 10% CO_2.

Chromatographic purification of murine neurotrypsin

For the purification of recombinant neurotrypsin, 14 L culture supernatant were concentrated using a Minisette tangential filtration cassette (Pall Corporation, USA). The retentate (500 ml) was adjusted to pH 8.0 by the addition of 20 mM Bis–Tris. Ammonium sulphate (Sigma–Aldrich) was added in small portions to a final saturation of 25% and afterwards stirred for 45 min at 4 °C. The solution was centrifuged for 30 min at 24,000*g*. The pellet was discarded, whereas the supernatant was subjected to another round of ammonium sulphate precipitation (a final saturation of 45%). After addition of the ammonium sulphate the pH of the solution was adjusted to 8.0 and once more stirred for 45 min at 4 °C. The solution was centrifuged and the pellet was resuspended in 200 ml loading buffer of the first chromatographic step. The resuspended protein was loaded onto a 60 ml heparin sepharose CL-B6 column equilibrated with the loading buffer (50 mM NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8). The column was washed with one column volume of loading buffer substituted with 100 mM NaCl and bound proteins were eluted in a gradient of three column volumes from 100 mM to 1 M NaCl in loading buffer. Fractions of the late peak shoulder of the elution at NaCl concentration of around 600 mM were pooled. For the HIC column 1 M NaCl was added to the neurotrypsin fractions. After dissolving, the sample was loaded onto a 12 ml butyl sepharose column equilibrated with 1 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8. Bound proteins were eluted in three column volumes with a gradient from 1.25 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8–0.1% PEG6000, 20 mM MOPS in 30% ethylenglycol, pH 7.0. The neurotrypsin-containing fractions were pooled, supplemented with 10 mM imidazole, and loaded onto a 1 ml column of copper-labeled chelating sepharose column equilibrated with 0.25 M NaCl, 10 mM imidazole, 0.1% PEG6000 in 20 mM MOPS, pH 7.0. Proteins were eluted in eight column volumes with a gradient from 10 to 250 mM imidazole, 0.25 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 7.0. All purification steps were performed on an Aekta Purifier Chromatography System (GE Healthcare).

The concentration of neurotrypsin in the pooled fractions was determined by measuring the absorption at 280 nm with a CARY 50 spectrometer and calculated using the specific extinction coefficient for murine neurotrypsin of $159280\,M^{-1}\,cm^{-1}$. Aliquots of 50 μl were frozen in liquid nitrogen and stored at −80 °C.